Aqueous Extract of Dried Flower Buds of *Syzygium aromaticum* Inhibits Inflammation and Oxidative Stress

Tasleem Ahmad[1], Tijjani Salihu Shinkafi[2], Indusmitha Routray[2], Amena Mahmood[2] and Shakir Ali[2]*

[1]Regional Research Institute of Unani Medicine, Guzari, Patna 800001, India
[2]Department of Biochemistry, Faculty of Science, Jamia Hamdard, Hamdard Nagar, New Delhi 110062, India

ABSTRACT

This study reports the beneficial effect of aqueous extract of dried flower buds of *Syzygium aromaticum* (clove) in acute and chronic inflammation. Inflammation was induced in rats by injecting carrageenan in hind paw or implanting cotton pellet in the axilla. Administration of the extract (1 g/kg body weight) inhibited the formation of oedema induced by carrageenan and decreased granuloma in cotton pellet granuloma model. The extract, when compared with the disease control, is reported to decrease the elevated levels of succinate dehydrogenase ($p<0.001$), xanthine oxidase ($p<0.05$) and lipid peroxidation, and increase the activity of catalase ($p<0.001$) and glutathione peroxidase ($p<0.01$) in the two animal models. Potential role of xanthine oxidase in inflammation and the ability of the extract to alleviate oxidative stress and inflammation is discussed. The study advocates the use of aqueous extract, rather than the isolated bioactive principle for various reasons.

INTRODUCTION

Dried flower buds of *Syzygium aromaticum* (Family *Myrtaceae*), an evergreen indigenous tree of India, Indonesia, Zanzibar, Mauritius and Sri Lanka, are esteemed as flavoring agent, and have been traditionally used as carminative and antispasmodic [1]. Extract prepared from the bud has been found to inhibit platelet aggregation, alter arachidonic acid metabolism [2], used in *Herpes simplex* infection [3], and has anticarcinogenic potential [4]. Essential oil extracted from the bud is used topically to relieve pain and promote healing [5]. Free eugenol, eugenol acetate, caryophyllene, sesquetrepene ester, phenyl propanoid, and β-caryophyllene are the pharmacologically active compounds isolated from the bud [3,6]. This study reports the anti-inflammatory activity of dried flower bud of *S. aromaticum* in acute and chronic inflammation. Injection of carrageenan in the hind paw of rat and implantation of cotton pellet in its dorsal skin has been used earlier to study acute and chronic inflammation, respectively [7,8]. Reduction in carrageenan-induced paw oedema (CIO) and cotton pellet granuloma (CPG) has significant predictive value for anti-inflammatory agents, and is commonly used to test the anti-inflammatory activity of natural and synthetic compounds.

METHODS

Materials and Preparation of Extract

Pathogen free adult female rats (Wistar strain) weighing 160-170 g were used in the study. Animals were used in accordance to the CPCSEA guidelines for the care and use of laboratory animals, as described earlier [9]. Chemicals and consumables for this study were obtained from standard commercial sources in India. Clove was purchased from local market

*Corresponding Author E-mail: sali@jamiahamdard.ac.in

and the extract was prepared as follows: 10 g crude powder was mixed well in 50 ml of warm distilled water, and the mixture was left overnight in a conical flask. The mixture was extracted again the following day, filtered through muslin cloth, and stored in pre-sterilized glass container at 4°C till further use. The amount of extract obtained from 100 g of crushed plant material (extractive value or percent yield) was calculated and found to be 40%. Extract was used within two days of preparation.

Experimental Protocol

Rats were divided randomly into the following groups: NC (normal control, the group that did not receive any treatment), DC (disease control, injected with carrageenan or implanted the cotton pellet; the groups were called CIO or CPG, respectively), AE (aqueous extract treated, treated with the extract prior to the induction of inflammation, and DF (diclofenac treated, the group treated with diclofenac 1 h prior to the induction of inflammation). Extract was administered orally at a dose level of 1 g/kg body weight. Diclofenac was administered to rats at a dose of 5 mg/kg, orally. In CIO model, carrageenan was injected into rat hind paw to induce acute inflammation [10], and in CPG, cotton pellet was implanted in the axilla of rat instead of carrageenan injection. Cotton pellet implantation leads to the formation of granuloma and inflammatory response.

Evaluation of Anti-inflammatory Activity of Aqueous Extract

The anti-inflammatory activity of the extract was determined *in vivo* in acute and chronic inflammation. In CIO, carrageenan (0.1 ml of 1% carrageenan suspended in normal saline) was injected into rat hind paw one hour after the drug administration, as described by Winter et al [7]. Oedema (swelling) was measured for three consecutive h by plathysmometer (Ugo Basile, Italy). Mean increase in paw volume was measured, and

percent inhibition was calculated as follows: V_c-V_t/V_c × 100; where V_c represents the oedema volume in control group, and V_t, the volume of oedema in treated group. In CPG, accurately weighed (10 ± 1 mg) pellet of sterile cotton, soaked in 0.1 ml distilled water containing 0.1 mg penicillin and 0.1 mg streptomycin, was implanted subcutaneously in the incision made in axilla [8]. Pellet was implanted under ether anesthesia and incision was stitched after implantation. The test drug was administered orally twice a day at 12 h interval for 10 days after implanting the cotton pellet. First dose of the drug was given an hour prior to cotton pellet implantation. Animals were sacrificed 12 h after administering the last dose, which was on day 10. Subsequently, the pellet was dissected out carefully, dried at 60°C and weighed. Difference in the weight of cotton pellet before and after implantation was determined, and % inhibition of granuloma formation in treated rats was calculated as follows: W_P-W_{P-t}/W_P × 100; where, W_P is the weight of the implanted cotton pellet, and W_{P-t}, the weight of pellet in treated group.

Evaluation of Biochemical Changes in Inflammation in Extract Treated Rats

All biochemical procedures were carried out on the hepatic tissue obtained from rats sacrificed four hours after administering carrageenan or 10 days after implanting cotton pellet. Samples were processed as described earlier [9] and kept at 0 to 4°C till final analysis, which was done on the same day. Succinate dehydrogenase, catalase, glutathione peroxidase, and xanthine oxidase were measured by the spectrophotomeric procedure and enzyme activity was calculated as described earlier [11-13,9]. Lipid peroxidation and glutathione were measured according to the method described by Wright et al [14] and Jollow et al [15], respectively. Protein was measured by Lowry's method [16].

Statistical analysis

Statistical analysis was carried out using Graph Pad Prism 4.0, and results were expressed as Mean ± S.E.M. (n = 6). Statistical significance was calculated by Student's *t*-test. The group of animals treated with the extract was compared with the respective disease control groups, either CIO or CPG; *p*<0.05 was considered as significant.

RESULTS AND DISCUSSION

Inflammation is a protective biological response of vascular tissue to harmful stimuli including pathogens, damaged cells, or irritants to remove the injurious stimuli and initiate the healing process. It is also considered as a mechanism of innate immunity. Persistent inflammation, however, may lead to a host of diseases including diabetes, rheumatoid arthritis and even cancer. This study reports inhibition of inflammation by the aqueous extract of clove. The extract could inhibit both acute and chronic inflammation in rat, and modulated a cascade of biochemical reactions that propagate and mature the inflammatory response. We observed inhibition in the formation of oedema and granuloma, and found the extract to decrease elevated levels of several biochemical parameters involved in inflammation and oxidative stress. The increase in average paw volume of rats injected with carrageenan was significantly less in the extract treated group. In the treated rats, paw volume, measured over a period of 3 h, was 84%, when compared to the untreated group. In the untreated rats, paw volume increased to 174% in comparison to normal control. Extract also caused a significant decrease in the weight of cotton pellet implanted in the animal skin. The weight of granuloma at the end of the experiment was 20.5 mg in the treatment group *vs* 33 mg in untreated control rats (Table 1). In both animal models of acute and chronic inflammation, pharmacological response of the extract was comparable to diclofenac, which was used as a standard drug.

One of the objectives of the study was to investigate the effect of extract on the levels of succinate dehydrogenase (SDH), which has been used as a biochemical marker in inflammation [17, 18]. This study reports a considerable increase in SDH activity in untreated rats in both acute and chronic inflammation (Table 2). SDH is a key enzyme linked with the energy yielding citric acid cycle in mitochondria. An increase in SDH would mean increased supply of ATP to the liver and possibly other tissues including the inflamed tissue.

Inflammatory process comprises of the nonphagocytic/phagocytic inflammatory response, inflammation mediators including neutrophil-derived reactive oxygen species or ROS, nitric oxide, prostaglandins, and cytokines. In inflammation, the inflammatory processes induce oxidative/nitrosative stress and lipid peroxidation (LPO), thereby producing excess ROS, reactive nitrogen species (RNS), and DNA-reactive aldehydes, which affect the tissue and organ. Persistent oxidative and/or nitrosative stress and increased LPO induced by inflammatory processes deregulate cell homeostasis. Neutrophils migrate to the site of inflammation and upon activation by a stimulus, generate ROS in abundance [19-21]. Prevention of inflammatory and nociceptive responses by preventing the generation of ROS has been reported earlier in acute and chronic inflammation. In animals, xanthine oxidase (XOD) is one of the en-

Table 1: Effect of clove extract on carrageenan-induced oedema and cotton pellet granuloma.

Group	Oedema				Granuloma	
	1 hr	2 hr	3hr	Change (%)	Pellet weight (mg)	Pellet weight (mg)
DC	0.34	0.50	0.59	0.59/0.34 × 100 (174%)	33.0	-
AE	0.25	0.26	0.21	0.21/0.25 × 100 (84%)	20.5	20.5/33 × 100 (62%)
DF	0.16	0.16	0.11	0.11/0.16 × 100 (69%)	19.0	19.0/33 × 100 (58%)

Oedema was measured in terms of the volume of the fluid displaced, expressed as ml, and the granuloma was measured as the weight of cotton pellet (mg) implanted in rat skin and excised after the completion of the treatment. Percent change is calculated with respect to the control value, which in the case of oedema, was compared with the volume of the fluid displaced initially. In the case of cotton pellet granuloma, the weight of the cotton pellet implanted in the skin of untreated rats was taken as control, and used to calculate the percent change. DC, disease control; AE, the group treated with the aqueous extract; DF, treated with diclofenac.

Table 2: Effect of clove extract on the marker of inflammation and oxidative stress in rats.

Group	SDH		XOD		LPO		GSH	
	CIO	CPG	CIO	CPG	CIO	CPG	CIO	CPG
NC	4.6±0.3[b]	5.60±0.4[b]	172±1[c]	209±18.3	100%	100%	100%	100%
DC	7.8±0.7	12.5±1.1	207±9.5	245±25.9	125%	270%	95%	110%
AE	3.7±0.5[a]	3.90±0.3[b]	170±4.9[b]	202±23.8	65%	150%	106%	140%
DF	4.3±0.4[b]	3.80±0.2[b]	201±11.3[ns]	169±06.7[c]	70%	120%	108%	115%

Values represent Mean ± S.E. (n=6). CIO, carrageenan-induced hind paw oedema; CPG, cotton pellet granuloma; NC, normal control; DC, disease control; AE, the group treated with the aqueous extract; DF, treated with diclofenac; SDH, succinate dehydrogenase; XOD, xanthine oxidase; LPO, lipid peroxidation; GSH, glutathione. The enzyme activities were measured as the respective substrate/product consumed/ formed per minute per mg protein. LPO and GSH were measured as nmol malondialdehyde/g tissue and μmol/g tissue, respectively. Animals treated with the aqueous extract showed no significant change in any of the parameters when compared with the normal control rats. Significant at [a]$p<0.001$, [b]$p<0.01$, and [c]$p<0.05$; [ns]: non-significant when compared to the DC. LPO and GSH are expressed as percent of control. Experiments on CIO and CPG were performed at different time points, which explain slight variations in the control values due to experimental conditions.

zymes involved in the generation of ROS and cause injury [9]. This study reports an increase in XOD activity in inflammation, and found that the extract could attenuate the elevated levels of enzyme in acute and chronic inflammation (Table 2). The finding assumes significance and opens up avenues to further investigate the role of inhibitors of xanthine oxidase in inflammation. Clove extract could almost normalize the enzyme activity. Diclofenac, however, did not decrease the enzyme activity in CIO rat, but reduced it in CPG animals. This study further correlates the level of XOD-derived ROS with lipid peroxidation (LPO), which has been reported to increase in inflammation. LPO indicates changes in membrane fluidity and permeability. This study reports increased LPO in both CIO and CPG, and the level reduced significantly in extract treated rats (Table 2). The extract was also found to normalize total leukocyte count, neutrophil, lymphocyte, and platelet count (data not shown), which are affected in inflammation. Recruitment and activation of leukocytes and neutrophils, which produce ROS upon activation, has been reported by others to contribute to tissue damage in inflammation [19].

Tissue damage related to oxidative stress and consequent LPO can be reversed by the reduced form of glutathione (GSH). In states of oxidative stress, glutathione is depleted leading to further peroxidation of lipids, and a fall in glutathione is usually accompanied by a rise in lipid peroxidation [22]. GSH has pleiotropic roles including the maintenance of cells in a reduced state, serving as an electron donor for certain antioxidative enzymes, for example, glutathione peroxidase, and the formation of conjugates with some harmful endogenous and xenobiotic compounds by glutathione s-transferase. Thus, the effect of the extract on GSH may further benefit its anti-inflammatory action. We observed a slight (nonsignificant) decrease in GSH in CIO, while it increased slightly in CPG. GSH, however, increased considerably in the extract treated rats (Table 2), which could be due to its rapid turnover by glutathione reductase (GR). The activity of GR was found to increase significantly in CPG rats, while it decreased in CIO rats (Table 3). The results correlate with the level of GSH, which showed a slight change in CIO rats. Increase in LPO in CIO group was also considerably less when compared to CPG group (Table 1). Higher level of GR in CPG could be due to increased GSH turnover that can be attributed to increased LPO. Oxidative stress seems

to play a more prominent role in CPG. GSH is particularly important because it neutralizes the effect of peroxides generated in oxidative stress. The process involves glutathione peroxidase (GPx), which converts glutathione to its oxidized form (GSSG). We observed an increase in GPx in both CIO and CPG rats. Catalase, another enzyme involved in peroxide metabolism, also increased in the two animal models, and clove extract attenuated the activity of these enzymes. Diclofenac, the drug used in inflammation, is also reported to increase the activity of catalase and GPx (Table 3). These results suggest the clove extract might exert its effect by ameliorating oxidative stress in inflammation.

A large number of phytochemicals, for example, the phenylpropanoids (carvacrol, thymol, eugenol and cinnamaldehyde) [5,23,24], alkaloids, amino acids, flavonoids, proteins, sterols, reducing sugars, tannins, phenols [25], ascorbic-acid, benzyl-acetate, β-carotene, β-caryophyllene, β-pinene, β-sitosterol-glucoside, carvone, furfural, kaempferol, mucilage, naphthalene, oleanolic acid, stigmasterol, aluminum, zinc, boron, calcium, and manganese [18] have been isolated from clove and several have been reported for various pharmacological actions [26]. Earlier, eugenol was reported to inhibit ROS generated by macrophages [27], inhibit prostaglandin biosynthesis, formation of thromboxane B2, and arachidonic acid-induced platelet aggregation *in vitro* [28]. β-Sitosterol present in clove is also reported for antiulcer, anticancer, anti-diabetic, and T-cell proliferative activity in human [29]. Thus, it is difficult to assign the reported effect of clove extract to any specific ingredient, and various bioactive principles present in the extract are proposed to act synergistically. It is proposed that the extract should be used in its crude form in order to achieve better results. The idea is not to negate the use of single purified compound, but highlight the relevance of using the extract in the form it has been used traditionally. The presence of more than one ingredient in the dose is well documented to potentiate the effect of purified active principle. Further, the presence of antioxidants in the herb is proposed to attenuate the oxidative stress and, thus, inflammation. In addition to this, the use of purified active principle is often limited due to its toxicity and cost, which is not the case when the whole extract is used. Eugenol, which is present in the clove, has been reported to induce cell death (apoptosis), and might not account for all beneficial effects of clove.

Table 3: Effect of clove extract on peroxide and glutathione metabolizing enzymes.

Group	CAT		GPx		GR	
	CIO	CPG	CIO	CPG	CIO	CPG
NC	3019±48[a]	2956±88[a]	527±17 [a]	503±31[b]	381±15[b]	394±26[ns]
DC	1971±11	2098±58	298±27	309±30	272±27	414±24
AE	3169±191[a]	3423±364[b]	423±12[b]	492±39[b]	335±26[b]	485±15[c]
DF	2726±245[c]	2837±199[b]	464±55[c]	394±25[b]	358±15[c]	502±28c

Values represent Mean ± S.E. (n=6). CIO, carrageenan-induced hind paw oedema; CPG, cotton pellet granuloma; NC, normal control; DC, disease control; AE, the group treated with the aqueous extract; DF, treated with diclofenac; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase. The enzyme activities were measured as the respective substrate/product consumed/formed per minute per mg protein. Animals treated with the aqueous extract showed no significant change in any of the parameters when compared with the normal control rats. Significant at [a]$p<0.001$, [b]$p<0.01$, and [c]$p<0.05$; ns: non-significant when compared to the group with induced inflammation. Experiments on CIO and CPG were performed at different time points, which explain slight variations in the control values due to experimental conditions.

CONCLUSION

Clove extract is reported to inhibit acute and chronic inflammation, possibly through a mechanism that ameliorates the oxidative stress possibly by inhibiting xanthine oxidase. The study further reveals significant anti-oxidative effect of clove *in vivo*, and suggests the effect to be the consequence of multiple phytochemicals present in the extract.

ACKNOWLEDGMENTS

REFERENCES

1. Pourgholami MH, Kamalinejad M, Javadi M, et al. Evaluation of the anticonvulsant activity of the essential oil of *Eugenia caryophyllata* in male mice. Journal of Ethnopharmacology. 1999; 64:167.

2. Srivastava KC, Malhotra N. Acetyl eugenol, a component of oil of cloves (*Syzygium aromaticum* L.) inhibits aggregation and alters arachidonic acid metabolism in human blood platelets. Prostaglandins, Leukotrienes and Essential Fatty Acids. 1991; 42:73.

3. Kurkawa M, Hozumi T, Basnet P, et al. Purification and characterization of eugeniin as an anti-herpesvirus compound from *Geum japonicum* and *Syzygium aromaticum*. The Journal of Pharmacology and Experimental Therapeutics. 1998; 284(2):728-35.

4. Zheng GQ, Kenney PM. Sesqueterpenes from clove (*Eugenia caryophyllata*) as potential anticarcinogenic agents. Journal of Natural Product.1992; 55:999.

5. Chaieb K, Hajlaoui H, Zmantar T, et al. The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzygium aromaticum* L. Myrtaceae). Phytotherapy Research. 2007; 21(6):501-6.

6. Miyazawa M, Hisama M. Antimutagenic activity of phenylpropanoids from clove (*Syzygium aromaticum*). Journal of Agric. and Food Chemistry. 2003; 51(22):6413-22.

7. Winter CA, Ristey EA, Nuss GW. Carrageenin-induced edema in hind paws of the rat as an assay for anti-inflammatory drugs. Proceedings of the Society for Experimental Biology and Medicine. 1962; 111:544-7.

8. Vetrichelvan T, Segadeesan M. Effect of alcoholic extract of *Achyranthes bidentata* Blume on acute and subacute inflammation. Indian Journal of Pharmacology. 2002; 34:115.

9. Ali S, Diwakar G, Pawa S, et al. Xanthine oxidase-derived reactive oxygen metabolites contribute to liver necrosis: protection by 4-hydroxypyrazolo[3,4-d] pyrimidine. Biochimica et Biophysica Acta. 2001; 1536(1):21-30.

10. Crunkhon P, Meacock SE. Mediators of the inflammation induced in the rat paw by carrageenan. British Journal of Clinical Pharmacology. 1971; 42:392-402.

11. Kun E, Abood LG. Colorimetric estimation of succinic dehydrogenase by triphenyltetrazolium chloride. Science. 1949; 109:144-6.

12. Claiborne A. Catalase Activity, in CRC Handbook of Methods for Oxygen Radical Research, eds. R.A. Greenwald, Boca Raton, FL: CRC Press; 1985; pp. 283.

13. Mohandas J, Marshall JJ, Duggin GG, et al. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney possible implications in analgesic nephropathy. Biochemical Pharmacology. 1984; 33:1801-1807.

14. Wright JR, Colby HD, Miles PR. Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. Archives of Biochemistry and Biophysics. 1981; 206:296-304.

15. Jollow DJ, Mitchell JR, Zampaglione N, et al. Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology. 1974; 11:151-169.

16. Lowry OH, Rosenbrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry. 1951; 193(1):265-75.

17. Naik SR, Kalyanpur SN, Sheth UK. Effects of anti-inflammatory drugs on glutathione levels and liver succinic dehydrogenase activity in carrageenin edema and cotton pellet granuloma in rats. Biochemical Pharmacology. 1972 Feb; 21:511.

18. Duke JA. Handbook of Phytochemical Constituents of GRAS Herbs and Other Economic Plants, Boca Raton, FL: CRC Press; 1992.

19. Cuzzocrea S, Costantino G, Mazzon E, et al. Protective effects of Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), a superoxide dismutase mimetic, in paw oedema induced by carrageenan in the rat. Biochemical Pharmacology 1999 July; 58(1):171-6.

20. Salvemini D, Wang ZQ, Bourdon BM, et al. Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema. European Journal of Pharmacology. 1996; 303(3):217-20.

21. Ialenti A, Ianaro A, Moncada S, et al. Modulation of acute inflammation by endogenous nitric oxide. European Journal of *Pharmacology*. 1995; 211:177.

22. Pandey S, Sharma M, Chaturvedi P, et al. Protective effect of Rubia cordifolia on lipid peroxide formation in isolated rat liver homogenate. Indian Journal of Experimental Biology. 1994; 32(3):180-3.

23. Criddle DN, Madeira SV, Soares-de-Moura R. Endothelium-dependent and-independent vasodilator effects of eugenol in the rat mesenteric vascular bed. The Journal of Pharmacy and Pharmacology. 2003; 359:55-65.

24. Kumarvelu P, Subramanyam S, Dakshinmurthy DP, et al. The antioxidant effect of eugenol on CCl4-induced erythrocyte damage in rats. The Journal of Nutritional Biochemistry. 1996; 7:23-8.

25. Jenkins GL, Knevel AM, Digangi FE. Quantitative Pharmaceutical Chemistry. New York: Mcgraw Hill Book Company; 1967:455-462.

26. Lee KG, Shibamoto T. Antioxidant property of aroma extract isolated from clove buds [*Syzygium aromaticum* (L.) Merr. Et Perry]. Food Chemistry. 2001; 74:443-448.

27. Joe B, Lokesh BR. Role of capsaicin, curcumin and dietary n-3 fatty acids in lowering the generation of reactive oxygen species in rat peritoneal macrophages. Biochimica et Biophysica Acta . 1994; 1224:255-63.

28. Rasheed A, Laekeman G, Totte J, et al. Eugenol and prostaglandin biosynthesis .The New England Journal of Medicine. 1984; 310(1):50-51.

29. Pegel KH. The importance of sitosterol and sitosterolin in human and animal nutrition. South African Journal of Science. 1997; 93:263-268.