CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The investigation of Ridsiduangmahakan preparation and its twenty-two plant ingredients were based on their use by Thai traditional doctors for treatment of hemorrhoid. The objectives of this study were aimed to investigate anti-inflammation and cytotoxic activities against colon and rectum cancer cell lines (LS174T and SW480). The ethanolic extracts of these plants, i.e., *Anacyclus pyrethrum* L., *Anethum graveolens* L., *Angelica sylvestris* L., *Artemisia vulgaris* L., *Cinnamomum bejolghota* (Buch.-Ham.) Sweet, *Cinnamomum zeylanicum* L., *Commiphora abyssinica* Berg., *Cuminum cyminum* L., *Foeniculum vulgare* Mill., *Lepidium sativum* L., *Myristica fragrans* L., *Nigella sativa* L., *Picrorrhiza kurroa* Royle. Ex Benth. *Piper chaba* Hunt, *Piper nigrum* L., *Piper ribesioides* Wall., *Platycladus orientalis* (L.) Franco, *Pouzolzia pentandra* J.J. Bennett, *Terminalia chebula* Retz gall, *Zingiber officinale* Roscoe and Ridsiduangmahakan formula were prepared. Effects of the ethanolic of these plants on anti-inflammation activities, i.e., inhibition of Nitric Oxide (NO), inhibitory effect on LPS-induced TNF-α release and inhibitory effect on LPS- stimulated PGE_2 release were determined. Moreover, cytotoxicity activity of Ridsiduangmahakan preparation and its plant ingredient extract against two types of colon cancer cell lines (LS174T and SW480) and one type of normal cell line (MRC5) was investigated. Results from cytotoxicity activity will support the use of this preparation by Thai folk doctors for reducing inflammation of hemorrhoids. Furthermore, it may be developed to treat colon and rectum cancer. In addition to anti-inflammation and cytotoxic activities, the quality control of these plant materials of Ridsiduangmahakan preparation was also determined. The quality control was investigated through physical standardization such as loss on drying, total ash and acid insoluble ash, extractive value, and contamination by danger heavy metals such as arsenic, cadmium and lead.

Percentage of loss on drying of each plant ingredients and the preparation were in a range of 8-13%, which falls within 10% limit set by the standard of Thai herbal pharmacopoeia. However loss on drying values of the preparation showed less than 10%. Thai herbal pharmacopoeia limit ranges for total ash and acid insoluble ash in 6-12% and 0.5-2%, respectively (these ranges concern with types of plants). Total ash and acid insoluble ash in almost all of our plant ingredients is less than 11% and 2%, respectively. Only total ash and acid insoluble ash in *C. abyssinica* was quantified and 22.34±0.0324% and 16.10±0.0009%, respectively, were obtained. In addition to total ash and insoluble ash, the presence of heavy metals in plant ingredients by ICP-MS indicate contamination. Less than 4 mg/kg of arsenic, 10 mg/kg of lead, and no cadmium were detected in each plant ingredient and the preparation, which fall in the limit range according to standardization of Thai herbal pharmacopoeia.

For percentage yield, the highest percentage yield was obtained from *Nigella sativa* (37.28%) was obtained followed by *Terminalia chebula* gall (33.96%) and *Commiphora abyssinica* (33.66%). The third-percentage lowest percentage of yield were obtained from *Piper rebesioides* (1.01%), *Pouzolzia pentandra* (white) (1.84%), and *Pouzolzia pentandra* (red) (3.71%), respectively. The ethanolic extract of preparation gave the percent yields of 16.30%, which is related with the ethanol extractive value of 16.89%.

The results of Ridsidaungmahakan preparation and its ingredients through inhibitory activity on LPS induced NO production revealed that RSD preparation (oil) and (solid) had IC_{50} values of 48.1±1.8 and 56.9±5.1, respectively. Its plant ingredients such as *Z. officinale, P. chaba, T. chebula,* and *A. vulgaris* exhibited inhibition of NO production with IC_{50} values of 21.2±1.8, 25.9±2.5, 27.8±0.3, and 28.7±3.3 μg/ml, respectively. There is no cytotoxicity observed at the concentrations used for the investigation. Moreover, these plant extracts exhibited NO production inhibitory effect less than Indomethacin (IC_{50} = 56.78 μM or 20.32 μg/ml). More over the RSD preparation was tested anti-inflammation by inhibitory effects on LPS-induced TNF-α release in RAW 264.7 cells. The results showed that the preparation,

preparation (oil) and indomethacin (positive control) had IC_{50} values of 31.86±1.86 μg/ml and 51.41±3.58 μg/ml (143.69±10.01 μM), respectively.

It is concluded that the preparation showed low anti-inflammatory activity through NO production inhibitory effect but potent LPS-induced TNF-α release activity with a better value than indomethocin. However the ingredients of this preparation such as *T. chebula, C. abyssinica* and *P. nigrum* showed good TNF-α release activity with IC_{50} values of 20.65±2.89, 34.41±5.00 and 40.11±1.53 μg/ml,, respectively.

Another mechanism of anti-inflammation the inhibitory effects on LPS-stimulated PGE_2 of Ridsiduangmahakan preparation and its plant ingredients were also determined. The results were found that the preparation extracts (oil) gives the best IC_{50} value 8.85±1.60 μg/ml followed by preparation (solid+oil) 16.80±4.04 μg/ml and Preparation (solid) 21.22±3.29 μg/ml. While indomethacin exhibited inhibitory effects with IC_{50} value of 1.00±0.43 μg/ml (2.80±1.20 μM) . The plant ingredients of this preparation such as *Z. officinale, M. fragrans* (seed), *P. nigrum* and *P. chaba* showed inhibitory effect with IC_{50} values as 5.22±1.20, 16.99±1.93 and 17.70±2.48 μg/ml, respectively.

It is concluded that RSD preparation used for hemorrhoid treatment showed anti-inflammatory effect through COX-2 as well as inhibitory effects on TNF-α and NO production.

For the results for cytotoxic activity by SRB assay, *Z. officinale, C. abyssinica* and *P. nigrum* exhibited cytotoxicity against LS174T ($IC_{50=}$ 12.53±0.13 , 27.17±0.65 and 27.17±0.65 μg/ml, respectively) but the preparation (solid), preparation (oil) and preparation (solid+oil) showed less potent cytotoxicity against LS174T (IC_{50} values of 66.62±3.08, 35.44±1.07, and 46.54±2.92 μg/ml, respectively). The ethanolic extract of preparation (oil and oil+solid) and showed specific cytotoxicity against LS174T but low cytotoxic activity on SW480. However this fraction showed no cytotoxicity against normal cells. *Zingiber officianale* an ingredient of this preparation was specifically cytotoxic to both colon cancer LS174T and SW480 but was less cytotoxic to normal cells.

These results suggest that oil of RSD preparation should be selected for hemorrhoid preparation because it showed anti-inflammation via three different pathways and also showed cytotoxicity against colon cancer cell but not normal cells. In addition, the ethanolic extract of ginger should be promoted as colon cancer drug because it showed cytotoxicity against two colon cancer cell but not normal cells.

Investigation of chemical compound of the preparation which exhibited anti-inflammation was done by bioassay-guided fractionation. We obtained six fractions containing different compounds, which were then tested for anti-inflammation activities. The fraction which exhibited the best result was isolated for anti-inflammatory compounds. Piperine isolated from active fraction was identified in comparison with authentic piperine by TLC and HPLC. Piperine is present in three plant ingredients from this preparation such as *Piper nigrum, Piper chaba* and *Piper ribesioides*. Then piperine was tested for anti-inflammatory activity and it showed inhibitory effect on LPS induced NO production, LPS-induced TNF-α release, and LPS-stimulated PGE_2 with IC_{50} values of 17.0±4.6, 63.05±7.86 and 20.36±1.89 μg/ml, respectively (59.57±16.12, 220.96±27.54 and 71.35±6.62 μM, respectively) . These results indicated that piperine is an anti-inflammatory compound and is the main compound (9.32 mg/g) on HPLC pattern of RSD preparation. However, there are another compounds such as gingerol, shogaol from Ginger in this preparation that exhibited anti-inflammation.

The study of fingerprint of the preparation can be done by GCMS method since parts of some plant ingredients have oil as component. Stability test of the ethanolic extract of RSD preparation was done under 45±2°C with 75±5% RH for 120 days. The chemical finger print by GCMS and determination of piperine by HPLC were determined at day 0, 15, 30, 60, 90, and 120. The results of GCMS showed that the quantity and types of the compounds by GCMS gradually changed from day 0 to day 120 because volatile oil vaporized under accelerated condition (45°C). This result was concluded that the detection of chemical fingerprint of RSD preparation using GCMS should not be recommended in quality control of this product. The stability of piperine was determined by HPLC , because GCMS method cannot detect its peak. The quantity of piperine significantly decreased at day 60, day 90 and day 120. It related with inhibitory activity on NO production. The amount of piperine

reduced as 61.34% within 4 months. However, the ethanolic extract of RSD preparation kept in accelerated condition did not change anti-inflammatory activity by using COX-2 inhibition method. Thus it can be concluded that this preparation showed anti-inflammatory activity via COX-2 inhibition. Hence, another marker in the preparation which exhibits anti-inflammation and is stable in accelerated condition should be identified in future.

From all experiments, it is concluded that Ridsiduangmahakan preparation is an anti-inflammation through inhibition of a COX-2 enzyme which is a catalyst of prostaglandin production. Prostaglandin is a mediator which causes various strong physical effects such as pain. The preparation also inhibited NO release and TNF-α release. Hence, this preparation should reduce inflammation of hemorrhoid and prevent colon cancer.