CHAPTER III

MATERIALS AND METHODS

Chemical substances

Acetic acid (glacial) (Merck KgaA, Germany)
Acridine orange (Sigma Chemical Co.)
Aloe vera spray dried powder
Sodium pentobarbital
Sucralfate suspension (Siam Bheasach Co., Ltd.)

Animal preparation

Male Spraque Dawley rats weighting about 200-280 grams purchased from The International Animal Research Center, Salaya, N=48, were used in this study.

Animal were fasted but allowed only water for 12 hours before gastric ulcer induction. The animals were divided into four groups of 12 animals each as follows:

1. Control group
   The animals were received distilled water 1 ml via orogastric tube twice a day. (n=12)
2. Gastric ulcer group
   The animals were induced gastric ulcer by the administration of 20% acetic acid 1 ml (Modified from Liu et al., 1990). via orogastric tube. After received 20% acetic acid 1 hour, they

were received distilled water 1 ml via orogastric tube twice a day. (n=12)

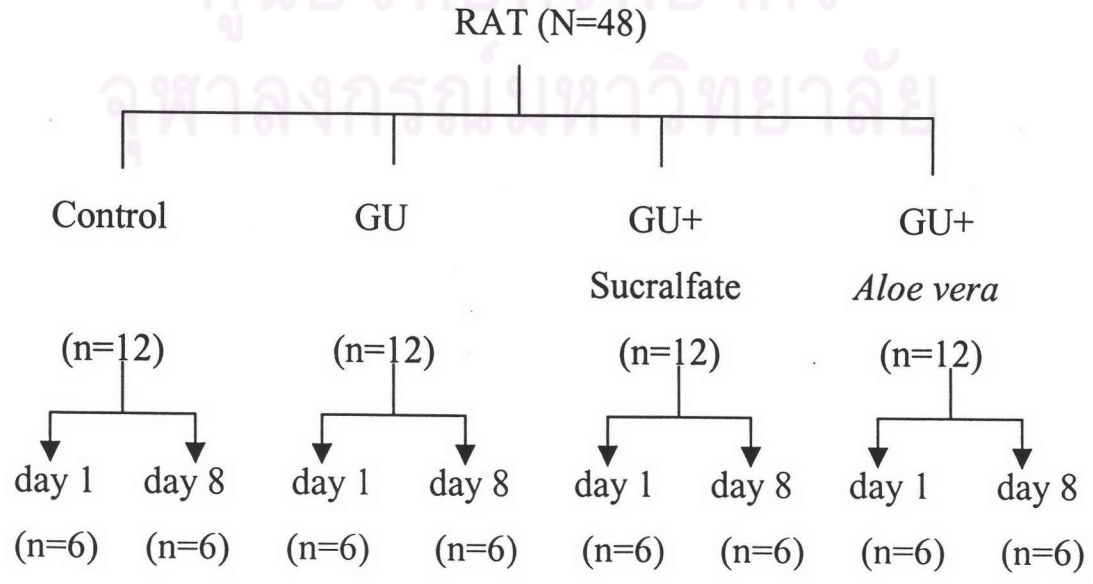
3. Gastric ulcer treated with sucralfate group

   The animals were induced gastric ulcer by the administration of 20% acetic acid 1 ml. via orogastric tube. After received 20% acetic acid 1 hour, they were received sucralfate suspension dose 200 mg/kg/dose via orogastric tube twice a day (Modified from Mahattadul, 1996). (n=12)

4. Gastric ulcer treated with *Aloe vera* group

   The animals were induced gastric ulcer by the administration of 20% acetic acid 1 ml. via orogastric tube. After received 20% acetic acid 1 hour, they were received *Aloe vera* dose 200 mg/kg/dose via orogastric tube twice a day (Modified from Mahattadul, 1996). (n=12)

After that, the animals were divided into two subgroups for day 1 and day 8 after received distilled water or treatment, as shown in this following diagram.



Methods

The animals were fasted but allowed only water 12 hours before experiment. On the day of experiment, the animals were weighed and anesthetised with intraperitoneal injection of sodium pentobarbital dose 50 mg/kg body weight. After tracheostomy, carotid artery and jugular vein were canulated for blood pressure measurement by using polygraph and for the administration of fluorescent marker respectively. As were shown in Figure 3.1. Next, the abdominal wall were incised and the stomach were extended and fixed on plastic state. After that, the animals were observed leukocyte adherence at body area of stomach by in vivo microscopic study (Figure 3.2).

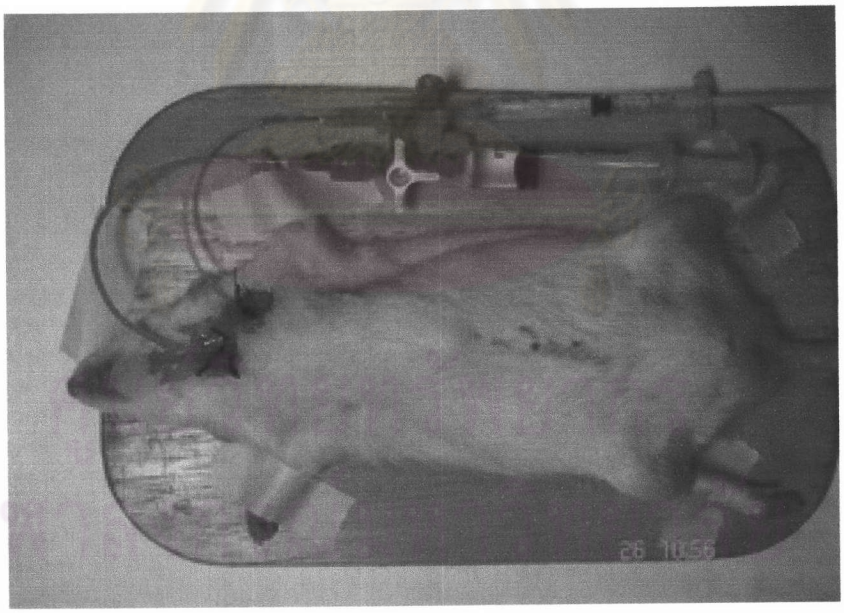


Figure 3.1. The tracheostomy was performed. After that, carotid artery and jugular vein were canulated for measuring blood pressure and for fluorescent marker administration, respectively.

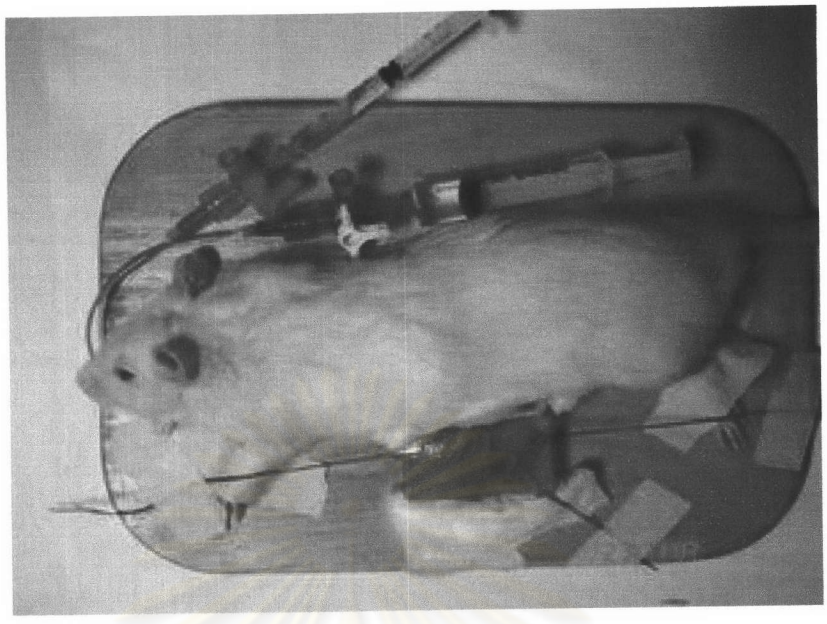


Figure 3.2 The abdomen was incised and opened.The stomach was extended and fixed on plastic state for intravital fluorescence microscope study at body of stomach.

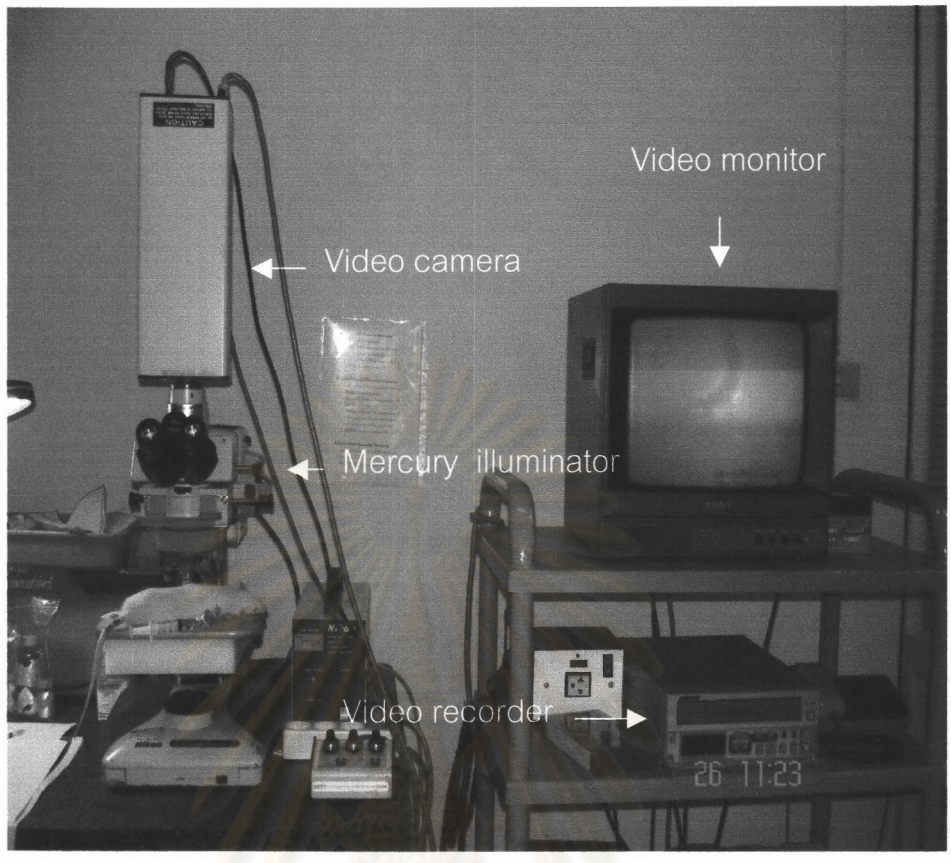
Intravital fluorescence microscopic study

After preparing the stomach, the animals were transferred to microscopic stage of the fluorescent microscope equipped with transillumination and epiillumination optics (Nikon Optiphot-2). After intravenous application of acridine orange (Sigma Chemical Co.) for label leukocyte, epiillumination was achieved with a 50 w, mercury lamp with a 488 nm attached to excitation filter and 515 emission barrier filter. An intravital microscope with the × 40 objective len were used to observe leukocyte adhesion in microvessel of stomach. The image of leukocyte adherence was shown on monitor (Sony) and stored on videotape (Sony) using SIT-video camera (Dage MIT) for playback analysis. A video was

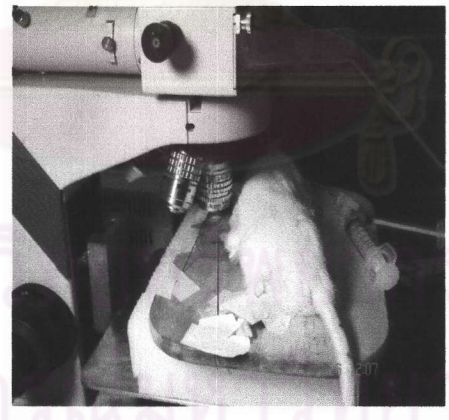
connected to a video timer (UTG 33) for time recorder. The picture demonstrates the set of intravital fluorescence microscope for leukocyte-endothelial cells interaction study was shown in Figure 3.3 (A).

Study of leukocyte-endothelial cells interaction in postcapillary venule

For visualization of the leukocyte, acridine orange was infused intravenously (0.5 mg/kg BW) (Lehr et al.,1993; 1991). Numbers of leukocyte adhesion were recorded by using video recorder. Videotape of each experiment was played back and then leukocyte adherence were monitored. Mostly the leukocytes were markly adherenced on postcapillary venule (diameter about 15-35 μm). The location of three areas were observed leukocyte adherence by randomize selection. A leukocyte was considered adherent to the vessel endothelium if it remained stationary for 30 seconds or longer. Adherent leukocytes were expressed as the means number of leukocyte adherence per field of view (Kalia N et al., 1997). The image of intravital fluorescence microscopic study was shown in Figure 3.3 (B). The calculation of mean number of leukocyte adherence were shown as Figure 3.4.



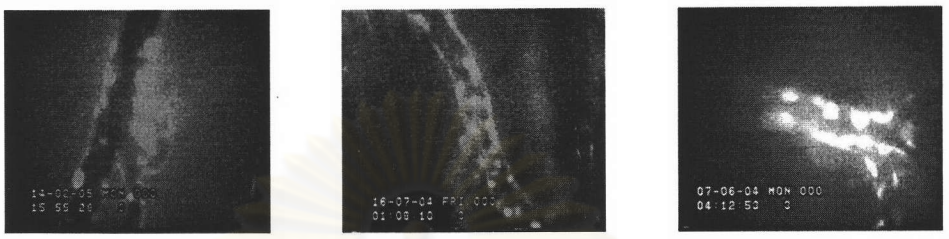
(A)



(B)

Figure 3.3 (A) The picture demonstrates the set of intravital fluorescence microscope. (B) The rat was observed leukocyte adherence on postcapillary venule at the "body" area of stomach.

Leukocyte adherence



Area 1 Area 2 Area 3

Mean number of leukocyte adherence

$$= \frac{\text{number of cells (Area 1+Area 2+Area 3)}}{3} \text{ cells/field}$$

Figure 3.4 The calculation of mean number of leukocyte adherence. The leukocyte adherence on postcapillary venule for 30 seconds or longer were observed per field. The areas were observed leukocyte adherence by randomize selection for three areas. The numbers of leukocyte adherence from each area were calculated for mean number of leukocyte adherence of each animal.

Determination of serum cytokines levels

After the experiment, a blood samples were taken by cardiac puncture, allow blood sample to clot for 2 hours at room temperature or overnight at 2-8°c before centrifuging for 20 minutes at approximately 2000 × g. Serum was separated and stored at about - 80°C for determining TNF α and IL-10 levels by Rat ELISA kit (Quantikine, R&D systems).

Determination of serum TNFα levels

Sample preparation

Rat serum samples required a 2-fold dilution into Calibrator Diluent RD5-17 prior to assay. A suggested 2-fold dilution is 75µl sample + 75µl Calibrator Diluent RD5-17, Mixed well.

Assay procedure

Reagents and sample were brought to room temperature before use. It is recommended that all sample, standards and controls be assayed in duplicate. The experimental procedure as following:

1. Prepare reagents, working standards, control and sample as directed in the previous section.
2. Remove excess microplate strips from the plate frame, return them to foil pouch containing the desiccant pack, reseal.
3. Add 50 µl of Assay Diluent RD1-41 to each well.

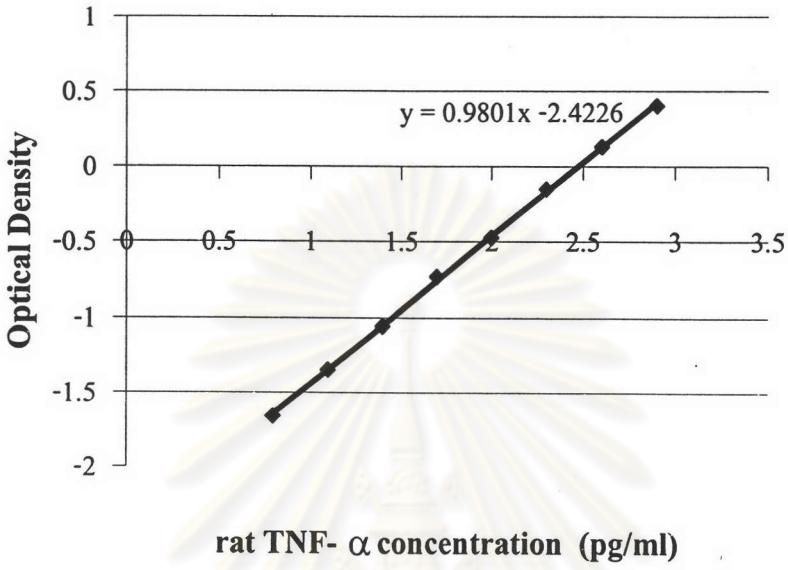
4. Add 50µl of Standard, Control or sample per well. Mix by gently tapping frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. Plate layout are provided to record standards and sample assayed.
5. Aspirate each well and wash, repeating the process four times for total five washes. Wash by filling each well with Wash Buffer (400µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
6. Add 100µl of diluted rat TNFα Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100µl of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.
9. Add 100µl of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract reading at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfection in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Calculation of results

Average the duplicate reading for each standard and sample and subtract the average zero standard optical density (O.D.) to correct O.D. Corrected O.D. of each standard was alternated to the log O.D and also alternated standard rat TNF-α concentration to the log of TNF-α concentration. Construct a standard curve by potting the log of O.D. for each standard on the y-axis against the log of rat TNF-α concentration for each standard on the x-axis and draw a best fit curve through the point on the graph. The standard curve and formula were created by using computer software. After that, the log of O.D. for each sample were calculated in formula for finding the log of TNF -α concentration of each sample. Finally, the log of TNF -α concentration from calculation was performed anti-log for TNF -α concentration (pg/ml.) of each sample. A standard curve must be generated for each set of samples assayed. The calculation of finding for TNF -α concentration (pg/ml.) of sample was shown as Figure 3.5.

Example.

Standard curve



sample examination

average zero standard = 0.037

average O.D.= 0.612

corrected O.D (average O.D.- average zero standard) = 0.575

log O.D = -0.240 (log y)

formula y = 0.9801x-2.4226

calculation

$$-0.240 = 0.9801x - 2.4226$$

$$\log x = \frac{2.4226 - 0.240}{0.9801} = 2.227$$

$\therefore$ anti-log x = 168.66 pg/ml

TNF-α concentration of this sample = 168.66 pg/ml.

Figure 3.5 The calculation of TNF-α concentration.

Determination of serum IL-10 levels

Reagents and sample were brought to room temperature before use. It is recommended that all sample, standards and controls be assayed in duplicate. The experimental procedure as following:

1. Prepare reagents and standard dilutions as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to foil pouch containing the desiccant pack, reseal.
3. Add 50 µl of Assay Diluent RD1-21 to each well.
4. Add 50µl of Standard, Control or sample per well. Mix by gently tapping frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. Plate layout are provided to record standards and sample assayed.
5. Aspirate each well and wash, repeating the process four times for total five washes. Wash by filling each well with Wash Buffer (400µl) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
6. Add 100µl of diluted rat IL-10 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100µl of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Add 100µl of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract reading at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfection in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Calculation of results

The calculation of IL-10 concentration (pg/ml.) is as same as the calculation of TNF-α concentration. Standard curve of IL-10 was shown in Figure 3.6.

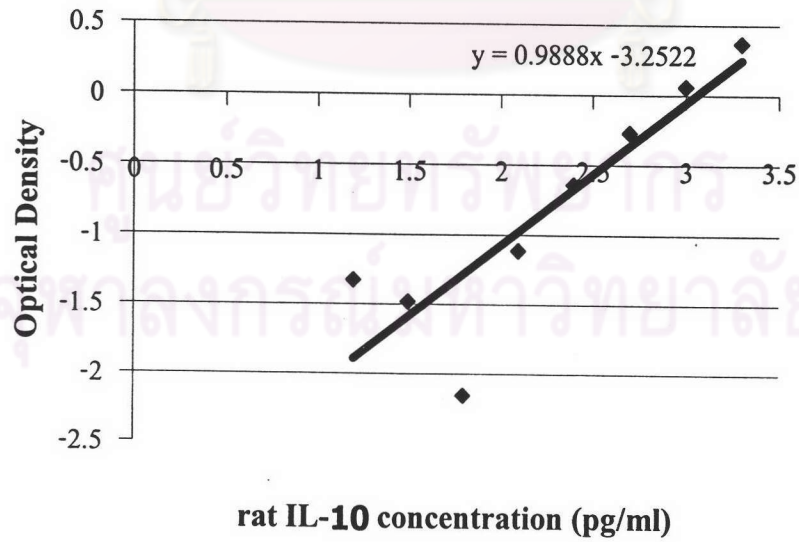


Figure 3.6 Standard curve of IL-10 levels.

Histological analysis

After the experiment, the stomach were incised and removed. The stomach were cut along greater curvature of stomach and wash with 0.9% normal saline. The stomach was observed gross pathology and photography by using digital camera (Sony cyber-shot DSB-W1). After that, the stomach were fixed in 10% formalin and embedded in paraffin. The section were cut at a thickness of 5μm and stained with hematoxylin and eosin (H&E) (Konturek, 2000). The histopathological changes and the maximum length of gastric ulcer were observed by using light microscope with magnification × 20. Histopathological examination was performed by pathologist. The data of histopathological changes and the maximum length of gastric ulcer (cms.) were reported by pathologist. The maximum length of gastric ulcer was demonstrated in Figure 3.7.

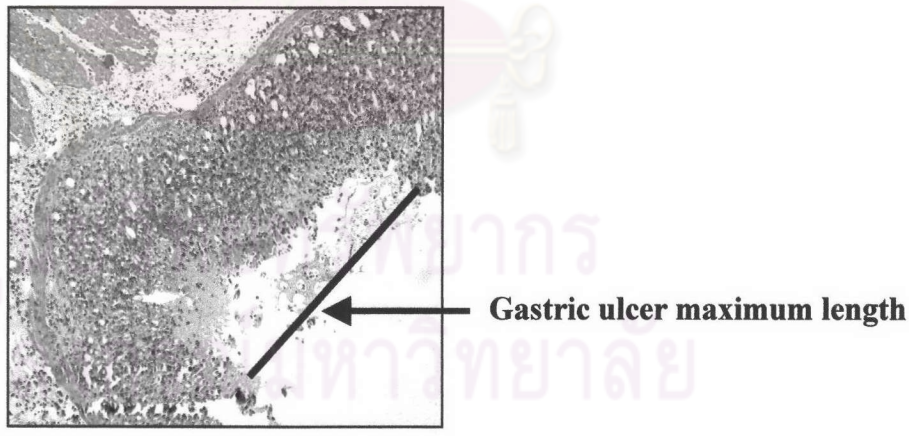


Figure 3.7 The maximum length of gastric ulcer was determined by using scale in light microscope (magnification × 20).

Limitation of this study

In this study, we plan to study the change of arterioles diameter in stomach after the administration of 20% acetic acid induced gastric ulcer and treatment. However, we could not define the arterioles diameter due to the arterioles run perpendicularly to the muscle fibers and deep into muscle layer of stomach. Therefore, we study only leukocyte adherence in postcapillary venules.

Data analysis

Data was expressed as mean ± standard error of mean (S.E). Statistical analysis was done by using one-way analysis of variance and the comparision of results between group was done using Post hoc test. A probability value (P) of less than 0.05 was considered to be statistically significant.