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The effect of *Syzygium aromaticum* (clove) on inflammatory markers (total leukocyte count, differential leukocyte count and tumor necrosis factor-alpha)

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Abstract

Background: Inflammation is involved in pathogenesis of many diseases. Anti-inflammatory chemicals can be used to treat such illness especially if they are derived from plant sources as they will have fewer side effects. To find out the outcome of ethanolic extract of *Syzygium aromaticum* flower buds on markers of inflammation in albino rats.

Methods: Anti-inflammatory activity was investigated in albino rats using an experimental model of inflammation, the "formalin test" (injecting 5% formalin into subplantar surface of right hind paw of the rat). Ninety rats were uniformly divided into three groups designated as control A, experimental B and reference C. Intraperitoneal injections of normal saline, *Syzygium aromaticum* flower bud extract and indomethacin were used. In these rats, foot edema was measured by Vernier caliper. Blood sampling was done through cardiac puncture under anesthesia to determine total and differential leukocyte counts and serum tumor necrosis factor-alpha (TNF-α) levels.

Results: The clove extract produced significant ($p=0.00$) decrease in inflammatory response initiated by 5% formalin. The extract significantly decreased ($p=0.009$) serum TNF-α. However, its effect on the total and differential leukocyte counts was non-significant ($p>0.05$).

Conclusion: The ethanolic extract of *Syzygium aromaticum* possess powerful anti-inflammatory effects. Anti-inflammatory effect of this herb is mainly through reduction in inflammatory cytokines level. By accepting effective herbaceous therapy, we can bring forth a revolutionary solution in management of inflammation with fewer side effects.

Introduction

Inflammation is a quick protective reaction of body tissues to injurious stimuli. Inflammatory process involves many chemicals like TNF alpha and raised leukocytes [1,2]. Out of leukocytes, neutrophils are the main cells which increase in number within few hours of acute inflammation [3,4]. Tumor necrosis factor-alpha (TNF-α) has been involved in the development of a wide spectrum of diseases [5]. It also increases pain receptors sensitivity by increasing tetrodotoxin (TTX)-resistant sodium current and substance P secretion [6]. This cytokine is secreted by the cells at the site of inflammation and in turn mediates inflammatory process through white blood cells chemo-attraction, activation of phagocytes, and production of interleukins and prostaglandins. It activates many other cytokines in inflammatory response. TNF-α acts via two receptors. Binding of tumor necrosis factor-alpha (TNF- α) to the type I TNF receptor (TNFR1) and activates signaling pathways that regulate inflammation [7,8]. When inflammation proceed unbridled, the affected cells start destroying nearby healthy cells leading to joint pain, cardiac diseases and even malignancies.

For years, researchers have focused on development of anti-inflammatory drug which have analgesic and antipyretic properties without any detrimental effect on surrounding normal cells. In this regard, many plants have been used for their medicinal properties. *Syzygium aromaticum* customarily known as clove is used usually as condiment. It has sharp pungent taste and phenolic smell [9]. It is known for its analgesic and anti-inflammatory properties evinced by its usage in rheumatic joints and swollen gums. It is also known to kills bacteria, fungi and viruses and is valued as herbal remedy.

Pharmacological anti-inflammatory drugs are creating financial oppression on mankind and have detrimental effects on stomach and kidney. The objective of this study was to fine out a reasonable, accessible and efficacious anti-inflammatory alternate with lesser complications and side effects.

Methods

Whole population of adult male albino rats each weighing 200-250 gram was randomly split up into 3 groups. Every group had 30 rats. Three groups were made to compare anti-inflammatory effects of clove with drug. Normal saline was used as control. Three-kilogram clove obtained from retail outlet Lahore and later, acknowledged by Dr. Zaheer ud din Khan, Professor and Head, Department of Botany, Government College University, Lahore. Ethanolic extract of *Syzygium aromaticum* was manufactured by utilizing the equipment at PCSIR Lab, Lahore.

Clove were put under shadow to get them dry and then grated with the help of electric grinder. The medicinal active components (eugenol, beta-caryophyllene) of clove were withdrawn with 99.9% ethanol for seventy-two hours in Soxhlet extractor. The attained extract was sifted, and the liquid (ethanol) vaporized with a rotary evaporator. After vaporization, we got a russet colored viscous, thick liquid called ethanolic extract. This was stored at 4˚C before use. The extract was reconstituted in sterilized distilled water at appropriate concentration for further use.

Group A: A shot of normal saline solution was administered in a dose of 10 ml/Kg of body weight, into peritoneal cavity of group A. Ethanolic extract of *Syzygium aromaticum* flower buds was injected into peritoneal cavity in a dose of 200 mg/Kg of body of Group B [10]. Indomethacin injection, in a dose of 3 mg/Kg of body weight, was given to Group C into peritoneal cavity [11].

"Formalin test" was used for the verification of anti-inflammatory effect of *Syzygium aromaticum* and indomethacin. This test is globally trusted for investigational studies of inflammation. To induce inflammation, diluted formalin shot was given into bottom of right hind paw of all rats using insulin syringe. Swelling of the feet was measured promptly before (0 hour) and at 1, 3, 10 and 25 hours after formalin shot with Vernier caliper [8,12]. Percent decrease in inflammation was estimated with the help of mathematical formula [10]. Blood sampling: 24 hours later, each rat was anesthetized using ether and 3 ml blood was drawn through cardiac puncture [12]. White blood cell counts (WBC counts) estimation was done by mixing one ml blood with anti-coagulant. Total and differential leukocyte counts were done by automated sysmex hematology analyzer in pathology Laboratory Services institute of medical sciences, Lahore. For determination of serum TNF-alpha, two mL blood of each sample was allowed to clot, serum was obtained through centrifugation at five thousand rpm for fifteen minutes and was preserved. Later estimation of serum TNF alpha was done by ELISA (Ref. No. K0331196P) [13].

Results

The percentage reduction of the inflammation (edema) caused by the *Syzygium aromaticum* (group B) and indomethacin (group C) at different time intervals is shown in Table 1. At 1 hour, it was 43.04% and 11.39%, at 3 hours, 36.27% and 15.69%, at 10 hour, 55.77% and 42.31% and at 25 hour, 64.52% and 64.52% respectively. Reduction of the inflammation (edema) caused by the *Syzygium aromaticum* was more than that of

indomethacin. However, at 25 hours both caused equal percent reduction in paw edema.

Percentage reduction of edema at different time intervals (%)	Time interval (hour)	B (*Syzygium aromaticum*)	C (Indomethacin)
	1 hour	43.04	11.39
	3 hours	36.27	15.69
	10 hours	55.77	42.31
	25 hours	64.52	64.52

Table 1: The comparison of percentage reduction of the inflammation (edema) in group B and C by ANOVA.

Total leukocyte count of experimental group was lesser than control and statistical analysis (ANOVA followed by Post hoc Tukey's test) revealed that it was not significantly different ($p>0.05$) $p=0.148$ as compared to control. While total WBC count of reference group was significantly different from the TLC of the control group ($p<0.05$) $p=0.005$.

Mean ± SEM TLC (cells/mm^3)	A (Control)	B (*Syzygium aromaticum*)	C (Indomethacin)
	6386.0±434.94	5510.69±184.61	4867.50±313.52

Table 2: Comparison of total WBC count (TLC) in groups Control, Experimental and Reference by Analysis of variance followed by Post hoc Tukey's test.

Regarding differential leukocyte counts (DLC), the percentages of the neutrophils of group B although less but was not markedly different from that of the control group ($p>0.05$) $p=0.864$ while indomethacin treated group showed raised number of neutrophils ($p<0.05$) $p=0.000$. The percentage of the neutrophils of group C was markedly raised from the respective cells of the group B ($p<0.05$).

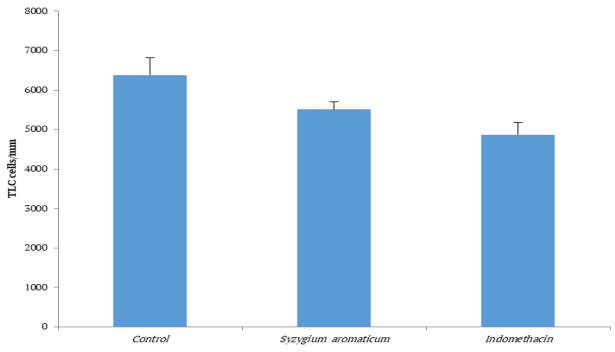

Figure 1: Total leukocyte counts (TLC) of the three groups.

Parameter Mean± SEM	Differential leukocyte count	A (Control) n=30	B (*Syzygium aromaticum*) n=30	C (Indomethacin) n=30
White Blood cells (%)	Neutrophils	16.87±1.82	15.88±0.69	25.63±1.38
	Lymphocytes	81.87±1.82	82.44±0.81	77.63±1.53
	Eosinophils	0.80±0.11	0.81±0.10	0.81±0.10
	Monocytes	0.20±0.11	0.19±0.10	0.19±0.10
	Basophils	0.19±0.10	0.69±0.12	0.69±0.12

Table 3: Analysis of differential WBC counts of rats by ANOVA followed by Post hoc test.

In Table 3, statistical analysis revealed significant decrease level of the TNF-α levels of group B and group C as compared to group A ($p<0.05$). Post hoc LSD test showed that the TNF-α level of the *Syzygium aromaticum* group was not significantly higher than that of the indomethacin group ($p=0.123$).

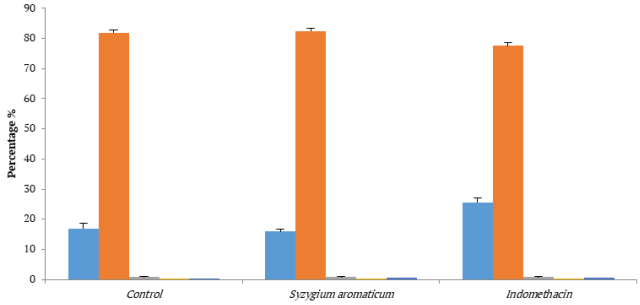

Figure 2: Differential leukocyte counts (neutrophils, eosinophils, basophils, lymphocytes, monocytes) of the three groups.

Mean ± SEM Serum Tumor necrosis factor alpha (pg/ml)	A (Control) (n=30)	B (*Syzygium aromaticum*) (n=30)	C (Indomethacin) (n=30)
	62.11±3.14	46.75±4.15**	37.07±3.84*

Table 4: Comparison of serum tumor necrosis factor alpha (TNF-α) levels among three groups of rats by ANOVA.
* $p=0.000$ as compared to control (highly significant)
** $p=0.064$ as compared to control (non-significant)

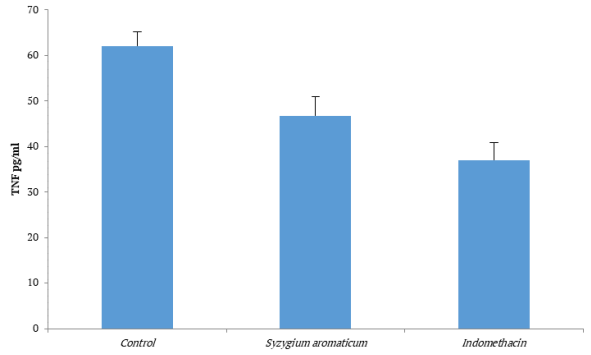

Figure 3: Mean ± SEM serum tumor necrosis factor alpha (TNF-α) levels of the three groups.

Discussion

The use of herbal remedies in health care system is well established. Medicinal plants are gaining popularity and acceptance due to their natural products with fewer side effects. Several reports have shown the antioxidant and antimicrobial properties of clove leading to inhibition of different degenerative diseases [14].

In this study, it was observed that total white blood cell count (TLC) of all groups (control, *Syzygium aromaticum*, indomethacin) were within the normal range (2000-11000 cells/mm3) and were also not markedly contrasting with one another. Raised neutrophil count was seen in all groups. The neutrophil count was least in *Syzygium aromaticum* treated group, yet it was not markedly dissimilar from that of the control ($p=0.864$). Very high neutrophil count was seen in group C in

comparison to group A and B (p=0.000). This shows that the acute anti-inflammatory activity of clove does not involve significant reduction of the leukocytes, but other mechanisms like decrease inflammatory cytokines [15].

Agbaje et al, examined aqueous extract of *Syzygium aromaticum* to see its effects on blood parameters in rats [16]. On oral administration of the extract, the total leukocyte count was decreased; while red blood cell count and indices improved markedly. It is noteworthy that in our study, the blood sample was taken just after 24 hours of inducing inflammation and administration of the ethanolic extract of *Syzygium aromaticum* while in the above-mentioned study, sampling was done after ninety days [12].

The eugenol (active component of the extract) is reported to decrease tumor necrosis factor-alpha, prostaglandin and leukotrienes in human macrophages in vitro [17]. Caryophyllene which is also an important constituent of clove extract, inhibits the nuclear factor-κB activation and neutrophil migration in rat paw edema. The nuclear factor-kappa B (NF-κB) has come to light as major mediator of inflammation. Therefore, chemicals that cause inhibition of NF-κB can be used in treatment of inflammatory disorders [18,19].

The analysis of serum tumor necrosis factor alpha levels of all groups revealed that TNF-α level of *Syzygium aromaticum* group was less than that of the control group although this was not significantly different (p=0.064) and was very decreased in indomethacin group (p=0.000). It seems that inhibition of TNF-α leads to reduction of pain [20]. Many reports have shown that analgesic effect of clove oil is CNS dependent, and its higher doses have sedative effect as well [21].

The present study has a few strengths and limitations. The main strength of the study is that it gives clear evidence on some pharmacological properties of clove extract while working on animal model. The limitation of the study is use of clove extract by intraperitoneal route as this route in not commonly used. This can limit extrapolation of our results. We also suggest in the light of our results that clove extract has very strong anti-inflammatory and analgesic properties and its therapeutic use in treatment of many conditions should be encouraged.

Ethanolic extract of *Syzygium aromaticum* flower buds have powerful anti-inflammatory activity as it reduces edema in rats. It also cause marked reduction in serum tumor necrosis factor alpha (a bio marker of inflammation). Based on the findings of our study we recommed that further studies and Clinical Trials should be conducted on the extract of *Syzygium aromaticum* flower buds. As it can be advantageous for the treatment of inflammatory disorders and can be beneficial for saving ICU patients and preventing the spread of resistant isolates in critical wards.

Competing Interests

The author declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contribution

Dr. Saima Tabassum: Original Research Idea, study design, Data Collection, writing
Dr. Ambreen Anjum: Literature Review, data collection
Dr. Sobia Manzoor: Data Collection, Literature Review
Dr. Wardah Toseef: Data collection, interpretation of results
Dr. Muhammad Hashim Ghouri PT: Data collection, Data analysis, final drafting and writing

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