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Full Length Research Paper

Histological effects of *Impatiens balsamina* Linn. crude extract and isolate to 2-methoxy-1,4-naphthoquinone on the pancreas, stomach, duodenum, and spleen of tumor-induced *Mus musculus*

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***Impatiens balsamina* Linn. is an ornamental plant with a wide range of bioactivities. This histological assessment was done as a continuation of a study that showed the anti-tumor promoting activity of its flower crude ethanol extract and isolate 2-methoxy-1,4-naphthoquinone (MeONQ) using the modified 2-stage mouse skin carcinogenicity assay. This evaluates any positive protective histological effects of the crude extract and isolate on the pancreas, stomach, duodenum and spleen of the same mice exposed to the tumor inducer dimethylbenzanthracene (DMBA) and promoter croton oil for 20 weeks. Standard paraffin technique of slide preparation was done and histological abnormalities were subjected to statistical analysis. ANOVA showed a significant difference between treatments A (DMBA and croton oil), B (DMBA and croton oil in acetone) and treatments C (DMBA, croton oil and crude extract), D (DMBA, croton oil and isolate). Treatment D showed no significant difference from the untreated. These results show that *I. balsamina* crude leaf extract and isolate MeONQ exhibit histoprotective effects on the pancreas, stomach, duodenum, and spleen of tumor-induced mice indicative of anti-tumor activity.**

Key words: *Impatiens balsamina* crude extract, 2-methoxy-1,4-naphthoquinone (MeONQ), mouse pancreas, stomach, duodenum, spleen.

INTRODUCTION

Impatiens balsamina Linn is an ornamental plant with several medicinal properties. This herbal plant has been used for treating rheumatism, isthmus and crural aches, fractures, superficial infections, fingernail inflammation, and has antifungal, antibacterial, antipruritic, antianaphylactic, and antitumor activities (Ding et al., 2008). Biologically active compounds such as peptides, quinones, and flavonoids were reported to be the ones responsible for these activities (Wang et al., 2009). *I. balsamina* has also been widely used in traditional Chinese, Taiwanese and Thai medicine for treating reheumatism, isthmus and crural aches, fractures, superficial infections, and fingernail inflammation etc. (Ding et al., 2008). Modern chemical and pharmacological studies have shown that this plant is capable of antifungal, antibacterial, antipruritic, antianaphylactic,

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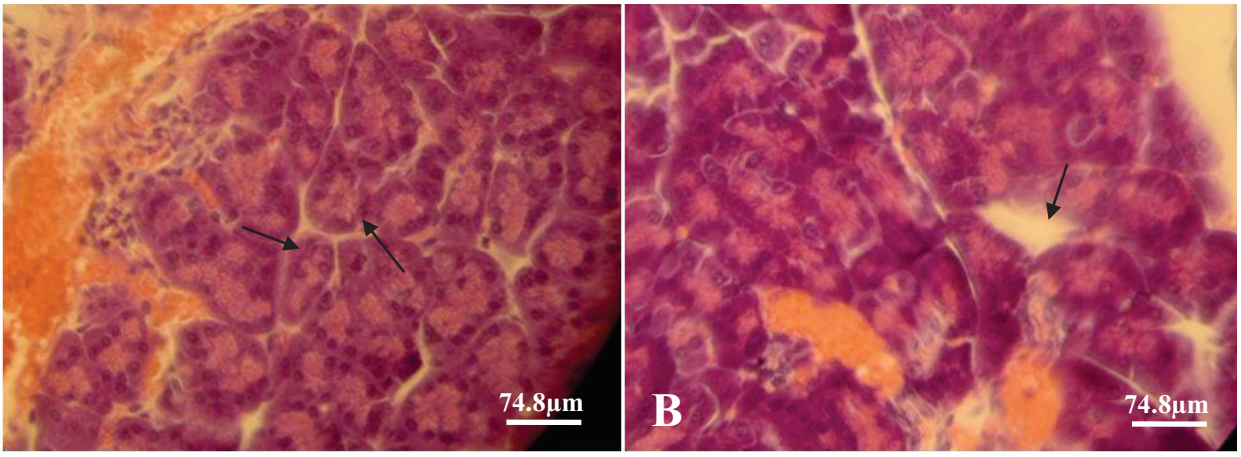

Figure 1. Cross sections of the untreated mice pancreas. (A) Compact pancreatic islets embedded within the acinar cells (arrows) with large, round basal nuclei. (B) Capillaries. H & E.

and antitumor activities. Moreover, active compounds such as peptides from seeds, quinones, balasquinone, flavonoids can be isolated from this plant (Wang et al., 2009). A recent study showed the anti-tumor promoting activity of the flower crude ethanol extract and the isolate 2-methoxy-1,4-naphthoquinone (MEONQ) using the 2-stage mouse carcinogenicity assay (Dr. Amelia P. Guevara, University of the Philippines-Diliman personal communication). Upon Dr. Guevara's endorsement, this follow-up study was done to assess any positive protective histological effects on some organs of the same mice used in her study. The results of this research can validate the efficacy and safety of the extract and isolate as anti-tumor promoters. It can provide lead towards a plant with pharmaceutical potential which is part of the continuing effort in the discovery of chemopreventive agents.

MATERIALS AND METHODS

The treated Swiss Webster albino mice were obtained from the Natural Product Research Laboratory of Dr. Amelia Guevara of the Institute of Chemistry, University of the Philippines, Diliman. Briefly, the crude ethanol extract was chormatographed in silica gel by rapid column in varying proportions of hexane: ethyl acetate: methanol solvent system to yield fractions 1 to 6 (F1-F6). The column fractions F2 was further fractionated using rapid column in varying increments of hexane: ethyl acetate: methanol: ethanol system to yield F2.1 and F2.2. The suspected compound was then co-spotted on thin layer chromatography (TLC) plate with the previously identified compound to prove similarity. To verify the structure, it was subjected to spectral characterization and compared with the spectral data of the previously isolated compound.

In the *in vivo* two-stage mouse carcinogenesis assay (Guevarra et al., 1999; Serrame and Syllianco, 1995; Beremblum and Shubik, 1978), the backs of the mice were shaved with surgical clippers and topically treated with dimethylbenzanthracene (DMBA). After one week of initiation, the carcinogenic growth was promoted twice a week by the application of croton oil on the skin. The mice were then divided into four groups. Group A received DMBA and croton oil alone, Group B received DMBA and croton oil in acetone, Group C received DMBA, croton oil, and topical applications of the plant crude ethanol extract while Group D received DMBA, croton oil, and topical applications of the plant isolate, 2-methoxy-1,4-naphthoquinone (MeONQ). Another set of Swiss Webster albino mouse was obtained for the negative control (untreated). After 20 weeks of treatment, the mice were brought to the Developmental Biology Laboratory of the Institute of Biology for paraffin processing of the pancreas, stomach, duodenum and spleen.

All observations from the slides were documented. The data gathered, which was the number of histological abnormalities, were log-transformed before applying any statistical test. After log-transforming, the data set was tested with Kruskal-Wallis. Those organs which showed significant results were then tested with ANOVA.

RESULTS

Pancreas control (Untreated)

The cross sections of the mice pancreas without application of chemicals produced compact pancreatic islets (islets of Langerhans). The light staining polygonal cells of these islets were seen around sinusoids. The pancreatic islets were embedded within the acinar exocrine tissue of the pancreas. The compact, pyramidal and polyhedral cells of the acinar tissue had noticeable large, round basal nuclei (Figure 1A). A very thin capsule of reticular fibers surrounded each islet to separate it from the adjacent acinar tissue. With Hematoxylin-Eosin staining, the four major islet cells, which secrete different hormones, appeared similar, although there are slight variations in cell size and basophilia. Capillaries were also apparent (Figure 1B).

Pancreas treatment A (DMBA + Croton oil)

The mice pancreas treated with DMBA and croton oil

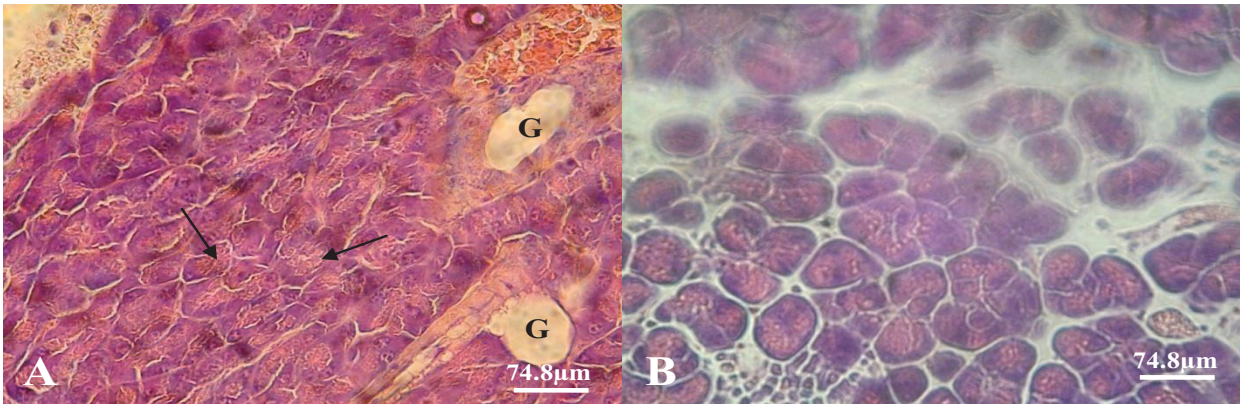

Figure 2. Cross sections of the mice pancreas treated with DMBA and croton oil (A); DMBA, croton oil, and acetone (B). (A) Islet cells (arrows) are no longer compact and irregular glands (G) are suspended in the dense fibrous stroma. (B) Big spaces between the acinar tissue. H & E.

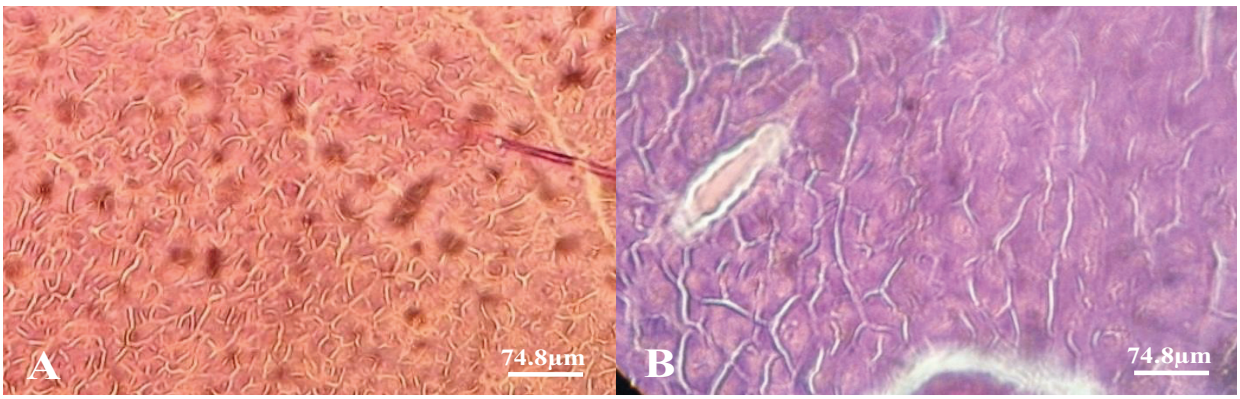

Figure 3. Cross sections of the mice pancreas treated with DMBA, croton oil, and plant crude extract (A); DMBA, croton oil, and plant isolate (B). Both show relatively compact islet cells and acinar tissue. H & E.

showed islet cells which were no longer compact. The pleomorphic tumor cells were seen lying in a densely fibrous stroma. Among these were some tortuous, irregular neoplastic glands. These observations were distinct to the rarely reported pancreatic adenocarcinoma (Figure 2A).

Pancreas treatment B (DMBA + Croton oil + Acetone)

Just like in treatment A, the mice pancreas treated with DMBA, croton oil, and acetone produced islet cells which are not compact. In a specific region of the pancreas, big spaces were seen separating the acinar exocrine tissue (Figure 2B).

Pancreas treatment C (DMBA + Croton oil + Plant crude extract)

The cross sections of the mice pancreas treated with DMBA, croton oil, and plant crude extract showed relatively compact islet cells as well as acinar exocrine tissue (Figure 3A).

Pancreas treatment D (DMBA + Croton oil + Plant isolate MeONQ)

There were no significant variations between the cross sections of the mice pancreas treated with plant crude extract and plant isolate. The cross sections also produced relatively compact islet cells and acinar exocrine tissue (Figure 3B).

Stomach control (Untreated)

The cross sections of the mice glandular stomach where no chemicals were applied showed all the four major wall layers. Rugae were seen at the mucosa and submucosa regions. Also, in the mucosa, tubular gastric glands were

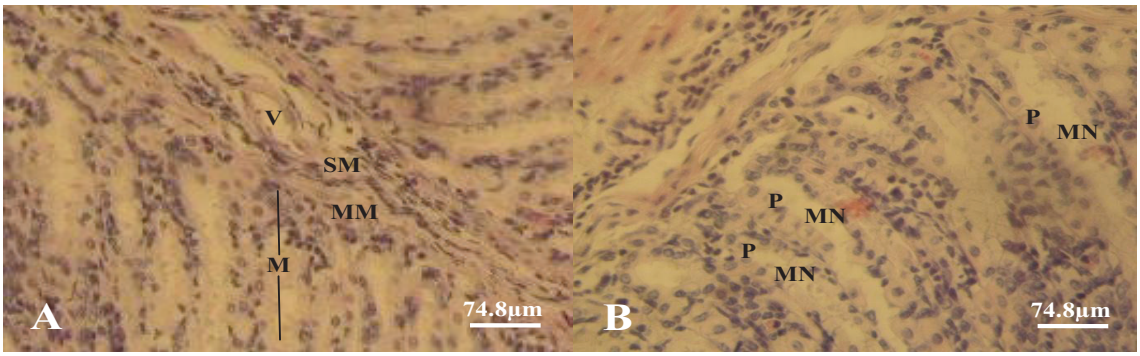

Figure 4. Cross sections of the untreated mice stomach. (A) A portion of a ruga showing the mucosa (M) and submucosa (SM) layers, the muscularis mucosa (MM), and the blood vessel (V). (B) The mucous neck cells (MN) of the fundic glands and the surrounding parietal cells (P). H & E.

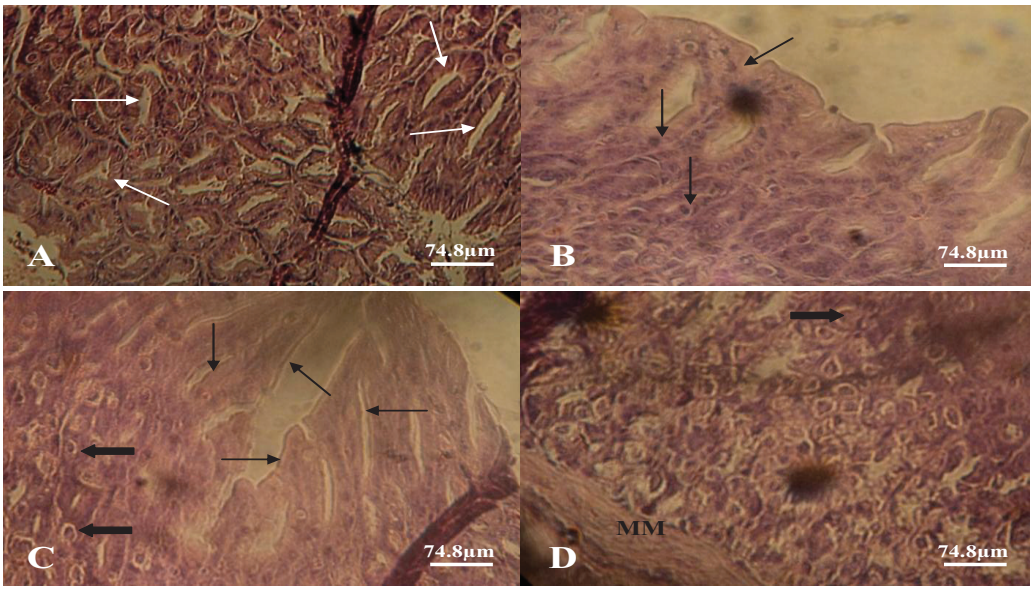

Figure 5. Cross sections of the mice stomach treated with DMBA and croton oil. (A) Glands. (B) Mitotic activity. (C) The glands lined by flat atrophic epithelium (thin arrows) and the signet ring cells (thick arrows). (D) Thickened muscularis mucosa (MM) and a few signet ring cells. H & E.

seen penetrating the full thickness of the lamina propria. Beneath the basal ends of these fundic glands, the muscularis mucosa could be distinguished. Blood vessels were also present in the submucosa (Figure 4A).

In the neck of the tubular fundic glands, mucous neck cells appeared as clusters. Surrounding the mucous neck cells were the parietal cells (Figure 4B).

Stomach treatment A (DMBA + Croton oil)

In the cross sections of the mice stomach treated with DMBA and croton oil, glands distinct to epithelial dysplasia were found in the antral mucosa. Increased mitotic activity and disturbed cell polarity were also observed. The tubular appearance of the gastric glands with surrounding parietal cells was no longer apparent (Figure 5A).

Adenocarcinomas of the intestinal and diffuse types were seen. Pleomorphic nuclei which showed considerable mitotic activity were evident. The well-differentiated glands at the upper gastric mucosa were lined by flat atrophic epithelial cells. The muscularis mucosa was thickened. Also, the signet ring cells were noticeable (Figure 5B, C, and D).

Stomach treatment B (DMBA + Croton oil + Acetone)

The abnormalities seen in treatment A of the stomach

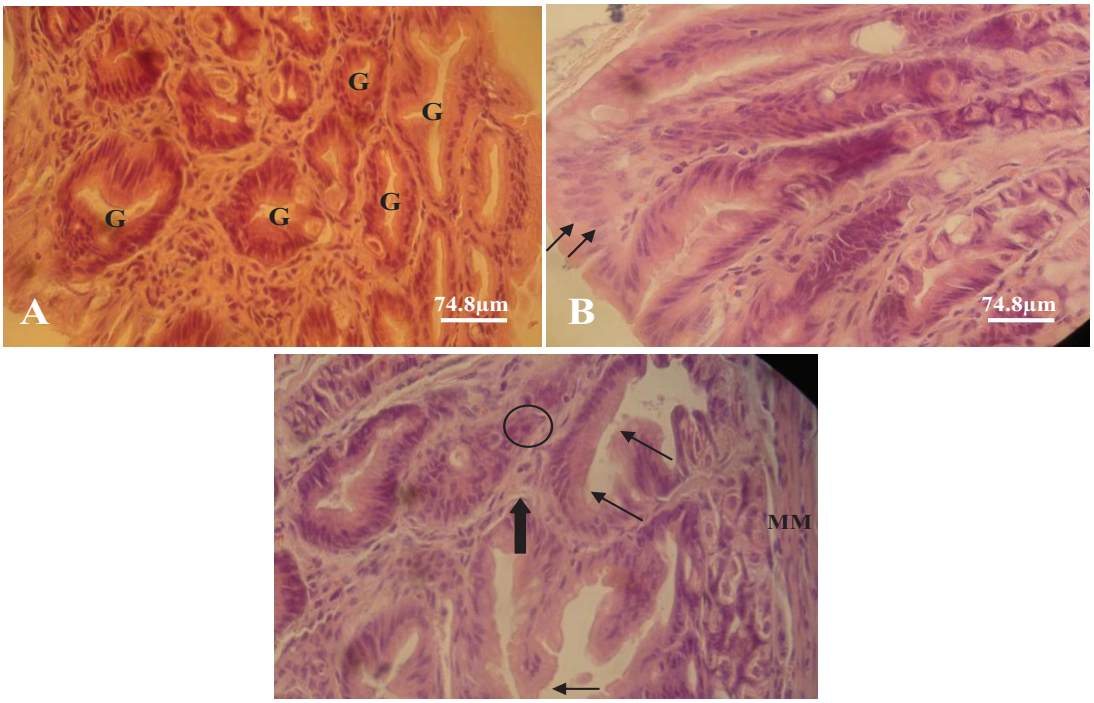

Figure 6. Cross sections of the mice stomach treated with DMBA, croton oil, and acetone. (A) The mucosal glands (G) lined by closely-packed columnar epithelial cells. (B) The epithelial lining composed of tall columnar cells. (C) Mitotic activity (encircled), flat atrophic epithelial cells (thin arrows), thickened muscularis mucosa (MM), and cell nuclei with signet-ring appearance (thick arrow). H & E.

were also observed in this treatment. Epithelial dysplasia was observed through the closely-packed columnar epithelial cells lining the glands of the mucosa. Also, the disturbed polarity of the cells was evident (Figure 6A).

For adenocarcinoma, it was also observed that the epithelial lining is composed of tall columnar cells with pleomorphic nuclei that form several layers (Figure 6B). Mitotic activity, flat atrophic epithelial cells, thickened muscularis mucosa, and cell nuclei with signet-ring appearance were all observed (Figure 6C).

Stomach treatment C (DMBA + Croton oil + Plant crude extract)

In the cross sections of the stomach treated with DMBA, croton oil, and plant crude extract, traces of epithelial dysplasia and adenocarcinoma were still present. A few mucosal glands lined by closely-packed columnar epithelial cells with large pleomorphic nuclei were present but other normal tubular glands were also observed (Figure 7A).

Although some properties of adenocarcinoma were observed, still, other characteristics distinct to this particular abnormality were not apparent. Such characteristics, which were not found, were the flat atrophic epithelial cells, the signet ring cells, and the malignant glands lined by a single layer of cuboidal cells with ovoid nuclei.

Stomach treatment D (DMBA + Croton oil + Plant isolate MeONQ)

In the cross sections of the stomach treated with DMBA, croton oil, and plant isolate, fewer glands distinct to epithelial dysplasia were observed. Normal tubular glands with surrounding parietal cells dominated the mucosal layer of the gland. The slide sections did not show any signs of adenocarcinoma. The mucosal arrangement appeared similar to that of the untreated stomach (Figure 7B).

Duodenum control (Untreated)

Just like in the stomach, the four major wall layers were also found in the slide containing cross sections of the mice duodenum. Plicae circulares was observed in the mucosa and submucosa layers (Figure 8A). The leaf-shaped intestinal villi were seen penetrating into the lumen. They were covered with a simple columnar epithelium. However, the absorptive enterocytes and goblet cells were no longer distinguished in the slide sections (Figure 8B).

In the submucosa of the duodenum, Brunner glands

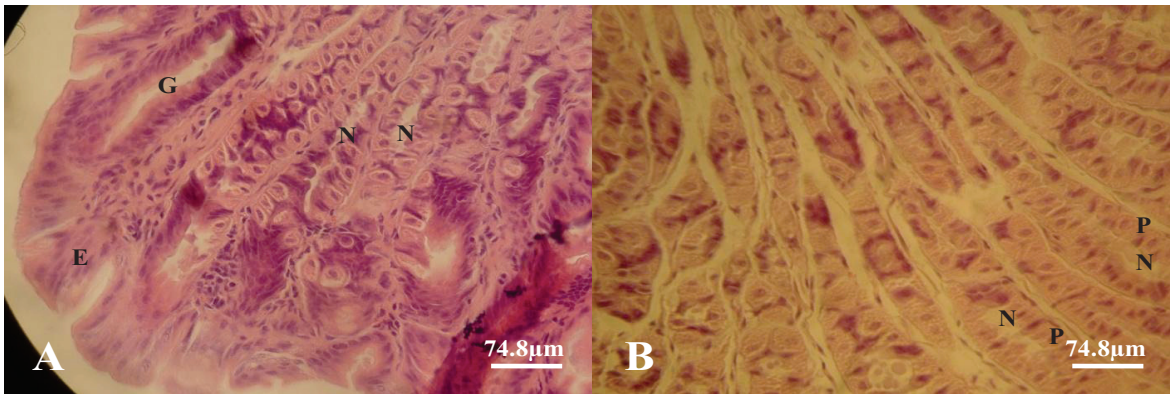

Figure 7. Cross sections of the mice stomach treated with DMBA, croton oil, and plant crude extract (A); DMBA, croton oil, and plant isolate (B). (A) The abnormal mucosal glands (G), normal tubular glands (N), and epithelial lining (E). (B) Mucosa dominated by normal tubular glands (N) with surrounding parietal cells (P). H & E.

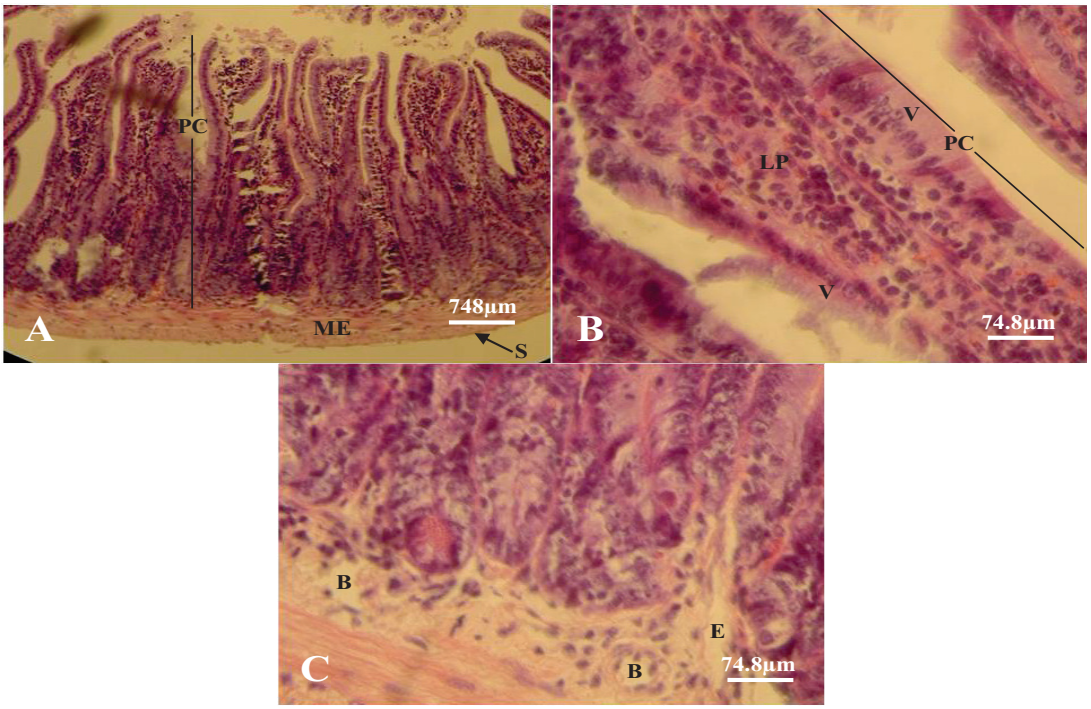

Figure 8. Cross sections of the untreated mice duodenum showing normal structures. (A) The plicae circulares (PC), muscularis externa (ME), and serosa (S). (B) The lamina propria (LP) and villi (V). (C) The Brunner gland (B) and excretory duct (E). H & E.

were seen. These glands were found extending into the lamina propria of the mucosa as small excretory ducts (Figure 8C).

Duodenum treatment A (DMBA + Croton oil)

The cross sections of the mice duodenum treated with DMBA and croton oil showed mucosal degeneration. The plicae circulares could still be distinguished but the lamina propria and villi were indistinct and destroyed (Figure 9A).

Carcinoid tumors were observed invading the muscle coats of the gland. These tumors were arranged in clusters (Figure 9A).

Duodenum treatment B (DMBA + Croton oil + Acetone)

In the cross sections of the mice duodenum treated with DMBA, croton oil, and acetone, a noticeable degeneration

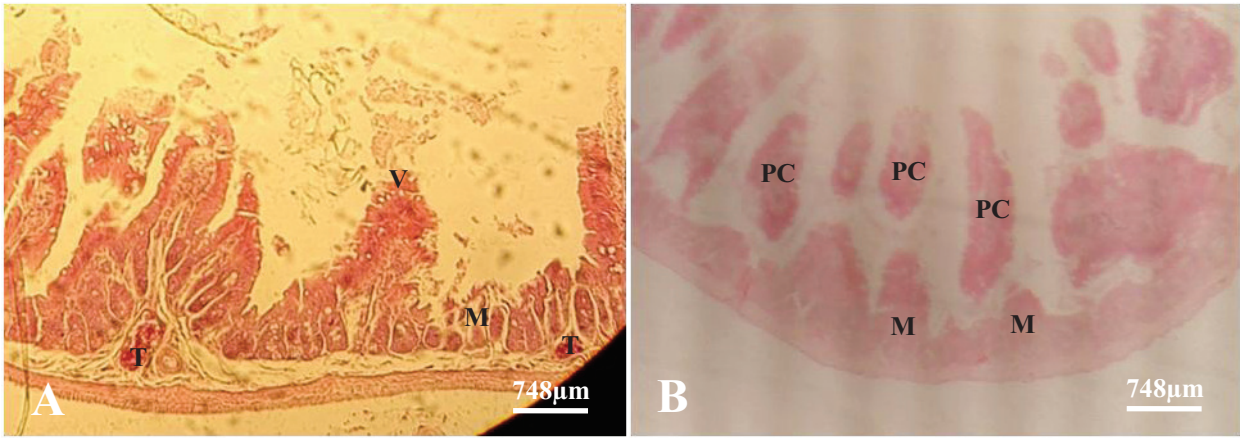

Figure 9. Cross sections of the mice duodenum treated with DMBA and croton oil (A); DMBA, croton oil, and acetone (B). (A) The degenerated mucosa (M), the indistinct and destroyed villi (V), and the carcinoid tumors (T). (B) The degenerated plicae circulares (PC) and mucosal base (M). H & E.

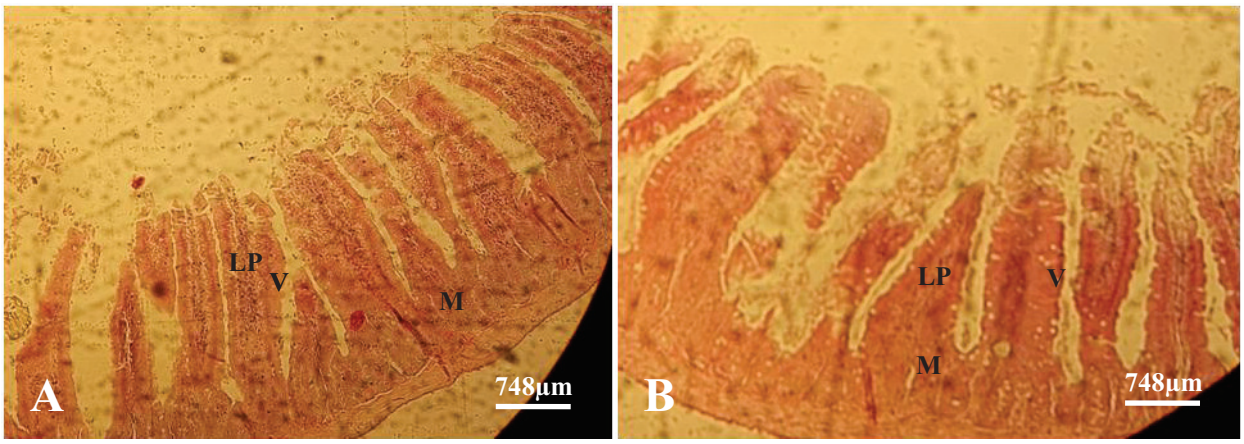

Figure 10. Cross sections of the mice duodenum treated with DMBA, croton oil, and plant crude extract (A); DMBA, croton oil, and plant isolate (B). (A) The distinct villi (V) and the intact mucosa (M) and lamina propria (LP). (B) The distinct and intact villi, mucosa, and lamina propria. H & E.

of plicae circulares was observed (Figure 9B). Just like in treatment A, carcinoid tumors were observed at the muscle coats of the gland.

Duodenum treatment C (DMBA + Croton oil + Plant crude extract)

Unlike the previous treatments, the cross sections of the mice duodenum treated with DMBA, croton oil, and plant crude extract did not show any carcinoid tumor or other types of tumor cells. Although the villi found at the upper mucosa were quite destroyed, the ones at the lower portion were distinct. Also, the mucosa, including the lamina propria, were intact and distinguishable. The overall arrangement of cells in this treatment was more intact and organized relative to that of treatments A and B (Figure 10A).

Duodenum treatment D (DMBA + Croton oil + Plant isolate MeONQ)

Just like in treatment C, the cross sections of the mice duodenum treated with DMBA, croton oil, and plant isolate did not produce any carcinoid tumor or other tumor cell types. The intact mucosa and lamina propria were also distinguished. The villi at the upper mucosa were more intact relative to those observed in treatment C (Figure 10B).

Spleen control (Untreated)

The cross sections of the mice spleen without application of chemicals showed the outermost capsule of dense connective tissue. Trabecula was seen emerging from this capsule and partially subdividing the parenchyma or

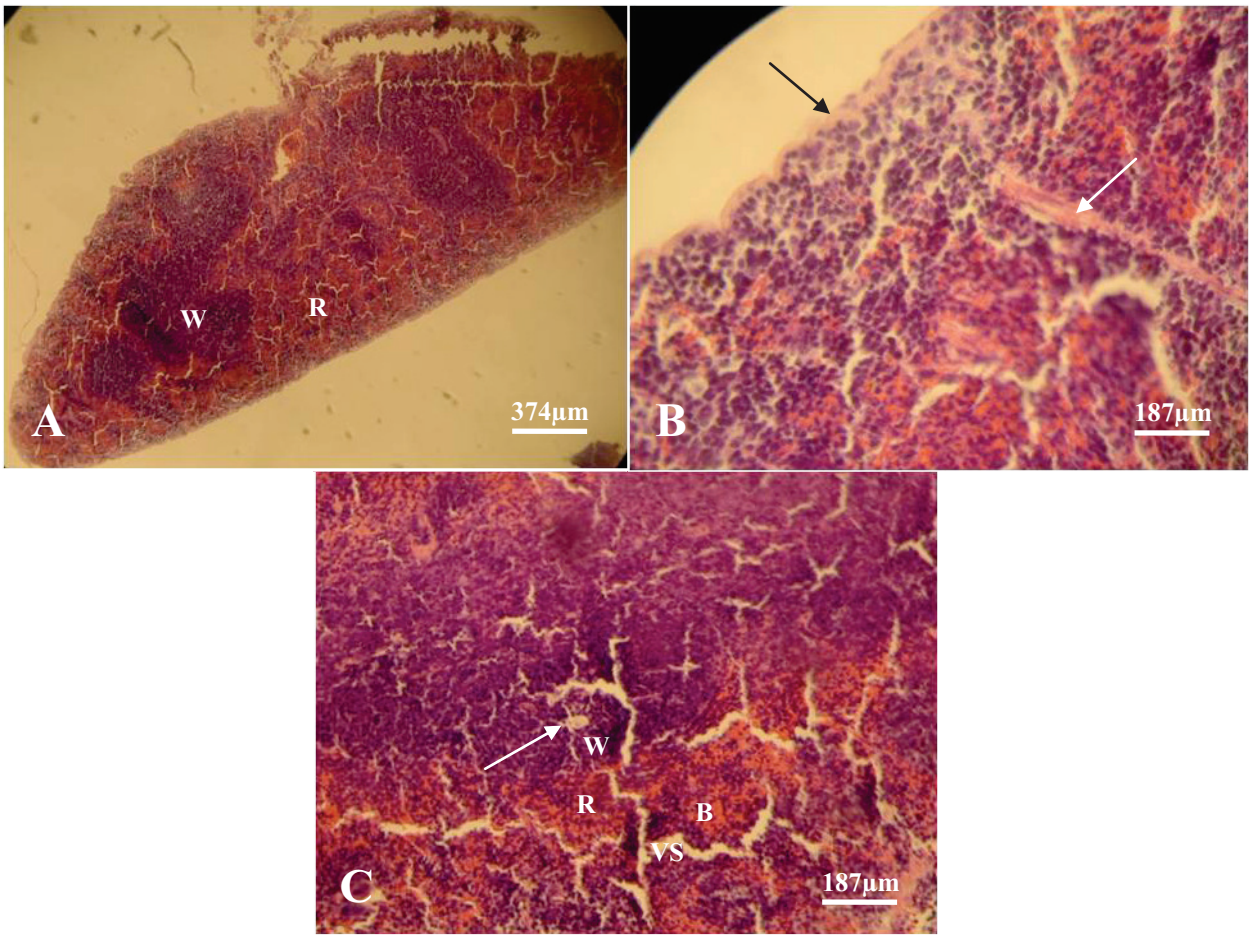

Figure 11. Cross sections of the untreated mice spleen. (A) The dominant red pulp (R) and the white pulp (W). (B) The capsule (black arrow) where the trabeculae (white arrow) emerges. (C) The white pulp (W), surrounding the central arteriole (arrow), and the red pulp (R), which consists of venous sinusoids (VS) and cords of Bilroth (B). H & E.

splenic pulp (Figure 11B).

The pulp-like interior of the organ consists of the red and white pulps. The red pulp was seen to be occupying most of the parenchyma while the white pulp was restricted to smaller areas, surrounding the central arterioles (Figure 11A). The splenic venous sinusoids and cords of Bilroth were also observed (Figure 11C).

Spleen treatment A (DMBA + Croton oil)

In the cross sections of the mice spleen treated with DMBA and croton oil, there was less red pulp relative to the white pulp. The venous sinusoids of the red pulp became less apparent. Damaged white pulp, which was not observed in the negative control sections, was seen extending throughout the splenic pulp (Figure 12A).

Noticeable hyaline changes were observed in the walls of the arterioles. Also, the lumen of these arterioles narrowed, implying the occurrence of secondary carcinoma (Figure 12B).

Spleen treatment B (DMBA + Croton oil + Acetone)

In the cross sections of the mice spleen treated with DMBA, croton oil, and acetone, the red pulp was difficult to distinguish from the white pulp, and vice versa. Just like in treatment A, the venous sinusoids of the red pulp were less apparent. The damaged white pulp observed in treatment A were also present in this treatment, but in an increased amount (Figure 13A).

Secondary carcinoma was still observed, marked by the hyaline change in the blood vessel wall (Figure 13C). Uniform sheets of pleomorphic histiocytes were also observed in the cross sections. The extensive growth of these multinucleated giant cells suggests the occurrence of hystiocytic sarcoma (Figure 13B).

Spleen treatment C (DMBA + Croton oil + Plant crude extract)

Compared to treatments A and B, the cross sections of

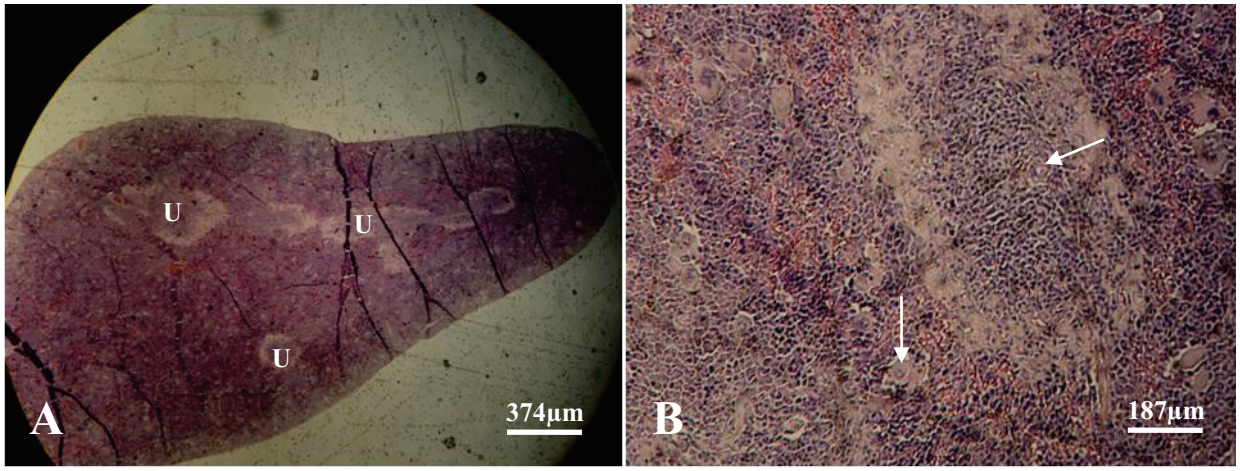

Figure 12. Cross sections of the mice spleen treated with DMBA and croton oil. (A) Less red pulp relative to the white pulp; Damaged white pulp sections (U). (B) The arteries and arterioles with narrowed lumen and hyaline changes in their walls. H & E.

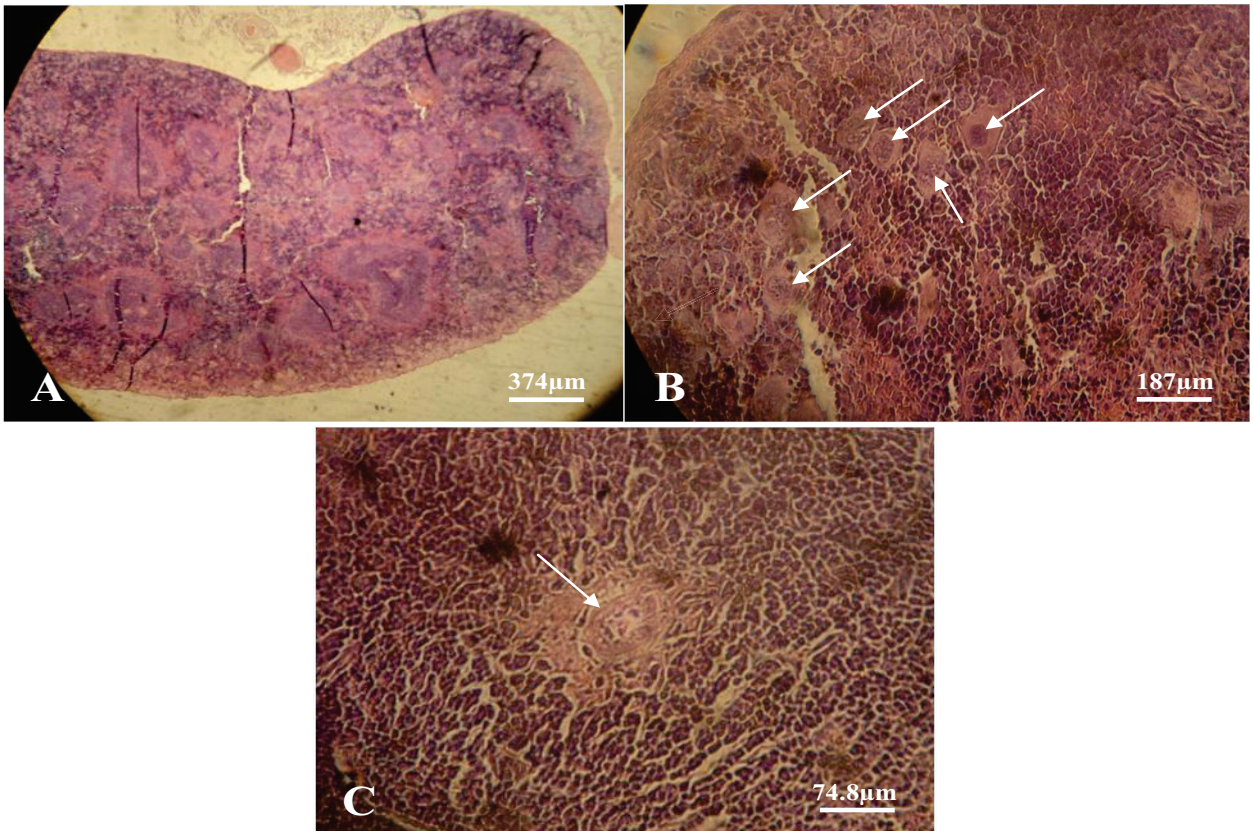

Figure 13. Cross sections of the mice spleen treated with DMBA, croton oil, and acetone. (A) The degenerative changes in the red and white pulps. (B) The histiocytes, which appeared as multinucleated giant cells. (C) The arteriole with marked hyaline change in its wall. H & E.

the mice spleen treated with DMBA, croton oil, and plant crude extract produced dominant red pulp. Damaged white pulp was not evident in this treatment (Figure 14A).

The cross sections did not show any signs of secondary carcinoma. Histiocytes were still present but in reduced amount and degenerated form (Figure 14B).

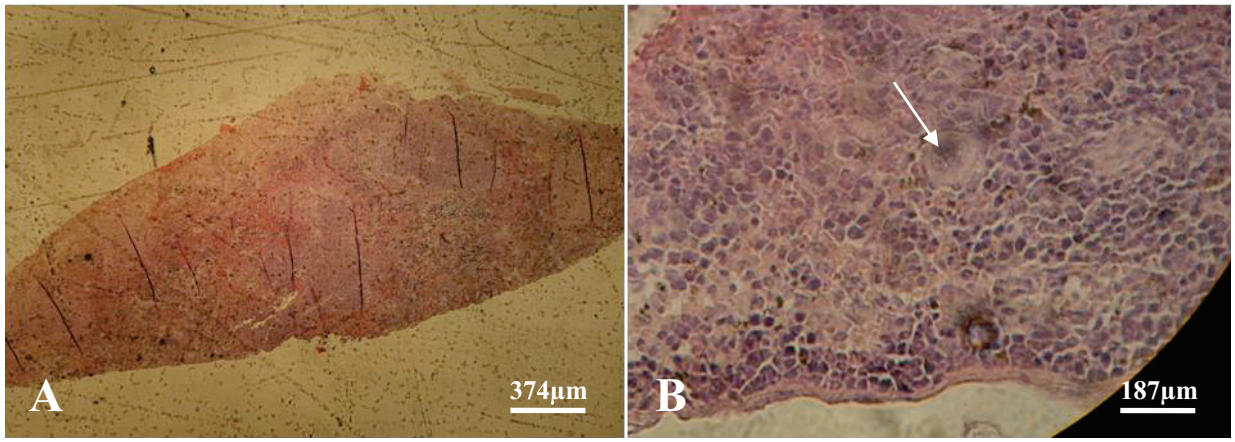

Figure 14. Cross sections of the mice spleen treated with DMBA, croton oil, and plant crude extract. (A) The splenic pulp dominated by the red pulp. (B) A single histiocyte, which is quite degenerated. H & E.

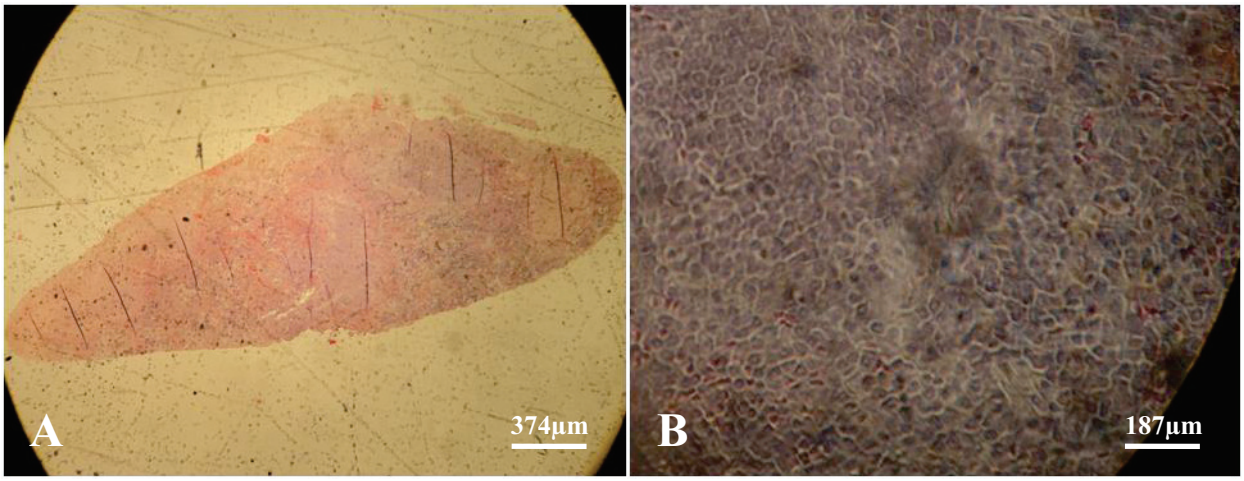

Figure 15. Cross sections of the mice spleen treated with DMBA, croton oil, and plant isolate. (A) The splenic pulp dominated by the red pulp. (B) No signs of secondary carcinoma, hystiocytic sarcoma. H & E.

Spleen treatment D (DMBA + Croton oil + Plant isolate MeONQ)

Just like in treatment C, the cross sections of the mice spleen treated with the plant isolate produced dominant red pulp. Also, the damaged white pulp was absent in this treatment (Figure 15A).

The cross sections did not show any signs of secondary carcinoma, hystiocytic sarcoma nor other types of abnormality (Figure 15B).

Statistical analyses

Kruskal-Wallis

The pancreas, stomach, and duodenum showed significance values of 0.000. The spleen, on the other hand, showed significance value of 0.001. The obtained values indicate that all organs can be tested further with ANOVA.

ANOVA

Analyses of the number of histological abnormalities in the pancreas, stomach, duodenum, and spleen were significantly different. All organs showed a *p*-value of 0.000. The following graphs were obtained for the four mouse organs (Figures 16 and 17).

DISCUSSION

Treatment A is the application of the tumor initiator, DMBA, and the tumor promoter, croton oil. Treatment B

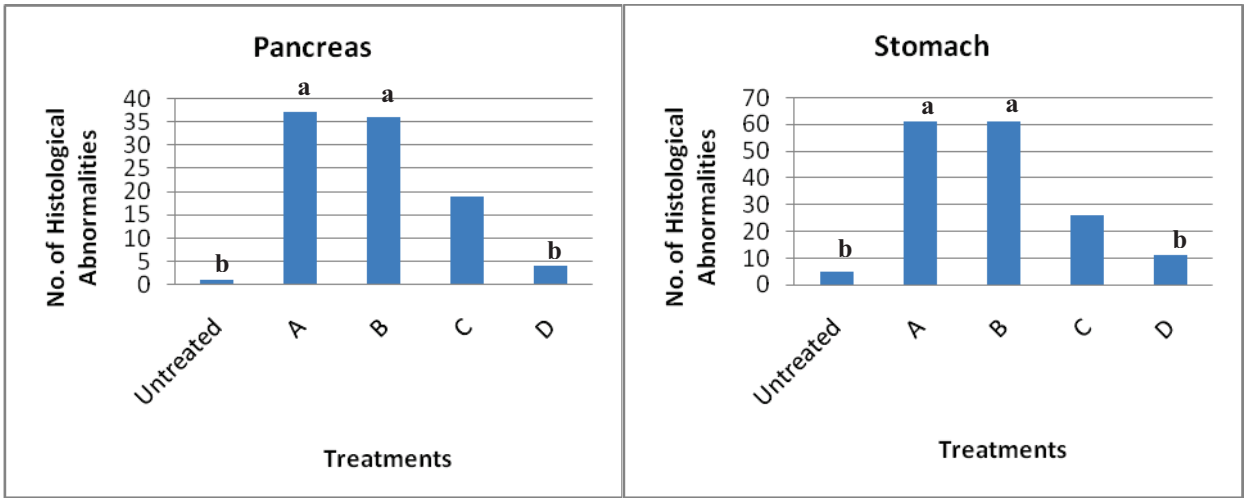

Figure 16. Frequency of histological abnormalities in the pancreas and stomach per treatment. Based on the number of trials, n = 5 (pancreas); n = 8 (stomach).

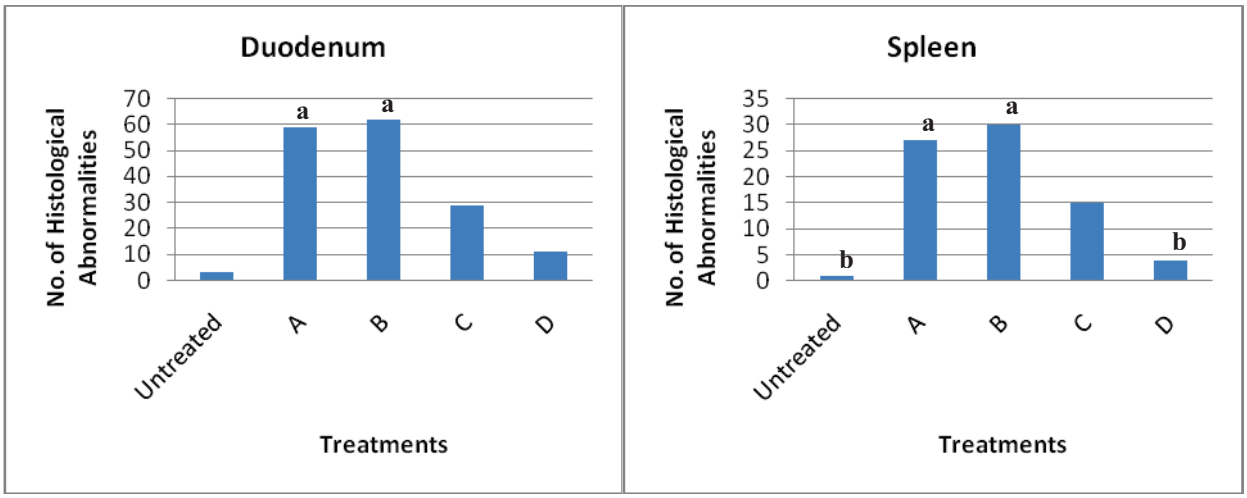

Figure 17. Frequency of histological abnormalities in the duodenum and spleen per treatment. Based on the number of trials, n = 8 (duodenum); n = 4 (spleen).

is the application of DMBA and croton oil in acetone. Treatment C is the application of DMBA, croton oil, and plant crude extract. Treatment D is the application of DMBA, croton oil, and plant isolate (MeONQ). The negative control is the untreated specimen.

In the pancreas, only one type of neoplasia was observed and that is pancreatic adenocarcinoma. As stated by Curran (1985), it is a common tumor which arises in the pancreas. No other type of neoplasia was observed. As discussed by Gold et al. (2001), tumor induction is rare in mouse pancreas. It occurs more frequently in rats.

In the stomach, epithelial dysplasia and adenocarcinomas of the intestinal and diffuse types were observed. According to Stevens et al. (2002), adenocarcinomas are the most common malignant tumors of the stomach. Also, the stomach is recorded to be one of the 8 most common target sites of chemical carcinogens in mice (Gold et al., 2001).

In the duodenum, only carcinoid tumor was observed. As stated by Stevens et al. (2002) and Gold et al. (2001), neoplasia in the small intestine is very rare except the carcinoid tumors and lymphomas.

In the spleen, secondary carcinoma and hystiocytic sarcoma were observed. Though induction of tumors is rare in the mouse spleen (Gold et al., 2001), according to Suttie (2006), some carcinogens still target the spleen.

A common trend was observed in all of the four organs, namely the pancreas, stomach, duodenum, and spleen. In both treatments A and B, tumor cells were produced

but they were more frequent in treatment B. Although some of the sections in treatments C and D produced traces of tumor, most of them did not display any tumor. Compared to treatment C, treatment D exhibited better antitumor potentials based on the frequency of tumor cells.

Tumor cells were produced in all of the organs because of the application of DMBA and croton oil. DMBA and croton oil are widely used as tumor initiator and promoter, respectively. In a study conducted by Sharma et al. (2004) and Das et al. (2005), two-stage skin carcinogenesis was induced in mouse with the use of these two chemicals. In this study, *in vivo* two-stage mouse carcinogenesis assay was done for the induction of tumors.

Treatment B involved the application of acetone in addition to DMBA and croton oil of treatment A. But statistical analysis based on the number of histological abnormalities showed that treatment B is not significantly different from treatment A in all organs (Figures 16 and 17). As stated by Chen et al. (1995), acetone only serves as a vehicle or solvent for all topically applied chemicals, which in this case are the DMBA and croton oil. Rapidly evaporating acetone, therefore, does not have any effect on the induction of tumor.

Reduction or absence of tumor cells was observed in treatments C and D. Statistical analysis based on the number of histological abnormalities showed that there is no significant difference between the negative control (untreated) and treatment D (Figures 16 and 17). This indicates that the plant isolate exhibited antitumor potential. This was observed in all of the organs except duodenum (Figure 17). In the duodenum, even though treatment D is significantly different from the negative control, it is still possible that it exhibited antitumor activity as seen from the decrease in the frequency of histological abnormalities (Figure 17). Treatment C, on the other hand, is also significantly different from the negative control. Despite of this, treatment C also resulted to decreased frequency of histological abnormalities (Figures 16 and 17). This was observed in all organs, indicating that the plant *I. balsamina*, in its crude extract form, also exhibits antitumor potential on the mice pancreas, stomach, duodenum, and spleen.

Statistical analysis showed that the plant isolate, MeONQ, has an efficient antitumor activity. As stated by Kamei et al. (1998), *I. balsamina* is capable of inhibiting human colon carcinoma cell growth in cultures. It has also been discussed by Villasenor and Domingo (2000) that the same plant has a great anticarcinogenicity potential when tested using the mouse skin tumor assay. In addition to this, *I. balsamina* has the potential to prevent the development of prostate cancer (Trump et al., 2001) and human breast carcinoma (Suzuki et al., 2001).

As stated by Ding et al. (2008), MeONQ is the main active component of *I. balsamina* that exhibits an intensive antitumor activity against human HepG2 cells *in vitro*. Also, as discussed by Wang et al. (2009), MeONQ exhibits strong potential for the eradication of *Helicobacter pylori*, which is classified as group 1 carcinogen.

CONCLUSIONS AND RECOMMENDATIONS

The *I. balsamina* crude leaf extract and the isolate, 2-methoxy-1,4-naphthoquinone (MeONQ), exhibited anti-tumor potential as seen from the results of the statistical analysis employed on the number of histological abnormalities seen in the mouse pancreas, stomach, duodenum, and spleen.

It is recommended that more trials in testing for the antitumor activity of *I. balsamina* be done in order to understand further its anticancer potential. Also, the antitumor activity of the said plant should be tested further on other mouse organs so as to determine the potency of the antitumor compound. Isolation and identification of other biological active compounds in the plant extract should also be conducted and experiments should be done to know more about the mechanisms by which compounds present in *I. balsamina* inhibit tumor formation.

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