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麒麟菜萃取物對發炎性腸病及骨關節炎之改善作用

The Ameliorative Effects of *Eucheuma cottonii* Extract on Inflammatory Functions: Bowel Disease and Osteoarthritis

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中華民國 108 年 12 月

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ABSTRACT

Inflammatory bowel disease (IBD) is a known medical burden in most developed countries and a significant cause of morbidity. The IBD label includes Crohn's disease and ulcerative colitis. Osteoarthritis (OA) is a common form of arthritis diseases, characterized by degeneration of articular cartilage, and leading to joint dysfunction. Some strategies have been used to manage these diseases including pharmacological and non-pharmacological. The first strategy uses drugs and seems effective for a short-time but have some side effects such as increased risk of gastrointestinal and cardiovascular diseases as well as seem not effective for a long time. Seaweeds have received much attention in the pharmacological application due to its various therapeutic properties, including the anti-inflammation and antioxidant effects. Therefore, this study aimed to investigate the ameliorative effects of red seaweed *Eucheuma cottonii* extract (ECE) in a mice model of colitis and an OA model in obese rat. Colitis was induced in male BALB/c mice by the administration of 2.5% (w/v) dextran sulfate sodium (DSS) for 7 days. After treatment, ECE administration protected against weight loss and decreased the colon weight per length ratio. It also decreased proinflammatory cytokine expressions, increased interleukin (IL)-10 level, and reduced colonic damage. Therefore, a dietary polysaccharide from ECE suppressed DSS-induced bowel inflammation, thereby becoming a promising candidate for the treatment of colitis. For OA model, an anterior cruciate ligament transection with partial medial meniscectomy surgery (ACLT+MMx) to induce osteoarthritis characteristics in obese rats. The male Sprague-Dawley rats were fed a high-fat diet for 12 weeks before the surgery. Treatment with ECE for 5 weeks decreased the body weight, triglyceride, and total cholesterol (TC) levels, and the TC/high-density lipoprotein-cholesterol ratio in obese rats. ECE also downregulated the expression of proinflammatory cytokines, suppressed nuclear factor-kappa B and extracellular-signal-regulated kinase-1/2 expressions, resulting in decreased levels of matrix metalloproteinase (MMP)-1 and MMP-13 and attenuated cartilage degradation. These results demonstrated that the dietary polysaccharide from ECE suppressed OA development in obese rats, suggesting its potential efficacy against OA.

Keywords: Colitis disease; *Eucheuma cottonii*; osteoarthritis; polysaccharide; seaweed

摘要

發炎性腸病 (Inflammatory bowel disease, IBD) 在已開發國家是已知的醫療負擔且是發病的重要原因，IBD 的標誌包含克隆氏症 (Crohn's disease) 及潰瘍性結腸炎。骨關節炎 (Osteoarthritis, OA) 是一種關節炎疾病常見的形式，其特徵為關節軟骨退化並造成關節功能障礙。目前已經運用了許多藥理性及非藥理性的策略控制這些疾病，第一種是使用藥物，在短期內似乎有效但通常有一些副作用，包含增加了胃腸道及心血管疾病的風險，且在長期來看效果有限。海藻在藥理學應用中備受關注，是由於其具有許多生物醫學特性，包含抗發炎及抗氧化作用，因此本研究目的為探討麒麟菜萃取物 (*Eucheuma cottonii* Extract, ECE) 對結腸炎小鼠及肥胖誘導骨關節炎大鼠之改善作用。給予雄性 BALB/c 小鼠 2.5% (w/v) 葡聚糖硫酸鈉 (Dextran sulfate sodium, DSS) 連續七天以誘導結腸炎，使用 ECE 後顯示其可防止體重減輕並降低單位結腸的重量比，ECE 還可降低促炎性細胞因子的表現、增加 IL-10 含量並減輕結腸損傷，因此，富含膳食多醣的 ECE 可以減輕 DSS 誘導的發炎性腸病，因此有望成為治療結腸炎的候選物。使用前十字韌帶橫斷及半月板切除術 (Anterior cruciate ligament transection with partial medial meniscectomy surgery, ACLT+MMx) 誘導肥胖大鼠 OA，且在手術前給予雄性 Sprague-Dawley 大鼠 12 周高脂飲食誘導，結果指出 ECE 治療五周可降低肥胖大鼠體重、三酸甘油酯、總膽固醇 (Total cholesterol, TC) 及 TC 與高密度脂蛋白膽固醇比例，且 ECE 可下調促炎性細胞因子表現、抑制核因子活化 B 細胞 κ 輕鏈增強子 (nuclear factor-kappa B, NF-κB) 及細胞外訊號調節激酶 (Extracellular signal regulated kinase, ERK1/2) 表現，造成基質金屬蛋白酶 (Matrix metalloproteinase, MMP)-1 及 MMP-13 含量降低並減弱軟骨降解。這些結果顯示富含膳食多醣的 ECE 可抑制肥胖大鼠 OA 發展，表示其有希望成為治療 OA 之候選物。

Keywords: Colitis disease; *Eucheuma cottonii*; osteoarthritis; polysaccharide; seaweed

PUBLICATIONS

This dissertation is based on the following published research articles:

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Sabri Sudirman
National Taiwan Ocean University
December 2019

Table of Contents

List of Appendices

List of Figures

List of Tables

List of Abbreviations

5-ASA	= 5-aminosalicylic acid
ACR	= American College of Rheumatologists
ACLT	= Anterior cruciate ligament transection
ADAMTS	= A disintegrin and metalloproteinase thrombospondin-like motifs
CD	= Crohn's disease
CFD	= Chow-fed diet
COX	= Cyclooxygenase
Cur	= Curcumin
DAI	= Disease activity index
DSS	= Dextran sulfate sodium
ECE	= *Eucheuma cottonii* extract
ELISA	= Enzyme-linked immunosorbent assay
ERK	= Extracellular signal-regulated kinase
EULAR	= European League against Rheumatism
GS	= Glucosamine sulfate
HDL-C	= High-density lipoprotein-cholesterol
HFD	= High-fat diet
HRP	= Horseradish peroxidase
H&E	= Hematoxylin and Eosin
IBD	= Inflammatory bowel disease
IL	= Interleukin
IFN	= Interferon
IPAA	= Ileal-J-pouch anal anastomosis
JNK	= c-Jun NH2-terminal kinase
MMP	= Matrix metalloproteinase
MMx	= Medial meniscectomy
MPAK	= Mitogen-activated protein kinase
MRI	= Magnetic resonance imaging
NF-κB	= Nuclear factor-kappa B
NSAID	= Non-steroidal anti-inflammatory drugs
OB	= Obesity
OA	= Osteoarthritis

OARSI	= Osteoarthritis Research Society International
PG	= Prostaglandin
TC	= Total cholesterol
TG	= Triglyceride
TMB	= Tetramethylbenzidine
TNF-α	= Tumor necrosis factor-alpha
SD	= Standard deviation
SF	= Synovial fluid
UC	= Ulcerative colitis

CHAPTER I

Dietary Polysaccharide from *Eucheuma cottonii* Extract Modulates the Inflammatory Response and Suppresses Colonic Injury in Dextran Sulfate Sodium-induced Colitis

I. INTRODUCTION

1.1. Background

Inflammatory bowel disease (IBD) is a known medical burden in most developed countries and a significant cause of morbidity (Cosnes *et al.*, 2011). Inflammatory bowel diseases including Crohn's disease (CD) and ulcerative colitis (UC) characterized by chronic and relapsed gut inflammation (Bitencourt *et al.*, 2015). Ulcerative colitis associated with intestinal inflammation and often results in weight loss, diarrhea accompanied by blood and mucus, fever, gastric dysmotility, and colon shortening (Hendrickson *et al.*, 2002; Bitencourt *et al.*, 2015). Since the middle of the twentieth century, the incidence of ulcerative colitis and Crohn's disease has increased in the Western world, which includes North America, Europe, Australia, and New Zealand (Molodecky *et al.*, 2012), but IBD was relatively rare in developing nations. However, over the past few decades, newly industrialized countries in Asia, South America, and the Middle East have documented the emergence of IBD (Kaplan, 2015). Furthermore, the newly industrialized countries of India and China each have a population exceeding 1 billion people. These large populations in conjunction with expanding urbanization and westernization might mean that the number of cases of IBD in newly industrialized countries could at some point overtake the number of cases in the Western world. Thus, IBD is a global disease (Molodecky *et al.*, 2012; Kaplan, 2015).

Currently, pharmacological and surgical interventions are the two main management approaches for IBD (Lean *et al.*, 2015). Traditional therapeutic agents, such as azathioprine and 6-mercaptopurine, as well as antibiotics, are becoming important in steroid-resistant and steroid-dependent patients (Cho *et al.*, 2011). Drugs such as corticosteroids, aminosalicylates, and immune-suppressants, which aim to decrease inflammation, show limited effectiveness for long-term remission and are associated with significant side effects (Triantafillidis *et al.*, 2011). Considering the severe side effects associated with the conventional treatment; natural products, including those from marine origin, have been studied to aid in the improvement of IBD clinical symptoms (D'Orazio *et al.*, 2012).

Seaweed or marine algae is a potential development as a source of IBD treatment. Some seaweeds used for management or treatment of IBD signs and symptoms, such as

Caulerpa mexicana (Bitencourt *et al.*, 2015), *Hypnea musciformis* (Brito *et al.*, 2016) and *Fucus vesicolosus* (Lean *et al.*, 2015). These seaweeds have many bioactive compounds, such as polysaccharides, terpenes, and flavonoids. These compounds have different pharmacological activities include antitumor, antiprotozoal, antiviral, antioxidant, anti-nociceptive, anti-inflammatory, and anticoagulant effects (De Souza *et al.*, 2009; da Matta *et al.*, 2011; Torres *et al.*, 2014; Bitencourt *et al.*, 2015).

Eucheuma cottonii is a red seaweed previously reported to demonstrate antioxidant, anticoagulant, anti-tumor, and anti-inflammation properties (Kumar *et al.*, 2008; Matanjun *et al.*, 2008; Namvar *et al.*, 2012; Liang *et al.*, 2014; Abu-Bakar *et al.*, 2015). *Eucheuma cottonii* is also known as *Kappaphycus alvarezii* (KA) or the "sea-bird nest" (Fayaz *et al.*, 2005; Lim *et al.*, 2015). Additionally, studies have reported that extracts produced from this seaweed can slow tumor cell growth rate (Chang *et al.*, 2017), promote wound healing (Fard *et al.*, 2011b), and upregulate cancer cell apoptosis (Abu-Bakar *et al.*, 2017). Moreover, KA extracts have been shown to improve cardiovascular, liver, and metabolic parameters in obese rat models (Wanyonyi *et al.*, 2017) and present anti-diabetic effects in streptozotocin-induced type 2 diabetic mice (Lee *et al.*, 2017). However, the effects of a dietary polysaccharide-rich extract from *Eucheuma cottonii* in a murine model of colitis has not been reported.

Various chemical agents can be used to induce colitis in rodent models, including dextran sodium sulfate (DSS), trinitrobenzene sulfonic acid (TNBS), oxazolone, acetic acid, carrageenan, indomethacin (a nonsteroidal anti-inflammatory drug [NSAID]), and peptidoglycan polysaccharides (Randhawa *et al.*, 2014). Acute or chronic colonic inflammation can be induced by DSS administration via drinking water, with its effects depending on dosage and duration (Park *et al.*, 2015). The DSS colitis model is popular owing to its controllability, reproducibility, simplicity, and rapidity (Chassaing *et al.*, 2015) and it has been confirmed to represent colitis both biochemically and morphologically (Jeon *et al.*, 2016).

1.2. Aims

In this study, the study aimed to investigate the ameliorative effects of *Eucheuma cottonii* extract (ECE) on inflammatory bowel disease induced by dextran sulfate sodium (DSS) in BALB/c mice.

II. LITERATURE REVIEWS

2.1. Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a known medical burden in most developed countries and a significant cause of morbidity (Cosnes *et al.*, 2011). Inflammatory bowel diseases including Crohn's disease (CD) and ulcerative colitis (UC) characterized by chronic and relapsed gut inflammation (Bitencourt *et al.*, 2015). The typical clinical features of IBD include persistent diarrhea, vomiting, hematochezia, unintentional weight loss, and pain (Nie *et al.*, 2017). The etiology of IBD remains unclear, but environmental factors, as well as infectious, immunological, and psychological ones, together with genetic susceptibility could be the major cause for the onset of UC (Moura *et al.*, 2015). UC is a chronic non-specific inflammatory bowel disease. UC occurs only in the large bowel, and the inflammation is confined to the mucosa, while CD may occur anywhere in the digestive tract, from mouth to rectum, and it affects the entire bowel wall to form abscesses and fistulas in the skin or internal organs (Nie *et al.*, 2017).

2.2. Definition, Epidemiology, and Classification of IBD

Inflammatory bowel disease is a chronic relapsing inflammation of the gastrointestinal tract (Valatas *et al.*, 2013). The cause of IBD is unknown; genetically susceptible individuals are thought to have a dysregulated mucosal immune response to commensal gut flora, which results in bowel inflammation (Abraham and Cho, 2009). In most cases, human IBD can be categorized, by clinical features and distinct intestinal pathology, into two separate clinical entities: Crohn's disease and ulcerative colitis (Valatas *et al.*, 2013). In 1875, Wilks and Moxon established the term ulcerative colitis into the medical vernacular. As the incidence of ulcerative colitis began to rise in the early twentieth century, regional ileitis' was formally recognized in 1932 following a paper by Crohn *et al*. (Kaplan, 2015) (Appendix 1). CD generally involves the ileum and colon, but it can affect any region of the intestine, often discontinuously. UC almost invariably involves the rectum and may affect part of the colon or the entire colon, but not the small intestine, in an uninterrupted pattern (Cottone *et al.*, 2008). The details comparison of the key features in Crohn's disease and ulcerative colitis shown in Appendix 2.

Since the middle of the twentieth century, the incidence of ulcerative colitis and Crohn's disease has increased in the Western world, which includes North America, Europe, Australia, and New Zealand (Molodecky *et al.*, 2012). The incidence of IBD rose steadily in the 20th century in the Western world, but IBD was relatively rare in developing countries. However, over the past few decades, newly industrialized countries in Asia, South America, and the Middle East have documented the emergence of IBD (Ng *et al.*, 2013; Park *et al.*, 2014; Ng, 2015). The newly industrialized countries of India and China each have a population exceeding 1 billion people. These large populations in conjunction with expanding urbanization and westernization might mean that the number of cases of IBD in newly industrialized countries could at some point overtake the number of cases in the Western world (Molodecky *et al.*, 2012; Kaplan, 2015). In Taiwan, IBD was relatively rare compared to Western countries (Kuo *et al.*, 2015). The global prevalence of IBD in 2015 shown in Appendix 3.

Before the first Rome classification in 1988, Farmer *et al.*, (1975) had reported on an anatomical classification of Crohn's disease into the ileal, colonic, and ileocolitis disease. Then, all three international working groups (Rome in 1988, Vienna in 1988 and Montreal in 2005) agreed that to classify Crohn's disease based on the age at onset of disease, the anatomic location and the clinical behavior (Appendix 4) (Vermeire *et al.*, 2012). Montreal classification for ulcerative colitis shown in Appendix 5.

2.3. Risk Factors, Symptoms, and Diagnosis of IBD

2.3.1. Risk Factors

Environmental influences and genetic primarily explain epidemiological differences in IBD between the Western world and newly industrialized countries on disease development. Environmental exposures in industrialized societies contribute to the etiology of IBD. Many of these environmental risk factors have been studied, but none fundamentally explains the pathogenesis of IBD (Frolkis *et al.*, 2013). Some environmental factors can affect IBD, such as smoking, microorganisms, diet, antibiotics, and appendectomy (Neurath, 2014). Smoking has long been known to affect IBD. A meta-analysis implicated smoking as a risk factor for CD (Calkins, 1989). In patients with CD, smoking worsens prognosis by increasing the frequency of disease flares and the

need for surgery, in addition to increasing postoperative recurrence (Cosnes *et al.*, 1996; Frolkis *et al.*, 2013).

IBD is believed to manifest in genetically predisposed individuals who mount an abnormal immune response to intestinal microbes after exposure to environmental triggers (Xavier and Podolsky, 2007). The intestinal epithelium at the interface between the intestinal microbiome and the gastrointestinal lymphoid tissue plays a critical role in shaping the mucosal immune response. The hallmark of active inflammatory bowel disease is a pronounced infiltration of innate immune cells and adaptive immune cells into the lamina propria. Increased numbers and activation of these cells in the intestinal mucosa elevate local levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, interferon (IFN)-γ, and cytokines of the IL-23-Th17 pathway (Abraham and Cho, 2009).

The human intestinal tract is colonized at birth by a complex microbiota consisting of altogether 10^{13} to 10^{14} microorganisms, including main bacteria, but also viruses, fungi, and protozoa (Dupaul-Chicoine *et al.*, 2013). Because IBD is an inflammatory disease of the gastrointestinal tract, it has speculated that luminal factors are involved. Therefore, gastrointestinal bacteria have frequently been suspected as the cause of IBD relapses (Rahimi *et al.*, 2006). The intestinal system has coevolved with the microbiota, which required for its normal development and function (Cader and Kaser, 2013). Microbiological findings in IBD patients show a reduction of the resident aerobic and anaerobic microbiota (as compared to healthy controls) and an increase in potentially pathogenic microorganisms such as *Klebsiella, Enterobacter, Proteus,* and fungi (Schultz *et al.*, 2004). Studies also show a significant reduction in the bacterial species *Lactobacilli* and *Bifidobacteria* as well as an increase in *Bacteroides* in the intestinal mucosa of IBD patients (Braun *et al.*, 2009).

Diet can influence gut inflammation through several mechanisms, including antigen presentation and alteration in the gut microbiota (De Filippo *et al.*, 2010; Hou *et al.*, 2011). High sugar intake is associated with the development of UC and CD (Sakamoto *et al.*, 2005; Amre *et al.*, 2006). A high intake of trans-unsaturated fats may also be associated with an increased risk of CD, while a high intake of dietary long-chain n-3 PUFAs may be associated with a reduced risk of UC. High fiber and fruit intakes are associated with a decreased risk of CD (Sakamoto *et al.*, 2005; Amre *et al.*, 2007; Ananthakrishnan *et al.*,

2013a). Moreover, a high vegetable intake with decreased risk of UC (Geerling *et al.*, 2000; Sakamoto *et al.*, 2005).

Several medications including antibiotics, nonsteroidal anti-inflammatory drugs (NSAIDs), postmenopausal hormone replacement, and contraceptives may increase the risk of IBD, although the mechanisms are poorly understood (Ananthakrishnan *et al.*, 2013b). Population-based studies have suggested an association between the use of antibiotics and the risk of IBD. The use of antibiotics 2-5 years before the onset of IBD may induce changes in the gut microbiome that could trigger IBD. The effect of antibiotics is more profound in CD than in UC (Hviid *et al.*, 2010; Shaw *et al.*, 2011; Virta *et al.*, 2012). Drugs such as corticosteroids, amino salicylates, and immune-suppressants, which aim to decrease inflammation, show limited effectiveness for long-term remission and are associated with significant side effects (Triantafillidis *et al.*, 2011; Lean *et al.*, 2015).

2.3.2 Symptoms and Diagnosis

Clinical features of UC consist of bloody diarrhea and chronic abdominal pain (Appendix 2) (Dignass *et al.*, 2012; Kaplan, 2015). UC is associated with intestinal inflammation and often results in weight loss, diarrhea accompanied by blood and mucus, fever, and colon shortening (Hendrickson *et al.*, 2002; Cho, 2008; Bitencourt *et al.*, 2015). Chronic diarrhea, defined as a decrease in fecal consistency for more than 4 weeks (Juckett and Trivedi, 2011) is the most common presenting symptom (Sands, 2004), abdominal pain, weight loss, and blood, as well as mucus or both in stools are also common findings in CD (Ha and Khalil, 2015).

Various techniques and medical tests are available to aid the physician in the diagnosis of IBD (Fakhoury *et al.*, 2014a). The diagnoses of Crohn's disease and ulcerative colitis made on clinical grounds supplemented with objective findings of radiological, endoscopic, and histological examination (Baumgart, 2009). The endoscopic images can localize small erosions and ulcerations that may be evident along the gastrointestinal tract and help physicians identify the location of inflammation (Tanaka *et al.*, 2006). Some endoscopy finding both of CD and UC shown in Appendix 2. An alternative to endoscopy is radiology tests that can aid in the diagnosis of the disease. In this technique, the patient drinks a solution containing barium sulfate that appears

white on X-ray and highlights the internal lining of the bowel (Eliakim *et al.*, 2004). Another diagnostic tool for IBD involves testing of blood samples from patients. Laboratory blood tests may show elevated sedimentation rates and white cell counts, both of which associated with intestinal inflammation. Complete blood counts from patients with IBD may reveal anemia caused by vitamin B12 deficiency and autoimmune hemolysis (Hruz and Eckmann, 2010).

X-ray computed tomography and magnetic resonance imaging scans are also commonly used to look for intra-abdominal complications of IBD, such as small bowel obstruction, abscesses or fistulae (Zissin *et al.*, 2005). Magnetic resonance imaging (MRI) has undergone the same technological advances as seen with computed tomography. Its spatial and temporal resolution of images allows evaluation of bowel wall contrast enhancement, wall thickening, and edema, which is useful for assessment of IBD activity (Vilela, 2012). Biopsies of the colon can be taken to confirm the diagnosis. This technique is very effective in diagnosing the disease and in differentiating the type of inflammation (Fakhoury *et al.*, 2014a). Under the microscope, biopsy of the affected colon may show mucosal damage, characterized by focal infiltration of leukocytes into the epithelium (Fakhoury *et al.*, 2014b).

2.4. Management of IBD

The goals of treatment for both varieties of chronic inflammatory bowel diseases are the rapid induction of a steroid-free remission, the prevention of complications of the disease itself and its treatment (Baumgart, 2009), mucosal healing, improved quality of life and avoidance of disability (Peyrin-Biroulet *et al.*, 2011). Currently, pharmacological and surgical interventions are the two main management approaches for IBD (Lean *et al.*, 2015).

2.4.1. Pharmacological Treatments

Medical therapy should be tailored individually depending on the diagnosis, extent, and severity of the disease. The age at onset and the disease duration are important factors in making decisions (Dignass *et al.*, 2012). Treatment success is dependent on the correct indication, induction or maintenance, optimization of the dose, and maximization of drug adherence (Ordás *et al.*, 2012). Treatment of the disease involves the use of anti-

inflammatory drugs that can significantly reduce the symptoms of the disease and help maintain its remission. Medications used to treat the symptoms of IBD include anti-inflammatory drugs, such as a 5-aminosalicylic acid (5-ASA) (Burger and Travis, 2011), and immunomodulatory, such as azathioprine, mercaptopurine, methotrexate, infliximab, adalimumab, certolizumab, and natalizumab. These compounds regulate the immune system by efficiently triggering a Th2-mediated response that dampens Th1-mediated inflammation. The results in the production of anti-inflammatory cytokines such as IL-4, IL-5, IL-6, and IL-10 that inhibit production of pro-inflammatory cytokines (Cassinotti *et al.*, 2008; Fakhoury *et al.*, 2014a). Anti-TNF-α antibodies are frequently used to treat IBD because they can effectively reduce the amount of TNF-α in the body. Recent studies suggest that infliximab, a monoclonal antibody, is a potential treatment for a CD because it neutralizes TNF-α by preventing it from interacting with its receptor (Cohen, 2001; Eggert *et al.*, 2007). Some pharmacological agents used for treating both CD and UC shown in Appendix 6.

2.4.2. Surgical Treatments

Emergency surgery is indicated for patients with life-threatening complications, such as intestinal perforation, refractory bleeding, or toxic mega colon, that do not respond to pharmacotherapy (Berg *et al.*, 2002). The most common surgical technique used to treat ulcerative colitis is total proctocolectomy with an ileal-J-pouch anal anastomosis (IPAA). Specific indications for surgery in Crohn's disease include the formation of fibrotic strictures causing partial or total intestinal obstruction, complicated internal fistulae, abdominal abscesses, and enterovesical, enterovaginal, and enterocutaneous fistulae (Larson and Pemberton, 2004). The majority of patients diagnosed with CD will have a surgical resection within ten years of their diagnosis (Bernell *et al.*, 2000). Surgical treatment is required for failed medical therapy, recurrent intestinal obstruction, malnutrition and for septic complications such as perforations and abscesses (Dasari *et al.*, 2011).

2.5. IBD and Inflammation

Altered patterns of cytokine production by immune cells from the periphery and the lamina propria of patients with IBD were initially described in the mid to late 1980s and

early 1990s (Neurath, 2014). However, the functional relevance of the observed changes in terms of the clinical activity of IBD remained unclear. In patients with IBD and in experimental mouse models of colitis, pro-inflammatory and anti-inflammatory cytokines have been shown to be produced by various cells of the mucosal immune system in response to environmental triggers, such as commensal microorganisms. Several attempts were made to treat patients with IBD using recombinant anti-inflammatory cytokines or antibodies specific for pro-inflammatory cytokines. The effects of treating patients with anti-inflammatory cytokines (such as IFN-β, IL-10, and IL-11) were disappointing (Sands *et al.*, 2002; Tilg *et al.*, 2002; Musch *et al.*, 2005), but a major breakthrough came with the first clinical use of a neutralizing antibody specific for TNF (infliximab) in patients with Crohn's disease (Van Dullemen *et al.*, 1995). Anti-TNF therapy resulted in marked clinical improvement and macroscopic healing of the inflamed mucosa on endoscopy (mucosal healing) in Crohn's disease. As there were similar findings in patients with ulcerative colitis, anti-TNF therapy with several chimeric, humanized or fully human antibodies (such as adalimumab, certolizumab pegol, golimumab, and infliximab) is now considered a crucial backbone of biological therapy in IBD (Danese and Fiocchi, 2011; Baumgart and Sandborn, 2012; Neurath and Travis, 2012; Danese *et al.*, 2013). The clinical efficacy of this approach has led to a new era of anti-cytokine therapies for the treatment of IBD. Some cytokines in pathogenesis of IBD shown in Appendix 7.

2.6. Seaweed Potential Development for IBD Treatment

Seaweeds have been used as a foodstuff in the Asian diet for centuries and are considered under-exploited resources. Seaweeds or marine algae are known to contain unique compounds that can find many uses in consumer products. Algae are a source of fiber, minerals, vitamins, pigments, steroids, lectins, halogenated compounds, polyketides, polysaccharides, mycosporine-like amino acids, proteins, polyunsaturated fatty acids and other lipids; thus, they largely consumed in many countries (Torres *et al.*, 2014). Seaweeds have many bioactive compounds, such as polysaccharides, terpenes, and flavonoids, which have different pharmacological activities with antitumor, antiprotozoal, antiviral, antioxidant, anti-nociceptive, anti-inflammatory and anticoagulant effects (De Souza *et al.*, 2009; da Matta *et al.*, 2011; Torres *et al.*, 2014; Bitencourt *et al.*, 2015).

Seaweed or marine algae is a potential development as a source of IBD treatment. Some seaweeds used for the treatment of IBD symptoms, such as *Caulerpa mexicana* (Bitencourt *et al.*, 2015), a sulfated polysaccharide from *Hypnea musciformis* (Brito *et al.*, 2016) and fucoidan extract from *Fucus vesicolosus* (Lean *et al.*, 2015) (Appendix 8). *Eucheuma* is a red algae belonging to family Solieraceae. *Kappaphycus alvarezii* was grouped under *Eucheuma* contains approximately 45 species, among which only 8-9 are commercially found, such as *K. alvarezii*, *K. denticulatum*, *K. gelatinae*, and *K. muricatum*. *Eucheuma cottonii* known in some communities as *Kappaphycus alvarezzi* or the 'sea bird nest' (Fayaz *et al.*, 2005; Lim *et al.*, 2015). *Eucheuma cottonii* is an edible species of Pacific red seaweeds obtained from Malaysian North Borneo Sabah waters which is as a potential source of a variety of compounds like dietary fibers, vitamin C, *α*-tocopherol, minerals, fatty acid and protein (Matanjun *et al.*, 2009). Appendix 9 – 11 shown the nutrient compositions, fatty acids and amino acids in *Euchuema cottonii* and Appendix 12 shown some experiments related with *Eucheuma cottonii*.

2.7. Curcumin

Curcumin is an active compound from turmeric (*Curcuma longa*). *C. longa* is a member of the ginger, Family Zingiberaceae (Chainani-Wu, 2003). Curcumin (diferuloylmethane) is approximately 90% of the curcuminoid in turmeric and followed by demethoxycurcumin and bisdemethoxycurcmin (Ruby *et al.*, 1995). Curcumin has been widely used for food ingredients, food preservatives, and also for disease treatment. Curcumin is a favorable functional food due to its antioxidant capacity and has observed in various experimental models, such as colitis disease model (Kao *et al.*, 2016b), arteriosclerosis, diabetic, hepatic, and pancreatic diseases (Hatcher *et al.*, 2008; Pan *et al.*, 2018). Curcumin has been demonstrated to prevent colitis by suppressing NF-κB (Brumatti *et al.*, 2014) and inhibiting STAT3 signaling (Yang *et al.*, 2013). Additionally, curcumin can also modulate certain inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α) and nitric oxide (Arafa *et al.*, 2009).

III. EXPERIMENTAL DESIGNS

Six-week-old male BALB/c mice were purchased from the National Laboratory Animal Center (Yilan, Taiwan). All mice were fed a standard chow-fed diet (Table 1) and water *ad libitum*. Mice were acclimatized for 1 week. Mice were housed 4 mice per cage in a room maintained at 25±2°C under a 12 h day/night cycle throughout experimentation. The Institutional Animal Care and Use Committee (IACUC Approval No. 107003) of the National Taiwan Ocean University reviewed and approved all protocols (Appendix 13).

Table 1. Standard chow-fed diet compositions

Compositions (Calorie, %)	Chow-Fed Diet*
Carbohydrate (%)	48.70 (58.00)
Protein (%)	23.90 (28.50)
Fat (ether extract, %)	5.00 (13.50)
Fat (acid hydrolysis, %)	5.70 (0)
Fiber (%)	5.10 (0)
Mineral (%)	7.00 (0)
Calories (kcal/g)	3.36

*Laboratory Rodent Diet 5001

Acute colitis was established using a previously established method (Jeon *et al.*, 2016). Briefly, mice were administered 2.5% (w/v) DSS in drinking water for 7 days by following previous method (Chassaing *et al.*, 2015). Forty-eight mice were weighed prior to experimentation and then divided into six groups (8 mice per group), one which received no DSS administration (control) and five which did (DSS groups). DSS groups were then administered either varying dosages of ECE by oral gavage (0.35 g/kg body weight, DSS+ECE1; 0.70 g/kg, DSS+ECE2; or 1.75 g/kg, DSS+ECE5), curcumin (0.10 g/kg, DSS+Cur), or water (DSS) (Figure 1). The dose of ECE was considered from some previous studies related to oral administration of seaweed extract in mice (Kong *et al.*, 2016; Wardani *et al.*, 2017). The dose of curcumin to prevent colitis disease was choose according to a previous method (Zhao *et al.*, 2016). Mice were examined daily for body weight, stool consistency, and the presence of fecal blood. ECE and curcumin were orally delivered once per day of DSS treatment for 7 days. The mice were sacrificed on the 8th day and fasted for 12 h prior to sacrifice. Mice were euthanized by CO_2 exposure in an empty chamber. The colon weight and length were measured on the day of sacrifice. Serum and colon tissue were stored at -20°C until further analysis.

IV. MATERIALS AND METHODS

4.1. Materials

4.1.1 Red Seaweed *Eucheuma cottonii*

Red seaweed *Eucheuma cottonii* (EC) was provided by a seaweed farm from Sabah (Famous Alpine Sdn. Bhd, Sabah, Malaysia).

4.1.2. Animal

Forty-eight male BALB/c mice (6 weeks-old) were purchased from the National Laboratory Animal Center (Yilan, Taiwan).

4.1.2. Chemical Reagents

a. Curcumin was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).
b. Dextran sulfate sodium (DSS, MW~40.000 Da) was purchased from Tokyo Chemical Industry (Tokyo, Japan).
c. Ethanol absolute was purchased from PanReac AppliChem (Darmstadt, Germany)
d. Tumor necrosis factor-α, interleukin-6, interleukin-1β, and interleukin-10 ELISA kits were purchased from Arigo Biolaboratories Corporation (Hsinchu, Taiwan).
e. Formaldehyde solution (4%) was purchased from Avantor Performance Materials, Inc. (Pennsylvania, USA).
f. Resorcinol reagent was purchased from Sigma-Aldrich (Missouri, USA).

4.2. *Eucheuma cottonii* Extraction

4.2.1. Seaweed extraction

Seaweed extraction was performed according to previously used methods (Hwang and Nhuan, 2014). Briefly, 40 g of *Eucheuma cottonii* dried powder was placed in an Erlenmeyer flask and macerated with 200 mL of 70% ethanol. Extraction was performed at 50°C for 3 h with stirring by a magnetic stirrer. The solution was filtered to separate the liquid ethanol extract from residue, with residue taken for repeated extraction by adding fresh solvent under the same conditions as the first extraction. Three extractions were performed in total. The ethanol extracts were then combined with the solvent then evaporated using a vacuum evaporator at 40°C, resulting in a concentrated ethanol extract.

Concentrated extract was transferred into fresh bottles and dried using a freeze dryer in order to obtain the final *E. cottonii* extract (ECE). The extraction yield (%) was calculated as extract weight (g) divided by dried seaweed powder weight (g) multiplied by 100%.

4.2.2. Seaweed extract characterization

a. Moisture content

Dry the empty dish and lid in the oven at 105ºC for 3 h, then transfer to desiccator. Weigh the empty dish and lid. Weigh about 0.5 g dried seaweed extract powder (sample) in the dish. Place the dish with the sample in the oven at 105ºC for 3 h. Transfer the dish to the desiccator and reweigh the dish. The method described by AOAC (2000).

Calculation:

$$\text{Moisture (\%)} = \frac{(W1-W2)}{W1} \text{ x } 100\%$$

Where:
W1 = Weight of sample before drying (g); W2 = Weight of sample after drying (g)

b. Crude Protein

Protein determination used Kjeldahl method. Place 0.5 g dried seaweed extract powder in digestion flask. Add 5 g Kjeldahl catalyst (K_2SO_4 : $CuSO_4.5H_2O$ = 9:1) and 15 ml of concentrate H_2SO_4. Prepare a tube with no sample added as blank. Place flasks in the heater and heated at 380ºC until the solution become clear pale aqua blue color (3.5 h). After cooling process, 70 mL of distilled water was added. Immediately connect the flask to digestion bulb on condenser immersed in 4% boric acid added with 2-3 drops of mix indicator in receiver bulb. Add 35% NaOH to digest bulb for 5 min. Titrate the receiver solution with 0.1 N H_2SO_4 (AOAC 2000, method 981.10).

Calculation:

$$\text{Protein (\%)} = \frac{(A-B)xNx14.007x6.25}{Wx1000} \text{ x } 100\%$$

Where:
A = volume (mL) of 0.1 N H_2SO_4 used sample titration
B = volume (mL) of 0.1 N H_2SO_4 used for blank titration
N = Normality of H_2SO_4
W = sample weight (g)
14.007 = atomic weight of Nitrogen
6.25 = the protein-nitrogen conversation factor

c. Crude Fat

Place the bottle into the incubator at 105ºC overnight to ensure that weight of the bottle is stable. Weigh 0.5 g sample to paper filter and wrap. Put the sample into extraction thimble and transfer into Soxhlet extraction. Fill the bottle with ethyl ether about 75 ml and take it on the heating mantle. Connect the apparatus and turn on the water to cool them and the switch on the heating mantle. Heat the sample about 6 h. Evaporate the solvent by using the vacuum condenser. Incubate the bottle at 105ºC until the solvent completely evaporated and bottle is completely dried, transfer the bottle to the desiccator. Reweigh the bottle with dried content (AOAC 2000, method 991.36).

Calculation:

$$\text{Fat (\%)} = \frac{B}{A} \text{ x } 100\%$$

Where:
A = Weight of sample (g); B = Weight of fat (g)

d. Ash content

Dry empty crucible and lid in the oven at 105ºC for 3 h, then transfer to desiccator and weigh the empty crucible and lid. Weigh about 0.5 g sample into crucible and put to the oven at 105ºC for 3 h. Heat at 550ºC for 8 h. Cool down in the desiccator. Weigh the ash in crucible and lid (AOAC 2000, method 930.05).

Calculation:

$$\text{Ash (\%)} = \frac{B}{A} \text{ x } 100\%$$

Where:
A = Weight of sample (g); B = Weight of ash (g)

e. 3,6-Anhydro-Galactose

Prepare the Resorcinol reagent by weigh 15 mg resorcinol powder and mix with distillated water to 10 mL. Then, add distillated water until the volume 100 mL. Fructose standard prepared by take 4.504 mg D-fructose (MW=180.16 g/mol) in 10 ml saturation benzoic acid. Add distillated water to 100 mL total volume (250 µmol/L). Take 1 mL sample or standard and cool in ice water, then add 5 mL Resorcinol reagent and put into ice water for 3 min. After that, put into 80ºC water for 10 min (colorful). Pipette 100 µL to 96 well and read at 520 nm to check. This method described by (Yaphe, 1960). The

absorbance of D-fructose was 55% of that of an equimolar amount of 3,6-anhydro-*α*-D-galactopyranoside (Matsuhiro and Zanlungo, 1983).

4.3. Disease Activity Index (DAI) Measurement

Disease activity index (DAI) was measured by previous methods (Soriano *et al.*, 2000; Gommeaux *et al.*, 2007; Jeon *et al.*, 2016) and calculated based on the presence of fecal blood, stool consistency, and weight loss percentage (Table 2).

Table 2. Disease activity index (DAI) scoring of DSS-induced colitis.

Score	Occult/gross bleeding	Weight loss (% of initial weight)	Stool consistency
0	None	< 1	Normal stools
1	Small spots of blood stool; dry anal region	1-5	Soft pellets not adhering to the anus
2	Large spots of blood in stool; blood appears through the anal orifice	5-10	Very soft pellets adhering to the anus
3	Deep red stool; blood spreads largely around the anus	10-15	Liquid stool in long streams; wet anus
4	Gross bleeding	> 15	Diarrhea

Table was adapted from the previous studies (Soriano *et al.*, 2000; Gommeaux *et al.*, 2007; Jeon *et al.*, 2016). DAI values were calculated as [(weight loss score) + (stool consistency) + (rectal bleeding score)]/4 and scored on a 0-4 scale.

4.4. Blood Collection and Supernatant of Homogenized Tissue Preparation

Blood serum was collected using a syringe and centrifuged for 15 min at 3,000 rpm and stored at -20°C. The supernatant of homogenized colon tissue was prepared by weighing 100 mg of colon tissue, suspending it in 900 μL of cold PBS, and then homogenizing using a micro-tube homogenizer. Preparations were stored at -20°C, then thawed at room temperature prior to use. Homogenized tissue was centrifuged at 5,000 rpm for 15 min and the supernatant was collected for the cytokine analysis (Kim *et al.*, 2010; Abdelali *et al.*, 2016).

4.5. Inflammatory Cytokines Analysis

Pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and anti-inflammatory (IL-10) cytokine were detected using enzyme-linked immunosorbent assay (ELISA) kits via

manufacturer's protocols (Arigo Biolaboratories). Briefly, added 100 µL of sample or cytokines standard into antibody-coated microplate and incubated for 1.5 h at 37°C. Washed with Wash buffer and added 100 µL of Antibody conjugated to each well and incubated for 1 h at 37°C. Washed and added 100 µL of Streptavidin conjugated to Horseradish peroxidase (HRP) and incubated for 30 min at 37°C. Washed and added 100 µl of Substrate solution (3,3',5,5'-Tetramethylbenzidine, TMB) and incubated for 15 min at 37°C. Added 100 µL of Stop solution and read at optical density (OD) 450 nm immediately.

4.6. Colonic Histopathology

The colon sections were removed and fixed with 4% formaldehyde solution and embedded in paraffin. After cutting in slices, tissue sections were stained with hematoxylin and eosin (H&E) (Fischer *et al.*, 2008). Hydrate the cells or tissue: rehydrated tissue sections fixed in either alcohol or an aldehyde-based fixative. Then, Immerse the slide for 30 secs with agitation by hand in water. Dip the slide into a Coplin jar containing Mayer's hematoxylin and agitate for 30 secs. Rinse the slide in water for 1 min. Stain the slide with 1% eosin Y solution for 10-30 sec with agitation. Dehydrate the sections with two changes of 95% alcohol and two changes of 100% alcohol for 30 secs of each. Extract the alcohol with two changes of xylene. Add one or two drops of mounting medium and cover with a coverslip. Colonic histology was scored according to previous methods (Hamamoto *et al.*, 1999; Kim *et al.*, 2013) as shown in Table 3.

Table 3. Colonic histology score in mice.

Score	Details
0	Normal colonic mucosa
1	Loss of one-third of the crypts
2	Loss of two-thirds of the crypts
3	Lamina propria covered with a single layer of epithelial cells with mild inflammatory cell infiltration
4	Erosions and marked inflammatory cell infiltration

Table was adapted from previous studies (Hamamoto *et al.*, 1999; Kim *et al.*, 2013).

4.7. Statistical Analysis

All data were expressed as the mean ± standard deviation (S.D.). The experimental data was analyzed by statistical package software such as Statistical Product and Services Solution (SPSS 22.0, IBM Corporation, New York, USA) for statistical analysis of variance (ANOVA). When the *F-value* is significant, Duncan's multiple range test was done to analyze the significant different; $p<0.05$ was considered as significant different. Another software, GraphPad Prism was used for made the line and bar graphics.

V. RESULTS

5.1. *Eucheuma cottonii* Extract (ECE) Yield and Chemical Compositions

The *Eucheuma cottonii* extract (ECE) yield was 17.79±0.90%. Proximate analysis revealed that ECE extract possessed high levels of carbohydrates (74.77±1.15%) and ash (19.66±0.16%), followed by proteins (2.88 ± 0.05%), moisture (1.44±0.48%), and fat (1.26±0.18%). The level of 3,6-anhydro-D-galactose was roughly 43.74±6.30 μmol/L.

5.2. Effects of ECE on Body Weight Changes and Disease Activity Index

Body weight loss was increased in DSS-treated mice, with ECE or curcumin administration attenuating body weight loss extent (Figure 2A). Disease activity index scores were higher in untreated DSS-treated mice versus ECE- and curcumin-treated counterparts (Figure 2B).

5.3. ECE Improves Colon Weight, Colon Length, and Spleen Weight

Colon length decreased in DSS-treated mice after 7 days of treatment. Administration of a medium- and high-dose of ECEs (ECE2 and ECE5) or curcumin significantly attenuated colon shortening (Figure 3A). Additionally, high-dose of ECE and curcumin treatments also resulted in decreased colon weight per length ratio (Figure 3B) and reduced splenic weight (Figure 3C). Figure 3D shown a representative colon for each group.

5.4. Impacts of ECE on Inflammatory Cytokines

After DSS treatment, proinflammatory cytokine levels (e.g., TNF-α, IL-6, and IL-1β) were significantly increased relative to control mice (Figure 4). Curcumin treatment significantly reduced TNF-α level. Treatment with ECEs or curcumin significantly reduced IL-6 level in serum. Whereas, IL-1β level was significantly decreased after treated with high-dose of ECE and curcumin. Additionally, medium- and high-dose of ECEs and curcumin also significantly reduced IL-1β expression in colon tissues (Figure 5A). IL-10 expression was high in healthy mice and decreased in DSS-treated mice. Treatment with ECE regulated IL-10 expression, however no significant difference with DSS group and curcumin significantly reduced it level in colon tissue (Figure 5B).

5.5. Effects of ECE on Colonic Histopathology

Under hematoxylin and eosin (H&E) observation, the normal colon shows good wall layer architecture and no loss of crypt cells (Score 0). However, DSS-treated mice presented thicker mucosal layers accompanied by erosion (Score 4) as shown in Figure 6. However, after treatment with ECE (especially in the high-dose) or curcumin, crypt cell loss was ameliorated (Score 1).

VI. DISCUSSION

Inflammatory bowel diseases, including Crohn's disease and ulcerative colitis, can be recognized by intestinal inflammation (Chassaing *et al.*, 2015). Various animal models have been developed for studying IBD pathogenesis, including dextran sodium sulfate (DSS), trinitrobenzene sulfonic acid (TNBS), oxazolone, acetic acid, carrageenan, indomethacin [a non-steroidal anti-inflammatory drug (NSAID)], and peptidoglycan-polysaccharide induced models (Randhawa *et al.*, 2014). DSS administration in the drinking water is a commonly used method for inducing colitis in mice owing to its simplicity, reproducibility, controllability, and rapidity. DSS dosages given to BALB/c mice have ranged between 2.5-5.0% (Chassaing *et al.*, 2015).

Body weight decreased in mice with DSS-induced colitis, with ECE or curcumin administration both helping to attenuate this body weight loss (Figure 2A). Additionally, the disease activity index scores were also increased in mice with colitis (Figure 2B). These results are in accordance with previous studies (Kim *et al.*, 2013; Chassaing *et al.*, 2015; Jeon *et al.*, 2016). Colitis induced by DSS was associated with acute histological changes such as weight loss, diarrhea, and rectal bleeding, resulting in increased disease activity index scores. The oral administration of either ECE or curcumin alleviated disease activity index scores in DSS-treated mice. Colitis was also characterized by colon shortening (Figures 3A and 3C) and increased colon weight/length ratios (Figure 3B), with ECE or curcumin treatment preventing this increase in colon weight. A previous study also observed increasing in splenic weight in an untreated-DSS group (Hwang *et al.*, 2019). Overall, treatment with ECE or curcumin ameliorated the clinical signs and symptoms of colitis induced in mice by DSS.

Molecular observations supported the beneficial effects of ECE and curcumin, as treatment with either reduced the levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in DSS-treated mouse sera. In contrast, untreated DSS-mice showed increased expression levels of these cytokines (Figure 4). TNF-α, IL-6, and IFN-γ are the main inflammatory mediators in murine colitis models (Myers *et al.*, 2003; Jeon *et al.*, 2016). TNF signaling has been found to induce the pleiotropic pro-inflammatory effects of colitis, including the activation of effector T cells and macrophages. TNF signaling also directed intestine epithelial cell (ICE) damage via myosin light chain kinase (MLCK)

activation (Su *et al.*, 2013; Neurath, 2014). Increased IL-6 production by the lamina propria and $CD4^+$ T cells has been reported in experimental colitis models (Kai *et al.*, 2005). A recent study reported that the administration of anti-TNF-α may be a strategy for managing colitis (Pugliese *et al.*, 2016). Colitis can also be prevented by regulating IL-10 expression (Li and He, 2004; Neurath, 2014). The present study showed that in DSS-treated mice, IL-10 expression in colon tissues was lower than in animals treated with ECE or curcumin (Figure 5B). A previous study has also reported that untreated IL-10 gene-deficient mice showed progressive histopathological injury, increased colon weight/length ratios, and elevated IFN-γ and IL-17 expressions (Ung *et al.*, 2009). Additionally, anti-inflammatory IL-10 secretion can promote by seaweed natural polysaccharide (Wen *et al.*, 2016). Colitis severity is related to colonic damage severity. Here, untreated mice presented thicker mucosal layers and erosion. ECE extract or curcumin treatment both reduced histopathological score severity and resulted in a high number of crypt cells in the mucosal layer (Figure 6). A previous study has reported that mice treated with 5% DSS presented mucosal thickness and epithelial injury as well as increased microscopic damage scores (Kim *et al.*, 2013).

Red seaweeds are mostly composed of sulfated galactans, such as carrageenan and agar (Jiménez-Escrig and Sánchez-Muniz, 2000), which are made up of repeating disaccharide units of alternating 3-linked *β*-D-galactopyranose and 4-linked *α*-D-galactopyranose or 4-linked 3,6-anhydro-*α*-D-galactopyranose. These can be extracted from red seaweeds (Rhodophyta), such as *Eucheuma*, *Hypnea*, *Gigartina*, and *Chondrus crispus* (Campo *et al.*, 2009; Ghani *et al.*, 2018). Dietary fibers include polysaccharides, oligosaccharides, lignins, and other compounds associated with plant substances. Dietary fiber, as edible parts of plants or analogous carbohydrates, are resistant to digestion and absorption in the human small intestine, resulting in complete or partial fermentation in the large intestine (Dhingra *et al.*, 2011). A recent review reported that dietary fermented oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) possessed beneficial effects for IBD patients by reducing levels of the pro-inflammatory markers C-reactive protein and fecal calprotein (Barbalho *et al.*, 2018).

The ECEs have shown antioxidant, anti-inflammation, and anti-cancer properties (Shamsabadi *et al.*, 2013; Lim *et al.*, 2015; Raman and Doble, 2015; Chang *et al.*, 2017), and have previously been demonstrated to slow tumor cell growth rate (Chang *et al.*,

2017), promote wound healing (Fard *et al.*, 2011b), and upregulate cancer cell apoptosis (Abu-Bakar *et al.*, 2017). Moreover, extracts of this seaweed improved cardiovascular, liver, and metabolic parameters in obese rat models (Wanyonyi *et al.*, 2017) and presented anti-diabetic effects in streptozotocin-induced type 2 diabetic mice (Lee *et al.*, 2017).

Used in this study as a positive control, curcumin has been demonstrated to prevent colitis by suppressing NF-κB (Brumatti *et al.*, 2014) and inhibiting STAT3 signaling (Yang *et al.*, 2013). Additionally, curcumin can also modulate certain inflammatory mediators such as TNF-α and nitric oxide (Arafa *et al.*, 2009). Future work should focus on microbiota variation in murine intestines, as previous studies have reported that the gut microbiota is altered in cases of IBD (Rooks *et al.*, 2014; Ilott *et al.*, 2016).

VII. CONCLUSION

Eucheuma cottonii extract (ECE) has demonstrated suppressive effects on colonic disease induced by DSS in mice. ECE treatment reduced weight loss and disease activity index scores, as well as regulated the levels of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β. ECE extract administration also reduced colon injury in DSS-treated mice. Therefore, the dietary polysaccharides found in the *E. cottonii* extract may be used for the future treatment of colitis.

CHAPTER II

A Dietary Polysaccharide from *Eucheuma cottonii* Downregulates Proinflammatory Cytokines and Ameliorates Osteoarthritis-associated Cartilage Degradation

I. INTRODUCTION

1.1. Background

Osteoarthritis (OA) is a common type of arthritis most prevalent in knees. OA conditions lead to pain and disability among adults, most commonly in females, and is recognized as a chronic health condition (Barve *et al.*, 2007; Cross *et al.*, 2014; Wallace *et al.*, 2017). There are numerous risk factors associated with OA, including older age, gender, joint injury, and repetitive use of the joints, as well as obesity and maintaining an overweight status (Zhang and Jordan, 2010; Heidari, 2011). Obesity is associated with OA progression, especially in the knee, through its increasing the mechanical stress on weight-bearing joints, potentially resulting in cartilage degradation (Felson, 2000; Yusuf *et al.*, 2009). Additionally, obesity increases the expression of proinflammatory markers, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and leptin (Tzanavari *et al.*, 2010; Collins *et al.*, 2016), with TNF-α and IL-1β reportedly mediating the regulation of matrix metalloproteinases (MMPs), such as MMP-1, MMP-3, MMP-10, MMP-12, and MMP-13 (Kelley *et al.*, 2007; Ahn *et al.*, 2014; Du *et al.*, 2016) by inducing nuclear factor-kappa B (NF-κB) signaling (Rigoglou and Papavassiliou, 2013). MMPs are enzymes involved in degradation and associated with articular cartilage breakdown (Vincenti and Brinckerhoff, 2002; Rigoglou and Papavassiliou, 2013). Moreover, MMP levels are regulated by pathways related to mitogen-activated protein kinases (MAPKs), including extracellular-signal-regulated kinase (ERK)1/2, c-Jun NH_2-terminal kinase (JNK), and p38 subfamilies (Loeser *et al.*, 2008).

Spontaneous and surgical induced OA have been used as an animal models (Kuyinu *et al.*, 2016), with anterior cruciate ligament transection (ACLT) used to induce posttraumatic OA based on its ability destabilize knee joints (Anderson *et al.*, 2011; Kao *et al.*, 2016a). Additionally, total or partial meniscectomy disturbs the natural loading mechanism of the knee joint, which in turn increases the strain on articular cartilage and mimics OA development (Song *et al.*, 2008; Roemer *et al.*, 2009; Little and Fosang, 2010). Pharmacological and non-pharmacological therapies have been used for OA treatment, with oral-drug therapy capable of ameliorating some OA signs and symptoms; however, these can be accompanied by side effects, such as severe gastrointestinal diseases, renal injuries, and increased risk of cardiovascular diseases (Trelle *et al.*, 2011; Fibel *et al.*, 2015). Therefore, our research has focused on preventing or alleviating OA signs and symptoms, as well as therapeutic side effects, through the use of functional foods. For example, seaweeds, such as *Actinotrichia fragilis* extract (Sayed *et al.*, 2016), *Cladosiphon*

okamuranus (Sudirman et al., 2018b), Lithothamnion calcareum (Aslam *et al.*, 2010), and *Caulerpa cupressoides* (Rivanor *et al.*, 2014) exhibit the potential to ameliorate OA progression.

Eucheuma cottonii is a type of red seaweed that exhibits diverse pharmacological properties, including antioxidant, anti-inflammatory, anti-diabetic, and anti-obesity effects (Kumar *et al.*, 2008; Abu-Bakar *et al.*, 2015; Lee *et al.*, 2017; Wanyonyi *et al.*, 2017). A previous study reported that sulfated oligosaccharides from *E. cottonii* upregulates regulatory T cell levels in an animal model (Xu *et al.*, 2017), and our previous study showed that this extract was non-toxic to RAW 264.7 macrophages, suppressed colonic injury, and regulated inflammatory responses in dextran sulfate sodium-induced colonic disease in mice (Sudirman *et al.*, 2018a). These findings lead us to hypothesize that *E. cottonii* extract might also ameliorate OA signs and symptoms. Therefore, this study investigated the ameliorative effects of dietary polysaccharide-rich *E. cottonii* extract (ECE) on the effects of ACLT accompanied by a partial medial meniscectomy (ACLT+MMx) in high-fat diet (HFD)-induced obese rats.

1.2. Aims

In Chapter II, this thesis aimed to investigate the ameliorative effects of *Eucheuma cottonii* extract (ECE) on anterior cruciate ligament transection (ACLT) and medial meniscectomy (MMx) induced osteoarthritis in high-fat diet (HFD)-induced obese Sprague-Dawley rats.

II. LITERATURE REVIEWS

2.1. Definition, Epidemiology, and Classification of Osteoarthritis (OA)

Osteoarthritis (OA) is the most common form of arthritis, involving inflammation and major structural changes of the joint, causing pain and functional disability (Cross *et al.*, 2014). OA is caused by progressive joint degeneration which includes loss of articular cartilage surface integrity, subchondral bone remodeling and sclerosis with osteophyte formation (Dieppe and Lohmander, 2005). OA is one of the most common chronic health conditions and a leading cause of pain and disability among adults (Allen and Golightly, 2015). Pain is the most prominent and disabling symptom of OA, resulting in reduced participation in activities and negative effects on mood, sleep, and overall quality of life (Dieppe and Lohmander, 2005; Lane *et al.*, 2011; Shimura *et al.*, 2013).

Osteoarthritis can arise in any synovial joint in the body, but is most common in the hands, hips, knees and spine (Dieppe and Lohmander, 2005). It is a major cause of disability and morbidity in people aged 45 and above in developed countries (Helmick *et al.*, 2008). The incidence of hand, hip and knee OA increases with age, with women having higher rates than men (Cooper *et al.*, 2013). Knee OA accounts for more than 80% of the disease's total burden (Wallace *et al.*, 2017) and affects at least 19% of American adults aged 45 y and older (Lawrence *et al.*, 2008; Wallace *et al.*, 2017). In Canada, OA affects approximately 4.4 million people and in worldwide, OA is the most common joint disorder (Storheim and Zwart, 2014). In England and Wales approximately 1.3 – 1.75 million people are affected by symptomatic OA, while in France about 6 million people are newly diagnosed each year (Reginster, 2002; Enohumah and Imarengiaye, 2008). Appendix 14 shown the global distribution of the knee OA available data in 2010 study.

Depending on the standards of care, elderly patients presenting with OA of the knee joint will have already undergone a clinical and radiographic assessment (Buckland-Wright *et al.*, 1995; Köse *et al.*, 2017). In more advanced stages of the disease, cartilage loss occurs, determined by narrowing of the joint space on radiographs. Other radiological findings which may be seen are subchondral bone cysts, bone collapse, loose bodies, subchondral radiolucency, wear, subluxation, and deformity (Köse *et al.*, 2017) and histological grading of OA by OARSI (Pritzker *et al.*, 2006) as shown in Appendix 15**.**

2.2. Risk Factors, Symptoms, and Diagnosis of OA

2.2.1. Risk Factor

Primary OA is OA in the absence of a known cause. Several large prospective cohort studies have provided a wealth of information on risk factors for the development of OA. The risk of developing OA is largely driven by systemic (non-modifiable) or local (modified) factors. The main systemic risk factors associated with the incidence of OA are age, race/ethnicity, gender, while family history and developmental conditions that affect bone growth or joint shape have also been identified (Felson, 2000; Blagojevic *et al.*, 2010; Chaganti and Lane, 2011). Local risk factors are obesity, previous knee injury/surgery, occupational and physical activities (Zhang and Jordan, 2010; Chaganti and Lane, 2011).

Age is one of the strongest risk factors for OA of all joints (Felson and Zhang, 1998; Felson, 2000; Lawrence *et al.*, 2008). Increased age is a risk factor for increased incidence of OA in weight-bearing joints (Chaganti and Lane, 2011). Women not only are more likely to have OA than men, but they also have more severe OA (Srikanth *et al.*, 2005). The definite increase in OA in women around the time of menopause has led investigations to hypothesize that hormonal factors may play a role in the development of OA (Hannan *et al.*, 1990; Nevitt, 1996; Wluka *et al.*, 2000).

The prevalence of OA and patterns of joints affected by OA vary among racial and ethnic groups. Both hip and hand OA were much less frequent among Chinese in the Beijing Osteoarthritis Study than in whites in the Framingham Study (Nevitt *et al.*, 2002; Zhang *et al.*, 2003). But, Chinese women in the Beijing Osteoarthritis Study had significantly higher prevalence of both radiographic and symptomatic knee OA than white women in Framingham Study (Zhang *et al.*, 2001). Results from the Johnston County Osteoarthritis Project have shown that the prevalence of hip OA in African American women (23%) was similar to that in white women (22%), and prevalence was slightly higher in African American men (21%) than that in white men (17%) (Nelson *et al.*, 2010). In genetic factor, results from several studies have shown that OA is inherited and may vary by joint site. Twin and family studies have estimated the heritable component of OA to be between 50 and 65% with larger genetic influences for hand and hip OA than for knee OA (Palotie *et al.*, 1989; Spector *et al.*, 1996a; Felson *et al.*, 1998).

Obesity and overweight have long been recognized as potential risk factors for OA, especially OA of the knee (Felson, 2000; Chaganti and Lane, 2011). The results from the Framingham Study demonstrated that women who had lost about 5 kg had a 50% reduction in the risk of development of symptomatic knee OA (Felson *et al.*, 1992). Weight-loss

interventions have been shown to decrease pain and disability in established knee OA (Messier *et al.*, 2004; Christensen *et al.*, 2007). Numerous studies have shown that knee injury is one of the strongest risk factors for OA. Severe injury to the structures of a joint, particularly a trans-articular fracture, meniscal tear requiring meniscectomy, or anterior cruciate ligament injury, can result in an increased risk of OA development and musculoskeletal symptomatology (Roos *et al.*, 2001; Lohmander *et al.*, 2004).

Repetitive use of joints at work associated with an increased risk of OA. Studies have found that farmers have a high prevalence of hip OA (Croft *et al.*, 1992). The risk of development of knee OA was more than two times greater for men whose jobs required both carrying and kneeling or squatting in mid-life had more than for those whose jobs did not require these physical activities. Studies examining the relationship between sports activities and subsequent OA have produced conflicting results. There is some evidence that elite long-distance runners are at high risk for the development of knee and hip OA (Kujala *et al.*, 1995; Spector *et al.*, 1996b); and elite soccer players are at higher risk of getting knee OA when compared with non-soccer players (Roos *et al.*, 1994; Kujala *et al.*, 1995).

2.2.2 Symptoms and Diagnosis

Symptoms of the patient were recognized as persistent knee pain, limited morning stiffness and reduced function. Signals are crepitation, restricted movement and bony enlargement. Joint degeneration represents the disease while symptoms of aching, discomfort, pain, and stiffness for which patients seek medical care represent the illness (Zhang *et al.*, 2009; Heidari, 2011). Pain and other symptoms of OA may have a profound effect on quality of life affecting both physical function and psychological parameters. Knee OA is not a localized disease of cartilage alone but considered as a chronic disease of the whole joint, including articular cartilage, meniscus, ligament, and periarticular muscle that may result from multiple pathophysiological mechanisms. It is painful and disabling disease that affects millions of patients (Heidari, 2011).

Osteoarthritis diagnosis can include self-reported osteoarthritis obtained from a questionnaire, radiographic definitions of osteoarthritis, and symptomatic osteoarthritis as defined by self-reported joint pain and radiographic evidence of osteoarthritis (Chaganti and Lane, 2011). Imaging for assessment of all the structures of the joint using conventional radiography is still the most widely used technique for evaluation of a patient with a known or suspected diagnosis of OA. However, recent magnetic resonance imaging (MRI) based knee OA studies have begun to reveal the limitations of radiography. The capability of MRI

to visualize structural lesions within the knee joint is great, and there is a growing body of work using MRI for the diagnosis of knee OA as well as to examine the correlation between structural findings and knee symptoms (Heidari, 2011). Radiography which is the simplest and least expensive imaging technique is used by physicians to frequently diagnose OA together with patient-reported symptoms, particularly pain and stiffness (Gignac *et al.*, 2006). From the radiographic abnormalities' osteophytes, narrow intra-articular space, subchondral sclerosis and subchondral cysts are signs of knee OA (Zhang *et al.*, 2009; Heidari, 2011).

The American College of Rheumatologists (ACR) developed classification systems for hand (Altman *et al.*, 1990), knee (Altman *et al.*, 1986) and hip (Altman *et al.*, 1991) OA with the aim of differentiating OA from other forms of arthritis. A similar criterion for diagnosing knee OA to those developed in 1986 by ACR was recommended in 2009 by the European League against Rheumatism (EULAR) (Zhang *et al.*, 2009).

2.3. Management of OA

OA has no cure as of yet, and although structure-modifying treatments remains a significant unmet need in OA, several symptomatic treatments are available. These treatments directed at modifying the signs and symptoms of the disease especially pain and inflammation, to improve quality of life, joint function and mobility and if possible, delay disease progression. There is no specific cure for OA exists and the severity of the condition varies from individual to individual. Hence, a more generic approach to current treatment methods revolves around some combination of non-pharmacological and pharmacological treatment modalities (Appendix 16) (Bhatia *et al.*, 2013).

The non-pharmacological treatments include patient education, exercise programs, injury prevention, weight loss, orthotic devices (special footwear, knee bracing, canes, etc.), all of which in combination to pharmacological treatments aid in managing OA (Roubille *et al.*, 2013). The alternative strategies are acupuncture and transcutaneous electrical stimulation (Zhang *et al.*, 2008; Zhang *et al.*, 2010; Bhatia *et al.*, 2013). Reducing knee OA severity on the WOMAC scale may further include pharmacological methods. Pharmacological treatments include analgesics (paracetamol, opioids, duloxetine, and capsaicin), anti-inflammatory agents with analgesic properties (non-selective non-steroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase (COX)-2 inhibitors, topical NSAIDs and intra-articular corticosteroids) (Michael *et al.*, 2010; Bhatia *et al.*, 2013).

2.4. Osteoarthritis and Inflammation

OA was originally believed to be caused by the wear and tear of the articular surfaces in the joint. Mechanical abrasion in the knee can lead to the progressive degenerative changes in the meniscus with loss of both type I and, more severely, type II collagen (Man and Mologhianu, 2014; Englund *et al.*, 2016). This effect initially occurs from the mid-substance of each meniscus rather than the articulating surface. More importantly, recent studies point to an inflammatory mechanism for the initial stages of the disease. This occurs mainly in response to injury caused by mechanical stimulation of the joint. The release of cytokines, such as interleukin (IL)-1, IL-4, IL-9, IL-13, and TNF-α, degradative enzymes, such as a disintegrin and metalloproteinase thrombospondin-like motifs (ADAMTS), and collagenases/matrix metalloproteinases (MMPs) by chondrocytes, osteoblasts, and synoviocytes triggers the process (Appendix 17) (Sulzbacher, 2013; Man and Mologhianu, 2014; Glyn-Jones *et al.*, 2015). Furthermore, the innate immune system plays a role in OA progression through the activation of both the complement and alternative pathways (Orlowsky and Kraus, 2015).

2.5. Seaweed Potential Development for OA Treatment

Seaweeds have many bioactive compounds, such as polysaccharides, terpenes, and flavonoids, which have different pharmacological activities with antitumor, antiprotozoal, antiviral, antioxidant, anti-nociceptive, anti-inflammatory and anticoagulant effects (De Souza *et al.*, 2009; da Matta *et al.*, 2011; Torres *et al.*, 2014; Bitencourt *et al.*, 2015). As described in Chapter I, beside have potential to treated IBD, seaweeds also have a potential for osteoarthritis (AO) treatment.

In arthritis, some seaweeds extract used to treat arthritis, such as *Actinotrichia fragilis* (Sayed *et al.*, 2016), fucoidan from *Hizikia fusiforme* (Lee *et al.*, 2015) and *Fucus vesiculosus* (Myers *et al.*, 2016), low-molecular weight of fucoidan from *Cladosiphon okamuranus* (Sudirman et al., 2018b), mineral-rich extract from *Lithothamnion calcareum* (Aslam *et al.*, 2010), and lectin from *Caulerpa cupressoides* (Rivanor *et al.*, 2014) (Appendix 18).

2.6. Glucosamine Sulfate

Glucosamine is an amino monosaccharide and natural compound of glycosaminoglycans in synovial fluid and cartilage matrix (Hamerman, 1989). Glucosamine possesses pharmacological effects when administrated exogenously on chondrocytes and

cartilage in osteoarthritis (Bruyère *et al.*, 2016). A previous study reported that glucosamine sulfate (GS) reduces the inflammatory and degenerative mediator productions resulting in attenuation of cartilage degradation and disease progression (Chiusaroli *et al.*, 2011). *In vitro* study demonstrated that GS inhibits nuclear factor kappa B (NF-κB) pathways and decreases prostaglandin E2 (PG-E2) production (Reginster *et al.*, 2012). Additionally, GS also downregulates joint-degenerative effects of interleukin-1 (IL-1) (Rovati *et al.*, 2012). IL-1β induces several proinflammatory expressions, such as tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2), and inducible for of nitric oxide (iNOS) .

III. EXPERIMENTAL DESIGNS

Male Sprague–Dawley (SD) rats (5-weeks old) were purchased from BioLASCO Co. Ltd. (Yilan, Taiwan), housed individually, and maintained at 25±2°C with 50±10% of humidity under a 12-h light/dark cycle throughout the experiments. The Institutional Animal Care and Use Committee (IACUC Approval No. 107003) of the National Taiwan Ocean University reviewed and approved all protocols (Appendix 13). Rats (N = 42) were acclimatization for 1 week and administered a standard chow-fed diet (Laboratory Rodent Diet 5001) and water *ad libitum*. After acclimatization, rats randomly divided into two groups: Sham [n = 7; feeding with chow-fed diet (CFD) and obesity (OB; feeding with high fat diet (HFD). The HFD composed of ~20% of fat in total diet or ~40% calories from fat by adding lard according to previous method (Woods *et al.*, 2003) as shown in Table 4.

Table 4. Chow-fed and high-fat diet compositions.

Compositions (Calorie, %)	Chow-Fed Diet*	High-Fat Diet**
Carbohydrate (%)	48.70 (58.00)	43.50 (40.21)
Protein (%)	23.90 (28.50)	21.53 (19.73)
Fat (ether extract, %)	5.00 (13.50)	19.26 (40.05)
Fat (acid hydrolysis, %)	5.70 (0)	5.09 (0)
Fiber (%)	5.10 (0)	4.56 (0)
Mineral (%)	7.00 (0)	6.25 (0)
Calories (kcal/g)	3.36	4.33

*Laboratory Rodent Diet 5001; **The diet formula was adapted from previous study (Woods *et al*., 2003).

The rats were fed for 12 weeks accordingly to induce obesity (OB), after which the OB group was subdivided into five groups (n = 7 rats/group): OB Sham (surgery with an opened knee-joint capsule without ACLT+MMx) and four groups receiving surgery with ACLT+MMx. Three of the OBOA groups were administered a daily oral gavage with one of two different doses of ECE [low-dose: 100 mg/kg body weight (OBOAECE/L); high-dose: 400 mg/kg (OBOAECE/H)] or a dose of glucosamine sulfate (GS) as a positive control (200 mg/kg body weight; OBOAGS). The dose of ECE was evaluated from some previous studies related with oral administration of seaweed extract in rats (Terpend *et al.*, 2012; Waly *et al.*, 2016; Patil *et al.*, 2018). The dose of GS was considered according to previous studies (Wen *et al.*, 2010; Rovati *et al.*, 2012). The final OBOA group was orally administered saline (OBOA).

Rats were euthanized by exposure to carbon dioxide (CO_2) in an empty chamber at week 17 of experiment (treatment with ECEs or GS for 5 weeks) (Figure 7). Rats fasted for 12 h before surgery and sacrifice, and on the day of sacrifice, the body weight and adipose tissues were measured using a weighing scale. Whole blood and knee joints were collected and stored for further analysis.

IV. MATERIALS AND METHODS

4.1. Materials

4.1.1. Red Seaweed *Eucheuma cottonii*

Eucheuma cottonii was obtained from a seaweed farm in Sabah (Famous Alpine Sdn. Bhd, Sabah, Malaysia).

4.1.2. Animal

Forty-two male Sprague–Dawley rats (5-weeks old) were purchased from BioLASCO Co. Ltd. (Yilan, Taiwan).

4.1.3. Chemical Reagents

a. Glucosamine sulfate (GS) was purchased from Chen Ta Plama Mfg, Co. Ltd. (Tainan, Taiwan).
b. The standard laboratory chow-fed diet (Laboratory Rodent Diet 5001) was purchased from PMI Nutrition International, Inc. (Brentwood, MO, USA).
c. Lard was purchased from MP Biomedicals (Santa Ana, CA, USA).
d. Total cholesterol (TC) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Randox Laboratories, Ltd. (Crumlin, UK),
e. Triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) ELISA kits were purchased from BioAssay Systems (Hayward, CA, USA).
f. Matrix metalloproteinase (MMP)-1 and prostaglandin (PG)-E_2 ELISA kits were purchased from Elabscience Biotechnology Inc. (Houston, TX, USA).
g. MMP-13, interleukin-1β, tumor necrosis factor-α, and leptin ELISA kits were purchased from USCN Life Science Inc. (Wuhan, China).
h. Nuclear factor (NF)-κB and extracellular signal-regulated kinases (ERK)-1/2 ELISA kits were purchased from Wuhan Fine Biotech Co., Ltd. (Wuhan, China).
i. Zoletil 50 was purchased from Virbac (Carros, France)
j. Lofalin injections (cefazolin sodium) was purchased from Gentle Pharm Co. Ltd. (Yunlin, Taiwan).
k. Formaldehyde solution was purchased from Avantor Performance Materials Inc. (Radnor, PA, USA).

4.2. *Eucheuma cottonii* Extraction

Eucheuma cottonii extract (ECE) was obtained according to previous methods (Hwang and Nhuan, 2014; Sudirman *et al.*, 2018a). Briefly, 40 g of *E. cottonii* dry powder was macerated with 200 mL of 70% ethanol at 50±1°C with stirring (250 rpm) on a hot plate-magnetic stirrer (DLAB Scientific Inc., Los Angeles, CA, USA) for 3 h, and the liquid (supernatant) and residue were separated using filter paper (Advantec 5C; Tokyo Roshi Kaisha Ltd, Tokyo, Japan). The residue underwent repeated extraction by adding fresh solvent under the same conditions as the first extraction, with three extractions performed in total. The supernatant containing the extract was dried using a freeze dryer (ScanVac-CoolSafe; Copenhagen, Denmark) to obtain *E. cottonii* extract (ECE) dried powder. A previous study reported an extraction yield of ~17.79% comprising a high level of carbohydrates (74.77±1.15%) and ash (19.66±0.16%), then following by proteins (2.88±0.05%), moisture (1.44±0.48%), and fat (1.26±0.18%) (Sudirman *et al.*, 2018a). This extract also presence of 3,6-anhydro-D-galactose (Sudirman *et al.*, 2018a) with a total sugar content of 28.46±0.77 mg galactose per g dry powder. The total sugar was determined by colorimetric assay after acid hydrolysis (2 M H_2SO_4) at 95°C for 1.5 h according to the method described by previous method (Abd-Rahim *et al.*, 2014).

4.3. Surgery-induced Osteoarthritis

The ACLT+MMx surgery to induce OA was performed according to previous methods (Hayami *et al.*, 2006). Briefly, rats were anesthetized by intraperitoneal injection of Zoletil (25 mg/kg body weight), and hair on the right knee rat was shaved using a digital hair clipper. In the Sham groups (Sham and OB Sham), surgery was performed by opening the knee-joint capsule without ACLT+MMx, whereas the OBOA groups (OBOA, OBOAECE/L, OBOAECE/H, and OBOAECEGS) underwent ACLT+MMx. The knee-joint capsule and skin were closed by sewing with chromic catgut sterile (4-0) and silk braided sterile sutures (3-0; Unik Surgical Sutures Mfg, Co. Ltd, Taipei, Taiwan), respectively. The rats were then intraperitoneally injected with cefazolin antibiotic (30 mg/kg/day) for 3 days after surgery to prevent surgery related infection.

4.4. Blood Sample Collection

The whole blood of rats was collected from abdominal aorta of the rats on the day of sacrifice using a heparinized syringe. The plasma or serum was separated from whole blood by centrifugation (Kubota Centrifuge 3500; Kubota Corp., Tokyo, Japan) at 3,000 rpm and

4°C for 15 min. The supernatant was gently collected by micropipette and stored at -20°C for future analysis (Mussbacher *et al.*, 2017).

4.5. Biochemicals Analysis

The total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL)-cholesterol in blood serum were analyzed by using commercial kits. The inflammatory markers, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, leptin, nuclear factor-kappa B (NF-κB), matrix metalloproteinase (MMP)-1, MMP-13, extracellular signal-regulated kinase (ERK)-1/2, and prostaglandin (PG)-E_2 were analyzed by using ELISA kits, with all analyses performed according to manufacturer instructions.

4.5.1. Total Cholesterol

The total cholesterol was determined according to the Randox Laboratory's protocol. Briefly, 2 µL of sample or standard was pipetted to 96-wells plate. Then, 200 µL of Cholesterol Reagent was added to each well. The solution was mixed and incubated at 25ºC for 10 min. Optical density was measured the absorbance at 500 nm immediately.

4.5.2. Triglyceride

The assay was conducted by following the Bioassay System's protocol. Briefly, 10 µL of sample or standard was added to 96-wells plate. The Working Reagent was prepared by mixing 100 µL of Assay Buffer, 2 µL Enzyme Mix, 5 µL Lipase, 1 µL ATP, and 1 µL Dye Reagent in a clean tube. Then, 100 µL of Working Reagent was added to each well, mixed, and incubated for 30 min at room temperature. The optical density was measured at 570 nm.

4.5.3. High-Density Lipoprotein-Cholesterol

The assay was conducted by following the Bioassay System's protocol. Briefly, the sample was prepared by transferring 20 µL of serum into a 1.5 mL centrifuge tube and added 20 µL of Precipitation Reagent. Vortexed to mix and centrifugated at 9,500× *g* for 5 min. 24 µL of the supernatant was transferred to a clean tube and added 96 µL of Assay Buffer. The standard was prepared by transferring 12 µL of Cholesterol standard (300 mg/dL) and mixed with 108 µL of Assay Buffer. 50 µL of sample or standard was pipetted to 96-wells plate, and then 60 µL of Working Reagent (50 µL Assay Buffer, 18 µL NAD Solution, 1 µL

Enzyme Mix) was added to each well. Then, incubated for 30 min at room temperature and read the optical density values at 340 nm.

4.5.4. Tumor Necrosis Factor-alpha, Interleukin-6, Leptin, and Prostaglandin-E2

The tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and leptin were measured by following USCN Life Science's protocols. Briefly, 100 µL of the sample, standard, or blank was added to the 96-wells plate and incubated at 37°C for 1 h. After incubation time, the liquid was removed without washing. One hundred µL of Detection Reagent A was added to each well and incubated 37°C for 1 h. Then, aspirated the solution and washed with Wash Solution (350 µL) for 3 times. One hundred µL of Detection Reagent B was added to each well and incubated 37°C for 30 min. Then, aspirated the solution and washed with Wash Solution (350 µL) for 5 times. Ninety µL of Substrate Solution was added to each well and incubated for at 37°C 15 min. The liquid will be turned blue by the addition of a Substrate Solution. This step should be avoided by light. Then, Stop Solution (50 µL) was added to each well. The liquid will be turned yellow by the addition of Stop solution. The solution was mixed by tapping the side of the plate and immediately measured optical density at 450 nm.

The prostaglandin-E_2 (PG-E_2) was calculated by following the Elabscience Biotechnology's protocol. Briefly, 50 µL of standard or sample was added to each well and immediately added 50 µL of Biotinylated Detection Ab working solution, then incubated at 37°C for 45 min. Then, aspirated the solution and washed with the Wash buffer (350 µL) for 3 times. 100 µL of Horseradish peroxidase (HRP) Conjugate working solution was added to each well and incubated at 37°C for 30 min. Then, aspirated the solution and washed with the Wash buffer (350 µL) for 5 times. Ninety µL of Substrate Reagent was added to each well and incubated at 37°C for 15 min. After incubation time, 50 µL of Stop solution was added to each well and determined optical density at 450 nm immediately.

4.5.5. Nuclear Factor-kappa B and Extracellular Signal-regulated Kinase 1/2

The nuclear factor-kappa B (NF-κB) and extracellular signal-regulated kinase 1/2 (ERK-1/2) were determined by following the Fine Biotech's protocols. Briefly, the plate was washed 2 times before used. 100 µL of standard or sample was added to each well and incubated at 37°C for 1.5 h. Then, the solution was aspirated and washed with Wash Buffer for 2 times. 100 µL of Biotin-labeled antibody working solution was added to each well and

incubated at 37°C for 1 h. Then, the solution was aspirated and washed with Wash Buffer for 3 times. 100 µL of HRP-Streptavidin Conjugate working solution was added to each well and incubated at 37°C for 30 min. Then, the solution was aspirated and washed with Wash Buffer for 5 times. Ninety µL of 3,3',5,5'-Tetramethylbenzidine (TMB) Substrate was added to each well and incubated at 37°C for 15 min. Then, 50 µL of Stop solution was added to each well, mixed them thoroughly, and read the optical density at 450 nm immediately.

4.5.6. Matrix Metalloproteinases

The matrix metalloproteinase 1 (MMP-1) was determined by following the Elabscience Biotechnology's protocol. Briefly, 50 µL of standard or sample was added to each well and immediately added 50 µL of Biotinylated Detection Ab working solution, then incubated at 37°C for 45 min. Then, aspirated the solution and washed with the Wash buffer (350 µL) for 3 times. 100 µL of Horseradish peroxidase (HRP) Conjugate working solution was added to each well and incubated at 37°C for 30 min. Then, aspirated the solution and washed with the Wash buffer (350 µL) for 5 times. 90 µL of Substrate Reagent was added to each well and incubated at 37°C for 15 min. After incubation time, 50 µL of Stop solution was added to each well and determined optical density at 450 nm immediately.

The matrix metalloproteinase 13 (MMP-13) was determined according to the USCN Life Science's protocols. Briefly, 100 µL of the sample, standard, or blank was added to the 96-wells plate and incubated at 37°C for 1 h. After incubation time, the liquid was removed without washing. 100 µL of Detection Reagent A was added to each well and incubated 37°C for 1 h. Then, the solution was aspirated and washed with Wash Solution (350 µL) for 3 times. 100 µL of Detection Reagent B was added to each well and incubated 37°C for 30 min. Then, the solution was aspirated and washed with Wash Solution (350 µL) for 5 times. 90 µL of Substrate Solution was added to each well and incubated for at 37°C 15 min. The liquid will be turned blue by the addition of a Substrate Solution. This step should be avoided by light. Then, Stop Solution (50 µL) was added to each well. The liquid will be turned yellow by the addition of Stop solution. The solution was mixed by tapping the side of the plate and immediately measured optical density at 450 nm.

4.6. Knee Joint Histopathology

The knee joint was collected and fixed with 4% formaldehyde solution and embedded in paraffin. After cutting in slices, tissue sections were stained with Safranin-O/Fast Green (Avwioro, 2011). The slices were deparaffinized and hydrated to distilled water, and then

immersed in Weigert's iron hematoxylin (ferric chloride, aqueous: 1% hematoxylin 1%, alcoholic = 1:1) for 5 min. Differentiated in 1% acid alcohol after washing the slices gently in distilled water. The slices were soaked in 0.1% fast green, 1% acetic acid and 1% Safranin-O for 5 min, 10 secs and 10 secs separately, rinsed in 95% alcohol in the end. Stained knee joints were then used to measure morphological changes and proteoglycan loss, and OA cartilage pathologies were graded according to Osteoarthritis Research Society International (OARSI) guidelines as described previously as shown in Table 5.

Table 5. The grading of OA cartilage according to the OARSI grade.

Grade	Descriptions
Grade 0	Normal cartilage; hyaline articular cartilage uninvolved with OA
Grade 1	Threshold in cartilage for OA and characterized by the retention of the articular cartilage surface layer
Grade 2	Focal discontinuity of the cartilage superficial zone
Grade 3	The extension of matrix cracks into the mid zone to form vertical fissures
Grade 4	Cartilage erosion
Grade 5	Denudation, the complete erosion of the hyaline cartilage to a level of mineralized cartilage or bone
Grade 6	Changes in the contour of the cartilage surface (deformation)

Table was adapted from previous methods (Pritzker *et al.*, 2006; Waldstein *et al.*, 2016).

4.7. Statistical Analysis

All data were expressed as the mean ± standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA with Duncan post-hoc test ($p<0.05$) using statistical software SPSS 22.0 (IBM Corporation, NY, USA). Another software, GraphPad Prism was used for made the line and bar graphics.

V. RESULTS

5.1. Body Weight and Adipose Tissues

After HFD feeding for 17 weeks, the body weight of untreated groups (OB Sham and OBOA) was significantly higher than that in Sham groups; however, ECE treatment for 5 weeks before the end of experiment decreased body weight relative to those observed in OB Sham and OBOA groups, with this difference significant between the OBOAGS and Sham groups (Figure 8A). Additionally, adipose tissues, including retroperitoneal fat and epididymal adipose, displayed differences in weight among groups. Treatment with high-dose ECE for 5 weeks before sacrifice significantly decreased retroperitoneal fat as compared with that found in the OB Sham and OBOA groups (Figure 8B). Moreover, the weights of epididymal adipose tissue also increased significantly relative to those in untreated-groups (OB Sham and OBOA), although low- and high-dose ECE treatments resulted in no significant difference from the Sham group (Figure 8C).

5.2. ECE Treatment Attenuates Levels of Obesity Parameters

Feeding an HFD increased TG and TC levels, as well as the TC/HDL-C ratio (Figure 9), with TC levels in untreated groups (OB Sham and OBOA) significantly higher than those in the Sham group (Figure 9A); however, treatment with low- and high-dose of ECEs significantly decreased TC levels, and low-dose ECE significantly reduced TG levels relative to those in the OB Sham and OBOA groups. Moreover, ECE treatment significantly reduced the TC/HDL-C ratio relative to that in the OB Sham and OBOA groups (Figure 9C), although high-dose ECE treatment resulted in no significant difference relative to the Sham group.

5.3. ECE Treatment Alters Inflammatory Markers *in Vivo*

We found significantly higher levels of TNF-α, IL-1β, and leptin and higher levels of PG-E_2 in the untreated groups (OB Sham and OBOA) relative to the Sham group (Figure 10); however, following treatment with low- and high-dose ECE or GS, TNF-α levels decreased significantly relative to the OB Sham and OBOA groups (Figure 10A). Additionally, treatment with high-dose ECE for 5 weeks significantly decreased IL-1β to levels lower than those in the OB Sham and OBOA groups (Figure 10B). Moreover, elevated leptin levels in the OB Sham and OBOA groups decreased significantly following treatment with low-dose ECE and GS for 5 weeks relative to levels in the OB Sham and OBOA groups

while treatment with high-dose ECE resulted in no significant difference with levels observed in the Sham group (Figure 10C). Furthermore, treatment with both low- and high-dose ECE and GS significantly decreased PG-E_2 levels relative to those in the OB Sham and OBOA groups (Figure 10D).

5.4. ECE Treatment Downregulates Levels of NF-κB and ERK1/2

We found that levels of NF-κB and ERK1/2 increased in untreated groups (OB Sham and OBOA) relative to levels in treated groups (Figure 11), with oral administration of low- and high-dose ECE significantly decreasing both NF-κB and ERK1/2 levels.

5.5. ECE Treatment Alters MMP Levels

The OBOA group showed elevated levels of MMP-1 and MMP-13 (Figure 12), with both levels significantly higher relative to those in the Sham group. However, following treatment with high-dose ECE or GS, MMP-1 and MMP-13 levels significantly decreased relative to those in the OBOA group.

5.6. ECE Treatment Improves Knee-Joint Histopathology

Representative Safranin-O staining for each group is shown in Figure 13A. Normal cartilage was observed in the Sham and OB Sham groups, with accompanying proteoglycan loss and reduced staining and cell count in the superficial zone of the cartilage observed in the OBOA group. Additionally, OA development was observed according to light microscopy and scored accordingly (Figure 13B), with significantly higher-grade OA found in the OBOA group relative to that observed in the Sham and OB Sham groups. Following treatment with high-dose ECE and GS, we observed significant suppression of OA progression accompanied by lower grading relative to that in the OBOA group.

VI. DISCUSSION

In this study we have demonstrated surgically-induced OA using male obese rats. In our models, both obese and osteoarthritis are inducing models. In the case of a high-fat diet (HFD)-induced obese rats, the previous study reported that male Sprague-Dawley (SD) rats were highly susceptible to obesity induced by an HFD when compared to female rats. The male rats gained around 68% over their initial weight, where female rats only 10% in the same period feed with HFD (Taraschenko *et al.*, 2011). In the case of surgical-induced OA, a review study reported that the sex differences in OA studies are not necessarily the principal factor on which the studies are based (Boyan *et al.*, 2013) According to these conditions, we have demonstrated our study using male SD rats.

Surgically-induced OA has been reported by the previous study that it similar or mimic to joint injury in humans (Kao *et al.*, 2016a). Whereas, obesity is also associated with OA development and especially with knee-related OA (Zhang and Jordan, 2010; Heidari, 2011). In addition to associated increases in joint loading or stress on the knee joint, obesity is related to upregulated levels of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, as well as adipokines, especially leptin (Pottie *et al*., 2006; Fernández-Sánchez *et al*., 2011). In this study we have also provided the Sham group (only fed with CFD) as a control parameter to the obese groups, especially the body and adipose tissues weight, biochemical properties including lipid properties and pro-inflammatory cytokines. Additionally, the sham operation can be defined as a control group to ensure that same incidental effects of operation with the true operation (Sutherland, 2007). In the case of drug or pharmaceutical developments, there are 3 different options for the control group, including no treatment (untreated), the active agent (positive control), and placebo control (Probst *et al*., 2016). In the case of experimental intervention, comparison against no treatment allows the efficiency conclusion (Tenery *et al*., 2002). In this study, sham operation as a control group means untreated group.

The results showed that the body weight of untreated rats fed an HFD in the total 17 weeks including the treatments for 5 weeks (OB Sham and OBOA) was higher than that in the Sham or CFD groups (Figure 8A). However, oral administration of low- or high-dose ECE for 5 weeks before sacrifice decreased body weight. Decreased body weight represents a non-pharmacologic therapy for OA based on the resulting reductions in mechanical stress or knee-joint loading (Puett and Griffin, 1994). Additionally, we observed increased levels of the obesity parameters TC and TG in untreated OB Sham and OBOA rats (Figure 9),

whereas daily oral supplementation with ECE decreased these levels, as well as the TC/HDL-C ratio. Obesity is characterized by increases in levels of TG and TG (Hussain *et al.*, 2019). An elevated TC/HDL-C ratio is associated with cardiovascular diseases (Luz *et al*., 2008). In the present study, we found that ECE exerted anti-obesity effects on HFD-induced obese rats, which was similar to a previous study reporting that *Kappaphycus alvarezii* showed anti-obesity effects and prevented metabolic syndrome in HFD-induced obese rats (Wanyonyi *et al*., 2017). As shown in Figure 10, TNF-α, IL-1β, and leptin levels were elevated in untreated groups (OB Sham and OBOA). In addition to leptin, adipocytes are a source of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, as well as other adipokines, such as adiponectin and resistin (Pottie *et al.*, 2006). Moreover, adipose tissues are comprised mostly of adipocytes. In the present study, we found that ECE treatment decreased adipose-tissue weight (Figures 8B and 8C). Additionally, these proinflammatory cytokines have positively correlation with the OA progression by inducing some transcriptions signaling such as NF-κB and MAPK pathways and resulting in increasing the catabolic enzyme such as MMPs (Vincenti and Brinckerhoff, 2002; Loeser et al., 2008). According to these finding, we also concluded that obesity condition can enhance the OA development by increasing the loading stress in knee joint and also promoting the signaling to trigger the degradation factor of the cartilage such as proinflammatory cytokines and it supported by previous studies.(Pottie *et al*., 2006; Fernández-Sánchez *et al*., 2011).

Mechanical or posttraumatic injury to cartilage is a risk factors for OA development and also associated with increases in proinflammatory cytokine levels (Stevens *et al*., 2009). Previous studies reported that proinflammatory cytokine levels positively correlate with OA development, and that overexpression of leptin might stimulate chondrocyte differentiation and osteophyte formation (Figenschau *et al*., 2001; Dumond *et al*., 2003; Mabey and Sittisak, 2015). Additionally, leptin induces activation of the transcription factor NF-κB and MAPK signaling (Loeser *et al*., 2008; Rigoglou and Papavassiliou, 2013), regulates chondrocyte metabolism, is positively correlated with OA pathophysiology, and upregulated in human OA knee joints (Dumond *et al*., 2003). In the present study, oral supplementation of ECE and/or GS for 5 weeks decreased TNF-α, IL-1β, and leptin levels previously upregulated in the OB Sham and OBOA groups (Figure 10). Additionally, upregulated PG-E_2 levels in OA cartilage is associated with the severity of OA lesions and a major catabolic mediator involved in cartilage degradation (Maldonado and Nam, 2013). The ECEs and GS treatments also decreased the PG-E_2 (Figure 10D).

NF-κB and MAPK levels (i.e., ERK1/2) were elevated in the OBOA and OB Sham groups (Figure 11); however, following ECE and GS treatment for 5 weeks, these levels decreased significantly (Figures 11A and 11B). A previous study also reported that seaweed polysaccharide inhibited the NF-κB-p65/p50 activation and phosphorylation of p38 and extracellular signal-regulated kinase (Sanjeewa *et al.*, 2017). Activation of NF-κB signaling is induced by proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 as well as leptin, and mechanical stress such as surgery (Rigoglou and Papavassiliou, 2013). These signaling molecules binding to their receptor in the surface of cell membrane, subsequently induce the activation of IKKβ. It mediated the NF-κB-bound IκB phosphorylation, resulting in the NF-κB become to it active form, and it translocated from the cytoplasm to the nucleus (Atreya *et al*., 2008). In turn, NF-κB activity induces the expression of MMPs, including MMP-1, MMP-3, MMP-13, and aggrecanases, as well as others proinflammatory cytokines (Goldring and Marcu, 2009). Additionally, ERK1/2 is associated with OA-related cartilage destruction with a previous study reporting that ERK inhibition was sufficient to reduce OA lesions, and that this condition was associated with reductions in MMP levels (Boileau *et al.*, 2005; Loeser *et al.*, 2008). ERK1/2 as a part of MAPK signaling pathway also can triggered by proinflammatory cytokines, matrix proteins, and growth factors (Loeser *et al*., 2008). In the present study, we observed significant increases in both MMP-1 and MMP-13 levels in the OBOA group, followed by significant decreases following treatment with ECE and GS (Figure 12). MMPs are reportedly involved in cartilage degradation targeting type-II collagen and proteoglycans, especially aggrecan, which comprise cartilage (Vincenti and Brinckerhoff, 2002). MMP-1 (collagenase 1) and MMP-13 (collagenase 3) are collagenase enzymes that degrade interstitial collagens, including types I–III collagen and aggrecan, thereby making MMP reductions a form of targeted therapy for arthritis-related diseases (Burrage, 2006; Wu *et al*., 2007). In the present study ECE treatment (especially in high-dose) suppressed biochemical evidences related to OA progression, including levels of proinflammatory cytokines, immune-related signaling molecules, and enzymes involved in cartilage degradation.

We have used Safranin-O staining in OA cartilage to confirm that the ability of ECE suppresses the OA development. According to previous study the Safranin-O staining result shows the cartilage matrix (orange to red), nuclei (black), and cytoplasm (bluish or grey-green) (Schmitz *et al*., 2010). Here, the loss of staining intensity signaled the loss of cartilage or proteoglycans (Pritzker *et al*., 2006), a condition potentially caused by activation of cartilage-degrading enzymes, such as MMP-1 and MMP-13 through induction of NF-κB

(Vincenti and Brinckerhoff, 2002; Rigoglou and Papavassiliou, 2013) or MAPKs signaling pathways (Loeser *et al*., 2008). As shown in Figure 13, the Safranin-O staining revealed high-grade OA histopathology in the OBOA group. One of primary feature of OA is cartilage degradation and leading to a progression of joint dysfunction. Healthy cartilage is characterized by a smooth cartilage layer, with matrix and associated chondrocytes present in well-ordered zones (Pritzker *et al*., 2006). Additionally, we observed cell hypotrophy and surface discontinuity in the superficial layer of cartilage in the OBOA group, with low-dose ECE treatment also resulting in maintenance of OA development. Moreover, treatment with high-dose ECE and GS successfully suppressed OA progression in this context. Overall, this study showed that Eucheuma cottonii extract (ECE) has ameliorated the biochemical properties related to OA development and suppressed cartilage-associated OA damage in obese rat.

In the case of ECE administration, a previous study reported that dietary polysaccharides had regulated the toll-like receptors (TLRs) in the gut intestine. TLRs are the initial interaction between the environment and the host (Beutler, 2004). The secretion of cytokines and chemokines was triggered by activation of these receptors and leading to the development of adaptive immunity. Intestinal epithelial cells and antigen presenting cells (such as macrophages and dendritic cell) express receptors involved in recognition of carbohydrate including dietary components and have a crucial role in the regulation of immune response, considering that they can induce cell subpopulations including Th1, Th2, Th17, and T regulatory (Wichers, 2009). Additionally, the polysaccharides immunomodulatory effects have involved B cells, natural killer cell, neutrophils, macrophages, and T cells (Santa *et al*., 2014).

Use of glucosamine sulfate (GS) as a positive control in this study was based on its demonstrated ability to improve OA signs and symptoms with an in vitro study indicating that GS decreases PG-E_2 production and inhibits NF-κB levels (Largo *et al.*, 2003; Reginster *et al.*, 2012). Moreover, both intra-articular and oral administration of GS significantly decreased histological signs of OA in a Pond-Nuki model (Wenz *et al*., 2016).

In this study, we have used the serum or plasma to be analyzed both biochemical properties of OA progression in obese rats. The serum has been used for biomarkers of cartilage and joint injuries (McAlindon *et al*., 2017). Together with serum, and synovial fluid also has been used to evaluate joint disease (Lindqvist and Saxne, 1997). Synovial fluid represents the local condition of synovium, cartilage, and bone. The previous study has used the serum and synovial fluid (SF) to compare some biomarkers for OA, such as aggrecan

and cartilage oligomeric matrix protein. The results showed the positive correlation between its levels in SF and serum with the level progressive of OA (El-Arman *et al*., 2010). Additionally, the previous study also reported that more osteocalcin (biomarker of bone formation) level present in circulation than knee joint fluid. This study also suggested that osteocalcin in SF may be derived from the blood (Salisbury and Sharif, 1997). According to this condition, although serum provides information on the systemic turnover metabolism in the body, the serum also can have used to as a biomarker of cartilage condition.

VII. CONCLUSION

In this study, we observed OA progression in an obese rat following increase in body weight and adipose-tissue weight, as well as a high degree of cartilage degradation. Additionally, the OBOA condition was confirmed according to elevated TC, TG, and leptin levels in the OBOA model along with increases in proinflammatory cytokines, catabolic mediators, and cartilage-degrading enzymes. Our result showed that ECE treatment reduced the biochemical properties related to OA progression and suppressed cartilage-associated OA degradation in obese rats. Therefore, the ECE may suggested as a potentially efficacious therapeutic agent for OA treatment.

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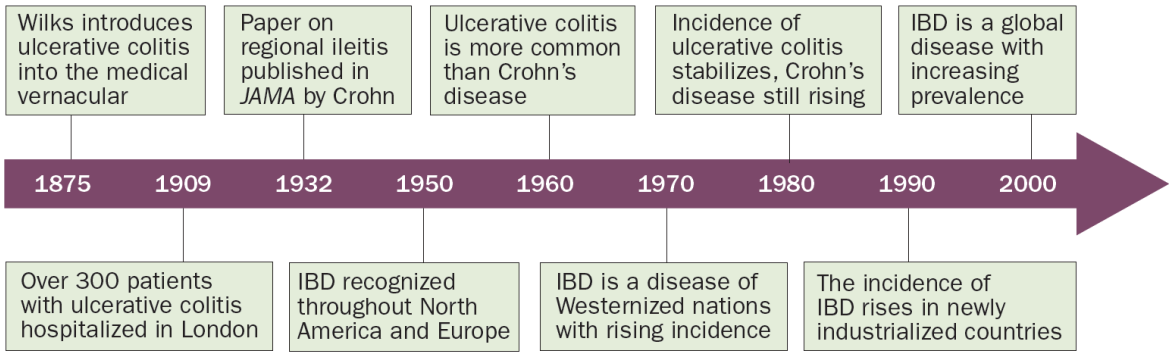
APPENDICES

Appendix 1. Historical timelines of Crohn's disease and ulcerative colitis throughout the world (Kaplan, 2015)

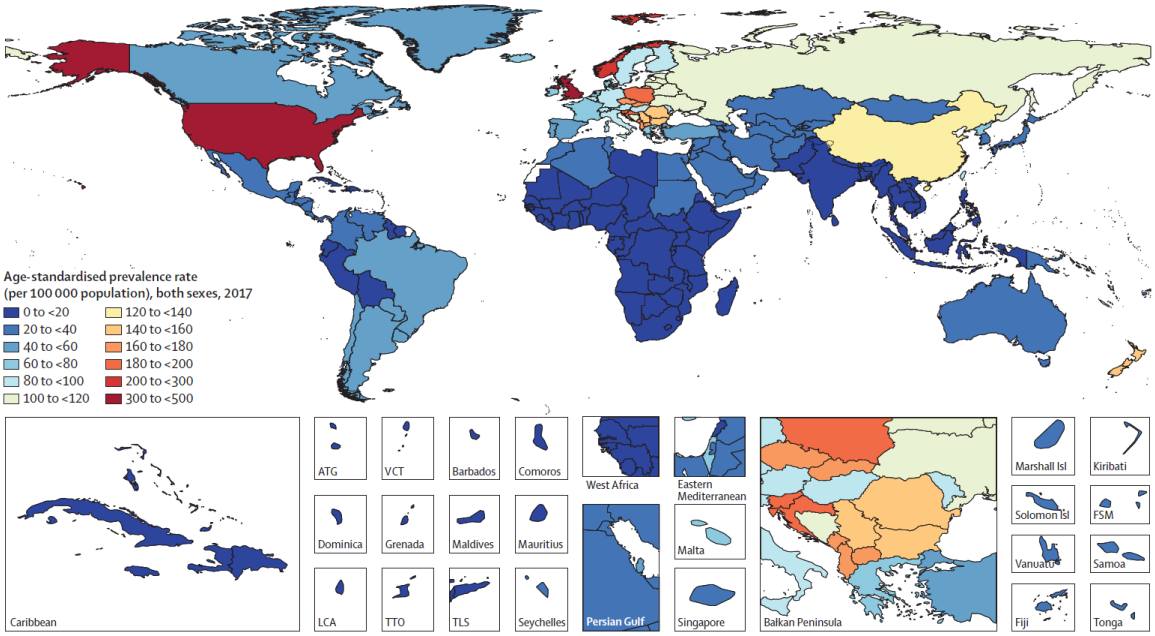


Appendix 2. Comparison of key features in Crohn's disease and ulcerative colitis

Key features	Crohn's disease	Ulcerative colitis
Location		
Upper parts of GIT*	Rarely	Never
Distal lleum	Very common	Never
Colon	Common	Always
Rectum	Rarely	Never
Signs and symptoms	Pain in the lower right abdomen, swelling, thickening of the bowel wall	Pain in the lower left abdomen, diarrhea, weight loss, rectal bleeding
Endoscopic findings	Discontinuous lesions, strictures, linear ulcerations	Continuous lesions, presence of crypts, formation of residual mucosal tissue

*GIT = Gastrointestinal tract; Source: Fakhoury *et al.*, (2014a).

Appendix 3. The global prevalence of IBD in 2017 for 195 countries (Alatab *et al.*, 2019)



Appendix 4. Vienna and Montreal classification for Crohn's disease (Vermeire *et al.*, 2012)

Variable	Vienna	Montreal
Age at diagnosis	A1 below 40 years	A1 below 16 years
	A2 above 40 years	A2 between 17 and 40 years
		A3 above 40 years
Location	L1 ileal	L1 ileal
	L2 colonic	L2 colonic
	L3 ileocolonic	L3 ileocolonic
	L4 upper	L4 isolated upper disease [a]
Behavior	B1 non-stricturing, nonpenetrating	B1 non-stricturing, nonpenetrating
	B2 stricturing	B2 stricturing
	B3 penetrating	B3 penetrating
		p perianal disease modifier [b]

[a] L4 is a modifier that can be added to L1-L3 when concomitant upper GI disease in patient;
[b] '*p*' is added to B1-B3 when concomitant perianal disease is present

Appendix 5. Montreal classification of extent and severity of ulcerative colitis (Vermeire *et al.*, 2012)

Score	Extent	Anatomy
E1	Ulcerative proctitis	Involvement limited to the rectum, *i.e.* proximal extent of inflammation is distal to the recto-sigmoid junction
E2	Left-sided ulcerative colitis (distal UC)	Involvement limited to a portion of the colon-rectum distal to the splenic flexure
E3	Extensive ulcerative colitis (pancolitis)	Involvement extends proximal to the splenic flexure
	Severity	**Definition**
S0	Clinical remission	Asymptomatic
S1	Mild ulcerative colitis	≤4 stools per day (with or without blood), absence of systemic disease, normal inflammatory markers (ESR*)
S2	Moderate ulcerative colitis	>4 stools per day, but with minimal signs of systemic toxicity
S3	Severe ulcerative colitis	≥ 6 bloody stools daily, pulse rate ≥90 beats/min, temperature ≥37.58C, hemoglobin <10.5 g/dl, and ESR ≥30mm/h

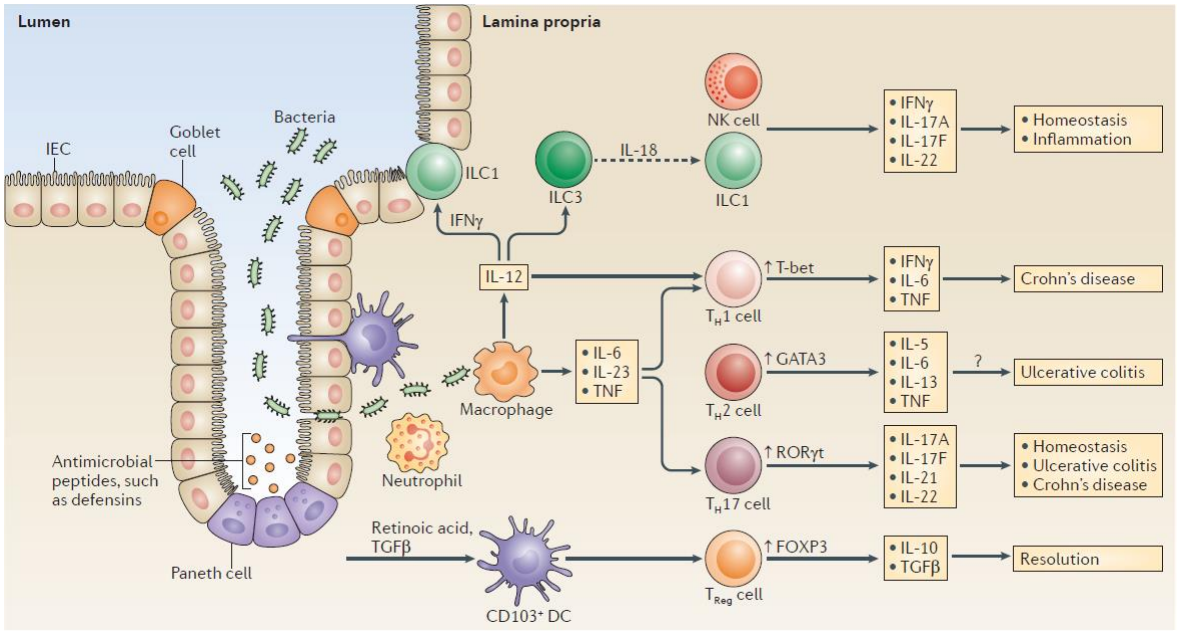
* ESR= erythrocyte sedimentation rate

Appendix 6. Pharmacological agents for treatment of Crohn's Disease and Ulcerative Colitis (Baumgart, 2009)

Medication (*)	Dosage	
	Crohn's Disease	**Ulcerative Colitis**
Sulfasalazine (oral)	Induction 3–6 g/d	Induction 2–6 g/d; Maintenance 2–4 g/d
Mesalazine (oral)		Induction 1.6–4.8 g/d; Maintenance 0.75–4 g/d
Mesalazine (suppositories)		Induction 0.5–1.5 g/d; Maintenance 0.5–1 g/d
Mesalazine (enema)		Induction 1–4 g/d; Maintenance 1–4 g/d
Prednisolone (oral)	Induction 0.25 mg/kg to 0.75 mg/kg	
Methyl- prednisolone (oral)	Induction 48 mg/d	
Prednisolone (IV)	Induction 60 mg/d	
Budesonide (oral)	Induction 9 mg/d; Maintenance 6 mg/d	
Metronidazole (oral)	Induction 10–20 mg/kg/d	
Azathioprine (oral)	2–3 mg/kg/d	
6-Mercaptopurine	Maintenance 1.5 mg/kg/d	
Methotrexate (IM)	Induction 25 mg/week; maintenance 15–25 mg/week	
Infliximab (IV)	Induction 5 or 10 mg/kg in weeks 0, 2, and 6; maintenance 5 or 10 mg/kg every 8 weeks	Induction 5 or 10 mg/kg in weeks 0, 2, and 6; maintenance 5 or 10 mg/kg every 8 weeks
Adalimumab (SC)	Induction 80 or 160 mg in week 0 and 40 or 80 mg in week 2; maintenance 40 mg every 2 weeks or weekly	
Olsalazine		Maintenance 1–2 g/d
Balsalazide		Induction 6.75 g/d (equivalent to Mesalazine 2.4 g/d); Maintenance 4 g/d (equivalent to Mesalazine 1.4 g/d)
Hydrocortisone (enema)		Induction 100 mg/d
Corticosteroids (oral cortisone)		Induction 100 mg/d
Azathioprine (oral)		Maintenance 2–2.5 mg/kg/d
Ciclosporin (IV)		Induction 2–4 mg/kg/d
Tacrolimus (oral)		Induction Serum trough level (5–15 ng/mL)

* Medication and injection methods

Appendix 7. Cytokines in the pathogenesis of IBD (Neurath, 2014)



Appendix 8. The kind of seaweeds for IBD treatment

Seaweed	Compounds	Experimental Design	Authors (Year)
Inflammatory Bowel Disease			
Hypnea musciformis, red algae	Sulphated polysaccharides extracted, *κ*-carrageenan	Colitis induced by trinitrobenzene sulfonic (TNBS) acid in rats	(Brito *et al.*, 2016)
Sargassum muticum, brown algae	Fucoxanthin-Rich Extract	DSS-induced colitis, colitis-associated colon cancer (CACC) in BALB/c mice	(Kong *et al.*, 2016)
Caulerpa mexicana, green algae	Methanolic extract	Ulcerative colitis was induced by DSS in BALB/c mice	(Bitencourt *et al.*, 2015)
Fucus vesiculosus, brown seaweed	Fucoidan extracts	DSS-induced mouse model of acute colitis	(Lean *et al.*, 2015)
Eisenia bicyclis, brown seaweed	Laminarin	DSS-colitis mice	(Tang *et al.*, 2015)

Appendix 9. Nutrient composition of *Eucheuma cottonii* (% dry weight of sample)*

Nutrient	Value	Nutrient	Value
Protein (%)	9.76±1.33	Total cations	15535.58±1.70
Lipid (%)	1.10±0.05	Na/K ratio	0.14
Ash (%)	46.19±0.42	Na (mg.100 g^{-1} DW)	1771.84±0.01
Crude fiber (%)	5.91±1.21	K (mg.100 g^{-1} DW)	13155.19±1.14
Carbohydrate (%)	26.49±3.01	Ca (mg.100 g^{-1} DW)	329.69±0.33
Moisture content (%)	10.55±1.60	Mg (mg.100 g^{-1} DW)	271.33±0.20
Soluble fiber (%)	18.25±0.93	Fe (mg.100 g^{-1} DW)	2.61±0.00
Insoluble fiber (%)	6.80±0.06	Zn (mg.100 g^{-1} DW)	4.30±0.02
Total dietary fiber (%)	25.05±0.99	Cu (mg.100 g^{-1} DW)	0.03±0.00
Vitamin C (mg 100 g^{-1} WW)	35.3±0.01	Se (mg.100 g^{-1} DW)	0.59±0.00
α-tocopherol (mg.100 g^{-1} DW)	5.85±0.27	I (μg.g^{-1} DW)	9.42±0.12

* Table was adapted from Matanjun *et al.*, (2009)

Appendix 10. Fatty acid content (% of total fatty acid content) of *Eucheuma cottonii**

Fatty acid (FA)	Carbon no.	Value (% of total fatty acid content
Capric	C10:0	0.17
Undecanoic	C11:0	3.67
Lauric	C12:0	0.88
Myristic	C14:0	1.65
Palmitic	C16:0	15.10
Palmitoleic	C16:1	11.10
Cis-10-Heptadecanoic	C17:1	8.16
Stearic	C18:0	2.11
Elaidic	C18:1ω9	0.11
Oleic	C18:1ω9	3.44
Linolelaidic	C18:2ω6	1.44
Linoleic	C18:2ω6	1.15
γ-Linolenic	C18:3ω6	0.80
α-Linolenic	C18:3ω3	3.88
Arachidic	C20:0	0.21
Cis-11-Eicosenoic	C20:1ω9	0.29
Cis-11,14,17-Eicosatrienoic	C20:3ω3	16.87
Arachidonic	C20:4ω6	1.29
Cis-5,8,11,14,17-Eicosapentaenoic	C20:5ω3	24.98
Erucic	C22:1ω9	0.20
Cis13,16-Docisadienoic	C22:2	0.86
Tricosanoic	C23:0	0.92
Lignoceric	C24:0	0.47
Nervonic	C24:1ω9	0.27
Saturated FAs		25.17±0.38
MUFAs		23.28±0.47
PUFAs		51.55±0.57
PUFAs ω6		4.68±0.05
PUFAs ω3		45.72±0.59
Ratio ω6/ω3		0.10

MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

* Table was adapted from Matanjun *et al.*, (2009)

Appendix 11. Amino acid content of *Eucheuma cottonii**

Amino acid	Value (mg/g dry weight)	Amino acid	Value (mg/g dry weight)
Aspartic acid (Asp)	2.65±0.15	Valine (Val)	2.61±0.07
Glutamic acid (Glu)	5.17±0.13	Methionine (Met)	0.83±0.17
Serine (Ser)	1.92±0.04	Isoleucine (Ile)	2.41±0.04
Glycine (Gly)	2.27±0.32	Leucine (Leu)	3.37±0.06
Histidine (His)	0.25±0.10	Phenylalanine (Phe)	19.07±2.48
Arginine (Arg)	2.60±0.14	Lysine (Lys)	1.45±0.48
Threonine (Thr)	2.09±0.01	Chemical score (%)	25.6
Alanine (Ala)	3.14±0.11	Most limiting amino acid	Lysine
Proline (Pro)	2.02±0.09	Essential amino acid	32.07±3.13
Tyrosine (Tyr)	1.01±0.12	EAA (%)	60.59±1.36

* Table was adapted from Matanjun *et al.*, (2009)

Appendix 12. Experiments related with red seaweed *Eucheuma cottonii*

Seaweeds	Compounds/ Extraction	Experimental Design	Authors (Year)
Kappaphycus alvarezii, IBIS Algoculture, Madagascar	Carrageenan	Ultrasound-assisted extraction and structural characterization by NMR	(Youssouf *et al.*, 2017)
Kappaphycus alvarezii, Vietnam	Carrageenan	Rheological properties	(Rhein-Knudsen *et al.*, 2017)
Eucheuma cottonii, Philippines	*κ*-carrageenan, gel-press method	Activity of κ-carrageenan in sucrose solution	(Leiter *et al.*, 2017)
Kappaphycus alvarezii, Semporna, Sabah, Malaysia	70% methanol extract	Breast cancer cell line (MCF-7), mammary carcinogenesis in rats	(Chang *et al.*, 2017)
Eucheuma cottonii, North coast of Sabah, Malaysia	-	Bioethanol production	(Tan and Lee, 2016)
Kappaphycus alvarezii, Sigma Aldrich product, Mumbai	Sulphated polysaccharide, carrageenan	*in vitro* antioxidant, anticancer and antidiabetic studies	(Suganya *et al.*, 2016)
Kappaphycus alvarezii, Karimun Jawa Islands, Indonesia	Refined *κ*-carrageenan	Extraction and characterization	(Manuhara *et al.*, 2016)
Kappaphycus alvarezii, Gorontalo Waters, Indonesia	-	Growth, morphology and growth-related hormone level	(Fadilah *et al.*, 2016)
Eucheuma cottonii, Fujian Lvqi Colloid Co., Ltd. Zhangzhou, China	Carrageenan oligosaccharides	Method for preparation	(Duan *et al.*, 2016)
Kappaphycus alvarezii, Simar, West coast of India	Carrageenan	Deep eutectic solvents as efficient solvent system	(Das *et al.*, 2016)
Kappaphycus alvarezii, North East Borneo (Sabah), Malaysia	50% methanol extract	Rats' breast tumor ultrastructure, immune and mRNA responses rats	(Abu-Bakar *et al.*, 2017)
Kappaphycus alvarezii, Bay of Bengal, India	*κ*-Carrageenan	Prevent colon carcinogenesis	(Raman and Doble, 2015)

Appendix 12. Experiment related with red seaweed *Eucheuma cottonii* (continued)

Seaweeds	Compounds/ Extraction	Experimental Design	Authors, Year
Eucheuma cottonii, Kuala Lumpur, Malaysia	Methanol extract	Antioxidant activity, HaCaT keratinocyte cell	(Lim *et al.*, 2015)
Eucheuma cottonii, North Borneo (Sabah)	50% aqueous methanol extract	Ovalbumin-induced allergic asthma animal model	(Abu-Bakar *et al.*, 2015)
Eucheuma cottonii, Tamiang, Kotabaru (South Kalimantan, Indonesia)	Ethanol extract	Chronically particulate matter 10 (PM_{10}) coal dust-exposed rats	(Saputri *et al.*, 2014)
Kappaphycus alvarezii, Tuticorin, Tamil Nadu, India	Dried seaweed	Physicochemical, structural characterisation	(Raman and Doble, 2014)
Eucheuma cottonii, North Borneo (Sabah), Malaysia	80% ethanol extract	Suppressing breast tumour	(Shamsabadi *et al.*, 2013)
Eucheuma cottonii, Tamiang, Kotabaru (South Kalimantan, Indonesia)	80% ethanol extract	Rat lungs chronically exposed to particulate matter 10 (PM_{10}) coal dust	(Kania *et al.*, 2013)
Kappaphycus alvarezii, Santa Catarina State, Brazil	Carrageenan	Molecular and rheological characterization	(Webber *et al.*, 2012)
Eucheuma cottonii Kudat (north coast of Sabah, Borneo)	Polyphenol, methanol extract	Suppresses breast tumor via hormone modulation and apoptosis induction	(Namvar *et al.*, 2012)
Kappaphycus alvarezii, Palk Bay coast, India	Methanol extract	Sub-chronic toxicity and heavy metal toxicity, albino rat	(Abirami and Kowsalya, 2012)
Eucheuma cottonii, North Borneo (Sabah)	80% ethanol extracts	*in vivo* hair growth and wound healing	(Fard *et al.*, 2011b)
Eucheuma cottonii, North Borneo (Sabah)	80% ethanol extracts	Wound healing properties, SD rat	(Fard *et al.*, 2011a)
Kappaphycus alvarezii (*Eucheuma cottonii*) Kudat (North Borneo)	Dried seaweed	in vivo cardiovascular protective effects, HFD, SD rats	(Matanjun *et al.*, 2010)
Eucheuma cottonii North Borneo, Malaysia	Dried seaweed	Nutrient content, fatty acid, amino acid	(Matanjun *et al.*, 2009)

Appendix 12. Experiments related with red seaweed *Eucheuma cottonii* (continued)

Seaweeds	Compounds/ Extraction	Experimental Design	Authors, Year
Eucheuma cottonii, Indonesia	Seaweed powder	SOD liver of hypercholesterolemic rats	(Wresdiyati *et al.*, 2008)
Eucheuma cottonii, North Borneo	Methanol, Diethyl ester	Antioxidant activities, phenolic content	(Matanjun *et al.*, 2008)
Kappaphycus alvarezii, Port Okha, north west coast of India	Ethanol, methanol, water, extract	Antioxidant potential, total phenol content	(Kumar *et al.*, 2008)
Kappaphycus alvarezii Kampot, Cambodia	50% methanol (v/v) extract	Antioxidant activity	(Chew *et al.*, 2008)
Kappaphycus alvarezii, Indonesia	Polyphenolic compounds	Antioxidant power	(Santoso *et al.*, 2004)
Kappaphycus alvarezii, Chubut, Argentina	Galactans	System of galactans	(Estevez *et al.*, 2004)
Kappaphycus alvarezii, Philippines	-	Growth condition, nitrate content	(Granbom *et al.*, 2004)
Kappaphycus alvarezii, Yucatan, Mexico	-	Mariculture	(Muñoz *et al.*, 2004)
Kappaphycus alvarezii, Philippines	-	The antioxidant adaptability	(Barros *et al.*, 2003)
Kappaphycus alvarezii,	-	Cultured seaweed	(Dy and Yap, 2001)
Kappaphycus alvarezii, Antique, Philippines	-	The seasonality and economic feasibility of cultivating	(Hurtado *et al.*, 2001)
Kappaphycus alvarezii Philippines	Carrageenan and agaroid	low-molecular-weight carrageenan and agaroids, the room-temperature-extracted	(Estevez *et al.*, 2000)

Appendix 13. The Institutional Animal Care and Use Committee (IACUC Approval No. 107003)

國立臺灣海洋大學生命科學院實驗動物照護及使用委員會審查同意書

Affidavit of Approval of Animal Use Protocol

College of Life Sciences, NTOU

動物實驗申請表暨同意書編號：107003

計畫主持人(PI)：龔瑞林　職稱：教授

單位：食品科學系　飼養/應用地點：陸生動物實驗中心/陸生動物實驗中心

計畫名稱：麒麟菜提取物對炎症性腸病（IBD）和骨性關節炎（OA）的改善作用

計畫執行期間：107 年 03 月 01 日至 107 年 07 月 31 日

本計畫之「動物實驗申請表」業經實驗動物照護及使用委員會 ☑實質□形式審查通過。本計畫預定飼養應用之動物如下：

動物種類	動物數量/年
小鼠/BALB/c	50 隻/6 週齡
大鼠/Sprague-Dawley (SD)	54 隻/5 週齡

另完成右列之審查項目　□實驗動物繁殖表；　☑申請動物實驗倫理 3R 說明

The animal use protocol listed below has been reviewed and approved by the Institutional Animal Care and Use Committee（IACUC）.

Protocol Title：Amelioration Effects of *Eucheuma cottonii* Extract on Inflammatory Bowel Disease (IBD) and Osteoarthritis (OA)

IACUC Approval No：107003

Period of Protocol: Valid From: 03/01/2018 To: 07/31/2018 （mm/dd/yyyy）

Principal Investigator（PI）：Zwe-ling, Kong

實驗動物照護及使用委員會主任委員 吳彰哲　日期 107.01.24

IACUC Chairman Chang-Jer Wu　Date 01.24.2018

共 1 頁，第 1 頁

Appendix 14. Osteoarthritis prevalence in 2017 for 195 countries*

Musculoskeletal disorders	Prevalence (thousands) 2017 counts	Percentage change in counts, 1990–2007	Percentage change in counts, 2007–2017
Osteoarthritis	303,096.5	63.1	31.4
Hip OA	40,010.0	59.1	35.3
Knee OA	263,086.5	63.7	30.8

* Table was adapted from James *et al.*, (2018)

Appendix 15. Histological and radiographic grading scale of osteoarthritis of the knee joint*

Histological Grading		
OARSI	**Description**	**Characteristic**
Grade 0	**Normal**: Hyaline articular cartilage **uninvolved** with OA	The cartilage surface is smooth; the matrix and associated chondrocyte are organized in three appropriately oriented
Grade 1	The **threshold** for OA in articular cartilage	Retention of the articular cartilage surface layer; mild abrasion; "superficial fibrillation"
Grade 2	Focal **discontinuity** of the cartilage superficial zone	Abrasion from shear forces leads to loss of small portions of superficial matrix parallel to the surface
Grade 3	Extension of matrix cracks into the mid zone to form **vertical fissures** (**clefts**)	Vertical fissures tend to extend and branch at angles from the vertical. The matrix texture is likely to become more heterogeneous, with adjacent domains of proteoglycan depletion and increased staining observed
Grade 4	Cartilage **erosion**	Delamination and excavation: Delamination involves the loss of a superficial zone fragment related to the action of shear forces; Excavation represents cavity formation related to matrix loss in a circumscribed cartilage volume
Grade 5	**Denudation**	Complete erosion of hyaline cartilage to level of mineralized cartilage and/or bone, whether or not the bone surface is accompanied by fibro cartilaginous repair
Grade 6	**Deformation**	Change in the contour of the articular surface; this result not only from articular plate fractures, but also from increased metabolic activity of the articular bone plate, as well as from activation of connective tissue at the lateral and sometimes, central cartilage/bone interfaces

OA, Osteoarthritis; OARSI, Osteoarthritis Research Society International
* Table was adapted from Pritzker *et al.*, (2006)

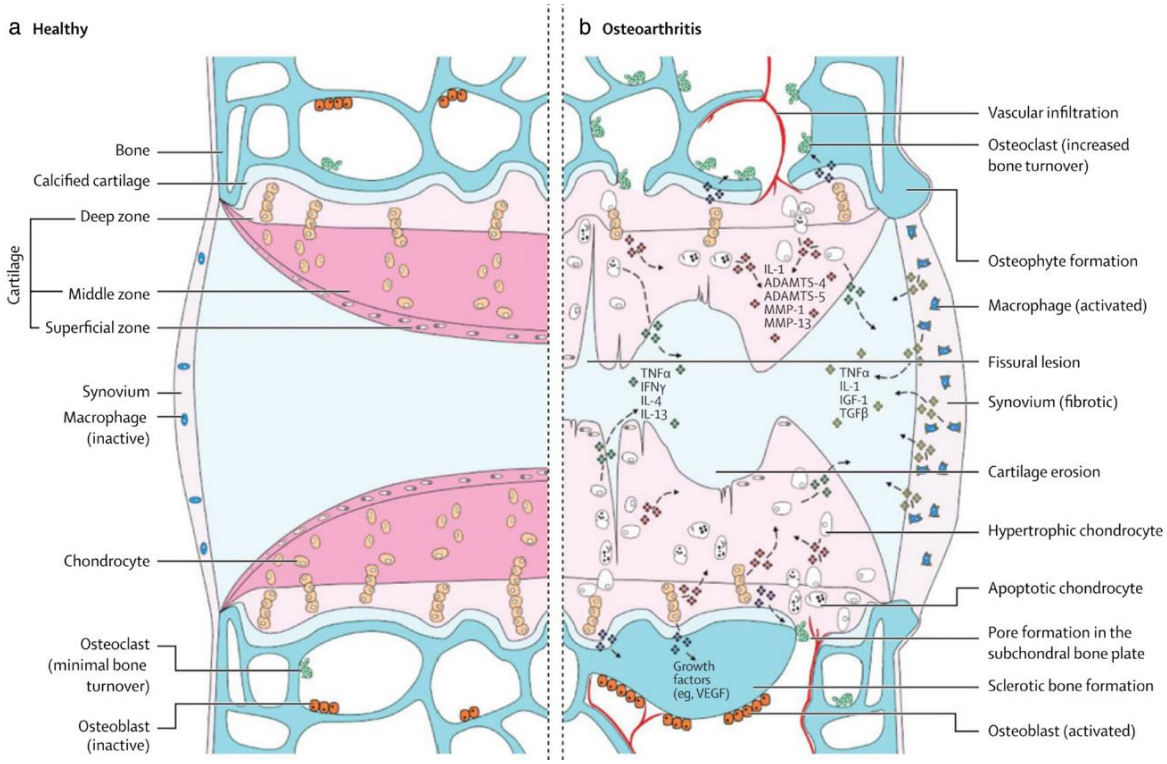
Appendix 16. Osteoarthritis management involving non-pharmacological, pharmacological, and surgical treatment options*

Treatment type	Component		Adverse effects/safety profile
Non-pharmacological	Education, exercise, injury prevention, weight loss, orthotic devices		
Pharmacological	Oral	Paracetamol	
		NSAIDS (co-therapy with proton pump inhibitor), COX-2 inhibitors	GI ulcer or bleeding, cardiovascular events, renal toxicity, worsening asthma
		Opioids	Constipation, vomiting, somnolence, increased risk of fracture, morbidity and mortality in the elderly, possible addiction
		Duloxetine	Constipation, nauseas, hyperhidrosis, dry mouth, fatigue
	Topical	Topical NSAIDS	Skin reactions, GI events
		Capsaicin	Skin burning sensation, long term skin desensitization
	Injectable	Intra-articular corticosteroids	Local infection, systemic effects, chondral degeneration
		Intra-articular hyaluronic acid or visco-supplementation	Local reactions at the site of injection, swelling, flares of pain
Surgical	Cartilage repair, osteotomy with axis correction, arthroplasty (UKA, TKR, THR)		Postoperative pain, infection, deep vein thrombosis, death

GI, gastrointestinal; NSAID, nonsteroidal anti-inflammatory drug, THR, total hip replacement; TKR, total knee replacement; UKA, unicondylar knee arthroplasty

* Table was adapted from Roubille *et al.*, (2013)

Appendix 17. Pathogenesis of osteoarthritis (Glyn-Jones *et al.*, 2015)



Appendix 18. The kind of seaweeds for IBD and OA treatment

Osteoarthritis			
Actinotrichia fragilis, red algae	Dried powder and gel	Female Wistar albino OA rats	(Sayed *et al.*, 2016)
Fucus vesiculosus, brown seaweed	Fucoidan	Knee and hip osteoarthritis in human	(Myers *et al.*, 2016)
Hizikia fusiforme, brown seaweed	Fucoidan	Osteoarthritis in rats	(Lee *et al.*, 2015)
Caulerpa cupressoides, green seaweed	Lectin	Rat temporomandibular joint during zymosan-induced arthritis	(Rivanor *et al.*, 2014)
Maritech® seaweed extract	Fucoidan	Osteoarthritis, human	(Myers *et al.*, 2010)
Fucus vesiculosis (85% w/w), *Macrocystis pyrifera* (10% w/w), *Laminaria japonica* (5% w/w)			
Fucus vesiculosus, brown algae	Fucoidan	Zymosan-induced arthritis, Male Wistar rats	(Cardoso *et al.*, 2009)
Seaweed	Natural seaweed derived mineral supplement, (Aquamin F)	Knee osteoarthritis, human	(Frestedt *et al.*, 2009)

FIGURES

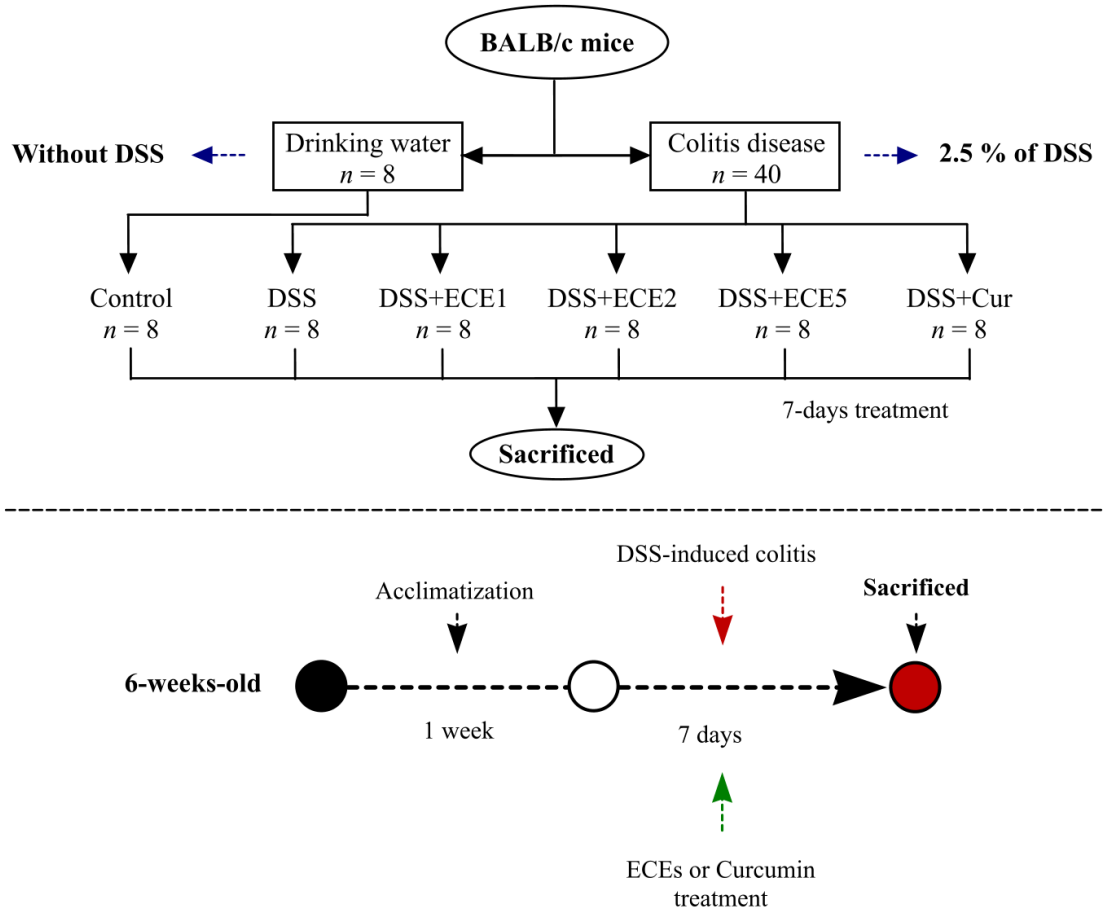


Figure 1. The flowchart of DSS-induced colitis disease in mice. Cur, curcumin; DSS, dextran sulfate sodium; ECE, *Eucheuma cottonii* extract; ECE1, 0.35 g/kg body weight; ECE2, 0.70 g/kg; ECE5, 1.75 g/kg; *n*, the number of mice per group.

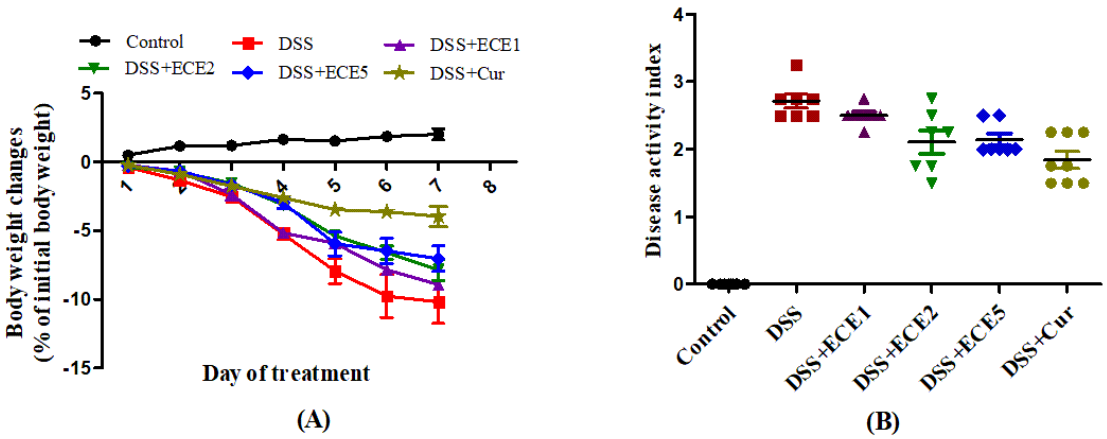


Figure 2. Effects of ECE on (A) body weight change and (B) disease activity index in DSS-treated mice during and after 7 days of treatment. Data are shown as the mean ± S.D. (n = 8). Cur, curcumin; DSS, dextran sulfate sodium; ECE, *Eucheuma cottonii* extract.

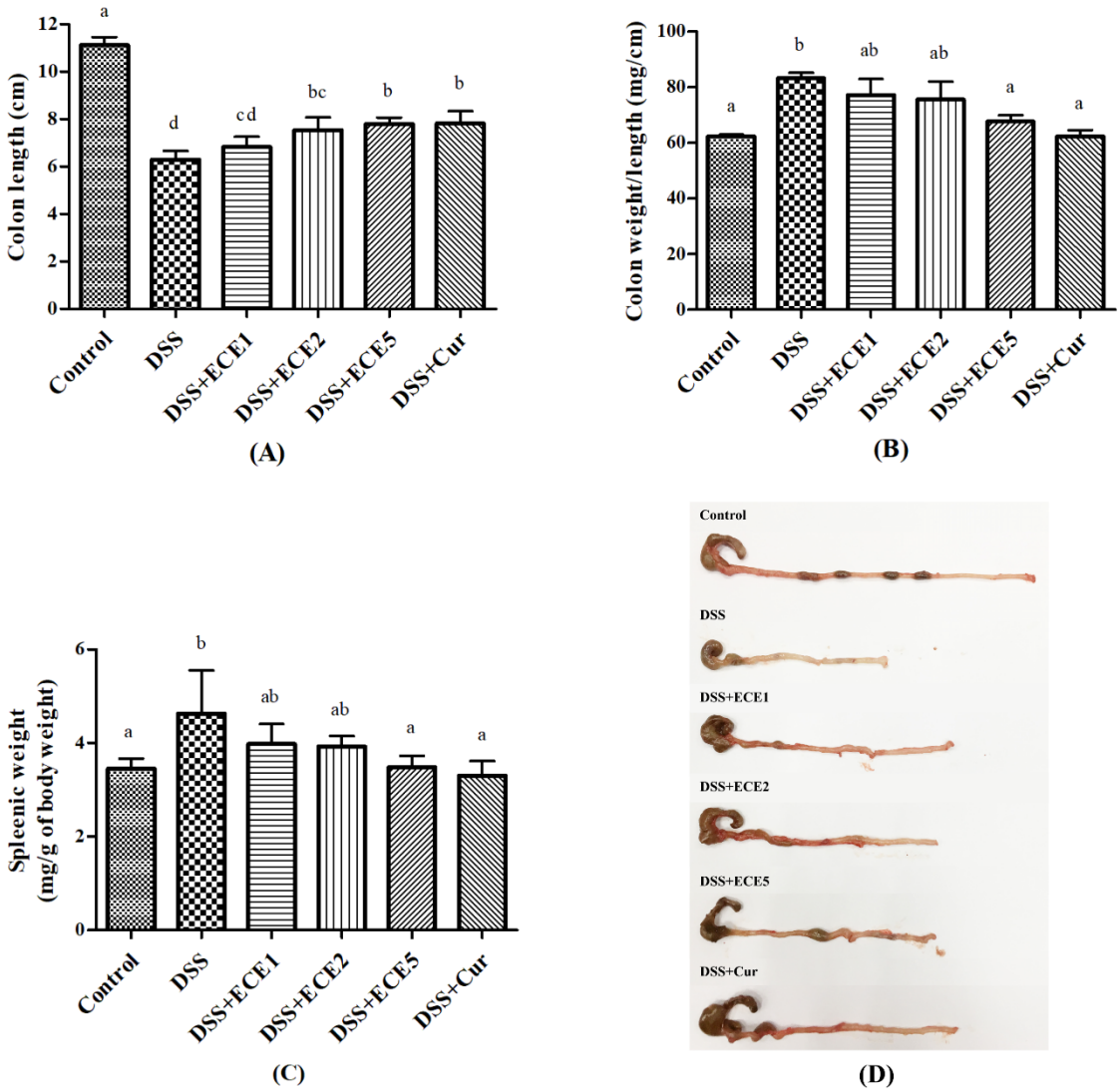


Figure 3. Effects of ECE treatment on colon health after 7 days of treatment. (A) colon length; **(B)** colon weight/length ratio; **(C)** splenic weight, **(D)** representative colons for each group. Data are shown as the mean ± S.D. (n = 8). Letters (a-d) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. Cur, curcumin; DSS, dextran sulfate sodium; ECE, *Eucheuma cottonii* extract.

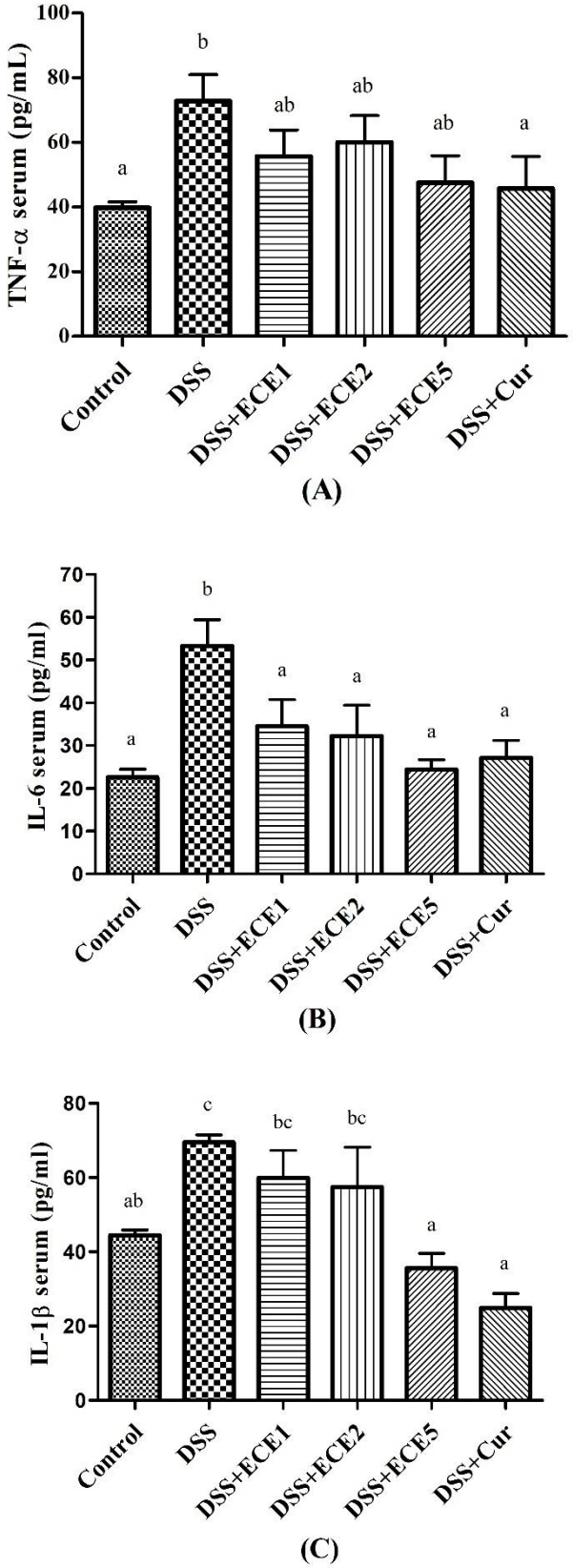


Figure 4. Effects of ECE on inflammatory cytokine expression in serum after 7 days of treatment. (A) TNF-α, (b) IL-6, and (c) IL-1β. Data are shown as the mean ± S.D. (n = 8). Letters (a-c) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. Cur, curcumin; DSS, dextran sulfate sodium; ECE, *Eucheuma cottonii* extract.

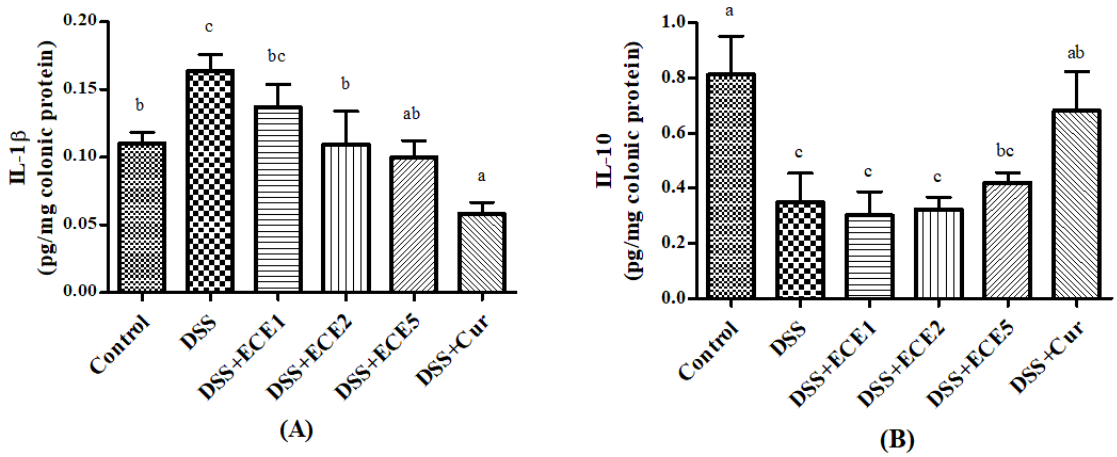


Figure 5. Effects of ECE on inflammatory cytokine expression in colon tissue after 7 days of treatment. (**A**) IL-1β and (**B**) IL-10. Data are shown as the mean ± S.D. (n = 8). Letters (a-c) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. Cur, curcumin; DSS, dextran sulfate sodium; ECE, *Eucheuma cottonii* extract.

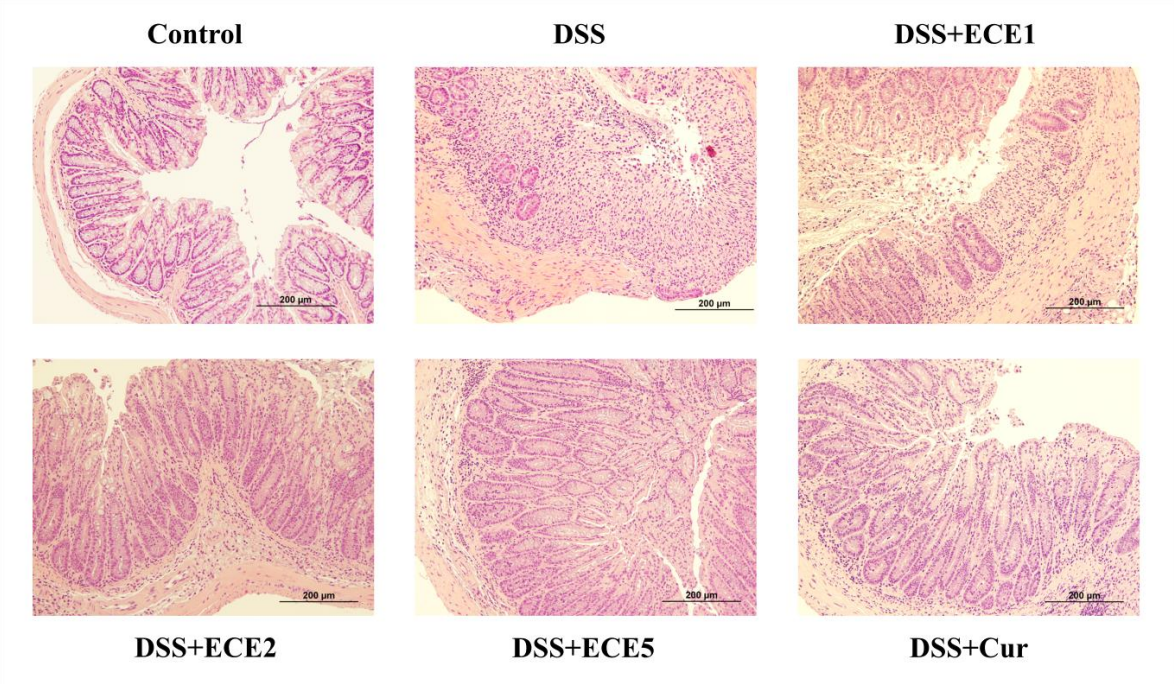


Figure 6. Representative colonic tissue histopathology for each group after 7 days of treatment. Cur, curcumin; DSS, dextran sodium sulfate; ECE, *Eucheuma cottonii* extract; ECE1, 0.35 g/kg body weight; ECE2, 0.70 g/kg; ECE5, 1.75 g/kg.

FIGURES

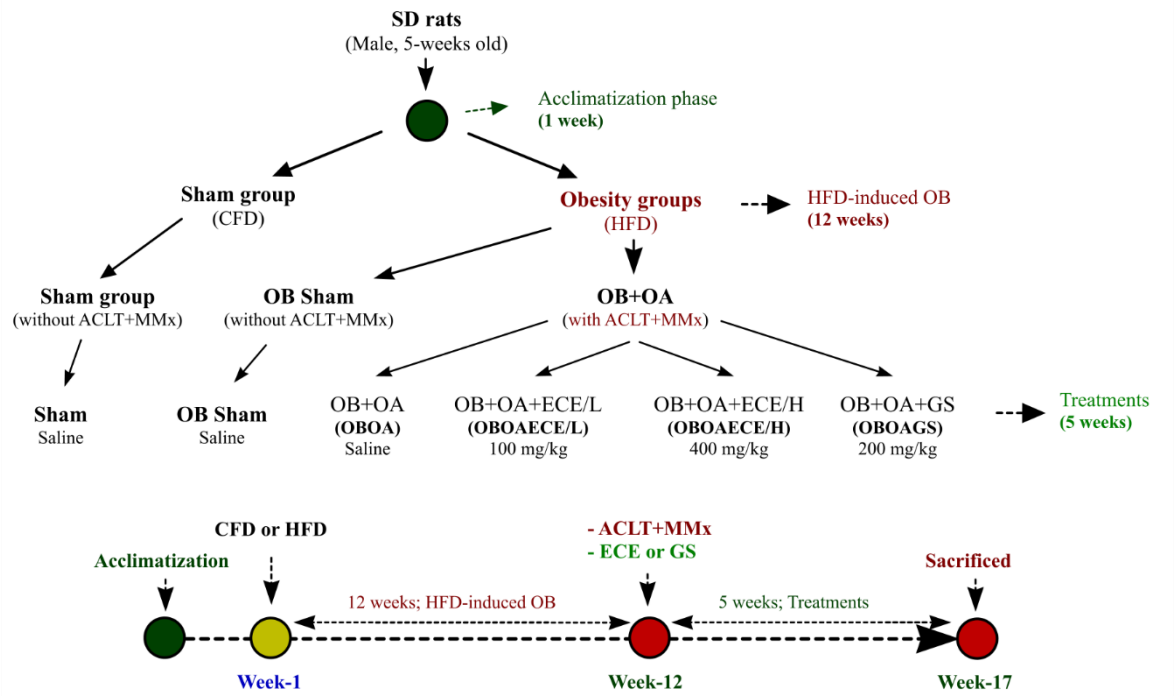


Figure 7. The flowchart of an anterior cruciate ligament transection and a partial medial meniscectomy in high-fat diet-induced OA rats. ACLT, an anterior cruciate ligament transection; CFD, standard chow-fed diet; MMx, a partial medial meniscectomy; ECE, *Eucheuma cottonii* extract; ECE/L, low-dose of ECE; ECE/H, high-dose of ECE; GS, glucosamine sulfate; HFD, high-fat diet; OB, obesity; OA, osteoarthritis; SD, Sprague-Dawley.

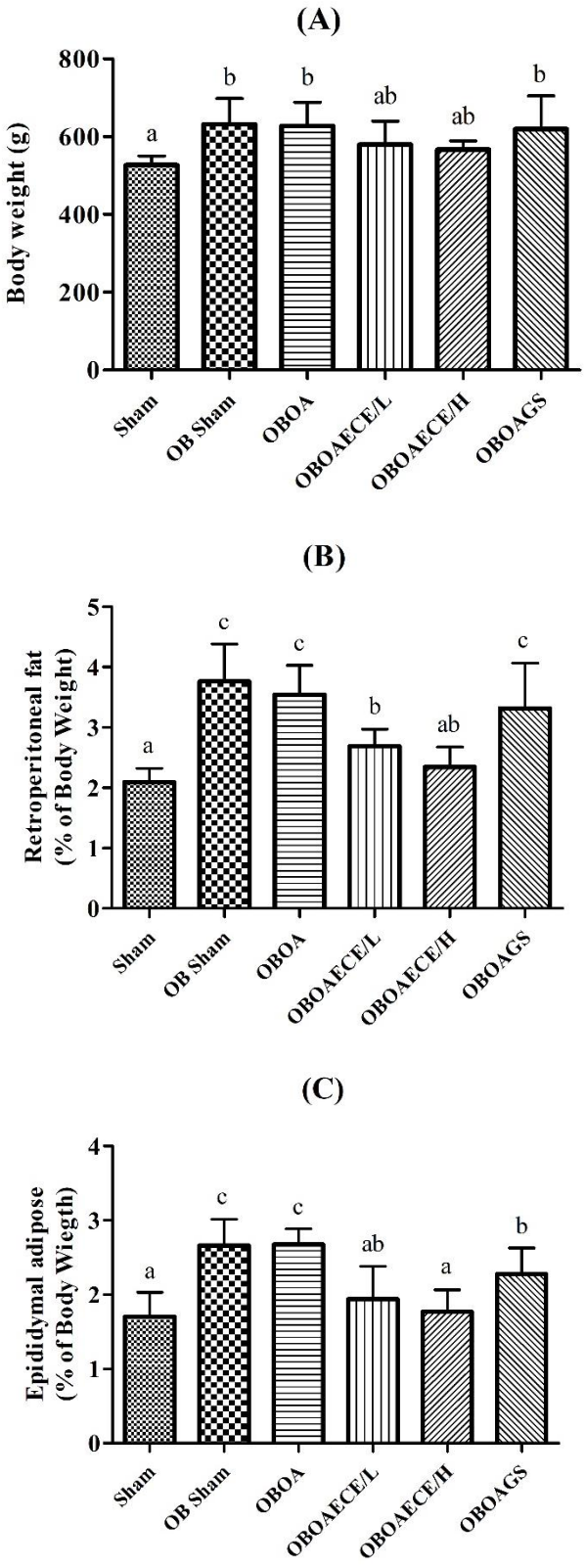


Figure 8. Effects of ECE and GS on (A) body weight, (B) retroperitoneal fat, and (C) epididymal adipose tissue of rats after treatment for 5 weeks. Data are shown as the mean ± S.E.M. (n = 7). Letters (a-c) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. ECE, *Eucheuma cottonii* extract; ECE/L, low-dose of ECE; ECE/H, high-dose of ECE; GS, glucosamine sulfate; OB, Obesity; OA, Osteoarthritis.

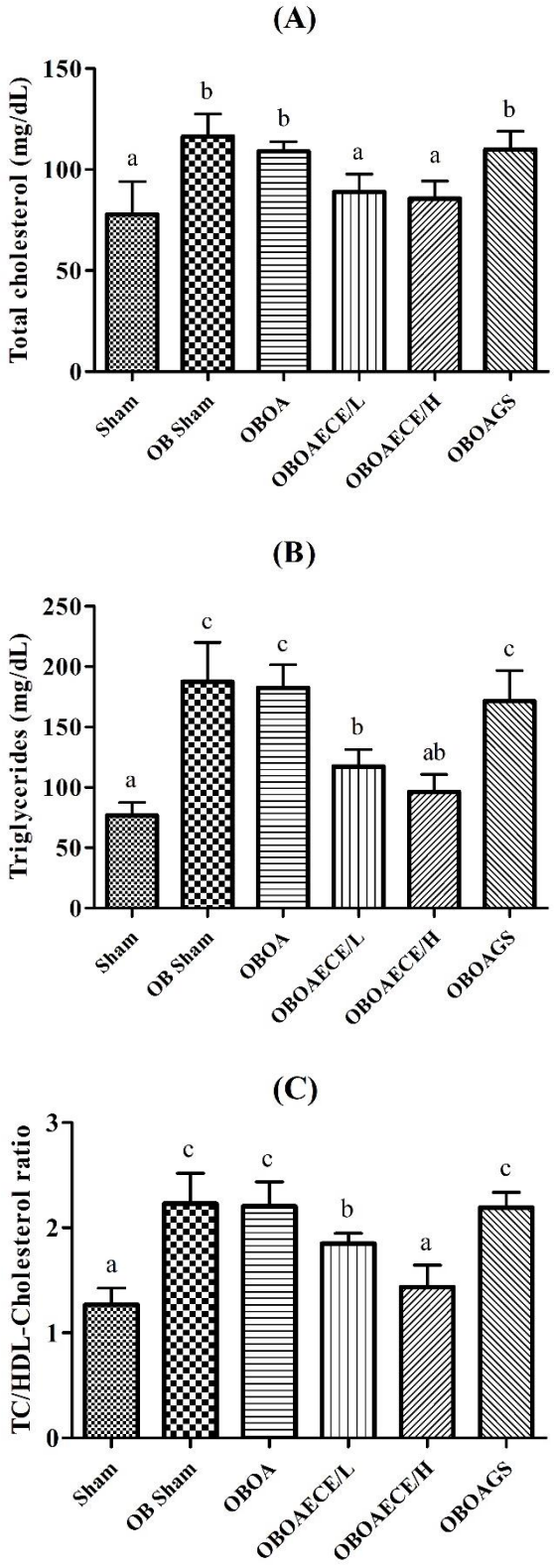


Figure 9. Effects of ECE and GS on (A) total cholesterol (TC), (B) triglycerides (TG), and (C) TC/HDL-cholesterol ratio of rats' serum after treatment for 5 weeks. Data are shown as the mean ± S.E.M. (n = 7). Letters (a-c) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. ECE, *Eucheuma cottonii* extract; ECE/L, low-dose of ECE; ECE/H, high-dose of ECE; GS, glucosamine sulfate; HDL-C, high-density lipoprotein-cholesterol; OB, Obesity; OA, Osteoarthritis.

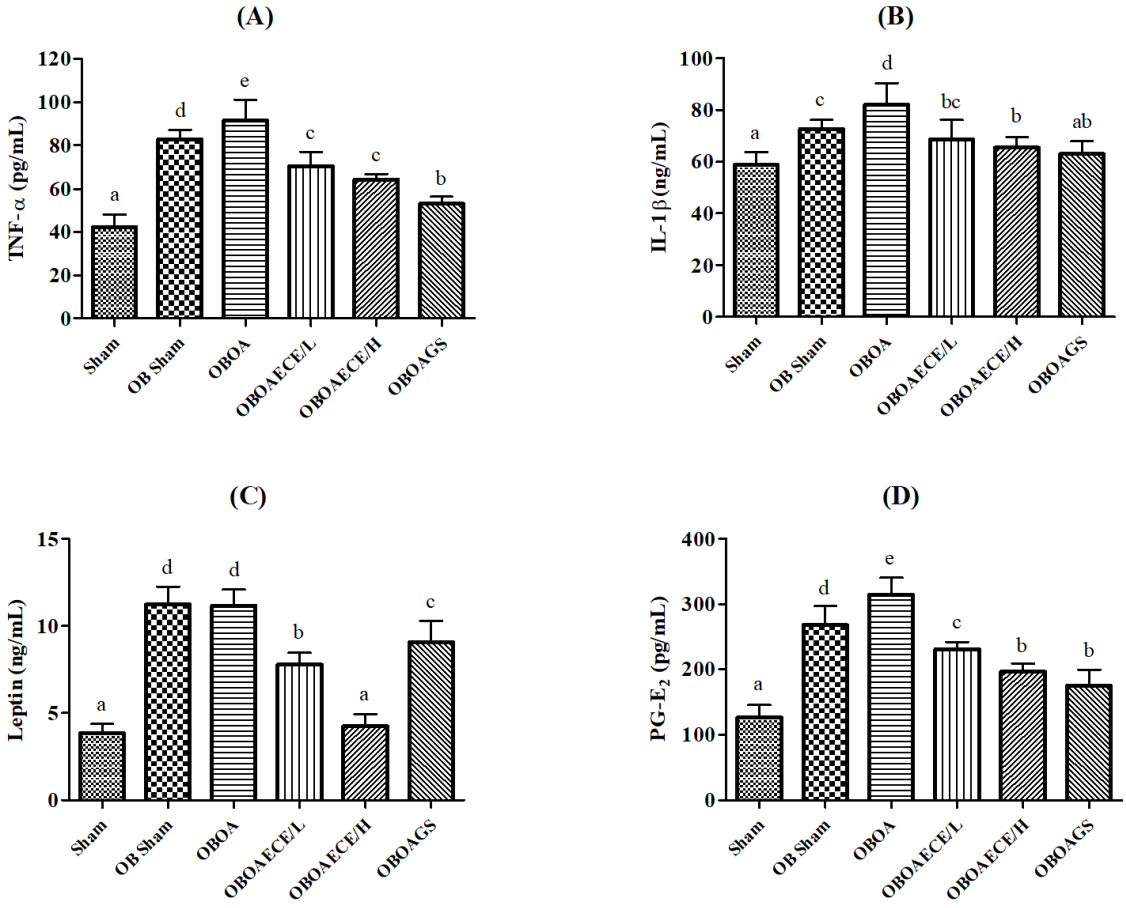


Figure 10. Effects of ECE and GS on (A) tumor necrosis factor (TNF)-α, interleukin (IL)-1β, leptin, and prostaglandin (PG)-E_2 of rats' plasma after treatment for 5 weeks. Data are shown as the mean ± S.E.M. (n = 7). Letters (a-e) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. ECE, *Eucheuma cottonii* extract; ECE/L, low-dose of ECE; ECE/H, high-dose of ECE; GS, glucosamine sulfate; OB, obesity; OA, osteoarthritis.

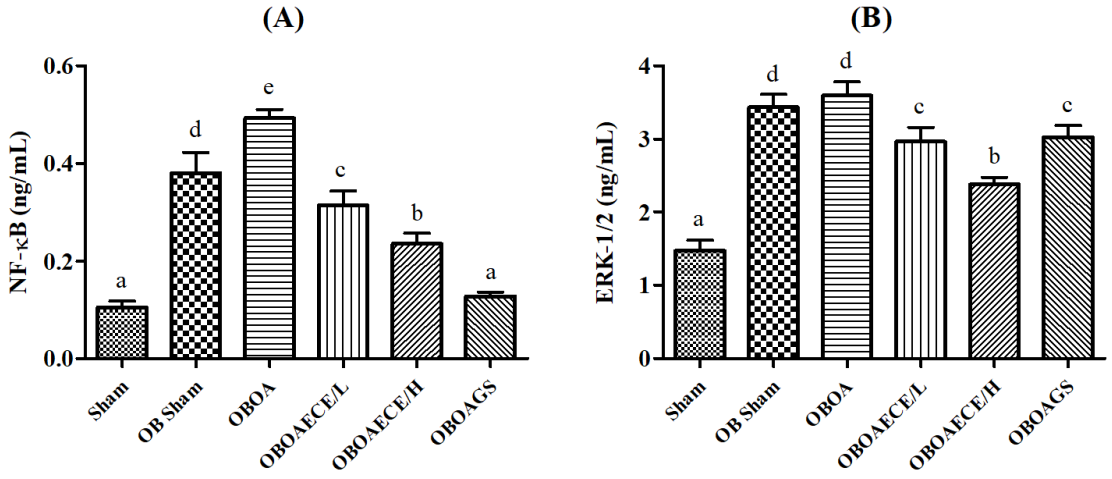


Figure 11. Effect of ECE and GS on (A) nuclear factor-kappa B (NF-κB) and (B) extracellular signal-regulated kinase (ERK)-1/2 expression of rats' plasma after treatment for 5 weeks. Data are shown as the mean ± S.E.M. (n = 7). Letters (a-e) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. ECE, *Eucheuma cottonii* extract; ECE/L, low-dose of ECE; ECE/H, high-dose of ECE; GS, glucosamine sulfate; OB, obesity; OA, osteoarthritis.

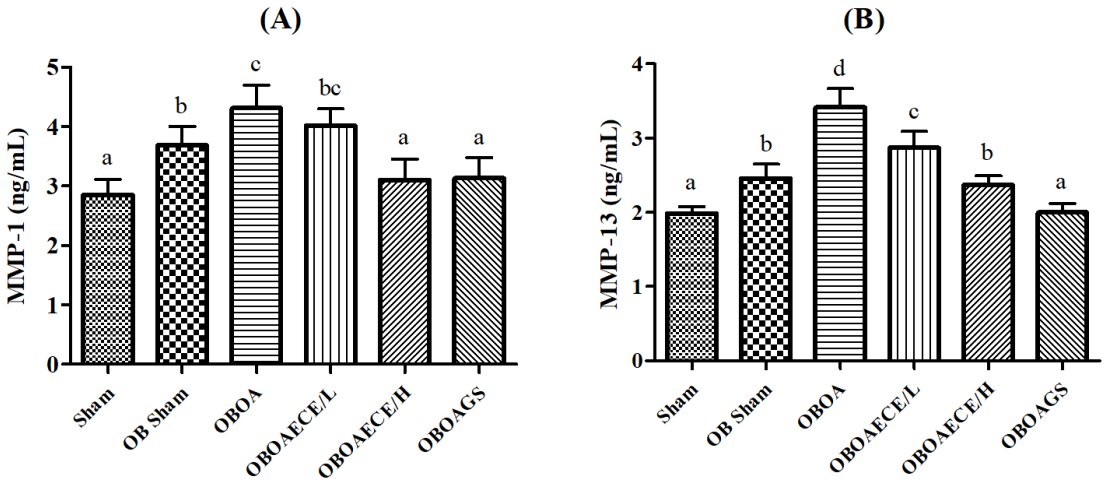


Figure 12. Effect of ECE and GS on (A) matrix metalloproteinase (MMP)-1 and (B) MMP-13 level of rats' plasma after treatment for 5 weeks. Data are shown as the mean ± S.E.M. (n = 7). Letters (a-d) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. ECE, *Eucheuma cottonii* extract; ECE/L, low-dose of ECE; ECE/H, high-dose of ECE; GS, glucosamine sulfate; OB, obesity; OA, osteoarthritis.

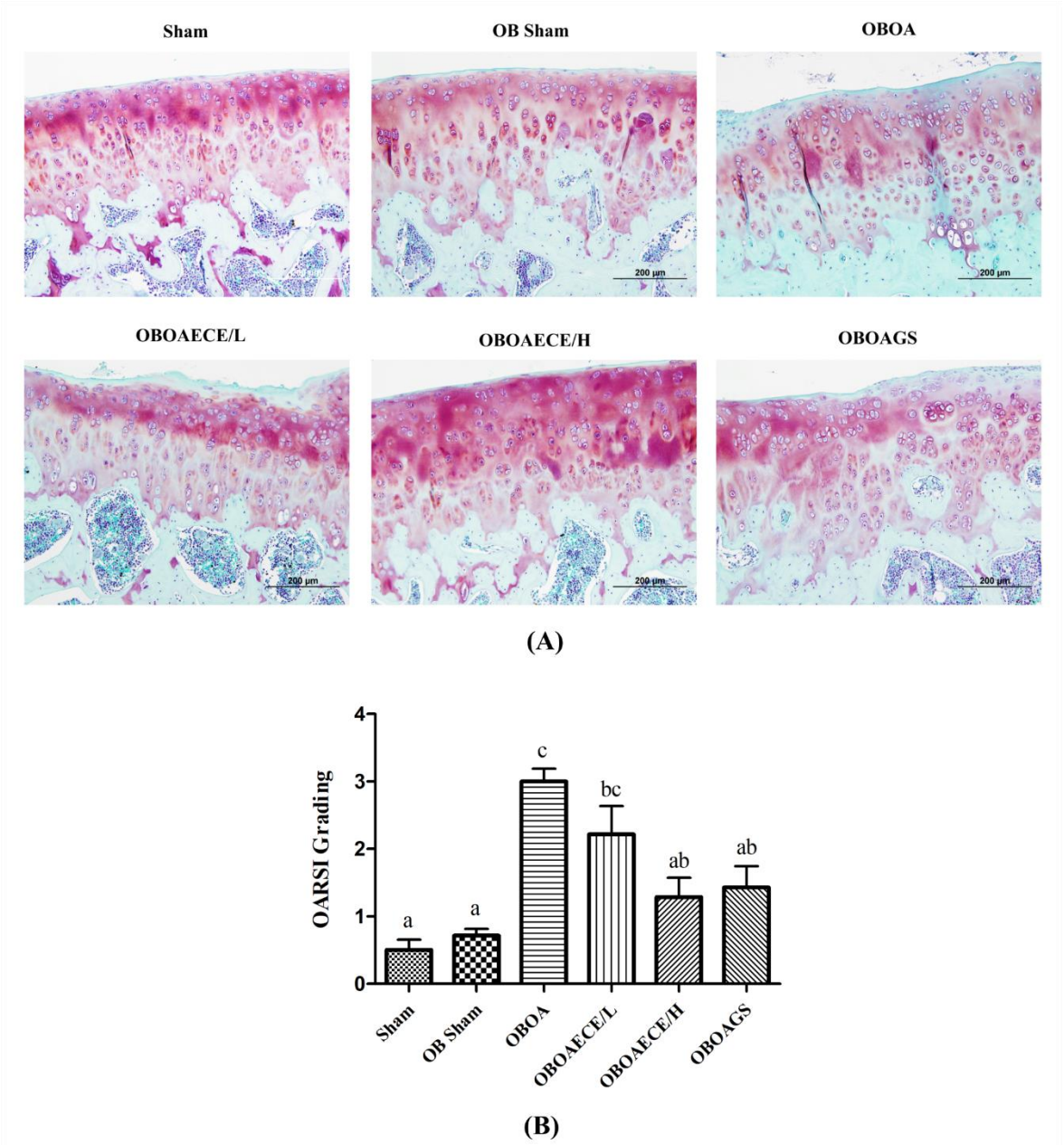


Figure 13. Knee joint histopathology. (A) Representative Safranin-O staining for each group. (B) OARSI grading. Data are shown as the mean ± S.E.M. (n = 7). Letters (a-c) indicate statistically significance (p<0.05) as analyzed by one-way ANOVA with Duncan's multiple range test. ECE, *Eucheuma cottonii* extract; ECE/L, low-dose of ECE; ECE/H, high-dose of ECE; GS, glucosamine sulfate; OB, obesity; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International.