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Screening for intestinal anti-inflammatory activity of *Alpinia galanga* against acetic acid-induced colitis in Mice (*Mus musculus)*

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Abstract
The objective of the study was to investigate the anti-inflammatory activity of *Alpinia galanga* rhizome extract against acetic acid induced colitis in male mice (*Mus musculus*). Twenty-eight (28) male albino mice were acclimatized for a total of two weeks prior to the actual experiment. Ten (10) of which were used in the preliminary experiment and the remaining eighteen (18) were used in the actual experiment. Each mouse (except for the control group, T0) was rectally instilled with 1 ml of 5% glacial Acetic acid. The treatments, distilled water, different concentrations of A. galanga extract (25%, 75% and 100%), and the positive control apple pectin were administered daily for 7 days via oral gavage. At study termination, the mice were sacrificed by cervical dislocation. Colon was fixed in 10% formaldehyde solution and was processed for histologic evaluation. Statistics showed that significant differences were found among the treatments in terms of depth of necrosis, (p=0.002) and extent of inflammation (p=0.046) and none as regards the extent of necrosis (p=0.107) and fibrosis (p=0.458). The above result suggests that 50% to 75% *Alpinia galanga* extract are potential anti-inflammatory agent to Acetic Acid induced colitis in mice, and have comparable effect to apple pectin exhibiting similar microscopic histoarchitecture to normal mice after being treated and appeared to be beneficial to mice in treating chemically induced colitis.

Keywords: *Alpinia galanga*, Acetic acid, Necrosis, Inflammation, Fibrosis

Introduction
Inflammatory Bowel Diseases (IBDs) are chronic inflammatory disorders of the gastrointestinal tract. Some of its characteristics include diarrhea, hemorrhage, lower abdominal pain and body weight loss. Crohn's disease (CD) and ulcerative colitis (UC) are categorized as typical IBD (Ito *et al*, 2006). The difference between these two forms is the location and nature of the inflammatory changes. Crohn's disease can affect any part of the gastrointestinal tract, most frequently the terminal ileum and colon. In contrast, ulcerative colitis exclusively affects the mucosal lining of the colon and rectum (Hale *et al.*, 2012) [12].
In the West, the incidence and prevalence of inflammatory bowel diseases has increased in the past 50 years, up to 8–14/100,000 and 120–200/100,000 persons, respectively, for ulcerative colitis (UC) and 6–15/100,000 and 50–200/100,000 persons, respectively, for Crohn's disease (CD). (Cosnes *et al.,* 2011). On the other hand, Philippine General Hospital in the year 1999 accommodated one (1) patient who was diagnosed with Ulcerative Colitis. After 4 years, the number of Inflammatory Bowel Disease-related cases grew up to twenty-two (22), which is diagnosed as either Ulcerative Colitis or Chron's Disease. (Feir, 2006) [8]. Both ends of the world share the same increasing trend as to the disease's increase in incidence.
The colon, also known as large bowel or large intestine is responsible for collecting and storing the waste products of digestion. It is a long muscular tube that pushes undigested food towards the anus for eventual elimination as a bowel movement. Food is digested in the stomach into liquid slurry that passes through the small intestine where the nutrients are absorbed into the body for use. When the liquid mixture enters the colon, it mixes with mucus and normal bacteria that reside in the colon. The wall of the colon has numerous layers. There is a smooth muscle layer that wraps the outside and is responsible for squeezing the undigested food through the length of the colon. The inner layer, or mucosa, comes into contact with the fluid and allows the absorption of water and electrolytes, which help to solidify the feces. The mucosal layer is where the colon inflammation occurs and is responsible for the symptoms of colitis (Boismenu, 2000) [1].

While a lot of things are discovered and is continued to be researched about the rhizome *Zingiber officinale,* another family of this plant is now making name as another helpful herb, the Galangal. The word galangal, in common usage can refer to four plant species, *Alpinia galanga* (greater galangal), *Alpinia officinarum* (lesser galangal), *Kaempferia galanga*, and *Boesenbergia rotunda*. Among these, the most common species found in the native land of Albay, Philippines is the *Alpinia officinarum* and *Alpinia galanga,* commonly called Lengkuas. (Department of Environment and Natural Resources R-V).

The rhizome of galangal resembles ginger in taste and appearance. It is also a source of sodium, iron, vitamins A and C. It contains the phytochemicals such as beta Sitosterol, Galangin, Emodin and Quercetin. It is commonly associated with a ginger which is a popular and more known spice, but a closer glance will give the difference between the two. Galangal has a tighter skin, lighter in color and can have pinkish portions too. This spice is popular in Asiatic cooking and was well-known in European medieval cooking. But aside from its use as a culinary ingredient, galangas have more to show in future researches. In other parts of the world, the rhizome is believed to be abortifacient, a substance that induces abortion. It is also used as carminative, a substance used to combat flatulence, anti-tuberculosis and stimulant properties. Aside from these internal uses, ground rhizome is also used as treatments for skin infections such as eczema, ringworm and other skin diseases. In Germany, herbalists use lesser galangal for dyspepsia biliary symptoms, bowel spasm and angina. In China, Galangal is used to relieve flatulence, dyspepsia, nausea, vomiting, loss of appetite, and motion sickness. The latest news about the galangal effect is that, people in Thailand now makes perfume out of this plant. (Indrayan *et al*., 2009) [13].

The study will enable further understanding on the efficacy of *A. galanga* extract to treat colitis, where chemical-induced models of gut inflammation are the most commonly used and best described models of the disease. This study will prove on the development of new drugs out of this indigenous rhizome found in our local land. This study could be used by future researchers as reference on the effects of *A. galanga* to Inflammatory Bowel Diseases related cases. There is currently no known cure for ulcerative colitis, so the aim of treatment is to relieve symptoms during an outbreak and prevent symptoms from returning during remission. A common drug used to treat Inflammatory Bowel Diseases (IBD) is Sulfasalazine, a drug also known to indulge rheumatoid arthritis. Aside from its expensive cost at the market, this drug has established side effects like loss of appetite and nausea (AHFS® Consumer Medication Information, 2010). People living in developing country like Philippines just do not have financial resources to pay for the high costs involved with manufacturing modern medicines. With this, a need to export foreign drugs raises which results to its expensive cost at the market. This is an extra burden to people from such countries. The study generally aims to determine the protective effect of *A. galanga* extract to Acetic Acid- induced colitis in mice. Specifically, it aims to determine the effective concentration of *A. galanga* extract as potential anti-inflammatory agent to Acetic Acid induced colitis in mice and conduct histological evaluation of the colon upon administration of *Alpinia galanga* rhizome extract.

Review of Related Literature

Increase interest in the botanical and herbal effects happens today. Researches all around takes place to see the potentials of the earths' creatures longed trusted by man. Medicinal plants and derived medicine are widely used in traditional cultures all over the world and they are becoming increasingly popular in modern society as natural alternatives to synthetic chemicals.

Alpinia galanga Plant

Alpinia galanga is commonly known as Greater galanga. Its root stocks are tuberous and slightly aromatic, Leaves are oblong, lanceolate, acute, glabrous, green above, paler beneath, with slightly callus white margins. Sheaths are long and glabrous while the ligules are short and rounded. Flowers are greenish white, 30 cm Panicles and bracts ovate are lanceolate. Calyx is tubular, irregularly 3-toothed. Corolla lobes are oblong, claw is green, and blade is white and striated with red, rather more than 1 cm long. It is broadly elliptic, shortly 2-lobed at the apex, and with a pair of subulate glands at the base of the apex, with a pair of subulate glands at the base of claw. Fruit size resembles that of the small cherry, which colors are orange and red. (Verma *et al.,* 2011) [28].

Alpinia galanga is locally known as langkuas. In Ayurvedic system, the rhizome is used to improve appetite, taste and voice. It is also useful in bronchitis and disease of the heart. In Unani system, rhizomes have been used for stomachic, aphrodisiac, tonic, diuretic, and expectorant, carminative, useful in headaches, rheumatic pains, sore throat, sour eructation, stuttering, pain in chest, diabetes, burning of the liver, tubercular glands and diseases of the kidney. In Thai folk system, the rhizomes of this plant are extensively used as carminative, ant flatulent, antifungal and anti-itching.

Biological and Pharmacological Action

During past several years, *Alpinia galanga* is gaining lot of interest. Recently, many pharmacological studies have been conducted on *Alpinia galanga*. A summary of the findings of these studies performed is presented below.

Active Components

A. galanga has been thoroughly studied by various researchers and a number of major as well as minor chemical constituents belonging to different classes of natural products have been isolated. The GC-MS analysis showed that the main compounds of galangal extract are 1, 8-cineole, β-bisaboline and β-selinene. Whereas α-selinene, farnesene, 1,2-benzenidicarboxylic acid, germacrene B and pentadecane are the minor components. Active components of *A. galanga* include its natural chemicals responsible for its unique aroma. Among these are hydroxyl-1-8-cineole, glucopyranosides, (1R, 2R, 4R) and (1S, 2S, 4R)-trans2-hydroxyl-1,8-cineole (Chudiwal *et al*., 2010) [4].

Gastric Anti-Secretory

Gastric anti secretory, and cytoprotective properties of ethanolic extract of *Alpinia galanga* Willd. in rats were studied. It is that said rhizomes of *A. galanga* are used widely in Arabian and Unani systems of medicine to treat stomach disorders. The ethanolic extract also significantly reduced gastric secretion and showed marked cytoprotective activity. It is suggested that these properties may be responsible for the antiulcer activity of *Alpinia galanga.*

Apple Pectin as Positive Control

Apple pectin is known to be used in treatment of overeating. Apple pectin reduces the rate of digestion by immobilizing food components in the intestine. This results in less absorption of food. The thickness of the pectin layer influences

the absorption by prohibiting contact between the intestinal enzyme and the food, thus reducing the latter's availability. In medicine, pectin increases viscosity and volume of school that is used against constipation and diarrhea. Pectin has a promising pharmaceutical uses and is presently considered as a carrier material in colon-specific drug delivery systems. The rationale for this is that, pectin and calcium pectinate will be degraded by colonic pectinolytic enzymes, but will retard drug release in the upper gastrointestinal tract due to its insolubility and because it is not degraded by gastric or intestinal enzyme. (Srivastava *et al.,* 2011) [24]

Acetic Acid as Inducing Agent

Acetic acid is also known as ethanoic acid, ethylic acid, vinegar acid, and methane carboxylic acid. It has the chemical formula of CH_3COOH (molecular weight of 60.05). Glacial acetic acid is the pure compound (99.8%), as distinguished from the usual water solutions known as acetic acid. It is a colorless liquid with a pungent, vinegar-like odor, and has an upper taste threshold of 1,000 parts per million (ppm), a lower taste threshold of 300 ppm, and an odor threshold of 24 ppm. The boiling point of acetic acid is 118°C and the melting point of its rhombic crystals is 16.6 °C. Glacial acetic acid is highly corrosive to metals. Acetic acid is soluble in alcohol, miscible with water, glycerol, ether, acetone, benzene, carbon tetrachloride, and practically insoluble in carbon disulfide. Acetates which are the salts of acetic acid are common constituents of animal and plant tissues and are formed during the metabolism of food substances. Typical concentrations of acetic acid occurring naturally in foods are 700 to 1,200 milligrams/kilogram (mg/kg) in wines, up to 860 mg/kg in aged cheeses, and 2.8 mg/kg in fresh orange juice. Estimated possible average daily intakes of acetic acid and sodium acetate, based on food intake concentrations for persons more than 2 years old are estimated at 2.1 grams (g)/day and 0.23 g/day, respectively. Acetic acid is absorbed from the gastrointestinal tract and through the lungs. It is readily metabolized by most tissues and may give rise to the production of ketones as intermediates. In vitro experiments have demonstrated that acetate is incorporated into phospholipids, neutral lipids, steroids, sterols, and saturated and unsaturated fatty acids in a variety of human and animal tissue preparations. (Virginia Department of Health.) Rats receiving acetic acid in their drinking water of up to 0.5 percent, for 2 to 4 months (daily doses up to 390 mg/kg) were found to lose body weight which is apparently due to anorexia, at the highest dose, but no such effects were observed up to concentrations equivalent to 195 mg/kg daily. There were no fatalities in any of these dose groups. In rats fed acetic acid (4.5 g/kg/day) in the diet for 30 days, gastric lesions occurred in some animals, whereas others revealed slight forestomach wall thickening or inflammatory changes (Virginia Department of Health).

Materials and Methods

Collection and Extraction of Plant Material

Fresh rhizomes *of Alpinia galanga* were collected in Taysan, Legazpi City where the plant is well established. It was peeled off and chopped into smaller cubes so it will fit the blender. After turbo mixing, filter paper was used to extract the pure juice of the rhizome. Different concentrations of extract were prepared. Low concentration (25%) was done by adding 25 ml of pure extract with 75 ml of distilled water. Medium concentration (50%) was fixed by adding 50 ml of pure extract with 50 ml distilled water. Lastly, the high concentration (75%) was prepared by adding 75 ml pure extract with 25 ml of distilled water. The sets of extract were refrigerated until the time they were used.

Test Animals

ICR strain male albino mice were the model for induction of Inflammatory Bowel Disease (IBD) using 5% Acetic Acid. In this study, twenty eight (28) seven-to-eight week old ICR strain mice were obtained from the Bureau of Animal Industry, Manila City, Philippines. Ten (10) of which were used in the preliminary experiment to assess the concentrations which are non-toxic to mice. The animals were kept in the Animal House Unit of Bicol University, College of Science, with *ad libitum* administration of food and water. Procedures that were made in the animal testing was in accordance with the existing guidelines of the Philippine Association of Laboratory Animal Science (PALAS) for care and proper use of laboratory animals and with the Administrative Order 40 of the Bureau of Animal Industry relative to Republic Act No. 8485.

Actual Experiment

Animals were acclimatized for 2 weeks prior to the start of the experiment. Eighteen mice were divided into six groups. The mice were fasted for a night before the induction of colitis but had free access to drinking distilled water. Each of the mice were tagged and weighed before the start of the experiment. The initial weight of each mouse was recorded.

Induction of Experimental Colitis

The mice were anesthetized using the open drop method, where the mice were subjected to inhalation of diethyl ether open drop in a jar with cotton soaked after the chemical. A 5 cm long plastic catheter was advanced 3 cm from the anus. Mice were in Trendelenburg position during this process and 1 ml of 5% Acetic Acid was slowly administered transrectally. The mice were maintained in head-down position for 30 seconds to prevent leakage, and the rest of the solution was aspirated. T0 group (normal control) animals received distilled water instead. (Popov *et al.,* 2006) [21]. After 24 hours, the five groups of mice were treated differently with distilled water, apple pectin and the various concentrations of *Alpinia galanga* extract given as gavage for seven days. The control group didn't receive any treatment but the usual distilled water and food pellet. All animals from each group were tagged for easy identification during observations. The weight on the 2nd and 5th day was recorded. On the 8th day of the study, all animals were weighed and later sacrificed by cervical dislocation, a commonly used and humane method of killing most small rodents, as it causes extensive damage to the brainstem resulting in immediate unconsciousness and death. This was done by pressing down the thumb and index finger on the sides of the neck at the base of the skull and the other hand quickly pulled the base of the tail. This caused the separation of the cervical vertebrae from the skull. Colon biopsies were removed for histopathological examination subsequently.

Assessment of Colonic Damage

The experimental units were sacrificed to harvest the organ to be obtained. Entire colon was isolated and rinsed with 10% formaldehyde solution to preserve for histological examination. For slide preparation, the colon was brought at the Histopathology laboratory of the Philippine Kidney Dialysis Foundation (PKDF), Quezon City Philippines. Reading of the prepared slides was done in Bicol Regional Training and teaching Hospital (BRTTH) guided after the professional expertise of Dr. Karlo Emir M. Tayson, the

resident pathologist. The histologic scoring of induced colitis was determined by examining each specimen for the following features and allocating increasing points according to the severity of the findings in depth of necrosis, extent of necrosis, extent of inflammation and fibrosis. The microscopic scoring of the colon damage was modified following the criteria described by Murat (2006) [17].

Table 1: Criteria for Microscopic Scoring of Colon

Score	Depth of Necrosis	Extent of Necrosis	Extent of Inflammation	Fibrosis
0	None	None	None	None
1	Mucosal	Small area	Mucosal	Mucosal
2	Mucosal and submucosal area	Moderate area	Mucosal and submucosal area	Mucosal and submucosal area
3	Mucosal, submucosal area, and muscularis propia	Large area	Mucosal, submucosal area, and muscularis propia	Mucosal, submucosal area, and muscularis propia
4	Full thickness	Extensive	Full thickness	Full thickness

Statistical Analysis

The data gathered was analyzed using ANOVA (Analysis of Variance) to determine the significant differences among treatments. Duncan's Multiple Range Test (DMRT) was used for multiple comparisons.

Results and Discussion

In this study, a total of eighteen (18) male albino mice of 8 weeks old with comparable weight were used as experimental units. The mice were treated with the varying dosage of the *A. galanga* fresh extract. T0 was the untreated group, fed regularly with standard feeds and ad libitum distilled water. The actual treatment concentrations were: T1 is 1 ml of distilled water for the Negative control, T2 is 1 ml of 25% *A. galanga* fresh extract, T3 is 1 ml 50% *A. galanga* fresh extract, T4 is 1 ml 75% *A. galanga* fresh extract and T5 is 1 ml Apple pectin for positive control. The data were recorded, tabulated and analyzed for comparison.

Effects on Body Weight

Five percent (5%) glacial Acetic Acid when instilled intra-rectally into the colon led to gradual decrease in body weight as observed in days 2, 5 and 7 in all groups of experimental animals, except for the untreated normal control group which remained constant with no significant change. After a week, untreated mice have shown to increase weight by 3.57% and 7.14% on the 5th and 7th day, respectively. This shows that normal mice would tend to gain weight when given with normal intake of food and water. On the other hand, the acetic acid induced colitis groups showed significant decrease in weight.

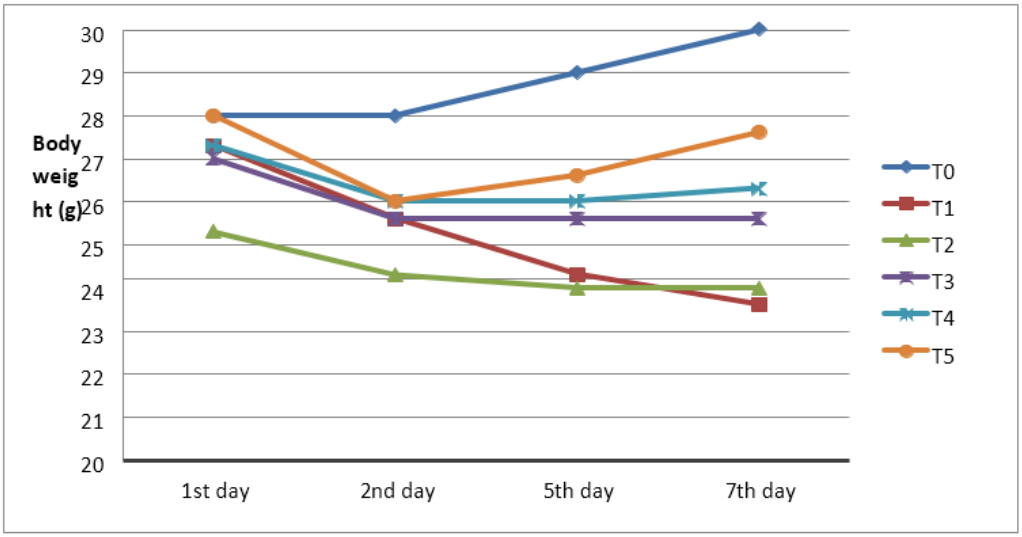

Fig 1: Mean body Weights of Albino Mice at Varying Experimental Treatments

It will be noted that in this graph, continuous decrease of body weight was observed to the T1 and T2 group, which received distilled water and a 25% *Alpinia galanga* extract respectively daily for one week. On the other hand 75% *Alpinia galanga* extract given to T4 showed a potential in reversing the decreasing trend which is in no significant difference as to the trend of animals' weight in group T5 which was treated positively with apple pectin.

Microscopic Scoring Results

In the study, colitis was successfully produced by intra-rectal instillation of 1 ml glacial 5% acetic acid. This was assessed microscopically with the inflammation of White Blood Cells and macrophages to the intestinal mucosal tissue.

Table 2: Microscopic Histoarchitecture of the Normal and the Pathologic Colon of Mice at Varying Concentrations of Galanga Extract (Mean±SEM)

Treatments	Depth of Necrosis	Extent of Necrosis	Extent of Inflammation	Fibrosis
To (Untreated)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T1 (Distilled water)	1.67±0.88	1.33±0.67	2.33±0.67	1.00±5.77
T2 (25% *Alpinia galanga* extract)	2.33±0.33	1.00±0.58	1.67±0.88	1.00±5.77
T3 (50% *Alpinia galanga* extract)	0.00±0.00	0.67±0.33	0.33±0.33	0.33±0.33
T4 (75% *Alpinia galanga* extract)	0.00±0.00	0.00±0.00	0.67±0.33	0.33±0.33
T5 (Apple Pectin)	0.00±0.00	0.00±0.00	0.33±0.33	0.33±0.33

Inflamed gut from IBD patients and animal models, are rich in activated macrophages and neutrophils and these inflammatory cells generate excess amounts of Reactive oxygen species (ROS) with subsequent increases in oxidative stress. These ROS include hydroxyl radicals, superoxide anions, hydrogen peroxide, and nitric oxide. ROS are extremely unstable species due to their high reactivity and may result in excessive generations of free radicals like lipid peroxidation and NO and the oxidation of DNA and proteins. Cellular antioxidants like SOD and NO are protective against the free radicals which are deleterious for the tissue milieu. This antioxidant system was deranged after treatment with acetic acid as found in the colonic mucosal homogenates. Acetic acid induced colonic injury leads to activation of arachidonic acid pathways leading to production of inflammatory mediators and formation of free radical or ROS (Ghatule *et al*, 2012) [10].

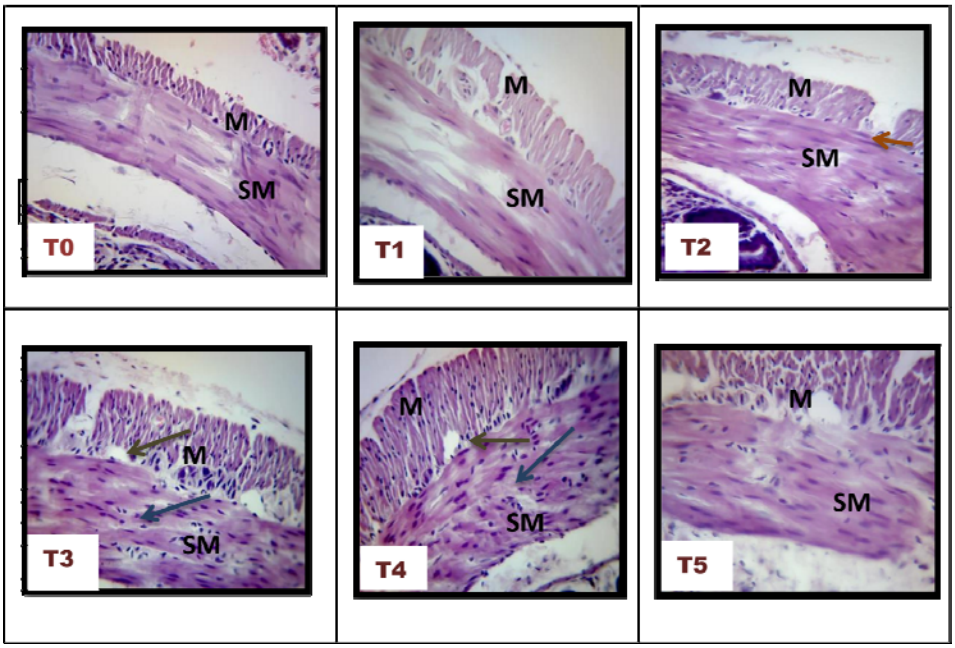

Fig 2: Histological Characterization of Male Albino Mice Colon at Different Experimental Treatments. Stained with H&E. (400x). T0, (control group), T1 (Distilled Water), T2 (25% *A. galanga* extract), T3 (50% *A. galanga* extract), T4 (75% *A. galanga* extract), T5 (Apple pectin). Blue arrows: fibrotic cells; Brown arrows: damage in basal crypt; Orange arrow; Crypt destruction. Mucosa (M); Submucosa (SM).

Inflammatory Bowel Disease is characterized by the involvement of ROS in tissue damage. Increased damage in mucosa and crypts are shown in Figure 3 (T1, T2 and T3) treated with various concentration s of *A. galanga* extract. With this, inflammatory mediators, such as toxic oxygen metabolites, lysosomal enzymes and derivatives of arachidonic acid metabolism, are released. It has been proposed that the inflammation of mucosa causes impairment of the antioxidant defense mechanism, and make tissue more susceptible to oxidative damage (Buffinton *et al.,* 1995). In turn, superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, secreted by neutrophils and phagocytes accumulating in the inflammatory lesions cause impairment of cellular membrane stability and cell death by leading lipid peroxidation. Colonic biopsy from specimens from patients with active IBD had enhanced level of lipid peroxidation products. These finding suggest that chronic gut inflammation promotes an imbalance activity between pro oxidant and antioxidant mechanism, leading to the net accumulation of oxidative modified proteins and lipids (Reifen *et al.,* 2000). Rhizomes of *A. galanga* have been found to contain galangin, which is a known anti-inflammatory agent that acts by inhibiting cyclooxygenase enzyme. Therefore this might be its probable mechanism of anti-inflammatory action. Also, flavonoids found in the *A. galanga* possess antiproliferate activity that causes decrease in the weight and volume of contents of granuloma in inflammation.

In a study made by Reddy in 2011, it is mentioned that galangin, a rich substance found in *A. galanga*, inhibits nuclear transcription factors in the expression of genes involved in inflammatory processes and cancer. Research suggests that galangin, activities, does via TNF-α. These effects are induced through the inhibition of phosphorylation and dysfunction tumor necrosis factor - alpha, which is a pre inflammatory agent. Tumor necrosis factor - alpha is an inflammatory agent with a significant role in the creation of inflammatory bowel disease. Increased serum levels of this factor is confirming in patients with intestinal ulcers.

Conclusions and Recommendations

Based on the statistical analysis and interpretations that were carried out, 5% glacial acetic acid in dose of 1 ml, produce colitis in mice. Mice administered with *A. galanga* extract employed by the T4 (75%) was verified to give the highest potential to reverse the decreasing trend of body weight after intra-rectal induction of 5% acetic acid. Histological assessment was also done to evaluate the possible effects of the treatments compared to the untreated group and the negative control. In conclusion, this study showed that 75% of A. galanga rhizome extract have intestinal anti-inflammatory potential that was proven by histopathological analysis. However, this claim needs further proof as to know the active constituents appearing to treat the inflammation caused by the induction of chemicals like Acetic Acid.

The present study did not focus much on the identification and isolation of the substances that may have effects on the colonic inflammation, so with this, the researcher recommends to do further analysis of the active components of *A. galanga* plant. It is also proposed that other forms of extraction be used to make sure that the active substance needed for the anti-inflammatory activity be isolated and be surely utilized. Different strains of albino mice like an inbred strain such as

Balb/C and other experimental units may be used. To better assess the inflammation, the researcher also endorse the need to have a macroscopic evaluation. This will add credibility to the claim that the colon has been inflamed without doing histologic or microscopic evaluation.

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