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Spice phenolics inhibit human PMNL 5-lipoxygenase

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Abstract

A wide variety of phenolic compounds and flavonoids present in spices possess potent antioxidant, antimutagenic and anticarcinogenic activities. We examined whether 5-lipoxygenase (5-LO), the key enzyme involved in biosynthesis of leukotrienes is a possible target for the spices. Effect of aqueous extracts of turmeric, cloves, pepper, chili, cinnamon, onion and also their respective active principles viz., curcumin, eugenol, piperine, capsaicin, cinnamaldehyde, quercetin, and allyl sulfide were tested on human PMNL 5-LO activity by spectrophotomeric and HPLC methods. The formation of 5-LO product 5-HETE was significantly inhibited in a concentration-dependent manner with IC_{50} values of 0.122–1.44 mg for aqueous extracts of spices and 25–83 μM for active principles, respectively. The order of inhibitory activity was of quercetin > eugenol > curcumin > cinnamaldehyde > piperine > capsaicin > allyl sulfide. Quercetin, eugenol and curcumin with one or more phenolic ring and methoxy groups in their structure showed high inhibitory effect, while the non-phenolic spice principle allyl sulfide showed least inhibitory effect on 5-LO. The inhibitory effect of quercetin, curcumin and eugenol was similar to that of synthetic 5-LO inhibitors—phenidone and NDGA. Moreover, the inhibitory potency of aqueous extracts of spice correlated with the active principles of their respective spices. The synergistic or antagonistic effect of mixtures of spice active principles and spice extracts were investigated and all the combinations of spice active principles/extracts exerted synergistic effect in inhibiting 5-LO activity. These findings clearly suggest that phenolic compounds present in spices might have physiological role in modulating 5-LO pathway.

Keywords: PMNL cells; 5-lipoxygenase; Inhibition; Spice active principles; Spice aqueous extracts

1. Introduction

5-Lipoxygenase (5-LO) belongs to the class of iron containing lipoxygenases that catalyze the incorporation of dioxygen into unsaturated fatty acid, preferentially arachidonic acid (AA), at C-5, giving rise to respective 5-hydroperoxy/5-hydroxy fatty acid derivatives. It is also the key enzyme in leukotriene (LT) biosynthesis, catalyzes initial steps in conversion of AA to biologically active leukotrienes (LTs) through lipoxygenase pathway. LTs are a group of highly potent molecules that mediate inflammatory and allergic reactions. Their pathophysiological role has been well defined in various disease conditions such as asthma, allergic rhinitis, psoriasis, inflammatory bowel disease, etc. The biological effects of LTs can be antagonized or prevented by targeting LT production through inhibition of 5-LO pathway and thus may have a therapeutical potential in a variety of inflammatory and allergic diseases.

Since ancient times, spices have been used in food preparations to improve the flavor and taste. In addition, spices are reported to contain bioactive compounds imparting antioxidant, preservative and antimicrobial properties to the food. Spices have shown to contain a number of phenolic and flavonoid compounds, which were shown to have antioxidant [1–4], antiinflammatory [5], antimutagenic [6] and anticarcinogenic activities [7,8]. Spice extracts especially that of turmeric and pepper are used in Indian traditional medicine to cure respiratory problems such as cough and cold attacks. Our earlier studies have demonstrated that spice active principle curcumin from turmeric and capsaicin from chilies exhibit antiinflammatory properties on carrageenan induced paw inflammation as well as adjuvant induced arthritis in rats [5,9]. As these inflammatory responses were mediated through LTs, in this study, we examined the inhibitory potential of

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aqueous extract of spices and their active principles namely quercetin (onion, garlic), eugenol (clove), curcumin (turmeric), piperine (pepper), capsaicin (chilies) cinnamaldehyde (cinnamon) and allyl sulfide (garlic) on human PMNL 5-LO, the key enzyme involved in biosynthesis of LTs.

2. Materials and methods

2.1. Materials

All chemicals used were of AR Grade. ATP, DTT, AA and all spice active principles, phenidone and NDGA were obtained from Sigma Chemical Co., USA. Cinnamaldehyde was purchased from ICN Biochemicals, USA. Solvents used for HPLC analysis were of HPLC grade from Ranbaxy Fine Chemicals Ltd., India. Spices—turmeric, cloves, pepper, cumin, cinnamon, chilies, onion and garlic were procured from local supermarket.

2.2. Isolation of 5-lipoxygenase enzyme from PMNLs of human blood

Human peripheral venous blood from healthy individuals who had not received any medication was collected into EDTA containing tubes. Polymorphonuclear leukocytes (PMNLs) were isolated from blood by Ficoll-Histopaque density gradient and hypotonic lysis of erythrocytes [10]. All the procedures were performed at 4°C. PMNL cells were resuspended in phosphate buffer saline and sonicated for 20–30 s at 20 kHz to release the cytosolic 5-LO enzyme into solution. This solution was centrifuged at 100,000*g* for 30 min at 4°C and the supernatant was directly used as a source of enzyme. Protein was estimated by Lowry method [11] using BSA as standard.

2.3. 5-lipoxygenase enzyme assay

The enzyme assay was performed according to the method of Aharony and Stein [12]. The standard reaction mixture for the of 5-LO assay contained 100 mM phosphate buffer pH 7.4, 50 μM of DTT, 200 μM of ATP, 300 μM of $CaCl_2$, 150 μM of AA and 5 μg protein. Enzymatic reactions were carried out at room temp. 5-LO activity was measured as 5-HETE formed at 234 nm using Shimadzu spectrophotometer. The molar extinction co-efficient of 28,000/M/cm was used to calculate specific activity of the enzyme. The enzyme activity was expressed as μmol of 5-HETE formed/min/mg protein.

2.4. Inhibitory studies with spice aqueous extracts and spice active principles on 5-LO

Spices namely pepper, cinnamon, cumin, turmeric, cloves, red chilies, dehydrated garlic and onion were powdered in a blender. Aqueous extract of the spices was prepared in known volume of distilled water, taking 0.50 g of the powdered spice. The extract was centrifuged at 1600 rpm for 10 min and the supernatant was used as spice extract to study the inhibitory effect on 5-LO activity.

The spice principles, viz., curcumin, piperine, capsaicin, eugenol, allyl sulfide, NDGA and phenidone were dissolved in DMSO at 2.0 mM concentration and used for inhibitory studies on 5-LO activity. The reaction mixture used was same as described above for enzyme activity assay, except that the spice active principles/spice aqueous extracts were incubated at different concentrations with the enzyme for 2 min prior to the initiation of reaction with AA. Formation of the product 5-HETE was followed spectrophotometrically at 234 nm.

2.5. Studies on synergistic effect of spice extracts and spice active principles on 5-LO

Synergistic effect of spice active principles were tested by combining IC_{50} concentration of quercetin with that of IC_{50} concentration of other spice active principles viz., eugenol, capsaicin, piperine, curcumin, cinnamaldehyde and allyl sulfide. Similarly, IC_{50} concentration of clove was combined with IC_{50} concentration of other spices. These mixtures of spices were tested on 5-LO activity as described above.

2.6. HPLC separation of 5-LO product 5-HETE and its inhibition by spice principles

5-LO product 5-HETE was resolved by HPLC method on a 4-μm Shim Pack straight phase silica column (2.5 mm × 15 cm). Enzyme assay was carried at room temperature as described above, in presence and absence of spice active principles. The typical reaction mixture contained 100 mM phosphate buffer pH 7.4, 50 μM of DTT, 200 μM of ATP, 300 μM of $CaCl_2$, 150 μM of AA and 5 μg protein. The reaction was stopped with addition of 0.5 ml of 6N HCl at the end of 5 min. The 5-LO product namely 5-HETE was extracted twice using organic solvent mixture of hexane and diethyl ether (1:1). The organic layers were pooled, dried under nitrogen and dissolved the sample in 50 μl of mobile phase. The sample (10 μl) was injected on a straight phase silica column and 5-HETE was resolved using a mobile phase of Hexane:Isopropanol:Acetic acid (100:2:0.1) with a flow rate of 0.5 ml/min at 234 nm. The product 5-HETE formed was confirmed by using an

authentic 5-HETE standard obtained from Sigma Chemical Co., USA. The percent inhibition of 5-HETE levels was calculated from peak area of the control and spice treated samples.

2.7. Statistical analysis

Data are represented as specific activity of 5-LO enzyme in presence of different concentrations of spice active principles. The IC_{50} values were calculated from dose response curves. Data are presented as percent inhibition of 5-LO activity in presence of different concentrations of spice aqueous extracts. The values are given as mean + SEM of two individual samples.

3. Results

The inhibitory effect of aqueous extracts of spices and their active principles on human PMNLs 5-LO activity was evaluated by measuring the formation of 5-LO product 5-HETE by spectrophotometric and HPLC methods. All the spice active principles tested namely curcumin, capsaicin, piperine, quercetin, cinnamaldehyde eugenol and allyl sulfide, inhibited human PMNLs 5-LO activity in a concentration dependent manner (Figs. 1 and 2). As expected, differences were observed in inhibitory potency among spice active principles. Quercetin, eugenol and curcumin showed maximum inhibition followed by piperine, cinnamaldehyde, capsaicin and allyl sulfide. The non-phenolic spice active principle allyl sulfide showed least inhibitory effect on

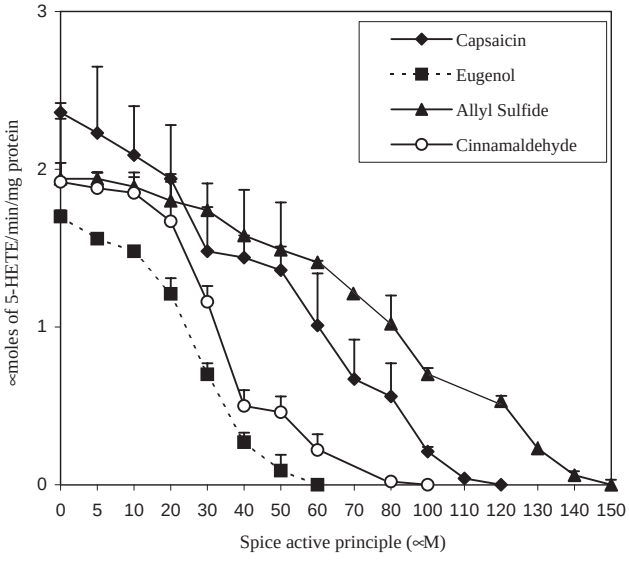

Fig. 1. Spice principles—capsaicin, eugenol, cinnamaldehyde and allyl sulfide were incubated with human PMNL 5-LO enzyme for 2 min before starting the reaction with substrate. The enzyme activity was followed spectrophotometrically at 234 nm. All the three spice principles dose dependently inhibited 5-LO activity. Values are mean ± SEM of two individual experiments.

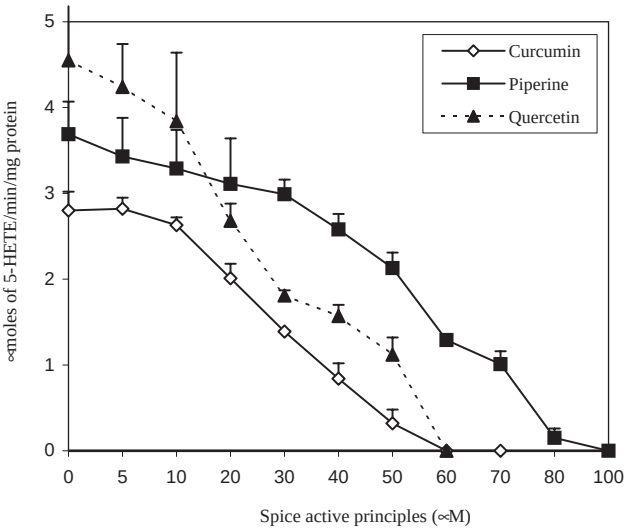

Fig. 2. Spice principles—curcumin, piperine and quercetin were incubated with human PMNL 5-LO enzyme for 2 min before starting the reaction with substrate. The enzyme activity was followed spectrophotometrically at 234 nm. All the spice principles dose dependently inhibited 5-LO activity. Values are mean ± SEM of two individual experiments.

Table 1
IC_{50} values of spice active principles for inhibition 5-LO activity of human PMNLs and comparison with synthetic 5-LO inhibitors

Compounds	IC_{50} value (μM)
Spice active principles	
Quercetin	25
Eugenol	26
Curcumin	30
Cinnamaldehyde	35
Piperine	54
Capsaicin	56
Allyl sulfide	83
Synthetic 5-LO inhibitors	
Phenidone	24
NDGA	28

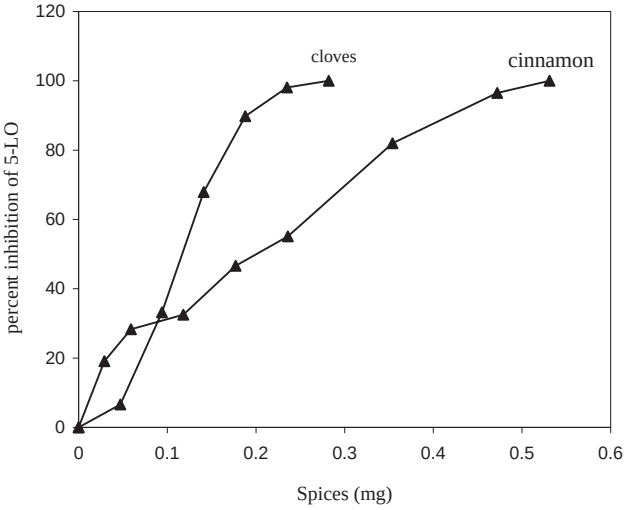

Fig. 3. Aqueous extracts of clove and cinnamon dose dependently inhibited human PMNL 5-LO.

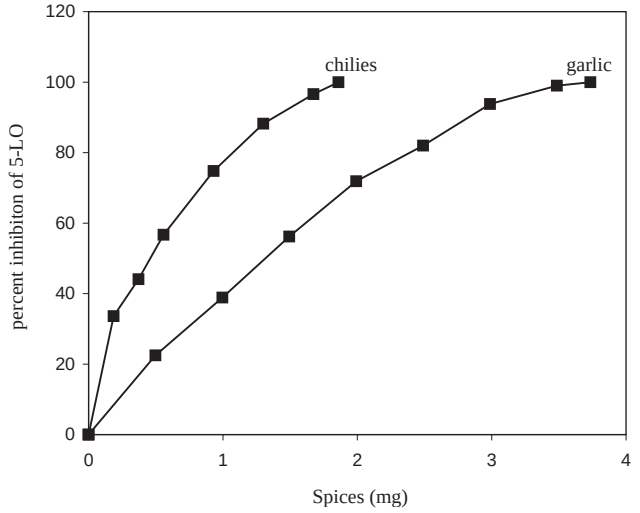

Fig. 4. Aqueous extracts of chilies and garlic dose dependently inhibited human PMNL 5-LO.

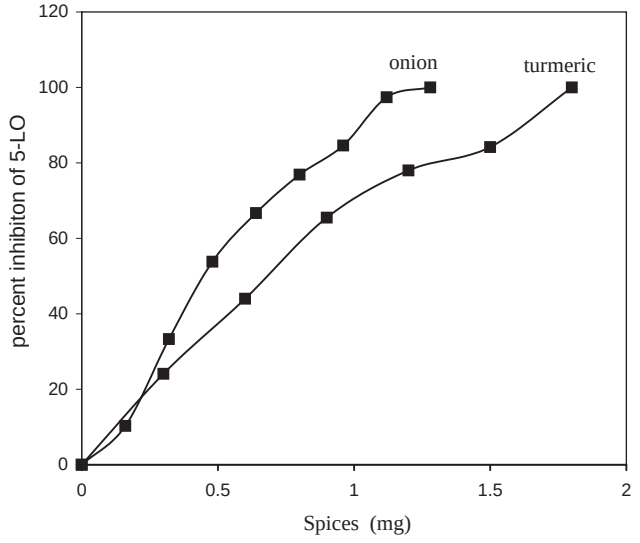

Fig. 5. Aqueous extracts of onion and turmeric dose dependently inhibited human PMNL 5-LO.

Table 2
IC_{50} values of spice aqueous extract for inhibition of 5-LO activity of human PMNLs

Spice	IC_{50} value (mg)
Clove	0.122
Cinnamon	0.207
Pepper	0.400
Onion	0.440
Chili	0.465
Turmeric	0.675
Garlic	1.400

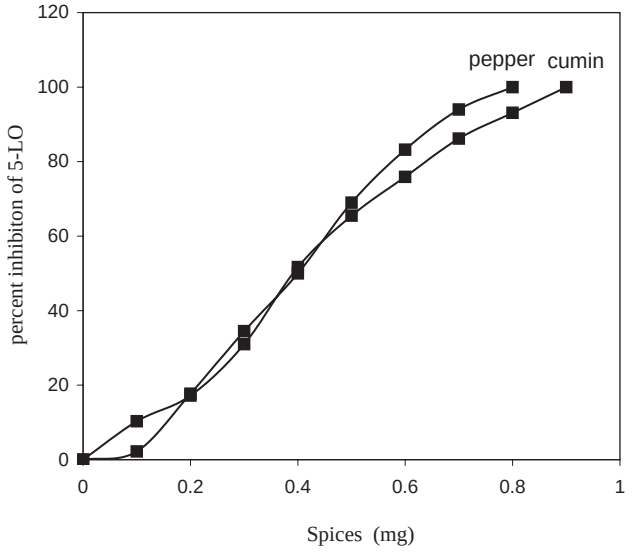

Fig. 6. Aqueous extracts of pepper and cumin dose dependently inhibited human PMNL 5-LO.

Table 3
Synergistic effect of spice extracts and active principle combinations on 5-LO activity of human PMNLs

Combinations	Expected inhibition (%)	Observed inhibition (%)
Spice active principles		
Quercetin + eugenol	100	89.3 ± 2.5
Quercetin + capsaicin	100	96.0 ± 4.5
Quercetin + piperine	100	98.0 ± 2.6
Quercetin + curcumin	100	91.0 ± 0.9
Quercetin + cinnamaldehyde	100	90.0 ± 1.3
Quercetin + allyl sulfide	100	99.3 ± 0.8
Piperine* + cinnamaldehyde*	50	53.6 ± 8.6
Spice aqueous extracts		
Clove + turmeric	100	100
Clove + onion	100	100
Clove + pepper	100	100
Clove + cumin	100	100
Clove + cinnamon	100	100
Clove + chili	100	100
Clove + garlic	100	100
Clove* + turmeric*	50	48.5 ± 7.8
Clove* + onion*	50	53.9 ± 1.7
Clove* + pepper*	50	55.2 ± 1.4
Clove* + cumin*	50	56.0 ± 2.2
Clove* + cinnamon*	50	49.2 ± 2.1
Clove* + chili*	50	58.6 ± 6.7
Clove* + garlic*	50	59.3 ± 3.7
Clove* + garlic*	50	59.3 ± 3.7

IC_{50} concentration of the individual spice active principles were combined and tested on 5-LO. *IC_{25} concentration of individual spice extracts were combined and tested on 5-LO. Values are mean ± SEM of two individual experiments.

5-LO activity. The IC_{50} values of quercetin, eugenol, curcumin and allyl sulfide were 25, 26 30 and 83 μM, respectively (Table 1).

Aqueous extracts of spices turmeric, chilies, pepper, onion, garlic, cloves and cinnamon also inhibited 5-LO activity dose dependent manner at micromolar

concentrations (Figs. 3–6). The inhibitory effect of spices decreased in the order of cloves > cinnamon > pepper > onion > chili > turmeric > garlic (Table 2). Except for turmeric and garlic, the inhibitory effect of spices viz., cloves, cinnamon, pepper, onion and chili correlated with the active principles present in the respective spices.

The 5-LO product 5-HETE was resolved on silica column by HPLC method at 234 nm 5-HETE eluted as a single, distinct peak at 1.45 min. Incubation of 5-LO with 32 μM of quercetin, 40 μM of curcumin and 45 μM of eugenol resulted in 74.4%, 64.6% and 62.5% decrease in 5-HETE levels, respectively (Fig. 7).

The effect of synthetic 5-LO inhibitors viz., phenidone and NDGA were tested and compared with that of spice active principles. The IC_{50} values of eugenol and quercetin were similar and comparable with that of above synthetic 5-LO inhibitors (Table 1).

The synergistic or additive effects of IC_{50} concentration of spice aqueous extracts/active principles were

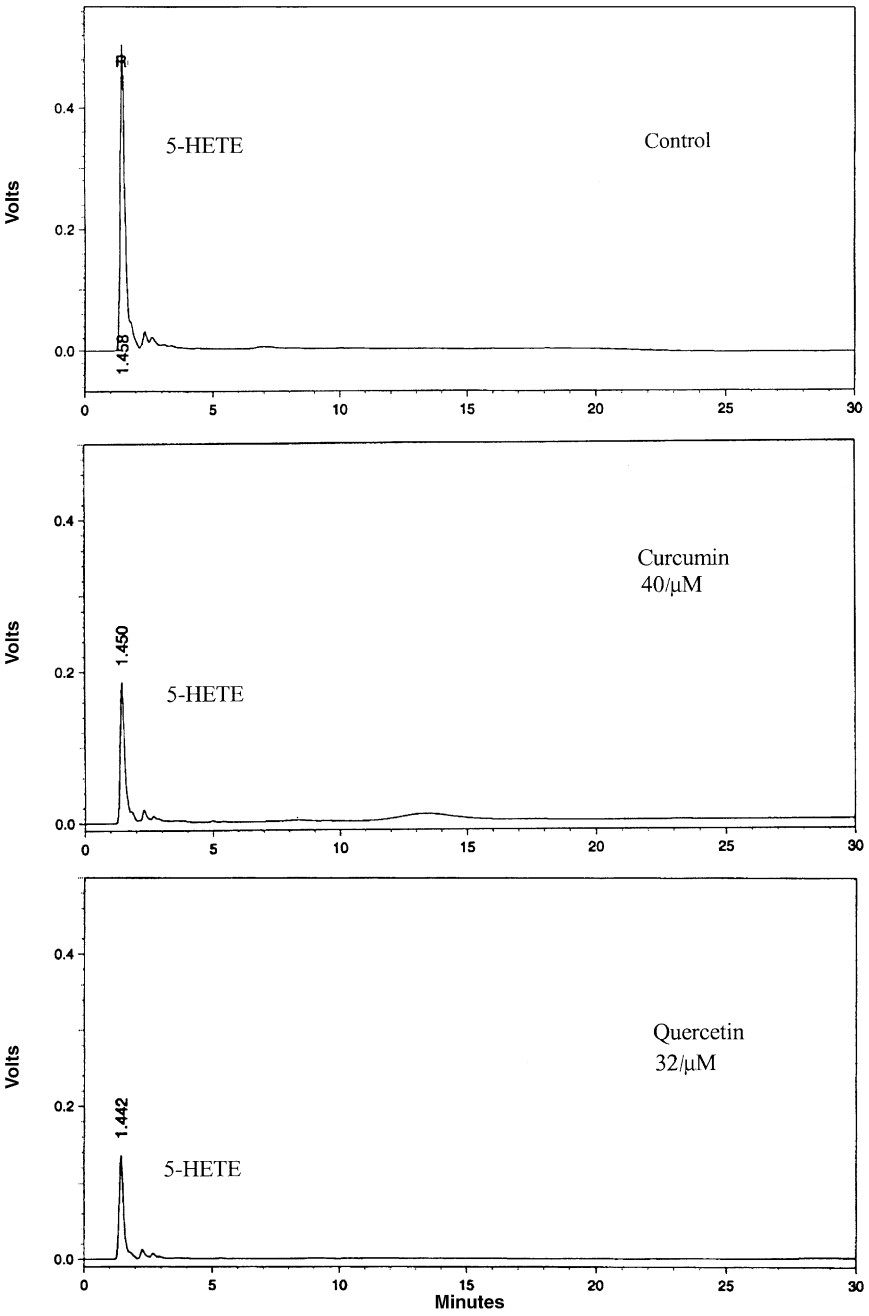

Fig. 7. HPLC chromatograph of HETE resolved on reverse phase HPLC using C18 column. A reduction in 5-HETE content was observed in presence of curcumin and quercetin.

evaluated by comparing the total inhibition obtained by the sum of the expected inhibition of individual compounds. All the combinations of spice extracts/active principles produced percent inhibition close to the expected percent inhibition levels (Table 3).

4. Discussion

Excess production of LTs has been implicated in several inflammatory diseases, there has been considerable interest in the development of 5-LO inhibitors for therapeutic use [13,14]. Although use of many antiinflammatory drugs are in vogue, the continued administration of these over a long period of time can have adverse side effects [15,16]. Therefore, there is a need to explore alternative strategies to lower the formation of inflammatory mediators with the help natural dietary products. Many phenolic/flavonoid compounds found in vegetables/fruits are shown to modulate 5-LO and prostaglandin H synthase pathways of AA [17–20].

In this study, we have examined the inhibitory effect of individual spice aqueous extracts and their spice active principles and also the mixture of spices extracts and active principles on human PMNLs 5-LO activity. All the spice extracts and their active principles significantly inhibited 5-LO activity in a concentration dependent manner. Among the spice active principles tested, quercetin, curcumin and eugenol showed maximal inhibition of 5-LO activity with IC_{50} values of 25, 28 and 30 μM, respectively. The order of inhibitory potency was quercetin > eugenol > curcumin > cinnamaldehyde > piperine > capsaicin > allyl sulfide (Table 1). All the combinations of spice extracts/active principles produced percent inhibition close to the expected percent inhibition levels suggesting the additive or synergistic effect of spices on 5-LO activity.

The inhibitory potency of quercetin and eugenol were found to be similar to that of synthetic 5-LO inhibitors indicating that these spice active principles from garlic and cloves are good inhibitors of 5-LO enzyme.

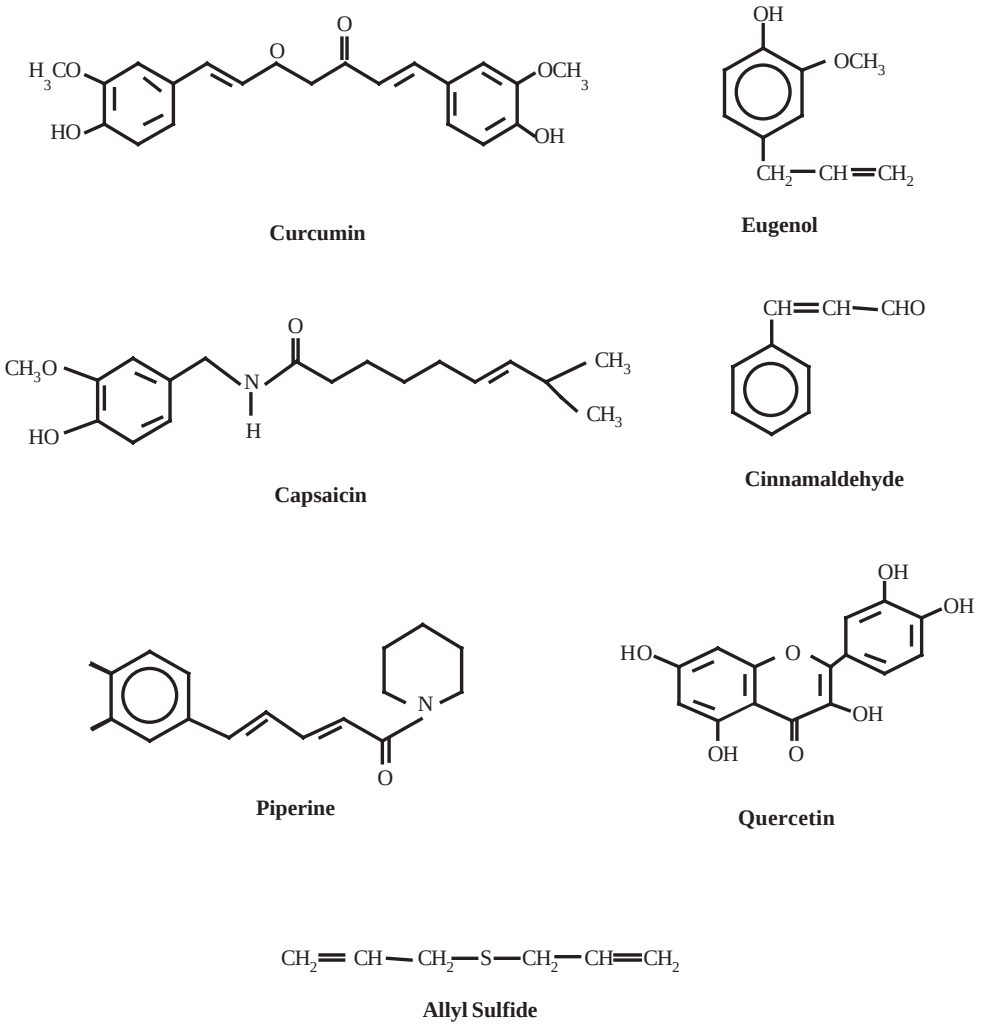

Fig. 8. Chemical structure of spice active principles.

The observed variability in the degree of inhibition of 5-LO by different spice active principles can be attributed to the differences in their structure and functional activity (Fig. 8). Quercetin with penta hydroxy phenolic structure, curcumin and eugenol with a phenolic ring and one or more methoxy groups has found to be nearly equipotent in inhibiting 5-LO activity. Capsaicin with a phenol and a methoxy group and long carbon chain is less potent compared to the above spice active principles. However, the non-phenolic straight chain spice active principle allyl sulfide from garlic has shown to be the least potent inhibitor of 5-LO. It has been suggested that the position and number of hydroxyl groups were most important in determining the inhibitory effect of phenolic compounds on 5-LO [17,20] (Fig. 8).

Many natural and synthetic compounds with redox and non-redox potential are known to be inhibitors of 5-LO [21]. A number of lipoxygenase inhibitors are considered as antioxidants and inhibit lipid peroxidation [22,23]. Inhibitors of lipid peroxidation can be classified as radical scavengers [22,24,25] and also chelators and reducers of ferric ion at the active site [26,27]. Our earlier studies have shown that spice extracts and active principles are inhibitors of hydroxy radical (Fe^{2+}-ascorbate) [1] and lipid hydroperoxy radical mediated lipid peroxidation reactions [2,28]. Quercetin was shown to reduce Fe^{3+} to Fe^{2+} and also chelate Fe^{2+} and render them inactive to participate in enzymatic reactions [29]. Thus, the lipid peroxy radical scavenging effect of curcumin, piperine, capsaicin and or ferric ion reducing and chelation capabilities of quercetin and eugenol could be responsible for the observed inhibitory effect of spice active principles on 5-LO. Further, we have reported earlier that curcumin and capsaicin significantly inhibit secretion of LTs in rat macrophages and reduce carrageenan induced rat paw inflammation, thus clearly indicating the ability of these spice principles to interfere with 5-LO pathway [5,30]. In this study, we confirm and extend the observation that spice aqueous extracts/active principles inhibit human 5-LO and block production of inflammatory mediators [5,30,31].

In conclusion, this study demonstrated that spice condiments used in food preparations contain phenolic/flavonoid compounds that can significantly inhibit (individually or in combination) human PMNLs 5-LO, the key enzyme involved in LT biosynthesis and thus can reduce the production of inflammatory mediators. Further studies are in progress to elucidate the mechanism of inhibitory effect of spice phenolic compounds on 5-LO enzyme.

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