Effects of *Rhodomyrtus tomentosa* (Aiton) Hassk. Leaf Extract on Human Neutrophils and Pathogens

Jutharat Hmoteh

A Thesis Submitted in Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Microbiology
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Rhodomyrtus tomentosa (Aiton) Hassk is the source of an ethanolic extract. The principal compounds of these products are bioactive substances extracted from the plant that are known to possess antioxidant, antibacterial, anti-inflammatory, and immunomodulatory properties.

Enterohemorrhagic *Escherichia coli* (*E. coli*) O157:H7 is one of the most virulent causative agents of foodborne disease. Use of antibiotics in the treatment of *E. coli* O157:H7 infection leads to hemolytic uremic syndrome. The present study evaluated the potential of the ethanolic leaf extract of the medicinal plant, *Rhodomyrtus tomentosa* extract to enhance the killing activity of human neutrophils against *E. coli* O157:H7. In addition, the effects of the extract on the membrane permeability of the organism were studied. The percentage survival of the bacterial cells after exposure to human neutrophils, in the presence of various concentrations of the extract, was determined by a killing assay. At 45 min, after exposure to neutrophils in the presence of the extract at concentrations of 125 to 250 µg/mL, the percentage survivals of *E. coli* O157:H7 and *E. coli* ATCC 25922 were 58.48% to 50.28% and 69.13% to 35.35%, respectively. Furthermore, upon treatment with *R. tomentosa* at 250 µg/mL, the uptake of crystal violet by *E. coli* O157:H7 and *E. coli* ATCC 25922 increased to 40.07% and 36.16%, respectively.

Oral candidiasis has become a major problem due to the increasing resistance of *Candida albicans* to antibiotics. *Rhodomyrtus tomentosa*, a medicinal plant possessing several phytochemical constituents, has been considered as a

potential source of antimicrobial and immunomodulatory agents. The aim of this study was to investigate the anti-virulence and immunostimulatory activities of *R. tomentosa* ethanolic leaf extract against *C. albicans*. The effects of the extract on *C. albicans* were assessed on germ tube production, adherence of the organisms to surfaces, biofilm formation, and mature biofilm. In addition, the effects of the extract on phagocytosis and the killing activity of neutrophils against the pathogen were investigated. Suppression of germ tube production following exposure to the extract at 256 μg/mL significantly increased in comparison with that of the unexposed cells ($p < 0.05$). The pathogens demonstrated a significantly reduced ability to adhere to surfaces in a dose dependent manner, compared with the control ($p < 0.05$). At 48 h, the extract at 512 to 1024 μg/mL had reduced the ability of the organism to form biofilm by up to 42.31 to 64.58% ($p < 0.05$). Compared with the control group, there was a significantly increased inhibition of mature biofilm after treatment with the extract at 256 μg/mL ($p < 0.05$). The extract at 50 μg/mL significantly enhanced phagocytosis and the killing activity of neutrophils against the organism, compared with the control ($p < 0.05$).

R. tomentosa extract displayed a specific mode of action as a membrane permeabilizing agent, inhibiting the virulence factors of *C. albicans*, and enhancing the killing activity of neutrophils against the organism. Further development of the extract as an alternative therapy for oral candidiasis might be useful.

ชื่อวิทยานิพนธ์ ผลของสารสกัดหยาบจากกระทุต่อเม็ดเลือดขาวชนิด neutrophil ของคนและเชื้อก่อโรค

ผู้เขียน นางสาวจุฑารัตน์ หมอเต๊ะ

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บทคัดย่อ

สาสกัดหยาบที่สกัดได้จากใบพืชกระทุ (*Rhodomyrtus tomentosa*) นั้นมีฤทธิ์ทางชีวภาพ โดยเป็นสารต้านอนุมูลอิสระ มีฤทธิ์ดีในการต้านแบคทีเรียชนิดแกรมบวก ฤทธิ์ต้านการอักเสบ รวมไปถึงการออกฤทธิ์ปรับระบบภูมิคุ้มกัน

Enterohaemorrhagic *Escherichia coli* (*E. coli*) O157:H7 เป็นเชื้อแบคทีเรียที่มีความสำคัญในการก่อโรคอาหารเป็นพิษ แต่การใช้ยาปฏิชีวนะ เพื่อรักษาจากการติดเชื้อจากแบคทีเรียชนิดนี้ มีรายงานพบว่าทำให้เกิด Hemolytic uremic syndrome เป็นโรคซึ่งทำให้มีการแตกของเม็ดเลือดแดง ไตวายเฉียบพลัน ทำให้มีของเสียคั่งในเลือดรวมไปถึงทำให้เกล็ดเลือดต่ำ ส่วนใหญ่พบในเด็ก การศึกษาในครั้งนี้จึงให้ความสนใจในการใช้สารกัดจาก *Rhodomyrtus tomentosa* นั้นมาทดสอบเพื่อหลีกเลี่ยงปัญหาดังกล่าวข้างต้น การศึกษาในครั้งนี้เป็นการศึกษาประสิทธิภาพของสารสกัดหยาบต่อการจับกินแบคทีเรียชนิด *E. coli* O157:H7 ของเม็ดเลือดขาวชนิด neutrophil ของคน และพบว่าสารสกัดหยาบจากพืชชนิดนี้สามารถไปเพิ่มศักยภาพในการทำงานของเม็ดเลือดขาวได้ โดยการทดสอบที่มีสารสกัดที่ความเข้มข้น 125-250 µg/mL และมี neutrophil นั้น แบคทีเรียชนิดนี้ได้ลดลง 58.48%-50.28% ตามลำดับ สารสกัดใบกระทุสามารถกระตุ้นระบบภูมิคุ้มกันชนิดเม็ดเลือดขาวโดยเหนี่ยวนำให้เกิดการเพิ่มการแสดงออกในการจับกินของ neutrophil รวมไปถึงการศึกษาผลของสารสกัดต่อคุณสมบัติของการยอมให้สารผ่านเข้าเซลล์ พบว่าสารสกัดมีผลให้สารจากภายนอกยอมผ่านเข้าสู่เซลล์ได้มากขึ้น โดยทดสอบสารสกัดที่ความเข้มข้น 250 µg/mL กับ *Escherichia coli* พบว่าสี crystal violet สามารถผ่านเข้าไปได้สูงถึง 40.07%

Candida albicans เป็นเชื้อราที่เป็นสาเหตุของการติดเชื้อฉวยโอกาสในคนที่มีภูมิต้านทานบกพร่อง เมื่อมีการติดเชื้อราชนิดนี้ทำให้เกิดการรักษายากขึ้นเนื่องจากชักนำให้เกิดการดื้อ

ต่อยาปฏิชีวนะ จึงมีความจำเป็นในการหาทางเลือกใหม่ที่ช่วยแก้ปัญหาข้างต้น สำหรับพยาธิกำเนิดกับความรุนแรงของโรคติดเชื้อรา พบว่ามีความสัมพันธ์กับ 2 ปัจจัย คือ ตัวเชื้อรา โดยเฉพาะ virulence factors และระบบภูมิคุ้มกันของโฮสต์ดังนั้นการใช้พืชก็เป็นทางเลือกที่น่าสนใจที่นำมาใช้ในการแก้ปัญหานี้ โดยการนำสารสกัดจากใบพืช *Rhodomyrtus tomentosa* มาใช้ในการศึกษาครั้งนี้เช่นกัน โดยศึกษาสารสกัดต่อ virulence factors ของจุลชีพชนิด คือ ความสามารถในการสร้าง germ tube การเกาะติดบนพื้นผิว รวมไปถึงการก่อตัวเป็นไบโอฟิล์ม พบว่า ความเข้มข้นของสารสกัดชนิดนี้ลดการสร้าง germ tube ของ *C. albicans* ที่ความเข้มข้นสูงสุดเพียง 256 μg/mL และยังมีผลต่อการลดการเกาะติดบนพื้นผิวได้อย่างมีนัยสำคัญเมื่อเปรียบเทียบกับที่ไม่มีสารสกัด ($p< 0.05$) เมื่อใช้สารนี้ที่ระดับความเข้มข้น 512-1024 μg/mL ทดสอบการเชื้อราที่เวลา 48 ชั่วโมง นั้นพบว่าสามารถลดการก่อตัวของไบโอฟิล์มได้ถึง 42.31-64.58% รวมไปถึงความสามารถในยับยั้งการเจริญเติบโตต่อไบโอฟิล์มที่สร้างแล้วทำให้ปริมาณไบโอฟิล์มลดลงอย่างเห็นได้ชัดเมื่อเปรียบเทียบกับกลุ่มที่ไม่ใช้สารสกัดอย่างมีนัยสำคัญ ($p< 0.05$) สำหรับปัจจัยที่สองที่ทำให้เกิดการติดเชื้อชนิดนี้คือระบบภูมิคุ้มกันของมนุษย์เอง จึงทำการศึกษาประสิทธิภาพของสารสกัดใบกระทุในการกระตุ้นระบบภูมิคุ้มกันแต่กำเนิดคือ เม็ดเลือดขาวชนิด neutrophil โดยดูความสามารถในการจับกินและฆ่าจุลชีพชนิดนี้ พบว่าสารสกัดที่ความเข้มข้น 50 μg/mL สามารถกระตุ้นให้เม็ดเลือดขาว ทำงานได้มีประสิทธิภาพมากขึ้น

ประสิทธิภาพของสารสกัดใบกระทุมีหลายอย่าง คือการกระตุ้นภูมิคุ้มกันชนิดที่มีมาแต่กำเนิด คือ เม็ดเลือดขาวชนิด neutrophil ลด virulence factor ของ *C. albicans* รวมไปถึงทำให้เซลล์จุลชีพยอมให้สารผ่านเข้าเซลล์ได้มากขึ้น ซึ่งผลทดสอบจากงานวิจัยนี้จะเป็นองค์ความรู้พื้นฐานในการพัฒนาสารสกัดใบกระทุเป็นผลิตภัณฑ์ที่สามารถนำไปใช้ในการป้องกันและรักษาโรคในคนที่ก่อโรคจากจุลชีพข้างต้น ส่งเสริมให้ใช้ผลิตภัณฑ์จากธรรมชาติเพื่อการหลีกเลี่ยงการใช้สารเคมีและยาปฏิชีวนะ

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Jutharat Hmoteh

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CHAPTER 1

INTRODUCTION

Background and Rationale

The epidemiology of infectious microorganisms has increased significantly, leading to morbidity and death. The ability of many organisms to become resistant to therapeutic agents calls for novel approaches to the treatment of some infectious diseases. Recently, natural products have been identified as effective alternatives to existing therapies. According to the World Health Organization, medicinal plants are considered rich sources of active constituents which can be used as alternative drugs.

The number of antimicrobial agents available at present is very small, production is much more difficult, many side-effects are noted, and the appearance of resistance is possible, as has happened with antibiotics in the treatment of bacterial infections. Plant materials used in traditional medicines are valuable sources of antimicrobial agents and the use of plant extracts and/or products that specifically target virulence factors could be used as a new alternative to conventional antifungal therapy (Gauwerky, *et al.*, 2009). The medicinal plant *Rhodomyrtus tomentosa* (Aiton) Hassk. has been used for various treatments including diarrhoea (Ong & Nordiana, 1999), gastroprotective strategies (Geetha, *et al.*, 2010, Geetha, *et al.*, 2010), and urinary tract infections (Wei, 2006). Moreover, the extract and its derived compound, rhodomyrtone, possess antibacterial, (Limsuwan, *et al.*, 2009, Voravuthikunchai, *et al.*, 2010, Saising, *et al.*, 2011), antioxidant (Lavanya, *et al.*, 2012), and immunomodulatory properties (Srisuwan, *et al.*, 2014, Hmoteh, *et al.*, 2016). Although a previous study reported that *R. tomentosa* extract exhibits antifungal activity against *C. albicans* (Limsuwan, *et al.*, 2014), the aim of this work was to investigate the immunomodulatory effect of *Rhodomyrtus tomentosa* leaf extract against major pathogens, including *Escherichia coli* O157:H7 and *Candida albicans*.

Enterohaemorrhagic *Escherichia coli* O157:H7 is a Gram-negative human pathogen that causes bloody diarrhea as well as severe abdominal cramps in infected patients. Moreover, it produces a Shiga-like toxin that could lead to haemorrhagic colitis in humans (Griffin & Tauxe, 1991, Nguyen & Sperandio, 2012). Although conventional antimicrobial treatment is generally prescribed for infections caused by various pathogenic bacteria, the use of antibiotics to treat enterohaemorrhagic *E. coli* O157:H7 infection is not generally recommended due to an increased risk of haemolytic uremic syndrome (Smith, *et al.*, 2012). Therefore, suitable alternative measures for the treatment of infections caused by *E. coli* O157:H7 are urgently required (Lim, *et al.*, 2010).

Literature Reviews

1. Introduction of *Escherichia coli* O157:H7

1.1 Enterohaemorrhagic *Escherichia coli* O157:H7

Enterohaemorrhagic *Escherichia coli* O157:H7, facultative anaerobic Gram-negative rods which produce Shiga toxins (Stxs) causes diarrhea, haemorrhagic colitis, and haemolytic uremic syndrome (HUS). The principal habitat of the organism is the bovine intestinal tract and the pathogen lives within the gastrointestinal tract. Pathogenic *E. coli* can be classified by serogroups, mechanisms of bacterial pathogenicity, and clinical symptoms (Nataro & Kaper, 1998, Kaper, *et al.*, 2004). Currently, there is no specific antibiotic for Stx-mediated HUS. A number of studies have identified antimicrobial agents as an important risk factor which increased toxin production from the pathogen leading to the development of HUS.

Escherichia coli O157:H7 is the most important serotype of enterohaemorrhagic *E. coli* and a major cause of foodborne disease outbreaks in North America, and Japan (Michino, *et al.*, 1999, Kaper, *et al.*, 2004, Rangel, *et al.*, 2005). The illnesses caused by the pathogen have always been a serious threat to public health and the economy of nations. The organism can produce Shiga toxigenic *E. coli* (STEC) which is most commonly mentioned as verotoxin (VT) in relation to the outbreaks. Ruminant animals are reservoirs of pathogenic bacteria and the consumption of contaminated meat and dairy products from cattle plays a significant role in the dissemination of organisms that can cause disease in the human community (Pennington, 2010). The organism colonizes the gastrointestinal tracts of cattle, but the animals display no symptoms and can tolerate Shiga toxin since ruminants lack globotriaosylceramides, which are receptors of the Shiga toxins (Pruimboom-Brees, *et al.*, 2000). Infection can also spread to humans through *Acanthamoeba*, which harbours the pathogen and is present in contaminated water and soil (Chekabab, *et al.*, 2012).

1.2 Mechanisms of bacterial pathogenicity

1.2.1 Adherence factors

Viable pathogens must attach to intestinal host cells to prevent being removed during peristaltic flow. Bacteria may have fimbriae and fimbrial adhesins that enable them to adhere to human mucosal cells (Law, 2000). Several adhesive surface structures, whether fimbriated or non- fimbriated, contribute to colonization by adhesion to cell surfaces. Fimbriae and fimbrial adhesins are thin, adhesive, thread-like surface organelles. *Escherichia coli* type 1 fimbriae are protein polymers on the bacterial surface. The adhesins mediate the binding of the pathogen to specific glycoprotein receptors on the surfaces of eukaryotic cells containing mannose (Hedegaard & Klemm, 1989). Moreover, the microorganisms express curli, thin fimbrial structures that form the major proteinaceous extracellular fibers. These fibers contribute directly to surface and cell-cell contacts that promote bacterial adhesion to host cells (Barnhart & Chapman, 2006, Fronzes, *et al.*, 2008).

A major virulence factor in human pathogens that cause severe gastroenteritis is the locus of enterocyte effacement (LEE), which contains genes responsible for attaching and effacing lesions, and possesses a type III secretion system (TTSS). TTSS is composed of translocator and effector proteins that can be secreted from the bacterial cytosol into the host cell's cytoplasm during the early stages of infection (Ghosh, 2004). The pathogen induces the attaching and effacing of lesions by secreting translocators such as EspA, EspB, and EspD and effector proteins such as Tir, Map, Nles, EspF, EspG, Cif, and Orf3 (Coburn, *et al.*, 2007). r characterized by localized destruction of brush border microvilli in intestinal epithelial cells (Franzin & Sircili, 2015).

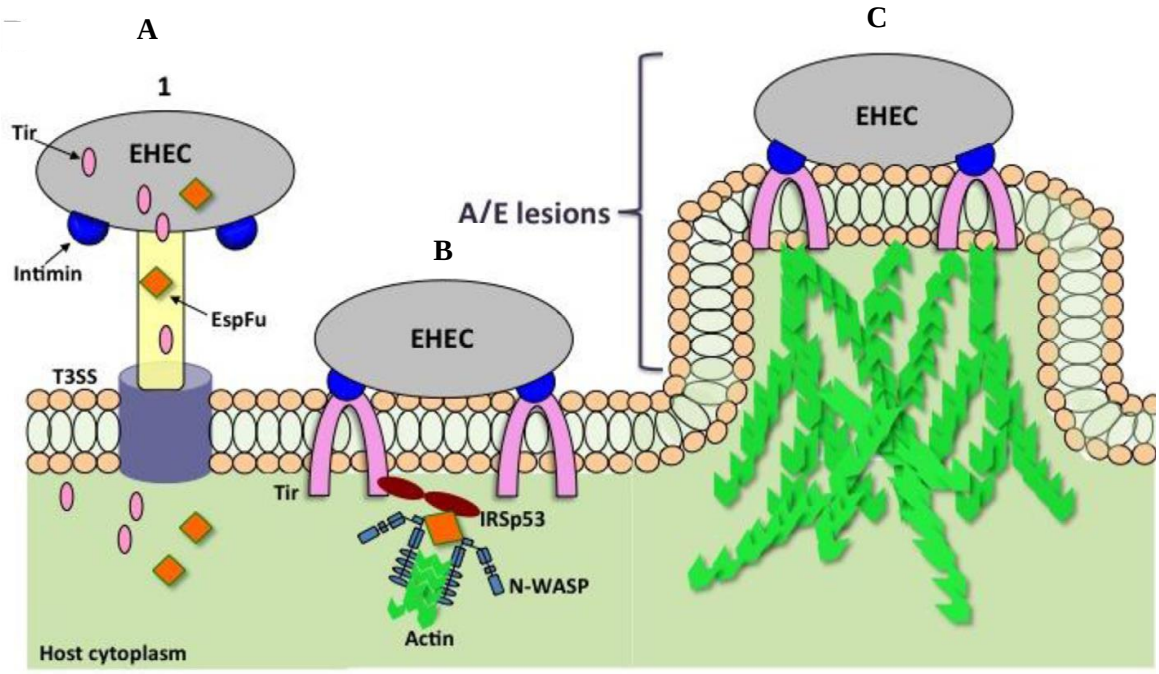


Figure 1. Schematic diagram showing formation of attaching and effacing lesions. *Escherichia coli* O157:H7 injects effector proteins such as Tir and EspFu into the host cytoplasm through the T3SS (A). Tir localizes to the host membrane and binds to intimin to intimately attach the bacteria to the cell. Tir and EspFu recruit host factors (B) to subvert host cytoskeleton and actin polymerization (C) (Nguyen & Sperandio, 2012).

1.2.2 Shiga-like toxin

Shiga toxin-producing *E. coli*, also known as a potent verocytotoxin-producing *E. coli*, have been associated with bloody diarrhea, haemorrhagic colitis, and haemolytic uremic syndrome. Moreover, the toxin is also found in *Shigella dysenteriae* serotype 1. Shiga toxin, one of the AB5 protein toxin family, can be divided into two different immunological groups, Shiga-like toxins 1 and 2 (Jacewicz, *et al.*, 1999). Verotoxins produced by the organism mainly destroy endothelial cells in small vessels of the intestine and kidney resulting in haemorrhagic colitis and haemolytic uraemic syndrome. The structures of all the verotoxins are composed of A and B subunits (O'Brien, *et al.*, 1992, Paton & Paton, 1998). The B subunit forms a

pentamer that binds to glycosphingolipid globotriaosylceramide 3 in host cell membranes and the pathophysiology is closely related to the structure (Lingwood, *et al.*, 1987, Tétaud, *et al.*, 2003). The catalytic subunit demonstrates RNA N-glycosidase activity which removes an adenine base from 28S RNA by inhibiting host protein synthesis and induces apoptosis (Endo, *et al.*, 1988, Sandvig, 2001, Karmali, *et al.*, 2010). The pathogen colonizes the human large intestine (Phillips, *et al.*, 2000). Shiga-like toxins released by the organism bind to globotriaosylceramide protein receptors on human endothelial cells and the absorbed toxins are then disseminated by blood vessels to other organs (Sandvig, 2001). Human tissue and cell types exhibit different levels of the specific protein receptor for the B subunit pentamer of the microorganism, for example renal glomerular endothelial cells possess high levels of globotriaosylceramide 3, and production of Shiga-like toxins results in haemolytic uraemic sysndrome including acute renal failure, thrombocytopenia, and microangiopathic haemolytic anemia (Karmali, *et al.*, 1983, Pruimboom-Brees, *et al.*, 2000).

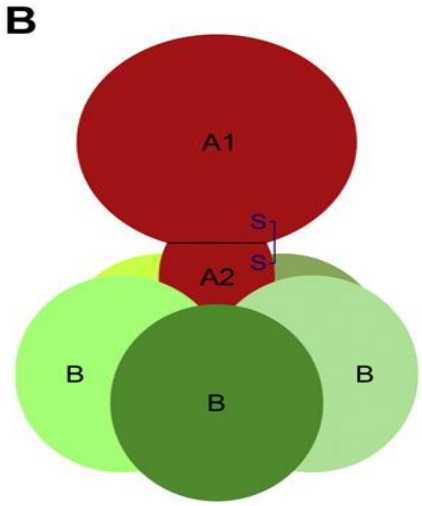


Figure 2. Schematic representation of the Shiga toxin structure (Bergan, *et al.*, 2012).

1.2.3 Plasmid O157 (pO157)

A plasmid is any DNA that is found in extrachromosomal elements of mammalian cells. Plasmid O157 or pO157, is an F-like, 92 to 104 kb plasmid (Nataro & Kaper, 1998). It is highly conserved and plays an important role in the pathogenesis of *E. coli* O157:H7. There are many proteins known to be involved in the pathogenesis of the infections including hemolysin, catalase-peroxidase , type II secretion system, serine protease , metalloprotease, putive adhesion, and eae gene-positive conserved fragment but the biological significance of pO157 in pathogenesis is not yet clearly understood (Lim, *et al.*, 2010).

1.2.4 Clinical pathology and symptoms of haemolytic uraemic syndrome

Escherichia coli O157:H7 infections can be entirely asymptomatic or present diarrhoea, bloody diarrhoea, haemolytic uraemic syndrome, thrombotic thrombocytopenic purpura, and leading causes of death. Diarrhoea usually appears 3 to 4 days after the initial infection. Approximately 25-75% of patients recover from the symptoms. In other cases, the diarrhoea subsequently becomes bloody, two to three days later. Some 5% to 10% of children develop haemolytic uraemic syndrome within a week of the initial infection (Besser, *et al.*, 1999). The literature on the disease confirms that it is more prevalent in children and old people (Griffin & Tauxe, 1991, Rangel, *et al.*, 2005, Pfaller & Diekema, 2007).

2. Introduction to *Candida albicans*

2.1 *Candida albicans*

Candida albicans are eukaryotic unicellular fungi that belong to the normal microbial flora. The organisms frequently colonize humans are commensal and certain body sites are skin, oropharynx, colon, and vagina (Levinson, 2016). Generally, the microorganisms are member of fungal kingdom and reproduce asexually by fission or by budding. The microorganisms have to several morphological structures including, oval shaped, budding yeast, pseudohypha, and true hypha. The transition from a commensal to a pathogen of the organisms associated with the number of normal flora, epithelial barriers and the innate immune response. A weak immune system in human or loss of normal flora is one of the main factors to infections by *C. albicans*. The opportunistic pathogen, the most common cause of candidiasis, expresses selectively several virulence determinants that contribute to pathogenesis. The factors include the ability to adhere to host cells or inert surfaces, yeast-to-hypha conversion that promotes resistance to phagocytic killing, and biofilm formation which also supports resistance to antifungal drugs.

2.2 Pathogenicity of *Candida albicans*

2.2.1 Cell wall

C. albicans cell wall has been shown to contain 90% carbohydrate and 10% protein. It is crucial both to the biology of the organisms and to interactions with host cells and composed mainly of three forms of polysaccharides including mannoprotein, β glucan containing β-1,3 and β-1,6 glucan, and chitin. The mannan form is found in the outer layers of the walls while the β-glucan and chitin are located in the inner layers (Figure 3) (Chaffin, *et al.*, 1998). Covalent attachment between mannoprotein and β-1,6-glucan are linked by the glycosylphosphatidylinositol remnant (Surarit, *et al.*, 1988).

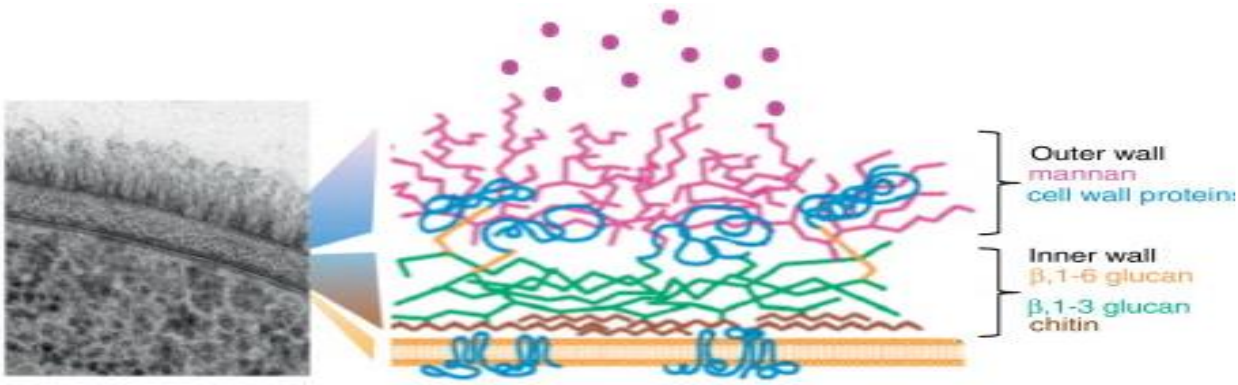


Figure 3. *C. albicans* cell wall structure as visualized by transmission electron microscopy and a model showing the arrangement of the major components (Gow & Hube, 2012).

2.2.2 Adhesion

C. albicans possess a remarkable capacity to adhere to abiotic surfaces surface and host cells that is important for a successful the pathogen. There are a number of specific adhesins which are critical step for biofilm formation (Kline, *et al.*, 2009). The factors that let the organisms firmly attached to the surface and aid to prevent them from being flushed away (Chaffin, *et al.*, 1998).

The immune response and the anti candidal activity of neutrophils play an important role in host defence against localized *C. albicans* infection (Ellepola & Samaranayake, 2000, Neto, 2007). The single-celled, budding yeast *C. albicans* are often visible on in 24 to 48 hours. The following show soft and moist colonies colony, which resemble the bacterial species. The fungal pathogens are found in 40 to 80% of healthy human individuals. It may be present as a commensal or a pathogenic organism. Thrush, oropharyngeal candidiasis, is assessed as white patches on the mucosa of the floor of the mouth, tongue and /or inner lip surface. The affected area can become inflamed which may cause difficulty in swallowing and spread to the esophagus. The infection is usually found in immuno-compromised human and it causes life-threatening invasive diseases. The recommended treatment consists of clotrimazole tablets or a nystatin suspension. Fluconazole or itraconazole is effective for esophageal infection. Amphotericin B is considered as effective drug for some

patients. *C. albicans* infections occur in immunocompromised patients who have also shown to cause reduced resistance to fungal infections. The establishment of the organism infection and inflammation appears to be affected by host. The microorganism can present in many forms such as germ tubes form and pseudohyphae that are reportedly associated with the pathogenicity of infection. The yeast cell is Gram positive and approximately 10 to 12 microns. Currently, itraconazole and fluconazole are the drugs of choice for systemic infection. Successful establishment of infection by the pathogens require colonization, dissemination to host tissues, adhesion, and biofilm formation (Donlan, 2001, Thompson, *et al.*, 2011). Germ tube formation is the precursor step to hyphal formation that is a key virulence determinant.

2.2.3 Hyphal formation

The transition between unicellular budding yeast to the filamentous form is thought to contribute significantly to the virulence of *C. albicans*. (Lo, *et al.*, 1997). Signal transduction pathways that regulate morphogenesis in the pathogen. It has been reported that in the organism is associated with adhesion as well as filamentous hyphae (Yang, 2003). These findings suggest that only strains that can both form filament and produce yeast form cells are capable of penetrating vital organs and proliferating sufficiently to kill the host. Many genes expression data shows that both growth results in formation of hyphal and the killing of *Caenorhabditis elegans* are associated with virulence upon *C. albicans* infection in *Caenorhabditis elegans* model (Pukkila-Worley, *et al.*, 2009). *Candida* hyphae are able to actively penetrate, destroy tissue surfaces, and escape from the host immune system (Kumamoto & Vinces, 2005). Hyphal growth is significant for the formation of *C. albicans* biofilms that is associated with tissue invasion. Although yeast cells are an important form for distribution in the host (Chandra, *et al.*, 2001, Richard, *et al.*, 2005, Nobile & Mitchell, 2006). The pathogen show the ability of to form germ tubes that is key feature of virulence factor (Gow, 1997). Prostaglandin E_2, the primary cyclooxygenase isoenzymes products of arachidonic acid, stimulates the yeast-to-hyphal transition in the yeast (Kalo-Klein & Witkin, 1990).

2.2.4 Biofilm formation

Biofilm formation is now recognized as a major virulence attributes of *C. albicans* and also a key factor contributing to the high mortality rate associated with candidiasis (Chandra, *et al.*, 2001). The formation is a protected safe from antibiotic treatment and can create a source of persistent fungal infection (Hasan, *et al.*, 2009). Initial the fungal adhesion to abiotic or biotic surfaces is the first step toward colonization and following biofilm growth that are embedded within an extracellular matrix. The dimorphic *Candida albicans* is able to switch between yeast and hyphal form Which provides a significant effect on the host immune response (Conti, *et al.*, 1990).

2.3 *Candida albicans* and neutrophils

The depletion of polymorphonuclear leukocytes increased the severity of oral infection in both BALB/c and CBA/CaH mice (Martinez, *et al.*, 2010).
Resistance of *Candida albicans* to phagocyte killing is an important virulence function of the organism (Luo, *et al.*, 2010). The infection are characterized by fungal invasion of host cells (Yuan, *et al.*, 2009). The organism is a diploid, polymorphic fungus, switching readily from yeast to hyphal (and pseudohyphal) growth and back. The attach yeast cells to and invade into the host obtain nutrients and evade the host immune response. The pathogen follows an aggressive strategy to subvert the host response and to obtain nutrients for its survival (Brunke & Hube, 2013). A contributing factor to these statistics is the ability of the organisms to develop resistance to antifungal agents. New strategies for combating fungal infections without toxicity to humans are a high medical priority. The opportunistic pathogen can trigger a variety infection in the immunocompromised host. Yeast cell wall is composed mainly conserved molecule structure that is Pathogen-Associated Molecular Patterns (PAMPs). The several fungal PAMPs bind to pattern-recognition receptors or PRRs stimulates innate immune and adaptive immune system.

3. Introduction to human neutrophils

3.1 Neutrophils

Polymorphonuclear neutrophils, immune cell, are types of leukocytes that have granules with enzymes are released during infections and inflammation. The granules of neutrophil are purple-blue following stained by neutral dyes. A normal neutrophil account for approximately 50 to 80 percent of all the white bloods cells and neutrophils vary in size from 9 to 15 microns in diameter. The nucleus is composed of two to five lobes connected by chromatin filaments and the cells are capable of amoeboid movement. In addition, nucleus and cytoplasm are enriched with granules and secretory vesicles, respectively.

The world is full of microbes. Fortunately, human immune system has the essential functions of protecting the body against diseases caused by the pathogenic organisms. Modulation of the immune system denotes any change in the immune response and can involve induction, expression, amplification or inhibition of any part or phase of the immune response. The system comprises both innate immunity and acquired immunity. The immune response of humans is complex and multi-leveled, involving many cell types with distinct but overlapping roles (Schepetkin, *et al.*, 2005). An important first line is responsible for non-specifically defending and eliminating to foreign invaders. Additionally, the system is able to response organisms immediately on infections. Both inflammation and antiviral infections are two major types of complex responses of body. Inflammation includes white blood cells and protein of plasma aggregation and action at the infections. Different types of cells in the body make up the arsenal of innate immune system and prevent, recognize, and destroy invading pathogens.

The neutrophil has a critical influence on innate immunity and the body's first line of defense against microorganisms (Burg & Pillinger, 2001). Phagocytosis and intracellular killing are major mechanisms by which the nonspecific immune system eliminates microorganisms. Neutrophils play a main role in this system. They are circulating scavengers and are found in extremely concentrated levels in blood, liver, lymph nodes, and spleen. Additionally, they are found in all

tissues, especially at sites of inflammation and infection. They are the first innate immune cells to migrate to the site of infection or inflammation for the containment and clearance of infectious particles.

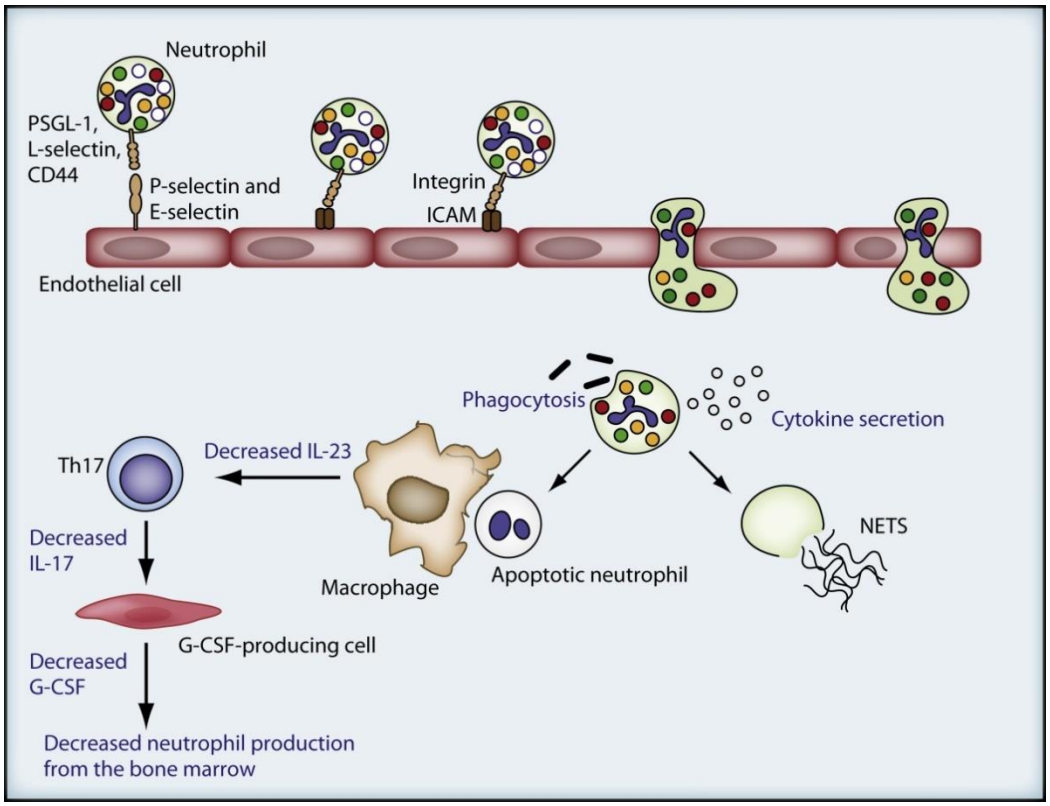


Figure 4. Mechanism of action of neutrophil (Mitchell, *et al.*, 2000, Burg & Pillinger, 2001, Ley, *et al.*, 2007, Pospieszalska & Ley, 2009).

3.2 Neutrophil granulocyte content, function, and transport

Human neutrophils change during development from bone marrow to circulation. Morphological stages of the cells are myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and polymorphonuclear cell. The mature neutrophils have at least four types of specialized granules and function as microbicidal mediators defense against microbial invaders. The granules have been classified as azurophilic granules, specific granules, gelatinase granules, and secretory vesicles. Peroxidase-positive azurophil granules or primary granules are formed at the

promyelocytic stage. The granules contain proteins, including myeloperoxidase, α-defensins, bactericidal/permeability-increasing protein, elastase, proteinase-3, and cathepsin G are found and released into the phagosome. The secondary or specific peroxidase-negative granules were exhibited at stage of maturation of neutrophil that comprise about two thirds of the granules. The granules contain collagenase, lactoferrin, and gelatinase, among other proteins. Gelatinase granules or tertiary granules are storage site of matrix metalloprotease 9 that is also called gelatinase B. In addition, the secretory vesicles contain human serum albumin that is extracellular fluid from endocytic origin (Borregaard & Cowland, 1997).

3.2.1 Granule content and host defense

The granules are important to the innate immunity in the host's defense and fuse with the plasma membrane. During phagocytosis, NADH oxidase activity of neutrophils is increased and degranulation, cationic proteinases, interacts with and disrupts bacterial cell surface negative charge. Bactericidal/permeability increasing protein, 55 kDa cationic protein, was produced largely by polymorphonuclear leukocytes (PMN) and can be released into the plasma in response to certain stimuli. Endotoxin neutralization occurs because BPI competitively antagonizes the binding of lipopolysaccharide to lipopolysaccharide-binding protein, with a subsequent inhibitory effect on the inflammatory response (Guerra-Ruiz, *et al.*, 2010). Bactericidal/permeability increasing protein is highly positively charged and obvious cytotoxicity against only Gram-negative bacteria that causes of the antimicrobial activity by increasing permeability of the bacterial cell, hydrolysis of bacterial phospholipids, and death of organisms. In addition, the protein has also been shown to neutralizing endotoxin activity through direct binding of LPS, and opsonic activity (Mannion, *et al.*, 1990, Levy, 2000). Thus, anti-infective properties of bactericidal/permeability increasing protein induced apoptosis is mediated by host cell detachment (Levy, 2000).

3.2.2 Defensins

Defensins are 29-34 amino acid peptides that comprise 30-50% of the total protein in the azurophilic granules of human neutrophils (Ganz, *et al.*, 1990). The synthesis and secretion of defensins are regulated by microbial signals, development signal cytokine, and neuroendocrine signaling. During phagocytosis, azurophilic granules fuse with phagocytic vacuoles and generate high concentrations of defensins (Ganz, 2003).

3.2.3 Proteinase 3

Proteinase 3/myeloblastin, a serine proteinase, is found both in azurophilic and secretory granules of neutrophilic granulocytes in man. (Pederzoli, *et al.*, 2005). Proteinase 3 is not only the antigenic target of antineutrophil cytoplasmic autoantibodies (ANCA) and also are widely thought to induce granulomatosis with polyangiitis (Campbell, *et al.*, 2000). Inactive precursors human neutrophil peptides are converted by proteinase 3 to active microbicides (Tongaonkar, *et al.*, 2012).

3.2.4 Human leukocyte elastase

Serine proteases human leukocyte elastase is found in the azurophilic granules of the neutrophils. It has been assigned a unique number by the Enzyme Commission of the International Union of Biochemistry (E.C. 3.4.21.37). The human leukocyte elastase may act as a key host defence protein released by activated neutrophil at sites of inflammation (Weinrauch, *et al.*, 2002). The physiologic proteolytic enzyme may be direct or indirect damages the extracellular matrix protein and alternate inflammation. The study reported that mice deficient in neutrophil elastase were shown to be more susceptible than normal mice to Gram-negative bacteria inoculations (Belaaouaj, *et al.*, 1998). Other worker has reported similar increases in defense against *Klebsiella pneumoniae* and *Escherichia coli*. Meanwhile, there were no the intracellular killing activity of neutrophil mediated killing of

Staphylococcus aureus (Belaaouaj, 2002). In addition, mice deficient in elastase gene showed susceptible to fungal infections (Tkalcevic, *et al.*, 2000).

3.2.5 Neutrophil peptide cathelicidins

Neutrophil peptide cathelicidin is a 19 k-Da cationic antimicrobial protein that is an essential element of the innate immunity (Cowland, *et al.*, 1995). The protein is synthesized in neutrophilic granulocyte progenitors in the bone marrow and stored in the specific granules that contain 0.627 μg /10^6 neutrophils (Sørensen, *et al.*, 1997). LL-37, C-terminal part, has potent antimicrobial activity against both Gram-positive and Gram-negative bacteria (Turner, *et al.*, 1998).

3.2.6 Matrix Metalloproteinase (MMPs)

Matrix metalloproteinases (MMPs) or matrixins, are implicated in the extracellular matrix degradation and removal from the tissue (Nagase, *et al.*, 2006). Human neutrophil granules contain twenty-three matrix metalloproteinase which are zinc-dependent protein and peptide hydrolases (Nagase & Woessner, 1999). Three classes of metalloproteases stored in granules include collagenase, gelatinase, and leukolysin. Neutrophil collagenase (MMP-8) is 75 kDa that is located in specific granules (Lazarus, *et al.*, 1968, Murphy, *et al.*, 1977). Gelatinase (MMP-9), 92 kDa, resides in the tertiary granules of neutrophils (Wilhelm, *et al.*, Kjeldsen, *et al.*, 1992). Leukolysin (MMP-25), 56 kDa, is localized to specific, tertiary granules, secretory vesicles, and on plasma membrane (Pei, 1999, Kang, *et al.*, 2001). Activated neutrophils secreted the granules enzymes by degranulation that are mobilized and released to the extracellular space. A number of inflammatory mediators, including bacterial lipopolysaccharide induce the polymorphonuclear neutrophil release gelatinase (Pugin, *et al.*, 1999). The methanol extract of *Tristaniopsis calobuxus* Brongniart & Gris (Myrtaceae) reduced MMP-9 secretion from mouse peritoneal macrophages (Bellosta, *et al.*, 2003). Surfaces proteins of *Staphylococcus aureus* possess important biological functions which are able to elude innate immune system by promoting adhesion and invasion of host cells, and interfering with human

complement system evasion of immune responses and biofilm formation (Itoh, *et al.*, 2010, Foster, *et al.*, 2013).

3.3 Phosphatidylinositol 3-Kinase (PI 3-K)

Phosphatidylinositol 3-Kinases are a family of lipid kinases involved in regulation of migration, degranulation, and superoxide anion production in neutrophils through extracellular signal-regulated kinase (Marshall, 1995, Sadhu, *et al.*, 2003). 5-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3,7- dimethoxy-4H-chromen-4-one, *Melicope Semecarpifolia* fruit extract, is a phosphatidylinositol 3-kinase inhibitor that is a potent inhibitor of neutrophil activation (Liao, *et al.*, 2011). The lipid kinase are necessary for chemoattractant-induced respiratory burst (Ahmed, *et al.*, 1995)

3.4 Mitogen Activated Protein Kinases (MAPKs)

Mitogen Activated Protein Kinases, serine/threonine kinases, are important signal transducing enzymes that include p38, Erk1, Erk2, and Jnk that participate in cell signaling for growth, differentiation, and responses to stress (Cobb, *et al.*, 1994). In the serine/threonine kinases signaling cascade in neutrophils, catalyze the phosphorylation and activation by MAPK-kinases (Kim & Haynes, 2013). Mitogen activated protein (MAP) kinase signal transduction pathway play an important role in various aspects of the immune and inflammatory responses. The p38 MAP kinase pathway was identified as a kinase activated in response to extracellular stimuli such as lipopolysaccharide, staphylococcal peptidoglycan, and staphylococcal enterotoxin B that are the stimulation of neutrophils (Hommes, *et al.*, 2003). cytoplasmic components to the membrane and their association with flavocytochrome b558 produces the functional complex. The cytochrome then transfers electrons from NADPH to O_2 to create O_2^-. Genetic defects in p47-, p67-, p22-, and gp91phox are the causes of chronic granulomatous disease (CGD) (Burg & Pillinger, 2001). CGD patients have deficient O_2 production and experience ineffective inflammatory

reactions to infection. They commonly suffer from repeated bacterial infections by organisms that are catalase positive such as *S. aureus*.

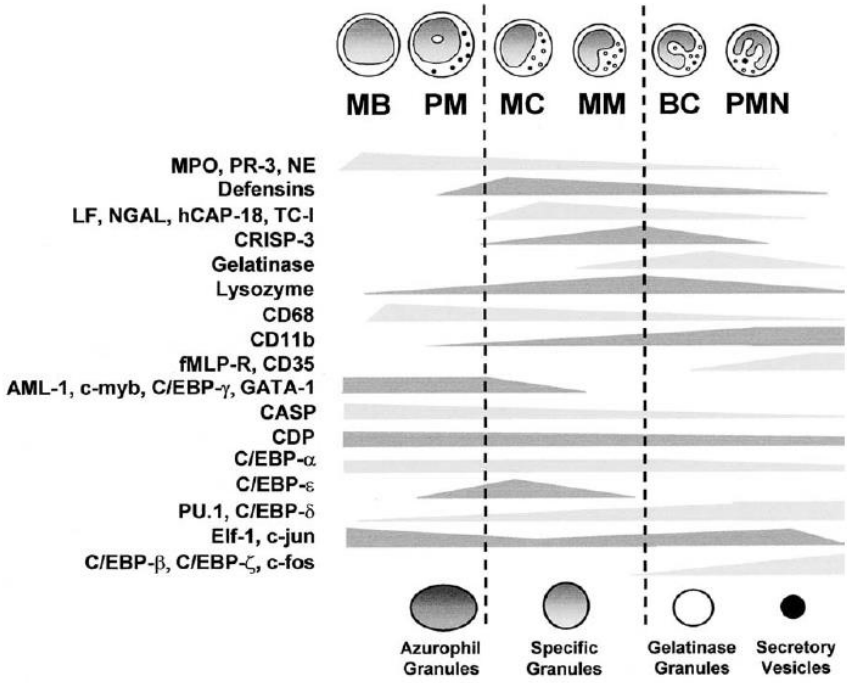


Figure 5. Biosynthetic windows of granules, granule proteins, and transcription factors (Faurschou & Borregaard, 2003).

3.5 Phagocytic activity of neutrophils

Phagocytosis by neutrophil expresses for interaction with opsonins Fcγ receptor type I, II, and III that are critical for the leukocyte surface receptors for initial inflammatory reaction to invading microbes (Kim, *et al.*, 2003). The uptke of IgG-coated pathogens or unusual substances is stimulated by the expression pattern of FcγRII and tyrosine kinase receptor, which results in an increased in actin polymerization (Zuccotti, *et al.*, 2009). The Syk Src homology 2 domains bind to specific phosphotyrosine that show mediate specificity sequences of phagocytosis. The signaling pathways lead to Rho protein activation that modulate actin dynamics during engulfs the pathogens (Swanson & Baer, 1995, Greenberg, *et al.*, 1996, Massol, *et al.*, 1998). The Rho family GTPases including Rac, Rho, and Cdc42 that involved in many functions of the cells such as organization of actin filament maintenance of cell shape, adhesion, motility, and trafficking from membrane

proteins (Hackam, *et al.*, 1997, Caron & Hall, 1998). Cytoskeleton changes during phagocytosis involve several steps among which are the actin depolymerizations. Phosphoinositide 3-kinase (PI3-kinase), phosphorylated lipids, are significantly enhances cell motility that are involved in polymerization of actin at the cellular membranes (Chitemerere & Mukanganyama, 2014). FcγRIII is expressed on human neutrophils that is receptor anchored through a phosphatidylinositol (PI) linkage (Mbah, *et al.*, 2012). FcγR-mediated phagocytosis of C3-opsonized target particles is involves in affected independently of an increase of cytosolic-free Ca^{++} and the magnitude of inositol phosphate production (Fällman, *et al.*, 1989). In addition, the mediated phagocytosis is accompanied by the activation of the respiratory burst and by the production of arachidonic metabolites and cytokines, this does not occur during C3bi-dependent uptake (Yamamoto & Johnston, 1984, Wright & Meyer, 1986). Both complement receptor 1 and 3, present exclusively on neutrophils, bind complement factor C3b/iC3b and result in the promotion of phagocytosis. Whereas extracellular matrix proteins and phorbol myristate acetate can increase the phagocytic activity (Wright & Silverstein, 1983, Brown, 1986). The trigger substances are known to stimulate complement receptor 3 on the surface of neutrophils for binding capacity. Fcγ receptor and complement receptor are shown to be directly activated by interacting with C3b/iC3b bearing targets, the presence of opsonizing antibodies, and displaying glycosylated CR3 ligands (Ehlenberger & Nussenzweig, 1977). There is report that CR3 neutrophils deficient patients have impaired antibody responses to phagocytosis (Dana, *et al.*, 1984).

3.6 Degranulation

3.6.1 Biogenesis of granules

The molecules are packed together in neutrophils granules that are secreated from cells during cellular activation (Spitznagel, 1990, Elsbach, 1998, Lehrer & Ganz, 1999). Several granules biogenesis pathways has been elucidated (Borregaard & Cowland, 1997). An azurophilic granule emerges during at the promyelocytic stage of neutrophil differentiation. The synthesis of myeloperoxidase,

serine proteases stored in the granules and are considered as the true microbicidal that activated upon phagocytosis (Egesten, *et al.*, 1994). The stage of metamyelocyte contains lactoferrin and collagenase that occur in specific granules followed by an exhibition gelatinase in tertiary granule. Several secretory vesicles, the fourth type of granules, were observed at the stage of mature neutrophil. The potentially causal mechanisms consisting in the stimulation of granule secretion can be separated and controlled. Several studies have reported that a congenital disorder with the specific granule deficiency characterized by impairment structure and function of neutrophils as a marker of severe bacterial infections. Abnormalities in neutrophil function such as migration and bacterial killing are characterized by impaired of specific granules and defensins. Chediak-Higashi syndrome, the specific granule deficiency, is an uncommon autosomal recessive disorder by leading to increase susceptibility to infections and diseases.

3.6.2 Mechanisms of Degranulation

Degranulation of neutrophils, a process of exocytosis, is generally considered key events for antimicrobial activity against microorganisms (Maia, *et al.*, 2011). Secretory vesicles of neutrophils fused with the plasma membrane during and after exocytosis. The docking and membrane fusion process involve protein-protein interactions between lipid bilayer-surrounded granules to target membranes.

3.6.3 Microbicidal Molecules

The antimicrobial efficiency of human neutrophils relies on two concurrent presences of two types appearing in initial phagolysosome of triggered human neutrophils. The release of reactive oxygen species (ROS) from polymorphonuclear leukocytes, part of physiologically metabolic processes, was generated by nicotinamide adenine dinucleotide phosphate oxidase. At the same time, the professional phagocytes release granule antimicrobial proteins that kill various microbes and play a role in the pathogenesis of disease. It is stimulated by several factors such as promoting adhesion or by phagocytic targets.

3.6.4 NADPH-Derived Oxidants

During phagocytosis of foreign particles, neutrophils increase the oxygen consumption through the regulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. The NADPH oxidase enzyme is a multicomponent enzyme complex that include of five subunits and the small GTPase Rac2 present in the vesicular and plasma membranes of neutrophils. The active phagocyte oxidase (phox) complex consists of three in the cytosol (p40phox, p47phox, and p67phox). Flavocytochrome b558 is the core catalytic enzymes in resting phagocytic cells a membrane-bound flavohemoprotein that contains two in the membrane p22phox and gp91phox (DeLeo & Quinn, 1996, Babior, 1999, Clark, 1999). The events occurred since upon activation of neutrophils by the antigens through the genertion the enzyme respiratory burst oxidase. In response to stimulation, p47phox, p67phox, p40phox and Rac2 migrate to the plasma membrane where are assembled together to form a functional enzyme and release oxygen radicals. The reactive oxygen species (ROS) from oxygen are chemically derived oxygen-containing ions and small molecules such as superoxide anion (O_2^-), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) (Nathan, 1987, Babior, 1999). In patients with chronic granulomatous disease are lack NADPH oxidase activity that lead to suppression of the bactericidal/bacteriostatic activity of host neutrophils against the pathogens (Heyworth, *et al.*, 2003).

3.6.5 The H_2O_2-Myeloperoxidase System

Myeloperoxidase (MPO) is involved in the activation of NADPH oxidase leading to superoxide generation in polymorphonuclear leukocytes (Klebanoff, 1968, Klebanoff, 1999). The enzyme is a heme protein present in azurophil granules that was released from neutrophils into the phagolysosome membrane. The peroxidase enzyme, microbicidal protein, catalyzes the formation of the highly reactive oxidant such as hypochlorous acid (HOCl), tyrosyl radicals, and cross-links proteins (Klebanoff, 2005). The antimicrobial systems formed MPO-H_2O_2-halide system that is a complex system composed of myeloperoxidase (MPO),

hydrogen peroxide (H_2O_2), and a halide, particularly chloride. The system, MPO-H_2O_2-chloride system is antimicrobial systems including hypochlorous acid, chloramines, hydroxyl radicals, singlet oxygen, and ozone.Both nitrite and hydrogen peroxide are substrates of myeloperoxidase activity to catalyze tyrosine nitration in proteins and can react with peroxinitrite (Floris, *et al.*, 1993, Podrez, *et al.*, 1999, Kumar & Varela, 2012). Several studies reported an important role of MPO activity from circulating neutrophils in microbicidal activity such as *Candida* (Nauseef, 1998, Petrides, 1998, Aratani, *et al.*, 1999).

3.6.6 Nitric Oxide-Synthase-Derived Reactive Nitrogen Intermediates

Nitric oxide synthase (NO) are unique among eukaryotic enzymes in being dimeric, calmodulin-dependent or calmodulin-containing cytochrome P450-like hemoproteins that combine reductase and oxygenase catalytic domains in one monomer. They bear both FAD and FMN, and carry out a 5-electron oxidation of a non-aromatic amino acid (L-arginine) with the aid of tetrahydrobiopterin (Nathan & Xie, 1994). Reactive nitrogen intermediates include nitric oxide (NO), which can react with oxygen to form much stronger oxidants such as nitrogen dioxide (NO2). The direct toxicity of NO is modest, but is greatly enhanced by reacting with superoxide to form peroxynitrite (ONOO-) (Beckman & Koppenol, 1996). Human phagocytes and especially neutrophils, appear to generate so little nitric oxide (Padgett & Pruett, 1995, Shane & Pickering, 2012). In neutrophil enriched fractions from urine, we demonstrate a 43-fold increase in nitric oxide synthase activity in patients with urinary tract infections compared with that in neutrophil (Wheeler, *et al.*, 1997).

3.7 Cytokine Synthesis

Human neutrophils are influence goals of several proinflammatory cytokines, chemokines, growth factors, and the response function will be triggered. The leukocytes can produced proinflammatory cytokines including tumor necrosis factor α (TNF-α) interleukin 1α, 1β, and 12 (IL-1α, 1β, and 12). Chemokines are IL-8,

granulocyte/monocyte colony stimulating factor (G-CSF and GMCSF), macrophage infiltrating protein-1α and 1β (MIP-1α and 1β), and, cytokine-induced chemoattractants (CINC). The biological properties of the cytokines and chemokines have been shown to be hig0hly effective in enhancing capacity to adhere to endothelial cells, generating reactive oxygen species and also leading to increase neutrophils migration toward the site of inflammation. The signaling proteins secreted by activated neutrophils may be functioning as priming of the leukocytes (Witko-Sarsat, *et al.*, 2000, Cassat, *et al.*, 2007). *In vitro*, the cytokine production is significantly different but the outputs are less evident *in vivo*. The production of cytokines is also principally influenced by the most potent effective inducing agents including lipopolysaccharide and lipoteichoic acid (von Aulock, *et al.*, 2003, Hattar, *et al.*, 2006).

3.8 Tumor necrosis factor α (TNF-α)

Tumor necrosis factor α, a proinflammatory cytokine, is a soluble homotrimer of 17 kDa polypeptide subunits that is secreted by phagocytes engulf and digests of the pathogens or foreign substances. For the first time in an experiment, the naturally occurring protein was produce by active monocytes and macrophages that inhibits the growth of cancer cells (Witko-Sarsat, *et al.*, 2000). Interestingly, the proinflammatory cytokine increase proliferation of usual cells including monocytes, macrophages, lymphocytes, eosinophils, and neutrophils. The proinflammatory effects of TNF-α express to increase the capacity of endothelial cell adhesion molecules and as a priming agent for neutrophil functions by promoting and stimulating adhesion, phagocytosis, degranulation, and oxidative burst. Though, TNF-α mRNA are expressed in neutrophils stimulated with LPS, *C. albicans* TNF-α, IL-1β, GM-CSF, and IL-2 as stimulating agents (Djeu, *et al.*, 1990, Mandi, *et al.*, 1991, Witko-Sarsat, *et al.*, 2000, Nathan, 2006). Neutrophils have exquisite specificity for TNF-α targeting and sensitivity for changes in inflammation. The effects are mediated via two different types of TNF-α receptors, type A and type B receptors, are 75 kDa and 55 kDa, respectively. Type B receptor is expressed in leukocytes of myeloid origin that binds to and neutralizes the activity of TNF-α (Nakamura, *et al.*, 1996).

TNF-α receptors is the receptors of neutrophil elastase that stimulate the leukocytes and upregulate in response to insults at sites of inflammation.

3.9 Interleukin 1 (IL-1) and Interleukin 1 Receptor antagonist (IL-1-Ra)

Neutrophils exhibit both IL-1α and IL-1β mRNA and secreted IL-1 proteins. IL-1β level showed up to 10-fold more than amount of IL-1α. Several stimuli of specific agents are able to trigger IL-1 production such as IL-1 β, TNF-α, and anti-neutrophil cytoplasm autoantibodies, by neutrophils. There are two classes of IL-1 receptors, IL-1-RI and IL-1-RII. Both receptors and IL-1 Receptor antagonist are expressed on the wide variety of cells. IL-1Ra, 23-25 kDa soluble protein, inhibits the effects of IL-1 by binding to the specific IL-1 receptors without stimulating signal activation. Both LPS-treated neutrophils and LPS-treated monocytes produce IL-1Ra mRNA. The level of the protein was up to 100-fold of LPS-treated neutrophils, compare with LPS-treated monocytes. Neutrophils could contribute to modulation of the IL-1 induced inflammatory and immune responses (Witko-Sarsat, *et al.*, 2000). Additionally, IL-4, IL-13, and TGF-β are potent stimulators of IL-1Ra mRNA (Witko-Sarsat, *et al.*, 2000).

3.10 Interleukin 8 (IL-8)

Interleukin 8 (IL-8) or chemokine (CXCL8) act as both potent neutrophil chemoattractant and activator that exhibited in response to LPS and *S. aureus* (Fujishima, *et al.*, 1993, Rennekampff, *et al.*, 2000, Caswell, *et al.*, 2001, Hattar, *et al.*, 2006). T lymphocytes, epithelial cells, keratinocytes, fibroblasts, endothelial cells, and neutrophils can produce proinflammatory chemokine (Monteseirin, *et al.*, 2004). Remarkably, the chemokine is the most abundantly protein expression of neutrophils that are the principal target cell for IL-8 (Godaly, *et al.*, 1997).

3.11 Toll-like receptors on neutrophils

Innate immune system acts as the first line of defense against invading organisms and the Toll-like receptors (TLRs) play a part of the innate immune system by recognizing conserved pathogen-associated molecular patterns in the microorganisms such as lipopolysaccharide, bacterial lipoproteins, peptidoglycan, and bacterial DNA (de la Morena, 2012). Subsequently, inflammatory response is initiated by the interaction between TLRs and specific microbial patterns (Lien & Ingalls, 2002). TLR1, 2, 4, 5, 6 and 10 are localize in the cell surface while TLR3,7, 8, 9, 11, 12 and 13 are are found in intracellular compartments (Kawai & Akira, 2010). Neutrophils expressed TLR family members including TLR1, 2, 4, 5, 6, 7, 8, 9, and 10 that allow the recognition microbe-associated molecular patterns (Hayashi, *et al.*, 2003). On activation, TLRs in neutrophils can activate cytokine release, superoxide production, and L-selectin shedding, whereas inhibition of release of chemotaxis and increase phagocytosis of opsonized latex beads (Hayashi, *et al.*, 2003). TLR2 recognizes a variety of microbial components which include lipoproteins, lipopeptides, peptidoglycan, lipoteichoic acid, lipoarabinomannan glycosylphosphatidylinositol anchors, a phenol-soluble modulin, zymosan, and glycolipids (Takeda, *et al.*, 2003).

3.12 Arachinodic acid

Arachidonate, polyunsaturated fatty acid of lipid bilayers, are rich in mammalian cells and tissues. Prostaglandins, mediators of inflammation synthesized from arachidonic acid, are secreted by neutrophils during phagocytosis (Sakamoto & Ooshima, 1985, Wright, *et al.*, 2010). Particulary, prostaglandin E_2 plays an important role in the pathogenesis of inflammatory. During inflammation, the mediator molecules in phagocytes were shown to be responsible for inducing immunosuppressive conditions (Medeiros, *et al.*, 2012). In addition, pathogens and their components act as a potent regulator factor that promote the synthesis of prostaglandin E_2 by activated neutrophils (Agard, *et al.*, 2013).

4. *Rhodomyrtus tomentosa* (Aiton) Hassk.

Rhodomyrtus tomentosa is a plant in the family Myrtaceae often used in traditional medicine in Malaysia and Indonesia (Voravuthikunchai, *et al.*, 2010). *Myrtus tomentosa* Aiton and *Rhodomyrtus parviflora* Alston are synonyms. Its various common names are downy rose myrtle, downy myrtle, rose myrtle, hill gooseberry, Ceylon hill cherry, and hill guava. The genus *Rhodomyrtus* includes about 20 species, from tropical Asia to New Caledonia and Australia. The name is derived from the Greek *rhodon* meaning red, referring to the rose-colored flowers that are common in plants of this genus, and *myrtos,* meaning myrtle. *R. tomentosa* is a small shrub or evergreen tree, two to three meters tall. It has dense, short, soft hairs on the young stem and simple, opposite, entire, elliptic-oval leaves, glossy green above, with dense soft hairs below and three conspicuous longitudinal veins. The rose-pink flower is solitary or in three-flowered dichasia in upper axils and possesses five hairy sepals and petals, and many stamens with pink filaments. The ripe fruit is dark purple, sweet, and has many seeds. The plant is native to Southeast Asia, India, Sri Lanka, and Southern China (Wagner, 1999). Nevertheless, it has been reported as a serious invader of the native plant communities in Florida (Winotai, *et al.*, 2005). Extracts and active pure compounds from the plant have been shown to inhibit the growth of pathogen microorganisms (Dachriyanus, *et al*., 2002; Limsuwan, *et al*., 2009; Limsuwan, *et al*., 2009) Extracts have also acted as a blood platelet aggregation inhibitor and calcium antagonist. In addition, it is used in formulations of skin-whitening, anti-aging and skin-beautifying agents. Among the substances derived from the species, rhodomyrtone is an active compound belonging to the group of acylphloroglucinols (Dachriyanus, *et al*., 2002; Hiranrat, *et al*., 2008). The flavonoids of the species are flavone glycosides and include myricetin-3-O-α-L rhamnoside, myricetin-3-O-α-L-furanoarabinoside, myricetin-3-O-α-D-glucoside, and 2, 3-hexahydroxydiphenyl-D-glucose (Hou, *et al*., 1999). Tannins have been isolated from the leaves and root of *R. tomentosa* and were identified from their structures as pedunculagin casuariin, castalagin and tomentosin (Liu, *et al.*, 1997, Liu, *et al.*, 1998).The identified triterpenes and steroids from this plant include lupeol, β-amyrin, β-amyrenonol, betulin, friedelin, α-amyrin, taraxerol, betulinic, ursolic, and aliphitolic

acids(Hui, *et al.*, 1975, Hui & Li, 1976). There are many studies of the antibacterial properties of the plant's ethanolic leaf extract. Its good activity on Gram-positive bacteria such as *Bacillus cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Streptococcus pyogenes*, and *S. aureus* has been well documented (Limsuwan & Voravuthikunchai, 2008, Limsuwan, *et al.*, 2009, Voravuthikunchai, *et al.*, 2010, Odedina, *et al.*, 2015). The extract showed remarkable activity on coagulase-positive and coagulase-negative of *S. aureus* with minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of 32-1024 μg/ml. At 4MIC of the extract decreased bacterial survival at 3 log-fold within 6-8 h (Saising, *et al.*, 2008). The extract killed both endospores and vegetative cells of *B. cereus* at 15.6-62.5 μg/ml within 105 min and 18 h, respectively (Voravuthikunchai, *et al.*, 2010). Acylphloroglucinols rhodomyrtone was isolated from *R. tomentosa* (Dachriyanus, *et al.*, 2002). The active compound exhibited strong antibacterial activity against *S. pyogenes* (Limsuwan, *et al.*, 2009). Interestingly, MIC of this compound on *S. aureus* was 0.5 μg/ml and this property was very close to that of vancomycin (Saising, *et al.*, 2008). This pure compound has similar myrtucommulone B to *Myrtus communis* (Hui & Li, 1976).

Objectives

1. To assess the potential of ethanolic leaf extract of a medicinal plant, *Rhodomyrtus tomentosa* in enhancing the killing activity of human neutrophils against pathogens.
2. To study the effects of the extract on membrane permeability of the organisms.
3. To determine the effect of *Rhodomyrtus tomentosa* extract on the inhibition of germ tube formation in *Candida albicans*.
4. To examine yeast cells were treated with the extract exhibited decreased adhesion to surfaces.
5. To investigate the effect of the extract on the reduction of *Candida albicans* biofilm formation and mature biofilm.

CHAPTER 2

RESEARCH METHODOLOGY

Materials and Methods

1. Microorganisms

1.1 *Staphylococcus aureus* ATCC 29213
1.2 Methicillin-resistant *Staphylococcus aureus* (MRSA) 001R
1.3 *Escherichia coli* ATCC 29522
1.4 Enterohaemorrhagic *Escherichia coli* O157:H7 050910788152
1.5 *Candida albicans* ATCC 90028
1.6 *Candida albicans* NCPF 3153
1.7 *Candida albicans* NPRCoE160101, NPRCoE160105, and NPRCoE160111

2. Antibiotic

2.1 Vancomycin (Kaiser Foundation Health Plan)
2.2 Amphotericin B (Cerner Multum)

3. Media

3.1 Saboaraud's dextrose broth (Difco)
3.2 Saboaraud's dextrose agar (Difco)
3.3 Tryptic soy broth (TSB)

3.4 Roswell Park Memorial Institute 1640 medium (RPMI-1640) (Sigma)

3.5 Human serum

3.6 Fetal Bovine serum (invitrogen)

4. Chemicals

4.1 Histopaque 1.077 (Sigma-Aldrich)

4.2 Dextran (Fluka)

4.3 *Rhodomyrtus tomentosa*

4.4 99% ethanol (Sigma-Aldrich)

4.5 2.5% glutaraldehyde (Polysciences)

4.6 1% glutaraldehyde (Polysciences)

4.7 Trypan blue (Difco)

4.8 Resazurin (Difco)

4.9 Crystal violet (Merck)

4.10 Wright Giemsa stain (Sigma-Aldrich)

4.11 Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich)

4.12 85% NaCl (NSS)

4.13 5% NaCl

4.14 Phosphate buffer saline (PBS)

5. Equipments

5.1 Blender (Sharp)

5.2 Rotary evaporator (BUCHI Rotavapor R-114)

5.3 Whatman size 1.25 mm (Whatman Schleicher and Schuell)

5.4 FilterPore size 0.45 micron (Fisher Scientific)

5.5 Scanning electron microscope (FEI, Brno)

6. *Rhodomyrtus tomentosa*

The leaves of *Rhodomyrtus tomentosa* were obtained from Sadao District, Songkhla Province in the southern part of Thailand. Classified reference voucher specimens were deposited at Herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkhla University, Hat Yai, Songkhla.

7. Buffy coat Samples

Buffy coat from normal donors was collected at Blood bank of Songklanagarind hospital, Hatyai, Songkhla, Thailand.

8. Human Serum

Fresh human serum was obtained from healthy blood.

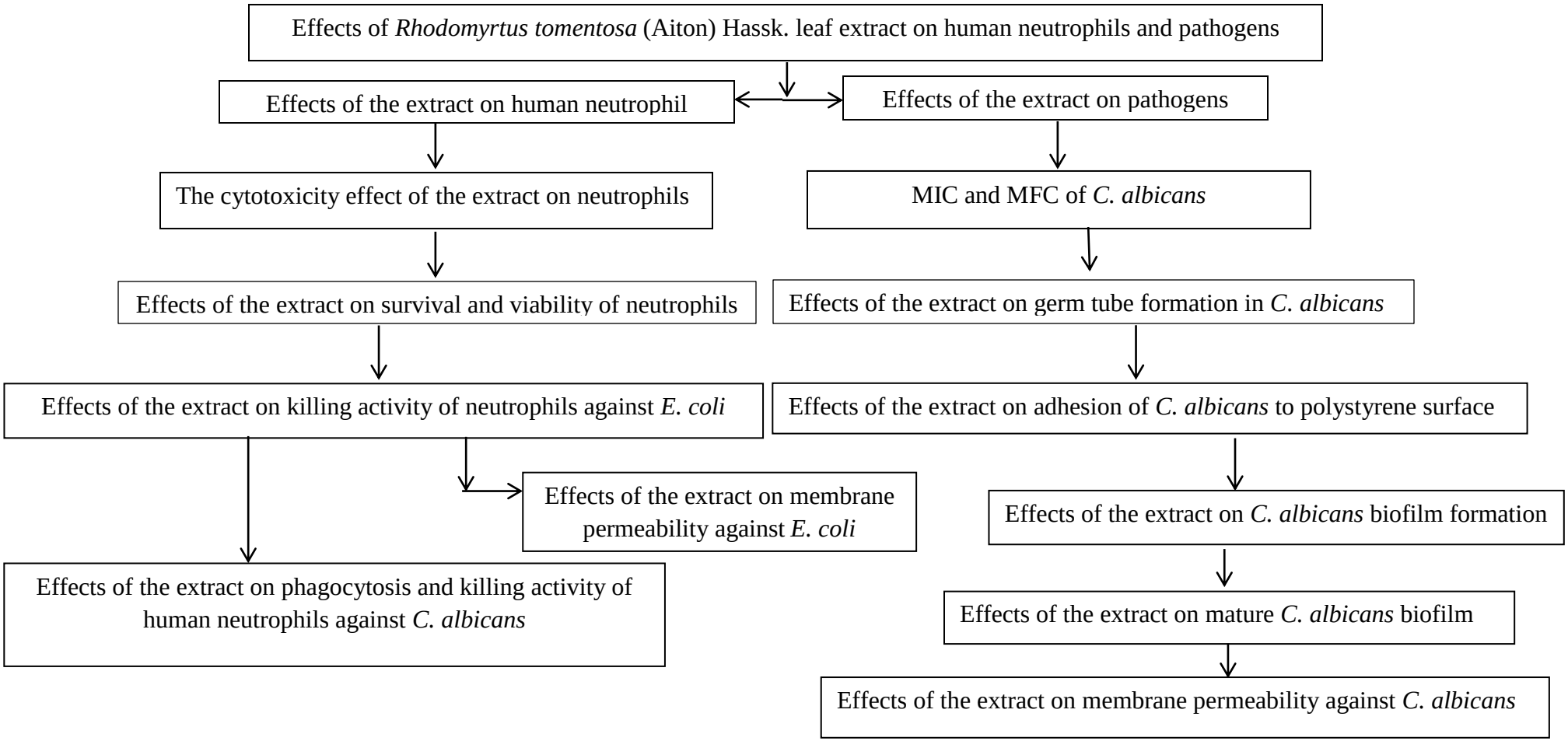


Figure 6. Experimental outline (flow chart) describing the overview of the study

Methods

1. Preparation of crude extract from *Rhodomyrtus tomentosa*

Leaves of *R. tomentosa* were harvested in April, 2011 from Sadao District, Songkhla Province in the southern part of Thailand. The obtained samples were washed with distilled water, dried, pulverized into powder, soaked in 95% ethanol (Lab –Scan Analytical Science, Thailand) at room temperature for seven days. After incubation, the ethanol layer was collected, filled through a Whatman filter paper 125 mm (Whatman Schleicher and Schuell), and concentrated to complete dryness under reduced pressure in a rotary evaporator at 45^{o}C. After evaporation, a green solid of ethanol was obtained and deposited at -20°C further experiments. The yield of the extraction was calculated by dividing the final weight of the dried extract with the weight of dry material and multiplying by 100.

$$\% \text{ Yield} = \frac{\text{Final weight of the dry extract}}{\text{Final weight of dry material}} \text{ X } 100$$

2. Serum preparation

Whole blood sample from donors were collected. (NCL method ITA-9, 2005). After collection, the blood was allowed to clot at room temperature for 1 h. Remove the clot blood by centrifuged at 2,000xg for 5 min and collect the serum (supernatant), and maintained at 4ºC for further study.

3. Opsonization

Opsonization was performed by incubating bacterial/fungal suspension in TSB/RPMI-1640 medium containing 10% human serum at 37ºC for 30 min. After conditioning, the cell suspensions were centrifuged at 2,000xg for 10 min and washed thoroughly with PBS buffer for two times. The cells were diluted with Roswell Park Memorial Institute 1640 medium (RPMI-1640, Sigma-Aldrich, USA) to reach cell density of 10^6cells/ml. The number of opsonized cells was estimated by counting in haemocytometer.

4. Isolation of human neutrophils

Five packs of buffy coat were extracted from healthy volunteers' whole blood that was kindly supplied from Blood bank of Songklanagarind hospital. Ten milliliters of each buffy coat was mixed with 6% dextran (Fluka, Switzerland). The combination was gently shaken and allowed to separate at room temperature for 1 h to sediment erythrocytes. White blood cells in the top layer were carried out in a sterile test tube. Leukocytes were performed slowly and carefully lay in Histopaque-1.077 (Sigma-Aldrich, USA) solution and centrifuged at 700xg for 35 min at 20°C for neutophils isolation (Strasser, et al., 1998). The erythrocyte was existent at the bottom of the tube and granulocyte was the next layer of red blood cell layer. Remove the upper of granulocyte layer with a pipette carefully avoid mixing with the layer. The viability and purity of neutrophils were assessed by trypan blue (Difco) exclusion assay (Strober, 2001) and Wright Giemsa stain (Sigma-Aldrich) respectively were greater than 95%.

5. Oral isolates and identification of *C. albicans*

A swab of an oral cavity from each individual was collected and consequently inoculating on to Sabouraud's dextrose agar (SDA, Difco, USA) (Ethical

Approval No. REC 59−241−19−6). Germ tube formation is identification and characterization of *C. albicans* and other *Candida* species. Then *Candida* isolates were cultured in human serum at 37°C for 3 h. The germ tube test associates to induce filamentous growth (Betzen, *et al.*, 2016).

6. Culture conditions

Microorganisms were used in the study, *Candida albicans* ATCC 90028, *C. albicans* NCPF 3153, *C. albicans* NPRCoE160101, NPRCoE160105, and NPRCoE160111 were cultured in Saboaraud's dextrose broth (SDB, Difco, USA) while *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 29522, and *Escherichia coli* O157:H7 RIMD 0509107 were grown in tryptic soy broth (TSB, Difco, USA) at 37^0C for overnight. The organism cells were pelleted at 2,000xg for 7 min, washed twice with phosphate buffer saline (PBS) (pH 7.4). The cell pellet was resuspended in RPMI-1640.

7. Determination of cytotoxic activity of *Rhodomyrtus tomentosa* extract against neutrophils

To determine the cytotoxic activity of *R. tomentosa* extracts on human neutrophils following the modified method for determination cell viability (Talwar, 1974). Neutrophils ($1x10^6$cells/ml) were incubated with various concentrations (0−2,048 µg/ml) of the extract for 30, 60, and 90 min at 37^0C. After incubation, the viability of cells was determined by trypan blue dye exclusion method (Wheeler, *et al.*, 2008).

8. Effect of *R. tomentosa* extract on phagocytosis of *C. albicans* ATCC 90028 and *C. albicans* NCPF 3153 by neutrophils

The examination was done by following the method of Bin-Hafeez et al (Bin-Hafeez, et al., 2003). One hundred microlitres of *R. tomentosa* extract (0−250 µg/ml) along with 100 µl of neutrophils (10^6cells/ml) were transferred to sterile 1.5 ml eppendorf tubes. One hundred microlitres of the diluted opsonized cell suspensions of *C. albicans* were separately inoculated into each of the tubes. In each assay tube, neutrophils treated with 0.5% DMSO alone were used as controls. The test tubes were incubated at 37^0C for 10, 30, and 60 min with moderate shaking to expose *C. albicans* cells to the extract and neutrophils. The mixture in the tube test was smeared onto a clean glass slide, air dried, and stained with Wright Giemsa stain (Sigma-Aldrich). The slides were examined under light microscopy (X1000). At least 100 neutrophils were counted and score of phagocytosis was expressed as percentage phagocytosis and phagocytic index (PI).

$$\text{Percentage phagocytosis} = \left[\frac{\text{Numbers of neutrophils engulf}}{\text{Total neutrophil count}} \right] \text{X } 100$$

$$\text{Phagocytic index} = \left[\frac{\text{Number of phagocyted particles}}{\text{Infected Neutrophils}} \right] \text{X } 100$$

9. Survival assay of the organisms

The methods described by Wanten (Saeedi, *et al.*, 2017) were followed. Equal volumes (100 µl) of opsonized cell suspensions of *S. aureus*, *E. coli* ATCC 25922, *E. coli* O157:H7, *C. albicans* ATCC 90028, and *C. albicans* NCPF 3153 were exposed to neutrophils suspension (10^7 cells/ml) with *R. tomentosa* extract or 0.5% DMSO were

added to sterile plastic tubes and incubated at 37^0C at various time with shaking. Ten microliters of treated and untreated pathogenic cell suspensions were plated on agar media. The number of colonies was counted after incubation at 37^0 C for 24 h.

10. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

Minimum inhibitory concentration of *R. tomentosa* extract was tested as previously by the two-fold serial dilution method. The dried extract was dissolved in 100% DMSO to obtain 1000 g/mL stock solutions. Diluted samples were then two-fold serially diluted in Sabouraud Dextrose Broth for the organism to obtain a concentration range of 1.024 mg/mL to 32.768 mg/mL. The pathogen was treated with various concentrations of extract on to each well, while the control contained only the organisms. Microtiter plate was incubated in incubators at 37 ˚C for 24 h. The lowest concentrations, which did not show any growth of the fungi after evaluation was demonstrated as MIC. To determine the MFC, the sample from the well defined as having the MIC and the wells with other higher concentrations were spotted on agar plate and incubated at 37 ˚C for 24. The MFC was considered as the lowest concentration of the extract that did not permit any visible fungal outgrowth on Sabouraud Dextrose agar plate after the period of incubation.

11. Germ tube formation assay of *Candida albicans*

Germ tube test was achieved by following the method of Mackenzie (Mackenzie, 1962). Briefly, *C. albicans* yeast cell suspensions ($1x10^6$ cells/mL) were grown in one ml of Sabouraud Dextrose Broth without and with the extract (16−256 µg/mL). The tubes were mixed gently incubated with human serum to induce yeast form to hyphal form at 37^0C for 180 min. Each cell was added 1% glutaraldehyde in PBS for fixation. Twenty five microliters of suspension test and control were dropped on a slide,

covered with a coverslip, and observed under microscope for germ tube formation. Three hundred cells were counted in each test, the numbers of germ tube form and yeast form were documented and the percentage germ tube formation was calculated. The germ tube forms were yeast cells with germ tube that outgrowth longer than the diameter of the mother cell without any constriction at the junction between the mother and the elongated cells (Vale-Silva, et al., 2007). Clumping of yeast form and/or germ tube forms, and pseudohyphal forms were excluded. All the tests were repeated two times. And germ tube assay was performed in a 96-well flat bottom microtitre plate (Krasowska, et al., 2009).

12. Mycelium formation of *Candida albicans*

C. albicans ATCC 90028 and *C. albicans* NCP 3135 were grown overnight in SDB. And then, 1×10^6 cells/mL with or without the extract were incubated in RPMI 1640 medium plus 10% FBS at 37°C for 16 h. Inhibition of the fungi mycelial development was quantified by counting the number of individual budded cells versus the number of hyphae in the population as previously expressed (Toenjes, *et al.*, 2005). More than 100 cells were counted for each well in duplicate. The experiments were performed in duplicate.

13. Adhesion assays on polystyrene surface of *Candida albicans*

The assay was carried out by following the method of Krasowska (Krasowska, *et al.*, 2009) and Pettit (Pettit, *et al.*, 2009). One hundred microliters of fungal suspensions ($1X10^7$cells/mL) were cultured with 100 µl of various concentrations for the extract (16−1024 µg/ml) or 1% DMSO was used as control at 37^0C for 4 h. After incubation, the extract and planktonic cells were eliminated and adherent cells in the wells were gently washed twice with NSS. In consequence, the adherent fungal cells in the plate were exposed to resazurin stain (Thermo Fisher Scientific Inc, USA) and incubated at 37^0C for 1 h. Following incubation, the absorbance reading was then taken

at 570 nm and 600 nm using spectrophotometer (Biotek PowerWaveX). The adhesion assays were performed in duplicate, percent difference in reduction between treated and control cells in adhesion assay that was amount of reduction of adhesion in the test well of that in the control (Pettit, *et al.*, 2005).

14. Biofilm formation of *Candida albicans*

Biofilm assay was achieved by following the method of Pettit (Pettit, et al.2005). Briefly, 100 µl of the cell suspensions ($1X10^7$cells/ml) were cultured with 100 µl of various concentrations of the extract (16−1,024 µg/ml) in 96-well flat bottom microtiter plate. One percentage of DMSO was used as solvent control. The plate was incubated at 37^0C for 24 and 48 h. Following incubation, the sample was rinsed with NSS to remove non-adherent cells and biofilm growth in the plate was quantified by resazurin assay. Briefly, 100 µl of freshly prepared resazurin solution (20 µl of resazurin with 80 µl NSS) was added to each well and incubated at 37^0C for 1 h. Following incubation, the absorbance of the solution was measured at 570 nm and 600 nm by the spectrophotometer. The biofilm formation assays were performed in three replicates at least twice, percentage difference in reduction between treated and control cells in biofilm formation assay that was amount of reduction of biofilm formation in the test well of that in the control.

15. Matured *Candida albicans* biofilm

To investigate the effects of the extract on matured biofilm, initially, the yeast cells were grown to develop biofilm in flat bottom well plate (Krasowska, et al., 2009) and incubated at 37^0C for 24 and 48 h. Following incubation, the plate was washed two times with NSS and 200 µl of fresh RPMI 1640 medium containing several concentrations (16-1,024 µg/ml) of the extract were added. RPMI 1640 medium with 1 % DMSO was retained as control. The sample was further incubated for 24 h at 37°C.

Following incubation, the cell was washed with NSS to remove non-adherent organisms. The biofilm in the plate was exposed to resazurin solution as mentioned above and incubated at 37^0C for 1 h. After incubation, the absorbance of the solution was measured at 570 nm and 600 nm by the spectrophotometer. The quantification of mature biofilm formation were performed in three replicates at least twice, percentage difference in reduction between treated and control cells in the assay that was amount of reduction of biofilm formation in the test well of that in the control (Pettit, *et al.*, 2005).

16. Scanning electron microscopy (SEM)

The morphological changes in formation of germ tube, biofilm and mature biofilm formation of yeast cells after treatment with the extracts were explored by scanning electron microscopy (Herrero, *et al.*, 1999). Briefly, changes in both treated (256 µg/ml) and untreated yeast cells form the germ tube induction test in serum, the biofilm architecture of yeast cells after treatment with the extracts in the absence and presence of the extract (512 and 1,024 µg/ml) on glass slide immersed in 24-well flat bottom containing RPMI 1640 medium. In addition, in order to assess the effects of the extract on matured biofilm, yeast cells were initially allowed to form biofilm on glass slide immersed in 24-well flat bottom (Krasowska, *et al.*, 2009) containing the medium for 24 and 48 h and after this condition treated with the extract (512 and 1,024 µg/ml). For SEM studies, the glass slides from each experimental set up were processed according to the previous method. All slides were washed thrice with PBS and air dried, fixed with 2.5% glutaraldehyde for 2 h, and gently rinsed with PBS. The slides were dehydrated with a graded series of ethanol and dried. The dried samples were coated with gold particles and analyzed via a scanning electron microscope (Quanta400).

17. Permeability of membrane of *S. aureus*, *E. coli*, and *C. albicans*

To determine permeability membrane of organisms when treated with the ethanolic extract by crystal violet assay (Vaara and Vaara, 1981). The cells ($1X10^7$cells/ml) were incubated with 1,024 and 2,048 µg/ml of the extract at 35^0C for 8 h. After incubation, the cell suspentions were centrifuge and resuspended in 0.5%NaCl solution containing crystal violet. They were incubated at 37^0C for 10 min, shaked and centrifuged. Supernatant were measured by spectrophotometer at OD590 nm.

$$\% \text{ uptake} = \left[\frac{\text{OD value of the sample}}{\text{OD value of crystal violet}} \right] X\ 100$$

18. Statistical analysis

Statistical significance was calculated by analysis of variance (Anova). Comparisons between means were carried out according to Dunnett test. *P* values lower than 0.05 were considered statistically significant.

CHAPTER 3

RESULTS AND DISCUSSION

The cytotoxicity effect of *R. tomentosa* extract on human neutrophils

The use of plant products as immunostimulants has a traditional history. Apart from natural mechanisms, there are additional factors that stimulate and suppress host immunity. However, there has been very little research reported on the effectiveness of *Rhodomyrtus tomentosa* leaf extract that are involved in host's immune system. The aim of this study was to evaluate the cytotoxicity of the extract. Human neutrophils were incubated with various concentrations (8−2,048 μg/mL) of the extract for 30, 60, and 90 min at 37°C. After incubation, the viability of cells was determined by trypan blue dye exclusion method (Talwar, 1974). After 90 min, more than 90% neutrophils survived when treated with various concentrations of the ethanol extract. The effectiveness of the extract was verified in figure 7 for all concentrations. The results showed that the extract exhibited nontoxic for human neutrophils. No significant differences were observed between treated and untreated cells ($p<0.05$). As shown in figure 8, 2−8 mg/mL of the extract did not cause any cytotoxic effect on the total cell population. Only the extract used at 10 mg/mL was found to induce a significant decrease of viability ($p<0.05$).

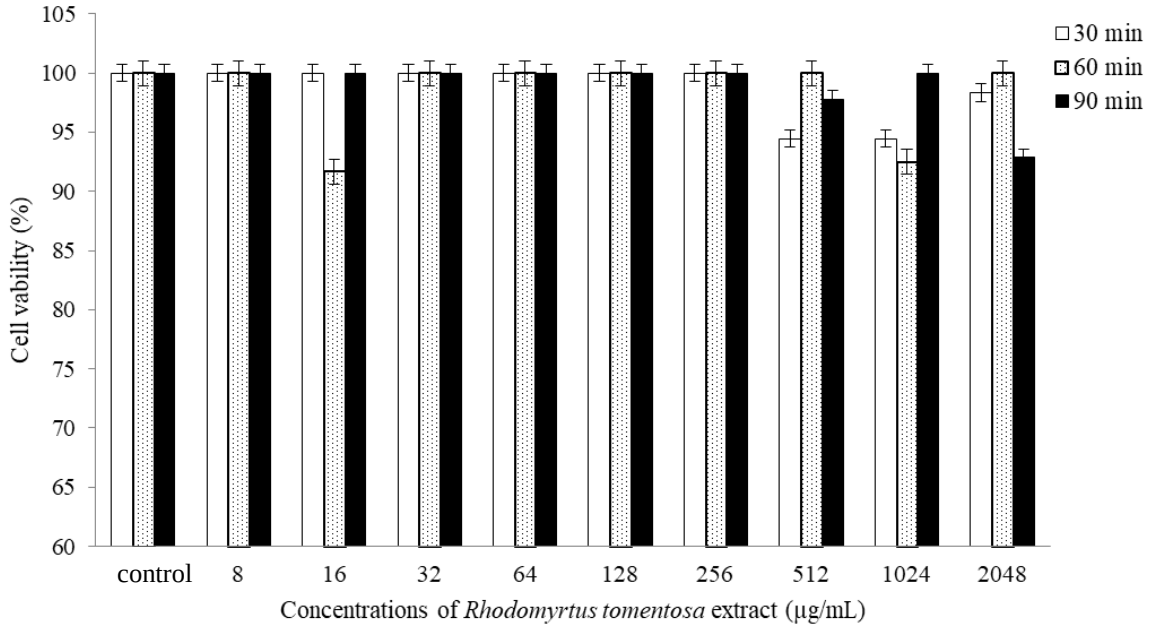


Figure 7. The cytotoxicity effect of *R. tomentosa* extract concentrations ranged from 8 to 2048 µg/mL on human neutrophils at 30, 60, and 90 min on survival of human neutrophils. * Statistical significance at $p < 0.05$.

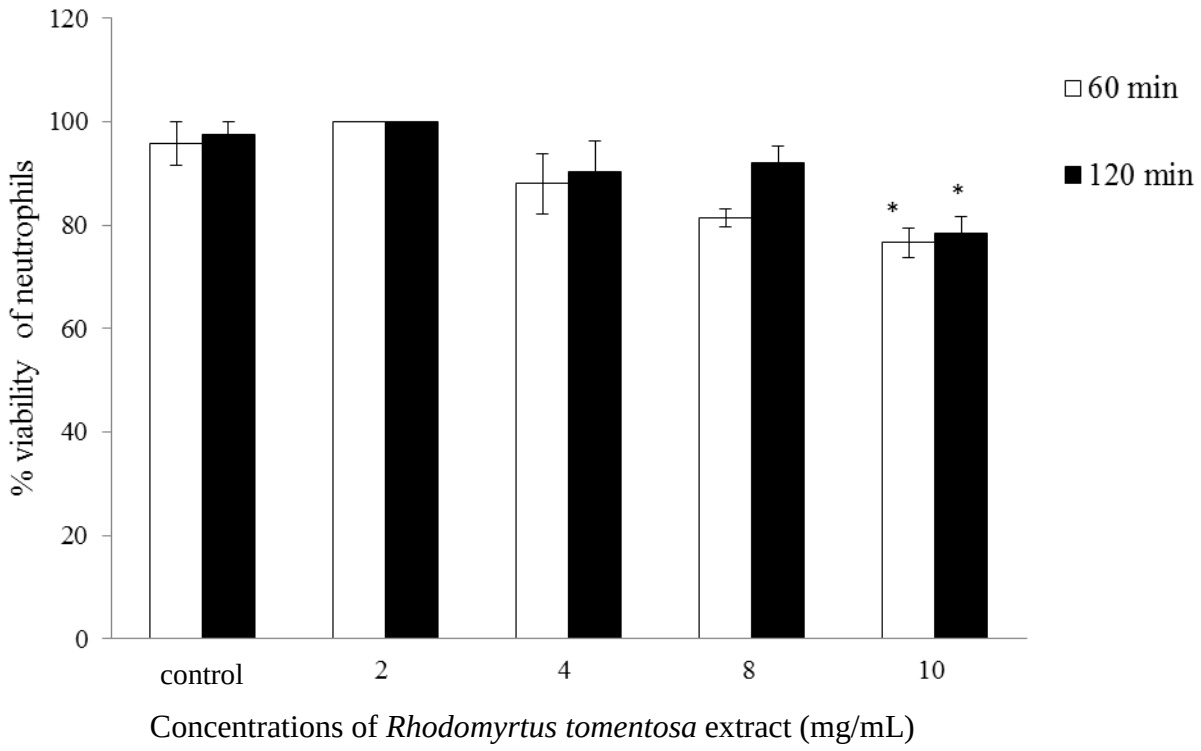


Figure 8. Effects of *Rhodomyrtus tomentosa* extract on human neutrophils. Neutrophils were maintained in suspension culture at 37°C with and without the extract at a concentration 0-10 mg/mL as designated above. After incubation for 60 and 120 min, aliquots of the neutrophil suspensions were prepared and scored for dead cell by microscopy after staining with tryphan blue. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as * $p<0.05$.

Effects of 0.5%, 1%, and 2% dimethyl sulfoxide (DMSO) on survival and viability of human neutrophils

Dimethyl sulfoxide (DMSO), an organic solvent, is used to dissolve *R. tomentosa* extract. DMSO has been shown to have pharmacological and pathological effects on immune system (Elisia, *et al.*, 2016). Human neutrophils play a key role in innate host defenses against invading pathogens but the effect and mechanism of DMSO on neutrophils has not been studied. The present study demonstrated that exposure of neutrophils to 0.5%, 1%, and 2% DMSO for 30, 45, and 60 min did not significantly affect cellular viability (Figure 9).

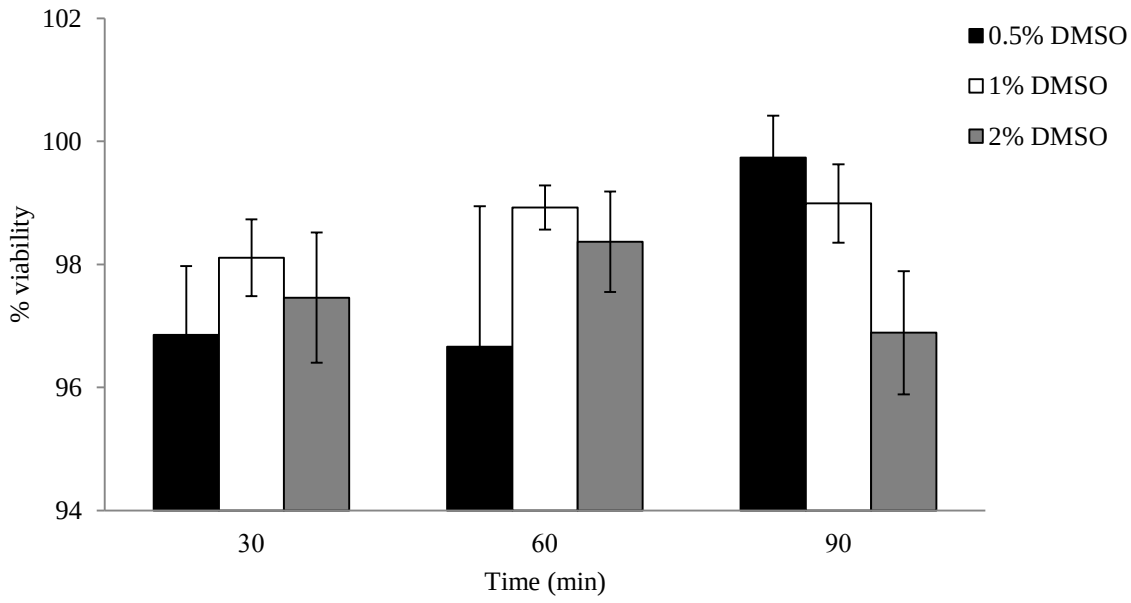


Figure 9. Effects of 0.5%, 1%, and 2% DMSO concentrations on viability of human neutrophils in culture. General growth profile of incubated with 0.5% concentrations of DMSO for 30, 60, and 90 min. Data are expressed as mean ± standard error from two independent experiments. Each measure was performed in triplicate. * Statistical significance at $p < 0.05$.

Effects of *R. tomentosa* extract on killing activity of neutrophils against *E. coli* strains

Immunostimulatory activity of plants in enhancing the killing effect of the host immune cells is an attractive approach to overcome the usage of antibiotics in controlling the infection caused by pathogenic bacteria (Ghule & Yeole, 2012, Chitemerere & Mukanganyama, 2014). Therefore, in the present study, potential of the ethanolic leaf extract of *R. tomentosa* was evaluated for its ability in increasing killing activity of human neutrophils against *E. coli* O157:H7. The percentage survival of *E. coli* after exposed to neutrophils in the presence of the extract is given in figure 10A and B. It was observed that the extract alone at the tested concentrations of 62.5–250 µg/mL had no direct killing activity on the organisms. However, the extract enhanced the killing activity of neutrophils towards *E. coli*. At 30 min, the extract at 62.5–250 µg/mL showed a moderate effect in enhancing the killing activity of neutrophils against both *E. coli* strains. However, a significant increase in the activity of neutrophils was observed after 45 and 60 min ($p<0.05$). At 45 min, survival percentage of *E. coli* O157:H7 and *E. coli* ATCC 25922 when exposed to neutrophils in the presence of 62.5, 125, and 250 µg/mL of the extract were 94.15%, 58.48%, 50.28%, and 73.15%, 69.13%, and 35.35 %, respectively. Similarly, at 60 min, survival percentage of *E. coli* O157:H7 and *E. coli* ATCC 25922 after being incubated with neutrophils in the presence of the same concentrations of the extract was 59.91%, 50.34%, 40.15% and 78.79%, 58.46%, and 47.02%, respectively. In the earlier study, it was demonstrated that DMSO up to 1% did not affect the killing activity of human neutrophils against *E. coli* (Czuprynski, *et al.*, 1984). In the present work, the solvent was used at the highest concentration of 0.5% (v/v) for control and treatment samples. From the results of cell survival assay, the killing activity of neutrophils increased with the increased addition of the extract. Therefore, it is envisaged that the solvent at the tested percentage did not interfere with the observed effects with neutrophils and demonstrated the ability of *R. tomentosa* extract to enhance the killing activity of human neutrophils against *E. coli*. In the earlier studies, other plant

resources have been reported to enhance the activity of host immune cells against pathogenic organisms. Serafino et al., (Serafino, *et al.*, 2008) reported that, upon treatment with *Eucalyptus* essential oil, the phagocytic ability of macrophages increased towards *Staphylococcus aureus*. In another study, iridoids fraction obtained from methanol extract of *Barleria prionitis* enhanced the intracellular killing activity of neutrophils against *Candida albicans* (Ghule & Yeole, 2012).

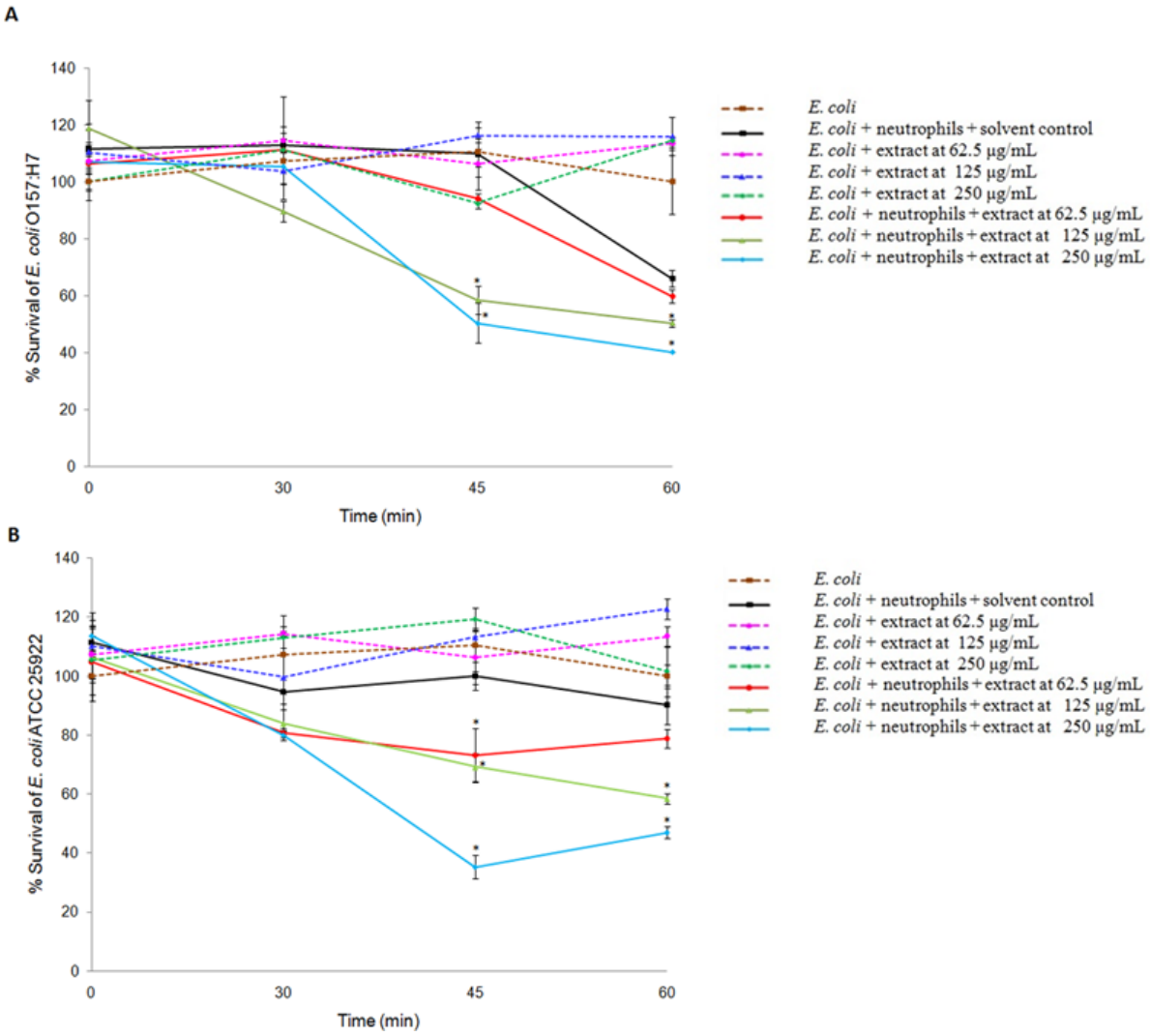


Figure 10. Effects of *R. tomentosa* extract on the killing activity of human neutrophils against *Escherichia coli* O157:H7 RIMD 05091078 (A) and *E. coli* ATCC 25922 (B). The bacterial cells were exposed to neutrophils in the absence and presence of *R. tomentosa* extract (62.5–250 µg/mL). *Escherichia coli* cells in the presence of the extract (62.5–250 µg/mL) alone were also maintained. Dimethyl sulfoxide (0.5% v/v) was used as solvent control. The percentage survival of the organisms was determined at 0, 30, 45, and 60 min. Data are expressed as mean ± standard error of the mean from two independent experiments. Each measure was performed in triplicate. * Statistical significance at $p < 0.05$.

Effects of *R. tomentosa* extract on membrane integrity of *E. coli*

Membrane permeability or integrity plays a critical role as a barrier in Gram-negative bacteria which protects the bacterial cells from the action of antimicrobial compounds (Ghule & Yeole, 2012, Chitemerere & Mukanganyama, 2014). Alteration in the membrane permeability could lead to easy passage of antimicrobial drugs into the cells resulting cell death (Ghule & Yeole, 2012, Li, *et al.*, 2013, Chitemerere & Mukanganyama, 2014, Yadav, *et al.*, 2015). In addition, defects in the membrane integrity may increase the susceptibility of the bacterial cells to host immune attack. In the present study, since the extract alone at the tested concentrations did not produce any considerable effects on the survival of *E. coli* cells, we evaluated the possible impact of *R. tomentosa* extract against the organisms by membrane permeability assay at the time intervals as those used in a similar set up mentioned in cell survival assay. It was observed that, upon treatment with the extract, uptake of crystal violet by the cell membrane of the organisms was significantly increased. Crystal violet uptake by *E. coli* O157:H7 and *E. coli* ATCC 25922 after 8 h was 4.89% and 1.95%, respectively. However, upon being treated with the extract at 62.5–250 µg/mL, uptake of the dye was 24.43%–40.07% in *E. coli* O157:H7 and 22.48%–36.16% in *E. coli* ATCC 25922. Even after short time exposure to *R. tomentosa* extract, the organisms showed alteration in their membrane permeability. The uptake of dye by *E. coli* O157:H7 at the similar time interval mentioned in the cell survival assay at 30, 45, and 60 min after the extract treatment at 62.5–250 µg/mL was 8.67%–14.67%, 8.00%–24.00%, and 18.47%–21.02%, respectively. Similarly, the uptake of dye by *E. coli* ATCC 25922 at 30, 45, and 60 min after the extract treatment at 62.5–250 µg/mL was 12.00%–22.00%, 8.67%–18.00%, and 19.11%–24.20%, respectively (Figure 11A and B).

Therefore, it is demonstrated that the extract exhibited membrane permeabilizing activity rather than antibacterial activity at the tested concentrations, and resulted in the alteration of membrane integrity of *E. coli*. The attained result is in accordance with the previous study, in which membrane permeability of *E. coli* was

disrupted after being treated with corilagin; a tannin group of compound resulted in increased uptake of crystal violet dye (Li, *et al.*, 2013). In another report, eugenol, a phytochemical from clove essential oil affected the membrane integrity of *S. aureus* (Yadav, *et al.*, 2015). We would also like to mention here that in our previous report, an acylphloroglucinol component, rhodomyrtone from the leaf extract of *R. tomentosa* showed immunomodulatory effects on THP-1 human monocyte cell line leading to enhanced killing of methicillin-resistant *Staphylococcus aureus* (Srisuwan, *et al.*, 2014). Therefore, it is anticipated that the extract possesses both membrane permeabilizing as well as immunomodulatory properties resulting in the increased susceptibility of *E. coli* cells to immune cell attack.

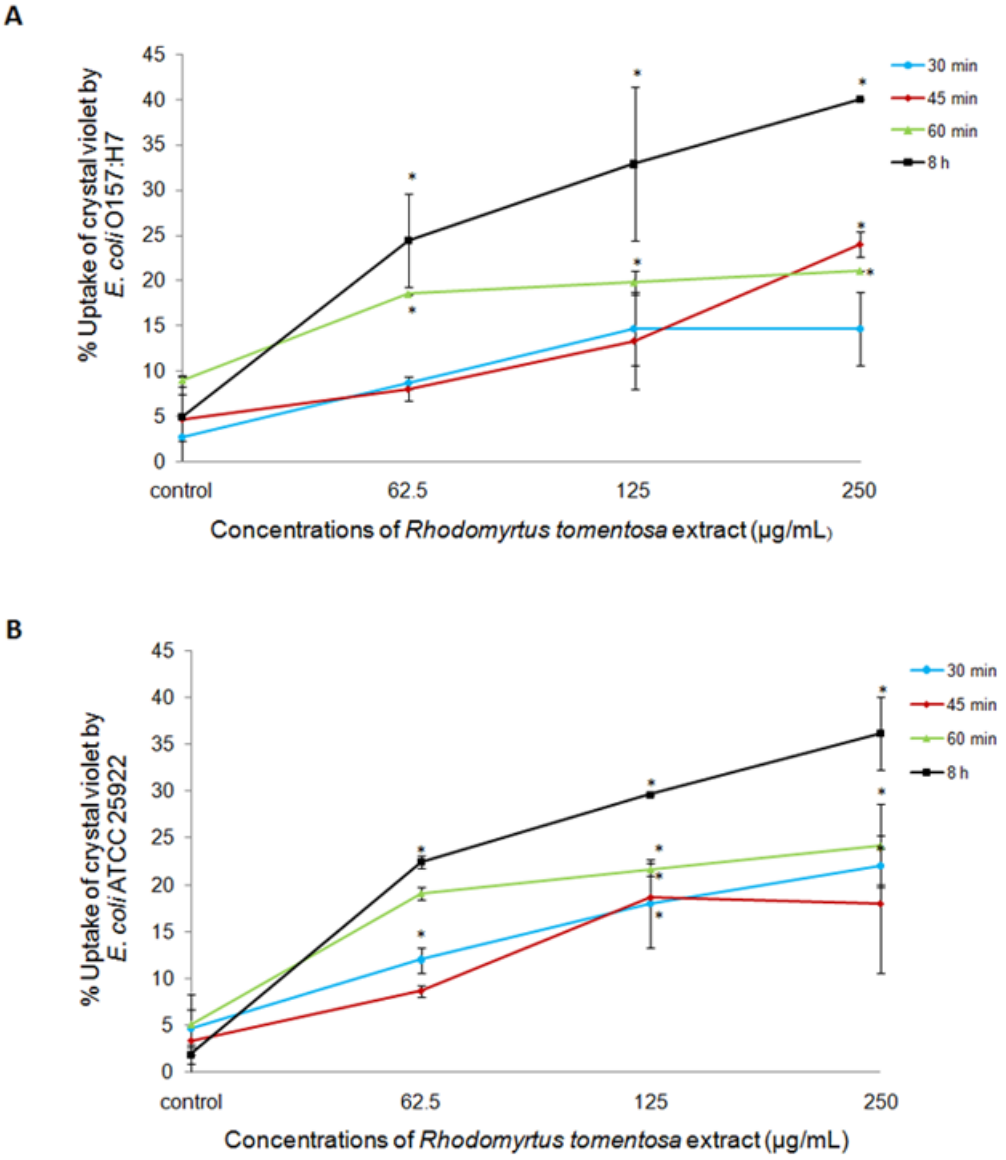


Figure 11. Effects of *R. tomentosa* extract on membrane permeability of *Escherichia coli* O157:H7 RIMD 05091078 (A) and *E. coli* ATCC 25922 (B) Crystal violet uptake by *E. coli* at 30, 45, 60 min, and 8 h after treated with the extract at 62.5, 125, and 250 µg/mL was determined. Dimethyl sulfoxide (0.5% v/v) was used as the solvent control. Data are expressed as mean ± standard error from two independent experiments. Each measure was performed in triplicate. * Statistical significance at $p < 0.05$.

Effects of *R. tomentosa* extract on phagocytosis and killing activity of human neutrophils against *C. albicans*

Modulation of the innate immune response by several medicinal plants may provide new approaches to the treatment of candidiasis. Human neutrophils, the most common leukocytes in peripheral blood, play a key role against *C. albicans*. In the present study, immunomodulatory effects of *R. tomentosa* extract on human neutrophil function against *C. albicans* were investigated. Following 30 min incubation, treatment of neutrophils with the extract at 50 and 250 µg/mL significantly enhanced phagocytic uptake of the organisms when compared with the control ($p<0.05$). Phagocytic index of neutrophils in the presence of the extract at 50 and 250 µg/mL was 2.21 and 2.30, compared with 1.61 in the control (Figure 12). However, no significant differences were observed for all concentrations of the extract at 10 and 60 min. As shown in figure 13, a significant increase in the killing activity of neutrophils were observed when yeast cells were treated with the extract at 50 and 250 µg/mL within 60 min ($p<0.05$). Meanwhile, there were no significant differences in the killing activity of neutrophils after exposure to all concentrations of the extract at 10 and 30 min when compared with the control ($p<0.05$). The results indicated that *R. tometosa* extract at 50 and 250 µg/mL enhanced phagocytic and killing activities of neutrophils against *C. albicans*. A report showed that neutrophils phagocytosed and killed *C. albicans* yeast form much more efficiently than germ tube form (Scherwitz & Martin, 1979). *C. albicans* biofilm formation and mature biofilm are resistant to neutrophil killing activity (Xie, *et al.*, 2012). Prostaglandin E_2 has been reported to inhibit leukotriene B_4 which is a potent neutrophil chemoattractant and promote their accumulation and adherence to inflammation (Ham, *et al.*, 1983). Neutrophils recruitment is an essential step of phagocytosis and killing activity (Sadik, *et al.*, 2011). In an earlier study, the leaf extract exhibited the ability to stimulate phagocytosis of human neutrophils towards *Escherichia coli* O175:H7 (Hmoteh, *et al.*, 2016). Moreover, an enhancing effect of carotenoid astaxanthin on phagocytic activity of human neutrophils against *C. albicans* has been documented (Macedo, *et al.*, 2010).

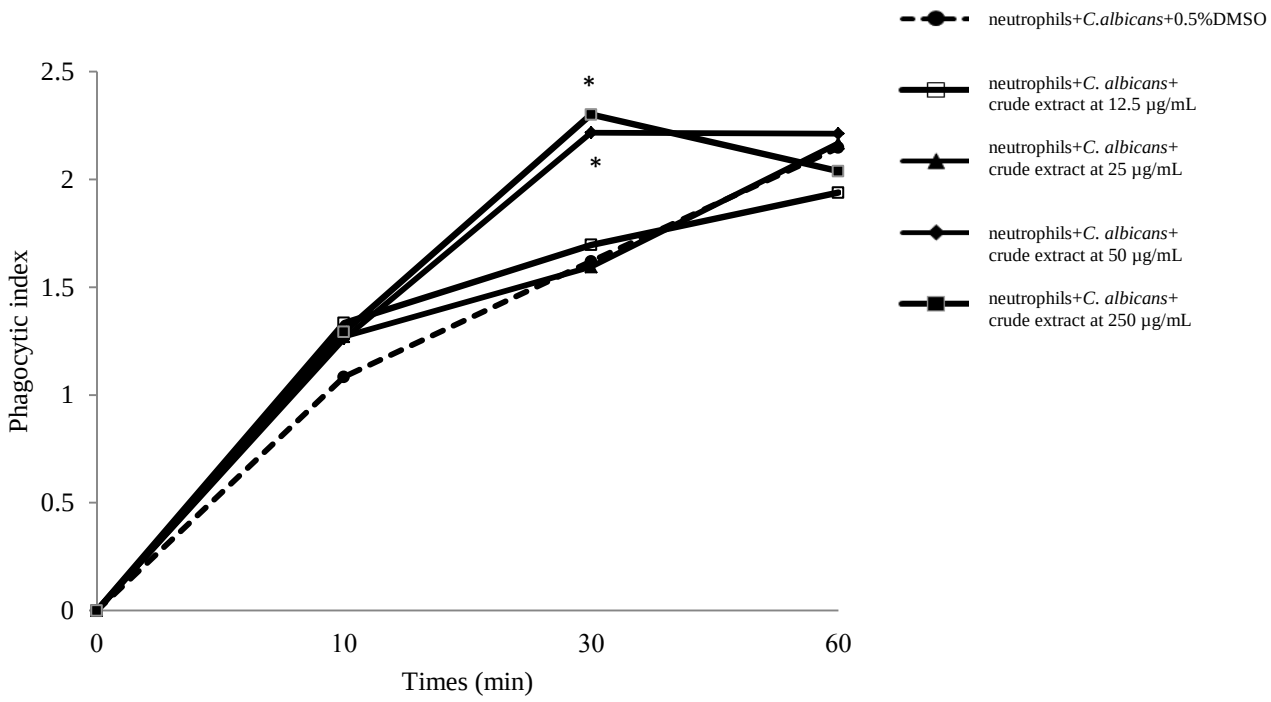


Figure 12. Effects of *Rhodomyrtus tomentosa* extract in enhancing phagocytic index of human neutrophils against *Candida albicans* ATCC 90028. Phagocytic index of the yeast cells was determined at 0, 10, 30, and 60 min after exposure to human neutrophils in the presence of the extract at 0−250 µg/mL. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as * $p<0.05$.

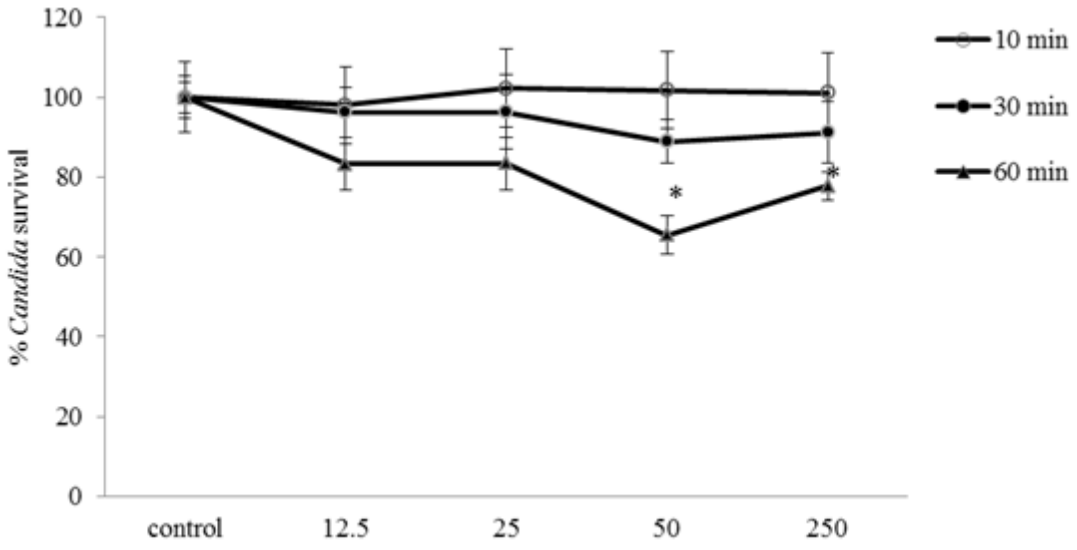


Figure 13. Effects of *Rhodomyrtus tomentosa* extract on phagocytosis of human neutrophils against *Candida albicans* ATCC 90028. Opsonised *C. albicans* and the extract at 0−250 μg/mL were coincubated with human neutrophils ($5x10^6$ cells/mL). Survival of the yeast cells were determined by enumerating colony number at 10, 30, and 60 min and percentage survival was subsequently calculated. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as * $p<0.05$.

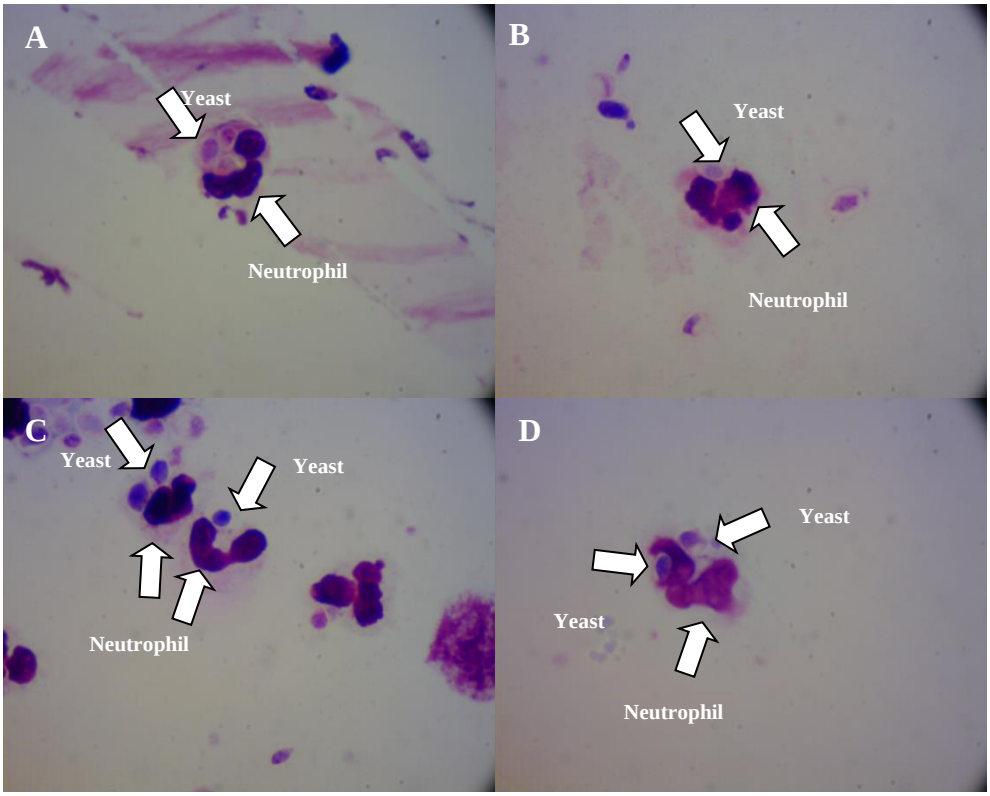


Figure 14. Phagocytosis by human neutrophils was exposed to *Candida albicans* at various concentrations of the extract. Within several minutes, a neutrophil engulfs several particles in succession.

Oral isolates and identification of *C. albicans*

To isolate *Candida* saliva was collected yeasts, mucosa with from the fungal sterile swabs. Duplicate samples were then seeded on Sabouraud dextrose agar (MERCK, Darmstadt, Germany). The standard laboratory method for identifying *C. albicans* is the germ tube test. The test involves the induction of hyphal outgrowths (germ tubes) when subcultured in human serum at 37°C for 2–4 h. Approximately 95% of *C. albicans* isolates produce germ tubes.

Antifungal activity of *R. tomentosa* extract

The result was showed on table 1 that the ethanol extract of *R. tomentosa* possessed antifungal activities against the organisms tested. Two yeasts including *Candida albicans* ATCC 90028 and NCPF 3153 were tested. The extract inhibited the visible growth at a concentration of 2.048 mg/mL and exhibited MFC of 8.192 mg/mL whereas amphotericin B exhibited MIC at 0.25 µg/mL and MFC value ranged between 0.5 and 1.0 µg/mL.

Table 1.

Antifungal activity of *R. tomentosa* extract

Fungi	**MIC (µg/mL)**		**MFC (µg/mL)**	
Candida albicans isolates	*R. tomentosa*	amphotericinB	*R. tomentosa*	amphotericinB
ATCC 90028	2,048	0.25	8,192	0.50
NCPF 3153	2,048	0.25	8,192	1.00

Effects of *R. tomentosa* extract on germ tube formation and mycelia formation in *C. albicans*

Morphological changes of *C. albicans* from yeast form to hyphal form through germ tube formation have been considered as a key role in pathogenesis of the organism. Germ tube development has been documented to be a crucial functional mechanism that enhances adherence of the pathogen to the host cell surfaces. The virulence factor could be an important therapeutic target for antifungal agent development.

In this study, the experiments were performed to verify germ tube production in *C. albicans* after exposure to *R. tomentosa* extract. The yeast cells were induced to form the germ tubes in the presence of the extract at concentrations ranging from 16 to 256 µg/mL. As shown in figure 15, the suppression of germ tube production following 180 min exposure to various concentrations of the extract was significant in comparison with that of the unexposed cells ($p<0.05$). All *C. albicans* isolates, at concentrations range from 128-256 µg/mL of the extract, significantly reduced germ tube formation ($p<0.05$). Nevertheless, an intra-species variation in germ tube inhibition after treatment of the extract was observed. This ranged from 1.36–63.67% for 16–32 µg/mL, 39.87–89.28% for 64–128 µg/mL, and 69.01–95.42% for 256 µg/mL. Certain antifungal agents have been earlier reported to elicit a wide spectrum of germ tube suppression, for example, 5-fluorocytosine, ketoconazole, fluconazole, and 0.0025% chlorhexidine gluconate (Ellepola & Samaranayake, 1998, Ellepola & Samaranayake, 2000).

Furthermore, the inhibitory effect of the extract on germ tube formation was confirmed by scanning electron microscopy. In the absence of the extract illustrated extended outgrowth from the mother cells, as shown in figure 16A and B and figure 17A and B for *Candida albicans* ATCC 90028 and *Candida albicans* NCPF 3153, respectively. In contrast, the treated *Candida albicans* ATCC 90028 and *Candida albicans* NCPF 3153 showed more yeast cells and reduction of germ tube form as shown in figure 16C and D and figure 17C and D, respectively. In addition, both isoltaes

Candida albicans ATCC 90028 and *Candida albicans* NCPF 3153 were almost the same and the growth rates at different concentration and significantly decreased mycelia formation ($p<0.05$). As shown in figure 18, *Candida albicans* ATCC 90028 and *Candida albicans* NCPF 3153 treatments at 256 µg/mL significantly decreased to 20%−40% of the formation.

The transition from yeast to hyphal form was interfered by the extract without affecting the viability of the pathogen. The results suggested that the effect of the extract might due to inhibition of prostaglandin E_2 production associated with germ tube formation (Jeong, *et al.*, 2013). Other anti-inflammatory agents have been reported to inhibit germ tube formation in *C. albicans* through prostaglandin synthesis (Abdelmegeed & Shaaban, 2013).

Certain plant extracts from Myrtaceae family have been reported to inhibit hyphal formation in *C. albicans*. For instance, the crude ethanolic extract of *Pimenta pseudocaryophyllus* with concentrations ranged from 128–256 µg/mL significantly reduced germ tube production in the organisms (Assal, *et al.*, 2014). In addition, essential oils from *Melaleuca alternifolia* and *Eucalyptus globulus* demonstrated inhibitory activity on the formation of germ tube upon treating the pathogen at 312 µg/mL (Noumi, *et al.*, 2010). Other phytochemical constituents in terpenoid group which present in *R. tomentosa* have been demonstrated to prevent dimorphic transition in the yeast cells (Raut, *et al.*, 2013).

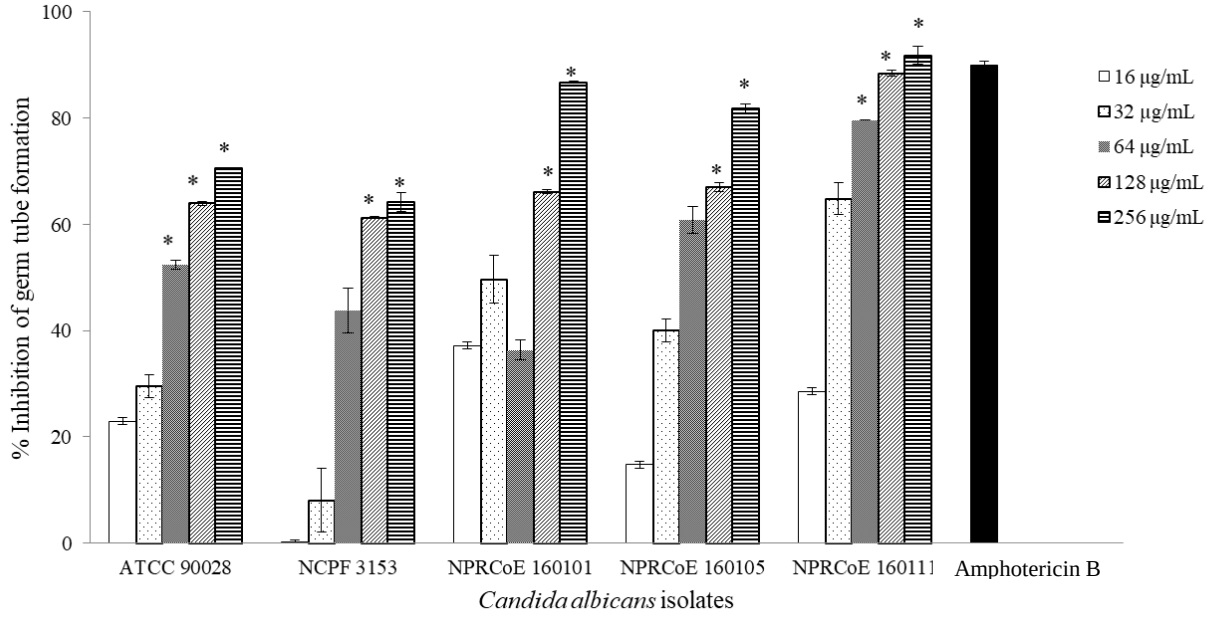
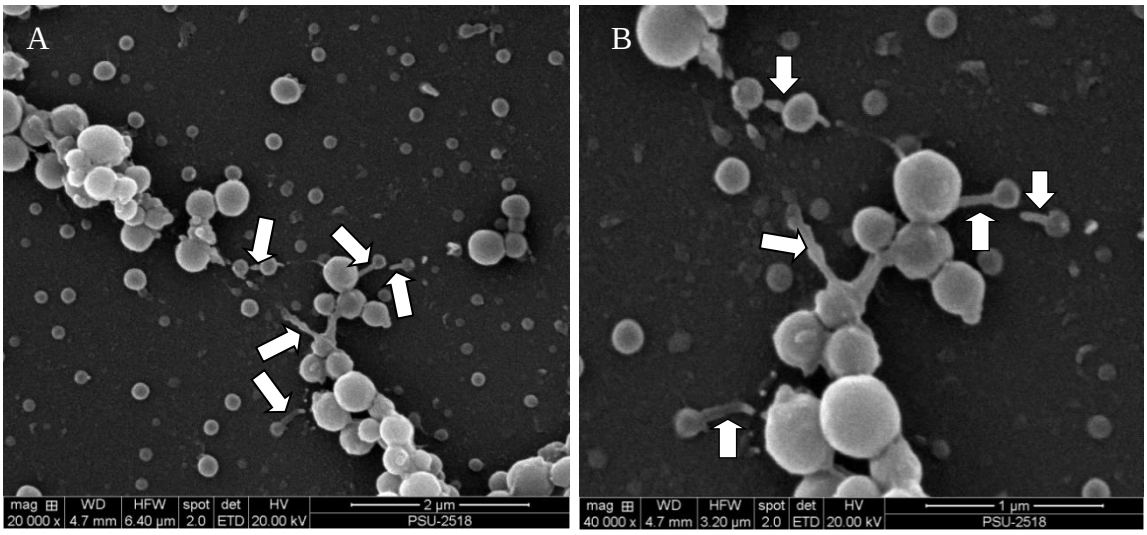


Figure 15. Inhibitory activity of *Rhodomyrtus tomentosa* extract at 16−256 µg/mL on germ tube formation in *Candida albicans*. Amphotericin B was included as positive control. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as * $p<0$.

Untreated *C. albicans* ATCC 90028



Treatment of *C. albicans* ATCC 90028 with *R. tomemtosa* extract at 256 µg/mL

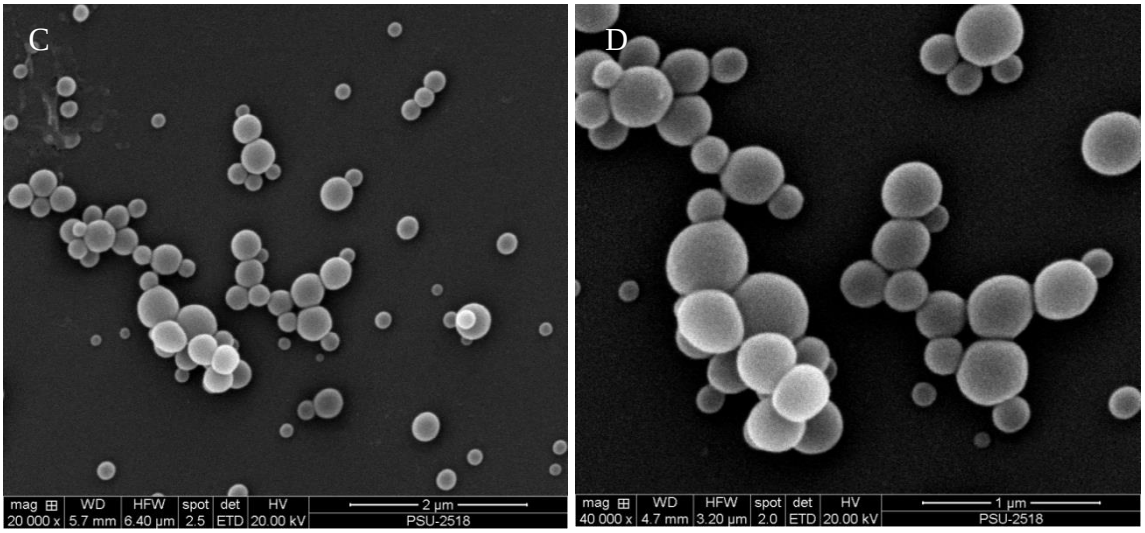
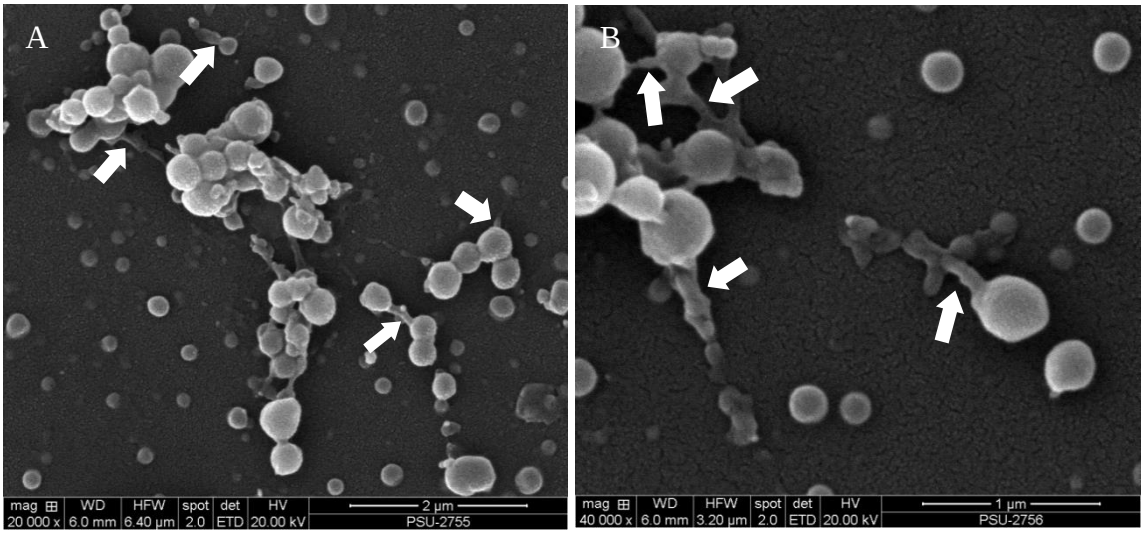


Figure 16. Scanning electron micrographs showed the effects of *Rhodomyrtus tomentosa* extract on germ tube formation in *Candida albicans* ATCC 90028. The yeast cells were grown in RPMI 1640 medium (A and B) and exposed to 256 µg/mL of the extract (C and D). White arrows indicated germ tube−like forms. Scale bars = 1 µm and 2 µm.

Untreated *C. albicans* NCPF 3135



Treatment of *C. albicans* NCPF 3135 with *R. tomemtosa* extract at 256 µg/mL

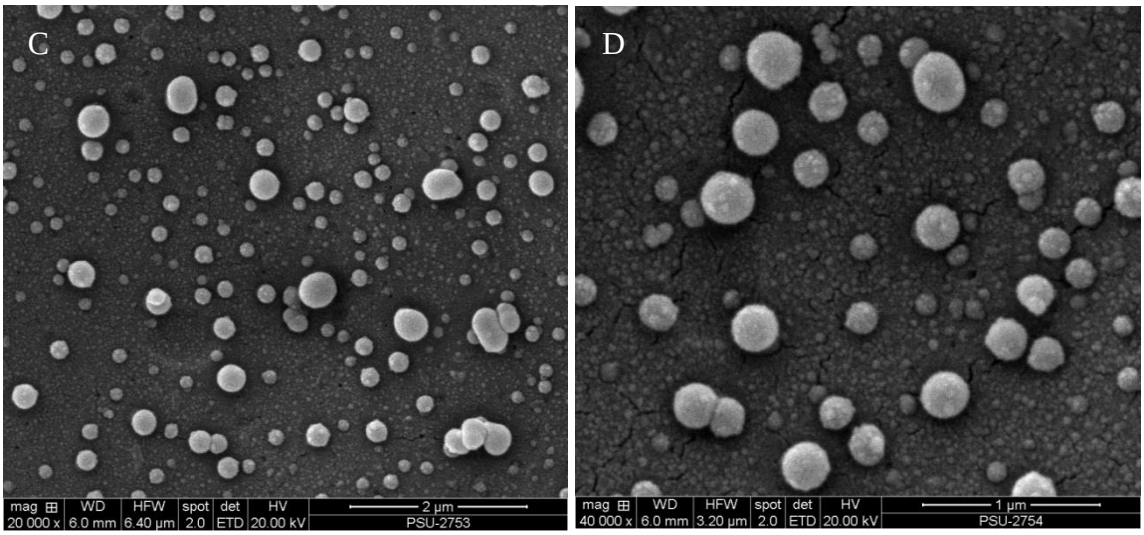


Figure 17. Scanning electron micrographs showed the effects of *Rhodomyrtus tomentosa* extract on germ tube formation in *C. albicans* NCPF 3153. The yeast cells were grown in RPMI 1640 medium (A and B) and exposed to 256 µg/mL of the extract (C and D). White arrows indicated germ tube−like forms. Scale bars = 1 µm and 2 µm.

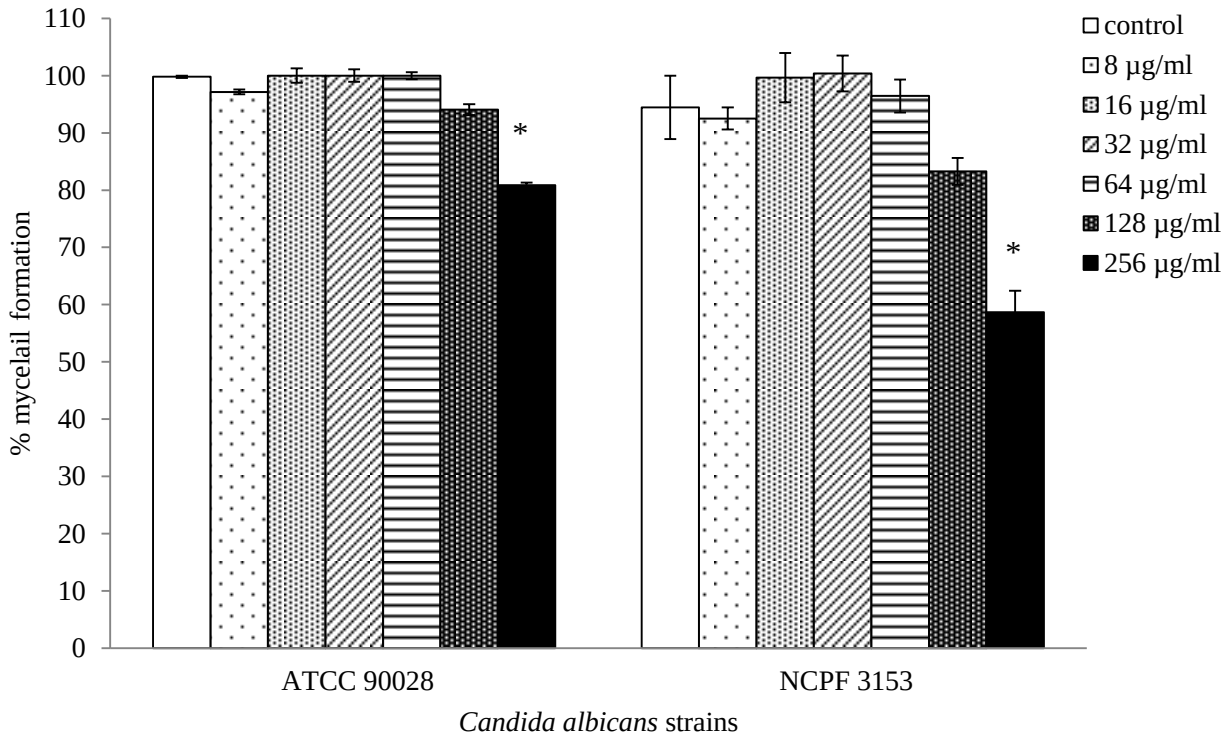


Figure 18. Effect of the *Rhodomyrtus tomentosa* extract on mycelia formation of *Candida albicans* ATCC 90028 and *C. albicans* NCP 3135 incubated with various concentrations (8-256 µg/mL) of plant extract and without which was 0.5% DMSO (control) in fetal bovine serum for 16 h at 37 ^{0}C using light microscope. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as * p<0.05.

Biofilm formation by *C. albicans* on surface of microtiter plates

Biofilm forms of the organism that attach to a substrate, followed by microcolonies formation, productions of multiple cell types including yeast cell, pseudohyphal cells, true hyphal cells, and an exopolymer matrix (Chandra, *et al.*, 2001).

In a preliminary experiment, biofilms formation by *C. albicans* ATCC 90028 demonstrated significant difference from each well of a 96-well microplate ($p< 0.05$). The fungal biofilm form on the surface is quantified by resazurin metabolic assay. The reduction assay showed increased metabolic activity with increase in incubation time. Biofilm growth shows how the increased colorimetric reading obtained by the resazurin reduction assay correlates with increased density cell in the biofilm. Percentage reduction of resazurin is associated with increased viability cell density in the biofilm as shown in figure 19. After 24 h and 48 h incubation time, the pathogens demonstrated a significant increase in biofilm formation in a time dependent manner, compared with other time periods ($p<0.05$).

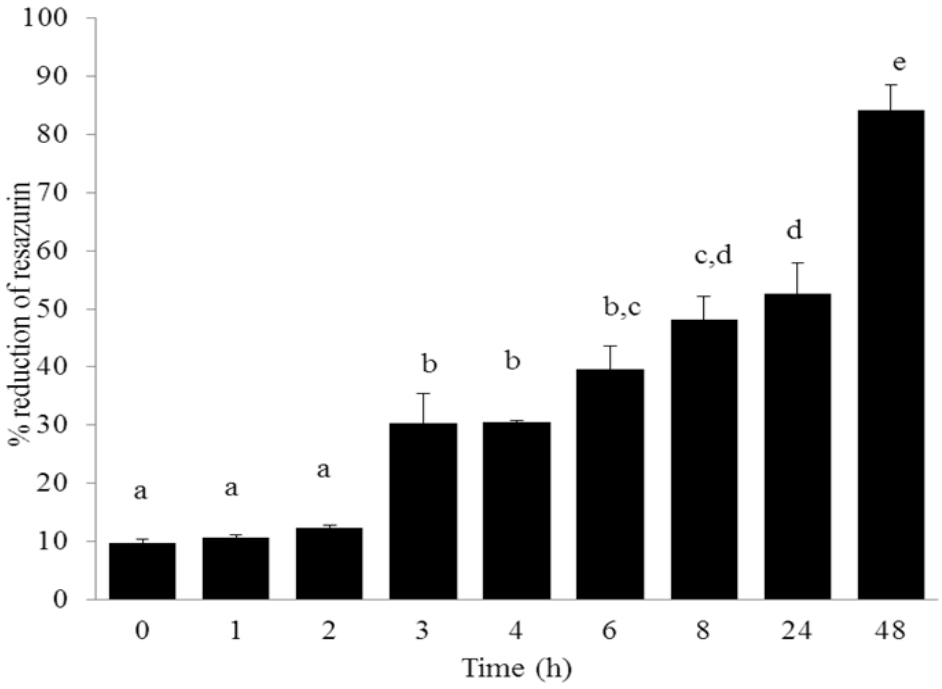


Figure 19. *C. albicans* biofilm formation in 96 microtiter plates as establishd by the colorimetric resazurin reduction assay. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Different letters (a, b, c, d, and e) are significantly difference (p<0.05).

Effects of *R. tomentosa* extract on adhesion of *C. albicans* to polystyrene surface

Adherence of *C. albicans* to oral mucous membranes is a key feature in oropharyngeal infections and their interaction can be regarded as an initial step leading to the disease outcome. The ability of the organisms to attach to medical devices may allow the pathogen entry into host cells (Lawaf & Azizi, 2009).

In this investigation, the effect of *R. tomentosa* extract on microbial adhesion to polystyrene surfaces was determined. The anti-adherence property of the extract was assessed against two representative isolates of *C. albicans*. Both strains demonstrated a significant decrease in their adherence ability to polystyrene surface in a dose dependent manner, compared with the control ($p<0.05$). As presented in figure 20, the percentage reduction resazurin of *C. albicans* ATCC 90028 and *C. albicans* NCPF 3153 after 4 h exposure to the extract at 16−1024 µg/mL was ranged from 83.26%−63.58% and 84.30%−68.31%, respectively. Untreated yeast cells were considered as 100% of resazurin reduction. The results indicated that the extract suppressed the fungal germ tube formation and its property was practically correlated with anti-adherence to cell surfaces. Other worker has previously reported an increase in adherence of the organisms after germ tube formation (Kimura & Pearsall, 1980). This finding was in agreement with previous work that *R. tomentosa* extract inhibited adhesion of the pathogens to buccal epithelial cells (Limsuwan, *et al.*, 2014). Another plant from Myrtaceae family, *Melaleuca alternifolia* has been found to reduce the adhesion of *C. albicans* to biotic and abiotic surfaces (Sudjana, *et al.*, 2012, Tobouti, *et al.*, 2016).

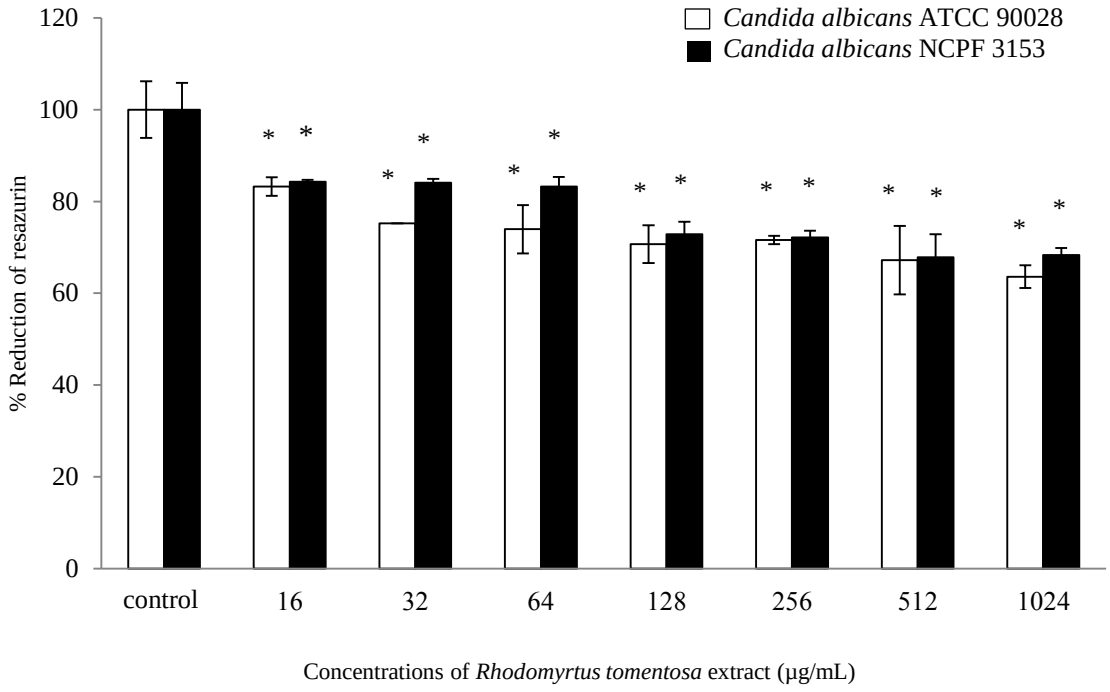


Figure 20. Percent reduction of resazurin at 4 h for adhesion of *Candida albicans* ATCC 90028 and *C. albicans* NCPF 3153 on polystyrene surface while treated with *Rhodomyrtus tomentosa* extract at 16−1024 µg/mL. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as * $p<0.05$.

Effects of *R. tomentosa* extract on *C. albicans* biofilm formation

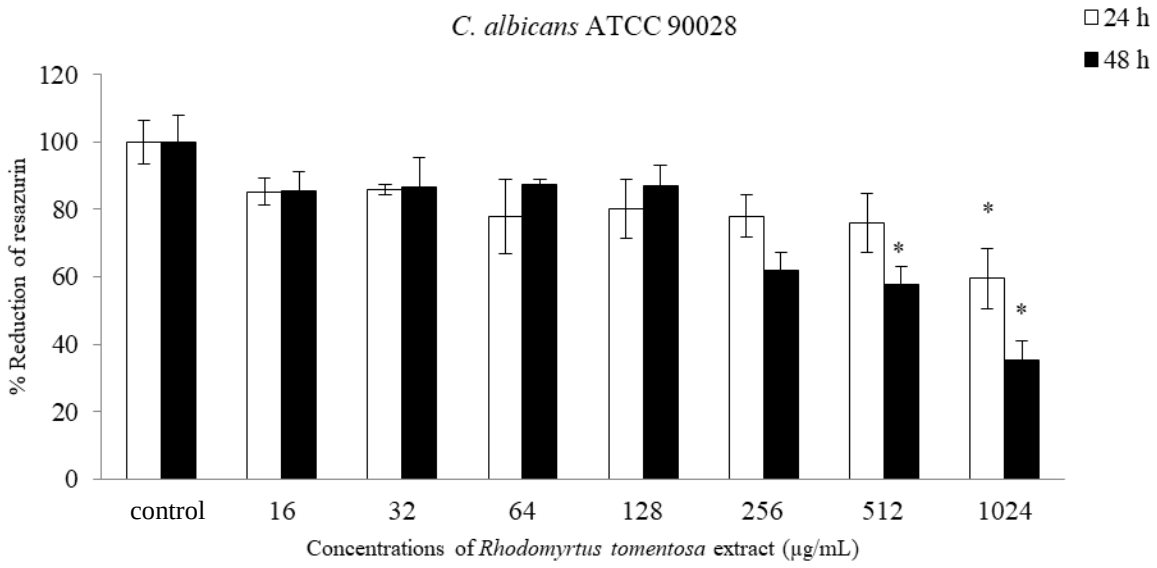
Candida albicans biofilm is composed of yeast cells and filaments that enclosed in an extracellular matrix which adhere to the mammalian cell surface as well as inert surface (Hawser & Douglas, 1994, Andes, *et al.*, 2004). The biofilm is an important virulence factor that contributes to antifungal drug resistance (Sherry, *et al.*, 2014). Further study was carried out to evaluate the inhibitory activity of *R. tometosa* extract on biofilm formation of *C. albicans*. It was found that percent resazurin reduction by the organisms after treated with the extract decreased in a somewhat dose dependent manner (Figure 21). Biofilm formation of the organisms treated with the extract was significantly reduced, compared with control ($p<0.05$). Percent reduction of resazurin at 24 h for *C. albicans* ATCC 90028 and NCPF 3153 biofilm when incubated with the extract at 1024 µg/mL were 59.45% and 62.00%, respectively. At 48 h, percentage reduction of resazurin in *C. albicans* ATCC 90028 and NCPF 3153 treated with the extract at 512 µg/mL to 1024 µg/mL were 35.42−57.69% and 35.84−54.37%, respectively.

The effect of *R. tomentosa* extract on biofilm formation of both isolates was further confirmed by scanning electron microscopy. Biofilm architecture of the untreated cells revealed a dense layer of yeast cells (Figure 22A, B, C and 23J, K, and L). In contrast, there was no aggregation of *C. albicans* cells when were grown in the presence of the extract at 512 µg/mL (Figure 22D, E, F, and 23M, N, and O) and 1024 µg/mL (Figure 22G, H, I, and 23P, Q, and R).

Morphological changes and adherence are critical properties of *C. albicans* biofilm. The organisms within biofilm produced higher amounts of prostaglandin than planktonic cells. Prostaglandin production may promote fungal colonization, thereby increase the biofilm formation (Alem & Douglas, 2005). Inhibitors of cyclooxygenase enzyme that suppress prostaglandin synthesis were found to decrease *C. albicans* biofilm formation (Alem & Douglas, 2004). Anti-biofilm activity of medicinal plants in the Myrtaceae family such as *Syzygium cumini* and *Syzygium aromaticum* against *C. albicans* has been earlier documented (Khan & Ahmad, 2012, Pereira, *et al.*, 2016). It was

reported that *R. tomentosa* extract decreased prostaglandin E_2 production in murine macrophage-like cell line RAW 264.7 (Jeong, *et al.*, 2013). *R. tomentosa* leaf extract may inhibit *C. albicans* biofilm formation by suppressing prostaglandin production.

A



B

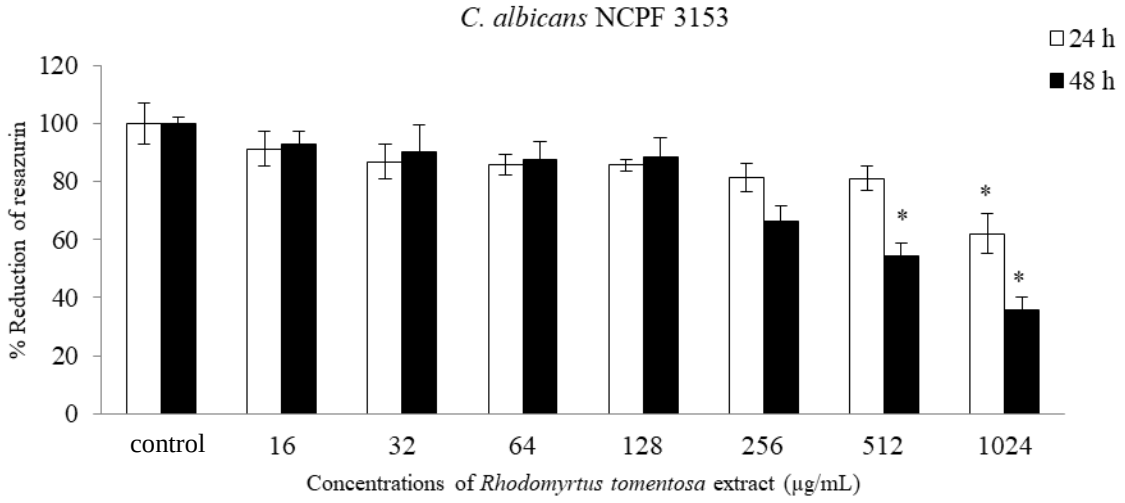
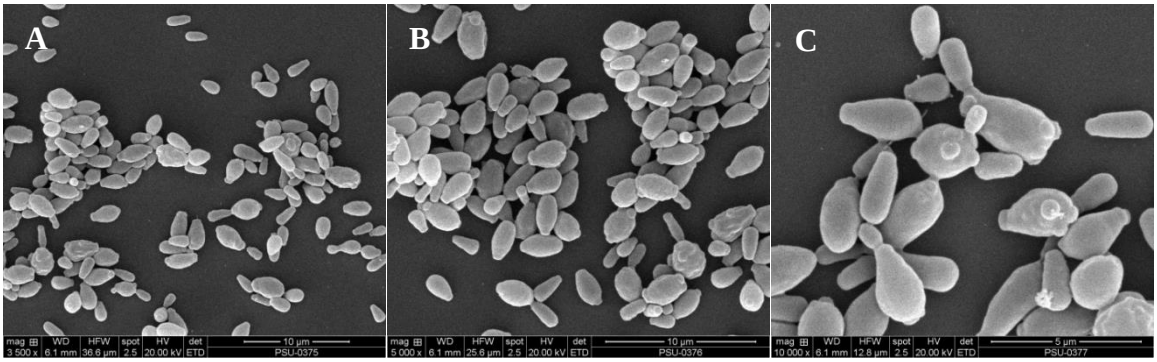
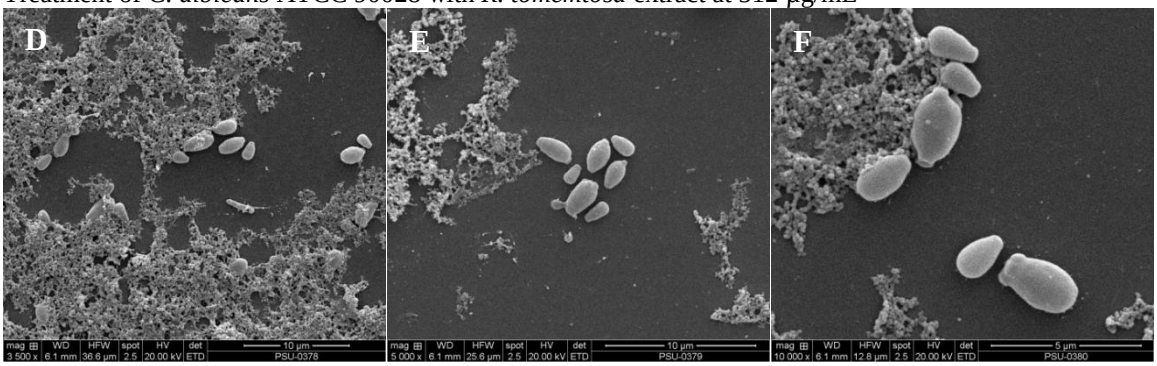


Figure 21. Effects of *Rhodomyrtus tomentosa* extract concentration on biofilm formation of *Candida albicans* ATCC 90028 (A) and *Candida albicans* NCPF 3153 (B), measured by percent resazurin reduction. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as $*p < 0.05$.

Untreated *C. albicans* ATCC 90028



Treatment of *C. albicans* ATCC 90028 with *R. tomemtosa* extract at 512 µg/mL



Treatment of *C. albicans* ATCC 90028 with *R. tomemtosa* extract at 1024 µg/mL

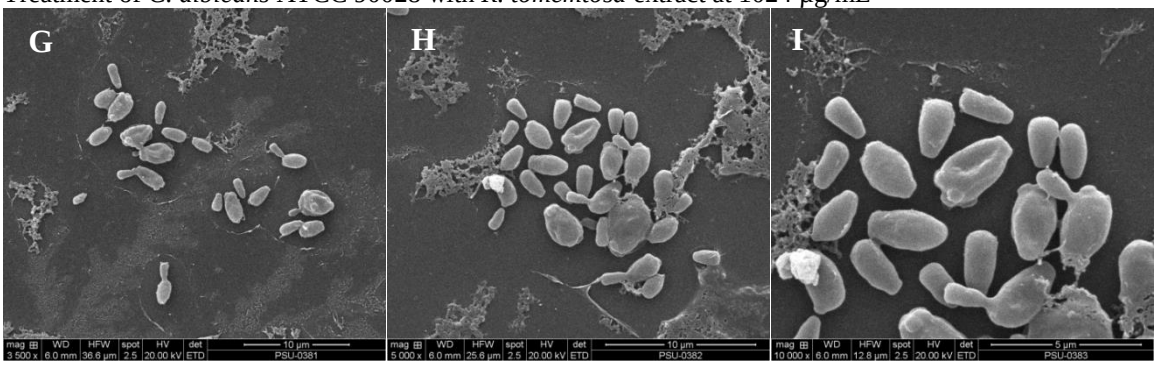
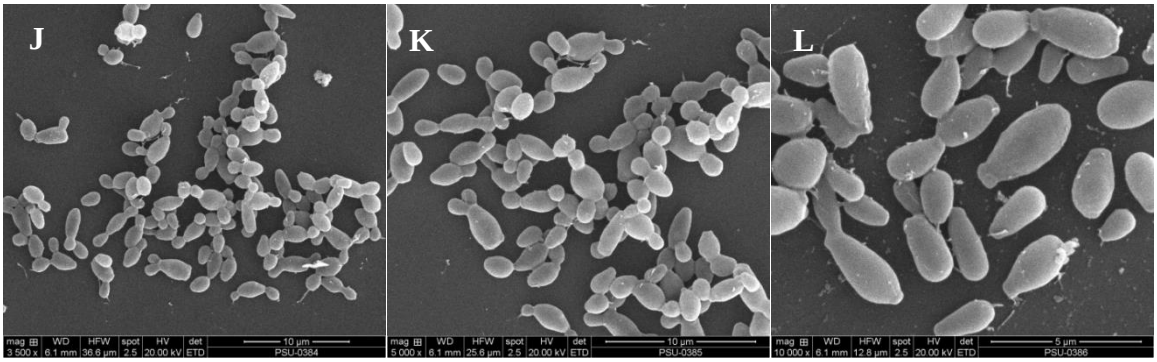
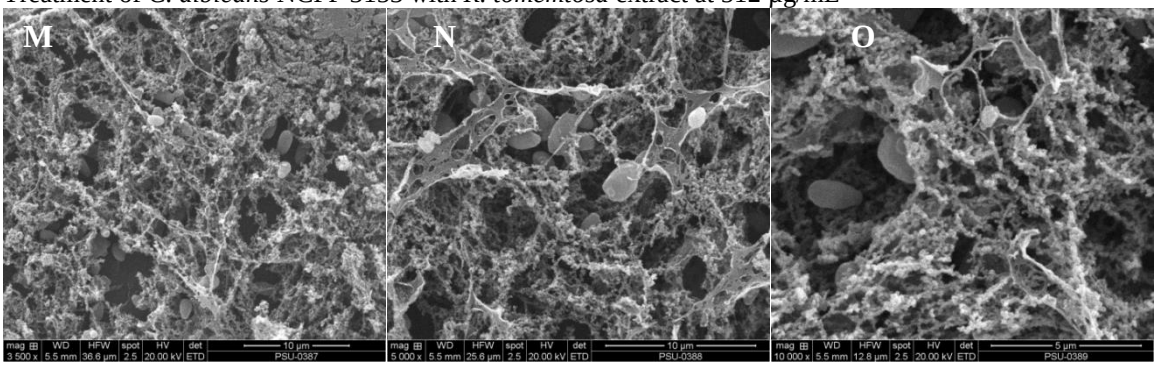


Figure 22. Effects of *Rhodomyrtus tomentosa* extract on *Candida albicans* biofilm formation was analysed by scanning electron microscopy. *C. albicans* ATCC 90028 was treated with 512 µg/mL (D, E, and F) and 1024 µg/mL (G, H, and I) of the extract. Control biofilm of *C. albicans* ATCC 90028 (A, B, and C). in the absence of the extract was shown. Scale bars = 5 and 10 µm.

Untreated of *C. albicans* NCPF 3153



Treatment of *C. albicans* NCPF 3153 with *R. tomemtosa* extract at 512 µg/mL



Treatment of *C. albicans* NCPF 3153 with *R. tomemtosa* extract at 1024 µg/mL

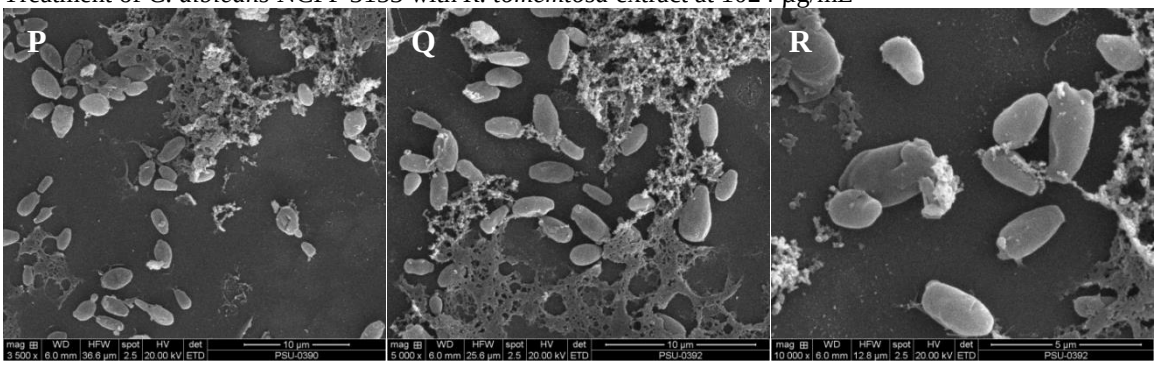


Figure 23. Effects of *Rhodomyrtus tomentosa* extract on *Candida albicans* biofilm formation was analysed by scanning electron microscopy. *C. albicans* NCPF 3153 was treated with 512 µg/mL (M, N, and O) and 1024 µg/mL (P, Q, and R) of the extract. Control biofilm of *C. albicans* NCPF 3153 (J, K, and L) in the absence of the extract was shown. Scale bars = 5 and 10 µm.

Effects of *R. tomentosa* extract on mature *C. albicans* biofilm

Mature *C. albicans* biofilm is not only able to resist various antifungal agents but can also prevent induction of immune response (Xie, *et al.*, 2012). In this study, an inhibitory effect of *R. tomentosa* extract on mature *C. albicans* biofilm was investigated by resazurin assay. Compared with the control group, a significant ($p<0.05$) decrease in mature biofilm after treated with the extract at 256, 512, and 1024 µg/mL was observed as shown in figure 24. The percentage reduction of resazurin for mature biofilm from *C. albicans* ATCC 90028 and NCPF 3153 after treated with the extract at concentrations ranging from 256 to 1024 µg/mL was 34.84−36.26% and 36.89−38.61%, respectively. Scanning electron microscopy demonstrated that mature biofilm architecture of the untreated control remained clumps of yeast cells as shown in figure 25A, B, C, and 26J, K, and L. In contrast, dispersal cells were observed when exposed to the extract at 512 µg/mL (Figure 25D, E, F, and 26M, N, and O) and 1024 µg/mL (Figure 25G, H, I, and 26P, Q, and R).

The results indicated that the extract could decrease *C. albicans* biofilm formation as well as promote biofilm detachment. Prostaglandin synthesis inhibitors have been demonstrated to increase dispersion of *C. albicans* biofilm (Abdelmegeed & Shaaban, 2013).

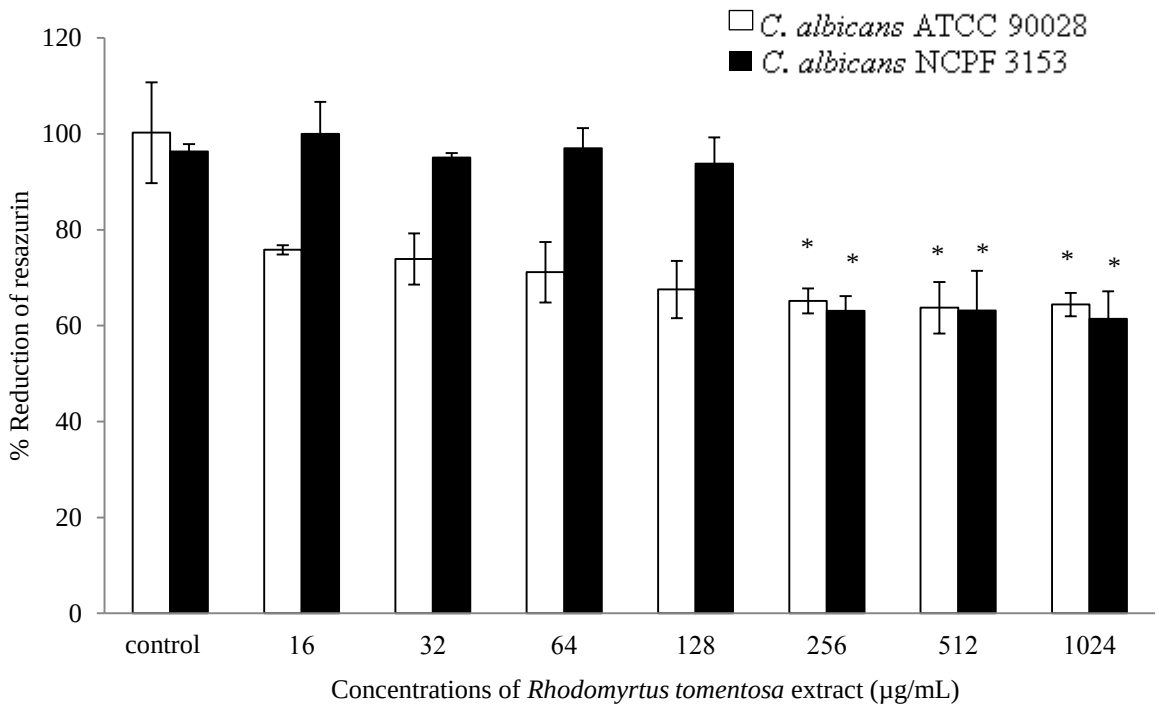
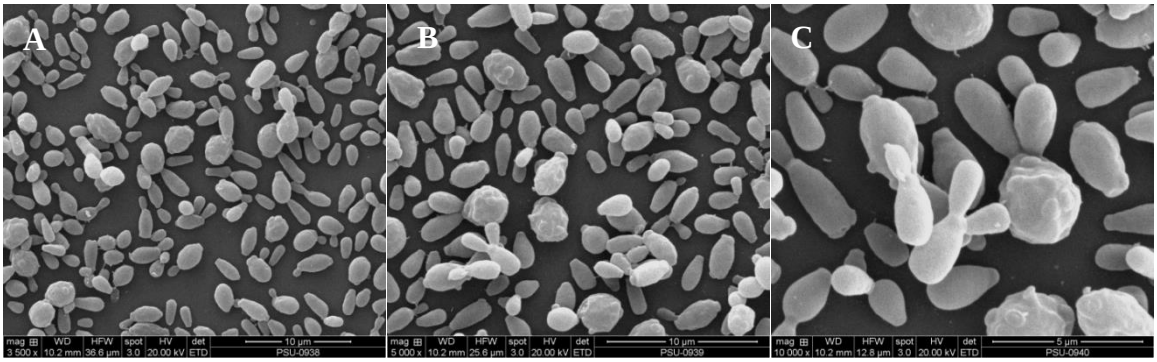
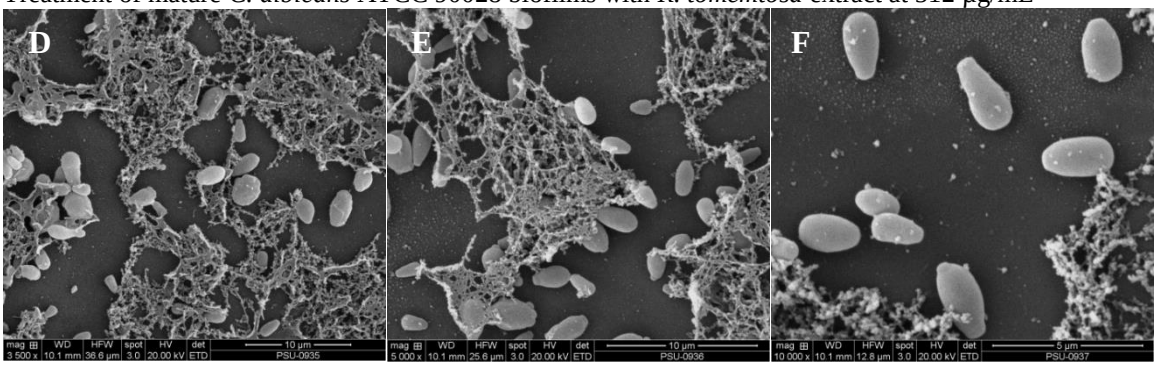


Figure 24. Percent reduction of resazurin for mature biofilm from *Candida albicans* ATCC 90028 and *C. albicans* NCPF 3153 treated with *Rhodomyrtus tomentosa* extract at 0−1024 µg/mL. Values are shown as means of triplicate ± standard error of the mean from two independent experiments. Statistically significant values were defined as * $p<0.05$.

Untreated mature biofilm of *C. albicans* ATCC 90028



Treatment of mature *C. albicans* ATCC 90028 biofilms with *R. tomemtosa* extract at 512 µg/mL



Treatment of mature *C. albicans* ATCC 90028 biofilms with *R. tomemtosa* extract at 1024 µg/mL

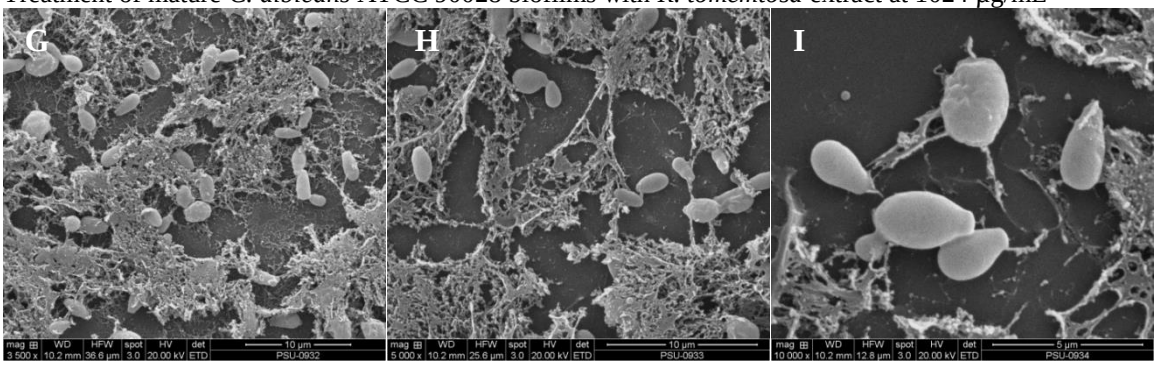
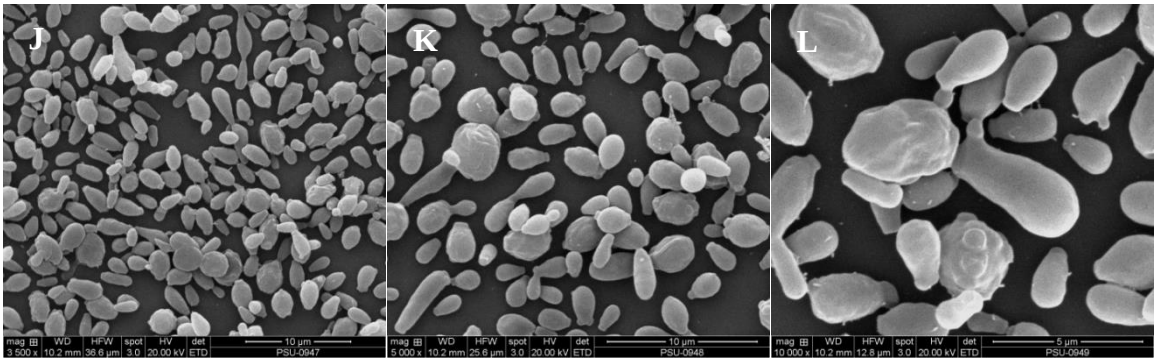
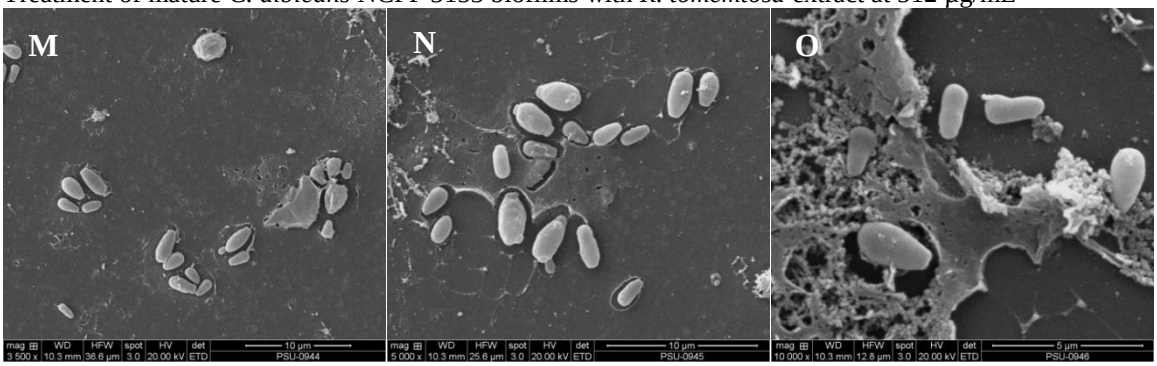


Figure 25. Effects of *Rhodomyrtus tomentosa* extract on mature *Candida albicans* biofilm was analysed by scanning electron microscopy. *C. albicans* ATCC 90028 (D, E, and F) was treated with 512 µg/mL of the extract. *C. albicans* ATCC 90028 (G, H, and I) was treated with 1024 µg/mL of the extract. Mature biofilm of *C. albicans* ATCC 90028 (A, B, and C) in the absence of the extract was shown. Scale bars = 5 and 10 µm.

Untreated mature biofilm of *C. albicans* NCPF 3153



Treatment of mature *C. albicans* NCPF 3153 biofilms with *R. tomemtosa* extract at 512 µg/mL



Treatment of mature *C. albicans* NCPF 3153 biofilms with *R. tomemtosa* extract at 1024 µg/mL

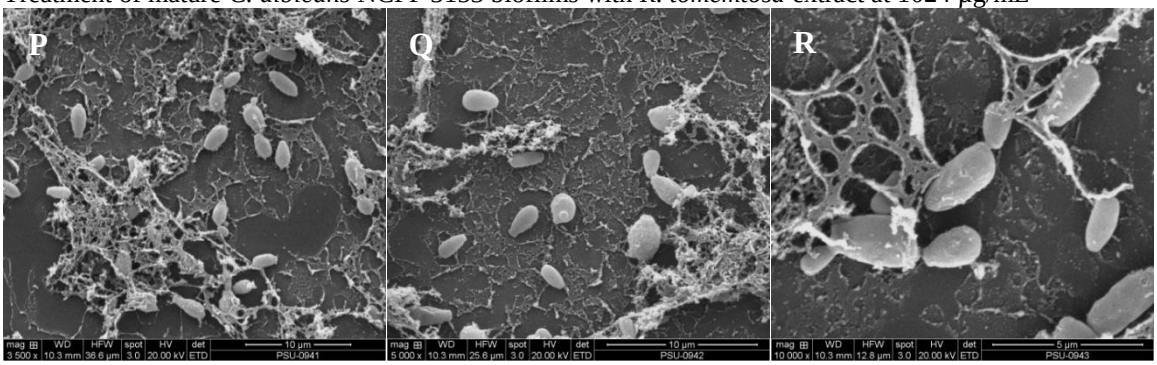


Figure 26. Effects of *Rhodomyrtus tomentosa* extract on mature *Candida albicans* biofilm was analysed by scanning electron microscopy. *C. albicans* NCPF 3153 (M, N, and O) was treated with 512 µg/mL of the extract. *C. albicans* NCPF 3153 (P, Q, and R) was treated with 1024 µg/mL of the extract. Mature biofilm of *C. albicans* NCPF 3153 (J, K, and L) in the absence of the extract was shown. Scale bars = 5 and 10 µm.

Effects of *R. tomentosa* extract on membrane integrity of *C. albicans*

Cell membrane is a thin semi-permeable membrane that surrounds the cytoplasm of a cell. The function of cell membrane is to protect the integrity of the interior of the cell by allowing certain substances into the cell, while keeping other substances out. Thus the cell membrane also serves to help support the cell and help maintain its shape. Changes in structural integrity and permeability of cell membrane and cell wall may have affected the cell metabolism and lead to cell death (Booth, 1985, Poolman, *et al.*, 1987, Trumpower & Gennis, 1994**).** Under stress conditions, increased membrane permeability can affect membrane integrity.

The result was shown that the extract possess the extract effects against *C. albicans*. In the present study, since the extract alone at the tested concentrations did not produce any considerable effects on the survival of yeast cells, we evaluated the possible impact of *R. tomentosa* extract against the organisms by membrane permeability assay at 8 h. It was observed that, upon treatment with the extract, uptake of crystal violet by the cell membrane of the organisms was significantly increased. Upon being treated with the extract at 1024–2048 µg/mL, uptake of the dye was 94.59%–95.38% in *Candida albicans* ATCC 90028 and 95.11%–95.48% in *Candida albicans* NCPF 3153 (Figure 27).

In another report, *Syzygium cumini* (L.) Skeels, a phytochemical from Myrtaceae family affected the membrane integrity of *C. albicans* (Pereira, *et al.*, 2016). The extract may be expected to increase permeability of cell membrane of of *C. albicans***.**

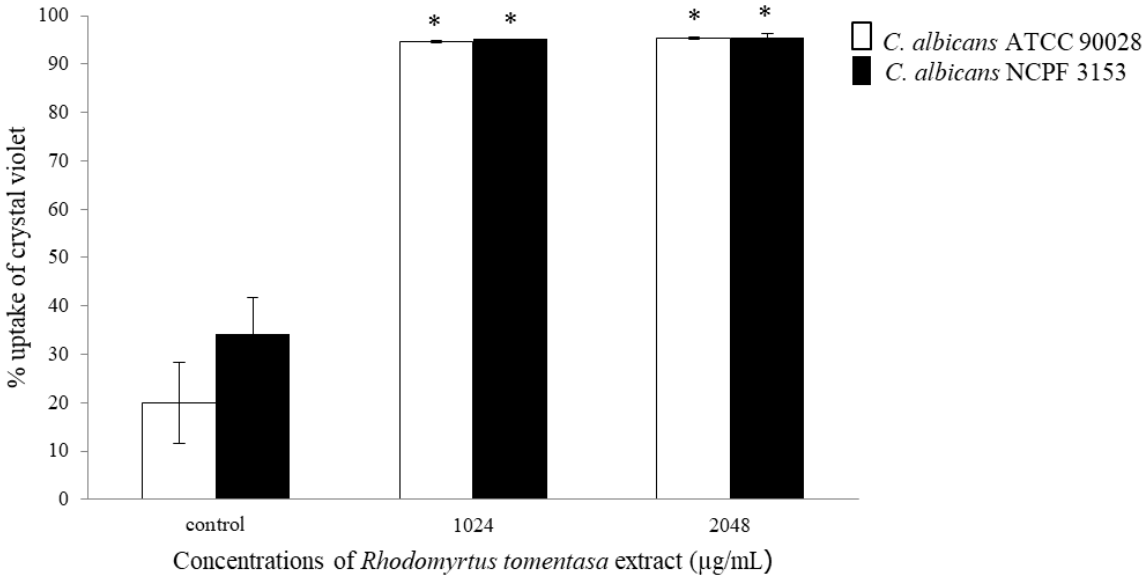


Figure 27. Effects of *R. tomentosa* extract on membrane permeability of *Candida albicans* ATCC 90028 and *Candida albicans* NCPF 3153. Crystal violet uptake by yeast cells at 8 h after treated with the extract at 1024 and 2048 µg/mL was determined. Dimethyl sulfoxide (0.5% v/v) was used as the solvent control. Data are expressed as mean ± standard error from two independent experiments. Each measure was performed in triplicate. * Statistical significance at $p < 0.05$.

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CHAPTER 4

CONCLUSIONS

Rhodomyrtus tomentosa ethanolic leaf extract exhibited nontoxic for human neutrophils. The observed effects with neutrophils and demonstrated the ability of the extract to enhance the killing activity of human neutrophils against *E. coli* and *C. albicans*. From the results of cell survival assay, the killing activity of neutrophils increased with the increased addition of the extract. The extract would stimulate phagocytosis and killing activity of human neutrophils. Therefore, it is demonstrated that the extract exhibited membrane permeabilizing activity rather than antibacterial activity at the tested concentrations, and resulted in the alteration of membrane integrity of the pathogens. Three isolates of *Candida albicans* was collected from the fungal mucosa with sterile swabs. The extract significantly reduced germ tube formation and adhesion in *C. albicans*. The results indicated that the extract suppressed the fungal germ tube formation and its property was practically correlated with anti-adherence to cell surfaces. The results revealed that *R. tomentosa* extract inhibited biofilm formation and mature biofilm. The leaf extract may inhibit *C. albicans* biofilm formation by suppressing prostaglandin production. In addition, the finding suggested that the extract may be used as an alternative therapeutic agent for microorganism infections.

Further studies are needed to assess the significance of the findings. *R. tomentosa* extract was shown to induce neutrophil activities against *E. coli* and *C. albicans*. The results demonstrated the important phagocytic function of neutrophils. Associated tests exhibited that yeast cells were killed more efficiently than *E. coli* by neutrophils. Future work could involve deeper analysis of particular mechanisms. A consideration of the role played by Toll-like receptors 2 and 4 and their signals in mediating the induction of inflammatory cytokines such as tumor necrosis factor –α,

interleukin 10, interleukin 12, and interferons –γ. Investigation of upregulated, downregulated, and steady levels of mRNA of selected Toll-like receptors and cytokines would help to elucidate some very complex problems.

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List of Publications and Proceeding

Hmoteh J, Syed Musthafa K, Pomwised R & Voravuthikunchai S. 2016. Effects of *Rhodomyrtus tomentosa* extract on killing activity of human neutrophils and membrane integrity of enterohaemorrhagic *Escherichia coli* O157:H7. Molecules 21: 692.

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