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Toxic Effects in Gaster of *Rhizoma Coptidis* (Huang Lian) Treated Mice using Histology Technique

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Abstract

The study was conducted to examine the effects of 65 mg/kg, 250 mg/kg and 500 mg/kg of *Rhizoma coptidis* (Huang Lian) on the changes in gastric epithelium tissue, submucosa tissue and muscularis tissue of mice. In the experiment, sample of gaster organ of control group mice, 65 mg/kg *Rhizoma coptidis* treated mice, 250 mg/kg *Rhizoma coptidis* treated mice and 500 mg/kg *Rhizoma coptidis* treated mice are collected. All samples are stored in 10% (v/v) neutral buffered formalin solution with pH 6.8-7.2 at 25˚C. In the first procedure, each sample underwent tissue processing and tissue sectioning. Then, data were collected by observing tissues under microscope for gross and microscopic examination. There was no significant change in gross appearance of gaster of control group, 65 mg/kg, 250 mg/kg and 500 mg/kg of *Rhizoma coptidis* treated mice. In additional, the microscopic examination in the gaster of 65 mg/kg and 250 mg/kg of *Rhizoma coptidis* treated mice, the layer of mucosa, submucosa and muscularis externa can be identified. Gastric pits can be identified clearly with no sign of erosion. However in the gaster of 500 mg/kg of *Rhizoma coptidis* treated mice, there were presence of black dots which indicates infiltration of inflammatory cells filling in lamina propria. There was high infiltration rate in mucosa layer indicates inflammation happens at the mucosa layer. Minor detachment of tissue in this 500 mg/kg treated mice can also be observed under microscope. It can be concluded that the gaster of 500 mg/kg of *Rhizoma coptidis* treated mice brings morphological changes to the tissue cells in gaster, specifically causes infiltration of inflammatory cells, and indicates that 500 mg/kg of *R. coptidis* carries toxic to the tissue cells at microscopic level.

Keywords

Epithelium, Gaster, Huang Lian, Inflammation, Microscope, Rhizoma coptidis, Toxic.

Introduction

Rhizoma coptidis refers as the dry rhizome of Coptis Chinensis Franch, Coptis Deltoidea C.Y. Cheng et Hsiao or Coptis teeta Wall (Chen, 2013). It was firstly recorded in Shennong's Meteria Medica (200-250AD) in Eastern Han dynasty and was classified as top grade herb (Liu et al., 2015). *Rhizoma coptidis*, which is also known as Huang Lian in China was first seen in The Divine

Farmer's Materia Medica, classified as upper class herbs. It mainly originated from Hubei, Hunan, Guizhou, Sichuan and Chongqin of China, found in forests or shady valleys with an altitude of 500 to 2000 m, pH 5.5-6.5 (Liu et al., 2015).

Figure 1: Processed *Rhizoma coptidis*

Figure 2: Fresh *Rhizoma coptidis*

In modern medicine, numerous studies on berberine, an alkaloid isolated from *Rhizoma coptidis* was reported to have anti-microbial effects (Kong et al., 2012) and anti-neoplastic effects (Tang et al., 2009), specifically on pancreatic cancer (Pinto-Garcia et al., 2010) and nasopharyngeal cancer (Li et al., 2014). It was also reported that berberine has hepatoprotective (Feng et al., 2011), nephroprotective (Domitrović et al., 2013), neuroprotective (Chai et al., 2014) and cardioprotective effects (Gong et al., 2012). Studies have also explored the effects of berberine in controlling metabolic syndrome (Pérez-Rubio et al., 2013), hyperlipidemia (Kong et al., 2008), and type II diabetes (Yin, Xing and Ye, 2008), highlighting berberine as multifaceted drugs with immerse therapeutic potential.

The main bioactive compounds found in *Rhizoma coptidis* are alkaloids which act as anti-bacterial agent, anti-virus agent, anti-inflammatory agent, anti-tumor agent and anti-arrhythmic agent. There are 6 major active alkaloids in *Rhizoma coptidis*, namely berberine, coptisine, palmatine, epiberberine, magnoflorine and jatrorrhizine (Han et al., 2011). Among the 6 active alkaloids, berberine (2,3-methylenedioxy-9,10dimethoxy-protoberberine) holds the greatest weightage. It is a quaternary ammonium salt from the group of isoquinoline alkaloid which presents to be yellow in color when isolated. Berberine has a molar mass of 336.36122 g/mol and has high concentration in roots, rhizomes and stem bark of various plants, including *Rhizoma coptidis* (Birdsall et al., 1997). *Rhizoma coptidis* is contraindicated with erythromycin

lactobionate, aminoglycosides antibiotics, gentamicin, streptomycin and Kanamycin A (Yu et al., 2006).

Various researchers had found out that *Rhizoma coptidis* has various potential functions. It could be a potent inhibitor of TNF-α induced inflammation in dermatological conditions (Enk et al., 2007), and potential effective drug in treatment of cyclophosphamide-induced cystitis (Xu and Malavé, 2008). In fact, some researchers believe that the over dosage of *Rhizoma coptidis* may lead to the adverse effects such as nausea, vomiting, diarrhea and liver damage (Zhu et al., 2015). However, the effects of different dosage of *Rhizoma coptidis* on normal cell especially in gaster as well as the toxicity of it have received little attention. This is a primarily exploratory study allowing the understanding of changes in the outlook and histological view of gaster in *Rhizoma coptidis* treated mice of different dosage, specifically 65 mg/kg, 250 mg/kg and 500 mg/kg. The findings are in qualitative data where data collected will be analysed on the histological changes at epithelial layer, submucosa layer and muscularis layer. Morphology of the tissue structure should be seen clearly.

Methodology

The study was conducted in Learning Resource Centre Level 4, Laboratory PL1 in INTI International University, Negeri Sembilan, Malaysia. All animal experiments were performed in accordance with the Universiti Kebangsaan Malaysia animal ethics guideline formulated in accordance to the guidelines of the National Health and Medical Research Council of Australia. Female BALB/c mice at the age of 4-6 weeks old which were obtained from the Animal House Facility, Universiti Kebangsaan Malaysia. Mice were maintained under specific pathogen-free conditions in a positive pressure environment, subjected to a 12h light/dark cycle and fed with protein-enriched diet and water *ad libitium*. Mice were force-fed by gavages with 1000 µl of *R. coptidis* or Huang Lian (65 mg/kg, 250 mg/kg and 500 mg/kg). The control group received the same volume of distilled water orally. The general appearance of mice was monitored daily and weight of mice were measured on 1st and 4th week. At the completion of treatment duration, mice were killed by cardiac puncture and gaster organs were collected and stored.

Gaster organ of control group mice (G-control), 65 mg *R. coptidis* treated mice (G65), 250 mg *R. coptidis* treated mice (G250) and 500 mg *R. coptidis* treated mice (G500) which have been collected and stored in 10% (v/v) neutral buffered formalin solution, pH 6.8-7.2 at 25˚C (room temperature). At least 5 gaster organs were collected from each group and were stored accordingly to the standard protocol prior to the analysis. The gross appearance of gaster organs were observed under stereo microscope. Frontal dissections were performed on G-control and were observed under stereo microscope and the gastric contents (if any) then removed from gaster organs. Similar procedures were carried out on G65, G250 and G500. Organ tissues were placed in cassettes followed by labeled identification number with lead pencil. Standard protocol of histology techniques was referred using Slaoui et al., (2011). Dehydration process were carried out where water was removed completely out from tissues. The tissues passed through a series of increasing concentration of alcohol. Ten Coplin staining jars were prepared and filled with 80% alcohol, 80% alcohol, 90% alcohol, 100% alcohol, 100% alcohol, 100% alcohol, xylene, xylene, paraffin and paraffin respectively. Cassettes containing G-control, G65, G250 and G500 were soaked into Coplin staining jar for an hour. The immersion of tissues in paraffin were carried out in paraffin

oven at 50-56˚C. Tissue then placed in Leuckhard embedding box, filled with warm liquid paraffin which forms a firm block after cooling and this was known as embedding process.

During the embedding process, Leuckhard embedding box was arranged on a glass slide of embedding machine. The specimens were placed at the bottom of cavity at right orientation while the identification number was placed adjacent to the tissue and care was taken so that it will not get in the way of knife blade. The mold then was kept in refrigerator for 5 days to ensure complete hardening of the wax. The molds were readied to be cut using a microtome. The block was first fixed in the block holder of microtome machine. Then knife was inserted tightly in knife holder with proper position. Adjustment was made so that the block almost touches the knife and was parallel to the edge of the knife to ensure a straight ribbon was produced during the sectioning. The gauze that controls the thickness of section was adjusted to 8mm and the microtome was operated until complete sections of tissues were obtained. As the microtome head moved with the blocks, section was cut and laid on knife, and then a ribbon was produced.

The ribbons undergone water bath with Microm SB 80 model of tissue floating bath at 55˚C. The structure of sectioning was 'fished' with microscopic slide and undergone deparaffinization by placing the slides on hotplate at 42˚C for 24 hours. After that, slides were immersed into xylene for 3-4 minutes agitationally. The slides were passed through a series of decrease concentration of alcohol, which are 100%, 90%, 80% and 70% for 30-60 seconds in each of the alcohol solutions. This was known as hydration process. After hydration process was completed, the slides were rinsed with tap water follow by distilled water. Afterwards, the sections subsequently drained well before staining. During the staining process, hematoxylin solution was prepared and slides were soaked in for 3-5 minutes follow by rinsed under running tap water. After that, the slides immediately dipped in and out of 0.5% (v/v) hydrochloric acid. Observation of slides under microscope is necessary to ensure the nuclei appeared dark purple while the rest of the tissue appeared pale. Then slides were rinsed quickly under running tap water for 30-60 seconds. Later on, slides were dipped several times in 30% dilute ammonia water then washed with tap water. Rinsing of slides then carried out in 95% alcohol follow by eosin solution in agitate manner for 10-60 seconds. Next, dehydration process was carried out by immersing microscopic slides into 70% alcohol, 95% alcohol and 100% alcohol in sequence for 30 to 60 seconds each. Subsequently, clearing process took placed by placing the slides in xylene twice for 30-60 seconds. The excess xylene was drained and mounted on DPX solution with cover slip. Lastly, microscopic slides were left for 48 hours at room temperature prior to microscopic examination.

Results

The effects of *R. coptidis* toward gaster cell in mice were observed using stereo microscope for the observations of significant changes such as color and structure on gross appearance on mice. Observation of gross appearance of control group and 500 mg/kg *R. coptidis* treated gaster are made. Further observations on tissue structure were examined and recorded under microscope including histological view of control group, 65 mg/kg *R. coptidis* treated gaster, 250 mg/kg *R. coptidis* treated gaster and 500 mg/kg *R. coptidis* treated gaster organs. Distribution of cells, changes of structure in gastric epithelium tissue, submucosa tissue and muscularis tissue were observed and recorded.

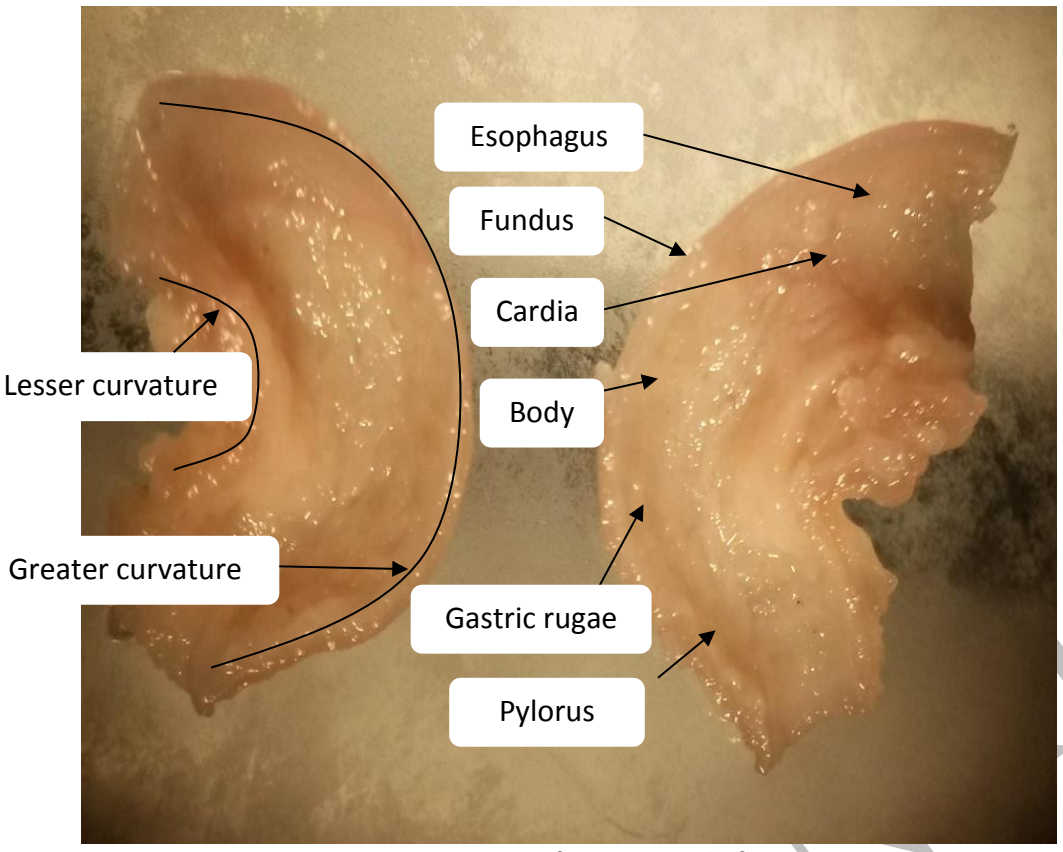

Figure 1: Gross appearance of G-control at frontal dissection.

Observed gaster organs showed the general J-shape of gaster organ which was brownish in color. The fundus is in dome shape. The lesser curvature extends between cardiac and pylorus which forms the medial border of stomach. Each part of gaster were identified clearly. Gastric rugae were observed clearly under stereo microscope. Frontal dissection showed no significant changes in structure or color of gaster organs. There were absent of abnormal appearance such as bleeding spot or black spot which indicates irreversible necrotic lesion. The same results were obtained for G-65 and G-250 gaster organs.

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The layer of mucosa, submucosa and muscularis externa were identifed. The mucosa layer were filled with gastric glands and pits. The prominent layer of smooth muscle, which was the muscularis mucosa were seen clearly. It also shown clearly each sections which were mucosa layer, submucosa layer, muscularis layer and serosa layer. There were no sign of rupture or infiltration of inflammatory cells in the tissue and absence of filling of blood vessels in lamina propria. Gastric pits were identified clearly with no sign of erosion. The same results are obtained for G-65 and G-250 gaster organs.

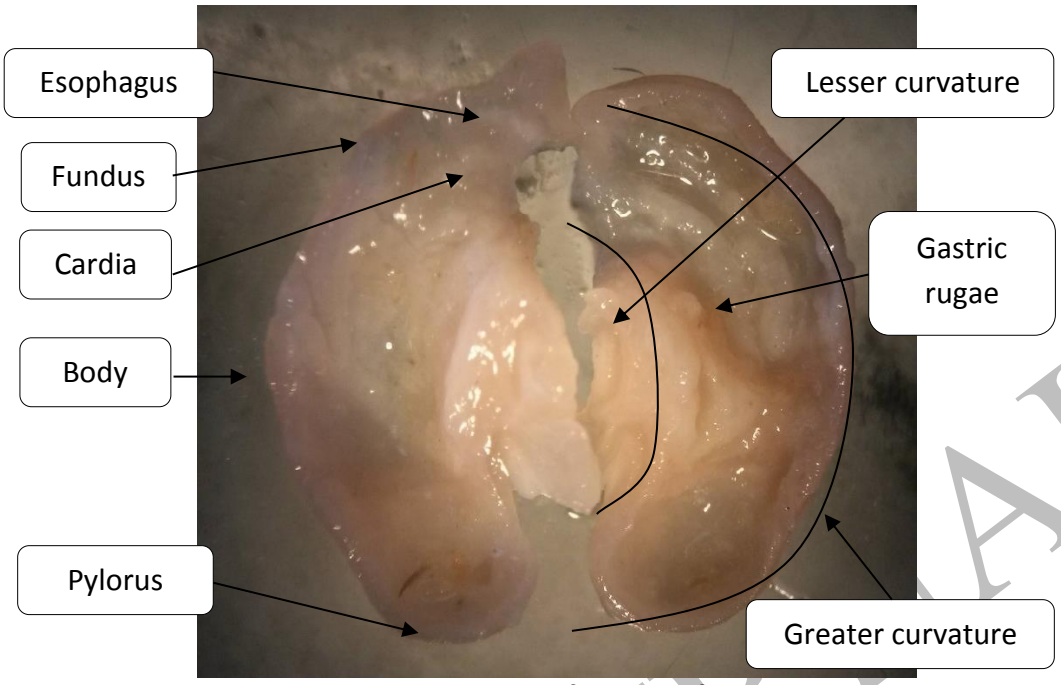

Figure 2: Gross appearance of G500 at frontal dissection.

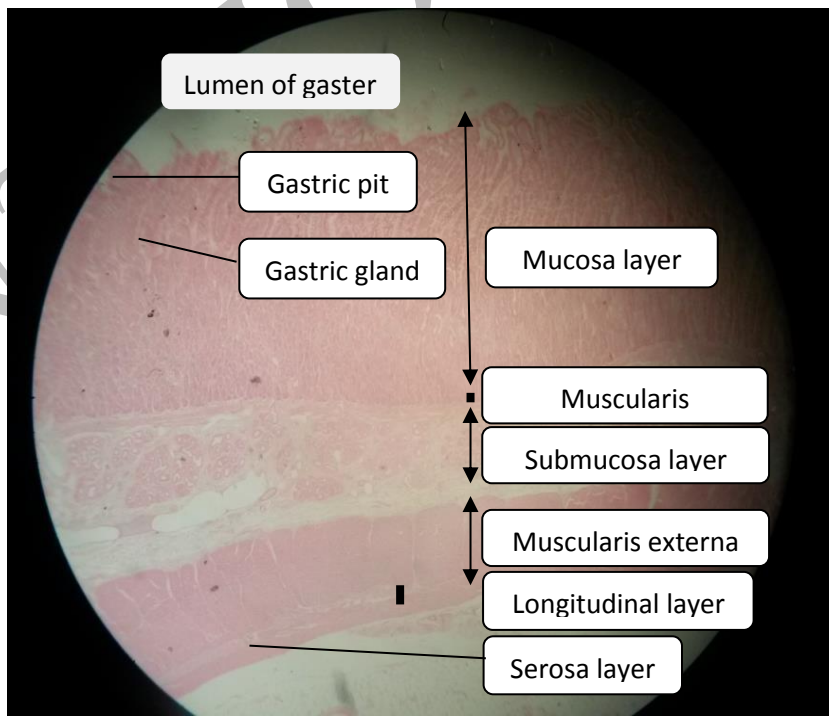

Figure 3: G-Control microscopic view at frontal dissection under 40X magnification.

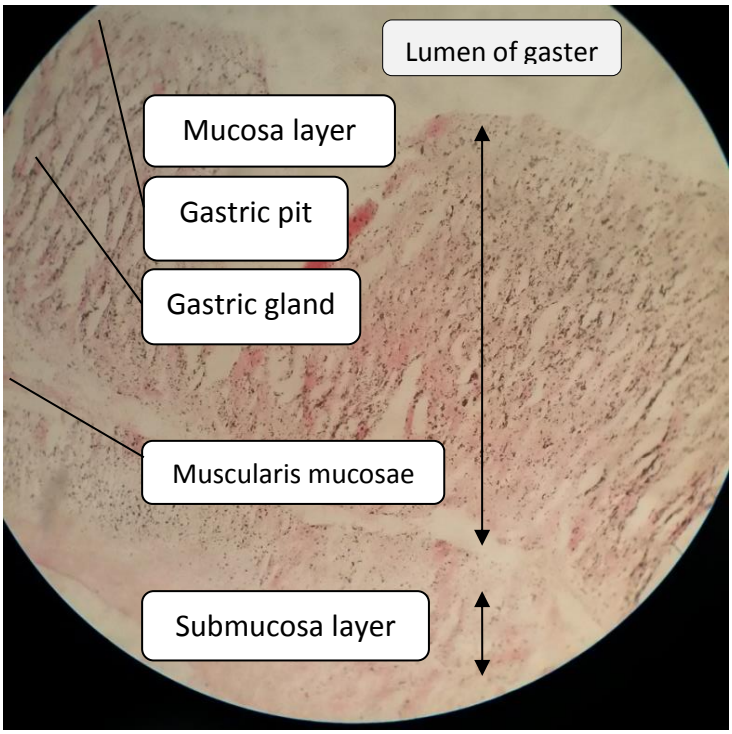

Figure 4: G-500 microscopic view at frontal dissection under 40X magnification.

The layer of mucosa and submucosa were identifed. The mucosa layer were filled with gastric glands and pits. The porminent layer of smooth muscle, which was the muscularis mucosa were seen clearly. There were no significant sign of rupture in the tissue. However there were presence of black dots that to be indicated as infiltration of inflammtory cells filling in lamina propria in other gaster organs samples of G-500. This steps were repeated as for confirmation on consistency. This G-500 gaster organs were showed high infiltration rate in mucosa layer indicates inflammation happens at the mucosa layer. Minor detachment of tissue were also observed under microscopic examination.

Discussion

Results showed that microscopic view of G-500 (500 mg/kg *R. coptidis* treated mice) at frontal dissection under 40X and 100X magnification were identified the layer and structure of mucosa filled with gastric pits and gastric glands and submucosa layer clearly. However, the presence of black dots were seen which can be indicated as infiltration of inflammatory cells filling in lamina propria. High infiltration rate in mucosa layer may indicates inflammation happens at the mucosa layer. Based on a research done to examine the effects of common-used bitter-cold herbs, from total of nine herbs decoction, *R. coptidis* was one of the bitter-cold herbs that were used in this study was showed that the amount of motilin in gaster decreased (an amino acid peptide that can be found in gaster and participated in controlling the pattern of smooth muscle contractions to stimulate gastrointestinal motility), gastrin increased (a peptide hormone that stimulates secretion of gastric acid by parietal cells of gaster and aids in gastric motility) while amount of prostaglandin E_2 in gaster decreased (a group of hormone-like substances that participate in

contraction and relaxation of smooth muscle, dilation and constriction of blood vessels and modulation of inflammation) (Li et al., 2007). This can be suggested that *R. coptidis* with cold-bitter property may decrease motility of gaster and may participate in lesion of structure and function of gaster organ.

From TCM point of view, based on a study done by Song and Zhang (2016) the average dosage of *Rhizoma coptidis* used by physicians in Ming dynasty is in between 0.7 g to 18.5 g per day. Since average body mass globally was 62 kg (Walpole et al., 2012), the dosage of 65 mg/kg indicates consumption of 4.03 g of *R. coptidis* in an adult each day, dosage of 250 mg/kg indicates consumption of 15.5 g of *R. coptidis* in an adult each day while dosage of 500 mg/kg indicates consumption of 31 g of *R. coptidis* in an adult each day. This shows that usage 250 mg/kg in this study were still in acceptable range of consumption. However the consumption of 500 mg/kg was exceeded the upper boundary of range of usage based on this previous reported study. There was no record mentioning the adverse effects of *R. coptidis* which approximately as 31 g each day. Thus, the usage of 500 mg/kg of *R. coptidis* may indicates the initial stage of inflammation in gaster organs at it cellular levels without observable changes on gross appearance.

One report of nausea, vomiting, enterocinetic sound, abdominal distortion, diarrhea, polyuria and erythropenia after administration of oral *R. coptidis* in human adults did not mention the dosage used (WHO monographs on selected medicinal plants, 1999). Based on Meteria Medica Lecture Series written by Yan Zheng Hua on year 2009, overdose or prolong consumption may impair stomach, resulting in poor appetite, nausea and vomiting (Yang, Duan and Qian, 2016). As mentioned by Bensky, Clavey and Stoger (2004) prolonged consumption of *R. coptidis* may impair spleen and stomach and harm Yin fluid. It suggests that patient with deficiency cold of spleen and stomach or deficiency of Yin shall restrain from taking *R. coptidis*. Symptoms of cold deficiency of stomach included vague pain on epigastria region of abdomen which aggravate during hunger and alleviate after taking food, pain alleviates when warming treatment or massage was applied, poor appetite, abdominal distension, acid regurgitation, lassitude, coldness of abdomen and distal part of limbs, loose stool, pale tongue and deep and thin pulse (Chen, 2008). Therefore, patient who consumed *R. coptidis* appeared to have the above stated symptoms was said to have cold deficiency of stomach due to the consumption of *R. coptidis* which was cold and bitter in nature. Thus, microscopic changes in G-500 gaster organs can suggest that patients with cold deficiency syndrome of gaster may have alteration of morphology of gaster tissue which can only be observed under microscopic examination. Yet, currently there was no study on the relationship between cold deficiency of gaster syndrome and histological changes of gaster organs in the field.

Conclusions

Chinese herbs have now been widely used and received great attention due to its effectiveness. Understanding of the therapeutic window as well as the toxic dosage can assist physicians in determining the dosage used during treatment and to use suitable herbs according to disease based on syndrome differentiation. Therefore, the effects of 65 mg/kg, 250 mg/kg, and 500 mg/kg of *R. coptidis* (Huang Lian) on histological changes of gaster in mice were studied in this research project. It can be concluded that the gaster of 500 mg/kg of *R. coptidis* treated mice brings morphological changes to the tissue cells in gaster, specifically infiltration of inflammatory cells,

indicates that 500 mg/kg of *R. coptidis* carries toxic to the tissue cells even though there is no significant changes on the gross appearance of gaster or physical appearance of mice. Chemical compound derived from plants have been used since the origin of human beings to counteract a number of diseases. Among them, the natural isoquinoline alkaloid berberine has been employed in *Ayurvedic and Chinese Medicine* for hundreds of years with a wide range of pharmacological and biochemical effects. Numerous reports have been published regarding the pharmacological effects and treatment uses of *R. coptidis* in complimentary medicine. However, the effects of over dosage of herbs are rarely been investigated. Thus, effects of over dosage of herbs can be study in the future so that integration of modern science and technology with Traditional Chinese Medicine herbs can be accomplished.

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