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Development of Gastrointestinal Distress in *Rattus rattus* Against Methyl Anthranilate: A Bird Repellent

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ABSTRACT: Methyl anthranilate (MA) is a known nociceptive bird repellent. The present study was conducted to determine the histopathological effect of methyl anthranilate causing gastrointestinal malaise and thus conditioned taste aversion in house rat, *Rattus rattus*, which is a known rodent pest worldwide. Both mature male and female rats were fed on 2.5% MA treated bait continuously for 30 days under laboratory conditions. Histomorphometric studies showed that consumption of MA treated bait causes a significant reduction in the thickness of different layers of the stomach and intestinal wall. Vacuolization in the stomach and intestinal mucosa was also recorded, which resulted in a significant reduction in the number of cells present in intestinal and gastric glands. Histopathological studies also revealed focal hyperplasia with keratin pearl, squamous cell papilloma, and mucosal cysts in the lumen of the non-glandular region of the stomach of rats. Present studies confirmed that gastrointestinal tract was severely affected in rats fed on 2.5% MA treated bait. In the future, methyl anthranilate based repellent formulations can be developed to induce condition taste aversion in the rats to repel them for long period in commensal situations.

Keywords: Methyl anthranilate; gastrointestinal tract; *Rattus rattus*; histopathology.

INTRODUCTION

Rodents are considered as the most significant mammalian agricultural pest responsible for considerable yield losses and food shortages in many countries (Fayenuwo *et al.*, 2007). In Asia, each year, rats consume crops worth feeding 200 million people (Singleton, 2003). In contrast, in Tanzania, they cause 5–15% crop loss each year, equivalent to around $45 million, which is sufficient to feed approximately 2 million people (Stenseth *et al.*, 2003). Generally, rodents damage in the Indian subcontinent ranges from 2-5% in crops, but it can increase to 25-100% during rodent outbreaks (Islam *et al.*, 2008). Rodents in India cause a 4.75% yield loss in wheat at the post-harvest stage. They contaminate environment and foodstuff with their urine, faecal pellets and hairs (Daniels *et al.*, 2003; Stejskal *et al.*, 2005) and also cause damage and economic losses to poultry farms, rural and urban residences and grain stores (Parshad, 1999). House rat *Rattus rattus* was found as the major pest of oyster mushrooms in Gujarat which directly influences the yield performance, morphological parameters, and characteristics of fruiting body of oyster mushroom (Chavan *et al.*, 2024). In order to kill rats farmers prefer to use acute rodenticides as these are fast-acting but persistent use of these rodenticides leads to the development of poison aversion and bait shyness in rats. In such circumstances, more advanced rat pest management strategies such as use of repellents are required that are environmentally benign (Bala and Babbar 2022).

Repellents are used for manipulation of animal behaviour. Repellents which cause illness/unpleasant physiological responses soon after ingestion (gastrointestinal tract malaise) are responsible to cause conditioned taste aversion in animals (CTA) (Johnson *et al.*, 1982). CTA inducing repellents are considered very effective, responsible for long term avoidance responses, depending upon the severity of gastrointestinal malaise caused by ingested compounds (Domjan, 1998). Once the post ingestive malaise established, avoidance response persists even in the absence of illness producing agents.

MA is a preingestive, trigeminal (Werner and Provenza 2011) nociceptive, non-lethal bird repellent (Clark, 1997). It has been used to protect fruit crops (Mason *et al.*, 1991; Clark *et al.*, 1991; Clark *et al.*, 1993). Earlier studies reported its repellent effect in red-winged

blackbirds at 2.5% concentration (Avery *et al.*, 1995) and in grazing waterfowl at 1 and 2% concentration (Cummings *et al.*, 1992). 0.25% concentration of MA was used as a repellent in pig feed, resulting in a significantly lower number of approaches and reduced feed intake, demonstrating its effectiveness in altering feeding behaviour (Ampode *et al.*, 2022). MA has been shown to repel various bird species, such as starlings, when applied to seeds or grass, thereby protecting crops from bird predation. It is marketed under trade names like ReJeX-iT and Bird Shield, highlighting its commercial viability as a bird repellent (Müller-Schwarze and Müller-Schwarze 2009). The study found that TP-40, a formulation containing MA, maintained low toxic levels for channel catfish. It is demonstrated that MA is repellent to western corn rootworm larvae in the soil environment and may have potential as a rootworm treatment (Bernklau *et al.*, 2019). Additionally, its role as a quorum sensing inhibitor in bacterial infections suggests potential therapeutic applications beyond its current uses (Gao *et al.*, 2023).

Our previous laboratory studies based on hourly bait consumption data and histological studies on tongue and nasal tissue of house rats exposed to 2.5% MA treated bait confirmed the existence of olfactory and gustatory repellent effects of MA against house rats (Kaur *et al.*, 2020). However, development of gastrointestinal malaise due to MA in house rat is not reported yet.

The present study was undertaken with the primary aim to evaluate the effects of 2.5% MA on histomorphology of gastrointestinal tract of *R. rattus* in order to confirm its effect on gastrointestinal tract causing gastrointestinal distress.

MATERIAL AND METHODS

The present work was carried out at Animal House Laboratory and Rodent Research Laboratory, Department of Zoology, Punjab Agricultural University (PAU), Ludhiana, India, located at an intersection of 30˚55' N parallel of latitude and 75˚54' E line of longitude.

A. Chemical used

MA was purchased from Loba Chemical Private Limited, Mumbai, India.

B. Animal collection and maintenance

For the present study, mature house rat, *R. rattus* of both sexes were trapped with the help of single/multi-catch rat traps from the storehouse, grocery shops and poultry farms in and around Ludhiana, India. Rats were individually acclimatized in laboratory cages (36 × 23 × 23 cm) for 15-20 days before the start of the experiment, with food and water provided *ad libitum.* Cracked wheat, powdered sugar, and vegetable oil (WSO bait) were mixed in a ratio of 96: 2: 2 to prepare the bait for rats. For the usage and maintenance of animals, guidelines of the Institutional Animal Ethics Committee were followed. Approval was obtained from the Institutional Animal Ethics Committee, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana's for the usage of animals during experimentation (vide memo no. GADVASU/2019/IAEC/52/03, during 52nd Meeting of IAEC). Guidelines on the regulation of scientific experiments on animals were followed. Proper hygienic conditions were maintained. Plastic trays were kept under each laboratory cage for collection and disposal of animal faeces and urine as well as for the collection of spilled food.

C. Exposure of R. Rattus to 2.5% methyl anthranilate

Rats were divided into two groups of 6 animals each (n=6, 3 males and 3 females for each group). Rats of one group were fed on 2.5% MA treated WSO bait for 30 days and this group was considered as treated group while the second group of rats were fed on plain WSO bait and considered as untreated group of rats.

D. Effect of 2.5% methyl anthranilate on the gastrointestinal tract of R. rattus

After the completion of the treatment period, rats of the treated and untreated groups were sacrificed. Their stomach and intestine were collected and weighed. Organs were washed in 0.9% saline. Pieces of anterior, middle and posterior parts of both stomach and intestine were fixed in 10% neutral buffered formalin for 48 hours. After complete fixation, the tissue was dehydrated in graded series of ethanol, cleared in benzene and embedded in paraffin wax with a melting point between 58-60˚C (Humason, 1966). Sections of tissues were obtained at 5µm thickness with a rotary microtome. They were stained with haematoxylin and eosin, dehydrated in ascending ethanol series and then cleared in xylene and mounted in DPX.

The anterior part of the stomach is composed of the non-glandular area, the middle part composed of both glandular and non-glandular area and posterior portion composed of the glandular area only. Four layers *i.e.* muscularis externa, muscularis interna, submucosa and mucosa of stomach (Sujin *et al.*, 2008) and intestine (AI- Qudah, 2012), were identified. Their thickness was measured at 100x magnification. In stomach, keratinised stratified squamous epithelium of non-glandular region of mucosa was identified (Chandana *et al.*, 2013) and its cells were counted at 400x magnification. Stratified squamous epithelium of non-glandular stomach transitioned abruptly into simple columner epithelium in glandular region. Columnar cells of epithelial layer/mucosal cells as well as chief cells, parietal cells, neuroendocrine cells and stem cells of gastric glands were identified (Chandana *et al.*, 2013) and counted at 400x magnification. Number and diameter of gastric glands was recorded at 100x magnification.

The epithelial lining of intestinal mucosa protrudes into finger-like projections called villi, which are covered by a simple columnar epithelium consisting of enterocytes and goblet cells. Intestinal glands or Crypts of Lieberkuhn are present in the mucosa layer. These glands consist of paneth cells, neuroendocrine cells, stem cells and goblet cells. Lymphocytes are present in lamina propria of the intestinal mucosa layer. All these

cells of the small intestine were identified and counted at 400x magnification. Diameter (µm) of the cells present in the glands and thickness (µm) of different layers of the stomach and intestine was measured using the auto grid bar system of Magvision software. The actual average number counted of nearly round cells was corrected by a modification of Abercrombie's formula:

$$\text{True count} = (\text{Observed count}) \times \frac{\text{Section thickness}}{\text{Section thickness} + \sqrt{(\text{Av.dia./2})^2 - (\text{Av.dia./4})^2}}$$

E. Statistical analyses

Values were calculated as mean ± SE. Data collected for the dependent variables (diameter, the thickness of different layers and number of different types of cells in the mucosa of stomach and intestine) using factorial completely randomized design was subjected to analysis of variance using Proc GLM procedure of statistical software SAS 9.3. Tukey's multiple comparison method was applied to compare the significant difference among parts of the stomach, intestine and treatments at $P<0.05$.

RESULTS

Effect of 2.5 % MA on the gastrointestinal (GI) tract of house rats

A. Histomorphometric and histomorphological studies of stomach

Consumption of 2.5% MA treated bait caused significant ($P<0.05$) reduction in thickness of all layers (muscularis externa, muscularis interna, submucosa and mucosa) of anterior (non-glandular), middle (non-glandular and glandular) and posterior (glandular) part of stomach in treated group of rats (Table 1). The mucosal layer of anterior (non-glandular) part of stomach consists of keratinized stratified squamous epithelium and posterior (glandular) region consists of gastric glands. Keratin layer was found in close association with stratified squamous epithelium of anterior (non-glandular) region in untreated group of rats but consumption of 2.5% MA treated bait caused separation, thinning and sloughing of this layer in treated group of rats (Fig. 1a). Other pathological changes like focal hyperplasia as there was a significant increase in the number of stratified squamous epithelial cells (Table 2), keratin pearl in mucosa and lumen, mucosal cyst in the lumen (Fig. 1b), squamous cell papilloma in the non-glandular region of the stomach (Fig. 1c) and invasion of the submucosal layer in glandular mucosa (Fig. 1d) were also observed in the treated group of rats. The number of stratified squamous epithelium cells/mm^2 in anterior (non-glandular) and middle (non-glandular) part of stomach, gastric glands/mm^2, mucosal cells/mm^2, parietal cells/mm^2, chief cells/mm^2, neuroendocrine cells/mm^2 in middle (glandular) and posterior (glandular) was significantly ($p\leq0.05$) less in the treated group of rats (Table 2).Vacuolization due to the degeneration of gastric glands was observed in the mucosal layer of the glandular stomach in the treated group of rats (Fig. 2).

B. Histomorphometric and histomorphological studies of small intestine

There was a significant difference ($p\leq0.05$) in villi length among different parts of the small intestine being significantly smaller in the ileum than duodenum and jejunum. Consumption of 2.5% MA treated bait caused degenerative changes in villi length in treated group of rats. Compared to the untreated group of rats, the villi length of different parts of the intestine was significantly ($p\leq0.05$) less in rats of treated group (Table 3). The thickness of mucosal layer in duodenum and jejunum also reduced significantly ($p\leq0.05$) in the treated group as compared to the untreated group of rats (Table 3). The thickness of submucosa also reduced significantly ($p\leq0.05$) in duodenum and ileum in the treated group of rats. There was however a non-significant difference in the thickness of submucosa among different parts of the small intestine (Table 3). Thickness of muscularis externa differed significantly among different parts of intestine and between treated and untreated groups of rats. The thickness of the muscularis externa layer of ileum and jejunum reduced significantly in the treated group as compared to the untreated group of rats (Table 3).

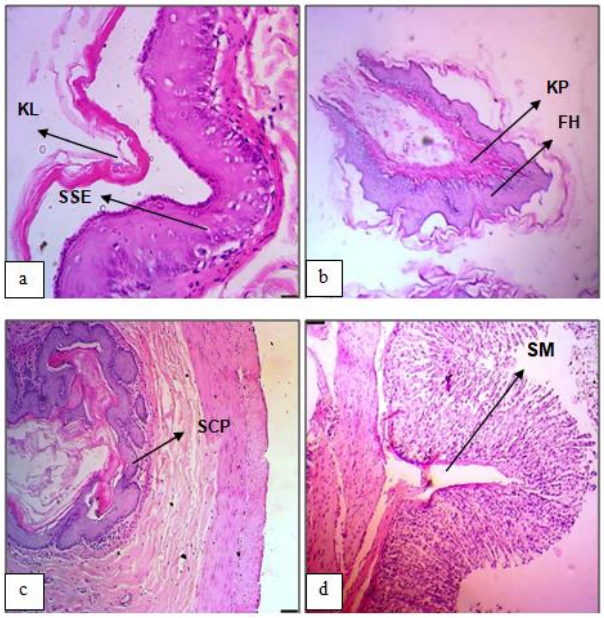
Significant reduction in the number of goblet cells and enterocytes (Fig. 3 a,b) and a significant increase in the lymphocytes (Fig. 4a, b) was also observed in the treated group as compared to the untreated group of rats (Table 4). Lymphocytes aggregates and their penetration in lamina propria were also recorded in the treated group of rats and there was a significant reduction in the number of crypts (Fig 3b), paneth cells, and neuroendocrine cells (Table 5). However, there was a significant increase in the number of stem cells in all parts of the intestine in the treated group compared to the untreated group of rats (Table 5).

DISCUSSION

Histomorphological studies of stomach showed thinning and separation of keratin layer which indicate reduced protection to the non-glandular part of the stomach in the treated group of rats. Reduction in keratin layer thickness might be because of its sloughing with 2.5% MA treatment. Sloughing of the keratinized layer was also noticed in the stomach of house rats treated with 1% chili powder (Kaur *et al.*, 2017). Submucosal layer of glandular portion comprises of loose connective tissue with plentiful collagen fibre, few elastic fibres, blood vessels and nerve fibres. Its main function is to provide support to the underlying muscular layer (Ghoshal and Bal 1989). Thinning of the submucosal layer in the treated group of rats may be due to the loss of loose connective tissue, blood vessels and nerve fibre present in this layer. The muscularis layer causes contraction and peristaltic movements in the GI tract. These muscles are responsible for movement and churning of food. Reduced thickness of this layer might also decrease the mechanical digestion of food in the stomach. Empty spaces observed in the gastric glands in the treated group of rats were similar to the vacuolization of the

mucosa layer seen in house rats treated with 1% chili powder (Usher *et al.*, 1988). Consumption of 2.5% MA treated bait caused deterioration of the gastric glands resulting in the formation of empty spaces in between which ultimately leads to reduction in the number of mucosal, chief, parietal and neuroendocrine cells lining these glands and thus reduction in the production of digestive juices and hormones. Abnormal reduction in the production of digestive hormones and juices was associated with the gastric distress (Hunt *et al.*, 2015). The house sparrows exhibited noticeable behavioral changes when consuming MA treated seeds and seedlings. Specifically, they displayed head-shaking and feather ruffling, which are indicative of discomfort or aversion to the treated food. This suggests that the MA caused a negative sensory response in the birds (Ahmad *et al.*, 2015).

In intestine, significant reduction in the villi length in treated group of rats indicated the reduction in intestinal absorptive area. Count of different types of cells, such as goblet cells, enterocytes and lymphocytes lining the villi were also affected by the consumption of 2.5% MA treated bait. Decreased count of enterocytes cells might be responsible for the reduction in uptake of ions, water, nutrients, resorption of bile salts, and secretion of immunoglobulins from digestive tract whereas increase in the number of lymphocytes indicated inflammation of the intestine due to abrasion caused by 2.5% MA. Crypts of Lieberkühn are situated in the mucosal lamina propria. Paneth cells, stem cells and neuroendocrine cells are present at the base of crypts. The stem cells count increased to compensate the deterioration of cells however the number of crypts, paneth cells, and neuroendocrine cells decreased because of the degeneration of intestinal glands and vacuolization of intestinal mucosa indicating the severe gastric distress caused by ingestion of 2.5% MA treated bait. Lymphatic intrusion in lamina propria and degenerative changes in crypts of Lieberkuhn were also observed in house rat treated with 1% chili powder (Usher *et al.*, 1988).



(a) Separation of keratin layer from stratified squamous epithelium; (b) Focal hyperplasia with keratin pearl in lumen; (c) Squamous cell papilloma in non-glandular region of stomach; (d) Submucosal (SM) invasion in glandular mucosa. KL: Keratin layer; SSE: Stratified squamous epithelium; FH: Focal hyperplasia; KP: Keratin pear; L: Lumen; SCP: Squamous cell papilloma; SM: Submucosa; M: Mucosa.

Fig. 1. Pathological changes in stomach of treated group of rats.

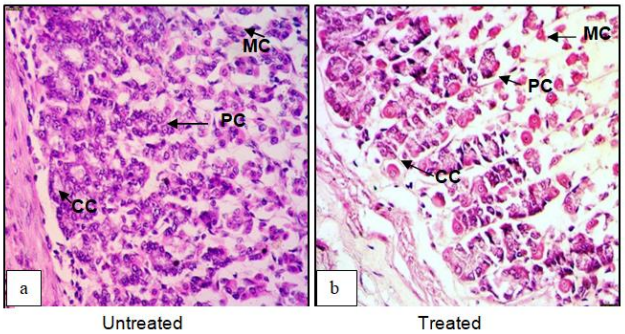


Fig. 2. (a) Gastric glands, mucosal cells, chief and parietal cells in untreated group of rats; **(b)** Reduction in number of gastric glands, mucosal cells, chief and parietal cells in glandular region of stomach in treated group in comparison to untreated group of rats. MC: Mucosal cells; PC: Parietal cells; CC: Chief cells.

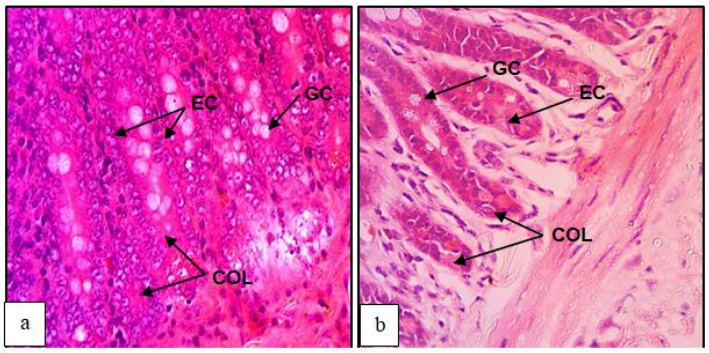


Fig. 3. (a) Crypts of Lieberkühn, goblet cells and enterocytes in the villi of small intestine in untreated group of rats; (b) Reduction in the number of crypts of Lieberkühn, goblet cells and enterocytes in the villi of small intestine in treated group of rats in comparison to untreated group of rats. COL: Crypts of Lieberkühn; GC: Goblet cells; EC: Enterocytes.

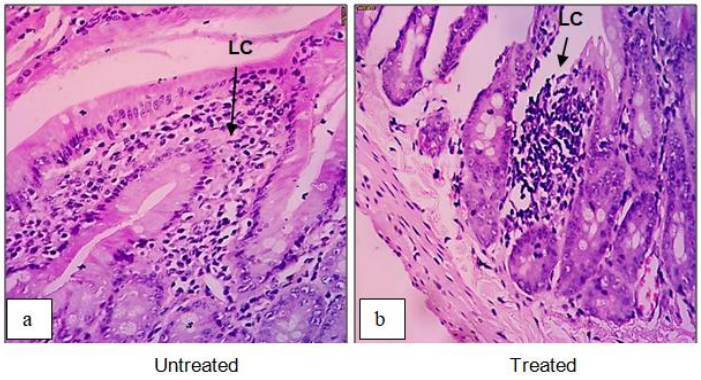


Fig. 4. (a) Lymphocytes in untreated group of rats; (b) Increase in the number of lymphocytes in treated group of rats in comparison of untreated group of rats. LC: Lymphocytes.

Table 1: Effect of 2.5% methyl anthranilate on the thickness of different layers of the stomach.

Parts of stomach	Untreated group	Treated group
Thickness of Keratin layer (µm)		
Anterior (Glandular)	45.53±1.38^{a}	21.75± 1.02^{b}
Middle (Non-glandular)	40.98±0.94^{a}	19.75±0.62^{b}
Thickness of mucosa (µm)		
Anterior (Glandular)*	49.52±0.96^{a}	41.61±0.61^{a}
Middle (Non-glandular)*	48.56±1.00^{a}	38.75±1.00^{a}
Middle (Glandular) **	609.20±0.96^{c}	399.87±2.03^{b}
Posterior (Glandular) **	612.55±2.79^{c}	413.06±1.44^{b}
Thickness of submucosa (µm)		
Anterior*	271.49±1.78^{a}	144.76±1.58^{c}
Middle**	247.52±2.04^{b}	209.55±1.16^{d}
Posterior**	240.06±2.20^{b}	194.96 ±1.02^{d}
Thickness of muscularis externa (µm)		
Anterior	304.85±1.30^{a}	299.76±4.10^{b}
Middle	306.75±3.16^{a}	278.70±2.87^{b}
Posterior	304.85±1.30^{a}	292.09±0.58^{b}

Values expressed are mean±SE based on three replications.
^{a,b,c,d} denotes the significant difference between groups($P \leq 0.05$).
*,**denotes significant difference among different parts of stomach ($P \leq 0.05$).

Table 2: Effect of 2.5% methyl anthranilate on the number of cells of glandular and non-glandular stomach mucosa.

Parts of stomach	Untreated group	Treated group
Number of cells of stratified squamous epithelium/mm2		
Anterior (Glandular)	2.79±0.15[a]	3.54±0.08[b]
Middle (Non-glandular)	2.78±0.10[a]	3.55±0.27[b]
Number of gastric glands/mm^2		
Middle	57.00±1.79[a]	28.87±3.21[c]
Posterior	57.37±1.63[a]	27.32±2.10[c]
Number of mucosal cells/mm^2		
Middle	3.50±0.37[a]	2.46±0.14[b]
Posterior	3.75±0.50[a]	2.37±0.14[b]
Number of parietal cells/mm^2		
Middle	1.67±0.27[a]	0.96±0.50[c]
Posterior	1.65±0.46[a]	0.87±0.67[c]
Number of chief cells/mm^2		
Middle	1.64±0.27[a]	0.76±0.50[c]
Posterior	1.67±0.46[a]	0.76±0.67[c]
Number of neuroendocrine cells/mm^2		
Middle	1.39±0.60[a]	1.14± 0.26[c]
Posterior	1.39±0.76[a]	1.14±0.33[c]

Values expressed are mean±SE based on three replications
[a,b,c] denote significant difference between groups (P≤0.05).

Table 3: Effect of 2.5% methyl anthranilate on the thickness of different layers of Intestine.

Parts of intestine	Untreated group	Treated group
Length of villi (µm)		
Duodenum*	92.46±1.63[a]	57.78±3.09[c]
Jejunum*	85.73±1.94[a]	69.59±2.49[b]
Ileum**	75.55±3.70[a]	20.82±0.75[d]
Thickness of mucosa (µm)		
Duodenum*	125.73±2.60[a]	119.36±1.69[b]
Jejunum*	123.33±1.33[a]	119.61±1.89[b]
Ileum**	117. 09±1.68[b]	50.93±3.07[c]
Thickness of submucosa (µm)		
Duodenum	8.85±0.43[a]	4.30±0.24[b]
Jejunum	8.60±0.35[a]	8.84±0.43[a]
Ileum	9.81±0.34[a]	4.10±0.52[b]
Thickness of muscularis externa (µm)		
Duodenum*	26.00±0.85[a]	23.85±0.72[a]
Jejunum**	22.24±0.61[a]	19.72±1.60[b]
Ileum***	23.05±0.47[a]	16.77±0.63[c]

Values expressed are mean±SE based on three replications.
*, **, *** values shows significance difference among different parts of intestine (P<0.05).
[a, b,c] shows significant between groups of rats (P≤0.05).

Table 4: Effect of 2.5% methyl anthranilate on the number of cells of villi.

Parts of intestine	Untreated group	Treated group
Number of goblet cells/mm^2		
Duodenum*	0.94±0.30[a]	0.32±0.42[b]
Jejunum**	0.95±0.24[a]	0.58±0.04[b]
Ileum***	1.02±0.53[a]	0.37±0.04[b]
Number of enterocytes cells/mm		
Duodenum	6.64±0.20[a]	3.62±0.18[b]
Jejunum	6.83±0.32[a]	3.86±0.20[b]
Ileum	7.27±0.32[a]	3.41±0.49[b]
Number of lymphocytes/mm^2		
Duodenum	3.50±0.11[a]	4.44±0.16[b]
Jejunum	3.38±0.16[a]	4.38±0.14[b]
Ileum	3.48±0.24[a]	4.43±0.08[b]

Values expressed are mean±SE based on three replications
*, **, *** values shows significance difference among different parts of intestine (P≤0.05).
[a, b] show s significant difference between groups of rats (P≤0.05).

Table 5: Effect of 2.5% methyl anthranilate on number of crypt of lieberkuhn and cells of intestinal mucosa.

Parts of intestine	Untreated group	Treated group
Number of crypts of lieberkuhn /mm^2		
Duodenum	0.51±0.06^a	0.35±0.07^b
Jejunum	0.50±0.07^a	0.28±0.08^b
Ileum	0.56±0.04^a	0.13±0.15^c
Number of paneth cells /mm^2		
Duodenum	1.18±0.17^a	0.51±0.07^b
Jejunum	1.13±0.09^a	0.58±0.09^b
Ileum	1.15±0.29^a	0.44±0.11^b
Number of stem cells /mm^2		
Duodenum	0.54±0.07^a	0.72±0.05^b
Jejunum	0.47±0.36^a	0.73±0.39^b
Ileum	0.51±0.08^a	0.81±0.38^b
Number of neuroendocrine cells /mm^2		
Duodenum*	1.90±0.10^a	0.97±0.12^b
Jejunum**	1.92±0.10^a	0.96±0.25^b
Ileum***	1.90±0.15^a	0.93±0.07^b

Values expressed are mean±SE based on three replications.
$^{a, b,c}$ shows significant difference between groups of rats ($P<0.05$)
*,**,*** shows significance difference among different parts of intestine ($P\leq0.05$)

CONCLUSIONS

From the present study, it is concluded that 2.5% MA severely affected the gastrointestinal tract causing gastric distress (secondary repellent effect) in house rats. Therefore, exposure of rats to 2.5% MA treated bait can induce condition taste aversion (CTA) against MA leading to avoidance towards its smell for a long time. Earlier studies conducted in our laboratory also reported its olfactory and gustatory repellent (Primary) effects.

FUTURE SCOPE

2.5% MA treated bait has both primary and secondary repellent effects and can be used to develop a formulation for long term prevention of rodent damage under commensal situations against house rats. While MA exhibits repellent properties in different animal models, its effects on other species and long-term impacts require further investigation. Future studies could explore long-term effects on rats behavior, and its efficacy in various environmental conditions.

Author contributions. Dr. B.K. Babbar contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Bindu Bala. The first draft of the manuscript was written by Bindu Bala. Dr. B.K. Babbar commented on the first version of the manuscript. Both authors read and approved the final version of the manuscript.

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Conflict of Interest. None.

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