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Evaluation of the effect of *Myrica sapida* on bronchoconstriction and bronchial hyperreactivity

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The present investigation was undertaken to evaluate the bronchodilator and bronchial hyperreactivity of the stem bark of *Myrica sapida*. Experimental models studied were histamine induced bronchospasm in guinea pigs, bronchoalveolar lavage fluid (BALF) in egg albumin sensitized guinea pigs, histamine release from the lung tissues of sensitized guinea pigs and histopathological studies. Ethanolic extract of *M. sapida* (75 mg/kg, p.o., for 7 days) showed significant protection against histamine aerosol induced bronchospasm. Significant decrease in the total and differential leukocyte counts in BALF and prevention of egg albumin induced histamine release from chopped lung tissues of sensitized guinea pigs was observed on chronic administration of ethanolic extract of *M. sapida* (75 mg/kg, p.o., for 15 days). Histological examination of the section of lung from sensitized guinea pigs treated with ethanolic extract of *M. sapida* (75 mg/kg, p.o., for 15 days) was comparable to that of the control group. These results suggest that *M. sapida* possesses not only bronchodilator activity but also decreases bronchial hyperresponsiveness by decreasing the infiltration of inflammatory mediators like eosinophils, neutrophils in BALF and inhibiting histamine release from lungs of sensitized guinea pigs.

1. Introduction

Bronchial asthma is characterized by both bronchoconstriction and airway inflammation which results in bronchial hyperresponsiveness to various stimuli (Boushey 1980). It is known that asthma can be triggered by various factors; allergens, drugs, respiratory infection, dust, cold air, exercise, emotions, occupational stimuli, chemicals, histamine etc. (DiPiro 2005). Airways inflammation has been demonstrated in all forms of asthma, and an association between the extent of inflammation and the clinical severity of asthma has been demonstrated in selected studies. Although the role of inflammation is still evolving as a concept, intensive research during the last several decades provides a much firmer scientific basis to indicate that asthma results from complex interactions among inflammatory cells, mediators, and the cells and tissues resident in the airways. Thus, asthma is now defined as a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli (NAEPP expert panel report).
The available treatment options have major limitations owing to low efficacy, associated adverse events and compliance issues. Ayurveda, an ancient system of Indian medicine, has recommended several drugs from indigenous plant sources for the treatment of bronchial asthma and allergic disorders. *M. sapida* (commonly known as Kaiphal, family Myricacea) is a small to moderate-sized tree varying from 3 to 15 m from place to place in sub-tropical Himalayas from the Ravi river eastward to Khasi, Jaintia, Naga and Lushai hills of India at altitudes of 900 m to 2100 m. The plant is known to possess various medicinal properties. The bark of this medicinal plant has been used in the treatment of asthma, fever, dyspnoea, throat and lung affections, chronic bronchitis, typhoid, dysentery, diuresis, cough, stomatitis, chronic gonorrhea, headache, piles, gleet, liver complaints, sores, uterine stimulant, excessive burning sensation, appetizer, toothache, ulcers (Kirtikar and Basu 1999). The oil from the bark is used in earache. The oil obtained from the flowers is tonic and useful in earache, diarrhoea, inflammation and paralysis. It is also used as bitter, astringent, carminative and tonic (Nadkarni's 2002). In the present study various *in vivo* experimental methods were studied for evaluating the effect of *M. sapida* on bronchoconstriction and bronchial hyperreactivity.

2. Investigations and results

2.1 Effect on histamine induced bronchospasm in guinea pigs

Acute study: Ethanolic extract (75 mg/kg, p.o.) and methanolic extract (120 mg/kg, p.o.) of *M. sapida* significantly prolonged the latent period of PCD ($p < 0.01$) as compared to the control, following exposure to a histamine aerosol (Table 1). The activity of the ethanolic extract was

Table 1: Acute study for effect of ethanolic and methanolic extract of *M. sapida* on histamine aerosol induced bronchospasm in guinea pigs

Group	Preconvulsion time (min)		Percent protection	
	Ethanolic extract (75 mg/kg, p.o.)	Methanolic extract (120 mg/kg, p.o.)	Ethanolic extract (75 mg/kg, p.o.)	Methanolic extract (120 mg/kg, p.o.)
Before treatment	2.83 ± 0.4736	2.34 ± 0.4197	–	–
After treatment	$7.65 \pm 1.202^{*}$	$5.88 \pm 1.077^{*}$	62.51%	60.20%

Values are mean ± SEM; n = 6 in each group
Significantly different from control group * $p < 0.01$

Table 2: Chronic study for effect of ethanolic extract of *M. sapida* on histamine aerosol induced bronchospasm in guinea pigs

Group	Preconvulsion time (min)	Percent protection
Before treatment	2.6 ± 0.3372	–
After treatment (Ethanolic extract, 75 mg/kg, for 15 days, p.o.)	$13.36 \pm 2.094^{**}$	80.54%

Values are mean ± SEM; n = 6 in each group
Significantly different from control group ** $p < 0.005$

found to be better than that of the methanolic extract; therefore the ethanolic extract was used for further studies.
Chronic study: Ethanolic extract of *M. sapida* (75 mg/kg, p.o., for 15 days) significantly prolonged the latent period of PCD ($p < 0.005$) following exposure to histamine aerosol (Table 2). It was found that the activity of the ethanolic extract in chronic treatment was significantly greater than in acute treatment.

2.2 Evaluation of bronchial hyperreactivity

All guinea pigs used in this study exhibited a marked reaction when challenged with antigen characterized by acute dyspnoea.
Effect on bronchoalveolar cell counts: There was a highly significant increase in the total and differential leucocyte count in the sensitized group as compared with control group. Ethanolic extract of *M. sapida* (75 mg/kg, p.o., for 15 days) significantly prevented the rise in total leucocyte count, neutrophil, eosinophil count but produced a non-significant decrease in monocyte and lymphocyte count as compared to sensitized group (Table 3).
Effect on histamine release from lung tissue of sensitized guinea pigs: Antigen challenge in sensitized guinea pigs produced a highly significant ($p < 0.001$) increase in the histamine release from the lungs of sensitized group as compared to control group. Ethanolic extract of *M. sapida* (75 mg/kg, p.o., for 15 days) significantly inhibited the rise in histamine release ($p < 0.001$) as compared to the sensitized group (Table 4).
Lung histology: Histopathological examination of control animals revealed no significant abnormalities (Fig. A). There was a low grade eosinophilia in some sections, but this was not associated with pathological changes. In sensitized animals, there was perivascular and peribronchiolar eosinophilia after challenge. This suggests a net movement of these cells from the vascular to the bronchiolar compartment. The presence of eosinophils in the bronchiolar area showed anatomical correlation with tissue oedema, epithelial cell hypertrophy and damage and airway lumen plugging by mucus and cells, and focal loss of integrity of the smooth muscle layer. The lumen size was reduced drastically (Fig. B). It is evident that treatment with *M. sapida* prevented the tissue oedema, epithelial cell hypertrophy and damage and airway lumen plugging thereby decreasing inflammation and bronchoconstriction which may lead to normal lumen size and normal lung function (Fig. C).

3. Discussion

Histamine induced bronchospasm of asthma is characterized by allergen induced immediate airway constriction and late airway reactivity to pharmacological vasoconstrictors such as histamine and leukotrienes. Histamine is a central mediator in the pathogenesis of allergic and inflammatory disorders (Gopumadhavan et al. 2005). In the present study, ethanolic and methanolic extract of *M. sapida* prolonged the time of preconvulsive dyspnoea (PCD) in guinea pigs following histamine aerosol induced bronchospasm. Therefore it may be concluded that protective effect of ethanolic and methanolic extract of *M. sapida* against histamine induced bronchospasm may be due to its anti-histaminic activity. Moreover the bronchodilating activity following treatment with *M. sapida* was found to be greater for ethanolic extract compared to methanolic extract. In the present study, it appears that *M. sapida* antagonized the histamine induced bronchospasm more in chronic treatment compared to acute treatment.

Table 3: Effect of ethanolic extract of *M. sapida* on BALF in egg albumin sensitized guinea pigs

	Control	Sensitized	Sensitized + *M. sapida*
Total leucocyte count/cmm	5500 ± 330.7	$14500 \pm 483^{*}$	$5866.7 \pm 721.4^{\#}$
Eosinophil count/cmm	148.2 ± 16.29	$375 \pm 32.15^{**}$	$158.2 \pm 36.55^{\#\#}$
Monocyte count/cmm	95.8 ± 17.14	$161 \pm 22.58^{***}$	125.7 ± 20.33
Neutrophil count/cmm	3416.7 ± 366.8	$7073 \pm 464.7^{*}$	$3489 \pm 671.4^{\#}$
Lymphocyte count/cmm	1746 ± 39.9	$2030.8 \pm 56.3^{**}$	2104.5 ± 276

Values are mean ± SEM, n = 6 in each group
Significantly different from control group * $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$
Significantly different from sensitized group $^{\#}$ $p < 0.01$, $^{\#\#}$ $p < 0.05$

Table 4: Effect of *M. sapida* on change in histamine levels of lung due to egg albumin induced histamine release in guinea pigs

Treatment	Histamine level in lungs (ng/g)
Control	295 ± 1.071
Sensitized (SEN)	412.5 ± 5.663*
SEN + *M. sapida*	212.5 ± 2.484#

Values are mean ± SEM, n = 6 in each group
Significantly different from control group *$p < 0.001$
Significantly different from sensitized group #$p < 0.001$

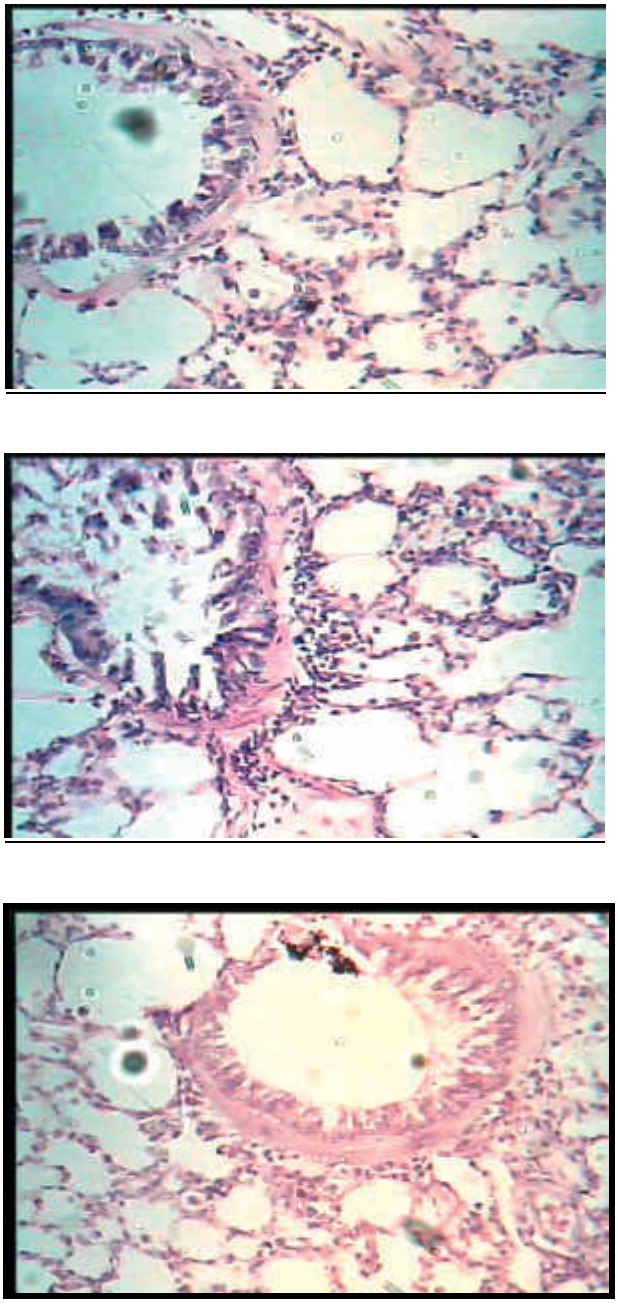

Fig.: Light micrographs of bronchiolar tissue from guinea pigs of the different experimental groups. A – Control group, B – Sensitized group and C – Sensitized + treated group

The concomitant presence of infiltrating eosinophils and T lymphocytes in the bronchi of asthmatics plays a major role in the development of airway inflammation and the accompanying bronchial hyperreactivity (Azzawi et al. 1990; Bentley et al. 1993; Jeffery et al. 1989; Corrigan and Kay 1992) may result from their common pathway of adherence to endothelial cells. Indeed, eosinophils bind to activated endothelial cells by adhering to VCAM-1 (Weller et al. 1991), a ligand also involved in lymphocyte adherence to the endothelium (Carlos et al. 1990). Furthermore, adhesion of eosinophils and lymphocytes to VCAM-1 is mediated by the binding of VLA-4 present on their surface (Weller et al. 1991; Hemler 1990) which underlines the selectivity of the mechanisms involved in their migration into inflamed tissues. Upon reaching the airways, eosinophils can release a variety of cationic proteins, such as EPO (eosinophil peroxidase) and MBP (major basic protein) that are responsible for injury and shedding of airway epithelium (Motojima et al. 1989; Gleich et al. 1979; Frigas et al. 1980). Disruption of the epithelium leads to the exposure of the underlying mucosal structures and sensory nerve endings to allergen and irritants, contributing to the developing of nonspecific bronchial hyperreactivity (Underwood et al. 1995). Moreover, it is also well-established that there are increased eosinophil numbers in the airways of patients with ongoing asthma, even those with mild disease (Beasley et al. 1989), whilst autopsy and biopsy studies have revealed a characteristic eosinophil infiltration of the airway mucosa (Djukanovic et al. 1990). In the present study, sensitization using egg albumin (1 ml, 10% w/v, i.p.) and then second exposure to same antigen i.e. egg albumin (0.5 ml, 2% w/v) through saphenous vein causes acute anaphylactic shock resembling the acute asthmatic attack resulting in the release of various mediators and cellular infiltration. Antigen challenge resulted in an approximately more than twofold increase in the number of eosinophils in BALF. This was accompanied by intense eosinophil infiltration in the bronchopulmonary tissue, eosinophil migration, accumulation and degranulation in the guinea pig lung as evident from histopathology which is in consistence with reports in the human asthmatic lung. The present findings show that in antigen challenged animals, treatment with *M. sapida* significantly inhibited antigen-induced bronchial hyperreactivity by preventing the increase in total leucocyte counts (Diaz et al. 1989). Airway hyperresponsiveness after antigen challenge is supported by the inflammatory pathology suggesting the involvement of other mediators in pathogenesis of asthma. As in human asthma, leukotrienes are also implicated in the induction of antigen-induced airway hyperresponsiveness in the guinea-pig. Neutrophil numbers have also been reported to increase in bronchial lavage fluid (Metzger et al. 1986, 1987) in asthmatics, but neutrophilia is generally of shorter duration than eosinophilia (Diaz et al. 1989; Metzger et al. 1987; Smith 1992). This finding fits well with the current observation that pretreatment of the sensitized animals with *M. sapida* resulted in a significant inhibition of antigen-induced bronchial hyperreactivity by decreasing neutrophil counts. The predominant cells in BALF recovered from unchallenged guinea-pigs were those of the monocyte. The numbers of these cells were increased after challenge (Tarayre et al. 1990). In contrast, other workers have not found an increased monocyte influx (Hutson et al. 1988; Walls et al. 1991; Coyle et al. 1988). Thus, unlike eosinophils and neutrophils, monocyte recruitment is not a consistent feature of the airway inflammatory response following antigen challenge in this species. In this context, it is worth noting that, treatment of the sensitized animals with *M. sapida* produced a nonsignificant decrease in monocyte count. Although an influx of lymphocytes does not appear to be a consistent feature of airway inflamma-

tion in this model, it remains possible that antigen specific T-lymphocytes resident in the airway wall become activated as a result of antigen challenge. Thus, T-cells may, by secreting interleukin-4 (IL-4), IL-5 and other proinflammatory cytokines, contribute to the orchestration of the ensuing allergic reaction. In line with this notion, the present findings show that treatment of the sensitized animals with *M. sapida* produced a nonsignificant decrease in lymphocyte count as compared to sensitized animals. In conclusion, the results of this study demonstrate that, in guinea-pig airways, antigen challenge induced eosinophil infiltration and activation is similar to that reported in human asthmatics. Changes in the numbers of other cell types (monocytes and lymphocytes) did not correlate directly with structural changes. We conclude that eosinophil products may be the major mediators of damage to the airway mucosa and enhancement of airway hyperresponsiveness in antigen challenged guinea-pigs. This indicates the protective effect of *M. sapida* by preventing release of several pre-formed mediators, thereby preventing the direct damage of airway epithelium.

Histamine is released from guinea pig lungs during anaphylaxis by an allergic or nonallergic insult resulting in bronchoconstriction which can be attenuated by H_1 receptor antagonists (Bartosch et al. 1932; Holgate et al. 1989). Histamine also stimulates sensitized afferent nerves and also activates eosinophils (Empey et al. 1976; Raible et al. 1994). The histamine levels which were increased due to sensitization with egg albumin were found to be significantly reduced in the *M. sapida* treated animals. This depicts that *M. sapida* inhibits the release of inflammatory mediator-histamine.

The understanding of various processes involved in bronchial asthma such as inflammatory response can explain various histopathological alterations observed. In asthma, there occurs chronic inflammation, which then leads to bronchoconstriction. This leads to airway narrowing and decrease in the lumen size of the bronchioles (Dipiro et al. 2002). This can be clearly seen by the histopathological studies of the lung tissue. In the present study, the sections of the lung tissues of animals sensitized with egg albumin depicted marked bronchitis and severe bronchoconstriction. Treatment with *M. sapida* prevented inflammation and bronchoconstriction which may lead to normal lumen size and normal lung function.

These results suggest that *M. sapida* possesses bronchodilator activity, inhibits the infiltration of different leukocyte, and prevents the release of histamine from lungs thereby decreasing bronchial hyperresponsiveness. All this findings reveal that *M. sapida* decreases bronchoconstriction and bronchial hyperreactivity indicating its beneficial use in asthma. More detailed phytochemical studies are, however, required to identify the active principle(s) and exact mechanism(s) of action.

4. Experimental

4.1. Plant material

Stem bark of *M. sapida* was obtained from a commercial supplier in Ahmedabad. The plant was identified and authenticated by Dr. Minoo Parabia, Head and Professor, Department of Bioscience, Veer Narmad South Gujarat University, Surat, Gujarat. A voucher specimen of the plant was deposited in the herbarium of the Department of Bioscience, Veer Narmad South Gujarat University, Surat, Gujarat.

4.2. Preparation of ethanolic and methanolic extract

The stem bark reduced to coarse powder was macerated with ethanol for 48 h, filtered and filtrate was evaporated under reduced pressure to obtain dry extract. The extract was stored in cool and dry place and used for pharmacological evaluation. Same procedure was followed for preparation of methanolic extract.

4.3. Animals

Hartley strain guinea pigs (350–500 g) of either sex housed under standard conditions of temperature (22 ± 2 °C), relative humidity ($55 \pm 5\%$) and light (12 h light/dark cycles) were used. Guinea pigs were fed with green vegetables. Animal studies were approved by the Institutional Animal Ethics Committee (protocol no 6012 dated 19/12/06), as per the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA).

4.4. Drugs and Chemicals

Histamine dihydrochloride (Sigma chemicals), egg albumin (Sigma chemicals), diazepam, ketamine, saline, calcium chloride, o-phthalaldehyde.

4.5. Histamine induced bronchospasm in guinea pigs (Sheth et al. 1972)

4.5.1. Acute study

Experimental animals selected randomly were divided in 2 groups each containing six animals. The animals of group I and group II placed in an aerosol chamber were exposed to 0.05% histamine dihydrochloride aerosol under constant pressure (1 kg/cm^2) on day 0 without any treatment and time for preconvulsive dyspnoea (PCD) was noted. The end point for PCD was determined from the time of aerosol exposure to the onset of dyspnoea leading to the appearance of convulsions. As soon as PCD commenced, the animals were removed from chamber and placed in fresh air to recover. This time for PCD was taken as day 0 value. After 15 days of wash out period, the animals of group I and group II received an ethanolic extract (75 mg/kg, p.o.) and a methanolic extract (120 mg/kg, p.o.) of *M. sapida* suspended in 0.5% CMC respectively. Two hours later the animals were exposed to 0.05% histamine dihydrochloride aerosol and time for PCD was noted. The % increase in time of PCD was calculated using following formula (Mitra et al. 1999)

$$\text{Percentage increase in time of PCD} = \left(1 - \frac{T_1}{T_2}\right) \times 100$$

Where: T_1 = time for PCD onset on day 0, T_2 = time for PCD onset on day 15

4.5.2. Chronic study

The animals placed in an aerosol chamber were exposed to 0.05% histamine dihydrochloride aerosol under constant pressure 1 kg/cm^2 on day 0 without any treatment and time for PCD was noted as day 0 value. The animals received an ethanolic extract of *M. sapida* (75 mg/kg, p.o.), suspended in 0.5% CMC for fifteen days. On fifteenth day, two hours later the animals were exposed to 0.05% histamine dihydrochloride aerosol and time for PCD was noted. The % increase in time of PCD was calculated using the above formula (Mitra et al. 1999).

4.6. Assessment of bronchial hyperreactivity

Sensitized guinea-pigs were challenged by exposure to antigen aerosol and following parameters were evaluated for assessment of bronchial hyperreactivity: A) cell populations in BALF, B) histamine estimation in the lung tissues, C) Histopathological changes in lung tissue.

4.6.1. Sensitization, challenge protocols and treatment

The animals were randomly divided into three groups of six animals each i.e. group-I (control), group-II (sensitized), group-III (sensitized + treatment). The animals of group II and group III were sensitized with egg albumin (1 ml, 10% w/v, i.p.) in saline on the first day. The animals of group I received 0.5% CMC for fifteen days. The animals of group III were dosed with ethanolic extract of *M. sapida* (75 mg/kg, p.o., for 15 days, once daily) suspended in 0.5% CMC. Fifteen days later, animals of Group II & III were challenged with egg albumin (0.5 ml, 2% w/v) through the saphenous vein. Animals exhibited labored breathing and coughing. Animals which did not respond were excluded from the study. After 3 h of the challenge of egg albumin or just prior to death of animals, which ever was earlier, the animals were anaesthetized with diazepam (8 mg/kg, i.p.) and ketamine (5 mg/kg, i.p.).

4.6.2. Bronchoalveolar cell counts and differentiation (Thomas et al. 1995)

The trachea was immediately cannulated after anaesthetization and the airways lavaged with saline at 25 °C (two aliquots of 1 mL/100 g body weight). To standardize the lavage technique, 60% of the instilled medium

(the maximum that could be consistently recovered) was withdrawn from each animal. Bronchoalveolar cells were collected in two successive lavages using saline and recovered through a tracheal cannula. The lavage fluid was stored on ice and total WBC cell counts were performed using an automated cell counter (Cell Dyn 3200SL). Dilutions of lavage fluid (1 in 10) were made in saline, and differential WBC cell counts were made by light microscopy stained with Leishman's stain. At least 200 cells were counted on each slide. Cells were differentiated using standard morphological criteria. All differential cell counts were performed blind and in randomized order at the end of the study.

4.6.3. Study of histamine release from the lung tissues (Singh et al. 2001)

The lungs of the guinea pigs were chopped into fragments. The chopped lung tissues were placed in tubes with 2 ml of ice cold calcium free Tyrodes solution and kept on ice until further used. 200 mg (wet weight) of lung tissues was incubated with 1.8 mM $CaCl_2$ for 10 min. at 37 °C. The lung tissues were further incubated with 2 mg/ml egg albumin for 15 min at 37 °C. After 15 min, the reaction was stopped by filtration through nylon mesh (100 μm). Histamine in the solution was measured spectroflourometrically by using o-phthalaldehyde as a substrate (Shimadzu Spectrophotoflourometer RF 5401) (Shore et al. 1959)

4.6.4. Histopathological study
(Djukanovic et al. 1990; Beasley et al. 1993)

To avoid possible traumatic damage due to BAL, histopathological assessment of inflammatory changes in lung tissue were made in separate animals. The lungs were removed intact from all the three groups and then fixed by slowly inflating with buffered formalin and subsequently embedded in paraffin. A transverse section (2–4 μm thick) was cut from each sample and stained with haematoxylin and eosin. Histopathological assessment (light microscopy) was performed blind on randomized sections.

4.7. Statistical analysis

The results of various studies were expressed as mean ± SEM and analyzed statistically using paired't'-test or one way ANOVA to find out the level of significance. Data were considered statistically significant at $p < 0.05$.

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