

Antimicrobial and Anti-Inflammatory Effects of Ethanol Extract of *Corylopsis coreana* Uyeki Flos

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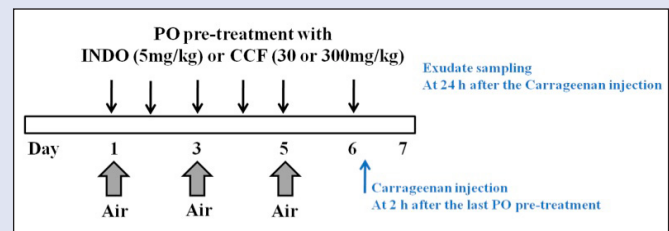
ABSTRACT

Background: *Corylopsis coreana* Uyeki (Hamamelidaceae) is a medicinal plant cultivated in Northeast Asia. Previously, we have reported that an ethanol extract of *Corylopsis coreana* Uyeki flos (ECCF) contains four active compounds with antioxidant activity. **Objective:** The aim of this study was to investigate the antimicrobial spectrum against infectious bacteria and anti-inflammatory effect of ECCF in a mouse model of acute local inflammation. **Materials and Methods:** *In vitro* antimicrobial susceptibility was evaluated using standard plate assay technique. Antimicrobial activities (minimum inhibitory concentration, MIC; $\mu\text{g}/\text{mL}$) were determined with the serial dilution method. *In vivo* anti-inflammatory activity was studied using a mouse model of carrageenan-induced air pouch inflammation. **Results:** The ECCF showed antimicrobial activities against general bacteria and drug-resistant bacteria including *Staphylococcus aureus*, *Micrococcus luteus* ATCC 9341, *Mycrobacterium smegmatis* ATCC 9341, *Mycrobacterium smegmatis* ATCC 9341, *Salmonella typhimurium* KCTC 1925, and nine methicillin-resistant *Staphylococcus aureus* strains, with MIC values ranging from 250 to 1000 $\mu\text{g}/\text{mL}$. In *in vivo* mouse model, inflammatory morphologic changes observed in the air pouch membrane were restored to its normal condition by the ECCF treatment. Moreover, the ECCF significantly reduced exudate volumes, protein contents, inflammatory cell counts, and pro-inflammatory cytokine levels in the exudates recovered from air pouches of the mouse model. Flavonoids in the ECCF were found to contain bergenin, quercitrin, and quercetin with reported anti-inflammatory activity via suppressing tumor necrosis factor- α production. **Conclusion:** To the best of our knowledge, this is the first report to demonstrate the antimicrobial and anti-inflammatory activities of ECCF. Our results suggest that the ECCF might potentially serve as an alternative or complementary medicine for treating inflammatory diseases caused by microbial infection.

Key words: Antimicrobial activity, *Corylopsis coreana* Uyeki flos, inflammation, TNF- α

SUMMARY

- ECCF showed antimicrobial activity against infectious bacteria and multidrug-resistant strains.
- ECCF exhibited anti-inflammatory activity in a carrageenan-induced air pouch mouse model.
- ECCF contained several active constituents such as bergenin, quercitrin, and quercetin.



Abbreviations used: *Corylopsis coreana* CCF: Uyeki flos, ECCF: ethanol extract of CCF

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INTRODUCTION

Inflammation is an immune response of damaged tissues to injury induced by physical, chemical, or biological attack. During an inflammatory process, neutrophils are the first cells that are attracted toward the injured area to cope with bacteria or antigens to remove them or release inflammatory mediators to attract macrophages to the site of inflammation.^[1] Specific immune responses against foreign antigens are generated by macrophages. Together with the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interferon (INF)- γ , interleukin(IL)-1, and IL-6, they can cause an influx of monocytes, granulocytes, lymphocytes, and mast cells.^[2] Pro-inflammatory cytokines and mediators can regulate the functional activities of immune cells. They are also involved in the pathogenesis of inflammatory diseases such as shock, hemorrhagic shock, multiple sclerosis, rheumatoid arthritis, ulcerative colitis, and atherosclerosis.^[3] These processes can initiate a multitude of immune pathological responses

to remove infectious bacterium or agents, leading to the restoration of homeostasis in acute and chronic inflammatory disorders.^[4]

Recently, a number of plant extracts and phytochemicals therein have attracted increasing attention for the treatment of various inflammatory diseases.^[5-8] *Corylopsis coreana* Uyeki (Hamamelidaceae) is a medicinal

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plant cultivated in Northeast Asia.^[9] Water extract, decoction, and infusion preparations of flowers of *C. coreana* Uyeki (CCF) has been used as a folk medicine in South Korea because of its antipyretic and antiemetic properties.^[10] Moreover, some species in the genus of *Corylopsis* such as witch hazel have been widely used as a traditional medicine for treating skin irritation and inflammatory disease.^[11] Previously, we have reported that the ethanol extract of the flower of *C. coreana* Uyeki (ECCF) contained four active components (i.e., quercetin, quercitrin, bergenin, and isosalipurposide) with antioxidant activity.^[10] Bergenin, quercetin, and quercitrin have been reported to possess pharmacologic activity against inflammation and microbial infection.^[12-14] However, to the best of our knowledge, the antimicrobial and anti-inflammatory effects of ECCF have not been reported.

Therefore, we herein investigated the antimicrobial activity against infectious microbes and multidrug-resistant (MDR) strains and anti-inflammatory activity of ECCF. A carrageenan-induced air pouch model was used as an *in vivo* mouse model of acute local inflammation. Various inflammatory cells and pro-inflammatory cytokines were monitored to evaluate the anti-inflammatory activity of ECCF.

MATERIALS AND METHODS

Plant materials and preparation of ECCF

CCF was collected in May 2013 near Jogye Mountain (Jeonnam, Korea). A voucher specimen (MNUCSS-CC-01) was deposited in the College of Pharmacy, Mokpo National University. The flower was separated for this study. The air-dried and powdered CCF (200 g) was extracted twice with 80% ethanol (1000 mL) at room temperature for 3 days. After filtration, the resultant ethanol extract was evaporated, freeze dried, and stored at -50°C. Crude extract was resuspended in distilled water and filtered using a 0.4 µm membrane.

Antimicrobial activity analysis

In order to find the inhibitory effect of ECCF against infectious and multi-antibiotics resistant microbes, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA), carbapenemase-producing strains (IMP), and extended spectrum β-lactamase-producing strains (ESBL) were used in this study. These strains were kindly provided by professor Jin-Cheol Yoo of Chosun University (Gwangju, Republic of Korea). All strains were stored in Mueller-Hinton broth (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with 15% glycerol at -80°C. The standard plate assay technique described by Iwasa *et al.*^[15] was employed to determine

the effect of CCF on test strains with slight modifications. Briefly, ECCF (1 mg/40 µL) were loaded onto paper disks (sterilized, Adventec, Toyo, Kaisha Ltd., Tokyo, Japan) and transferred to Mueller-Hinton agar plate containing test strains of 10⁶ colony-forming units.^[15] The susceptibilities of the microbes to ECCF were expressed as plus or a minus. If the width of the transparent zone was 1.2 mm or greater, the susceptibility was a plus. However, if the width of the transparent zone was less than 1.2 mm, the susceptibility was a minus. The *in-vitro* antimicrobial activities expressed with minimum inhibitory concentration (MIC, µg/mL) were determined with the serial dilution method.^[16]

Carrageenan-induced air pouch inflammation model in mice

Male ICR mice (20-25 g) were purchased from Orient Bio Inc. (Seongnam, Korea). Animal study was scheduled as shown in Figure 1. To make space (or air pouch) under skin, 2-mL air was inserted three times into the intrascapular area of mice's back for 6 days. Animals were divided into two groups. Animals (*n* = 6) in the first group were not treated with carrageenan. Animals (*n* = 24) in the other group were treated with carrageenan. The animal study was repeated once with the same protocol. Carrageenan-treated group consisted of four subgroups: (1) inflammation-induced group was injected with carrageenan after normal saline oral administration, (2) 5 mg/kg indomethacin-treated group (indomethacin was used as an anti-inflammatory drug), (3) 30 mg/kg ECCF-treated group, and (4) 300 mg/kg ECCF-treated group. Indomethacin and ECCF were orally administered for 6 days. At 2 h after treatment, carrageenan solution (1 mL, 2 w/v% dissolved in saline) was injected into the air pouch.

Pro-inflammatory cytokine measurement and histopathological analysis

At 24 h after carrageenan insertion, all mice were sacrificed, and exudate was collected. The numbers of total and differential cells of the exudate in the pouch were counted using Hemavet Multispecies Hematology System (Drew Scientific Inc., Waterbury, CT, USA). The exudate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used as test sample. Protein concentration was determined using Bradford method.^[17] Supernatant was analyzed for TNF-α and IL-6 levels using enzyme-linked immunosorbent assay (ELISA) with commercial kits (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The lower limit of detection of TNF-α and IL-6 were determined to be 1.07 and 3.8 pg/mL, respectively. After harvesting the

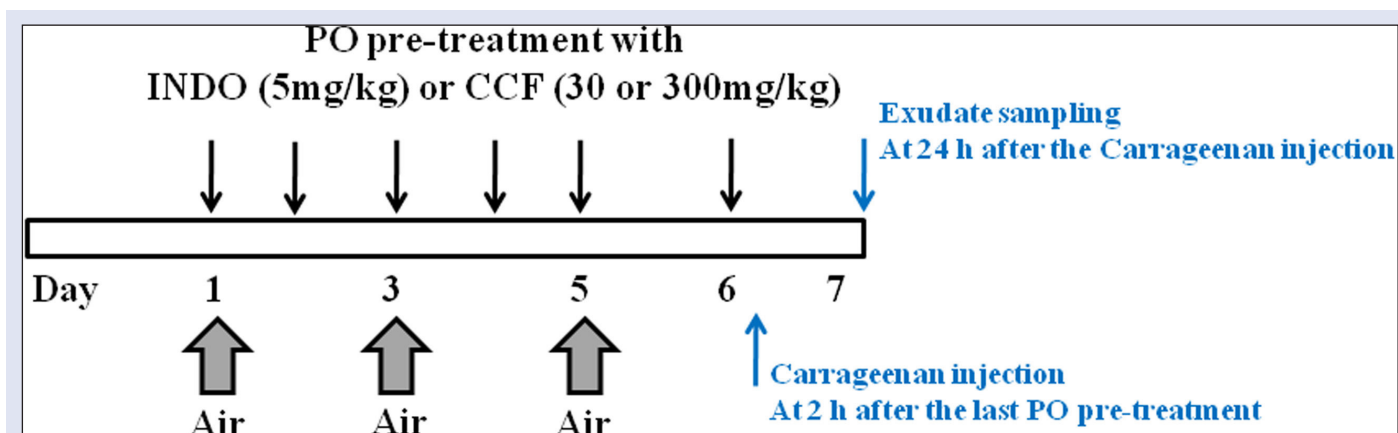


Figure 1: Schematic diagram illustrating the schedule for the preparation of carrageenan-induced air pouch inflammation mouse model and oral pretreatment with indomethacin (5 mg/kg) or ECCF (30 or 300 mg/kg)

exudate, the skin tissues were collected, fixed in 10% (v/v) formaldehyde solution, dehydrated in a graded ethanol series (99.9, 90, 80, and 70%), and embedded in paraffin. Paraffin-embedded skin tissues were then sectioned (4 µm) and stained with hematoxylin and eosin.

Ethics statement

All animals were maintained according to the guidelines of the Institutional Animal Care and Use Committee of Dongshin University. Experiments were performed after obtaining approval for the study protocol (Approval No. 2014-08-03).

HPLC conditions

All HPLC analyses were performed on a Waters Alliance 2695 HPLC system with photodiode array detector. An Agilent Zorbax extended C18 (5 µm, 150 mm × 4.6 mm) column was used as the analytical column. Mobile phase was a mixture of solvent A (methanol) and B (water containing 0.1% acetic acid) employing gradient elution (from 10:90 to 100:0, v/v) at a flow rate of 1 mL/min. HPLC conditions are summarized in Table 1. Column temperature was maintained at 25°C. Detection wavelength was set at 270 nm for bergenin and 350 nm for quercetin and quercitrin. Solvent was filtered through a 0.22 µm filter and degassed. Sample injection volume was 10 µL.

Statistical analysis

All data were expressed as mean ± standard deviation. Student's *t*-test was used for statistical analysis. A *P* value of less than 0.01 or 0.05 was considered as statistically significant.

RESULTS

Antimicrobial effects of ECCF

The susceptibilities of various microorganisms to ECCF and their corresponding MIC values are shown in Table 2. The ECCF was found to exhibit significant antimicrobial activities against *Staphylococcus aureus*, *Micrococcus luteus* ATCC 9341, *Mycobacterium smegmatis* ATCC 9341, *Mycobacterium smegmatis* ATCC 9341, *Salmonella typhimrium* KCTC 1925, and nine MRSA strains, with MIC values ranging from 250 to 1000 µg/mL.

Table 1: Chromatographic conditions of HPLC for the determination of four phytochemicals

Parameter	Condition		
Column	Zorbax extended-C18 (C18, 4.6 mm × 150 mm, 5 µm)		
Flow rate	0.8 mL/min		
Injection volume	10 µL		
UV detection	270 nm and 350 nm		
Run time	40 min		
Gradient	Time (min)	Acetonitrile (%)	0.2% Phosphoric acid (%)
	0	10	90
	10	10	90
	11	20	80
	27	20	80
	30	100	0
	35	100	0
	36	10	90
	40	10	90

Exudate volumes and protein contents

The effects of ECCF on the volumes and protein contents of the exudates recovered from air pouches of normal mice and carrageenan-induced air pouch inflammation mouse model are shown in Figure 2. Subcutaneous injection of carrageenan into air pouches without pretreatment (PC) significantly increased the volumes and protein levels of exudate (by about 4.7- and 5.6-fold, respectively) compared with those in normal mice (NC). Pretreatment with ECCF at doses of 30 and 300 mg/kg (ECCF-30 and ECCF-300, respectively) significantly reduced the volumes of exudates recovered from air pouches and carrageenan-induced protein leakage in a dose-dependent manner.

Inflammatory cell infiltration

The effects of ECCF on the numbers of white blood cells, neutrophils, mononuclear cells, and eosinophils in the exudates recovered from air pouches of normal mice and carrageenan-induced air pouch inflammation mouse model are shown in Figure 3. The numbers of white blood cells, neutrophils, monocytes, and eosinophils were markedly increased in the carrageenan-injected air pouch exudates (PCs)

Table 2: Susceptibilities of various microorganisms to ECCF and their corresponding MIC values

Gram	Organisms	Susceptibility	MIC (µg/mL)
-	<i>Alcaligenes faecalis</i> ATCC 1004	-	>1000
+	<i>Enterococcus faecalis</i> ATCC 29212	+	>1000
+	<i>Bacillus subtilis</i> ATCC6633	+	>1000
+	<i>Staphylococcus aureus</i> KCTC 1928	+	250
+	<i>Micrococcus luteus</i> ATCC 9341	+	250
+	<i>Mycobacterium smegmatis</i> ATCC 9341	+	250
-	<i>Salmonella typhimrium</i> KCTC 1925	+	1000
-	<i>Escherichia coli</i> KCTC 1923	+	>1000
-	<i>Pseudomonas aeruginosa</i> KCTC	+	>1000
	MRSA 693E	+	250
+	MRSA 4-5	+	500
+	MRSA 5-3	+	500
+	VRSA(MRSA2-32)	+	500
+	MRSA S1	+	1000
+	MRSA S3	+	1000
+	MRSA U4	+	1000
+	MRSA P8	+	1000
+	MRSA B15	+	500
+	VRE 82	+	>1000
+	VRE 89	+	>1000
+	VRE 98	+	>1000
+	VRE 2	+	>1000
+	VRE 3	+	>1000
+	VRE 4	+	>1000
+	VRE 5	+	>1000
+	VRE 6	+	>1000
	IMP 100	+	>1000
	IMP 102	+	>1000
	IMP 120	+	>1000
	IMP 123	+	>1000
	IMP 129	+	>1000
	ESBL LMH-B1	+	>1000
	ESBL LMH-P3	+	>1000
	ESBL LMH-S1	+	>1000
	ESBL LMH-U4	+	>1000
+	<i>Enterococcus faecium</i>	+	>1000
-	<i>Enterococcus cloacea</i>	+	>1000
-	<i>Klebsiella oxytica</i>	-	>1000
-	<i>Klebsiella pneumonia</i>	-	>1000

compared with those in normal mice (NC). Pretreatment with ECCF at doses of 30 and 300 mg/kg (ECCF-30 and ECCF-300, respectively) significantly reduced the numbers of white blood cells (by about 29 and

70%, respectively [Figure 3A]) and the numbers of neutrophils (by about 58.2 and 75.4%, respectively [Figure 3]). Additionally, the numbers of monocytes and eosinophils were significantly reduced (by about 62.3

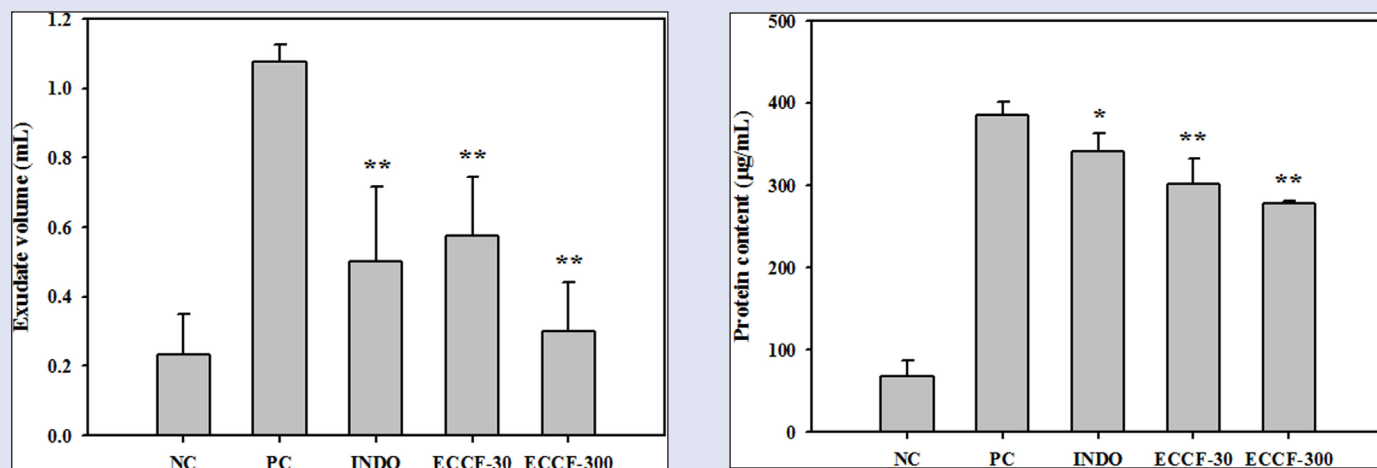


Figure 2: The volumes (A) and protein contents (B) of the exudates recovered from air pouches of normal mice (NC) or carrageenan-induced air pouch inflammation mouse model after oral administration of saline (PC), 5-mg/kg indomethacin (IND-5), 30-mg/kg ECCF (ECCF-30), or 300-mg/kg ECCF (ECCF-300) to mice ($n = 6$)

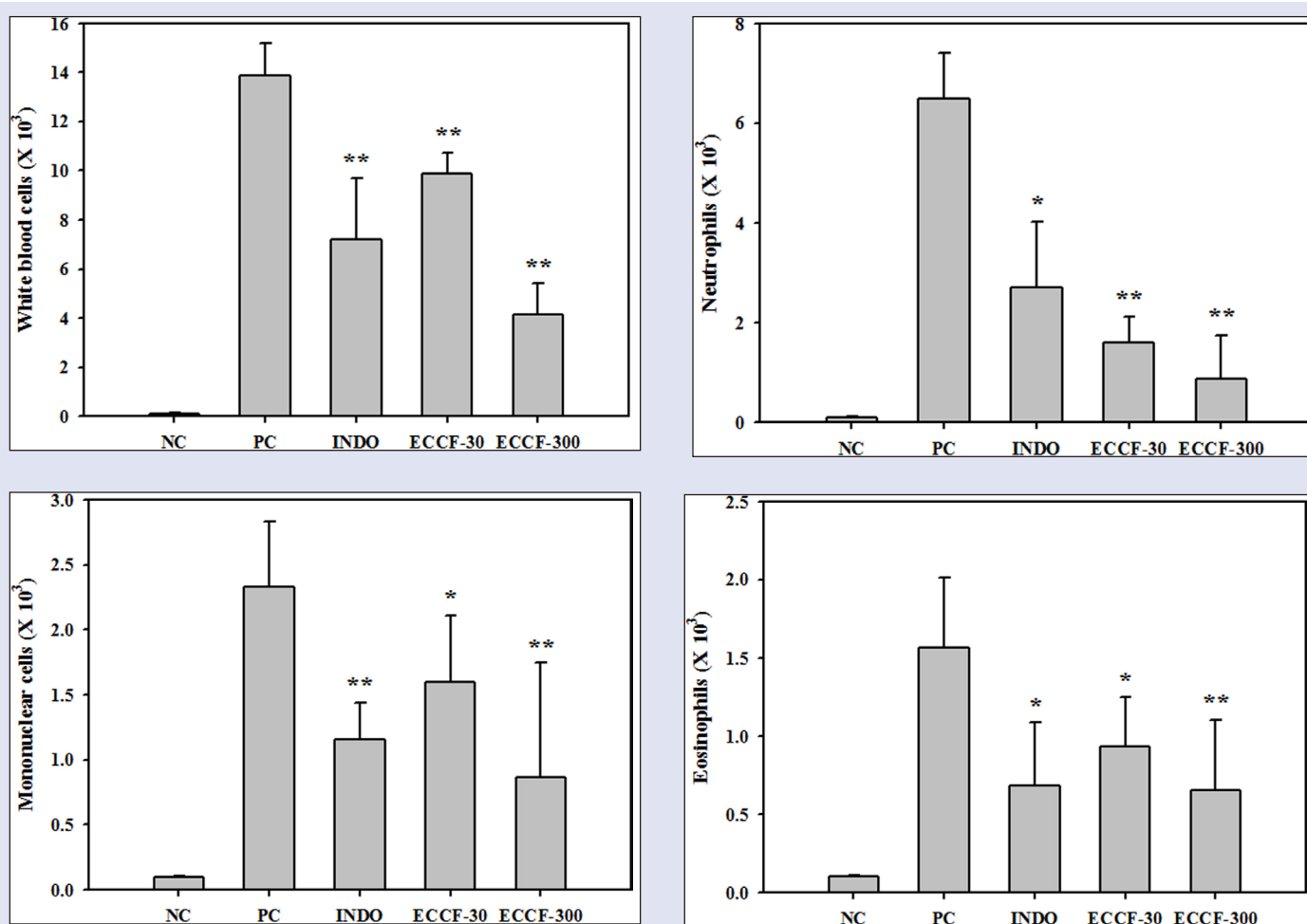


Figure 3: The numbers of white blood cells (A), neutrophils (B), mononuclear cells (C), and eosinophils (D) in the exudates recovered from air pouches of normal mice (NC) or carrageenan-induced air pouch inflammation mouse model after oral administration of saline (PC), 5-mg/kg indomethacin (IND-5), 30-mg/kg ECCF (ECCF-30), or 300-mg/kg ECCF (ECCF-300) to mice ($n = 6$)

and 59.1%, respectively) by pretreatment with ECCF at dose of 300 mg/kg [Figure 3C] and [Figure 3D].

Pro-inflammatory cytokines

The effects of CCF extract on the levels of TNF- α and IL-6 in the exudate recovered from air pouches of normal mice and carrageenan-induced air pouch inflammation mouse model are shown in Figure 4. Subcutaneous injection of carrageenan into air pouches without pretreatment (PC) significantly increased concentrations of TNF- α and IL-6 in the exudates compared with normal mice (NC). Pretreatment with ECCF at doses of 30 and 300 mg/kg significantly reduced the exudate levels of TNF- α and IL-6. Pretreatment with indomethacin at a dose of 5 mg/kg significantly reduced the exudate levels of TNF- α by 81.3% [Figure 4A]. However, it did not significantly change the exudate levels of IL-6 [Figure 4B].

Histopathological analysis

The thickness of skin in the carrageenan treatment group was greater than those in the control and indomethacin-treated group as the size of the air pouch membrane (AM) increased [Figure 5]. The thickness of skin in the control group was similar to that in the indomethacin-treated group. This could have been due to that indomethacin treatment blocked carrageenan-induced inflammatory responses in the air pouch membrane [Figure 5A] and [Figure 5C]. The thickness of AM in the carrageenan-treated group was smaller than that in all the other groups, indicating that the size of AM was restored to its control level by ECCF treatment in a dose-dependent manner [Figure 5B].

HPLC analysis of ECCF

Representative HPLC chromatograms of crude ethanol extract of CCF monitored at UV wavelength of 270 or 380 nm are shown in Figure 6. From the ECCF, we identified three flavonoids (bergenin, quercetin, and quercitrin) and one isocoumarin (isosalipurposide). The contents of bergenin, isosalipurposide, quercitrin, and quercetin in the ECCF were determined to be 17.01, 6.10, 1.46, and 0.04% (w/w), respectively.

DISCUSSION

Infections caused by drug-resistant bacteria remain a major problem.^[18] Some herbal sources are reported to have the potential to modify resistance of *S. aureus* and enhance the antibacterial action of antibiotics.^[19,20] Herein, we evaluated the antimicrobial activity of ECCF against general infectious

and MDR microbes through antimicrobial susceptibility test and MIC method. The results of this study showed that the ECCF inhibited the growth of MRSA, VRSA, IMP, and ESBL. This is the first report on the antimicrobial activity of ECCF against drug-resistant strains.

Subcutaneous carrageenan-air pouch model was employed as an acute local inflammation model in this study. The use of air pouch model in mice can mimic inflamed synovial membrane of patients with rheumatoid arthritis. In this model, subcutaneous injections of air into the back resulted in formation of a cavity similar to synovium. In addition, the injection of carrageenan in this cavity induced inflammation. This site can serve as a reservoir of cells and mediators such as TNF- α and IL-6 that can be measured in locally accumulating fluid.^[21] As shown in Figure 2, carrageenan markedly increased both the exudate volumes and protein contents, suggesting a vascular leakage of serum contents as reported previously. Such carrageenan-induced exudate production and protein leakage were significantly reduced by pretreatment with indomethacin (5 mg/kg) and ECCF (at doses of 30 and 300 mg/kg). These results could be attributed to disruption to inflammatory mediators' formation or to inhibition of receptor function.

The results shown in Figure 3 and Figure 4 clearly indicate that the ECCF significantly reduces inflammatory cell counts (white blood cells, neutrophils, mononuclear cells, and eosinophils) and pro-inflammatory cytokine levels (TNF- α and IL-6) in the carrageenan-induced air pouch inflammation mouse model. TNF- α is known to play an important role in the inflammatory process. TNF- α is released from activated monocytes and macrophages. It can increase vascular endothelial permeability,^[22] which might increase the exudate volumes and protein contents. Neutrophils are the main cellular components recruited to acute inflammatory sites induced by carrageenan. They also play central roles in the inflammatory process by secreting cytokines including TNF- α .^[4,23] Therefore, our results suggest that the anti-inflammatory effects of ECCF might be mediated by the inhibition of pro-inflammatory cytokine TNF- α . Among diverse inflammatory mediators that can induce vascular permeability, nitric oxide (NO) is well known as a major factor involved in various inflammatory diseases and pain induction/perception.^[24,25] Therefore, it is speculated that TNF- α /NO pathway might be involved in the induced inflammatory processes in this study, which merits further study.

We identified and quantified bergenin, quercitrin, quercetin, and isosalipurposide from the ECCF. The three flavonoids (i.e., bergenin, quercitrin, and quercetin) are widely found in food products derived from plant sources. It has been reported that they possess antioxidant,

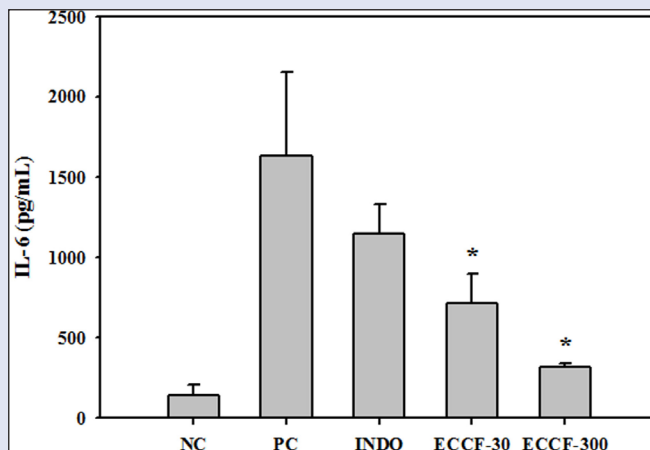
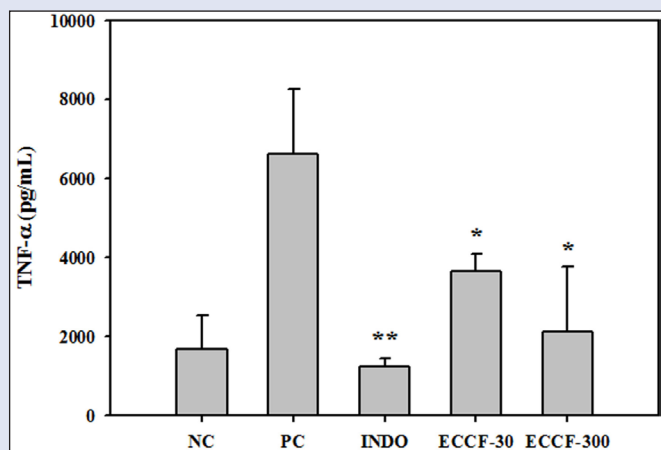


Figure 4: Levels of TNF- α (A) and IL-6 (B) in the exudates recovered from air pouches of normal mice (NC) or carrageenan-induced air pouch inflammation mouse model after oral administration of saline (PC), 5-mg/kg indomethacin (INDO), 30-mg/kg ECCF (ECCF-30), or 300-mg/kg ECCF (ECCF-300) to mice ($n = 6$)

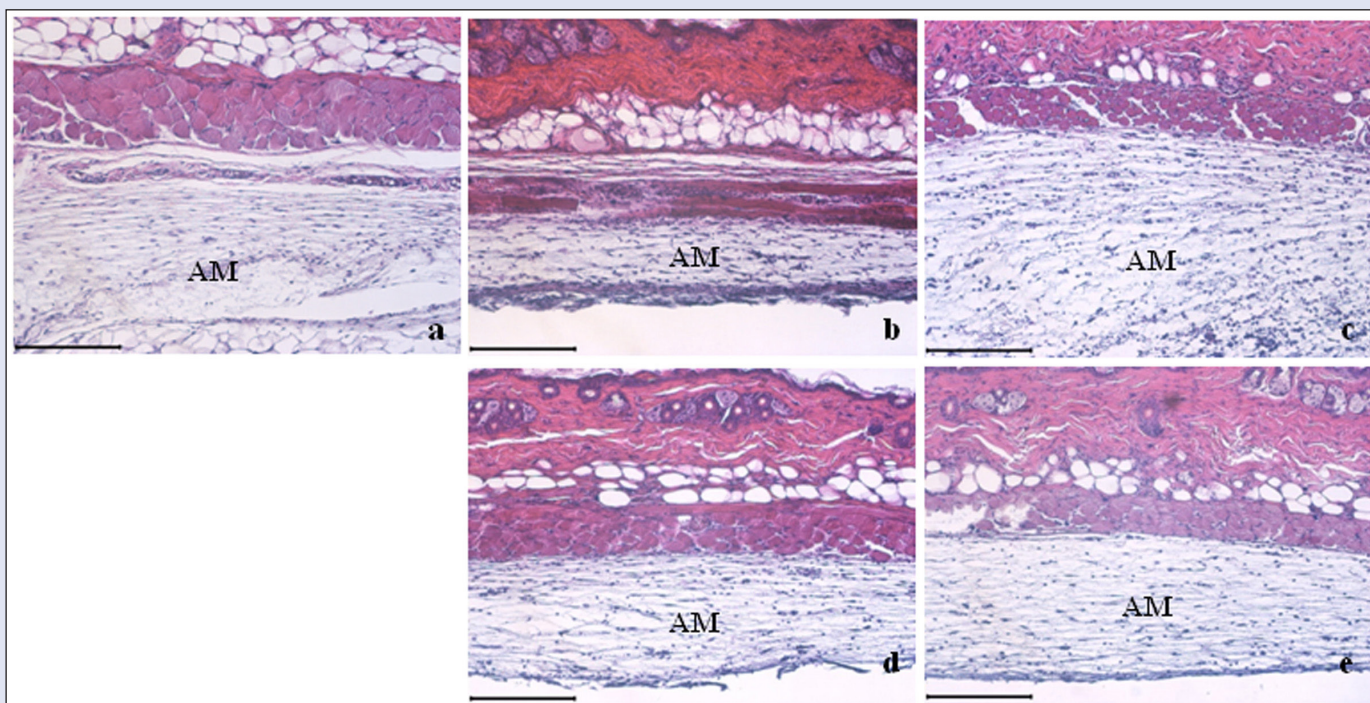


Figure 5: Representative histologic sections of air pouch membranes (AM). The scale bar represents 200 μm . (A) Control; (b) Carrageenan-treated group; (c) 5-mg/kg indomethacin-treated group; (d) 30-mg/kg ECCF-treated group; (e) 300-mg/kg ECCF-treated group

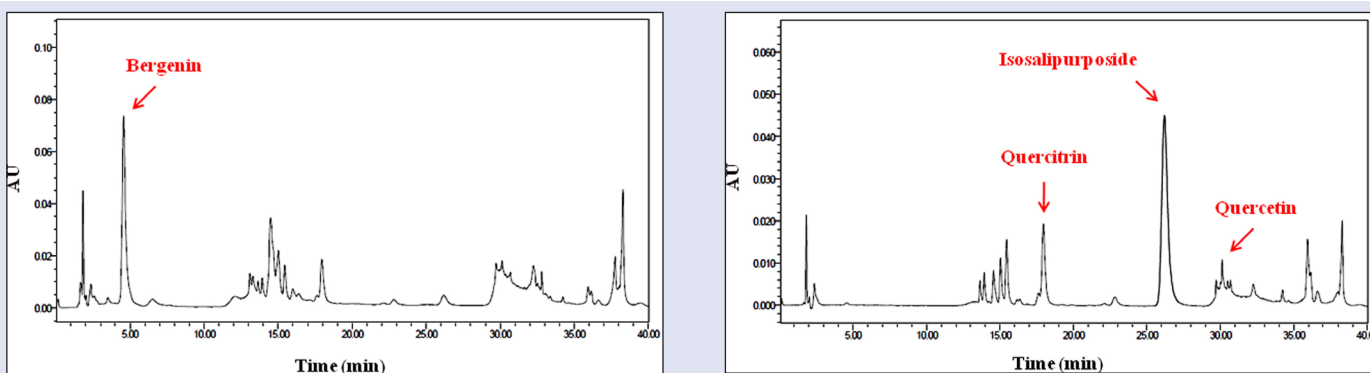


Figure 6: Representative HPLC chromatograms of ECCF monitored at UV wavelength of 270 nm (A) or 380 nm (B)

antimicrobial, and anti-inflammatory activities.^[26–29] Gao *et al.*^[30] have reported that bergenin can attenuate inflammatory cell infiltration and decrease the concentration of NO, TNF- α , IL-1 β , and IL-6 in a mouse model of lipopolysaccharide-induced mastitis. Moreover, bergenin can downregulate the phosphorylation of nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinases (MAPK) signaling pathway proteins in mammary glands with mastitis.^[30] Garcia-Mediavilla *et al.*^[31] have reported that quercetin can significantly inhibit the mRNA levels of iNOS, COX-2, and CRP (reactive C-protein). Quercitrin can specifically suppress the nuclear localization step of NF- κ B p65 in lipopolysaccharide-mediated NF- κ B activation, which can downregulate lipopolysaccharide-induced NO production and iNOS expression.^[32] Taken together, these flavonoids might have contributed to the anti-inflammatory effects of ECCF in the mouse model of carrageenan-induced air pouch inflammation.

CONCLUSION

In this study, the antimicrobial and anti-inflammatory effects of ECCF were evaluated. Antimicrobial assay demonstrated that the ECCF showed broad antimicrobial susceptibilities against infectious microbes including drug-resistant strains. The *in vivo* mouse study demonstrated that the ECCF suppressed the exudate volume, protein leakage, inflammatory cell counts, and pro-inflammatory cytokine levels in a dose-dependent manner. In addition, the ECCF was found to contain several well-known anti-inflammatory phytochemicals such as bergenin, quercetin, and quercitrin. To the best of our knowledge, this is the first report to demonstrate the antimicrobial and anti-inflammatory activities of ECCF. Our results suggest that the ECCF might potentially serve as an alternative or complementary medicine for treating inflammatory diseases caused by microbial infection.

Financial support and sponsorship

Nil

Conflicts of interest

There are no conflicts of interest

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