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รายงานวิจัยฉบับสมบูรณ์

โครงการฤทธิ์ของผลยอและสารเครื่องหมายในผลยอ scopoletin ต่อการเคลื่อนไหวและการอักเสบในท่อทางเดินอาหารส่วนต้น

Effect of *Morinda citricfolia* fruit and a biomarker scopoletin on upper gastrointestinal motility and inflammation

โดย นางศิริมา มหัทธนาดุลย์
คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์

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คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

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Abstract

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Investigator :

นักวิจัยผู้รับทุน: ผศ. ดร. ศิริมา มหัทธนาดุลย์

คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อ. หาดใหญ่ จ. สงขลา

นักวิจัยที่ปรึกษา: รองศาสตราจารย์ นพ. วิบูลย์ ฤทธิทิศ

สำนักวิชาแพทยศาสตร์ มหาวิทยาลัยวลัยลักษณ์ จ. นครศรีธรรมราช

E-mail Address :sirima.m@psu.ac.th

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Aims of the study: The present study was carried out to develop a rapid and economic analytical method for quantitative determination of scopoletin in *Morinda citrifolia* (noni) fruit products and to assess the effects of the fruit extracts and scopoletin on acute gastro-esophageal inflammatory models in rats.

Methods: Noni fruit extracts in KH_2PO_4 (pH 3, 0.01M) (4 mg/ml) was applied on solid phase extraction (SPE) cartridge and then purified by a cleanup procedure consists of KH_2PO_4 (pH 7, 0.01M) followed by 100% methanol. Chromatographic separation was achieved on a C_{18} column using 0.01 M sodium acetate (pH 3.0, 0.01 M) and acetonitrile (80:20 v/v) as a mobile phase and a UV-Vis detector at wavelength of 350 nm. The pharmacological activity of the fruit extracts and scopoletin at the same equivalent dose present in the fruit extracts was investigated in rat on gastrointestinal motility, gastric acid and pepsin secretion, and acute gastroesophageal inflammatory models (acid reflux esophagitis, gastritis induced by ethanol).

Results: A SPE procedure, followed by an RP-HPLC assay was successfully developed for a simple; rapid (retention time about 5 min); good sensitivity (LOD = 2.6 ng/ml; LOQ = 7.9 ng/ml); high accuracy (% recovery > 80%); high precision (% RSD's intra- and inter-day precision < 2%); high reliability (total peak purity > 0.99) and economic quantitative analysis of scopoletin in noni fruit extract. The scopoletin content in aqueous and ethanolic fruit extracts was between 0.86±0.01 and 1.99±0.04 mg/g, respectively. Both these noni fruit extracts exerted a prokinetic activity with a higher potency than cisapride. The fruit extracts also significantly inhibited gastric acid secretion and pepsin activity in rats. Additionally, the fruit extracts prevented the formation of acid reflux esophagitis and ethanol-induced acute gastric lesions in rats with equal potency to those of ranitidine and lansoprazole. Pure scopoletin, at the same equivalent dose present in the fruit extracts, exhibited similar antisecretory and antiulcer properties to those of noni fruit extracts though it exerted a less prokinetic activity.

Conclusion: The advantage of an established method as a rapid, simple and low cost analysis may be of significant economic values for routinely quality-control testing of noni fruit juice. The finding of the high correlation between the prokinetic and antiulcer properties and scopoletin content also allows for a quality evaluation of any noni fruit products used to prevent gastroesophageal inflammation. Further study is necessary to investigate the curative efficacy of scopoletin against chronic gastric ulcer including its antisecretory and prokinetic mechanisms.

Keywords : *Morinda citrifolia* fruit, noni, solid phase extraction, scopoletin, prokinetic agent, reflux esophagitis, gastritis

วัตถุประสงค์ การศึกษานี้เพื่อทำการพัฒนาวิธีที่รวดเร็วและประหยัดในการวิเคราะห์ปริมาณสารสโคโปเลตินในสารสกัดผลยอ และประเมินประสิทธิผลของสารสกัดผลยอและสารสโคโปเลตินต่อภาวะหลอดอาหารและกระเพาะอาหารอักเสบเฉียบพลันในหนูขาว

วิธีการศึกษา นำสารสกัดผลยอใน KH_2PO_4 (pH 3, 0.01 โมลาร์) (4 มก./มล.) มาสกัดด้วยเทคนิคการสกัดด้วยเฟสของแข็งในตัวกลางที่เป็นสารละลาย KH_2PO_4 (pH 7, 0.01 โมลาร์) ตามด้วยสารละลาย 100% เมธานอล วิเคราะห์หาปริมาณสารสโคโปเลตินด้วยระบบโครมาโทรกราฟฟีที่ประกอบด้วย คอลัมน์ขนาด C_{18} สารละลาย sodium acetate (pH 3.0, 0.01 โมลาร์) เป็นเฟสเคลื่อนที่ และเครื่องตรวจจับแบบใช้รังสีอัลตร้าไวโอเลตที่คลื่นความยาว 350 นาโนเมตร ทดสอบฤทธิ์ทางเภสัชวิทยาของสารสกัดผลยอและสารสโคโปเลตินในปริมาณเทียบเท่าปริมาณที่มีอยู่ในสารสกัดผลยอต่อการเคลื่อนไหวของท่อทางเดินอาหาร การหลั่งกรดและเปปซินของกระเพาะอาหาร ภาวะหลอดอาหารอักเสบเฉียบพลันเนื่องจากการไหลย้อนกลับของกรดจากกระเพาะอาหารและภาวะกระเพาะอาหารอักเสบเฉียบพลันเนื่องจากสารเอธานอลในหนูขาว

ผลการศึกษา วิธีวิเคราะห์หาปริมาณสารสโคโปเลตินในสารสกัดผลยอด้วยเทคนิคการสกัดด้วยเฟสของแข็งตามด้วยระบบโครมาโทรกราฟฟีแบบผันกลับเป็นวิธีที่สะดวก รวดเร็ว (เวลาที่สารเคลื่อนที่ผ่านคอลัมน์ประมาณ 5 นาที) มีความไวสูง (LOD = 2.6 นก./มล. LOQ = 7.9 นก. /มล.) มีค่าความถูกต้องสูง (% การกลับคืน > 80%) มีค่าความเที่ยงตรงสูง (% ความเที่ยงตรงภายใน 1 วันและระหว่างวัน < 2) มีความน่าเชื่อถือสูง (ค่าความบริสุทธิ์ทั้งหมดของสารที่แยกได้> 0.99) และประหยัดค่าใช้จ่าย ปริมาณสารสโคโปเลตินในสารสกัดผลยอด้วยน้ำและเอธานอลมีประมาณ 0.86 ± 0.01 และ 1.99 ± 0.04 มก./กรัม ตามลำดับ สารสกัดผลยอทั้งสองชนิดมีประสิทธิภาพกระตุ้นการเคลื่อนไหวของท่อทางเดินอาหารในหนูขาวสูงกว่ายาซิสซาไพรด์ และมีฤทธิ์ยับยั้งการหลั่งกรดและเป็ปซินในหนูขาวอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ยังมีประสิทธิภาพป้องกันการเกิดภาวะหลอดอาหารอักเสบเนื่องจากการไหลย้อนกลับของกรดจากกระเพาะอาหารและภาวะกระเพาะอาหารอักเสบเนื่องจากสารเอธานอลเทียบเท่ากับยารานิทิดีนและยาแลนโซพราโซล สารสโคโปเลตินในขนาดเทียบเท่ากับขนาดที่มีในสารสกัดผลยอมีประสิทธิภาพยับยั้งการหลั่งกรดและเป็ปซินและต้านการเกิดแผลใกล้เคียงกับสารสกัดผลยอถึงแม้ว่าจะมีฤทธิ์กระตุ้นการเคลื่อนไหวของท่อทางเดินอาหารน้อยกว่า

สรุปผล ข้อดีของวิธีวิเคราะห์ที่พัฒนาขึ้นสามารถวิเคราะห์หาปริมาณสารสโคโปเลตินได้รวดเร็วและค่าใช้จ่ายต่ำซึ่งน่าจะให้ความคุ้มค่าทางเศรษฐกิจในการใช้วิเคราะห์เพื่อควบคุมปริมาณสารสโคโปเลตินในน้ำผลยอ ความสัมพันธ์ระหว่างฤทธิ์กระตุ้นการเคลื่อนไหวและยับยั้งการเกิดแผลอักเสบของท่อทางเดินอาหารกับปริมาณสารสโคโปเลตินสามารถใช้เป็นแนวทางในการควบคุมคุณภาพของผลิตภัณฑ์น้ำผลยอสำหรับใช้ป้องกันภาวะหลอดอาหารและกระเพาะอาหารอักเสบ อย่างไรก็ตามจำเป็นที่จะต้องมีการศึกษาเพิ่มเติมในผลรักษาแผลกระเพาะอาหารแบบเรื้อรังและกลไกการยับยั้งการหลั่งกรดและกระตุ้นการเคลื่อนไหวของท่อทางเดินอาหารของสารสโคโปเลติน

คำหลัก ผลยอ โนนิ การสกัดด้วยเฟสของแข็ง สโคโปเลติน สารกระตุ้นการเคลื่อนไหวของท่อทางเดินอาหาร หลอดอาหารอักเสบ กระเพาะอาหารอักเสบ

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CHAPTER 1

INTRODUCTION

Background and rationales

Reflux esophagitis and gastritis are common upper gastrointestinal diseases found in all ages of humans and are increasingly recognized as significant health problems. Reflux esophagitis refers to the inflammation of the esophagus that results from repeated exposure for prolonged periods of time to regurgitated stomach contents usually containing acid and pepsin. With increasing severity, it may be associated with erosions, ulceration and formation of strictures. The development of reflux esophagitis is commonly associated with a decrease in the lower esophageal sphincter (LES) tone or esophageal clearance. This requires acid-suppressive therapy (with or without prokinetic agents) for medical management (Holzer, 2001; Kradjan, 2001). Nevertheless, a considerable number of patients do not achieve complete mucosal healing or suffer from either sustained symptoms or complications. Therefore, this disease continues to be investigated in order to develop effective treatment regimens. Since the presence of acid is still one fundamental characteristic in the pathogenesis of *Helicobacter pylori*-negative gastritis, its standard regimen involves acid-suppressive therapy but most of the effective drugs exhibit serious adverse effects such as impotence, gynaecomastia, hypergastrinemia and hemopoeitic changes. Hence, currently used effective regimens should be reinvestigated. Recently, several studies have shown that the presence of reactive oxygen species; pro-inflammatory cytokines [such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6]; and pro-inflammatory mediators [such as prostaglandins (PGE_2) and nitric oxide (NO) generated from the induction of inducible nitric oxide synthase (iNOS)]; all may play some part in the pathophysiology of both reflux esophagitis and gastritis (Holzer 2001; Oh et al., 2001). In addition, abnormal gastric motility, associated with rapid or delayed gastric emptying also contributes to gastric lesions development (Holzer 2001).

Morinda citrifolia Linn. (Rubiaceae), commonly known worldwide as “noni”, or so called in Thai as “Yor”, is regularly consumed as food and is one of the 66 Thai medicinal plants used in primary health care. Safety aspects from preclinical safety tests and human clinical safety reports have been published. In an acute toxicity test, the LD_{50} of an aqueous fruit extract and ethanol fruit extract were found to be greater than

7500 mg/kg and 3500 mg/kg, respectively when injected intraperitoneally into mice (Chearskul et al., 2004). Thus, The LD_{50} of crude noni fruit extracts is greater than the minimum criteria for nontoxic (acute oral LD_{50} > 5000 mg/kg and acute intraperitoneal LD_{50} > 2000 mg/kg). High doses of pureed noni fruit from Tahiti (15,000 mg/kg) administered orally to Sprague-Dawley rats also showed no signs of toxicity, induced behavioral changes or pathological changes within 2 weeks. Oral toxicity tests with Tahitian noni juice also revealed no adverse effects at a dose equivalent to 80 ml/kg/d in rats (West et al., 2006). In addition, no genotoxicity or allergenic responses to noni juice was found after oral, intraperitoneal or intravenous administration of 6 ml of Tahitian noni juice or puree or concentrate (10% w/w in distilled water) in guinea pigs for three days per week for two weeks (Product Safety Labs, 2000). Subchronic oral toxicity studies of noni fruit juice at a dose equivalent to 80 ml/kg/d for 13 wk in Sprague-Dawley rats demonstrated no adverse effects, with a normal chemistry examination, and no pathological changes in any of the 55 organs examined. Furthermore, a clinical randomized double-blind placebo-controlled safety study of Tahitian Noni Juice in 96 healthy subjects at a dose of 750 ml for 28 days, showed no clinically significant differences in the measured parameters including hematological, biochemical, and urological measurements that were made at wk 0, 2, 4, and 6 (Davies and Mugglestone, 2003). The determination of toxicities and maximum tolerated dose of noni in twenty-nine patients has also been performed. Patients were divided into 5 groups, each subject received capsules containing 500 mg ripe noni fruit extract for 28 days. The initial dose group ingested 4 capsules (2,000 mg). When no adverse effects were observed, the dose for the next group was increased by 2,000 mg and so on, with the final group consuming 10 g daily. Ingestion of the noni capsules was well tolerated at all doses and did not result in any adverse events (West et al., 2006). The maximum dose of the phase-I clinical study corresponds to approximately 200 ml (almost 1 cup) of noni fruit juice. Thus the no observable adverse effect level (NOAEL) was subsequently determined to be greater than 80 ml/kg (West et al., 2006). Nowadays, noni fruit juice has become a popular beverage for use as a diet supplement with the approval as a novel safe food from the European Commission in 2002 (European Commission, 2002; EFSA, 2006).

Noni fruit contains 90 % of water and 10 % soluble solids, dietary fibers, proteins (mainly aspartic acid, glutamic acid and isoleucine), minerals (mainly potassium, sulfur, calcium, phosphorus, and selenium) and vitamins (mainly ascorbic acid and provitamin A) (Chan-Blanco et al., 2006). The principal chemical constituents

consist of phenolic compounds (mainly damnacanthal, morindone, alizarin, aucubin, nordamnacanthal, rubiadin, rubiadin-1-methyl ether, asperuloside, and scopoletin); organic acids (mainly caproic and caprylic acids) and alkaloids (xeronine) (Wang and Su, 2001; Chan-Blanco et al., 2006). Several classes of metabolites have also been described, including polysaccharides; fatty acid glycosides; iridoids acid (mainly asperuloside, asperulosidic and deacetylasperulosidic acid); anthraquinones (mainly damnacanthal, morindone, and morindin); coumarins (mainly scopoletin); flavonoids (mainly rutin, narcissoside and nicotifloroside); lignans (mainly 3,3'-bisdemethylpino--resinol, americanol A, americanin A americanoic acid A, morindolin, isoprincepin and balanophonin); phytosterols; carotinoids; alcohols (mainly 3-methyl-3-buten-1-ol); ester (mainly methyl octanoate, methyl decanoate); ketones (mainly 2-heptanone); lactones [mainly (E)-6-dodeceno-glactone]; and a range of volatile constituents including monoterpenes, short chain fatty acids and fatty acid esters (Farine et al., 1996; Sang et al., 2002; Kamiya et al., 2005; Pawlus et al., 2005). In addition, miscellaneous compounds such as β-sitosterol and its 3-O-glucoside; ursolic acid and 19-hydroxyursolic acid; cytidine; borreriagenin and epiborreriagenin; iridoid derivatives; succinic acid diesters; 4-hydroxy-3-methoxycinnamaldehyde; β-hydroxypropiovanillone and vanillin had been isolated (Potterat and Hamburger, 2007).

Recently, noni fruit extract has been claimed to have several biological properties such as anti-microbial, anti-carcinogenic, analgesic, would-healing and anti-inflammatory activities (Wang et al., 2002; Nayak et al., 2007). Administration of 10 or 200 mg noni fruit juice extract, intraperitoneally, can reduce the carrageenan- or bradykinin-induced rat paw edema (McKoy et al., 2002). Noni fruit juice extract also exerted antioxidative activities through free-radical-scavenging activity (Su et al., 2005; Yang et al., 2007), inhibition of copper-induced low-density lipoprotein oxidation (Kamiya *et al*., 2004) nitric oxide scavenging activity (Basu and Hazra, 2006) and quenching of H_2O_2 (Chong et al., 2004). These properties are attributed to the main phenolic compounds especially scopoletin (7-hydroxy-6-methoxycoumarin) (Chan-Blanco et al., 2006).

Scopoletin, a coumarin derivative, is a yellow to beige crystalline powder with the molecular weight 192 and the melting point 204–206 ° C (Vasconcelos et al., 1998). Levand and Larson had reported its ability to control serotonin levels in the body (Levand and Larson, 1979). Scopoletin was also reported to have many other biological effects such as anti-microbial activity against 3 Gram-positive bacteria (*Staphylococcus aureus, Streptococcus pneumoniae*, and beta-hemolytic *Streptococcus* 1451), 5 Gram-

negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Haemophilus influenzae*, *Bacillus subtilis, and fungi Aspergillus niger* and *Cladosporium cladosporioides)* (Kayser O and Kolodziej, 1997; Yong et al., 2002); hypotensive effects in anaesthetized rats (Ojewole and Adesina, 1983); it has induced negative chronotropic and inotropic responses on guinea-pig isolated atria (Ojewole and Adesina, 1983); inhibitory activities against acetylcholine-induced contractures of the toad rectus abdominis muscle, electrically-evoked twitches of the chick isolated biventer-cervicis muscle or rat isolated phrenic-nerve hemidiaphragm muscle preparations (Ojewole and Adesina, 1983), the contractions of isolated rat aortic rings induced by various substances (phenylephrine, potassium chloride, serotonin and $PGF_2\alpha$) (Oliveira et al., 2001), and acetylcholinesterase (AChE) activity (Jae et al., 2004); hepatoprotective activity by decreasing the release of glutamic pyruvic transaminase and sorbitol dehydrogenase from carbon tetrachloride-intoxicated primary cultured rat hepatocytes (So et al., 1998) and hepatic lipid peroxidation (Panda and Kar, 2006); inhibitory activity on uric acid production and a uricosuric mechanism (Ding et al., 2005). Additionally, it was found to possess antioxidant activities by increasing superoxide dismutase and catalase activity (Panda and Kar, 2006) and scavenging for superoxide anions in the xanthine/xanthine oxidase reaction system (Shaw et al., 2003), and anti-inflammatory activities through inhibition of the release of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) (Kang et al., 1999; Kim et al., 2004; Deng et al., 2007; Moon et al., 2007), myeloperoxidase (Yu et al., 2008) and proinflammatory mediators (nitric oxide and PGE_2) (Zin et al., 2002; Kim et al., 2004; Ikeda et al., 2009). Moreover, scopoletin (1 mg/kg, p.o.) administered daily for 7 days to levo-thyroxine-treated animals decreased the levels of serum thyroid hormones and glucose as well as hepatic glucose-6-phosphatase activity, demonstrating its potential ability to regulate hyperthyroidism and hyperglycemia (Panda and Kar, 2006).

According to the claimed efficacies in Thai traditional textbooks, the fruit is also used for treatment of various gastrointestinal disorders as a carminative, appetite stimulant, and reliever of gum diseases, heartburn or stomachache (Farnsworth and Bunyapraphatsara, 1992). The decoction or infusion of roasted mature unripe fruits is also recommended to relieve the symptoms of nausea and vomiting, if it is not too severe (Ekpalakorn et al., 1987). Nevertheless, scientific evidence for these benefits is still limited with only one study demonstrating the antiulcer activity of an ethyl acetate extract of the fruit against acute gastric lesions induced by ethanol, aspirin and pyloric ligation, and acute duodenal ulcer induced by cysteamine in rats (Muralidharan and

Srikanth, 2009). This extract was claimed to exhibit potent antioxidant properties and the active components are thought to be non-polar lignans (Zin et al., 2002; Kamiya et al., 2004). Moreover, a few previous studies of an aqueous fruit extract on gastrointestinal motility reported controversial results with increase (Chuthaputti et al., 1996) and delayed (Pu et al., 2004) gastric emptying action. It should be noted, however, that the type of fruit extract and the concentration used in these experiments was quite different. In addition, an appropriated quality assessment of noni products for such purposes is lacking. Recently, noni fruit extract has been claimed to have several biological properties such as wound healing, anti-inflammatory and anti-oxidative activities (McKoy et al., 2002; Zin et al., 2002; Potterat and Hamburger, 2007; Ikeda et al., 2009). Among a number of major components identified in the aqueous fruit juice, scopoletin, a coumarin derivative is one of main compounds that has known pharmacological activities especially an ability to control serotonin level in the body (Levand and Larson, 1979), together with anti-inflammatory (Kang et al., 1999; Kim et al., 2004; Deng et al., 2007; Moon et al., 2007) and antioxidative activities (Ikeda et al., 2009). Scopoletin is also recommended as a marker constituent for the quality control and pharmacokinetic study of noni products (Samoylenko et al., 2006; Ikeda et al., 2009). Therefore, it will be interesting to elucidate the efficacy of dried mature unripe noni fruit extract and its biomarker: scopoletin on gastro-esophageal disorder models that are related to its claimed pharmacological properties and the recent pharmacological viewpoints. Given this, the chemical structure of scopoletin will be potentially used as a model for designing a new approach as a natural therapeutic agent with antiulcerogenic, anti-inflammatory and prokinetic properties. Additionally, the pharmacological results from animal study will also be useful for the standardization of noni fruit dosage for treatment of various upper gastrointestinal disorders.

There are three marker compounds in noni fruit used for quality assurance of commercial noni fruit juice products. One inorganic compound potassium and two organic compounds: scopoletin and (2E, 4Z, 7Z)-decatrienoic acid or DTA. Potassium is normally the most dominant mineral found in noni fruit juice (1000-3000 mg/L). To make it less than 1,000 mg/L dilution of noni fruit juice with water (Basar and Westendorf, 2010) is recommended. DTA is rarely observed in noni fruit juice, and its amount is decreased by the heating. If the hydrolysis occurs in fresh squeezed noni juice, DTA will be present as a major constituent and can indicate if the noni fruit juice is untreated or hydrolyzed. In contrast to DTA, scopoletin is always present in noni fruit collected from any region and is recently recommended as a marker constituent for the quality control

and pharmacokinetic study of noni (Samoylenko et al., 2006; Ikeda et. al., 2009). Therefore, it is also important to measure the amount of scopoletin in a dried mature unripe noni fruit extract. Several methods have been published for determination of scopoletin in different medicinal plants such as thin layer chromatography (TLC) (Li et al., 1996), ethanol modified supercritical fluids extraction (Tzeng et al., 2007), micellar electrokinetic capillary chromatography (Wang et al., 2007), spectroscopic methods, including 1 D- and 2D-NMR techniques (Bina et al., 2007), HPLC-MS assay (Potterat et al. 2007), and recently high-performance liquid chromatography (HPLC) (Ikeda et al., 2009). Since, a solid phase extraction (SPE) procedure is widely used as a sample-preparation technique for isolation and purification of selected interesting analytes due to its several advantages (simple, low cost, low consumption of organic solvents and high recovery) (Rodriguez et al., 2000), a SPE procedure on conventional C_{18} sorbents followed by an RP-HPLC assay was thus developed and validated for a rapid, low cost, precise and reliable analytical method to determine the quantity of scopoletin in noni fruit commercial or medical products.

Objectives

1. To develop and validate an analytical method based on solid-phase extraction (SPE) and RP-HPLC for quantitative and qualitative determination of scopoletin in *Morinda citrifolia* fruit products.
2. To determine the effects of *Morinda citrifolia* fruit extract and its biomarker, scopoletin, on the upper gastrointestinal tract motility in rats compared with a standard agent: cisapride.
3. To determine the effects of *Morinda citrifolia* fruit extract and its biomarker, scopoletin, on reflux esophagitis and gastritis in rats compared with standard agents: ranitidine and lansoprazole.

Benefits

1. To obtain a rapid, simple, low cost, precise and reliable analytical method to determine the quality of scopoletin in *Morinda citrifolia* fruit product.
2. To provide a pharmacological basis for the standardization of *Morinda citrifolia* fruit or pure scopoletin dosage for treatment of various upper gastrointestinal disorders in primary health care and clinical application
3. To provide a chemical structure basis as a model for designing a new approach as a natural therapeutic agent with antiulcerogenic, anti-inflammatory and prokinetic properties.

CHAPTER 2

RESEARCH METHODOLOGY

1. Plant materials, animals, drugs and chemicals

Plant materials

The fresh mature unripe noni fruits were harvested from Chana district, Songkhla province, Thailand. Voucher specimens (SKP 165130301) were retained for future reference at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Aqueous noni fruit extract (AFE)

19 kg of fresh mature unripe noni fruits were washed with tap water to remove dust, sliced into thin pieces and allowed to dry at 50°C in a hot air oven. The dried materials (1.3 kg) were then ground to powder using an electric grinder. 1 kg of ground powder was boiled in 4 l of water until it became a sticky paste, and then dried into a powder by lyophilization. The obtained powder was stored at -20°C. The percentage yield was about 28.8% w/w. The lyophilized powder was freshly dissolved in distilled water before use.

Ethanolic noni fruit extract (EFE)

1 kg of dried mature unripe noni fruits powder was immersed in 4 l of 95% ethanol and left for 7 days at room temperature. The three times extract was then filtered and concentrated by using a rotary evaporator in vacuum at 40°C. The residues were kept and dried overnight on water bath at 60°C until a constant weight was achieved and stored at -20°C. The percentage yield was about 14.2% w/w.

Animals

Male Wistar rats weighing 180–200 g were housed under normal laboratory conditions at 25$\pm$1°C with a controlled 12-h light-dark cycle and maintained on standard rodent chow and tap water *ad libitum*. When necessary, the rats were deprived of food but with access to water *ad libitum* 24 h before the experiments.

All animals received humane care in compliance with the guideline of the Animal Care and Use Committee of Prince of Songkla University, Thailand. The experimental procedures were approved by the Committee on Animal Care and in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Prince of Songkla University (MOE 0521.11/098).

Drugs and chemicals

Activated charcoal, bovine serum albumin, cisapride, lansoprazole, phenol red, pentobarbital sodium, pepsin from hog stomach, potassium dihydrogen phosphate (KH_2PO_4), ranitidine, scopoletin, serotonin hydrochloride and sodium acetate (CH_3COONa) were purchased from Sigma–Aldrich (USA). Sodium hydroxide was purchased from Ajax Finechem (Australia). Supelclean™ (57062) ENVI-18 SPE Tube (bed wt. 100 mg, volume 1 ml), and solid phase extraction (SPE) vacuum manifold 57030-U were purchased from Supelco (USA). HPLC grade 95% ethanol, methanol and acetonitrile (ACN) were purchased from RCI Lab-Scan (Thailand). Other chemicals used were of analytical grade. Water was obtained using a Milli-Q Plus water purification system (Millipore™, USA).

2. Quantitative analysis of scopoletin in noni fruit extracts

2.1 Determination of suitable eluting solvent and solvent volume for eluting scopoletin

To obtain a suitable solvent for elution of scopoletin from cartridge, different percentages of methanol in 0.01 M KH_2PO_4 buffer pH 3 and pH 7 solutions were examined. For determination of a suitable volume of elution solvent, different volumes (2, 4, 6, 8, 10 ml) of those solvents were used for elution of retained scopoletin from the cartridge. First of all, the sorbent was conditioned with 4 ml of methanol followed by 2 ml of water. 1 ml of standard scopoletin (10 μg/ml) in 0.01 M KH_2PO_4 buffer pH 3 and pH 7 were then passed through the SPE cartridges. The single or mixed solvent system consisting of any of three solvents: 100% MeOH; 0.01 M KH_2PO_4 buffer pH 3 and 0.01 M KH_2PO_4 buffer pH 7; and 20% MeOH in 0.01 M KH_2PO_4 buffer pH 3 and 20% MeOH in 0.01 M KH_2PO_4 buffer pH 7 in various volumes from 1-10 ml were used to disrupt hydrophobic interactions between the analyte and sorbent functional groups. Next, the eluates were evaporated and reconstituted with 1 ml of mobile phase prior to analysis by HPLC. Finally, the graphs were plotted between the volume of eluting solvent (ml) and the percent recovery of scopoletin in each eluting solvent. The smallest volume of solvent that can elute scopoletin from the SPE cartridge with maximum percentage will be selected as for eluting scopoletin from the SPE sorbent.

2.2 Determination of breakthrough volume

The breakthrough volume was determined according to the following procedures. Standard scopoletin was dissolved in 0.01 M KH_2PO_4 buffer pH 3 and pH 7 to give concentrations of 10 μg/ml. The solution was then passed through the cartridge in

aliquots of 5 ml. The effluent from each aliquot was collected in separate containers and analyzed by HPLC. The breakthrough curve which is the relationship between the concentration of the analyte in the effluent (μg/ml) and the total volume of sample (ml) passed through the SPE cartridge was plotted. The breakthrough volume was determined as the volume of sample at which 1% of the analyte was eluted from the cartridge. Next, the eluents were dried in a vacuum oven and reconstituted with 1 ml of the mobile phase prior to analysis by HPLC.

2.3 Determination of maximum loading capacity of SPE cartridge for scopoletin

The maximum loading capacity of SPE device refers to the maximum amount of the analyte that can be retained in the SPE device. The maximum loading capacity of the SPE cartridge for scopoletin was determined by passing different volumes ranging from 10-100 ml of 10 μg/ml aqueous standard solutions of scopoletin through the cartridge. Retained scopoletin was eluted with 5 ml of 100% methanol solution, dried in a vacuum oven. The residue was dissolved in 1 ml of the mobile phase and analyzed by HPLC.

2.4 Sample preparation

100 mg of aqueous noni fruit extract was reconstituted with 25 ml of 0.01M KH_2PO_4 buffer (pH 3) to yield a concentration of 4 mg/ml. The mixture was vortexed for 2 min to ensure homogeneity, sonicated at room temperature for 30 min, then filtered through a membrane filter (0.20 um x 47 mm, Whatman, USA). All noni fruit extract samples were prepared in three replicates.

2.5 Solid Phase Extraction (SPE) Procedure

The SPE sorbent was conditioned with 4 ml of methanol followed by 2 ml of Milli-Q water. Appropriate volumes of each aqueous noni fruit extract in 0.01M KH_2PO_4 buffer pH 3 (4 mg/ml) was then loaded into the cartridge and allowed to flow through the column at a flow rate of approximately 1 ml per min (~1 drop/sec). After the column was rinsed with 4 ml of 0.01M KH_2PO_4 buffer pH 7, 4 ml of 100 % methanol was used to elute the analyte from the cartridge. The eluate was evaporated to dryness using a vacuum oven (NAPCO model5861, USA) at room temperature for 3 h and then reconstituted in 1 ml of mobile phase. A 20 μl of the resulting solution was injected into the HPLC system.

2.6 The recovery of Solid Phase Extraction

The recovery of the solid phase extraction was evaluated by analyzing six replicates of 3 concentrations of standard scopoletin solution in 0.01M KH_2PO_4 buffer pH 3 and pH 7 (1, 5, 10 μg/ml) that were passed through the SPE cartridges, and quantitative determination of scopoletin by HPLC. The extraction recoveries were calculated as shown below:

Recovery = measured value/true value ×100

2.7 HPLC Apparatus and chromatographic conditions

The HPLC system (Waters HPLC Separation Module, USA) consisted of a 600 LCD pump, a 717 autosampler, a Jusco-UV-975 UV-detector (Jasco, Japan) operated at 350 nm, and a Waters® integrator (model 4400). Chromatographic separations were performed at room temperature (25±2° C) on the Express C_{18} analytical column (5.0 μm, 4.6 mm x 150 mm, Supelco Ascentis®) with isocratic elution at a flow rate of 1.0 ml/ min. The mobile phase was a mixture of 0.01 M sodium acetate (adjusted to pH 3 with 100% acetic acid) and acetonitrile (80:20 v/v). The column was equilibrated with the mobile phase at 1.0 ml/min for at least 60 min prior to sample analysis. The mobile phase was prepared freshly on the day of sample analysis, filtered through a 0.45 um membrane (Sartorius, Germany) and degassed by sonication for 30 min prior to use. The aqueous noni fruit extract samples were filtered through a 0.20 μm membrane filter (Whatman, USA) and 20 μl of the final processed samples was injected into the HPLC system. The run time was set at 10 min.

2.8 Preparation of calibration standards

Primary stock solutions of standard scopoletin (1,000 μg/ml) were prepared by dissolving 1 mg of scopoletin in 10 ml methanol. These stock solutions were sealed and stored at 4°C in a clear glass volumetric flask and wrapped with aluminum foil to protect from light. The working standard solution was freshly prepared by diluting the primary stock solution with methanol. Calibration standards of scopoletin were freshly prepared by serial dilution with mobile phase [0.01 M sodium acetate pH 3: acetonitrile (80:20 v/v)] to yield final concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, and 10 μg/ml.

2.9 Method Validation

The method was validated according to the International Conference on Harmonization (ICH) guidelines for the validation of analytical procedures. The following validation characteristics were addressed: linearity and range, sensitivity, selectivity, accuracy and precision.

Linearity of calibration curves

Calibration curves were constructed from working standard solutions of scopoletin in the concentration range of 0.05-10 µg/ml by plotting the mean peak areas (Y) versus scopoletin concentration (X). The calibration curve was prepared in three replicates from three separate scopoletin stock solutions. The regression parameters, i.e., slope; intercept and coefficientsa of determination (R^2) were evaluated. The coefficient of determination is used in the context of statistical models whose main purpose is the prediction of future outcomes on the basis of other related information between mean peak areas and concentrations.

Sensitivity

Sensitivity of the method was assessed from limit of detection (LOD) and limit of quantitation (LOQ). LOD was defined as the lowest concentrations of analytes in a sample that can be detected. LOQ was defined as the lowest concentrations of analytes in a sample that can be accurately and precisely measured. The LOD and LOQ were determined on the basis of signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively. The preliminary values of LOD and LOQ were determined from the calibration curve and the standard deviation of five replicate determinations according to the equation below.

Limit of Detection (LOD) = 3.3 (SD/Slope)

Limit of Quantitation (LOQ) = 10 (SD/Slope)

The preliminary calculated values of LOD and LOQ were then was confirmed by analyzing spiked aqueous noni fruit extract samples at these values in three replicates. The LOD was evaluated by measuring the S/N ratio. The accuracy and precision of the method at LOQ were determined as stated below under accuracy and precision.

Selectivity

The selectivity of the method was assessed by comparing the chromatograms of the samples to those of the blank (extracting solvent) and the standard scopoletin solutions. Peak shapes, retention times and spectral purity of the chromatographic peak were considered to detect possible interferences using HPLC with diode array detector. The purity index is a measure of the peak's relative purity measured using a full comparison of spectral data for the leading and training edge of the peak.

Accuracy (recovery)and precision

Each 100 mg of aqueous noni fruit extract was reconstitututed with 100 ml of 0.01M KH_2PO_4 buffer (pH 3) to yield the concentration of 1 mg/ml, and then spiked with three concentrations of scopoletin (2, 8 and 12 µg/ml) in five replications. Each of the mixed sample was vortexed for 2 min to ensure homogeneity, sonicated at room

temperature for 30 min and then filtered through a membrane filter (0.20 um x 47 mm, Whatman, USA). 1 ml of spiked sample was passed through the SPE procedure followed by a quantitative assay with the developed HPLC method. The accuracy (recovery) of the method was assessed by comparing the measured concentration of scopoletin in the spiked sample to the known spiked concentration as shown below:

$$\% \text{ Recovery} = \frac{\text{Scopoletin concentration in spiked sample - Scopoletin concentration in unspiked sample}}{\text{Known spiked concentration}} \times 100$$

The intra-day precision or repeatability is the degree of agreement of results when experimental conditions are maintained as constant as possible, and expressed as the percentage coefficient of variation (% CV) or relative standard deviation (RSD). It was determined from relative standard deviation of three replicate determinations of the samples within the same day. The same procedure was performed for five days to determine inter-day precision. The % RSD was calculated by

$$\% \text{ CV or (RSD)} = (\text{SD/mean}) \times 100$$

Stability-indicating capability of the method

In order to establish whether the proposed method was stable, the standard solutions of scopoletin were stressed under various conditions to conduct forced degradation studies. Scopoletin is poorly water soluble, but freely soluble in methanol. Most solutions prepared for use in forced degradation studies were prepared by dissolving scopoletin in small volumes of methanol and later diluted with aqueous hydrochloric acid, aqueous sodium hydroxide, and aqueous hydrogen peroxide, to give a concentration of 1 mg/ml. The conditions for forced degradation were as follow:

(1) Acid degradation: Scopoletin at a concentration of 1.5 mg/ml in 0.1 M HCl were heated at 60^{o}C for 0, 1, 2, 3, and 4 weeks.

(2) Base degradation: Scopoletin at a concentration of 1.5 mg/ml in 0.05 M NaOH were heated at 60^{o}C for 0, 1, 2, 3, and 4 weeks.

(3) Oxidative degradation: Scopoletin at a concentration of 1.5 mg/ml in 0.3% H_2O_2 were heated at 60^{o}C for 0, 1, 2, 3, and 4 weeks.

The samples were analyzed by HPLC and the results were compared with those obtained for freshly prepared samples. The peak from scopoletin must not be interfered with by any of the degradation products formed. A peak purity test was performed by a photodiode array detector to demonstrate that the scopoletin peak did not contain more than one substance.

3. Pharmacological evaluation of noni fruit extracts and scopoletin in rats

3.1 Effect of noni fruit extracts and scopoletin on gastric emptying rates (GER) of phenol red meals in rats

The GER was determined in conscious rats by measuring the disappearance of phenol red from the stomach (Lee, et al., 2005). Thirty minutes after the pretreatment with each test drug, a phenol red meal (phenol red 0.05% w/w in 1.5% carboxymethyl cellulose 1200, CMC) was administered orally to the rats at a volume of 0.75 ml/100 g body weight. After 20 min, the rats were sacrificed by cervical dislocation and the gastroesophageal junction and the pylorus was ligated. The stomach was then extirpated, rinsed in normal saline, and homogenized in 100 ml 0.1 N NaOH. The suspension was allowed to settle for 1 h at room temperature, and 5 ml of the supernatant was then added to 0.5 ml 20% trichloroacetic acid (w/v) and centrifuged at 3000 rpm at 4°C for 20 min. The supernatant was mixed with 4 ml of 0.5 N NaOH, and the absorbance of the sample was read at 560 nm (A_{560}) using a spectrophotometer. The phenol red recovered from the animals that had been killed immediately after the administration of CMC solution was used as the control (0% emptying). The GER in the 20-min period was calculated according to the following equation:

% GER = [1- (A_{560} of test/ A_{560} of control)] x 100

3.2 Effect of noni fruit extracts and scopoletin on intestinal transit rates (ITR) of charcoal meals in rats

Thirty minutes after the pretreatment with each test drug, a charcoal meal (10% w/v activated charcoal in 1% w/v CMC) was administered orally to the rats at a volume of 0.05 ml/100 g body weight. The rats were killed at 60 min. The distances of charcoal movement along the intestine and the total length of the small intestine were measured. The ratio of the distance traveled by the charcoal divided by the total length of the small intestine was expressed as % ITR.

3.3 Effect of noni fruit extracts and scopoletin on gastric acid secretion and pepsin activity

A pylorus ligation was carefully done in fasted rats under anesthesia with pentobarbital sodium (50 mg/kg, *i.p.*). The test drugs were administered intraduodenally immediately after the ligation. Four hours following the treatment, the rats were killed and the gastric juice was collected and centrifuged. After measuring the volume of the supernatant, the total acid output was analyzed by titration with 2 mM NaOH using 2% phenolphthalein as an indicator and expressed as μEq/ml. Pepsin activity was

determined by a slight modification of the Anson method (Anson, 1938), using bovine hemoglobin as a substrate.

3.4 Effect of noni fruit extracts and scopoletin on acid reflux esophagitis

Rats were laparotomized under light ether anesthesia to ligate the pylorus and the junction between the forestomach and the corpus (the limiting ridge) (Nakamura et al., 1982). The test drugs were administered immediately after the ligation. The rats were then deprived of food and water. Six hours later, the gastroesophageal portion of the digestive tract was excised and the severity of esophagitis was scored macroscopically, using an ulcer index according to the following criteria: 0, no injury; 1, erosion of mucosal epithelium; 2, the length of hemorrhagic ulcer area <20 mm; 3, the length of hemorrhagic ulcer area 20–30 mm; 4, the length of hemorrhagic ulcer area 30–40 mm; 5, the length of hemorrhagic ulcer area >40 mm or perforation.

3.5 Effect of noni fruit extracts and scopoletin on gastric mucosal lesions induced by ethanol

Thirty minutes after the oral administration of either test drugs, 80% ethanol (1 ml/200 g body weight) was orally administered to the fasted rat. One hour later, the stomach was excised and the sum of the length (mm) of all lesions for each stomach was used as a lesion index.

4. Statistical analysis

All data in quantitative determination of scopoletin in noni fruit extracts were expressed as mean $\pm$ standard deviation (SD). All data in pharmacological evaluation of noni fruit extracts and scopoletin were expressed as mean $\pm$ standard error of means (S.E.M). Comparisons between groups were made by one-way analysis of variance (ANOVA) followed by the Dunnett test. Values of $p<0.05$ was considered to be statistically significant.

CHAPTER 3

RESULTS

1. Quantitative analysis of scopoletin in noni fruit extracts

1.1 The optimization of SPE procedure

Selection of appropriate loading solution and volume is the first key factor that makes a good recovery of analyte from SPE. The result showed that scopoletin is better retained on adsorbent in 0.01 M KH_2PO_4 pH 3 buffer solution than in 0.01 M KH_2PO_4 pH 7 buffer solution due to a less breakthrough volume (Fig. 1). However, detectable scopoletin content was not found in washing effluent when a 0.01 M KH_2PO_4 pH 7 buffer solution was used to wash undesired component in noni fruit extract. Therefore, a 0.01 M KH_2PO_4 pH 3 buffer solution was selected for loading whereas a 0.01 M KH_2PO_4 pH 7 buffer solution was selected to rinse the sorbent. The high recovery of scopoletin (84.52 $\pm$ 3.65%) indicated that SPE procedure is suitable for the extraction.

1.2 Method validation

A typical HPLC chromatogrms of scopoletin was shown in Fig. 2A. It was found that the mixture of sodium acetate (pH 3; 0.01 M): acetonitrile (80:20, v/v) produced a good peak for scopoletin which was well separated from the solvent fronts at short retention time (about 5 min) and run time (10 min). The linear regression equation was calculated as Y= 263502.75X + 105171.49, where Y is the peak area of the standard, and X is the concentration (Fig. 3). The result showed an acceptable linearity with correlation coefficients (R^2) higher than 0.9998 within the range of concentration investigated. The LOD and LOQ of the method were 0.0026 and 0.0079 ug/ml, respectively.

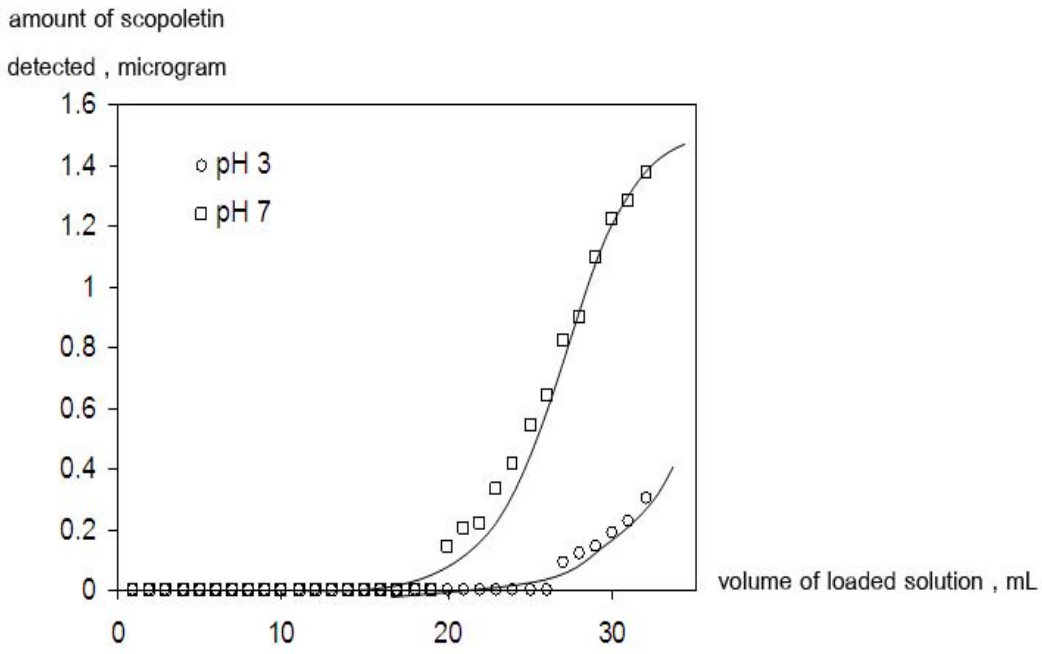

Figure 1. The breakthrough profile of scopoletin from SPE cartridge. Two loading solutions were used: 0.01 M KH_2PO_4 pH 3 and 0.01M KH_2PO_4 pH 7.

The extraction recoveries = measured value/true value x 100

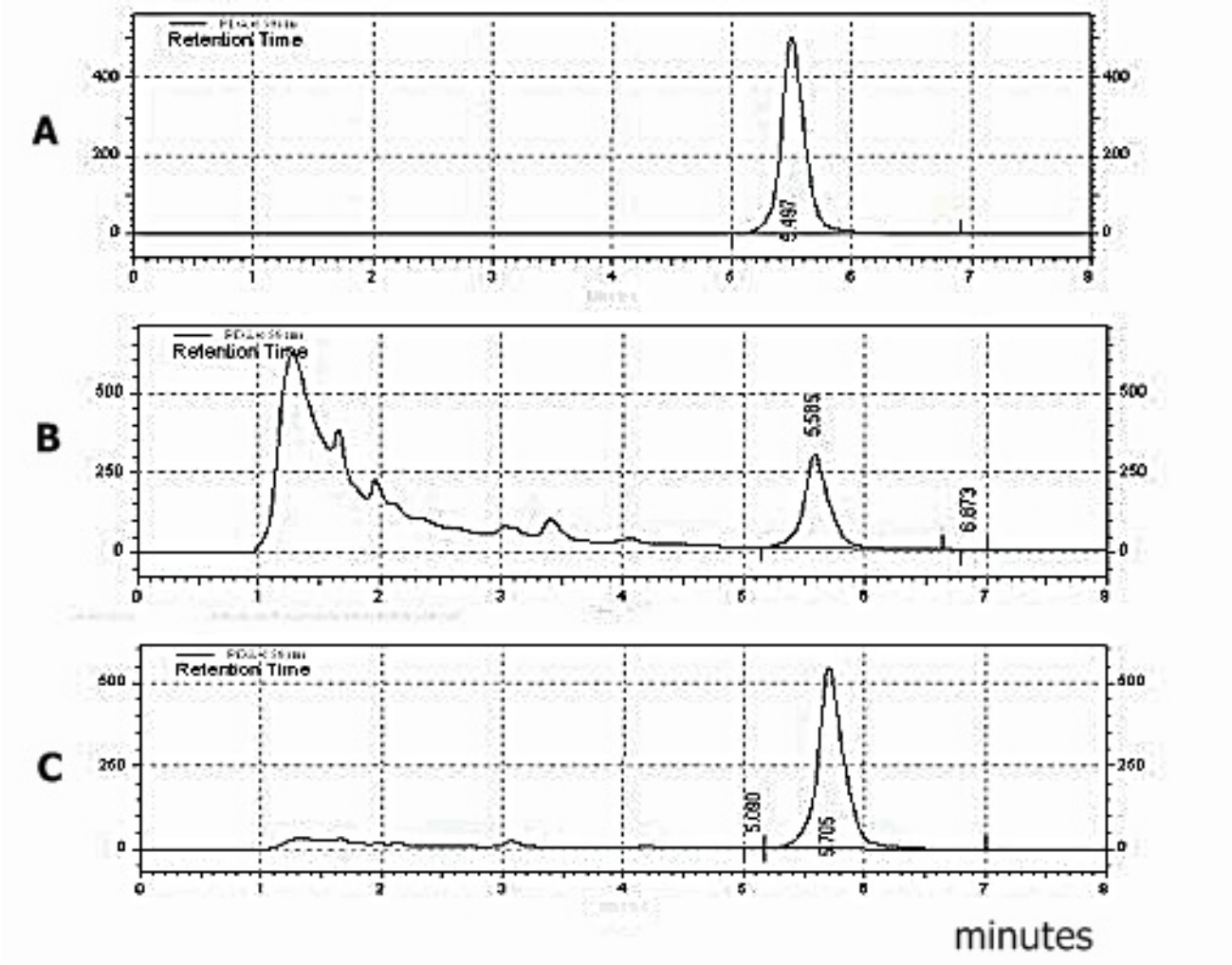

Figure 2. The HPLC chromatogrms of scopoletin in noni fruit extracts. The experimental conditions were described in chapter 2. The x- and y-axes represent the running time (min) and peak absorbance, respectively.

(A) chromatograms of scopoletin in standard solution

(B) chromatograms of scopoletin in AFE

(C) chromatograms of scopoletin in EFE

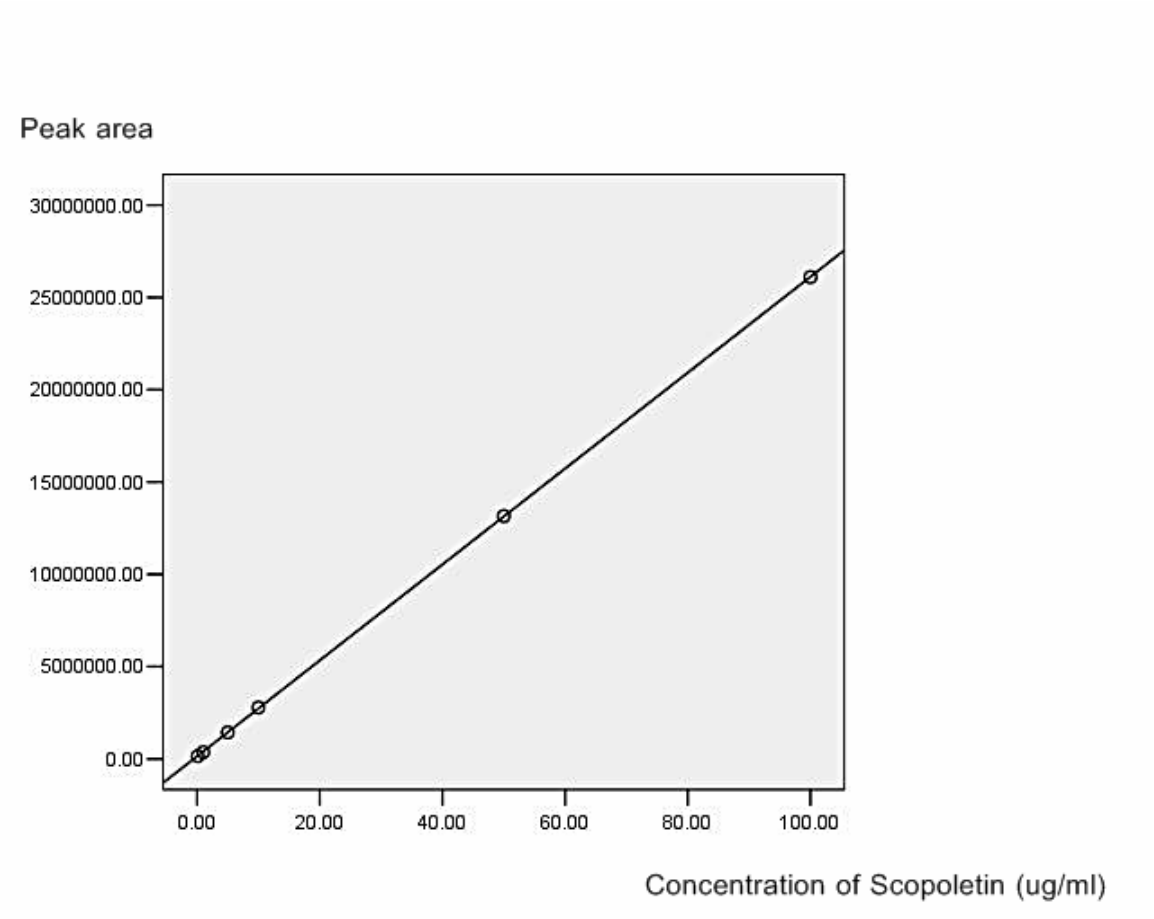

Figure 3. Calibration curve of scopoletin (10-100 ug/ml) in noni fruit extract

X = the concentration of scopoletin

Y = the peak area of the standard calculated as 263502.75X + 105171.49

Selectivity

The SPE extraction of noni fruit extracts exhibited a clear chromatogram as shown in Fig. 2B and 2C. The identification of the peaks corresponding to scopoletin in the chromatogram of noni fruit extracts after SPE was done by comparing the retention time, standard spiking and purity confirmation using diode array detector program with those of standard scopoletin. High peak purity of scopoletin was observed in AFE (with the total peak purity 0.90) and EFE (with the total peak purity 0.96) (Fig. 4-6).

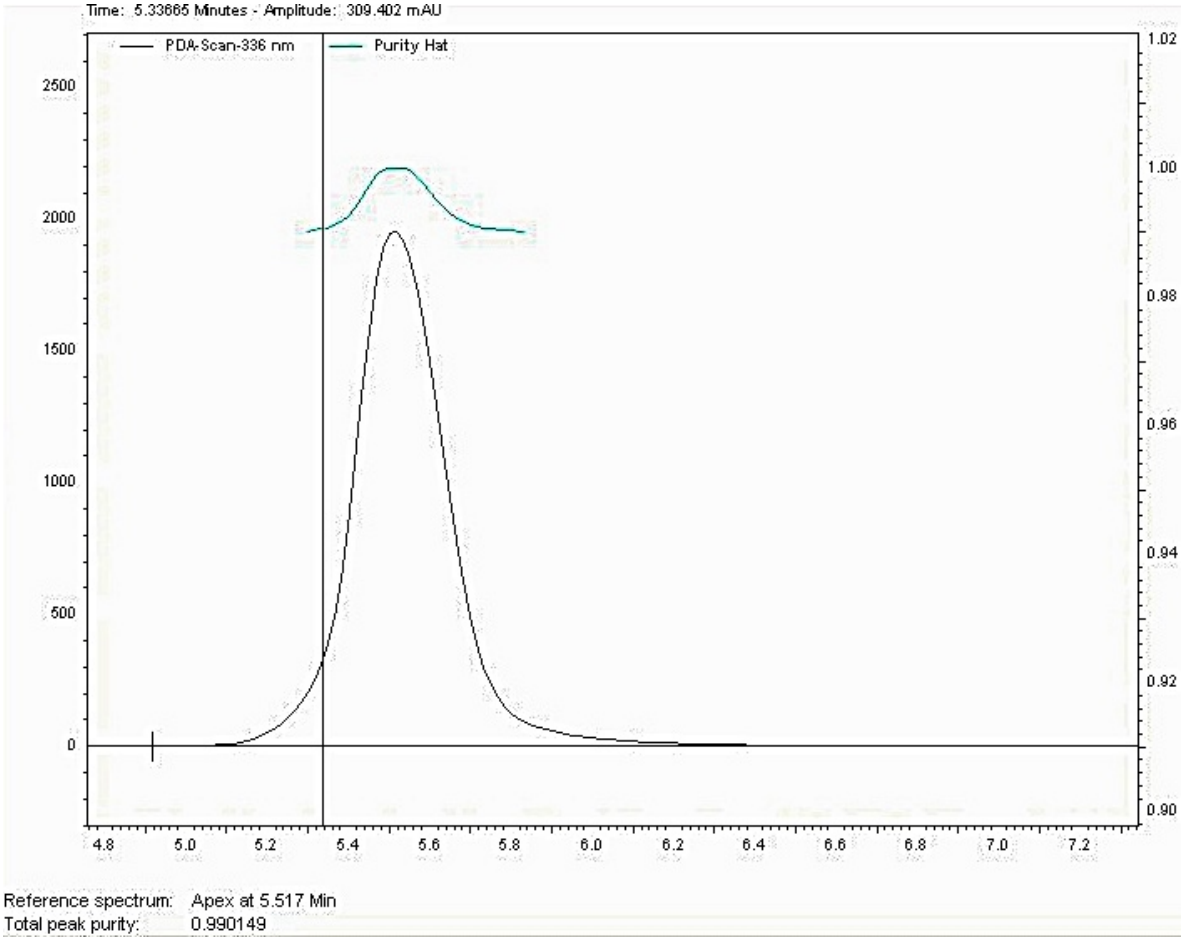

Figure 4. Peak purity of scopoletin in standard solution. The scopoletin peak purity was detected using a multi-wavelength of photo diode array detector
(total peak purity =0.99).

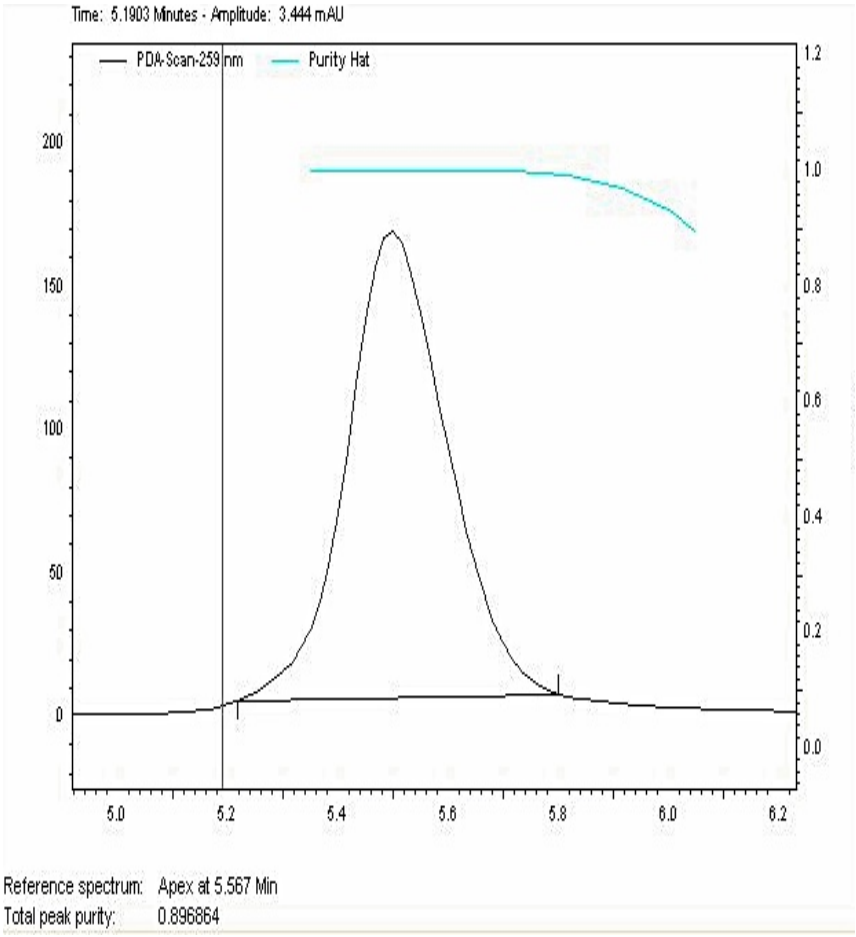

Figure 5. Peak purity of scopoletin in AFE. The scopoletin peak purity was detected using a multi-wavelength of photo diode array detector (total peak purity =0.90).

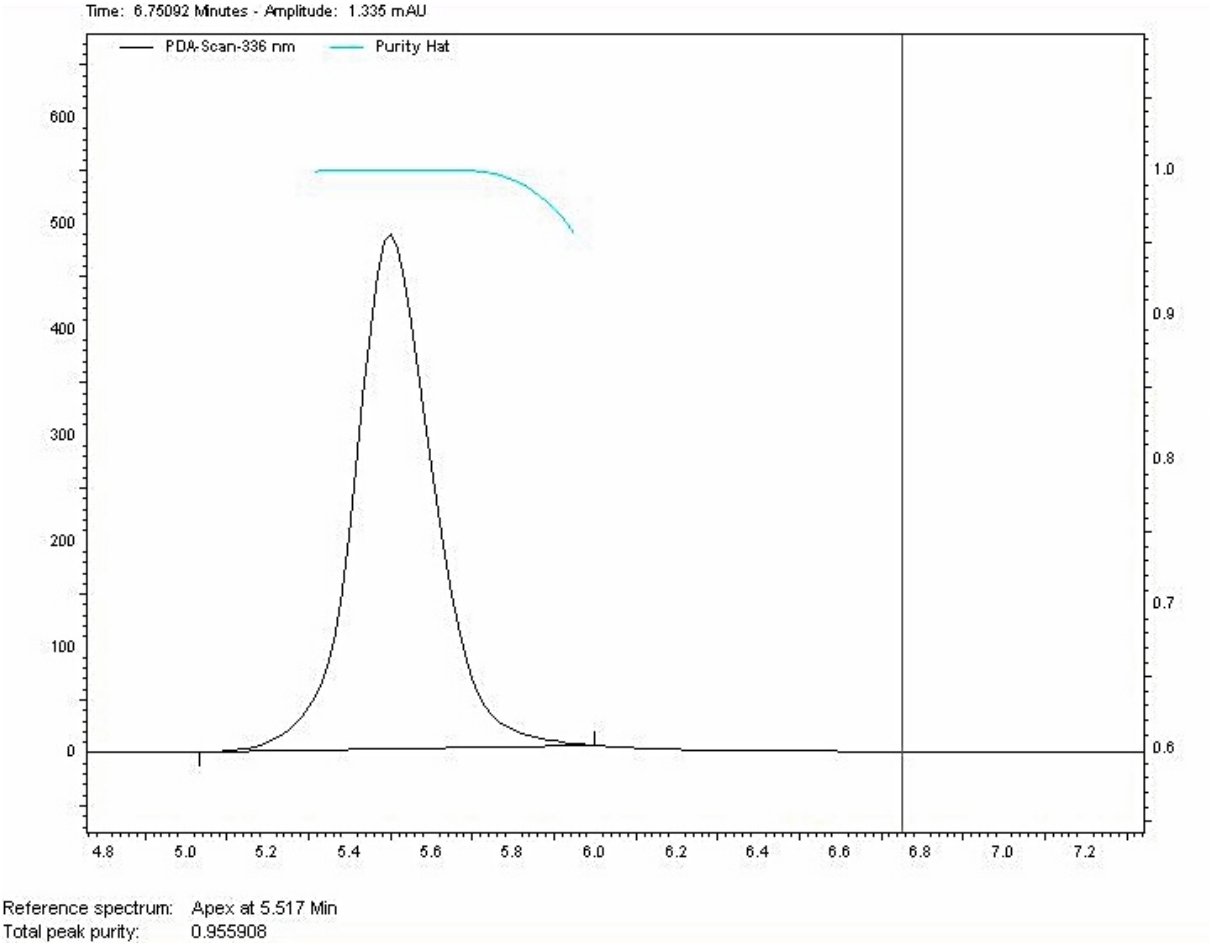

Figure 6 Peak purity of scopoletin in EFE. The scopoletin peak purity was detected using a multi-wavelength of photo diode array detector (total peak purity =0.96).

Accuracy (recovery) and precision

The data showed that the RSD's intra- and inter-day precisions were less than 2% for AFE and EFE (Table 1). The accuracy, expressed as a recovery percentage, was determined by spiking standard solution of scopoletin at three concentrations (2, 8, 12 ug/ml) in the noni fruit extracts. The percent recoveries of scopoletin in both fruit extracts was 95.84, 96.78 and 96.80 % for AFE and 94.67, 94.59 and 94.40 % for EFE (Table 2).

Table 1. The intra-day (repeatability) and inter-day (intermediate) precision for the quantitative determination of scopoletin in noni fruit extracts

Noni Matrix	SCP conc. (ug/ml)	SCP founded in sample (ug/ml)											
		Intra-day precision									Inter-day precision		
		Day 1 (n=9)			Day 2 (n=9)			Day 3 (n=9)			(n=27)		
		mean	SD	%RSD	mean	SD	%RSD	Mean	SD	%RSD	mean	SD	%RSD
AFE	Blank	2.00	0.004	0.22	2.03	0.001	0.06	2.00	0.004	0.19	2.01	0.017	0.84
	2	2.95	0.007	0.23	2.99	0.009	0.31	2.95	0.002	0.08	2.96	0.019	0.64
	8	5.87	0.022	0.37	5.91	0.023	0.39	5.81	0.010	0.17	5.86	0.047	0.80
	12	7.74	0.108	1.39	7.84	0.009	0.11	7.79	0.060	0.78	7.79	0.081	1.04
EFE	Blank	5.53	0.002	0.03	5.50	0.013	0.23	5.50	0.012	0.22	5.51	0.017	0.30
	2	6.48	0.044	0.68	6.45	0.055	0.86	6.44	0.053	0.82	9.30	0.052	0.56
	8	9.31	0.070	0.75	9.30	0.043	0.46	9.30	0.043	0.46	9.30	0.052	0.56
	12	11.26	0.089	0.79	11.17	0.072	0.64	11.17	0.012	0.11	11.20	0.078	0.70

Each value represents the mean ± SD from 9 replicates for intra-day precision and from 27 replicates for inter-day precision.

AFE = aqueous fruit extract EFE = ethanolic fruit extract conc. = concentration

SCP = scopoletin % RSD = % relative standard deviation

Table 2. The Accuracy (% recovery) assay for the quantitative determination of scopoletin in noni fruit extracts

Scopoletin spiked in noni matrix (n=9)	Recovery (%)			
	AFE		EFE	
	Mean	% RSD	Mean	% RSD
2 ug/ml	95.84	0.10	94.67	0.39
8 ug/ml	96.78	0.19	94.59	0.32
12 ug/ml	96.80	0.39	94.40	0.12
Average	96.47	0.23	94.55	0.28

Each value represents the mean ± SD from 9 replicates

% Recovery = (Scopoletin concentration in spiked sample - Scopoletin concentration in unspiked sample) x 100

Known spiked concentration

Stability (Degradation studies

The stability results of standard scopoletin determined by exposing to stress conditions, i.e. 0.1 M HCl, 0.1 M NaOH, and 3% H_2O_2 was also showed that neither degradation of scopoletin nor significant change in peak area and retention time of scopoletin was found in various stress conditions as shown in Table 3. It was only found a substantial decrease in the peak area of scopoletin but not in the retention time in the presence of 0.1 M NaOH in working solution.

1.3 Quantification

The quantitative data of scopoletin in noni fruit extracts was summarized in Table 4. EFE contained larger amount of scopoletin than AFE. The amount of scopoletin in 1 g of lyophilized AFE and EFE powder was calculated to be 0.85-0.87 and 1.95-2.03 mg, respectively.

Table 3. Summary of scopoletin stability in various stress conditions

Stability indicating method of scopoletin in various conditions						
Scopoletin concentration in each solution (ug/ml)	**Initiation concentration (ug/ml)**		**Percent amount left Time (week)**			
	Mean	**SD**	**1**	**2**	**3**	**4**
1) 0.1 M HCl 1.5	1.51	0.002	100.00	100.03	99.67	99.43
3	2.97	0.015	99.94	99.11	98.76	98.23
5	4.99	0.028	99.52	99.44	99.18	97.97
2) 0.01N NaOH 1.5	1.49	0.002	81.23	ND	ND	ND
3	2.89	0.003	81.14	40.98	ND	ND
5	4.71	0.014	80.37	39.23	26.49	ND
3) 0.3% H_2O_2 1.5	1.50	0.002	99.59	97.60	96.05	95.30
3	2.99	0.011	98.09	96.83	96.14	94.68
5	4.98	0.026	99.75	96.80	95.36	94.93

Each value represents the mean $\pm$ SD from 5 replicates.

Table 4. HPLC-UV determination of scopoletin in noni fruit extracts

Noni fruit extracts	Scopoletin concentration (ug/ml)	Scopoletin amount (mg/g)
	Mean ± SD	Mean ± SD
AFE (15 mg/ml) (n=9)	4.55 ± 0.14	0.86±0.01
EFE (15 mg/ml) (n=9)	10.26 ± 0.22	1.99±0.04

Each value represents the mean ± SD from 9 replicates.

2. Pharmacological evaluation of noni fruit extract and scopoletin in rats

2.1 Effect of noni fruit extracts and scopoletin on GER of phenol red meals and on ITR of charcoal meals in rats

AFE (0.63-2.50 mg/kg) significantly accelerated the GER of phenol red meals in a dose dependent manner (Table 5). In addition, AFE fed at the same dose range exerted the potent efficacy in accelerating the ITR of charcoal meals. EFE at the dose of 0.5 g/kg which contained the same equivalent dose of scopoletin as presented in 1.25 g/kg of AFE, also exerted the similar prokinetic efficacy to that of AFE. The prokinetic efficacy of AFE or EFE in both models appeared to be higher compared to cisapride (10 mg/kg) and pure scopoletin (at the same equivalent dose of scopoletin containing in the fruit extract) ($p < 0.05$).

Table 5. Effect of noni fruit extracts and scopoletin on GER and ITR in rats

Treatment	Dose	GER (%)	ITR (%)
Water control	5 ml/kg *p.o.*	47.17±0.43	53.06±0.66
1% CMC	5 ml/kg/ *p.o.*	42.92±0.24	55.12±0.71
AFE	0.63 g/kg/5ml *p.o.* (equivalent to 0.5 mg scopoletin)	76.91±0.65[a]	87.65±0.57[a]
	1.25 g/kg/5ml *p.o* (equivalent to 1 mg scopoletin)	82.63±0.39[a]	92.41±1.25[a]
	2.5 g/kg/5ml *p.o.* (equivalent to 1.5 mg scopoletin)	97.21±0.57[a]	95.59±0.14[a]
EFE	0.5 g/kg/5ml *p.o.* (equivalent to 1 mg scopoletin)	80.97±0.82[a]	85.06±1.36[a]
Scopoletin	0.5 mg/kg/5ml *p.o.*	58.54±0.61[b]	61.17±0.98[b]
	1 mg/kg/5ml *p.o.*	77.31±0.69[b]	83.73±0.86[b]
Cisapride	10 mg/kg/5 ml *p.o.*	78.57±0.26[b]	78.58±0.32[b]

Each value represents the mean ± S.E.M. from 8 rats. GER, gastric emptying rate; ITR, intestinal transit rate

All measuring parameters were determined as described in Chapter 2.

[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test)

[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test)

2.2 Effect of noni fruit extracts and scopoletin on gastric acid secretion and pepsin activity

Intraduodenal injection of AFE (0.63-2.50 mg/kg) had a dose dependent potency on the decrease of acid secretion and pepsin activity (Table 6) but had a less acid inhibitory efficacy than those of ranitidine (50 mg/kg) and lansoprazole (1 mg/kg) ($p< 0.05$). EFE at the dose of 0.5 g/kg which contained the same equivalent dose of 1 mg scopoletin as presented in 1.25 g/kg of AFE, also exerted the comparable antisecretory efficacy to that of AFE (NS). Additionally, the antisecretory efficacy of pure scopoletin was not significantly different from those of the fruit extracts, when compared to the same equivalent dose of scopoletin.

Table 6. Effect of noni fruit extracts and scopoletin on gastric acid secretion and pepsin activity in rats

Treatment	Dose	Total acidity (μEq/ml)	Pepsin activity (unit/ml)
Water control	5 ml/kg *i.d.*	0.0135±0.0005	384.01±0.79
1% CMC	5 mg/kg/ *i.d.*	0.0153±0.0004	372.69±1.32
AFE	0.63 g/kg/5ml *i.d.* (equivalent to 0.5 mg scopoletin)	0.0084±0.0004[a]	235.23±2.46[a]
	1.25 g/kg/5ml *i.d.* (equivalent to 1 mg scopoletin)	0.0069±0.0003[a]	209.51±1.16[a]
	2.5 g/kg/5ml *i.d.* (equivalent to 1.5 mg scopoletin)	0.0061±0.0002[a]	194.49±2.18[a]
EFE	0.5 g/kg/5ml *i.d.* (equivalent to 1 mg scopoletin)	0.0053± 0.001[a]	222.82±1.93[a]
Scopoletin	0.5 mg/kg/5ml *i.d.*	0.0073±0.0003[b]	208.92±2.23[b]
	1 mg/kg/5ml *i.d.*	0.0064±0.0008[b]	201.89±2.54[b]
Ranitidine	50 mg/kg/5ml *i.d.*	0.0028±0.0001[b]	199.74±2.70[b]
Lansoprazole	1 mg/kg/5 ml *i.d.*	0.0033±0.0002[b]	137.82±1.62[b]

Each value represents the mean ± S.E.M. from 8-9 rats. *i.d.*, intraduodenally

[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test)

[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test)

2.3 Effect of noni fruit extracts and scopoletin on acid reflux esophagitis

The severity of esophageal damage in the middle part of the esophagus increased with the duration of the ligation, and a 6 h schedule was suitable for the evaluation of drug efficacy. In the control group, the incidence of esophageal hemorrhagic lesions was 100%. Perforation was observed in about 62.5-75% of the rats and the mean severity of esophagitis (ulcer index) was 5.00±0.00 (Table 7). In the AFE (0.63-2.50 g/kg), EFE (0.5 g/kg, containing the same equivalent dose of 1 mg scopoletin as presented in 1.25 g/kg of AFE), ranitidine (50 mg/kg) or lansoprazole (1 mg/kg)-treated group, no perforation was observed and the severity of the ulcer index was

reduced with a similar inhibitory efficacy (NS). The preventive effect of pure scopoletin against the esophagitis formation was also not significantly different from that of the fruit extract when compared to the same equivalent dose of scopoletin.

Table 7. Effect of noni fruit extracts and scopoletin on acid reflux esophagitis in rats

Treatment	Dose	UI	Total ulcer area (mm^2)	Inhibition (%)
Water control	5 ml/kg *i.d.*	5.00±0.00	91.75±1.58	-
1% CMC	5 ml/kg *i.d.*	5.00±0.00	84.00±1.41	-
AFE	0.63 g/kg/5ml *i.d.* (equivalent to 0.5 mg scopoletin)	2.00±0.00[a]	25.50±1.13[a]	72.21[a]
	1.25 g/kg/5ml *i.d.* (equivalent to 1 mg scopoletin)	2.00±0.00[a]	23.44±0.75[a]	74.45[a]
	2.50 g/kg/5ml *i.d.* (equivalent to 1.5 mg scopoletin)	2.00±0.00[a]	21.19±1.48[a]	76.91[a]
EFE	0.5 g/kg/5ml *i.d.* (equivalent to 1 mg scopoletin)	2.00±0.00[a]		74.46[a]
Scopoletin	0.5 mg/kg/5ml *i.d.*	2.25±0.46[b]	27.56±2.58[b]	67.19[b]
	1 mg/kg/5ml *i.d.*	2.00±0.00[b]	24.81±1.00[b]	70.46[b]
Ranitidine	50 mg/kg/5ml *i.d.*	2.00±0.00[b]	16.69±0.60[b]	80.13[b]
Lansoprazole	1 mg/kg/5 ml *i.d.*	2.00±0.00[b]	18.00±0.75[b]	78.57[b]

Each value represents the mean ± S.E.M. from 8-9 rats. *i.d.,* intraduodenally UI, ulcer index

% inhibition = (total ulcer $area_{control}$-total ulcer $area_{treatment}$)/total ulcer $area_{control}$ x 100

[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test)

[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test)

2.4 Effect of noni fruit extracts and scopoletin on gastric mucosal lesions induced by 80% ethanol

Intragastric administration of 80% ethanol produced brand-like hemorrhagic lesions in the glandular portion of the stomach. Oral pretreatment of rats with either AFE (0.63-2.50 g/kg), EFE (0.5 g/kg, containing the same equivalent dose of scopoletin as presented in 1.25 g/kg of AFE), ranitidine (50 mg/kg), lansoprazole (1 mg/kg) or pure

scopoletin (at the same equivalent dose of scopoletin containing in the fruit extracts) prevented the development of gastric lesions with a similar potency (NS) (Table 8).

Table 8. Effect of noni fruit extracts and scopoletin on gastric mucosal lesions induced by ethanol in rats

Treatment	Dose	LI (mm)	Inhibition (%)
Water control	5 ml/kg *p.o.*	40.13+0.97	-
1% CMC	5 ml/kg *p.o.*	38.75+0.77	-
AFE	0.63 g/kg/5 ml *p.o.* (equivalent to 0.5 mg scopoletin)	8.38+0.50[a]	79.13[a]
	1.25 g/kg/5 ml *p.o.* (equivalent to 1 mg scopoletin)	6.25+0.41[a]	84.43[a]
	2.5 g/kg/5 ml *p.o.* (equivalent to 1.5 mg scopoletin)	5.50+0.19[a]	86.29[a]
EFE	0.5 g/kg/5 ml *p.o.* (equivalent to 1 mg scopoletin)	6.13±0.70[a]	84.74[a]
Scopoletin	0.5 mg/kg/5 ml *p.o.*	9.00+0.27[b]	76.77[b]
	1 mg/kg/5 ml *p.o.*	6.88+0.38[b]	82.25[b]
Ranitidine	50 mg/kg/5 ml *p.o.*	4.00+0.33[b]	89.68[b]
Lansoprazole	1 mg/kg/5 ml *p.o.*	4.13+0.35[b]	89.34[b]

Each value represents the mean ± S.E.M. from 8-9 rats. LI, lesion index

All measuring parameters were determined as described in Chapter 2.

% inhibition = $(LI_{control} - LI_{treatment})/ LI_{control} X 100$

[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test)

[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test)

CHAPTER 4

DISCUSSION & CONCLUSION

Several methods have been published for separation and quantification of scopoletin in different medicinal plants such as thin-layer chromatography (TLC) (Li, Liu & Xu, 1996), ethanol modified supercritical fluids extraction (Tzeng et al., 2007), micellar electrokinetic capillary chromatography (Wang et al., 2007), high-performance liquid chromatography with mass spectrometric (HPLC-MS) assay (Potterat et al., 2007) and HPLC-UV or -fluorescence detection (Ikeda et al., 2009). HPLC with different detection systems is the predominant analytical tool for quantitative determination of scopoletin in noni fruit extract. However, HPLC methods do not perform very well for samples containing small amounts due to their disadvantages of low detection limit and sensitivity. Using gas chromatography combined with mass spectrometric (GC-MS) can overcome such problems with its advantage of high separation capacity and extremely sensitive and selective. Nevertheless, polyphenol compound needed to be derivatized before analysis to reduce polarity and boiling point which is usually time-consuming. HPLC with UV detection also required long analysis time and a gradient of the mobile phase. Moreover, several related compounds of natural coumarins are frequently found together making their isolation difficult. In general, the conventional isolation method of the coumarins used solvents of increasing polarities (such as ether, acetone or methanol) through acid base treatment. Nevertheless, such isolation method made some degradation of the coumarins and resulted to the decrease overall yield of recovery. Nowadays, a solid phase extraction or SPE procedure is widely used as a sample-preparation technique for isolation and purification of selected interesting analytes due to its several advantages (simple, low cost, low consumption of organic solvents and high recovery) (Rodriguez et al., 2000). SPE procedure consists of KH_2PO_4 (pH 7, 0.01M) followed by 100% methanol on conventional C_{18} sorbents was chosen to eliminate the impurities matrix including non-phenolic compounds and reduce HPLC column loading from other existing complex constituents in AFE and EFE. Chromatographic separation was achieved by RP-HPLC assay on a C_{18} column using a mixture of sodium acetate (pH 3, 0.01 M): acetonitrile (80:20, v/v) as a mobile phase. More specificity was done by using high wavelength UV detection at 350 nm. The obtained analytical results indicated that the established method has been successfully

developed as a rapid, simple and validated analytical method for quantitative determination of scopoletin in both noni fruit extracts with high percent recovery over than 80% of pure scopoletin before the chromatography, high accuracy with the percent recovery of sample more than 80%, high precision (% RSD of repeatability or intra-day variation and intermediate precision or inter-day variation less than 2%], and high selectivity or reliability with a total peak purity more than 0.99. Furthermore, the linear correlation coefficient (R^2) was more than 0.999, within the range of concentration investigated (10-100 µg/ml). The LOD and LOQ of the established method indicated its good sensitivity that can be used over a very wide range of scopoletin concentrations. Interestingly, the established method provided more rapid phase separation and shorter retention and analytical time (about 5 min and 10 min, respectively) than those reported in previous studies (about 13.96 to 28.6 min) (Ikeda et al., 2009; Deng et al., 2010). Moreover, the high peak purities of scopoletin observed in noni fruit extract samples indicated the high selectivity of the established method for quantitative determination of scopoletin in both AFE and EFE. Although the scopoletin content in AFE is less than in that of EFE, the amount was in the same concentration level as found in other global noni fruits juice including Tahiti and Moorea of French Polynesia, Tonga, Dominica Republic, Okinawa, and Hawaii (Deng et al., 2010).

The present pharmacological study has shown that both fruit extracts (AFE and EFE) effectively inhibited the appearance of reflux esophagitis and the development of acutely induced gastric lesions in rats with an efficacy comparable to that of a standard potent antisecretory proton pump inhibitor (lansoprazole) and also exerted a stronger prokinetic efficacy than a standard prokinetic agent (cisapride, a serotonin 5-HT_4 receptor agonist) in accelerating gastric emptying and intestinal transit in rats. The results also showed that these observed beneficial effects of the fruit extracts may be accounted for by one of its major active biological components scopoletin.

Reflux esophagitis induced by the 6 h simultaneous ligation of the pylorus and the limiting ridge was used as an experimental model for human acute esophagitis (Nakamura et al., 1982). The reflux of gastric contents containing enough pepsin activity has been implicated as the principal causative factor for ulcer formation, while the presence of acid aggravates the ulcer formation by its corrosive property and by keeping an optimum environment for pepsin activity. Although there is still much controversy about the identities of the main pathophysiological factors that induce reflux esophagitis, the incompetence of the lower esophageal sphincter together with insufficient esophageal clearance are considered to be the key factors responsible for

the gastric juice's refluxing into the esophagus and remaining in contact with the esophageal mucosa for a sufficient time to cause inflammation (Holzer, 2001). Therefore, potent antisecretory agents and/or prokinetic agents are used in the management of human reflux esophagitis. In our study, the obtained results showed that the fruit extract produced a similar high inhibitory potency against the formation of reflux esophagitis but it exerted a less acid inhibitory potency than ranitidine and lansoprazole. As the fruit extract also exerted a more potent prokinetic activity than cisapride, it is strongly suggested that the potent inhibitory efficacy of the fruit extract against the formation of reflux esophagitis can be caused mainly by its antisecretory and prokinetic activity. It has been reported that not only gastric acid but also free radicals generated from the xanthine oxidase (Mutoh et al., 1990) and neutrophils (Tepperman and Soper, 1990) in the gastric mucosa play an important role in the pathogenesis of ethanol-induced acute gastric lesions. The important free radicals in producing acute gastric lesions are well known: superoxide radicals, hydroxyl radicals, hypochlorous acid (HOCl), and a cytotoxic peroxynitrite compound formed in the presence of excessive NO generated from the induction of iNOS activity and superoxide radicals. H_2 receptor antagonists including ranitidine have been shown to be a powerful scavenger of hypochlorous acid (Van Zyl et al., 1993) and hydroxyl radicals (Lapenna et al., 1994). The administration of ranitidine also increased the constitutive NOS (cNOS) activity and lowered the iNOS activity in damaged gastric tissues induced by indomethacin (Bayir et al., 2006). In contrast to ranitidine, lansoprazole exhibited a powerful hypochlorous acid and hydroxyl radical scavenging activity but did not exert any effects against prostaglandins or the endogenous nitric oxide-mediated pathway (Chandranath et al., 2002; Biswas et al., 2003). Our findings showed that the anti-ulcerogenic effect of the fruit extract against ethanol induced gastric mucosal damage was found to be almost equal to that of lansoprazole although it produced a lower acid inhibitory efficacy. Since noni fruit extract has recently shown anti-inflammatory activity in suppressing COX-2 enzyme activity (McKoy et al., 2002) and antioxidative activities in quenching all types of harmful free radicals mentioned above (Ikeda et al., 2009), it is possible that the anti-ulcerogenic effect of the fruit extract is due to its antisecretory, antioxidant, anti-inflammatory, and prokinetic properties.

Among the various classes of ingredients reported so far in the noni fruit, scopoletin, a coumarin derivative, is claimed to be one of the ingredients that contribute to its anti-inflammatory and antioxidative activity (Deng et al., 2007; Ikeda et al., 2009). It has been shown that scopoletin affects the expression of inflammatory cytokines by

inhibiting the nuclear factor (NF)-κB transcription factor (Moon et al., 2007) that results in inhibition of the production and release of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and pro-inflammatory mediators (PGE_2, iNOS-derived NO, and myeloperoxidase) (Kang et al., 1999; Kim et al., 2004; Deng et al., 2007). Recently, scopoletin was found to be a potent quencher of peroxynitrite compound production (Ikeda et al., 2009). It is also of interest, that in this study, for the first time, it was shown to be a powerful antisecretory and prokinetic agent. Therefore, the observed beneficial effects of AFE against gastro-esophageal inflammation seemed to be mainly from the presence of its biomarker, scopoletin. An earlier study has reported that natural coumarin compounds can interact with a proton pump such as an H+/K+-ATPase enzyme, the final step of gastric acid secretion (Reyes-Chilpa et al., 2006). The inhibitory potency of this compound on this enzyme depends on the presence, position and number of hydroxyl groups in the molecule. Thus, we can speculate that scopoletin (7-hydroxy-6-methoxycoumarin) may reduce gastric acid secretion through the suppression of H+/K+-ATPase. Further study is necessary to explain the precise role of scopoletin on gastric acid secretion and gastrointestinal motility. The reasons why the fruit extract produced a stronger prokinetic efficacy than that of pure scopoletin comparing at the same equivalent amount of scopoletin, was probably due to other contributions from other compounds with prokinetic activity in the fruit extract. Thus further studies will be needed to isolate and determine which compounds are the major active prokinetic constituents in noni fruit extract

In conclusion, the findings demonstrate that the extract of dried mature unripe noni fruit as well as its biomarker: scopoletin, may be beneficial as a potential preventive agent for gastro-esophageal inflammation, mainly through its antisecretory and prokinetic activities including its ability to enhance the mucosal defensive mechanisms through suppression of free radical and cytokine-mediated inflammation. Owing to the lack of prokinetic and anti-inflammatory activities of currently standard antiulcer agents, the regimen of combining an aqueous noni fruit extract and H_2 receptor antagonists or proton pump inhibitors may be beneficial in the treatment of reflux esophagitis and peptic ulcer. Additionally, scopoletin might be one of the biomarker constituents for quality assessment of noni fruit products used for treatment of upper gastrointestinal disorders.

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Appendix

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Effects of *Morinda citrifolia* aqueous fruit extract and its biomarker scopoletin on reflux esophagitis and gastric ulcer in rats

Sirima Mahattanadul[a,*], Wibool Ridtitid[b], Sawpheeyah Nima[a], Narubodee Phdoongsombut[c], Pranee Ratanasuwon[d], Srirat Kasiwong[a]

[a] *Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[b] *School of Medicine, Walailak University, Thasala, Nakhonsithammarat 80160, Thailand*
[c] *Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[d] *Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*

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ABSTRACT

Aims of the study: The present study was carried out to evaluate the effect of dried mature unripe *Morinda citrifolia* L. (Rubiaceae) fruit, commonly known as "Noni", in an aqueous extract preparation (AFE) as used in Thai traditional medicine and its biomarker scopoletin on gastro-esophageal inflammatory models that are related to the claimed pharmacological properties of AFE and/or resembled the human esophagitis or gastric ulcer.
Materials and methods: The powder of dried mature unripe Noni fruit was boiled in water until it became a sticky paste and was then dried into a powder by lyophilization. The pharmacological activity of AFE and pure scopoletin at the same equivalent dose present in AFE was investigated in rat on gastro-esophageal inflammatory models (acid reflux esophagitis, acute gastritis induced by ethanol and serotonin, and chronic gastric ulcer induced by acetic acid); gastric biochemical parameters and gastrointestinal motility.
Results: AFE (0.63–2.50 g/kg) significantly prevented the formation of acid reflux esophagitis, reduced the formation of ethanol-induced acute gastric lesions, suppressed the development of gastric lesions in response to serotonin, and accelerated the healing of acetic acid-induced chronic gastric ulcer in rats with equal potency to those obtained by standard antisecretory agents (ranitidine and lansoprazole). AFE also significantly inhibited gastric acid secretion and pepsin activity in pylorus ligated rats. Additionally, AFE strongly increased the gastrointestinal transit of charcoal meal with a higher potency than cisapride. Pure scopoletin, when compared at the same equivalent dose containing in AFE, possessed similar antiulcer and antisecretory properties to that of AFE although it exerted a less prokinetic activity than AFE.
Conclusion: The findings indicated that AFE as well as its biomarker: scopoletin may be beneficial as a potential preventive and therapeutic agent for gastro-esophageal inflammatory diseases, mainly through its antisecretory and prokinetic activities including an inhibitory activity on serotonin, free radicals, and cytokine-mediated inflammation. Additionally, scopoletin might be one of the biomarker constituents to use for the quality assessment of Noni fruit products used for treating gastro-esophageal inflammatory diseases.

1. Introduction

Reflux esophagitis and gastric ulcer are common chronic upper gastrointestinal diseases found in all ages of humans and are increasingly recognized as significant health problems. Reflux esophagitis refers to the inflammation of the esophagus that results from repeated exposure for prolonged periods of time to regurgitated stomach contents usually containing acid and pepsin. With increasing severity, it may be associated with erosions, ulceration and formation of strictures. The development of reflux esophagitis is commonly associated with a decrease in the lower esophageal sphincter (LES) tone or esophageal clearance. This requires acid-suppressive therapy (with or without prokinetic agents) for medical management (Holzer, 2001; Kradjan, 2001). Nevertheless, a considerable number of patients do not achieve complete mucosal healing or suffer from either sustained symptoms or complications. Therefore, this disease continues to be investigated in order to develop effective treatment regimens. The most common types of peptic ulcer are chronic gastric ulcer and chronic duodenal ulcer. Since the presence of acid is still one fundamental characteristic in the pathogenesis of *Helicobacter pylori*-negative chronic peptic ulcer, its standard regimen involves

* Corresponding author. Tel.: +66 74 428222; fax: +66 74 428222.
E-mail address: sirima.m@psu.ac.th (S. Mahattanadul).

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acid-suppressive therapy (Kradjan, 2001) but most of the effective drugs exhibit serious adverse effects such as impotence, gynaecomastia, hypergastrinemia and haemopoeitic changes. Ulcer relapse after long-term treatment has also been reported. Hence, currently used effective regimens should be reinvestigated. Recently, several studies have shown that the presence of reactive oxygen species; pro-inflammatory cytokines [such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6]; and pro-inflammatory mediators [such as prostaglandins (PGE_2) and nitric oxide (NO) generated from the induction of inducible nitric oxide synthase (iNOS)], all may play some part in the pathophysiology of both reflux esophagitis and gastric ulcer (Holzer, 2001; Oh et al., 2001). In addition, abnormal gastric motility, associated with rapid or delayed gastric emptying also contributes to gastric ulcer development.

Morinda citrifolia L. (Rubiaceae), commonly known worldwide as "Noni" or so called in Thai as "Yor" is regularly consumed as food and is one of the 66 Thai medicinal plants used in primary health care. The decoction or infusion of roasted mature unripe fruits is recommended to relieve the symptoms of nausea and vomiting, if it is not too severe (Ekpalakorn et al., 1987). According to the claimed efficacies in Thai traditional textbooks, the fruit is also used for treatment of various gastrointestinal disorders as a carminative, appetite stimulant, and reliever of gum diseases, heartburn or stomachache (Farnsworth and Bunyapraphatsara, 1992). Nevertheless, scientific evidence for these benefits is still limited with only one study demonstrating the preventive activity of an ethyl acetate extract of the fruit against acute gastric lesions induced by ethanol, aspirin and pyloric ligation; and acute duodenal ulcer induced by cysteamine in rats (Muralidharan and Srikanth, 2009). This extract was claimed to exhibit potent antioxidant properties and the active components are thought to be non-polar lignans (Zin et al., 2002; Kamiya et al., 2004). Moreover, a few previous studies of an aqueous fruit extract on gastrointestinal motility reported controversial results with increase (Chuthaputti et al., 1996) and delayed (Pu et al., 2004) gastric emptying action. It should be noted, however, that the type of fruit extract and the concentration used in these experiments was quite different. In addition, an appropriated quality assessment of Noni products for such purposes is lacking. Recently, an aqueous Noni fruit extract has been claimed to have anti-inflammatory (McKoy et al., 2002) and antioxidative activities (Ikeda et al., 2009) in several *in vitro* test systems. Among a number of major components identified in the aqueous fruit juice, scopoletin, a coumarin derivative, is one of main compounds that has known pharmacological activities especially an ability to control the serotonin level in the body (Levand and Larson, 1979), together with anti-inflammatory (Kang et al., 1999; Kim et al., 2004; Deng et al., 2007; Moon et al., 2007) and antioxidative activities (Ikeda et al., 2009). Scopoletin is also recommended as a marker constituent for the quality control and pharmacokinetic study of Noni products (Samoylenko et al., 2006).

The present study, therefore, was carried out to evaluate the effect of dried mature unripe *Morinda citrifolia* fruit in an aqueous extract preparation (AFE) as used in Thai traditional medicine and its biomarker scopoletin at the same equivalent dose as found in AFE on gastro-esophageal disorder models that are related to the claimed pharmacological properties of AFE and/or resembled the human esophagitis or gastric ulcer, compared to a standard prokinetic agent (cisapride) and standard antisecretory agents (ranitidine and lansoprazole).

2. Materials and methods

2.1. Plant material

The fresh mature unripe Noni fruits were harvested from Songkhla province, Thailand. Voucher specimens (SKP 165130301) were retained for future reference at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. 19 kg of fresh fruits were cut into thick slices and allowed to dry at 50 °C in a hot air oven. The dried materials were then ground to powder. 1 kg of ground powder was boiled in 4 l of water until it became a sticky paste, and then dried into a powder by lyophilization and stored at −20 °C. The percentage yield was about 28.8% (w/w). The lyophilized powder was freshly dissolved in distilled water before use.

2.2. Animals

Male Wistar rats weighing 180–200 g were housed under normal laboratory conditions at 25 ± 1 °C with a controlled 12-h light–dark cycle and maintained on standard rodent chow and tap water *ad libitum*. All animals received humane care in compliance with the guideline of the Animal Care and Use Committee of Prince of Songkla University, Thailand that closely follow the European Community guidelines (EEC Directive of 1986; 86/609/EEC). The experimental procedures were approved by the Committee on Animal Care and in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Prince of Songkla University (MOE 0521.11/098). When necessary, the rats were deprived of food but with access to water *ad libitum* 24 h before the experiments.

2.3. Drugs and chemicals

Acetylcholine chloride, activated charcoal, bovine serum albumin, cisapride, lansoprazole, phenol red, pentobarbital sodium, pepsin from hog stomach, ranitidine, scopoletin and serotonin hydrochloride were purchased from Sigma–Aldrich (USA). All chemicals were of analytical grade.

2.4. Quantitative analysis of scopoletin in Morinda citrifolia aqueous fruit extracts (AFE)

100 mg of aqueous noni fruit extract was reconstitututed with 25 ml of 0.01 M KH_2PO_4 buffer (pH 3) to yield a concentration of 4 mg/ml. The mixture was vortexed for 2 min to ensure homogeneity, sonicated at room temperature for 30 min, then filtered through a membrane filter (0.20 μm × 47 mm, Whatman, USA). All noni fruit extract samples were prepared in three replicates. The sample solution was then purified on C_{18} solid phase extraction cartridges and analyzed using HPLC-UV.

The HPLC system consisted of a Water® HPLC system (Toronto, Canada) equipped with a Waters 600 HPLC pump, a Waters 600 LCD controller, a 717 plus autosampler, a Water® 2489 UV/vis detector and an Water® integrator (model 4400). Chromatographic separations were performed on a C_{18} analytical column (5.0 μm, 4.6 mm × 150 mm; Supelco, USA) with a guard column (20 mm × 4.0 mm, particle size 5 μm; Supelco, USA). The mobile phase, an isocratic mixture of 0.01 M sodium acetate: acetronitrile (80:20, v/v), was delivered at a flow rate of 1.0 ml/min. Scopoletin content in the eluent was monitored at 350 nm. The concentration of scopoletin in AFE was calculated using a calibration curve prepared from a series of scopoletin solution (0.05–10 μg/ml).

2.5. Effect of AFE and scopoletin on acid reflux esophagitis

Rats were laparotomized under light ether anesthesia to ligate the pylorus and the junction between the forestomach and the corpus (the limiting ridge) (Nakamura et al., 1982). The test drugs were administered immediately after the ligation. The rats were then deprived of food and water. Six hours later, the gastroesophageal portion of the digestive tract was excised and the

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severity of esophagitis was scored macroscopically, using an ulcer index according to the following criteria: 0, no injury; 1, erosion of mucosal epithelium; 2, the length of hemorrhagic ulcer area <20 mm; 3, the length of hemorrhagic ulcer area 20–30 mm; 4, the length of hemorrhagic ulcer area 30–40 mm; 5, the length of hemorrhagic ulcer area >40 mm or perforation.

2.6. Effect of AFE and scopoletin on gastric mucosal lesions induced by ethanol

Thirty minutes after the oral administration of either test drugs, 80% ethanol (1 ml/200 g body weight) was orally administered to the fasted rats. One hour later, the stomach was excised and the sum of the length (mm) of all lesions for each stomach was used as a lesion index.

2.7. Effect of AFE and scopoletin on gastric mucosal lesions induced by serotonin

Gastric mucosal lesions were induced by serotonin treatment in rats according to the method of Yasuhiro et al. (1997). The rat was given serotonin (20 mg/kg, s.c.) once daily for 4 days. The test drugs were given once daily for 4 days, 30 min before the administration of serotonin. The rat was killed 24 h after the final administration of serotonin, and the sum of the lesions area (mm^2) per stomach was used as a lesion index.

2.8. Effect of AFE and scopoletin on gastric ulcer induced by topical application of acetic acid

Gastric ulcer was induced by acetic acid treatment in rats according to the method of Okabe et al. (1971). The abdomen of a rat anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*), was opened and a cylindrical plastic mold (6 mm diameter) was tightly placed upon the anterior serosal surface of the stomach wall (antrum). Acetic acid (100%, 0.06 ml) was then poured into the mold and allowed to remain for 1 min. The acetic acid remaining on the surface was wiped away with a filter and then gently washed with normal saline. The opened abdomen was then closed and the rat was fed normally. The test drugs were administered to the rat for 10 consecutive days, beginning on the 4th day after the operation. The rat was killed on the 14th day after the operation and examined macroscopically and histologically for ulcer by the pathologist for the following parameters: Ulcer index (UI) (mm^2) = length (mm) × the width of the ulcer (mm)

$$\%\,\text{Curation} = \frac{\text{UI}_{\text{control at 4}^{\text{th}}\text{ day}} - \text{UI}_{\text{treatment at 14}^{\text{th}}\text{ day}}}{\text{UI}_{\text{control at 4}^{\text{th}}\text{ day}}} \times 100$$

$$\%\,\text{Mucosal regeneration index (MRI)} = \frac{\text{regeneration of the mucosal layer}}{\text{defect of the mucosa} + \text{regeneration of the mucosal layer}} \times 100$$

$$\%\,\text{Healing index (HI)} = \left(1 - \frac{\text{defect of the mucosa}}{\text{distance of ruptured muscularis propia}}\right) \times 100$$

2.9. Effect of AFE and scopoletin on gastric biochemical parameters

A pylorus ligation was carefully done in fasted rats under anesthesia with pentobarbital sodium (50 mg/kg, *i.p.*). The test drugs were administered intraduodenally immediately after the ligation. Four hours following the treatment, the rats were killed and the gastric juice was collected and centrifuged. After measuring the volume of the supernatant, the total acid output was analyzed by titration with 2 mM NaOH using 2% phenolphthalein as an indicator and expressed as μEq/ml. Pepsin activity was determined by a slight modification of the Anson method (Anson, 1938), using bovine hemoglobin as a substrate.

2.10. Effect of AFE and scopoletin on gastric emptying rates (GER) of phenol red meals in rats

The GER was determined in conscious rats by measuring the disappearance of phenol red from the stomach (Lee et al., 2005). Thirty minutes after the pretreatment with each test drug, a phenol red meal (phenol red 0.05%, w/w in 1.5% carboxymethylcellulose 1200, CMC) was administered orally to the rats at a volume of 0.75 ml/100 g body weight. After 20 min, the rats were sacrificed by cervical dislocation and the gastroesophageal junction and the pylorus was ligated. The stomach was then extirpated, rinsed in normal saline, and homogenized in 100 ml 0.1 N NaOH. The suspension was allowed to settle for 1 h at room temperature, and 5 ml of the supernatant was then added to 0.5 ml 20% trichloroacetic acid (w/v) and centrifuged at 3000 rpm at 4 °C for 20 min. The supernatant was mixed with 4 ml of 0.5 N NaOH, and the absorbance of the sample was read at 560 nm (A_{560}) using a spectrophotometer. The phenol red recovered from the animals that had been killed immediately after the administration of CMC solution was used as the control (0% emptying). The GER in the 20-min period was calculated according to the following equation:

$$\%\,\text{GER} = \left[1 - \frac{A_{560\,\text{of test}}}{A_{560\,\text{of control}}}\right] \times 100$$

2.11. Effect of AFE and scopoletin on intestinal transit rates (ITR) of charcoal meals in rats

Thirty minutes after the pretreatment with each test drug, a charcoal meal (10%, w/v activated charcoal in 1%, w/v CMC) was administered orally to the rats at a volume of 0.05 ml/100 g body weight. The rats were killed after 60 min. The distances of charcoal movement along the intestine and the total length of the small intestine were measured. The ratio of the distance traveled by the charcoal divided by the total length of the small intestine was expressed as % ITR.

2.12. Statistical analysis

All data were expressed as mean ± standard error of means (S.E.M.). Comparisons between groups were made by one-way analysis of variance (ANOVA) followed by the Dunnett test. Values of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Content of scopoletin in AFE

Typical HPLC chromatograms for scopoletin and AFE were shown in Fig. 1. The identification of the scopoletin peak in the chromatogram of AFE was made by comparing its retention time (5.585 min) with that of standard scopoletin (5.497 min). The amount of scopoletin in 1 g of lyophilized AFE powder was calculated to be 0.85–0.87 mg.

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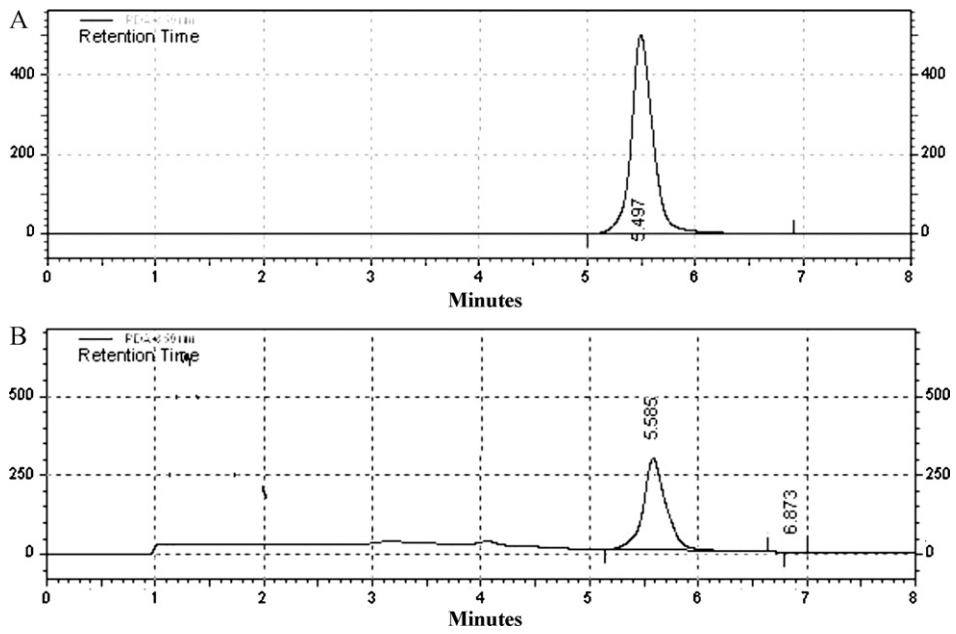

Fig. 1. Seperation of scopoletin by HPLC following the solid phase extraction method. The *X* and *Y* axes represent the running time (min) and peak absorbance, respectively. (A) Chromatogram of standard scopoletin (10 μg/ml). (B) Chromatogram of *Morinda citrifolia* aqueous fruit extract (AFE) (15 mg/ml).

3.2. Effect of AFE and scopoletin on acid reflux esophagitis

The severity of esophageal damage in the middle part of the esophagus increased with the duration of the ligation, and a 6 h schedule was suitable for the evaluation of drug efficacy. In the control group, the incidence of esophageal hemorrhagic lesions was 100%. Perforation was observed in about 62–75% of the rats and the mean severity of esophagitis (ulcer index) was 5.00 ± 0.00 (Table 1). In the AFE (0.63–2.50 g/kg), ranitidine (50 mg/kg) or lansoprazole (1 mg/kg)-treated group, no perforation was observed and the severity of the ulcer index was reduced with a similar inhibitory efficacy (NS). The preventive effect of pure scopoletin against the esophagitis formation was also not significantly different from that of AFE when compared to the same equivalent dose of scopoletin.

3.3. Effect of AFE and scopoletin on gastric mucosal lesions induced by 80% ethanol

Intragastric administration of 80% ethanol produced brand-like hemorrhagic lesions in the glandular portion of the stomach. Oral pretreatment of rats with either AFE (0.63–2.50 g/kg), ranitidine (50 mg/kg), lansoprazole (1 mg/kg) or pure scopoletin (at the same equivalent dose of scopoletin containing in AFE) prevented the development of gastric lesions with a similar potency (NS) (Table 2).

3.4. Effect of AFE and scopoletin on gastric mucosal lesions induced by serotonin

Repeated subcutaneous administration of serotonin once daily for 4 days caused varioliformed-like lesions in the whole corpus mucosa, with severe edema and hemorrhage in the submucosa. Simultaneous oral administration of either AFE (1.25 mg/kg), pure scopoletin (at the same equivalent dose of scopoletin containing in AFE) or ranitidine (50 mg/kg) significantly prevented the development of gastric lesions with similar potency (NS) (Table 2) whereas lansoprazole (1 mg/kg) was only a weak inhibitor of lesion formation.

3.5. Effect of AFE and scopoletin on gastric ulcer induced by topical application of acetic acid

Topical application of acetic acid produced a round ulcer in the stomach. The ulcer area on the 4th and 14th day after the induction was about 54.75 ± 1.36 mm^2 and 31.38 ± 0.89 mm^2, respectively. Oral administration twice daily of AFE (0.63–2.5 g/kg) for ten days significantly decreased the ulcer index and

Table 1
Effect of *Morinda citrifolia* aqueous fruit extracts (AFE) and scopoletin on acute acid reflux esophagitis in rats.

Treatment	Dose	UI	Total ulcer area (mm^2)	Inhibition (%)
Water control	5 ml/kg *i.d.*	5.00 ± 0.00	91.75 ± 1.58	–
1% CMC	5 ml/kg *i.d.*	5.00 ± 0.00	84.00 ± 1.41	–
AFE	0.63 g/kg/5 ml *i.d.* (equivalent to 0.5 mg scopoletin)	2.00 ± 0.00[a]	25.50 ± 1.13[a]	72.21[a]
	1.25 g/kg/5 ml *i.d.* (equivalent to 1 mg scopoletin)	2.00 ± 0.00[a]	23.44 ± 0.75[a]	74.45[a]
	2.50 g/kg/5 ml *i.d.* (equivalent to 1.5 mg scopoletin)	2.00 ± 0.00[a]	21.19 ± 1.48[a]	76.91[a]
Scopoletin	0.5 mg/kg/5 ml *i.d.*	2.25 ± 0.46[b]	27.56 ± 2.58[b]	67.19[b]
	1 mg/kg/5 ml *i.d.*	2.00 ± 0.00[b]	24.81 ± 1.00[b]	70.46[b]
Ranitidine	50 mg/kg/5 ml *i.d.*	2.00 ± 0.00[b]	16.69 ± 0.60[b]	80.13[b]
Lansoprazole	1 mg/kg/5 ml *s.c.*	2.00 ± 0.00[b]	18.00 ± 0.75[b]	78.57[b]

Each value represents the mean ± S.E.M. from 8 to 9 rats. *i.d.*: intraduodenally; UI: ulcer index determined as described in Section 2. % inhibition = (total ulcer area$_{control}$ − total ulcer area$_{treatment}$)/total ulcer area$_{control}$ × 100.

[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test).

[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test).

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Table 2
Effect of *Morinda citrifolia* aqueous fruit extracts (AFE) and scopoletin on acute gastric mucosal lesions induced by ethanol and serotonin in rats.

Treatment	Dose	LI (mm)	Inhibition (%)
Ethanol induced acute gastric mucosal lesions			
Water control	5 ml/kg *p.o.*	40.13 ± 0.97	–
1% CMC	5 ml/kg/*p.o.*	38.75 ± 0.77	–
AFE	0.63 g/kg/5 ml *p.o.* (equivalent to 0.5 mg scopoletin)	8.38 ± 0.50[a]	79.13[a]
	1.25 g/kg/5 ml *p.o.* (equivalent to 1 mg scopoletin)	6.25 ± 0.41[a]	84.43[a]
	2.5 g/kg/5 ml *p.o.* (equivalent to 1.5 mg scopoletin)	5.50 ± 0.19[a]	86.29[a]
Scopoletin	0.5 mg/kg/5 ml *p.o.*	9.00 ± 0.27[b]	76.77[b]
	1 mg/kg/5 ml *p.o.*	6.88 ± 0.38[b]	82.25[b]
Ranitidine	50 mg/kg/5 ml *p.o.*	4.00 ± 0.33[b]	89.68[b]
Lansoprazole	1 mg/kg/5 ml *p.o.*	4.13 ± 0.35[b]	89.34[b]

Treatment	Dose	LI (mm^2)	Inhibition (%)
Serotonin induced acute gastric mucosal lesions			
Water control	5 ml/kg *p.o.*	168.00 ± 2.21	–
1% CMC	5 ml/kg/*p.o.*	163.17 ± 1.72	–
AFE	1.25 g/kg/5 ml *p.o.* (equivalent to 1 mg scopoletin)	29.50 ± 2.55[a]	82.44[a]
Scopoletin	1 mg/kg/5 ml *p.o.*	30.32 ± 8.83[b]	81.42[b]
Ranitidine	50 mg/kg/5 ml *p.o.*	45.00 ± 1.30[b]	72.42[b]
Lansoprazole	1 mg/kg/5 ml *p.o.*	86.50 ± 3.42[b]	46.99[b]

Each value represents the mean ± S.E.M. from 8 to 9 rats. LI, lesion index. All measuring parameters were determined as described in Section 2. %inhibition = $(LI_{control} - LI_{treatment})/LI_{control} \times 100$.
[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test).
[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test).

Table 3
Effect of *Morinda citrifolia* aqueous fruit extracts (AFE) and scopoletin on acetic acid-induced gastric ulcer in rats.

Treatment	Dose	UI (mm^2)	Curation (%)	HI (%)	MRI (%)
Water control	5 ml/kg *p.o.* bid	31.38 ± 0.89	42.68	26.88 ± 0.41	27.56 ± 0.39
1% CMC	5 ml/kg/*p.o.* bid	31.63 ± 0.92	42.23	27.74 ± 0.87	27.77 ± 0.80
AFE	0.63 g/kg/5 ml *p.o.* bid (equivalent to 0.5 mg scopoletin)	7.38 ± 0.50[a]	86.52[a]	39.14 ± 0.88[a]	39.08 ± 0.32[a]
	1.25 g/kg/5 ml *p.o.* bid. (equivalent to 1 mg scopoletin)	3.31 ± 0.21[a]	93.95[a]	48.60 ± 1.13[a]	48.01 ± 0.62[a]
	2.5 g/kg/5 ml *p.o.* bid (equivalent to 1.5 mg scopoletin)	2.00 ± 0.25[a]	96.35[a]	51.71 ± 0.43[a]	50.04 ± 1.27[a]
Scopoletin	0.5 mg/kg/5 ml *p.o.* bid	6.00 ± 0.37[b]	89.04[b]	50.88 ± 1.04[b]	50.06 ± 0.53[b]
	1 mg/kg/5 ml *p.o.* bid	3.19 ± 0.33[b]	94.18[b]	55.96 ± 1.99[b]	55.90 ± 1.53[b]
Ranitidine	50 mg/kg/5 ml *p.o.* bid	1.53 ± 0.18[b]	97.21[b]	57.88 ± 1.36[b]	57.23 ± 0.72[b]
Lansoprazole	1 mg/kg/5 ml *p.o.* bid	2.97 ± 0.26[b]	94.58[b]	52.74 ± 1.53[b]	47.10 ± 1.63[b]

Each value represents the mean ± S.E.M. from 8 to 9 rats.
UI, ulcer index; HI, Healing index; MRI, Mucosal regeneration index; $UI_{control\ at\ 4th\ day} = 54.75 \pm 1.36\ mm^2$.
All measuring parameters were determined as described in Section 2.
[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test).
[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test).

promoted mucosal regeneration in the ulcerated portion as shown in Table 3 and Fig. 2. The curative potency of AFE was not significantly different from those of ranitidine (50 mg/kg), lansoprazole (1 mg/kg) and pure scopoletin (at the same equivalent dose of scopoletin containing in AFE).

3.6. Effect of AFE and scopoletin on gastric biochemical parameters

Intraduodenal injection of AFE (0.63–2.50 mg/kg) had a dose dependent potency on the decrease of acid secretion and pepsin activity (Table 4) but had a less acid inhibitory efficacy than those of ranitidine (50 mg/kg) and lansoprazole (1 mg/kg) ($p < 0.05$). However, the antisecretory efficacy of pure scopoletin was not significantly different from that of AFE, when compared to the same equivalent dose of scopoletin.

3.7. Effect of AFE and scopoletin on the GER of phenol red meals and on the ITR of charcoal meals in rats

AFE (0.63–2.50 mg/kg) significantly accelerated the GER of phenol red meals in a dose dependent manner (Table 5). In addition, AFE fed at the same dose range exerted a potent efficacy in accelerating the ITR of charcoal meals. The prokinetic efficacy of AFE in both models appeared to be higher compared to cisapride (10 mg/kg) and pure scopoletin (at the same equivalent dose of scopoletin containing in AFE) ($p < 0.05$).

4. Discussion

The present study has shown that AFE effectively inhibited the appearance of reflux esophagitis and the development of acutely and chronically induced gastric ulcer in rats with an efficacy comparable to that of a standard potent antisecretory proton pump inhibitor (lansoprazole) and also exerted a stronger prokinetic efficacy than a standard prokinetic agent (cisapride, a serotonin 5-HT_4 receptor agonist) in accelerating gastric emptying and intestinal transit in rats. The results also showed that these observed beneficial effects of AFE may be accounted for by one of its major active biological components scopoletin.

Reflux esophagitis induced by the 6 h simultaneous ligation of the pylorus and the limiting ridge was used as an experimental model for human acute esophagitis (Nakamura et al., 1982). The reflux of gastric contents containing enough pepsin activity has

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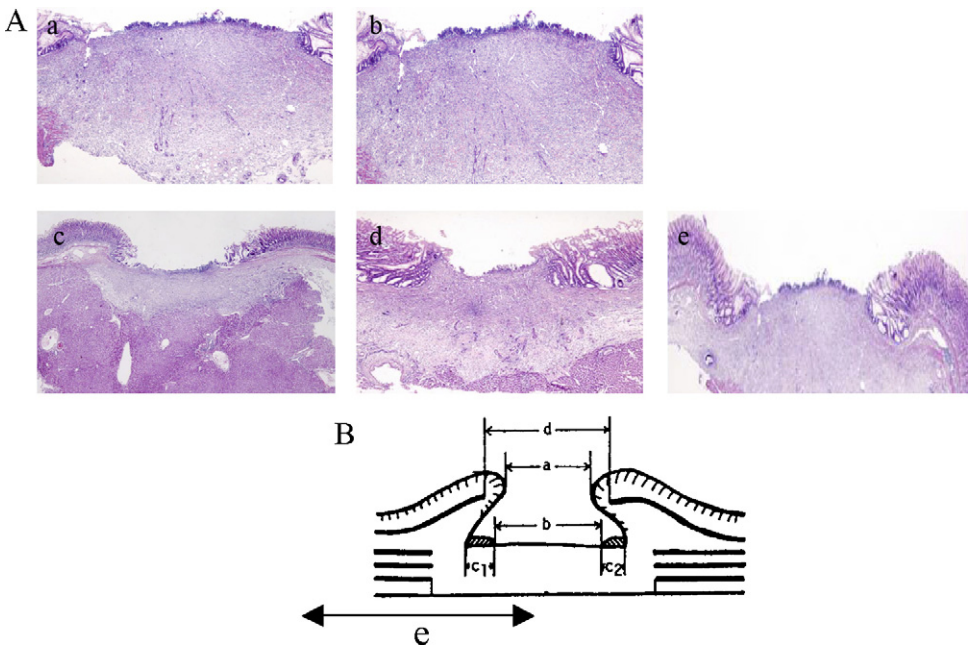

Fig. 2. (A) Histological appearance of the acetic acid-induced gastric ulcer in a rat that received water (a), 1% CMC (b), *Morinda citrifolia* aqueous fruit extract (AFE) at 1.25 g/kg (c) scopoletin at 1 mg/kg (d) or lansoprazole at 1 mg/kg (e) twice daily for 10 days. H.E. stain (40×). (B) Histological measurement of acetic acid-induced gastric ulcer. a: Apparent defect in the mucosa, b: True defect in the mucosa, c: The regeneration of the mucosal layer, d: Distance of the ruptured muscularis mucosa, e: Distance of the ruptured muscularis propia. MRI = $(c_1 + c_2)/b + c$; HI = $1 - [a/e]$.

been implicated as the principal causative factor for ulcer formation, while the presence of acid aggravates the ulcer formation by its corrosive property and by keeping an optimum environment for pepsin activity. Although there is still much controversy about the identities of the main pathophysiological factors that induce reflux esophagitis, the incompetence of the lower esophageal sphincter together with insufficient esophageal clearance are considered to be the key factors responsible for the gastric juice's refluxing into the esophagus and remaining in contact with the esophageal mucosa for a sufficient time to cause inflammation (Holzer, 2001). Therefore, potent antisecretory agents and/or prokinetic agents are used in the management of human reflux esophagitis. In our study, the obtained results showed that AFE produced a similar high inhibitory potency against the formation of reflux esophagi-

Table 4
Effect of *Morinda citrifolia* aqueous fruit extracts (AFE) and scopoletin on gastric acid secretion and pepsin activity in rats.

Treatment	Dose	Total acidity (μEq/ml)	Pepsin activity (unit/ml)
Water control	5 ml/kg *i.d.*	0.0135 ± 0.0005	384.01 ± 0.79
1% CMC	5 mg/kg/*i.d.*	0.0153 ± 0.0004	372.69 ± 1.32
AFE	0.63 g/kg/5 ml *i.d.* (equivalent to 0.5 mg scopoletin)	0.0084 ± 0.0004[a]	235.23 ± 2.46[a]
	1.25 g/kg/5 ml *i.d.* (equivalent to 1 mg scopoletin)	0.0069 ± 0.0003[a]	209.51 ± 1.16[a]
	2.5 g/kg/5 ml *i.d.* (equivalent to 1.5 mg scopoletin)	0.0061 ± 0.0002[a]	194.49 ± 2.18[a]
Scopoletin	0.5 mg/kg/5 ml *i.d.*	0.0073 ± 0.0003[b]	208.92 ± 2.23[b]
	1 mg/kg/5 ml *i.d.*	0.0064 ± 0.0008[b]	201.89 ± 2.54[b]
Ranitidine	50 mg/kg/5 ml *i.d.*	0.0028 ± 0.0001[b]	199.74 ± 2.70[b]
Lansoprazole	1 mg/kg/5 ml *s.c.*	0.0033 ± 0.0002[b]	137.82 ± 1.62[b]

Each value represents the mean ± S.E.M. from 8 to 9 rats. *i.d.*, intraduodenally.
[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test).
[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test).

Table 5
Effect of *Morinda citrifolia* aqueous fruit extracts (AFE) and scopoletin on GER and ITR in rats.

Treatment	Dose	GER (%)	ITR (%)
Water control	5 ml/kg *p.o.*	47.17 ± 0.43	53.06 ± 0.66
1% CMC	5 ml/kg/*p.o.*	42.92 ± 0.24	55.12 ± 0.71
AFE	0.63 g/kg/5 ml *p.o.* (equivalent to 0.5 mg scopoletin)	76.91 ± 0.65[a]	87.65 ± 0.57[a]
	1.25 g/kg/5 ml *p.o.* (equivalent to 1 mg scopoletin)	82.63 ± 0.39[a]	92.41 ± 1.25[a]
	2.5 g/kg/5 ml *p.o.* (equivalent to 1.5 mg scopoletin)	97.21 ± 0.57[a]	95.59 ± 0.14[a]
Scopoletin	0.5 mg/kg/5 ml *p.o.*	58.54 ± 0.61[b]	61.17 ± 0.98[b]
	1 mg/kg/5 ml *p.o.*	77.31 ± 0.69[b]	83.73 ± 0.86[b]
Cisapride	10 mg/kg/5 ml *p.o.*	78.57 ± 0.26[b]	78.58 ± 0.32[b]

Each value represents the mean ± S.E.M. from 8 rats. GER, gastric emptying rate; ITR, intestinal transit rate. All measuring parameters were determined as described in Section 2.
[a] $p < 0.05$ compared to the water control-treated rats (Dunnett test).
[b] $p < 0.05$ compared to the vehicle (1% CMC) control-treated rats (Dunnett test).

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tis but it exerted a less acid inhibitory potency than ranitidine and lansoprazole. As AFE also exerted a more potent prokinetic activity than cisapride, it is strongly suggested that the potent inhibitory efficacy of AFE against the formation of reflux esophagitis can be caused mainly by its antisecretory and prokinetic activity. It has been reported that not only gastric acid but also free radicals generated from the xanthine oxidase (Mutoh et al., 1990) and neutrophils (Tepperman and Soper, 1990) in the gastric mucosa play an important role in the pathogenesis of ethanol-induced acute gastric lesions. The important free radicals in producing acute gastric lesions are well known: superoxide radicals, hydroxyl radicals, hypochlorous acid (HOCl), and a cytotoxic peroxynitrite compound formed in the presence of excessive NO generated from the induction of iNOS activity and superoxide radicals. Similarly, it has also been found that both gastric acid and free radicals derived from infiltrated neutrophils can exert an inhibitory effect on the healing of ulcers induced by acetic acid in rats, the model that resembles the human chronic gastric ulcer both grossly and histologically (Okabe et al., 1971; Motilva et al., 1996). H_2 receptor antagonists including ranitidine have been shown to be a powerful scavenger of hypochlorous acid (Van Zyl et al., 1993) and hydroxyl radicals (Lapenna et al., 1994). The administration of ranitidine also increased the constitutive NOS (cNOS) activity and lowered the iNOS activity in damaged gastric tissues induced by indomethacin (Bayir et al., 2006). In contrast to ranitidine, lansoprazole exhibited a powerful hypochlorous acid and hydroxyl radical scavenging activity but did not exert any effects against prostaglandins or the endogenous nitric oxide-mediated pathway (Chandranath et al., 2002; Biswas et al., 2003). Consequently, our findings showed that lansoprazole produced a higher inhibitory potency than ranitidine in providing preventive and curative activity against gastric mucosal damage induced by ethanol and acetic acid but not by serotonin. It has been found that the pathogenesis of repeated subcutaneous administration of serotonin for 4 days in rats is mediated through the upregulation of the iNOS/NO system, the formation of oxygen radicals and the generation of the cytotoxic free radical nitric oxide (peroxynitrite) compound. In contrast, gastric acid secretion has been found to play only a minor role in the pathogenesis (Yasuhiro et al., 1997). The anti-ulcerogenic effect of AFE against ethanol and acetic acid induced gastric mucosal damage was found to be almost equal to that of lansoprazole although it produced a lower acid inhibitory efficacy. Since Noni fruit extract has recently shown anti-inflammatory activity in suppressing COX-2 enzyme activity (McKoy et al., 2002) and antioxidative activity in quenching all types of harmful free radicals mentioned above (Ikeda et al., 2009), it is possible that the anti-ulcerogenic effect of AFE is due to its antisecretory, antioxidant, anti-inflammatory, and prokinetic properties including its ability to control serotonin mediated gastric mucosal inflammation.

Among the various classes of ingredients reported so far in the Noni fruit, scopoletin, a coumarin derivative, is claimed to be one of the ingredients that contribute to its anti-inflammatory and antioxidative activity (Deng et al., 2007; Ikeda et al., 2009). It has been shown that scopoletin affects the expression of inflammatory cytokines by inhibiting the nuclear factor (NF)-κB transcription factor (Moon et al., 2007) that results in inhibition of the production and release of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and pro-inflammatory mediators (PGE_2, iNOS-derived NO, and myeloperoxidase) (Kang et al., 1999; Kim et al., 2004; Deng et al., 2007). Recently, scopoletin was found to be a potent quencher of peroxynitrite compound production (Ikeda et al., 2009). In the present study, the beneficial effect of scopoletin against serotonin-induced gastric lesions confirmed its oxygen-free radicals and peroxynitrite compound quenching activity and its ability to control the serotonin levels in the body. It is also of interest, that in this study, for the first time, it was shown to be a powerful antisecretory and prokinetic agent. Therefore, the observed beneficial effects of AFE against gastro-esophageal inflammation seemed to be mainly from the presence of its biomarker scopoletin. An earlier study has reported that natural coumarin compounds can interact with a proton pump or H+/K+-ATPase enzyme, the final step of gastric acid secretion (Reyes-Chilpa et al., 2006). The enzyme inhibitory potency of this compound depends on the presence, position and number of hydroxyl groups in the molecule. Thus, we can speculate that scopoletin (7-hydroxy-6-methoxycoumarin) may reduce gastric acid secretion through the suppression of H+/K+-ATPase. Further study is necessary to explain the precise role of scopoletin on gastric acid secretion and gastrointestinal motility. The reasons why AFE produced a stronger prokinetic efficacy than that of pure scopoletin comparing at the same equivalent amount of scopoletin, was probably due to other contributions from other compounds with prokinetic activity in the aqueous fruit extract. Thus further studies will be needed to isolate and determine which compounds are the major active prokinetic constituents in an aqueous extract of Noni fruit.

5. Conclusion

The findings from this publication demonstrated that an aqueous extract of dried Noni fruit as well as its biomarker: scopoletin may be beneficial as a potential preventive and therapeutic agent for gastro-esophageal inflammation, mainly through its antisecretory and prokinetic activities including its ability to enhance the mucosal defensive mechanisms through suppression of serotonin, free radicals and cytokine-mediated inflammation. Owing to the lack of prokinetic and anti-inflammatory activities of currently standard antiulcer agents, the regimen of combining an aqueous Noni fruit extract and H_2 receptor antagonists or proton pump inhibitors may be beneficial in the treatment of reflux esophagitis and peptic ulcer. Additionally, scopoletin might be one of the biomarker constituents for quality assessment of Noni fruit products used for treatment of upper gastrointestinal disorders.

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