UNIVERSITI PUTRA MALAYSIA

ANTIMICROBIAL ACTIVITY, PHYTOCHEMICAL AND TOXICITY ANALYSES OF JAMBU MAWAR [Syzygium jambos (L.) Alston] LEAF EXTRACT ON SHRIMP AND CHERRY TOMATO SAMPLES

SALAR KADHUM ALI

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By

SALAR KADHUM ALI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

June 2021

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DEDICATION

This thesis is wholeheartedly dedicated to my beloved parents and siblings, who have been my source of inspiration and gave me strength when I thought of giving up, who continually provide their moral, spiritual, emotional and financial support.
To my supervisor who always guided and motivated me to do my best
To my friends who stood beside me, for their assistance and support

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

ANTIMICROBIAL ACTIVITY, PHYTOCHEMICAL AND TOXICITY ANALYSES OF *JAMBU MAWAR* [*Syzygium jambos* (L.) Alston] LEAF EXTRACT ON SHRIMP AND CHERRY TOMATO SAMPLES

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June 2021

Chairman : Yaya Rukayadi, PhD
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Infections caused by foodborne and waterborne pathogens have brought a serious attention in the last decades. In the last two decades, the discovery and development of new antimicrobial agents have been unprecedented activity. Since most of these antimicrobial agents are not applicable to apply in food productions that led most of the food manufacturers to find the alternative and that is a natural antimicrobial agent, which has no effect on the nutritional and sensory aspect of the foodstuffs and satisfy the consumer demand. *Jambu mawar* [*Syzygium jambos* (L.) Alston] is a species of the genus *Syzygium*, which has belonged to the family of Myrtaceae and has been reported for its medicinal uses as anti-inflammatory, antioxidant, antibacterial and hepatoprotective, and treatment of a variety of illnesses as toothache, diabetes, diuretic, diarrhea, dysentery and leprosy. The objectives of this study were to determine the antimicrobial activity and mechanism of *S. jambos* (L.) Alston leaf extract against foodborne pathogens namely *V. parahaemolyticus* ATCC17802, *L. monocytogenes* ATCC19112, *K. pneuomoniae* ATCC13773, *S. aureus* ATCC29737, *P. mirabilis* ATCC21100 and *P. aeruginosa* ATCC9027, and food spoilage microorganisms *C. albicans* ATCC10231, *C. krusei* ATCC32196, *C. glabrata* ATCC2001, *C. parapsilosis* ATCC22019, *Asp. fumigatus* ATCC26430, *Asp. niger* ATCC9029, *Rh. oligosporus* ATCC22959 and *Rh. oryzae* ATTC 22580, to analyses the phytochemicals, its stability and toxicity of the extract, and to evaluate the effect of the extract on microbial population in food samples. The dried leaf were extracted using ethanol as a solvent with maceration method. Antimicrobial activity against foodborne pathogens were determined using Clinical and Laboratory Standard Institute (CLSI) methods. The stability of antimicrobial activity of the extract was conducted at different pHs and temperatures. Cell constituent release and crystal violet analysis were the methods used to investigate the mechanism of action of the extract. The phytochemicals compounds in the extract were analyzed using GC-MS and LC-MS. The toxicity level of the extract was assessed using brine shrimp lethality assay (*Artemia salina* spp.). Finally, the effect of the extract on natural microflora populations on shrimp and cherry tomato samples via washing treatment were conducted at different concentrations (0.05, 0.5 and 5%) and soaking time (5 and 15 min), and different storage

temperatures. The results showed that the extract can inhibit all microbial tested with inhibition zone ranged between 7.00 ± 0.00 to 10.25 ± 0.29 mm. The minimum inhibitory concentrations (MICs) of the extract were ranged between 0.01 to 2.5 mg/mL, whereas the MB/FC ranged 0.01 to 5.00 mg/mL. Time-kill curve assay demonstrated that all microbial tested can reduced ≥3 Log_{10} up to 4× MIC for 2 h. Meanwhile, quantitative analysis of conidia inhibition showed all conidia of fungi tested were killed or reduced in the range of 89% to 100% inhibition after 48 h of incubation and no filamentous growth after treated with extract between 0.5× MIC and 2× MIC for 14 days. Cell constituent release and crystal violet analysis of representative pathogens treated with the extract at MIC values showed disruption and leakage of the cell cytoplasm. Generally, the antimicrobial activity of the extract against all tested microorganisms at different pHs (3, 6, 7 and 11) and temperatures (30˚C, 50˚C and 80˚C) was not significantly different, meaning the extract was stable. GC-MS analysis identified the presence of 35 compounds in the extract and the antimicrobial active compounds includes methyl 5,11,14,17-eicosatetraenoate, phytol, ethyl linoleate, phenol, 3-pentadecyl, squalene, 9,12,15-octadecatrienoic acid, methyl ester, neophytadiene, gamma-tocopherol and alpha-amyrin. LC-MS analysis were identified 36 compounds such as ferulic acid, rutin, 3,4-Dihydrocadalene, curcumene, and ethyl myristate. The toxicity analysis of the extract demonstrated that the LC_{50} was 4.51 mg/mL; it might consider being safe. *Syzygium jambos* (L.) Alston leaf extract as a washing treatment solution demonstrated a significant reduction of natural microflora in tested food samples were started at 0.05% (v/v) of extract at 15 min. In storage study, 0.5% (v/v) of the extract exhibited a better effect in controlling the microbial survival throughout the storage time. In conclusion, *S. jambos* (L.) Alston leaf extract exhibits antimicrobial activities against wide spectrum of foodborne pathogens and food spoilage microorganisms with no significant affect by exposure to different pHs and temperatures, and no toxicity effect, thus it can be developed as a natural sanitizer for washing raw food materials.

Keyword: Antimicrobial activity, natural food sanitizer, pathogenic and spoilage microorganisms, stability, *Syzygium jambos* (L.) Alston leaf extract, toxicity.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

ANALISIS AKTIVITI ANTIMIKROB, FITOKIMIA DAN KETOKSIKAN EKSTRAK DAUN JAMBU MAWAR [*Syzygium jambos* (L.) Alston] KE ATAS SAMPEL UDANG DAN TOMATO CERI

Oleh

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Jangkitan yang disebabkan oleh patogen bawaan makanan dan air membawa perhatian serius dalam beberapa dekad terakhir. Walau bagaimanapun, beberapa jangkitan yang disebabkan oleh mikroorganisma patogen didokumentasikan dengan baik, tetapi dianggap muncul kerana ia sering berlaku. Dalam dua dekad terakhir, penemuan dan pengembangan agen antimikroba baru merupakan aktiviti yang belum pernah terjadi sebelumnya. Oleh kerana sebilangan besar agen antimikroba ini tidak dapat digunakan dalam pengeluaran makanan yang menyebabkan kebanyakan pengeluar makanan mencari alternatif dan itu adalah agen antimikroba semula jadi, yang tidak berpengaruh pada aspek nutrisi dan deria makanan dan memuaskan pengguna permintaan. *Jambu mawar* [*Syzygium jambos* (L.) Alston] adalah spesies genus *Syzygium*, yang telah tergolong dalam keluarga Myrtaceae dan telah dilaporkan untuk kegunaan perubatannya dan rawatan pelbagai penyakit. Objektif kajian ini adalah untuk menentukan aktiviti antimikroba *S. jambos* (L.) ekstrak daun Alston terhadap patogen bawaan makanan iaitu *V. parahaemolyticus* ATCC17802, *L. monocytogenes* ATCC19112, *K. pneuomoniae* ATCC13773, *S. aureus* ATCC29737, *P. mirabilis* ATCC21100 dan *P. aeruginosa* ATCC9027, dan mikroorganisma merosakkan makanan *C. albicans* ATCC10231, *C. krusei* ATCC32196, *C. glabrata* ATCC2001, *C. parapsilosis* ATCC22019, *Asp. fumigatus* ATCC26430, *Asp. niger* ATCC9029, *Rh. oligosporus* ATCC22959 dan *Rh. oryzae* ATTC 22580, fitokimia dan ketoksikan ekstrak dan untuk menilai kesan ekstrak terhadap populasi mikrob pada sampel makanan. Daun mawar Jambu kering diekstraksi menggunakan etanol sebagai pelarut dengan kaedah maserasi. Aktiviti antimikroba terhadap patogen bawaan makanan ditentukan menggunakan kaedah Institut Standard Klinikal dan Makmal (CLSI). Kestabilan aktiviti antimikroba ekstrak dilakukan pada pH dan suhu yang berbeza. Pembebasan konstituen sel dan analisis kristal ungu adalah kaedah yang digunakan untuk menyiasat mekanisme tindakan ekstrak. Sebatian fitokimia dalam ekstrak tersebut dianalisis menggunakan GC-MS dan LC-MS. Tahap ketoksikan ekstrak daun *S. jambos* (L.) Alston dinilai menggunakan ujian kematian udang air garam (*Artemia salina* spp.). Akhirnya, kesan ekstrat pada populasi mikroba dalam sampel makanan dilakukan pada kepekatan dan suhu penyimpanan berbeza.

Kata kunci: Aktiviti antimikrob, pembersih semula jadi, mikroorganisma patogen dan kerosakan, kestabilan, daun *Syzygium jambos* (L.) Alston, ketoksikan. Hasil kajian menunjukkan bahawa ekstrak dapat menghambat semua mikroba yang diuji dengan zona penghambatan berkisar 7,00 ± 0,00 hingga 10,25 ± 0,29 mm. Kepekatan inbibitoy minimum (MIC) ekstrak pada semua mikrob yang diuji berkisar antara 0,01 hingga 2,5 mg / mL sedangkan ekstrak dapat membunuh semua mikrob yang diuji dengan MB / FC antara 0,01 hingga 5,00 mg / mL. Uji keluk time-kill menunjukkan bahawa semua mikrob yang diuji dapat mengurangkan ≥3 Log_{10} hingga 4 × MIC selama 2 jam. Sementara itu, analisis kuantitatif perencatan konidia menunjukkan semua kulat konidia yang diuji mati atau dikurangkan dalam lingkungan 89% hingga 100% perencatan setelah 48 jam inkubasi dan tidak ada pertumbuhan filamen setelah dirawat dengan ekstrak antara 0,5 × MIC dan 2 × MIC selama 14 hari. Pembebasan konstituen sel dan analisis kristal ungu patogen perwakilan yang dirawat dengan ekstrak pada nilai MIC menunjukkan gangguan dan kebocoran sitoplasma sel. Secara amnya, aktiviti antimikrob ekstrak terhadap semua mikroorganisma yang diuji pada pH yang berbeza (3, 6, 7 dan 11) dan suhu (30˚C, 50˚C dan 80˚C) tidak berbeza secara signifikan, yang bermaksud ekstraknya stabil. Analisis GC-MS mengenal pasti adanya 35 sebatian dalam ekstrak dan sebatian aktif antimikroba termasuk metil 5,11,14,17-eicosatetraenoate, phytol, ethyl linoleate, phenol, 3-pentadecyl, squalene, 9,12,15-octadecatrienoic asid, metil ester, neophytadiene, gamma-tokoferol dan alpha-amyrin. Analisis LCMS dikenal pasti 36 sebatian seperti asid ferulat, rutin, 3,4-Dihydrocadalene, curcumene, dan ethyl myristate. Analisis ketoksikan ekstrak menunjukkan bahawa LC50 adalah 4.51 mg / mL; ia mungkin dianggap selamat. Syzygium jambos (L.) Ekstrak daun Alston menunjukkan pengurangan mikroflora semula jadi yang signifikan pada sampel makanan yang diuji dimulakan pada 0,05% (v / v) ekstrak pada 5 min. Dalam kajian penyimpanan, 5% (v / v) ekstrak menunjukkan kesan yang lebih baik dalam mengawal kelangsungan hidup mikroba sepanjang masa penyimpanan. Kesimpulannya, ekstrak *S. jambos* (L.) Alston menunjukkan aktiviti antimikrobial terhadap spektrum mikroorganisma patogen bawaan makanan dan kerosakan makanan tanpa pengaruh yang signifikan dengan pendedahan kepada suhu dan pH yang berbeza dan tidak ada kesan toksisitas, sehingga dapat dikembangkan sebagai pengawet semula jadi atau pembersih untuk mencuci bahan makanan mentah.

Kata kunci: Aktiviti antimikrobial, pembersih makanan semula jadi, mikroorganisma patogen dan kerosakan, kestabilan, ekstrak daun *Syzygium jambos* (L.) Alston, toksikan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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TABLES OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ANOVA	Analysis of variance
CFU	Colony forming unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethylsulfoxide
GC-MS	Gas Chromatography – Mass Spectrometry
GRAS	Generally Regarded as Safe
LC_{50}	Lethality concentration
LC-MS	Liquid Chromatography – Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum Inhibitory Concentration
MOH	Ministry of Health
m/z	Mass/charge ratio
NIST	National Institute of Standards and Technology
OD	Optical density
PBS	Phosphate buffered saline
PCA	Plate Count Agar
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Rpm	Revolutions per minute

SDA	Sabouraud Agar
SDB	Sabouraud Broth
spp.	Species
TCBS	Thiosulfate citrate bile salts sucrose
TPC	Total Plate Count
UPM	Universiti Putra Malaysia
WHO	World Health organization

CHAPTER 1

INTRODUCTION

1.1 Background

Foodborne diseases can be defined as the consumption of raw or cooked food that have been contaminated by pathogens or their toxins. These diseases could be spread easily, and therefore, they become a considerable worldwide public health burden. In developing countries, the foodborne pathogens become a real threat to food safety with poor hygiene and decontamination practices. These illnesses occurred usually even though the large-scale of cases are unreported or did not undergo diagnosis due to a complex of chains of events should be happen prior the food borne diseases officially take down. Any cross at any point of this chain will lead the case will not being reported (Olsen *et al*., 2000)

Foodborne illnesses mostly occur due to the contamination of food with bacteria and fungi, but also some viruses, prions and protozoa, and this can be happen during food production, processing, storage and transport before consuming. These microorganisms can secrete different components during their growth, including toxins, into the extracellular environment (Martinović *et al*, 2016).

Overall, there were 5196 foodborne outbreaks reported in the European Union in 2013, which infected 43,183 people, led to 5946 cases admitted to hospital and 11 death cases reported (EFSA, 2015). While in the United States, almost 9.4 million outbreaks of foodborne illness annually are due to foodborne diseases, along with 55,961 admissions to hospital and 1351 deaths (Scallan *et al.*, 2011). Furthermore, approximately 5,400,000 cases had been reported annually in Australia with 15,000 people admitted to hospital and 120 deaths (Soon *et al*., 2011). In Malaysia, food is most likely to be sold everywhere with less concern to be paid to the food hygiene, where food safety found to be less consideration as a real issue today (The World Bank, 2017). According to the reports, the foodborne illness cases in Malaysia increased along the year, that reflects the fact of food safety situation and that leads to increase the foodborne illness burden. In 2017, the reported foodborne poisoning incidence rate was 42.25 per 100,000 populations, compared to the incidence rate in 2009 and in 2016 where it was 36.17 and 55.21per 100,000 populations, respectively (MOH, 2010; Department of statistics Malaysia, 2017). On the other hand, most of the cases were reported in schools and the available data showed that the foodborne disease cases rising since 2010.

In general, food contamination with pathogens may occur during any stage of the farm-to-fork, by means during processing, packing, transporting or storagging due to inappropriate food handling or poor hygiene practices (Taban and Halkman, 2011). The most common examples of widely reported pathogens, which are considered the causative agents of foodborne illnesses include *Bacillus cereus, Clostridium perfringens, Campylobacter*, Shiga-toxin producing *Escherichia coli* O157 and non-O157, *Listeria*

monocytogenes, *Salmonella*, *Staphylococcus aureus*, *Vibrio* spp. and *Shigella* spp. (Gilliss *et al*., 2013; New *et al*., 2017). In addition, raw food, fruits and vegetables subjected to microbial spoilage caused by fungi (yeast and mold). Fungal food spoilage are able to produce a variety of metabolic by-products, which may cause off-odors and flavors and accompanied visible changes in color or texture as well (Rawat, 2015). Spoilage may occur during food processing and production and subsequently affect the supply chains to the consumers. In developing countries, fungal food spoilage estimated to be responsible for 5-10% of all food losses (Pitt and Hocking, 2009). The formations of mycotoxins represent the most important figure of fungal spoilage of foods. Many studies and reports revealed that *Alternaria* spp., *Rhizopus* spp., *Aspergillus* spp., *Botrytiscinerea*, *Cladosporium* spp., *Fusarium* spp. and *Penicillium* spp. related to the deterioration of perishable products (Tournas, 2005; Birhanu *et al*., 2014; Khokhar and Bajwa, 2014).

Food was proven to exposure to contamination from microbiological origin and based on that, consumers become more concerned about the food quality. As well known, contamination may occur during pre-harvest period as it should be associated to the farm environment, water irrigation system, fertilizer, transport or any other reasons, or it may occur during post-harvest processing (Doyle and Erickson, 2006; Nerin *et al*., 2016). Proper decontamination during food processing and good storage conditions are key parameters in food quality and safety that facilitate the elimination of the presence of possible microorganisms and extends the shelf life of food and therefore, they are crucial to minimize food contamination. On the contrary, inadequate decontamination and poor storage will allow the pathogens to grow and cause food contamination (Scott, 2003; Nerin *et al*., 2016). Chemical cleaning with detergents, together with manual or mechanical cleaning of food contact surfaces and the application of a sanitizer and food preservatives, are an effective way to control microbial growth in every raw food and fresh produced (Abushelaibi *et al*., 2012). These treatments including but not limited to sugar, salt, chlorine, organic acids, hydrogen peroxide, diacetyl, ozonation, thermal and irradiation (Leistner, 2000; Tiwari *et al*., 2009), nevertheless, it was important to eradicate or reduce the microbial population at the early level of food processing. Therefore, washing food materials with any food sanitizer was critical above all for ready-to-eat foods such as fruits and vegetables. A number of chemical sanitizers were used in the food industry, including chlorine, hypochlorite, iodine, quaternary ammonium, hydrogen peroxide and fatty acid sanitizer. However, these chemicals have the potential to cause health and environmental harm (Neo *et al*., 2013). In addition, chlorine-based sanitizers are widely used in fresh produce industry. Chlorine-based sanitizers such as sodium hypochlorite (NaOCl) have been extensively used as disinfectants in fresh and fresh-cut products after harvest (Luo *et al*., 2011). Moreover, the antimicrobial potency was predominately depended on the free chlorine residual in processing water (Gil *et al*., 2009). Furthermore, immoderation amount of free chlorine may react with organic matter such as decayed leaves leads to the formation of carcinogenic halogenated by-products such as trihalomethanes, haloacetic acids, haloketones and chloropicrin (Fan and Sokorai, 2015).

Many studies and efforts are being made to find a natural antimicrobial agent from plants is the alternatives to prevent the growth of bacteria and fungi in food. In recent years, and due to the high consumer awareness and concerns about synthetic chemical additives, foods preserved with natural additives have become more demanding. In this

context, plant antimicrobials are increasingly interested in the food industry for their potential as decontaminating agents, as they are classified as Generally Recognized as Safe (GRAS) (Del Nobile *et al*., 2012). Many researches have been taken to discover out the antimicrobial potency of medicinal plants, specially the herbs and spices because they are enriched with vast of compounds having a strong antimicrobial activity. Nowadays, over 1350 plants possessed antimicrobial potency and more than 30,000 antimicrobial components were extracted from medicinal plants (Arshad and Batool, 2017).

1.2 Problem Statements

Plant-based biological products had a wide spectrum of antimicrobial properties against microorganisms, including pathogenic microbes. Several current studies on antimicrobial elements in food products have made it possible to eradicate microbes responsible for food deterioration, thereby prolonging the expiration date of food products (Tajkarimi *et al*., 2010). Due to their safety and pleasant fragrance, natural products have been used in food and their use has generated consumer interest. It is essential to find natural additives with antimicrobial activities that have the ability to prevent microorganisms from contaminating food. This inspired the current studies to determine the antimicrobial activity of plants with a wide variety of medicinal properties.

Previous research showed that crude extract from leaves of *Jambu Mawar* [*Syzygium jambos* (L.) Alston] showed exhibiting antibacterial activity against pathogenic bacteria. The antibacterial activity was determined through various combinations of polyphenols, anthraquinones, tannins, flavonoids, flavonols, phenolic acids, anthocyanins, ellagitannins and steroids (Sobeh *et al*., 2016; Wamba *et al*., 2018). The microflora of the *S. jambos* (L.) Alston leaf had not been investigated in the current study since the leaves after collecting them were undergo several steps in the extraction process including soaking in ethanol as an extraction solvent. As it is well known that ethanol is widely used for general surface disinfecting and has reported biocidal efficacy against bacteria, fungi and viruses in the concentration range of 50%–90% (Rogawansamy *et al*., 2015) and this mean, ethanol may kill the existed microflora of the leaves. Unfortunately, the antimicrobial activity of ethanolic or other solvents' extract of *S. jambos* (L.) Alston leaf against foodborne pathogens (bacteria) and spoilage microorganisms (fungi) have not been determined yet. Moreover, to the best of our knowledge, the phytochemical compounds in the *S. jambos* (L.) Alston leaf extract which are responsible for the antimicrobial activity against foodborne pathogens and spoilage microorganisms have not been analyzed. Over and above, the *S. jambos* (L.) Alston leaf extract have not been investigated as a sanitizer on food and food products, since the previous studies proved its antibacterial against clinical pathogenic bacteria only. For our knowledge, some of the tested foodborne pathogens and spoilage microorganisms in the current study have association with the selected raw food samples (*Vibrio parahaemolyticus*, *K. pneumoniae*, *P. mirabilis*, *Pseudomonas aeruginosa*., *S. aureus*, and *Asp. niger* were associated with tomato samples, while *Vibrio* spp., *K. pneumoniae*, *Pseudomonas* spp. and *S. aureus*. were the most predominant with shrimp samples). Therefore, this study was conducted to evaluate the antimicrobial activity of *S. jambos* (L.) Alston leaf extract against several types of foodborne pathogens and food spoilage microorganisms.

1.3 Objectives

1. To determine the antibacterial activity of *S. jambos* (L.) Alston leaf extract against foodborne pathogens.

2. To determine the antifungal activity of *S. jambos* (L.) Alston leaf extract against food spoilage microorganisms.

3. To evaluate the effect of *S. jambos* (L.) Alston leaf extract on cell constituents' release of foodborne pathogens and to identify the present of bioactive compounds in *S. jambos* (L.) Alston leaf extract by Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS).

4. To evaluate the effect of different pHs and temperatures on stability of antimicrobial activity of *S. jambos* (L.) Alston leaf extract.

5. To determine toxicity of *S. jambos* (L.) Alston leaf extract by using brine shrimp (*Artemia salina*) lethality assay and to evaluate the effect of *S. jambos* (L.) Alston leaf extract on natural microflora in food samples at different concentrations, exposures times and storage temperatures.

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