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Total Flavonoid Content, Total Phenolic Content, Antimicrobial and Antioxidant Activities of Acacia Rigidula Leaf Extracts

Pablo Arenaz

TOTAL FLAVONOID CONTENT, TOTAL PHENOLIC CONTENT, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *ACACIA RIGIDULA* LEAF EXTRACTS

A Thesis

by

PABLO ARENAZ III

Submitted to Texas A&M International University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

May 2021

Major Subject: Biology

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Approved as to style and content by:

Chair of Committee,	Ruby Ynalvez
Committee Members,	Monica Mendez
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ABSTRACT

Total Flavonoid Content, Total Phenolic Content, Antimicrobial and Antioxidant Activities of *Acacia rigidula* Leaf Extracts (May 2021)

Pablo Arenaz III, B.S., Texas A&M International University;

Chair of Committee: Dr. Ruby Ynalvez

The growing incidence of multidrug resistant bacteria has prompted a need for alternatives to traditional antibiotics. The Fabaceae family including the *Acacia* species have been reported to contain secondary metabolites known to exhibit antibacterial and antioxidant activities. Specifically, *A. ridigula* was previously shown to be effective against a range of bacterial species. When extracting secondary metabolites from plants, the method and choice of solvent is important in order to efficiently extract a high yield. This study aims to: 1) determine whether Soxhlet or maceration is the more efficient extraction method; 2) determine whether acetone and ethanol will yield more bioactive compound extracts; 3) quantify flavonoids and total phenolics in our extracts; and 4) determine if our extracts contain antimicrobial and antioxidant properties. The degree of antibacterial activity was evaluated through a disc diffusion assay. Quantitative chemical analyses were done to determine total flavonoids and total phenolic acids. Antioxidant activity was determined by inhibition of linoleic acid peroxidation. There was no statistically significant difference between Soxhlet and maceration extracts, as well as between ethanol and acetone extracts based on mean zone of inhibition (mZOI). All extracts showed antimicrobial activity against our bacteria of interest. Furthermore, in our study, phytochemical analysis was carried out to determine differences in total phenolic and total flavonoid content between extracts. Soxhlet method extracted statistically more flavonoids

($p<0.01$), and ethanol as a solvent significantly extracted more flavonoids compounds ($p<0.01$). On the other hand, no statistical difference was found between Soxhlet and maceration as well as between acetone and ethanol for total phenolic acids. No statistically significant difference was found between the antioxidant activity of maceration and Soxhlet extracts nor between acetone and ethanolic extracts. These findings can serve as a basis for further isolation and purification and the utilization of secondary metabolites in *A. ridigula* for novel drug treatments.

DEDICATION

I would like to dedicate this thesis to my family for all their love and support.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my mentor Dr. Ruby Ynalvez for allowing me to join her laboratory. She cared enough to give me that “extra push” that I needed in order to complete this thesis. I would also like to thank Dr. Marcus Antonius Ynalvez for his help with all the statistical analysis, his encouragement, and his mentorship. They make a great team. Together they encouraged me, taught me, were patient with me, and brought me one step closer to becoming a physician.

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1 INTRODUCTION

Antibiotic resistant bacteria are becoming a serious problem in health care. According to the World Health Organization (WHO), the consistent misuse of antibiotics has exacerbated this problem (Avorn, 2001). Consequently, many pathogenic bacteria are becoming resistant to current antibiotics. These bacteria are often the cause of hospital associated infections (HAI). Without intervention, HAI can become a serious health crisis as no treatment will be available. Novel drugs and treatment must be identified to help avert this urgent health hazard.

Plants have historically been used for its medicinal purposes. According to the WHO, 80% of developing countries rely on folk medicine. Even with technological advancements in medicine, plants are still widely used for its medicinal purposes (Ali et al., 2018). It is well documented that plants possess defensive mechanisms to protect against microorganisms. Literature suggests that plants contain secondary metabolites which provides the plants with antimicrobial properties useful for medicinal purposes (Gabr et al., 2018). These secondary metabolites including alkaloids, phenols, steroids, and tannins, are natural compounds that can be evaluated for its pharmacological use as an alternative to antibiotics.

The *Acacia* spp. are shrubs and trees that encompass over 1,200 species and are part of the Fabaceae family (Seigler, 2003). While literature suggests that many Acacia species contain secondary metabolites known to have antimicrobial activities, few reports on antimicrobial activity in *Acacia rigidula*, also referred to as *Vachellia rigidula*, have been documented. Thus, we seek to extract bioactive compounds from *Acacia rigidula* and test it for its antimicrobial activity. If indeed antimicrobial activity is found, it can be a promising alternative to traditional antibiotics.

This thesis follows the journal model of *SN Applied Sciences*

When extracting bioactive compounds from plants, the choice of solvent and extraction method is critical and important in attaining maximum yield. That said, the aim of the study is to determine the biological activities present in the leaves of *Acacia rigidula.* Specifically, the proposed study aims to: 1) determine whether Soxhlet or maceration is the more efficient extraction method; 2) determine whether acetone and ethanol will yield more bioactive compound extracts; 3) quantify flavonoids and total phenolics in our extracts; and 4) determine if *Acacia ridigula* extracts contain antimicrobial and antioxidant properties.

2 REVIEW OF LITERATURE

2.1 Antibiotic resistance: emergence and causes

Bacteria contain extrachromosomal circular DNA molecules called plasmids, which carry genes that provide antibiotic resistance. Antibiotic resistant bacteria can easily transfer their plasmids to antibiotic susceptible bacteria through conjugation (Blackwell et al., 2020). Consequently, these once susceptible bacteria become resistant to antibiotics. Thus, the research of bacteria and their plasmids are important in scientific research because the transfer of antibiotic resistant genes is alarming. As such, there is a renewed interest in finding novel drugs.

According to the World Health Organization (WHO), antimicrobial resistance (AMR) is fast becoming a global threat (The World Health Organization, 2015). AMR is the ability of bacteria to survive exposure to medications that would normally inhibit or kill them. The emergence of these microorganisms, referred to as multidrug resistant (MDR) bacteria, has been attributed to excessive use of antibiotics, use of antibiotics in animals, antibiotics being sold over the counter, and use in anthropogenic activity such agriculture and aquaculture. For example, when antibiotics are used in healthy animals, bacteria living in them can acquire resistance. These resistant bacteria can be passed to humans when the animal is consumed (Davies & Davies, 2010).

The repercussions of AMR on health implications is considerable. It not only affects the treatment of primary bacterial infection, but also the preventative measures used for invasive surgeries. According to the WHO, without intervention it is estimated that by 2050, AMR will cause 10 million preventable deaths per year (The World Health Organization, 2015). The transfer of antibiotic resistant genes is exacerbated by the improper use of antibiotics. People can

misuse antibiotics by not finishing their prescription, overusing antibiotics, or taking them when it is not needed. The continuous misuse of antibiotics can lead to antibiotic resistant bacterial strains (Izadi et al., 2020). This is concerning as it can lead to "superbugs," such as methicillin-resistant *Staphylococcus aureus* (MRSA). Superbugs are difficult to treat with antibiotics and an outbreak can become a public health problem.

Many antibiotic resistant bacteria are commonly found in hospitals and hospital equipment (Izadi et al., 2020). These nosocomial pathogens are a serious issue, as many patients are already immunocompromised. With limited treatment available, Hospital Acquired Infections (HAI) can be fatal. It is therefore important to study nosocomial associated bacteria to develop new treatments and drugs. The microorganism that we will investigate are bacteria associated with HAI including *S. aureus, Y. enterocolitica, P. aeruginosa,* and *P. alcalifacien*s, and, *E. faecium*.

2.2 Importance of the microorganisms in this study

Nosocomial bacterial infections are acquired in hospitals through invasive procedures. These infections can be caused by either Gram-positive or Gram-negative bacteria. When catheters are used, patients have a 20% chance of acquiring nosocomial bacterial meningitis (Rodríguez-Lucas et al., 2020). *P. aeruginosa* has been mainly associated with neurosurgical meningitis related to intraventricular catheters. Infections have a high mortality rate and cost. Limited drugs are available to treat patients suffering from drug resistant *P. aeruginosa.*

Enterobacteriaceae are considered the leading bacteria that cause hospital acquired infections. *Enterococcus faecium* has transformed from a harmless commensal organism of the GI tract to a dangerous nosocomial bacterium. During the second half of the 1990s, US hospitals

saw an emergence of hospital associated *E. faecium* infections (Willems & Van Schaik, 2009). Handwerger et al., (1993) reported an outbreak of glycopeptide, penicillin, and aminoglycoside resistant *E. faecium* at a medical-surgical ICU. Cultures showed that 22% of patient and 6.5% of staff members were colonized by *E. faecium* over a 3-month period. This globally rapid spread makes *E. faecium* an important microorganism.

Yersinia entercolitica causes a wide range of gastrointestinal diseases. It has also been known to cause acute arthritis. It is transmitted via fecal-oral route. From 1980 to 1990, the Hospital Infection Program of the Centers for Disease and Control and Prevention investigated nosocomial outbreaks, and reported that 62% were caused by bacteria. Outbreaks associated with *Y. enterocolitica* were caused by contamination from red blood cell transfusion by the donor (Jarvis, 1991). Although, *Y. enterocolitica* infections are mainly associated with food poisoning, it is worth investigating because of its high virulence factors and ability to acquire resistance (Lucero-Estrada et al., 2020).

Staphylococcus aureus has been associated with many nosocomial infections. In Germany, the rate of methicillin-resistant *S. aureus* (MRSA) has increased from 8% to 34% between 1997 and 2004. MRSA has been known to cause worse outcomes in patients compared to methicillin-susceptible *S. aureus* (MSSA). The increase of MRSA hospital infections has been associated with increased hospital stays and higher costs. Additionally, MRSA causes more deaths compared to MSSA (Ott et al., 2010). MRSA has also increased the incidence of toxic shock syndrome in burn patients (Matsushima et al., 2020). The outbreaks these aforementioned bacteria have caused, as well as the high cost and the limited treatment associated with their infections, make these microorganisms important for our study.

2.3 Antimicrobial activity of Fabaceae plants

Fabaceae is a large family of angiosperms. Several species have traditionally been used to treat different illnesses such as cough, diarrhea, and toothache (Dzoyem et al., 2018). Furthermore, antimicrobial properties have been reported in different species. For example, *Pseudarthria hooker* has been shown to exhibit antimicrobial activity. *P. hooker* is a medicinal plant native to Africa. It has been used to treat pneumonia, cough, abdominal pains, and diarrhea. Dzoyem et al. (2018) isolated flavonoids from *P. hooker* and tested its antibacterial and cytotoxicity activity. All of their flavonoid extracts exhibited antibacterial, bactericidal, and bacteriostatic activities against both Gram-positive and Gram-negative bacteria. Additionally, extracts inhibited the growth of human tumor cell lines. A similar study tested the antibacterial activity of several species of Fabaceae against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Listeria monocytogenes.* Phytochemical analyses were performed on extracts to identify their content. Araya-Cloutier et al. (2017) reported that extracts containing prenylated phenolic aglycones are significantly correlated with inhibition of MRSA and *L. monocytogenes.*

Another species of Fabaceae showing antimicrobial activity was identified by Azzouzi et al. (2014). Aerial parts of *Bituminaria bituminosa* were used for extraction and tested against different bacteria through disc diffusion methods. It was determined that *B. bituminosa* showed inhibitory effects against all bacteria tested, including microorganisms we will use in this study- *S. aureus and P. aeruginosa*. Plants belonging to Fabaceae have also been used to treat peptic ulcers. For example, *Erythrina speciosa* was shown to provide gastroprotective effects in rats suffering from gastric ulcers. In Fahmy et al. (2020), rats were pretreated with methanolic leaf extract (ELSE) of *E. speciosa* and were subsequently administered ethanol in order to induce ulcers. ELSE pretreated rats showed a 98% gastroprotection compared to control group.

2.4 Antimicrobial activity of Acacia

The *Acacia* spp. has been traditionally used in bush medicine to treat a variety of illnesses such as treat colds, bronchitis, pneumonia, dysentery, diarrhea and sore throat. Phytochemical analysis has shown that many Acacia species contain secondary metabolites including alkaloids, fatty acids, cyanogenic glycosides, amino acids, terpenes, hydrolysable and condensed tannins, and flavonoids (Seigler, 2003). These phytoconstituents are responsible for Acacia's antimutagenic, anti-fungal, anti-bacterial, antioxidant, anti-cancer, vasoconstrictor, anti-platelet aggregatory, and anti-hypertensive activities (Ali et al. 2018). Several *Acacia* species have been reported to exhibit antimicrobial activities.

The antimicrobial activity of methanolic extracts of *Acacia aulacocarpa* leaves and *Acacia complanta* leaves were evaluated (Cock, 2012). *A. aulacocarpa* was reported to inhibit the growth of 6 out of the 14 bacteria tested, including S. *aureus.* In a similar study, acetone and methanol extracts of *Acacia arabica* were evaluated for its antimicrobial activities against both gram-negative and gram-positive bacteria. Results showed inhibition against the different multi-drug resistant microorganisms used in the study (Jeyakumar et al., 2015). These results warrant further investigation into the different species and genus of the Fabaceae family to test for its antimicrobial properties.

In a study by Alajmi et al. (2017), anticancer and antimicrobial activities of *Acacia salicina, Acacia laeta, Acacia hamulosa,* and *Acacia tortillis* were evaluated. Ethanolic extracts of aerial parts of the four different species were evaluated for its antimicrobial activity against *S. aureus*, *E. coli, P. aeruginosa,* and *C. albicans* using a disc diffusion assay. Results showed zones of inhibition (ZOI) ranging from 14 to 20 mm for all extracts. In a similar study, *A. nilotica* was evaluated for its antimicrobial activity against several gram-positive and gram-negative bacteria.

Results from the disc diffusion assay showed varying levels of inhibition against the microorganisms used including S. *aureus* (Ali et al., 2018). Various species of Acacia have been found to inhibit a wide range of bacteria. *Acacia mellifera* leaf extracts are found to be active against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Mutai et al., 2009). *Acacia nilotica* has been shown to possess antimicrobial activity against gram positive cocci and gram-negative bacilli (Mustafa et al., 1999).

It has been reported that *Acacia rigidula* exhibits anti-fungal and anti-mycobacterial activities. In a study conducted by Alicia et al., (2017) bioactive compounds were extracted from *A. rigidula* to evaluate its antimicrobial activity against *Mycobacterium tuberculosis* and isolates of the *Candida* species. Minimum inhibitory concentration (MIC) was used to assess the antifungal and antimycobacterial activity. *A. rigidula* displayed a low MIC against *Candida species* and *M. tuberculosis.*

This provides strong evidence that species belonging to *Acacia* exhibit antimicrobial properties. Our study will focus on *Acacia ridigula*, which grows in the southwest, west Texas, and Northern Mexico (Clement et al, 1998). There is limited report on the biological activities, i.e., antimicrobial activity of *A. rigidula*. Our research group previously reported on its antimicrobial activity (Cavazos et al., 2020).

This research study will further investigate the biological activities of A. *rigidula*. *A. ridigula* has also been known as *Vachellia ridigula. Vachellia* is another genus of plants under the Fabaceae family. It is worth noting that other species of *Vachellia* has also been reported to contain antimicrobial properties. For example, *Vachellia. karroo* has been used to treat sexually transmitted diseases in South Africa. In Maposa et al. (2019), *V. karroo* pods were evaluated for its antimicrobial properties. Methanolic extracts were found to strongly inhibit Gram-positive

bacteria. It has been documented that *Vachella farnesiana* exhibits antimicrobial activity against *Vibrio cholerae (*Maposa et al., 2019).

2.5 Antioxidant activity of the Fabaceae and Acacia plants

Oxidative stress from different biological processes can lead to cell death. The liver is the main organ affected from free radicals associated with drugs and toxins. Plants in the Fabaceae family have also been known to have antioxidant properties. Extracts of flavonoids from *Astragalus spruneri* were shown to have antioxidant and hepatoprotective effects *in vivo* against CCl_4-induced liver damage in rats (Kondeva-Burdina et al., 2018). Antioxidant activity has also been seen in nine different species of Fabaceae native to Balkans and Serbia. Of the tested plants, all of them were shown to exhibit antioxidant activity. Three of them, *Lahtryus binatus, Trifolium pannonicum, and Anthyllis aurea* had the highest activity (Gođevac et al., 2007). Literature suggests that the antioxidant properties seen in many plants are due to the presence of phenolic compounds.

Other species of *Acacia* are also known to have antioxidant properties. Free radicals are involved in many processes that can cause damage to membranes, lipids, and proteins. In particular, reactive oxygen species (ROS) are associated with cancer, cardiovascular diseases, and atherosclerosis (Lin et al., 2018). Tung et al. (2007) evaluated the antioxidant properties of *Acacia confusa*. Ethanolic extracts of its bark were evaluated by α, α-diphenyl-β-picrylhydrazyl (DPPH) assay. *A. confusa* extracts were shown to inhibit DPPH, a proton free radical. In a similar study, ethanolic extracts of four different species, *Acacia salicina, Acacia laeta, Acacia hamulosa,* and *Acacia tortillis* were evaluated for its antioxidant effect against DPPH and β-carotene-linoleic acid. All four species inhibited DPPH and β-carotene-linoleic acid. *Acacia salicina* had the highest IC_{50} at 250.13 µg/ml and 747.5 µg/ml respectively (Alam et al., 2017).

These results provide evidence that species belonging to Acacia contain phytoconstituents that possess antioxidant properties. Therefore, *A. ridigula* warrants investigation to evaluate its antioxidant properties.

2.6 Secondary metabolites and the roles of secondary metabolites

Vascular plants synthesize a diverse number of chemical compounds, including secondary metabolites. They are an important class of polyphenolic compounds found in plants (Bennett & Wallsgrove, 1994). Secondary metabolites unlike primary metabolites, do not play a direct role in the growth and development of plants. Instead, they play a role in protecting plants against pathogens, herbivores, and fungi. Not only do they protect plants against microbes, but also protect against abiotic stress, such as UV-B radiation, and oxidative stress (Takshak & Agrawal, 2019). Secondary metabolites are well known to have antimicrobial, and antioxidant properties. In addition, secondary metabolites are essential in providing the odors, colors, and tastes of plants. These make them a valuable source for the production of antibiotics, food additives, and pesticides. The major types of secondary metabolites are phenolics, terpenes, and alkaloid glycosides.

Phenolic compounds contain an aromatic hydrocarbon group bonded to a hydroxyl group. They are involved in enzyme inhibition and DNA alkylation. Its hydroxyl group forms ionic and hydrogen bonds with peptides and protons disrupting the target protein's structure (Bhattacharya et al., 2010). Secondary metabolites can change the protein structure by forming covalent bonds with free SH, OH-, or amino groups (Cantrell et al., 2001). Plant phenolic compounds have been studied for their bioactive activities. They have been used as an antioxidant agent to extend shelf life. Additionally, they have been shown to exhibit anticancer, anti-inflammatory, antimicrobial, and anti-neurodegenerative properties.

Tannins are found in most plant species including many plant-based foods. They are naturally occurring polyphenol in the bark and leaves of plants. Tannins function to protect plants and to regulate its growth. They can be classified as either hydrolysable or condensed tannins. Hydrolysable tannins contain a polyalcohol structure that is esterified with polyphenolic carboxylic acids. Condensed tannins are made up of flavin units. Plants contain a much higher content of condensed tannins; and, thus are used for industrial purposes such as leather production (Das et al., 2020). Plants rich in tannins have been reported to have been used for traditional medicinal purposes due to its antimicrobial activity. A study by Nakashima et al. (1992) showed tannins also exhibit anti-HIV properties.

Flavonoids are a class of polyphenolic secondary metabolites. They are often found in fruits, vegetables, nuts, flowers, and other plants. They exhibit antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties. Flavonoids provide flowers with their colors. It is also believed to protect leaves from fungal infections and UV-B radiation. They are also involved in photosynthesis, morphogenesis, and sex determination (Jash & Mondal, 2014). The basic structure of flavonoid 2 phenyl-benzo(α) pyrane, contains two benzene rings (A and B) linked through a heterocyclic pyrane ring (C). Flavonoids can be divided into different subgroups based on the carbon on the C ring and its degree of unsaturation (Cushnie & Lamb, 2005). The subgroups are flavones, flavanols, flavanones, flavanonols, flavanols, catechins, anthocyanins, and chalcones. A previous study in our lab carried out phytochemical analysis on leaf extracts of *A. rigidula* (Cavazos, et al., 2020). Extracts showed high levels of flavonoid content. Given that flavonoids exhibit antimicrobial activity, *A. rigidula* extracts warrant further evaluation.

Alkaloids are derived from amino acids and contain nitrogen and a simple heterocyclic ring (Choudhary et al., 2021). They are classified into indole alkaloids, pyridine alkaloids,

benzylisoquinoline, and alkaloids. These structures make alkaloids poorly soluble in water. Alkaloids are largely found in 25% of angiosperms (Rosales et al., 2020). Indole alkaloids contain a tryptophan or tryptamine structure in their precursor. They contain a bicyclic structure that are classified into three groups- corynanthe, aspidosperma, and iboga. These structures differ by the presence of carbonyl, methoxyl, and hydroxyl groups at different positions (Rosales et al., 2020). These differences account for their different functions i.e. cerebral vasodilators, anticancer, pesticides, antiarrhythmics, and hypertensives (Zhang et al., 2018).

Terpenoids are the most abundant and structurally diverse group of plant secondary metabolites (Li et al., 2012). More than 23,000 different structures have been identified. Five major groups of terpenoids are monoterpenes, sesquiterpenes, diterpenes, and triterpenes. It has been reported that terpenoids play a direct role in plant defense. The most biologically active class of terpenoids is norditerpenoid 12-demethylmulticauline. Two terpenoids, limonoid morenolide and diterpene 17-hydroxy-sandaracopimar-8, 15-dien-11-one were isolated from the roots of Azadirachta *indica*. Limonoid morenolide was found to inhibit *Mycobacterium tuberculosis* H37Rv and M299 (Passos et al., 2019). Terpenoids have also been reported to exhibited anti-inflammatory activity. Sinulatumolins terpenoids were isolated from *Sinularia tumulosa* and were reported to show significant TNF-α inhibitor activity (Cai et al., 2020). In another study, Wang et al., (2020) isolated 14 terpenoids from *Heteroscyphus coalitus* that showed anti-virulence against *Candida albicans.*

2.7 Antimicrobial activity of selected secondary metabolites

2.7.1 *Phenols*

Phenols have been shown to exhibit antimicrobial activities against *X. fastidiosa*. In Alves et al. (2013) several phenolic compounds were shown to inhibit the growth of *S. epidermidis*, *E. coli*, *N. gonorrhoeae,* and MRSA. Phenols have also been shown to inhibit growth of *K. pneumoniae*, *B. cereus, A. flavus, and A. parasiticus.* In another study, phenolic compounds extracted from berries of V*accinium meridionale* significantly inhibited *S. aureus (*Garzón et al., 2020). Finally, Li et al. (2020) showed that two of the three phenolic compounds extracted from pickled radish showed activity against *E. coli* and *B. subtillis.*

2.7.2 *Flavonoids*

Flavonoids have previously been reported to exhibit antimicrobial activity. Gutiérrez-Venegas et al. (2019), reported the antimicrobial activities of eight flavonoids: apigenin, luteolin, catechin, morin, myricetin, quercetin, rutin, and naringin. Rutin, quercetin, and morin showed inhibition against *Actinomyces naelundii* and *Actinomyces viscosus,* while apigenin, rutin, naringin, and morin showed activity against *Enterococcus faecalis*. Rutin, quercetin, naringin, morin, and luteolin were also effective against *Escherichia coli*, *Lactobacillus casei*, and *Staphylococcus aureus.* (Gutiérrez-Venegas et al., 2019) Three flavonoid glycosides extracted from *Ginkgo bilboa*, quercetin, kaempferol, and isorhamnetin, all showed activity against all selected bacteria (Sati et al., 2019). *Rhynchosia beddomei,* a shrub belonging to the Fabaceae, has been reported to contain flavonoids. Rammohan et al. (2019) isolated four flavonoids 5,7-dihydroxy-4′-methoxyisoflavone (RB1), quercetin-7-O-methylether (RB2), isovitexin (RB3), and 5,7,3′,4′-tetrahydroxy-6-C-β-D-glucopyranosyl (RB4). Extracts were subjected to an

antimicrobial assay against drug resistant gram positive and negative bacteria and fungi. Results showed that RB2 and RB4 exhibited the highest minimal inhibition concentration against *Pseudomonas aeruginosa* and *Candida albicans*.

2.7.3 *Saponins*

Plants that are rich in saponins have also been reported to show antimicrobial activity. For example, Borah et al. (2016) extracted saponins from the tender shoots from *C. leptospadix* and tested its antimicrobial activity against *E. coli, P. putida, S. aureus,* and *Bacillus toyonensis*. The extracts showed inhibitory effects against all bacterial strains. In another study, steroidal saponins were isolated from the stems and leaves of *Paris polyphylla var.* yunnanensis. The isolated saponins were active against *Propionibacterium acnes* (Qin et al., 2012).

2.8 Antioxidant activity of selected secondary metabolites

Reactive oxidant species (ROS) such as hydrogen peroxide, hydroxyl ion, and superoxide ion are highly reactive and toxic molecules. An imbalance of these free radicals can cause oxidative stress (Azizi et al., 2021). In humans, this causes DNA damage, lipid peroxidation, and oxidative inhibition of proteins. This has been linked to cancer, neurodegenerative diseases, and inflammation (Lin et al., 2018). Flavonoids and phenols are recognized to contain antioxidant properties and are important for development of new treatments. Baba and Malik (2015), observed the antioxidant activity of phenolic and flavonoid compounds in the roots of *Arisaema jacquemontii.* The authors quantified the total phenolic and flavonoid content in the extracts and tested their ability to oxidize free radicals. Results showed a positive correlation between total flavonoid and phenolic content and antioxidant activity. Another study showed that *Acacia*

confusa contained an abundant amount of phenolic and flavonoid compounds that were responsible for its antioxidant activity (Lin et al., 2017).

A study by Ali et al., (2012) extracted phytochemicals from *Acacia nilotica* and investigated their pharmacological properties. Results indicated that *A. nilotica* contains a variety of secondary metabolites. These metabolites, including phenols and gallic acid, were shown to exhibit antioxidant properties. Terpenes, tannins, flavonoids, and saponins have also been reported in *A. berlandieri* and *A. rigidula* and contribute to their antioxidant properties (Cavazos et al., 2020).

2.9 Extraction of plant secondary metabolites

2.9.1 *Sample preservation*

Three types of plant preservation known to be effective in preparing for solvent extraction are sun drying, freeze drying, and oven drying. Each of these methods influence the quality of the dried product. Thus, it is important to choose the appropriate drying method. Freeze drying takes place in a low vacuum tube under low temperature environment. As a result, freeze drying is more advantageous because it limits the impact of oxidative damages. Freeze drying has been shown to preserve phytochemicals and keep contamination of the samples at a minimum (Asami et al., 2003; Sarker, 2006; Papoutsis et al., 2017). Furthermore, the samples can be stored in the freezer for an extended period of time without losing its phytochemical properties. A study by Mondal et al., (2019) compared the effects of oven and air flow assisted drying on leafy and non-leafy vegetables. Plants that were dried by the air flow drying method contained higher antioxidant, proximate, and vitamin C levels. It is important to note that effective drying methods are species dependent. A study compared hot air drying, microwave drying, microwave-assisted

hot air hybrid drying, and freeze drying on the extraction of phytochemical and antioxidant activities of *Aloe vera, Cymbopogan citratus, Psophocarpus tetragonolobus,* and *Centella asiatica*. Effectiveness was evaluated based on browning index and recovery of phytoconstituents. It was found that microwave drying was most effective for *C. asiatica,* microwave-assisted hot air hybrid drying for *P. tetragonolobus,* and freeze drying for *A. vera* (Ng et al., 2020).

2.9.2 *Method of extraction*

Efficient extraction of secondary metabolites from plant material is necessary. It is the first step to separate natural product from raw materials. While several extraction methods exist, two are well established; these are Soxhlet and maceration. These extraction methods are documented to be able to extract plant material. Soxhlet extraction uses an apparatus to continuously extract the herb with fresh solvent. It has a high extraction efficiency and is more time saving. However, due to long exposure time to high temperatures, there is a possibility for thermal degradation. Maceration is a simple extraction method that involves exposing plant material to a solvent for 48 hours.

In a study by Clement et al. (1998), amines and alkaloids from *Acacia rigidula* were extracted by either Soxhlet or by a more traditional method of soaking the material in acid solution. Compared to the traditional method, Soxhlet extraction was found to produce a purer and more complete isolation of amines and alkaloids. A similar study evaluated maceration extraction. Ćujić et al. (2016) yielded high counts of total phenols and anthocyanins from chokeberry fruit. This suggest that maceration is an effective method for extraction of phenols from plants. Both extraction methods have been shown to successfully extract secondary metabolites that exhibit antimicrobial activities. No current study has yet compared the efficiency of Soxhlet versus

maceration method. Identifying a more efficient method is necessary in order to yield the highest quantity of secondary metabolites.

Modern methods of extraction include microwave assisted extraction (MAE) and ultrasound-assisted extraction (UAE). MAE uses microwave energy to partition analytes from the matrix to the solvent. The microwaves interact with the dipoles of polar compounds, disrupting hydrogen bonding. While this technique reduced traction time and solvent volume, the risk of thermal degradation limits this technique to only small-molecule phenolic compounds (Azwanida, 2015). UAE uses ultrasound that range from 20 kHz to 200 kHz. The ultrasound waves disrupts the cell wall and increases surface contact between solvent and sample. While this technique reduces extraction time, the ultrasound waves may affect phytochemicals through free radical formation (Abubakar & Haque, 2020; Azwanida, 2015).

2.9.3 *Solvents used in the extraction*

Selection of a solvent for extraction is crucial in order to extract the desired secondary metabolite. The solubility and selectivity should be considered carefully when selecting a solvent. Polar solvents such as acetone, ethanol, and methanol are typically used to extract secondary metabolites (Do et al., 2014). Evaluating the efficiency of these solvents is critical. For example, evaluation of extraction solvents on total phenolic content (TPC) and total flavonoid (TFC) was reported in a study by Ngo et al. (2017). Investigators extracted TPC using 50% methanol, 50% ethanol, and 50% acetone. Results showed the highest TPC extract yield with 50% methanol, ethanol, and acetone compared to absolute concentration. These findings indicate that the choice of solvents play a role in extraction of phenolic compounds. A study by Chavan et al. (2012) reported that methanol yielded the highest extract of TPC from *S. chinensis*, while Dailey & Vuong (2015), showed 50% acetone extracted the most phenolic content from

macadamia skin. In a similar study, the highest TFC was extracted from acetone and ethanol (Do et al., 2014).

On the other hand, a study by Awouafack et al. (2013), investigated the antimicrobial activity of ethanolic extracts of *Eriosema robustum* twigs. Crude ethanol extract showed inhibitory effects against several bacteria. A similar study extracted secondary metabolites using 80% methanol, 80% ethanol, and 80% acetone from the bark of *Azadirachta indica, Terminalia arjuna, Acacia nilotica,* and *Eugenia jambolan*a. The extracts were evaluated for its antioxidant, TPC, and TFC. The highest amount of TFC and TPC were recovered from ethanolic extracts (Sultana et al., 2007). Both acetone and ethanol have been used to successfully extract secondary metabolites. The comparison of the effectivity of 50% acetone and 50% ethanol to extract flavonoid and phenolic compounds will be investigated in this study.

3 RESULTS AND DISCUSSION

3.1 Comparison of the antimicrobial activities between Soxhlet and maceration leaf extracts

The secondary metabolites in *Acacia* are known to be responsible for its antimicrobial properties (Cock, 2012; Jeyakumar et al., 2015). In this regard, the higher its antimicrobial activity, the greater amount of extracted secondary metabolites. It is therefore important to establish the extraction method that will optimize the quantity of secondary metabolites extracted. Soxhlet (Sox) and maceration (Mac) have been traditionally and typically used to extract plant material for determination of antimicrobial activities. Both have been shown to be effective in extracting various metabolites from plant material. In this regard, we study and compare these two methods because we do not know which one is more efficient for the specific plant under study. There is no universal nor ideal extraction method because each extraction procedure is unique to the plant (Azwanida, 2015). However, previously reported methods can be used for optimization and be selected as a suitable method for *A. rigidula* leaves. We sought to compare Sox and Mac extraction methods to determine any differences between these and to choose the method of extraction for the next experiments. Here, we simply use zone of inhibition (ZOI) as the basis of efficiency between the two methods. We extracted secondary metabolites from *A. rigidula* leaves by either Sox and Mac extraction methods. Extracts were tested for its activity against two representative bacteria, *S. aureus* and *Y. enterocolitica*. These were bacteria already used from our previous study and were known to be inhibited by *A. rigidula* extract (Cavazos et al., 2020). Effectiveness of each method was evaluated by measuring the mean zone of inhibition (mZOI) which is computed as the average between the longest and shortest

diameter of the ZOI. A larger mZOI indicates greater antimicrobial activity and is assumed to contain more secondary metabolites.

An analysis of variance (ANOVA) associated with a split unit arrangement in randomized complete block design (RCBD) was performed to test if there was any significant difference for each of our main sources of variation (i.e., bacteria, extract, bacteria x extract) with respect to mZOI. In Table 1, results indicate that both bacteria ($p<0.05$) and extracts ($p<0.001$) significantly impacted mZOI. Furthermore, we can assert that there are differences between bacteria (*S. aureus* and *Y. enterocolitica*) and among extracts (i.e., Sox, Mac and positive control) in terms of mZOI. We also determined that there was a significant interaction effect between extract and bacteria on mZOI ($p<0.01$). These results indicate that there are statistically significant differences between extracts and bacteria and they have an impact on mZOI. This significant interaction implies that there are differences in mZOI among the six treatment combinations of bacteria x extracts; Fig. 1 shows graphically the nature of this interaction. In this same figure, both Mac and Sox extracts exhibited antimicrobial activity based on mZOI that were significantly different from 6 mm. As shown in Table 2, Mac extracts inhibited both bacteria: the mZOI for S. *aureus* was 15.3 mm and 11.7 mm for *Y. enterocolitica.* Likewise, the Sox extract inhibited *S. aureus* with mZOI, 14.9 mm and *Y. enterocolitica* with mZOI, 11.0 mm. The negative control, DMSO, did not exhibit any inhibition. Therefore, any inhibition was attributed to the positive control or the extracts (Sox or Mac).

From Fig. 1 it is also clear that there was no difference between Mac and Sox for each bacteria type. Marsoul et al. (2020) also compared Sox and Mac for the for the extraction of secondary metabolites from the flowers of *Papaver rhoeas*. They, on the other hand, reported Sox method to be significantly more efficient in terms of total flavonoids and total polyphenol

recovery, and gave a higher antioxidant and antimicrobial activities compared to maceration. While, Vongsak et al. (2013), reported Mac to extract a higher phenolic and flavonoid content compared to Soxhlet extraction.

Table 1. Analysis of Variance results for ZOI associated with a Split-Unit Arrangement in Randomized Complete Block Design.[1]

Source	SS	df	MS	F-value	p-value
Blocks	24.10	3	8.03	5.48	0.015*
Bacteria	12.47	1	12.47	8.51	0.014*
Blocks x Bacteria	11.10	3	3.70	2.53	0.111
Extracts	141.79	2	70.90	48.37	0.000***
Bacteria x Extracts	31.57	2	15.78	10.77	0.003**
Error	16.12	11	1.47		
Total	5494.16	23			

[1]Note: significance levels are denoted by: * if $p<0.05$; ** if $p<0.01$; *** if $p<0.001$; negative control showed no variation at 6mm and hence was excluded from analysis

Mac and Sox are both simple techniques used in various extraction methods. Mac exposes plant material to a solvent and aggregates it for an extended period of time. This breaks down the cell membrane, releasing soluble phytochemicals. Sox, on the other hand, uses a thimble, containing the extract, that is placed inside a Soxhlet apparatus. A round bottom flask containing the solvent is heated. This causes the solvent to vaporize into the thimble, condense, and then drip back (Azwanida, 2015). When choosing a method, it is important to consider the heat stability of the extracted plant material, the solvent, cost, final volume, duration of extraction, and the intended use. It is recommended to use Sox when extracting from heat stable plants, using a volatile solvent, and for experimental testing. Conversely, Mac is suitable for longer exposure times, larger volume products, and if a cheaper method is needed (Abubakar & Haque, 2020).

While there is no general consensus on a universal extraction method, is it important to choose the method that best suits the plant material (Uddin et al., 2018). Heat stable plant material are extracted using Soxhlet extraction while non-heat stable ones are extracted by

maceration. *A. rigidula* leaves based on preliminary experiments performed in our laboratory is heat stable. If the solvent of extraction is water, maceration is a suitable method; for volatile solvents, Soxhlet extraction is more appropriate (Abubakar and Haque, 2020). Since our solvent is 50% water and 50% volatile solvent (ethanol/acetone) either Mac or Sox will be an appropriate choice.

For our next experiment, Mac was chosen as the extraction method. It is because in addition to the given considerations mentioned above, we find maceration as more convenient, less tedious, applicable for both small- and large-scale extractions, and provided more reproducible results.

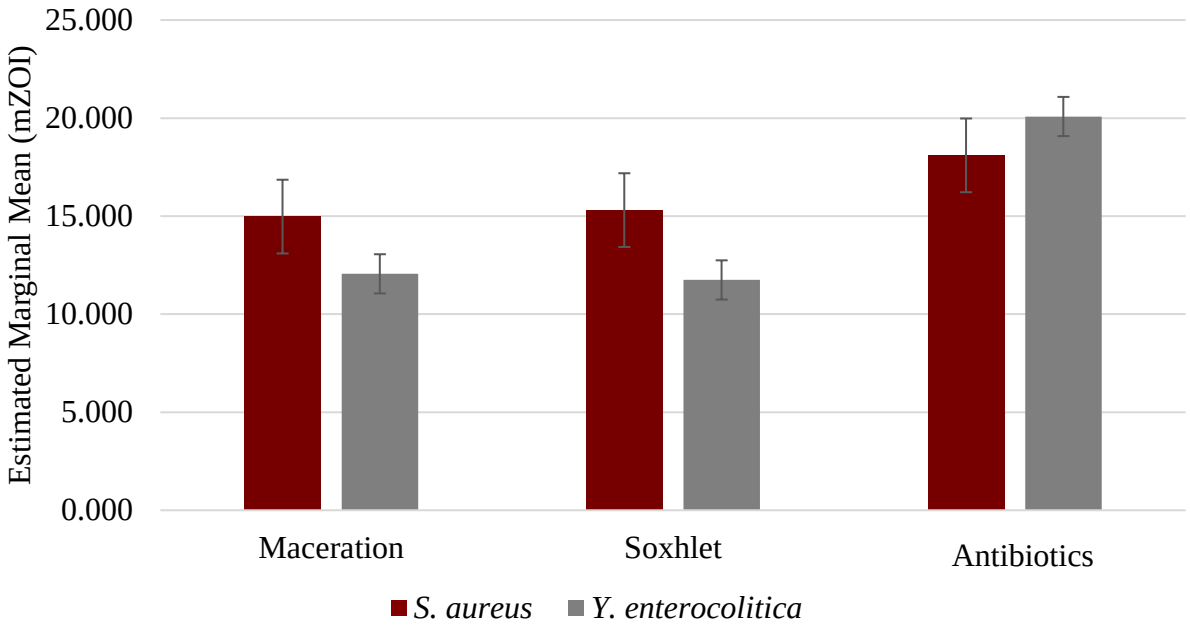

Fig. 1. Estimated mZOI differences between maceration and Soxhlet *A. ridigula* leaf extract. Error bars indicate 99% confidence intervals (CI). Non-overlapping CIs for side-by-side bars indicate significant difference in mZOI.

3.2 Comparison of the antimicrobial activities between acetone and ethanol leaf extracts

Efficiency of the solvent depends on the polarity and properties of the secondary metabolites. Polar solvents were reported to extract more secondary metabolites than non-polar solvents

(Yusnawan, 2013). This experiment aimed to determine the impact of two common solvents (50% ethanol, and 50% acetone) on the extraction efficiency of secondary metabolites. The combined use of water and organic solvents may facilitate the extraction of chemicals that are soluble in water and/or organic solvents (Do et al., 2014). Ethanol is more polar than acetone; with polarity index (PI) 0.654 and 0.355 respectively; water's PI is 1.000 (Abubakar and Haque, 2019). The extracts were then tested for their antimicrobial activity against five different bacteria (*S. aureus, Y. entercolitica, P. aeruginosa, E. faecalis,* and *P. alcalifaciens).* We used the mZOI as our initial basis to determine extraction efficiency.

Table 3 shows the result of an analysis of variance (ANOVA) of a split unit arrangement in randomized complete block design (RCBD). For these results, we can assert that the two sources of variation- bacteria ($p<0.05$), and extraction solvent ($p<0.001$) are statistically significantly different. This indicates that there are differences in mZOI among bacteria types, and among extraction solvents, negative and positive controls. But comparison of mZOI between ethanolic and acetone extracts for all bacteria tested did not show significant differences ($p>0.05$) (Fig. 2, Table 4). Both ethanolic and acetone extracts were able to inhibit all bacteria tested.

Table 2 Average zone of inhibition (mm ± SE) of Soxhlet and maceration extracts against gram-positive and gram-negative bacterial strains.[1]

Pathogen	Soxhlet	Maceration	Positive Control
S. aureus	14.98 ± 0.60	15.30 ± 0.60	18.10 ± 0.60
Y. enterocolitica	12.06 ± 0.60	11.75 ± 0.60	20.08 ± 0.741

[1]Measurements of greater than 6 mm were considered having antimicrobial activity and reported. The negative control, DMSO, was not included.

Table 3. Analysis of Variance results for acetone and ethanol extract ZOI associated with a Split-Unit Arrangement in Randomized Complete Block Design.[1]

Source	SS	df	MS	F-value	p-value
Blocks	0.125	3	0.042	0.914	0.446
Bacteria	0.527	4	0.132	2.888	0.040 *
Blocks x Bacteria	0.323	12	0.027	0.590	0.832
Extract	2.197	2	1.099	24.096	0.000 ***
Bacteria x Extract	0.698	8	0.087	1.912	0.096
Error	1.322	29	0.046		
Total	431.846	59			

[1]Note: significance levels are denoted by: * if p<0.05; ** if p<0.01; *** if p<0.001; negative control showed no variation at 6mm and hence was excluded from analysis[1]

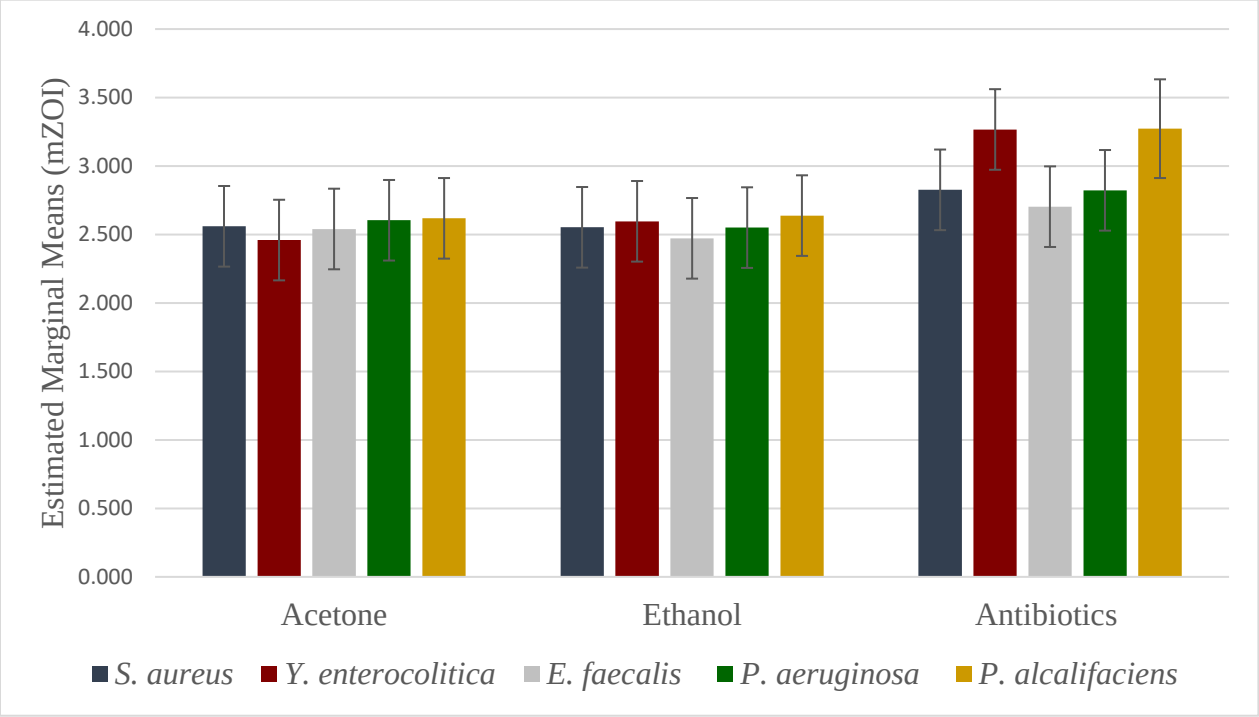

Fig. 2. Estimated mZOI differences between acetone and ethanol leaf extracts. Error bars indicate 99% confidence intervals (CI). The antibiotics are positive controls. Vancomycin served as the positive control for *S. aureus* and *E. faecalis* while Ciprofloxacin served as positive control for *Y. enterocolitica*, *P. aeruginosa*, and *P. alcalifacien*s. DMSO is the negative control (not shown).[2]

[1] The values for the negative control were removed. The negative values created severe non-normality even after data transformation.

[2] A more nuanced and detailed comparison test is shown the appendix which uses a Bonferroni pairwise comparison test.

Table 4. Average zone of inhibition (mm ± SE) of acetone and ethanol extracts against gram-positive and gram-negative bacterial strains.[1]

Pathogen	Acetone	Ethanol	Antibiotic
S. aureus	13.005 ± 1.112	12.815 ± 1.085	16.879 ± 1.093
Y. enterocolitica	11.697 ± 1.093	13.414 ± 1.093	26.231 ± 1.093
P. aeruginosa	13.517 ± 1.093	12.811 ± 1.093	16.820 ± 1.093
E. faecalis	13.581 ± 112	11.525 ± 1.085	14.927 ± 1.093
P. alcalifaciens	13.708 ± 1.093	13.985 ± 1.093	26.444 ± 1.113

[1]Measurements of greater than 6mm were considered having antimicrobial activity and reported. DMSO was used as the negative control and was not included. Vancomycin served as the positive control for S. *aureus* and *E. faecalis* while Ciprofloxacin served as positive control for *Y. enterocolitica, P. aeruginosa, and P. alcalifaciens*

Research in this field focuses on investigating and identifying the optimal extraction solvent for secondary metabolites of different plant species. When choosing a solvent, it is important to consider the suitability for the solvent in the extraction method, how the solvent can react with target compounds and the quality of the final product to be used (Chaves et al., 2020). Organic solvents (acetone, ethanol, methanol) mixed with various proportions of water have been widely used for the removal of significant quantities of secondary metabolites, e.g., phenolics and flavonoids, from plant species (Ngo et al., 2017; Chaves et al., 2020) Our study has focused on the total flavonoids and total phenolic acids. It is recommended to use an alcohol to extract more polar flavonoids, such as flavonoid glycosides with pure alcohol and aqueous alcoholic mixtures. Literature suggests that higher phenolic and flavonoid yields are usually produced in an acidic solvent (Chaves et al., 2020). This trend corresponds to the acidity supporting the cleavage of phenolics and flavonoids bound to proteins and carbohydrate polymers allowing for easier extraction from plant extracts (El-Abbassi et al., 2014). Thus, to extract a significant number of secondary metabolites in extracts, acidic organic solvents are favored.

The results of our study focused on the effects acetone and ethanol had on extracting secondary metabolites from *A. rigidula*. We observed no difference between acetone and ethanol

as solvents (Table 4; Fig. 2). In a study conducted by Ngo et al., it was determined that the mixtures of 50% (v/v) water with methanol, ethanol, and acetone had the highest extraction yield of total phenolic content (TPC) from the root of *Salacia chinensis* L. (2017). Additionally, a study conducted by Do et al., determined that 50% acetone extracted the highest yield of secondary metabolites from *L. aromatica* (2014). The concentrations of acetone and ethanol in our study are comparable to those reported in the aforementioned studies. Therefore, we can confer the efficacy of our solvents to exhibit presence of secondary metabolites via the inhibition of our five bacterial species. The extraction efficacy of acetone and ethanol can be attributed to their molecular structure. Ethanol is more polar than acetone due to its hydroxyl group; however, because of acetone's structure it has a higher dipole moment (Jadhav et al., 2017). As such, it is possible that this dipole moment made the solubility of metabolites in acetone comparable to that of ethanol.

Our results show that our solvents (50% acetone, 50% ethanol) did extract secondary metabolites that inhibited the growth of five bacterial species as compared to the control, DMSO (Fig. 2). Although there was no significant difference among the five bacteria, it is important to mention the presence of antimicrobial activity found in the Fabaceae family. The Fabaceae family has been reported to exhibit antimicrobial activity against both classes of bacteria: gram-positive and gram-negative. A study conducted by Ali et al. (2018) found that *A. nilotica* extracts possessed significant antimicrobial activity against *S. aureus* and *P. aeruginosa*. Dzoyem et al. (2014) found that leaf extracts of six species of the Fabaceae family had significant antimicrobial activity as deemed by having a minimum inhibitory concentration (MIC) of less than 100 μg/mL. A significant antimicrobial activity of an extract is a highly promising result, since an extract

consists of a mixture of compounds. Profiling and isolating the biologically active compounds from our extracts can further characterize their efficacy.

It is important to note the antimicrobial properties our extracts had against the two gram-positive strains (*S. aureus, E. faecalis*) and the three gram-negative strains (*Y. enterocolitica, P. aeruginosa, P. alcalifaciens)* used in our study. The *Acacia* species has been previously established to inhibit microbial growth. As discoveries of plant antimicrobial and antioxidant properties are found, their mechanisms of inhibiting bacterial growth must be established. The impact these compounds have on microorganisms is essential knowledge to the development of antibiotics. The mode of action of plant secondary metabolites relies on their chemical structure and properties (Garlenko et al., 2020). Generally, bacterial cells can be affected by disruption of cytoplasmic membrane function (including efflux system), interactions with membrane proteins, disruption of proton motive force and prevention of enzyme synthesis (Anand et al., 2019; Garlenko et al., 2020). For instance, flavonoid's mechanism of action on *S. aureus* is to inhibit the efflux pump located on the cytoplasmic membrane of the bacterial cell (Hong et al., 2006; Gorlenko et al., 2020). A study by Jeong et al. screened flavonoids as a candidate for antibiotics against *E. faecalis* (2009). They found that flavonoids present in their samples inhibited a key enzyme involved in fatty acid biosynthesis located in the membrane. The inhibition was caused via the interaction of the hydroxyl groups of the flavonoids and the sidechains of the two amino acids of the enzyme, arginine and phenylalanine (Jeong et al., 2009). The effect of phenols on bacterial cells is due possibly to the inactivation of cell envelope proteins, enzyme inhibition, or disruption of membranes (Pandey and Kumar, 2013; Garlenko et al., 2020). Literature suggests that the interaction of phenols with the membrane bilayer enhances the permeability in both gram-positive and gram-negative bacteria (Dzoyem et al., 2014; Tyagi et al., 2015).

Although the inhibition of bacterial growth in our study did not differ among the extracts nor bacteria, the mechanisms of action should be further investigated. This is essential in order to compare the mechanism of action of secondary metabolites to Vancomycin and Ciprofloxacin. Understanding how these modes of action relate, can provide a basis on the mechanism of bacterial resistance. To further characterize our extracts against the five bacteria investigated, a minimum inhibitory concentration (MIC) should also be established to further define their efficacy against the bacteria.

It is important to note that the inhibition of four of the bacterial species tested were not significantly different from their respective antibiotics. Specifically, both acetone and ethanol extract inhibited *S. aureus* and *E. faecalis* at a mZOI comparable to Vancomycin. Similarly, acetone and ethanol extracts were just as effective in inhibiting *P. aeruginosa and P. alcalifaciens* as Ciprofloxacin. Increasingly, many pathogenic bacteria including *S. aureus* and *E. faecalis* are acquiring resistance to Vancomycin (Werner et al., 2008). Vancomycin is often used as a last resort for *S. aureus* infections (Vivas et al., 2019). As such, this is a serious challenge for treatments of Gram-positive pathogens worldwide. Furthermore, Costa et al. (2016) observed an overall Ciprofloxacin resistance of 20% among gram-negative bacteria studied. It is estimated that by 2050, there will be no effective antibiotics available (Vivas et al., 2019). These findings are highly valuable as it serves as a basis for finding alternatives to treatments with Vancomycin and Ciprofloxacin.

3.3 Phytochemical analysis

We carried out total flavonoid content (TFC) and total phenolic content (TPC) on our leaf extracts to quantify the concentration of flavonoids and phenolic compounds in our extracts. We sought to determine if there are any correlations between antimicrobial activity and the

concentration of flavonoids and phenolics present in our extracts. Additionally, we sought to compare the efficiency of extraction of secondary metabolites between maceration and Soxhlet, as well as between acetone and ethanolic extracts.

3.3.1 *Total Flavonoid Content*

Total Flavonoid Content (TFC) was carried out on *A. ridigula* leaf extracts that underwent maceration or Soxhlet extraction, as well as extracts treated with either 50% acetone or 50% ethanol. TFC of the crude extracts was determined based on the standard quercetin and expressed as its equivalent (mg·QE/g freeze-dried extract). Table 5 presents the results of an analysis of variance of a randomized block design for extracts assigned to Mac or Sox extraction method. There was significant difference between extracts ($p<0.01$). Sox extracts (31.23 mg QE/g) contained a higher concentration of flavonoids compared to Mac (24.64 mg QE/g) (Fig. 3). Similar results were observed in TFC of Mac and Sox extracts from flowers of *Papaver rhoeas L.* Marsoul et al. (2020) reported higher TFC in Sox extracts compared to Mac extracts with the values of 21.7 ± 2.05 mg QE/g and 8.67 ± 0.024 mg QE/g respectively. On the other hand, Mac has been reported to extract a higher phenolic and flavonoid content compared to Sox extraction by Vongsak et al. (2013).

We expected no statistically significant difference between Sox and Mac extracts based on mZOI obtained from our antimicrobial assays. However, we cannot disregard the contribution of the other classes of secondary metabolites that are also present in our extracts. Acetone and ethanol leaf extracts of the genus *Acacia* have been reported to have presence of phenols, flavonoids, saponins, terpenoids and tannins (Cavazos et al, 2021; Deshpande 2013). Flavonoids, phenolic acids and terpenoids are the predominant secondary metabolites in the

genus *Acacia* (Madjid et al. 2020). Any of these secondary metabolites may have contributed to antimicrobial activity amounting to no significant difference in antimicrobial activity. Although Soxhlet extracts have higher flavonoids, the maceration extracts may have higher amounts of another class/es of secondary metabolite/s. The quantification of the other secondary metabolites especially terpenoids is recommended for further investigation.

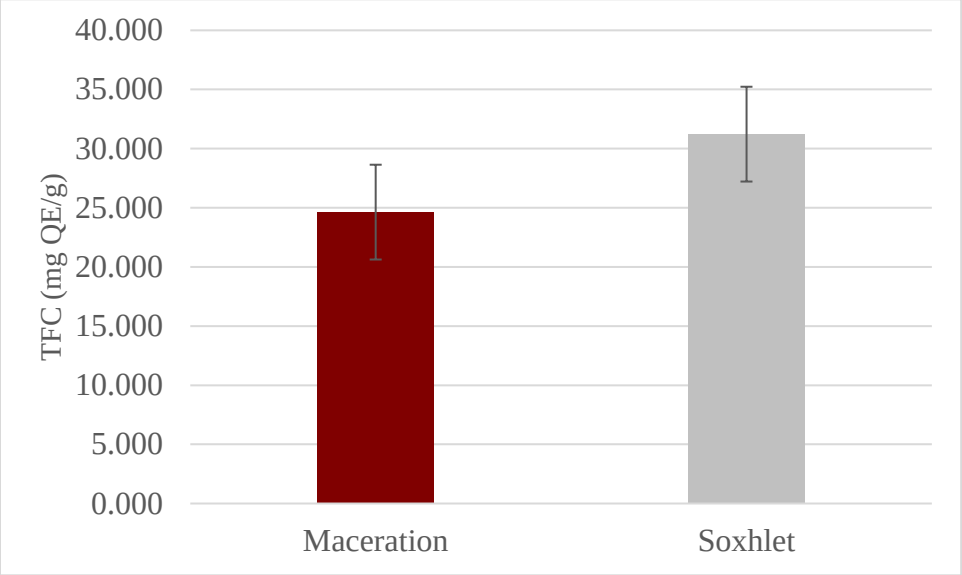

Fig 3. Mean TFC between maceration and Soxhlet extracts. Error bars indicate 99% confidence intervals (CI). Overlapping CIs for side-by-side bars indicate no significant difference in TFC. (However, Tukey test showed significant difference in TFC between methods of extraction, Table 5)

Our results showed a significant difference between extracts ($p<0.01$) (Table 6). Ethanol extract (26.55 mg QE/g) contained a higher concentration of flavonoids compared to acetone extract (24.17 mg QE/g) (Fig. 4). Our results were similar to other studies whereby the more polar the solvent, the higher the flavonoid content. In a study by Do et al. (2014) of *L. aromatica* extracts, TFC was significantly higher in 50% ethanol (19.22 mg QE/g ± 0.68) compared to 50%

acetone (17.19 mg QE/g ± 0.15). Polarity of solvents proved to have an effect in the extraction of TFC. Another example is the ethyl acetate bark extract of *Acacia ataxacantha*, it had the highest total flavonoid content (26.65 mg QE/100g ± 0 .68) and the hexane extract had the lowest (12.14 mg QE/100g ± 0.06) (Amoussa et. al, 2015). On the other hand, no significant difference in TFC was observed in 50% acetone and 50% ethanol with 100 and 89 mg catechin equivalent (CE)/g respectively for *S. chinensis* root extracts (Ngo et al., 2017).

Table 5 Analysis of Variance results for TFC of maceration and Soxhlet Extracts associated with a Randomized Complete Block Design.

Source	SS	df	MS	F-value	p-value
Blocks	178.29	3	59.43	4.46	0.028*
Extract	173.80	1	173.80	13.05	0.004**
Error	146.49	11	13.32		
Total	12980.43	16			

Note: significance levels are denoted by: * if p<0.05; ** if p<0.01; *** if p<0.001; negative control showed no variation at 6mm and hence was excluded from analysis

Table 6 Analysis of Variance results for TFC of acetone and ethanol associated with a Randomized Complete Block Design.

Source	SS	df	MS	F-value	p-value
Blocks	0.119	3	0.040	22.948	0.000***
Extract	0.017	1	0.017	9.689	0.004**
Error	0.060	35	0.002		
Total	79.011	40			

Note: significance levels are denoted by: * if p<0.05; ** if p<0.01; *** if p<0.001; negative control showed no variation at 6mm and hence was excluded from analysis

With comparison with other Acacia species Sox extracts, *A. rigidula* (31.23 mg TFC)/g) leaf extracts showed lower TFC compared to other Acacia species Sox extracts, *A. catechu* (170.8 mg QE/g), *A. sinuata* (73.0 mg QE/g), *A. nilotica* (157.2 mg QE/g) (Sulaiman and Balachandran, 2012) and *A. ataxacantha* (266.5 QE mg/g) (Amoussa et al., 2015). The observed differences

could be accounted for by the differences in the species, plant part used, and solvent used in the different studies.

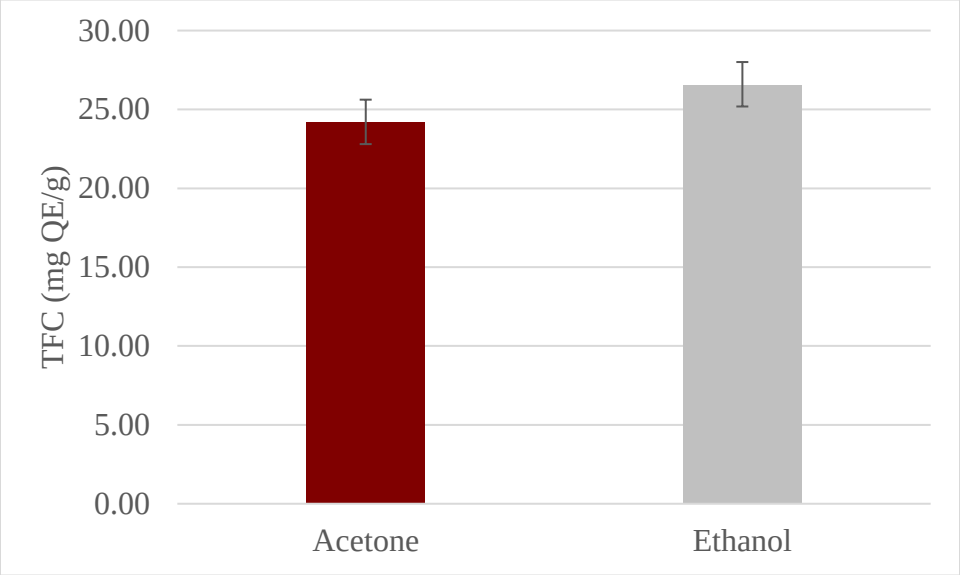

Fig. 4 Mean TFC between acetone and ethanolic extracts. Error bars indicate 99% confidence intervals (CI). Overlapping CIs for side-by-side bars indicate no significant difference in TFC. (However, Tukey test showed significant difference in TFC between solvents of extraction, Table 6)

3.3.2 *Total Phenolic Acid Content*

Total Phenolic Acid Content (TPC) was also carried out on *A. ridigula* leaf extracts (Mac, Sox, acetone and ethanol). TPC of the crude extracts was determined with reference to the standard gallic acid and expressed as its equivalent (mg·GAE/g freeze-dried extract). An analysis of variance of a randomized block design was carried out (Table 7). All extracts are rich in total phenolic acids. No statistical significance was found between Mac (119.25 mg GAE/g) and Sox extracts (125.60 mg GAE/g) ($p>0.05$) (Table 7, Fig. 4). On the other hand, Sox extracts had

higher TPC compared to Mac extracts in *Papaver rhoeas L.* with the values of 165.4 ± 3.84 mg GAE/g and 95.4 ± 2.42, respectively (Marsoul et al., 2020).

Table 7. Analysis of Variance results for TPC of maceration and Soxhlet extracts associated with a Randomized Complete Block Design.[1]

Source	SS	df	MS	F-value	p-value
Blocks	0.101	3	0.034	57.225	0.000***
Extract	0.002	1	0.002	3.506	0.088
Error	0.006	11	0.001		
Total	69.854	16			

[1]Note: significance levels are denoted by: * if p<0.05; ** if p<0.01; *** if p<0.001; negative control showed no variation at 6mm and hence was excluded from analysis

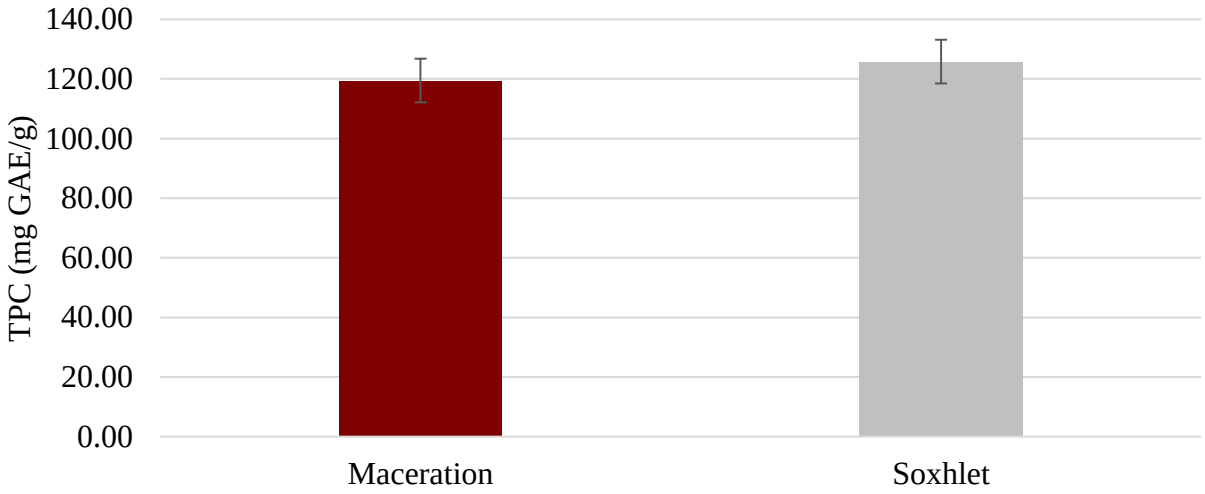

Fig. 5. Mean TPC between maceration and Soxhlet extracts. Error bars indicate 99.9% confidence intervals (CI). Overlapping CIs for side-by-side bars indicate no significant difference in TPC.

Different plant material requires different solvent types for maximum extraction of phenolic compounds (Ngo et al., 2017). Our results showed no statistically significant difference between acetone (124.98 mg GAE/g) and ethanol (125.93 mg GAE/g) extracts (p>0.05) as shown in Table 8 and Fig. 6. Our results were similar to *S. chinensis* root TPC whereby no significant

difference in TPC was observed with 60 mg GAE/g for both 50% acetone and 50% ethanol extracts (Ngo et al., 2017). No significant difference in TPC was also observed between 50% acetone (29.60 mg GAE/g ±0.15) and 50% ethanol (30.30 mg GAE/g ±0.54) of *L. aromatica* extracts (Do et al., 2014). On the other hand, Bhebhe et al. (2016) have reported that acetone at 50% with water could extract the highest TPC from *Camellia sinensis*, *Lippia javanica,* and *Ilex paraguariensis,* whereas Jamroz et al. (2014) have reported that 50% of ethanol with water is required to get the highest TPC from *Cuphea carthagenensis*. The amount of TPC was observed to be dependent on solvent whereby nonpolar solvents extracted less TPC compared to polar solvents (Amoussa et al., 2015). However, no generalization can be made when comparing polar solvents.

Many phenolic acid derivatives have been reported in the Genus Acacia. In addition, phenolic derivatives, gallic acid and coumaric acid were predominantly isolated from *A. nilotica* and A. *arabica* (Amoussa et al., 2020). Our results showed higher TPC in *A. rigidula*,125.60 mg GAE leaf extracts compared to other Acacia species Sox extracts, *A. nilotica*, 80.63 mg GAE, *A. catechu* 78.12 mg GAE (Sulaiman and Balchandran, 2012).

Table 8. Analysis of Variance results for TPC of acetone and ethanol associated with a Randomized Complete Block Design.[1]

Source	SS	df	MS	F-value	p-value
Blocks	0.217	3	0.072	73.508	0.000***
Extract	0.000	1	0.000	0.109	0.743
Error	0.034	35	0.001		
Total	176.395	40			

[1]Note: significance levels are denoted by: * if $p<0.05$; ** if $p<0.01$; *** if $p<0.001$; negative control showed no variation at 6mm and hence was excluded from analysis

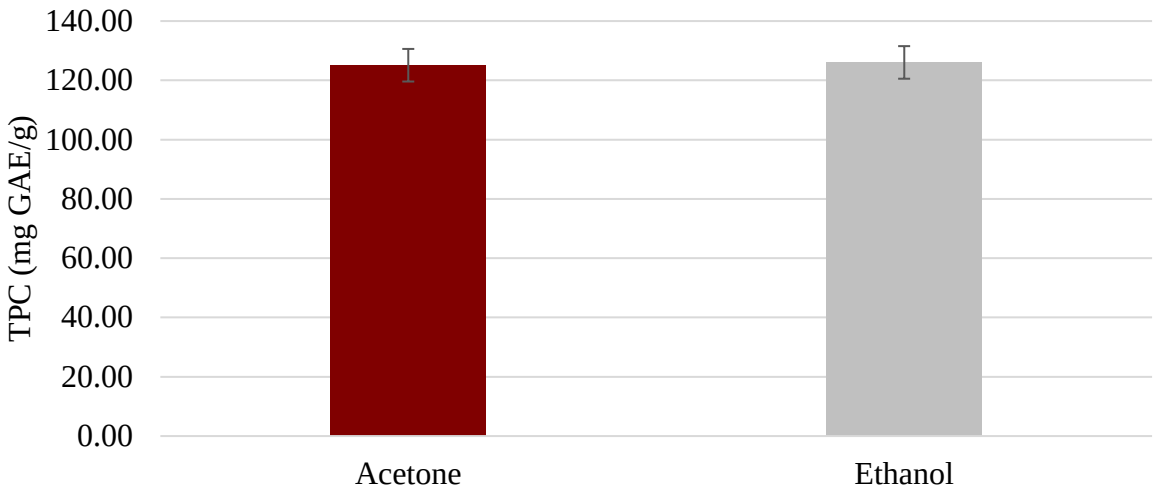

Fig. 6 Mean TPC between acetone and ethanol extracts. Error bars indicate 99.9% confidence intervals (CI). Overlapping CIs for side-by-side bars indicate no significant difference in TPC.

3.4 Antioxidant assays

An imbalance between reactive oxygen species (ROS) and antioxidant agents causes oxidative stress. ROS can damage DNA, deactivate protein, affect lipid peroxidation and are implicated in various cancers, cardiac disease, neurodegeneration, and other diseases. Synthetic antioxidants such as butylated hydroxytoluene (BHT) can manage oxidative stress but can be toxic and carcinogenic (Uzunuigbe et al., 2019). Therefore, alternatives to BHT and other synthetic antioxidants are needed to overcome the damaging effects.

Plants containing phenolic compounds act as natural antioxidants that stabilize free radicals by hydrogenation or forming complexes with oxidizing species (Afsar et al., 2018). Many *Acacia* species including *A. nilotica* (2.57 ± 0.03) at 1,000 µg/mL are reported to contain phenolic compounds that have reducing capabilities similar to ascorbic acid (2.62 ± 0.07) at 1,000 µg/mL(Afsar et al., 2018; Sadiq et al., 2017). Several species of *Acacia* have been reported to contain high levels of flavonoids and phenolic acids. Catechin and quercetin are the main

flavonoids reported, while gallic acid and coumaric acid are frequently isolated phenolic acids in different *Acacia* species. These secondary metabolites are reported to exhibit antioxidant activity (Madjid et al., 2020).

The antioxidant activity of *A. ridigula* leaf extracts was determined. We tested extracts (Soxhlet and maceration; acetone and ethanol) for their ability to prevent oxidation. The antioxidant activity of extracts and controls (BHT and ascorbic acid) was determined by the percent inhibition of linoleic acid oxidation. Linoleic acid is a polyunsaturated fatty acid that forms super radicals that oxidize Fe^{2+} to Fe^{3+} when undergoing peroxidization. This forms a complex with SCN^- that can be measured at 500nm (Sultana et al., 2007).

Our results of an analysis of variance (Table 9) shows no statistically significant difference of level of peroxidation inhibition between Soxhlet and maceration extracts ($p>0.05$). Soxhlet extract showed an inhibition of 2.2% and maceration showed a comparable inhibition of 2.4%. The controls, BHT and ascorbic acid, showed an inhibition of 10% and 30% respectively (Fig.7). All extracts were statistically significant from each other ($p<0.001$). A study by Cavazos et al. (2020) found a higher antioxidant activity for *A. ridigula*, reporting a peroxidation inhibition of 42%. Differences in our results can be due to uncontrolled conditions such as genetic variation, plant maturity, environmental conditions such as pH, growth season, climate differences, and plant population (Bajalan et al., 2016). Nobossé et al. (2018) cited age of plant leaves as significantly affecting antioxidant levels among *Moringa oleifera* L leaf extracts. It is also recommended to repeat the antioxidant assays in order to further investigate the observed difference between results.

Analysis of variance showed no significant difference were observed among treatments (positive controls, negative control, acetone and ethanolic extracts) (Table 10). Acetone extracts

showed a percent inhibition of 2.3% and ethanolic extracts showed an inhibition of 5.4%. This is less than the controls, BHT and ascorbic acid, showing an inhibition of 10% and 31% respectively (Fig. 8). Both acetone and ethanolic extracts were statistically significantly different from both positive and negative controls ($p<0.001$). Our results differ from Cavazos et al. (2020) who reported an inhibition of 70% for BHT and 69% for ascorbic acid. Additionally, their *A. rigidula* acetone extracts were observed to inhibit peroxidation at 42% and *A. berlandieri* acetone extract inhibition was observed at 19%. Similarly, Sultana et al. (2007) reported a higher antioxidant activity in *A. nilotica* acetone extracts. They observed a peroxidation inhibition of 75-86%. The antioxidant activity observed can be attributed to the secondary metabolites reported in our extracts namely flavonoids and phenolic compounds.

Table 9. Analysis of Variance results for antioxidant activity of maceration and Soxhlet extracts associated with a Randomized Complete Block Design

Source	SS	df	MS	F-value	p-value
Blocks	1.096	3	0.365	1.347	0.306
Extracts	67.892	4	16.973	62.560	0.000***
Error	3.256	12	0.271		
Total	198.351	20			

[1]Note: significance levels are denoted by: * if $p<0.05$; ** if $p<0.01$; *** if $p<0.001$; negative control showed no variation at 6mm and hence was excluded from analysis

Table 10. Analysis of Variance results for antioxidant activity of acetone and ethanol extracts associated with a Randomized Complete Block Design

Source	SS	df	MS	F-value	p-value
Blocks	0.705	3	0.235	1.259	0.336
Extracts	70.378	4	17.594	94.224	0.000***
Error	2.054	11	0.187		
Total	182.962	19			

[1]Note: significance levels are denoted by: * if $p<0.05$; ** if $p<0.01$; *** if $p<0.001$; negative control showed no variation at 6mm and hence was excluded from analysis

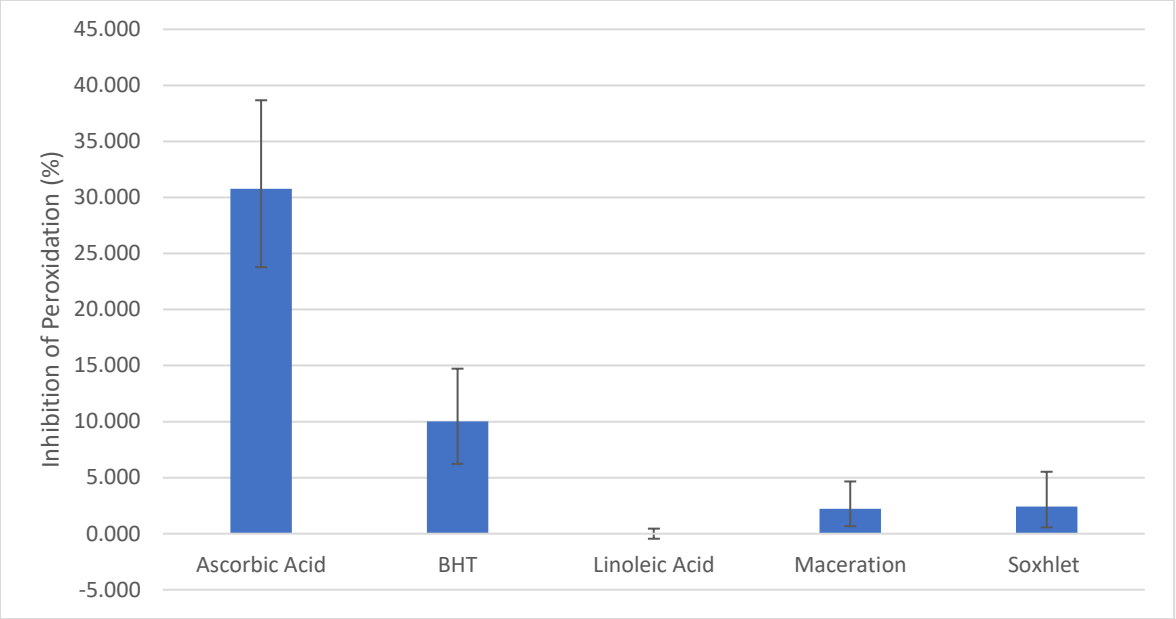

Fig. 7 Antioxidant activity of maceration and Soxhlet extracts. Positive controls (BHT and ascorbic acid) and negative control (linoleic acid) were used to compare extracts. Error bars indicate 99% confidence intervals (CI). Non-overlapping CIs for side-by-side bars indicate significant difference in peroxidation inhibition.

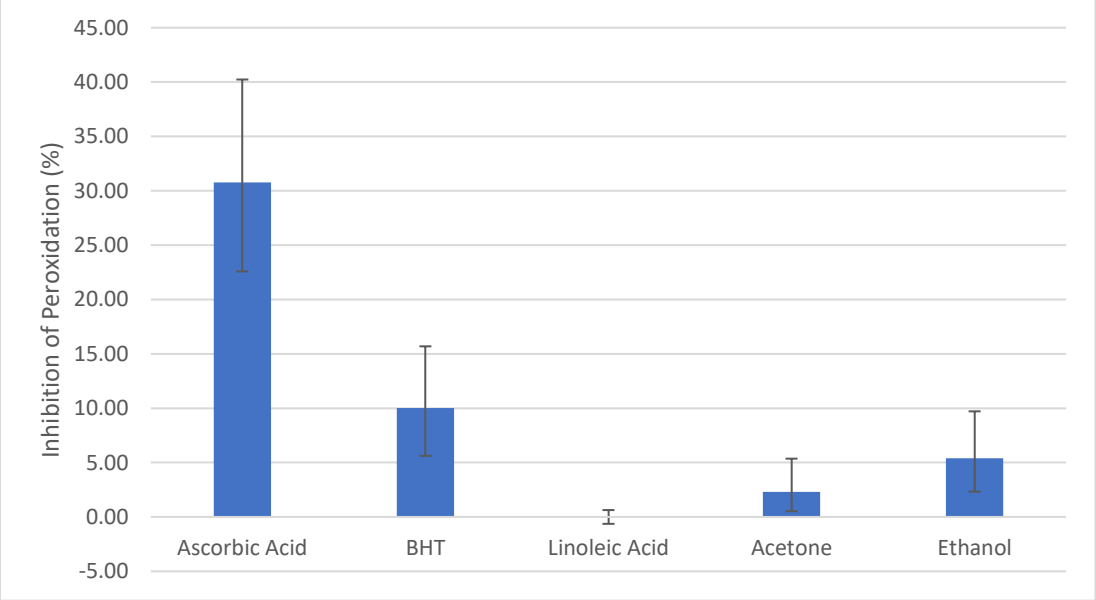

Fig. 8 Antioxidant activity of acetone and ethanolic extracts. Positive controls (BHT and ascorbic acid) and negative control (linoleic acid) were used to compare extracts. Error bars indicate 99% confidence intervals (CI). Non-overlapping CIs for side-by-side bars indicate significant difference in peroxidation inhibition.

4 EXPERIMENTAL SECTION

The methods were carried out in three parts, and will be referred to as experiment 1, 2, and 3. In experiment 1, we determined the efficiency of extraction of secondary metabolites by either maceration or Soxhlet extraction. After determining no differences in extraction efficiency between methods, we proceeded with maceration for experiment 2, as it was the more convenient method. In experiment 2, we determined whether 50% ethanol or 50% acetone will give higher antimicrobial activity and antioxidant activity. Finally, in experiment 3, the phytoconstituents namely flavonoids, total phenolics was quantitatively determined.

4.1 Plant Collection

Fresh leaves from *Acacia rigidula* were collected from four separate trees at Texas A&M International University. Tree one was located at 27°57’15’’N, 99°43’55”W, tree two was located at 28°34’25” N, 99°25’55’’W, tree three was located at 27°34’26’’ N, 99°25’55” W, and finally tree four was located at 27° 34’31’’N, 99° 26’4’’W. Trees were selected based on similar maturity and soil pH. Age was determined by using a caliper to measure trunk diameter. For each sample, 60 g of plant leaves was collected and washed using tap water and oven dried at 80°C for 90 minutes. Samples were ground into a powder consistency using a coffee grinder. This is done to increase surface area for more efficient extraction. Soil from the area around the trees were collected and 15g will be placed in a beaker with 30mL of distilled water. Samples were then shaken for 1 hour and centrifuged before being tested with a pH meter.

4.2 Crude extraction

Literature suggests that maceration is a well-established simple extraction method but has a long extraction time and low efficiency. Soxhlet extraction has a high extraction efficiency and

requires less time and uses less solvent (Zhang et al., 2018). Therefore, to save time and solvent, we investigated Soxhlet extraction as an alternative to maceration.

4.2.1 Maceration

A sample of 30g of powdered *A. rigidula* leaves was treated with 50% acetone (BeanTown Chemical 216290) at 1:10 mass: volume ratio in experiment 1, and 50% acetone and 50% ethanol in experiment 2. As suggested in the literature, acetone was chosen because of its ability to extract compounds from a range of polarity, and is easily removed from extracts (Elisha et al., 2017). Each sample were homogenized separately in acetone with shaking (VWR S-500 Orbital Shaker) for 48 hours at 350 rpm. Following the 48-hour shaking, the homogenate was centrifuged at 3000 rpm for 20 minutes (Beckman GS-15 Series Centrifuge) and the supernatant was collected and evaporated for the respective solvent using a Hei-VAP Precision rotary evaporator and finally lyophilized (Labconco 2.5L). The product after lyophilization was designated as the freeze-dried extract. The extract was tested for its antimicrobial activity and stored in a -20 ^{o}C freezer for future use.

4.2.2 Soxhlet extraction

Grounded leaf samples were subjected to a Soxhlet extractor apparatus (Chemglass® CG-1368-03). Thirty grams of ground leaves were packed into a glass Soxhlet thimble and extracted with 210 mL of 50% acetone or 50% ethanol (BeanTown Chemical 216290). The bottom flask containing the solvent was heated to boil the solvent (60^{o}C for 50% acetone and 70°C for 50% ethanol). Soxhlet extraction was ran for 24 hours.

4.3 Plant extract solvent removal

Two methods were employed to remove the excess solvent from the Soxhlet extract, rotary evaporation, and air-drying. Rotary evaporation (Heidolph® Laborota 4000) was used to remove most of the excess solvent (heated below the boiling temperatures of the solvents) from the Soxhlet extract. After most of the solvent was removed from the Soxhlet extracts, it was lyophilized using the Labconco® FreeZone 2.5 Liter Benchtop Freeze Dry System (Catalog 7670520). The 50% acetone or 50% ethanol extracts was frozen at -80℃ prior to freeze-drying. The freeze-dried extracts, dissolved in dimethyl sulfoxide (DMSO), were used for the disc diffusion assay.

4.4 Antimicrobial Assay

4.4.1 Microbial cell culturing

In experiment 1, we tested *Yersinia enterocolitica* and *Staphylococcus aureus*. In experiment 2, we tested two additional bacteria, *P. aeruginosa, and E. faecalis,* and *P. alcalifacien*s for a total of five bacteria. These bacteria were obtained from the bacterial stocks from Department of Biology and Chemistry at Texas A&M International University. A Mueller Hinton (MH) agar was used to grow our bacteria for the disc diffusion assay. On the other hand, LB Broth was used to maintain master plates. A single colony of our microorganism was used to prepare cultures for the disc diffusion assay.

5.4.2 Bacterial inoculum preparation

Miller's LB Broth was prepared based on instructions from the manufacturer (Amresco). Test tubes containing 2 mL of LB were autoclaved. A colony of each bacterium was transferred from the LB master plate to an LB Broth tube. The test tubes containing the bacterium in LB Broth

was incubated in a water bath shaker at 37°C for 16-18 hrs. After incubation, the turbidity of each LB Broth was diluted with deionized water to an absorbance level of 0.132 ± 0.005 at 625 nm. A spectrophotometer was utilized for the adjustment of the suspension. The bacterial suspension is equivalent to 0.5-1.0 x 10^8 CFU/mL.

4.4.3 Testing for antibacterial activity

Antibacterial activity of leaf extracts was evaluated by using the disc diffusion method as described by Cavazos et al. (2020). The Zone of Inhibition (ZOI) was measured to indicate any antibacterial activity by the extracts. The bacteria (20 µg) was spread on the MH agar plates using a sterile L-shaped glass rod, while rotating clockwise. Plant extracts were dissolved in DMSO to make a concentration of 0.5 mg/µL plant extracts. Sterile Whatman 6mm antibiotic assay discs was soaked with either the dissolved extract (20 µL) or the negative control, DMSO (20 µL). Vancomycin (10µg) served as the antibiotic positive control for *S. aureus, E. faecalis* and Ciprofloxacin (10µg) served as the antibiotic positive controls for *Y. enterocolitica, P. aeruginosa,* and *P. alcalifacien*s. .

Following the disc diffusion assay, plates were incubated at 37°C for 18-20 hrs. After incubation, the diameter of the zone of inhibition was measured in millimeters using a Vernier caliper. This was replicated three more times for a total of four replicates.

4.5 Antioxidant Assay

The antioxidant activity was be determined by inhibition of peroxidation in linoleic acid system using the ferric thiocyanate (FTC) method described in Sultana et al (2007). In the FTC method, the level of peroxide is measured at the beginning of lipid peroxidation. Peroxide reacts

with ferrous chloride, forming a ferric ion. The ferric ion interacts with ammonium thiocyanate, forming ferric thiocyanate and a red color (Zahin et al., 2009).

Briefly, 5 mg of extracts was treated with a solution of linoleic acid (0.13 mL), 99.8% ethanol (10 mL), and 0.2M pH 7 sodium phosphate buffers (10 mL). The mixture was diluted to 25 ml with DI water and incubated for 15 days at 40^{o}C. The degree of oxidation was measured by adding 0.2 mL of an aqueous solution of ammonium thiocyanate (30%),10 mL of ethanol (75%), 0.2 mL of sample solution, and 0.2 mL of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HCl). Absorbance was measured after 3 minutes of stirring at 500nm. The absorbance value indicated peroxide content and the percent inhibition of linoleic acid peroxidation was calculated to show antioxidant activity.

4.6 Quantitative phytochemicals analysis

Previous qualitative phytochemical analysis done in our laboratory had reported for presence of phenols, flavonoids, saponins, terpenoids and tannins. NMR, IR, and UV-Vis spectroscopy revealed structures found in flavonoids, saponins, and tannins, supporting the results of phytochemical testing (Cavazos et al., 2021). This study quantitatively determined total phenolic, total flavonoids and total saponins in *A. rigidula* leaf extracts.

4.6.1 Total phenol content

Total phenol content was determined using the Folin-Ciocalteu (FC) method laid out in Bakasso et al. (2008). The FC method is a colorimetric method, whereby a reaction occurs between hydroxyl groups of phenolic acids and a phosphomolybdate reagent (Way et al., 2020). Briefly, 0.5 mL of the extract was diluted to a concentration of 100 µg/mL. Then, it was mixed with 2.5 mL of Folin Ciocalteu reagent and incubated at room temperature for 5 min.

Subsequently, 2 mL of 75g/L sodium carbonate was added and was left to incubate for 2 hrs. The absorbance was measured at a wavelength of 760nm. The calibration curve was plotted using gallic acid.

4.6.2 Total flavonoid content

Total flavonoid content of our extract was quantified by the aluminum chloride colorimetry method as described in Do et al. (2014). In principle, acid stable complex form with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavanols and with aluminum chloride. Additionally, the orth-dihydroxyl groups in A or B rings of flavonoids form acid labile complexes with aluminum chloride (Kumar et al., 2013). The extract was diluted with ethanol with to a concentration of 100 µg/mL and subsequently diluted with 2 mL of quercetin (100 µg/mL). Then 0.1mL of 10% aluminum chloride and potassium acetate was added and left at 26°C for 30 min. A UV-VIS spectrophotometer was used to measure the absorbance at a wavelength of 415nm. Quercetin served as standard to construct a standard curve.

4.7 Experimental design and statistical analysis

4.7.1 Experimental Design 1

In experiment 1, the factors hypothesized to determine *percent inhibition* (PINH) are *treatments* (two maceration-derived extracts, two Soxhlet-derived extracts, the positive, and the negative control) and *bacterial type* (S. *aureus*, Y. *enterocolitica*). The design was a 2 x 6 factorial experiment laid out in a split-unit/split-plot randomized complete blocks design.[3] Within a complete block, the main experimental units (MEU) are the six media-prepared petri dishes; the sub-experimental units (SEU) are the six sectors within each of the six petri dishes

[3] There are four complete blocks/replication.

(MEU). In other words, there are six MEU where three were randomly assigned S. *aureus*, and the other three assigned *Y. enterocolitica.* The six treatments were randomly allocated to the six sectors (SEU) of the petri dishes (MEU). The sources of variation associated with the analysis of variance (ANOVA) derived from the following: blocks, bacterial types, residual, treatment, bacterial types X treatments, and error (treatments). Type-I error rates (α) of 0.05, 0.01, and 0.001 was used to evaluate the statistical significance. A planned comparison using linear contrasts technique was used to compare the two maceration-derived extracts and the two Soxhlet-derived extractions. Multiple comparison procedure such as the Bonferroni t-statistics or least significant difference test was used to evaluate the statistically significance associated with treatments, and bacterial types x treatments. Statistical Package for the Social Sciences (SPSS) was used for all statistical analyses. The design in shown in Table 11.

Table 11. Outline of the Analysis of Variance for Experiment Design #1 (Split Unit in RCBD)

Sources of Variation	df	SS
Replications/Blocks	r-1	SS(R)
Bacteria (or Factor A)	a-1	SS(A)
Error (A)	(r-1)(a-1)	SS(Ea)
Extract (or Factor B)	b-1	SSB
Bacteria x Extract	(a-1)(b-1)	SS(A x B)
Error (B)	(r-1)a(b-1)	SS(Eb)
Total	rab-1	

Blocks=Replication; r=4 replications, a=2 bacterial types, b=6 treatments

4.7.2 Experimental Design 2

The resulting "superior treatment" (ST) between maceration-derived and Soxhlet-derived extracts from experiment 1 was further evaluated in experiment 2. This part compared two new forms of treatment, ST with 50% acetone (ST-50A) and ST with 50% ethanol (ST-50E). The experimental layout was similar to Experiment 1, but included five bacterial types randomized into the MEU, and treatments randomized into SEU. The six treatments were still randomly

assigned to six petri dishes with sectors within a petri dish assigned to two ST-50A, two ST-50E, the positive control, and negative control. All aspects and test procedures from Experiment 1 was followed in Experiment 2. The outcome of interest were antibacterial activity, antioxidant activity, and bio-compound identification. Experiment 2 followed a 5 x 6 factorial experiment in split-unit in randomized complete blocks design (Table 12). SPSS was used for all statistical analyses.

Table 12. Outline of the Analysis of Variance for Experiment Design #2 (Split Unit in RCBD)

Sources of Variation	df	SS
Replications/Blocks	r-1	SS(R)
Bacteria (or Factor A)	a-1	SS(A)
Error (A)	(r-1)(a-1)	SS(Ea)
Extract (or Factor B)	b-1	SSB
Bacteria x Extract	(a-1)(b-1)	SS(A x B)
Error (B)	(r-1)a(b-1)	SS(Eb)
Total	rab-1	

Blocks=Replication; r=4 replications/blocks; a=5 bacterial types/MEU; b=6 treatments/SEU.

5 CONCLUSIONS

The efficiency of extracting secondary metabolites from *A. ridigula* leaf extracts between two methods (Soxhlet and maceration) and between two solvents (ethanol and acetone) was compared using a disc diffusion assay. Results showed no significant differences between mZOI between extracts indicating that both extraction methods and solvents were able to extract similar levels of bioactive compounds. All extracts were able to inhibit a range of Gram-positive and Gram-negative bacteria. Phytochemical analysis was preformed to compare the total phenolic content and total flavonoid content among extracts. Results showed that Soxhlet method and ethanol statistically extracted a higher level of total flavonoids. There was no difference in total phenolic acid content between methods or solvents. Our extracts showed antioxidant activity, however not statistically different from each other. These results show the importance of choosing the appropriate methods and solvent when extracting secondary metabolites to obtain a high yield. We have shown that both Soxhlet and maceration, as well as acetone and ethanol extracted comparable secondary metabolites. Thus, either extraction method and solvent is appropriate for extracting a high yield of secondary metabolites from *A. ridigula*.

The results of this study also provide a basis for further isolation and purification of specific secondary metabolites. Future studies will explore the isolation of specific flavonoids and phenolic to understand their biological activity in order to determine how they can be used in treatments against drug resistant bacteria. Mass spectrometry combined with gas/liquid chromatography or capillary electrophoresis, and nuclear magnetic resonance (NMR) allows for the isolation and characterization of the secondary metabolites (Gorlenk et al., 2020). Extracted secondary metabolites can used in conjugation with current antibiotics to increase their effectiveness and potentially overcome bacterial resistance. Additionally, the quantification of

other secondary metabolites, such as saponins and terpenoids present in *A. ridigula extracts* will be explored.

REFERENCES

Abubakar, A., & Haque, M. (2020). Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. *Journal of Pharmacy And Bioallied Sciences*, *12*(1), 1. https://doi.org/10.4103/jpbs.jpbs_175_19

Afsar, T., Razak, S., Shabbir, M., & Khan, M. R. (2018). Antioxidant activity of polyphenolic compounds isolated from Ethyl-acetate fraction of acacia hydaspica R. Parker. *Chemistry Central Journal*, *12*(1). https://doi.org/10.1186/s13065-018-0373-x

Alajmi, M. F., Alam, P., Alqasoumi, S. I., Ali Siddiqui, N., Basudan, O. A., Hussain, A., Mabood Husain, F., & Ali Khan, A. (2017). Comparative anticancer and antimicrobial activity of aerial parts of acacia salicina, acacia laeta, acacia hamulosa and acacia tortilis grown in Saudi Arabia. *Saudi Pharmaceutical Journal*, *25*(8), 1248-1252. https://doi.org/10.1016/j.jsps.2017.09.010

Alam, P., Alajmi, M. F., Arbab, A. H., Parvez, M. K., Siddiqui, N. A., Alqasoumi, S. I., Al-Rehaily, A. J., Al-Dosari, M. S., & Basudan, O. A. (2017). Comparative study of antioxidant activity and validated RP-HPTLC analysis of rutin in the leaves of different acacia species grown in Saudi Arabia. *Saudi Pharmaceutical Journal*, *25*(5), 715-723. https://doi.org/10.1016/j.jsps.2016.10.010

Ali, A., Akhtar, N., Khan, B. A., Khan, M. S., Rasul, A., Zaman, S., Khalid, N., Waseem, K., Mahmood, T., & Ali, L. (2012). Acacia nilotica: A plant of multipurpose medicinal uses. *Journal of Medicinal Plants Research*, *6*(9). https://doi.org/10.5897/jmpr11.1275

Ali, M. T., Haque, S. T., Kabir, M. L., Rana, S., & Haque, M. E. (2018). A comparative study of in vitro antimicrobial, antioxidant and cytotoxic activity of Albizia lebbeck and acacia

nilotica stem bark. *Bulletin of Faculty of Pharmacy, Cairo University*, *56*(1), 34-38. https://doi.org/10.1016/j.bfopcu.2017.10.002

Alicia, Blanca & Garza, Alanis & Arroyo, Joel & González, Gloria & Garza Gonzalez, Elvira & Torres, Noemí & Salazar-Aranda, Ricardo. (2017). Anti-fungal and Anti-Mycobacterial activity of plants of Nuevo Leon, Mexico. Pakistan journal of pharmaceutical sciences. 30. 17-21.

Alves, M., Ferreira, I., Froufe, H., Abreu, R., Martins, A., & Pintado, M. (2013). Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *Journal of Applied Microbiology*, *115*(2), 346-357. https://doi.org/10.1111/jam.12196

Anand U, Jacobo-Herrera N, Altemimi A, Lakhassassi N. (2019) A comprehensive review on medicinal plants as animirobial therapeutics: Potential avenues of biocompatible drug discovery. Metabolites. 9, 258.

Araya-Cloutier, C., Den Besten, H. M., Aisyah, S., Gruppen, H., & Vincken, J. (2017). The position of prenylation of isoflavonoids and stilbenoids from legumes (Fabaceae) modulates the antimicrobial activity against Gram positive pathogens. *Food Chemistry*, *226*, 193-201. https://doi.org/10.1016/j.foodchem.2017.01.026

Asami, D. K., Hong, Y., Barrett, D. M. and Mitchell, A. E., 2003, Processing-induced changes in total phenolics and procyanidins in clingstone peaches. J. Sci. Food Agric. 83, 56-63.

Avorn, J., 2001. Antibiotic resistance: Synthesis of recommendations by expert policy groups. Boston, Mass.: World Health Organization.

Awouafack, M. D., McGaw, L. J., Gottfried, S., Mbouangouere, R., Tane, P., Spiteller, M., & Eloff, J. N. (2013). Antimicrobial activity and cytotoxicity of the ethanol extract, fractions and eight compounds isolated from Eriosema robustum (Fabaceae). *BMC Complementary and Alternative Medicine*, *13*(1). https://doi.org/10.1186/1472-6882-13-289

Azizi, S., Dalli, M., Berrichi, A., & Gseyra, N. (2021). Quantification of secondary metabolites and the evaluation of the in vitro antioxidant activity of the argan tree of eastern Morocco. *Materials Today: Proceedings*. https://doi.org/10.1016/j.matpr.2020.12.1214

Azwanida, N. (2015). A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Medicinal & Aromatic Plants*, *04*(03). https://doi.org/10.4172/2167-0412.1000196

Azzouzi, S., Zaabat, N., Medjroubi, K., Akkal, S., Benlabed, K., Smati, F., & Dijoux-Franca, M. (2014). Phytochemical and biological activities of Bituminaria bituminosa L. (Fabaceae). *Asian Pacific Journal of Tropical Medicine, 7,* S481-S484. https://doi.org/10.1016/s1995-7645(14)60278-9

Baba, S. A., & Malik, S. A. (2015). Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of Arisaema jacquemontii Blume. *Journal of Taibah University for Science*, *9*(4), 449-454. https://doi.org/10.1016/j.jtusci.2014.11.001

Bakasso, S., Lamien-Meda, A., Lamien, C.E., Kiendrebeogo, M., Millogo, J., Ouedraogo, A.G., and Nacoulma, O.G. (2008). Polyphenol contents and antioxidant activities of five Indigofera species (Fabaceae) from Burkina Faso. Pakistan Journal of Biological Sciences, 11(11), pp. 1429-1435.

Bennett, R. N., & Wallsgrove, R. M. (1994). Secondary metabolites in plant defence mechanisms. New Phytologist, 127(4), 617-633. https://doi.org/10.1111/j.1469-8137.1994.tb02968.x

Bhattacharya, A., Sood, P., & Citovsky, v. (2010). The roles of plant phenolics in defense and communication during Agrobacterium and Rhizobium infection. Molecular Plant Pathology, no-no. https://doi.org/10.1111/j.1364-3703.2010.00625.x

Blackwell, G. A., Doughty, E. L., & Moran, R. A. (2020). Evolution and dissemination of L and M plasmid lineages carrying antibiotic resistance genes in diverse Gram-negative bacteria. *Plasmid*, 102528. https://doi.org/10.1016/j.plasmid.2020.102528

Borah, B., Phukon, P., Hazarika, M. P., Ahmed, R., Sarmah, D. K., Wann, S. B., Das, A., & Bhau, B. S. (2016). Calamus leptospadix griff. a high saponin yielding plant with antimicrobial property. *Industrial Crops and Products*, *82*, 127-132. https://doi.org/10.1016/j.indcrop.2015.11.075

Cai, Y., Cui, W., Tang, W., & Guo, Y. (2020). Uncommon terpenoids with anti-inflammatory activity from the Hainan soft coral Sinularia tumulosa. *Bioorganic Chemistry*, *104*, 104167. https://doi.org/10.1016/j.bioorg.2020.104167

Cantrell, C., Franzblau, S., & Fischer, N. (2001). Antimycobacterial Plant Terpenoids. Planta Medica, 67(08), 685-694. https://doi.org/10.1055/s-2001-18365

Cavazos, P. D., Lanorio, J., & Ynalvez, R. A. (2020). Evaluation of antimicrobial activity & phytochemical analysis of leaf extracts of acacia rigidula and acacia berlandieri. *The FASEB Journal*, *34*(S1), 1-1. https://doi.org/10.1096/fasebj.2020.34.s1.06311

Cavazos, P., Gonzalez, D., Lanorio, J., & Ynalvez, R. (2021). Secondary metabolites, antibacterial and antioxidant properties of the leaf extracts of acacia rigidula benth. and acacia berlandieri benth. *SN Applied Sciences*, *3*(5). https://doi.org/10.1007/s42452-021-04513-8

Chavan, J. J., Jagtap, U. B., Gaikwad, N. B., Dixit, G. B., & Bapat, V. A. (2012). Total phenolics, flavonoids and antioxidant activity of Saptarangi (Salacia chinensis L.) fruit pulp. *Journal of Plant Biochemistry and Biotechnology*, *22*(4), 409-413. https://doi.org/10.1007/s13562-012-0169-3

Choudhary, S., Zehra, A., Mukarram, M., Wani, K. I., Naeem, M., Hakeem, K. R., & Aftab, T. (2021). Potential uses of Bioactive compounds of medicinal plants and their mode of action in several human diseases. *Medicinal and Aromatic Plants*, 143-158. https://doi.org/10.1007/978-3-030-58975-2_5

Clement, B. A., Goff, C. M., & Forbes, T. A. (1998). Toxic amines and alkaloids from acacia rigidula. *Phytochemistry*, *49*(5), 1377-1380. https://doi.org/10.1016/s0031-9422(97)01022-4

Cock, I. (2012). Antimicrobial Activity of Acacia aulacocarpa and Acacia complanta Methanolic Extracts. Pharmacognosy Communications, 2(1), 66-71. https://doi.org/10.5530/pc.2012.1.12

Ćujić, N., Šavikin, K., Janković, T., Pljevljakušić, D., Zdunić, G., & Ibrić, S. (2016). Optimization of polyphenols extraction from dried chokeberry using maceration as

traditional technique. Food Chemistry, 194, 135-142. https://doi.org/10.1016/j.foodchem.2015.08.008

Cushnie, T. T., & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, *26*(5), 343-356. doi:10.1016/j.ijantimicag.2005.09.002

Dailey, A., & Vuong, Q. V. (2015). Effect of extraction solvents on recovery of bioactive compounds and antioxidant properties from macadamia (Macadamia tetraphylla) skin waste. *Cogent Food & Agriculture*, *1*(1). https://doi.org/10.1080/23311932.2015.1115646

Das, A. K., Islam, M. N., Faruk, M. O., Ashaduzzaman, M., & Dungani, R. (2020). Review on tannins: Extraction processes, applications and possibilities. *South African Journal of Botany*, *135*, 58-70. https://doi.org/10.1016/j.sajb.2020.08.008

Davies, J., & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. Microbiology and Molecular Biology Reviews, 74(3). https://doi.org/10.1128/MMBR.00016-10

Deshpande, S. (2013). Preliminary Phytochemical Analysis and In Vitro Investigation of Antibacterial Activity of Acacia nilotica against Clinical Isolates. *Journal of Pharmacognosy and Phytochemistry*, *1*(5).

Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., & Ju, Y. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. *Journal of Food and Drug Analysis*, *22*(3), 296-302. https://doi.org/10.1016/j.jfda.2013.11.001

Dzoyem, J., Tchamgoue, J., Tchouankeu, J., Kouam, S., Choudhary, M., & Bakowsky, U. (2018). Antibacterial activity and cytotoxicity of flavonoids compounds isolated from

Pseudarthria hookeri Wight & Arn. (Fabaceae). *South African Journal of Botany*, *114*, 100-103. https://doi.org/10.1016/j.sajb.2017.11.001

El-Abbassi A, Kiai H, Raiti J, Hafidi A. (2014) Could point extraction of phenolic compounds from pretreated olive mill wastewater. J. Environ. Chem. Eng. 2, 1480-1486.

Elisha, I. L., Jambalang, A. R., Botha, F. S., Buys, E. M., McGaw, L. J., & Eloff, J. N. (2017). Potency and selectivity indices of acetone leaf extracts of nine selected South African trees against six opportunistic Enterobacteriaceae isolates from commercial chicken eggs. *BMC Complementary and Alternative Medicine*, *17*(1). https://doi.org/10.1186/s12906-017-1597-3

Fahmy, N. M., Al-Sayed, E., Michel, H. E., El-Shazly, M., & Singab, A. N. (2020). Gastroprotective effects of erythrina speciosa (Fabaceae) leaves cultivated in Egypt against ethanol-induced gastric ulcer in rats. *Journal of Ethnopharmacology*, *248*, 112297. https://doi.org/10.1016/j.jep.2019.112297

Gabr, S., Nikles, S., Pferschy Wenzig, E. M., Ardjomand-Woelkart, K., Hathout, R. M., El-Ahmady, S., Motaal, A. A., Singab, A., & Bauer, R. (2018). Characterization and optimization of phenolics extracts from acacia species in relevance to their anti-inflammatory activity. *Biochemical Systematics and Ecology*, *78*, 21-30. https://doi.org/10.1016/j.bse.2018.03.001

Garzón, G. A., Soto, C. Y., López-R, M., Riedl, K. M., Browmiller, C. R., & Howard, L. (2020). Phenolic profile, in vitro antimicrobial activity and antioxidant capacity of vaccinium meridionale Swartz pomace. *Heliyon*, *6*(5), e03845. https://doi.org/10.1016/j.heliyon.2020.e03845

Gođevac, D., Zdunić, G., Šavikin, K., Vajs, V., & Menković, N. (2008). Antioxidant activity of nine Fabaceae species growing in Serbia and Montenegro. *Fitoterapia*, *79*(3), 185-187. https://doi.org/10.1016/j.fitote.2007.10.001

Gorlenko, C. L., Kiselev, H. Y., Budanova, E. V., Zamyatnin, A. A., & Ikryannikova, L. N. (2020). Plant secondary metabolites in the Battle of drugs and drug-resistant bacteria: New heroes or worse clones of antibiotics? *Antibiotics*, *9*(4), 170. https://doi.org/10.3390/antibiotics9040170

Gutiérrez-Venegas, G., Gómez-Mora, J. A., Meraz-Rodríguez, M. A., Flores-Sánchez, M. A., & Ortiz-Miranda, L. F. (2019). Effect of flavonoids on antimicrobial activity of microorganisms present in dental plaque. *Heliyon*, *5*(12), e03013. https://doi.org/10.1016/j.heliyon.2019.e03013

Handwerger, S., Raucher, B., Altarac, D., Monka, J., Marchione, S., Singh, K. V., Murray, B. E., Wolff, J., & Walters, B. (1993). Nosocomial outbreak due to Enterococcus faecium highly resistant to Vancomycin, penicillin, and Gentamicin. *Clinical Infectious Diseases*, *16*(6), 750-755. https://doi.org/10.1093/clind/16.6.750

Hong H, Landauer MR, Foriska MA, Ledney GD. (2006) Antibacterial activity of the soy isoflavone genistein. J. Basic Microbiol. 46, 329-335.

Izadi, N., Eshrati, B., Etemad, K., Mehrabi, Y., & Hashemi-Nazari, S. (2020). Rate of the incidence of hospital-acquired infections (HAIs) in Iran based on the data of the national nosocomial infections surveillance. New Microbes and New Infections, 100768. https://doi.org/10.1016/j.nmni.2020.100768

Jadhav D, Karthick NK, Kannan PP, Shanmugan R, Elangovan A, Arivazhagan G. (2017) Molecular interaction forces in acetone ethanol binary liquid solutions: ftir and theoretical studies. Journal of Molecular Structure. 1130, 497-502.

Jarvis, W. R. (1991). Nosocomial outbreaks: The centers for disease control's hospital infections program experience, 1980–1990. *The American Journal of Medicine, 91*(3), S101-S106. https://doi.org/10.1016/0002-9343(91)90352-x

Jash, S. K., & Mondal, S. K. (2014). Bioactive flavonoid fisetin - A molecule of pharmacological interest. *Journal*

Jeong KW, Lee JY, Kang DI, Lee JU, Shin SY, Kim Y. (2009) Screening of flavonoids as candidate antibiotics against Enterococcus faecalis. J. Nat. Prod. 72, 719-724.

Jeyakumar E (2015) Antibacterial activity of Acacia arabica (Bark) extract against selected multi drug resistant pathogenic bacteria. Int J Curr Microbiol Appl Sci 1:213-222.

Kondeva-Burdina, M., Shkondrov, A., Simeonova, R., Vitcheva, V., Krasteva, I., & Ionkova, I. (2018). In vitro/in vivo antioxidant and hepatoprotective potential of defatted extract and flavonoids isolated from astragalus spruneri Boiss. (Fabaceae). *Food and Chemical Toxicology*, *111*, 631-640. https://doi.org/10.1016/j.fct.2017.12.020

Kumar, P. T., Barman, K. P., Chatterjee, T. K., & Maity, S. (2013). Quantification of Total Flavonoid Content and antioxidant activity in comparison to a reference flavonoid as in vitro quality evaluation parameter for assessing bioactivity of biomarkers in herbal extracts or formulations. *JPR:BioMedRx: An International Journal*, *1*(8), 757-766.

Li, J., Huang, S., Deng, Q., Li, G., Su, G., Liu, J., & David Wang, H. (2020). Extraction and characterization of phenolic compounds with antioxidant and antimicrobial activities

from pickled radish. *Food and Chemical Toxicology*, *136*, 111050. https://doi.org/10.1016/j.fct.2019.111050

Li, S., Zhang, P., Zhang, M., Fu, C., Zhao, C., Dong, Y., Guo, A., & Yu, L. (2012). Transcriptional profile of taxus chinensis cells in response to methyl jasmonate. *BMC Genomics*, *13*(1), 295. https://doi.org/10.1186/1471-2164-13-295

Li, W., Zhao, F., Pan, J., & Qu, H. (2020). Influence of ethanol concentration of extraction solvent on metabolite profiling for Salviae Miltiorrhizae radix et Rhizoma extract by 1H NMR spectroscopy and multivariate data analysis. *Process Biochemistry*, *97*, 158-167. https://doi.org/10.1016/j.procbio.2020.06.008

Lin, H., Chang, T., & Chang, S. (2018). A review of antioxidant and pharmacological properties of phenolic compounds in acacia confusa. *Journal of Traditional and Complementary Medicine*, *8*(4), 443-450. https://doi.org/10.1016/j.jtcme.2018.05.002

Lucero-Estrada, C., Favier, G. I., & Escudero, M. E. (2020). An overview of Yersinia enterocolitica and related species in samples of different origin from San Luis, Argentina. *Food Microbiology*, *86*, 103345. https://doi.org/10.1016/j.fm.2019.103345

Madjid O., A., Sanni, A., & Lagnika, L. (2020). Chemical diversity and pharmacological properties of genus acacia. *Asian Journal of Applied Sciences*, *13*(2), 40-59. https://doi.org/10.3923/ajaps.2020.40.59

Maposa, S., Afolayan, A., & Otunola, G. (2019). Evaluation of the antimicrobial properties of Vachellia karroo Hayne Banfi and Galasso pods used traditionally for the treatment of venereal diseases. *International Journal of Pharmacology*, *15*(6), 772-776. https://doi.org/10.3923/ijp.2019.772.776

Marsoul, A., Ijjaali, M., Oumous, I., Bennani, B., & Boukir, A. (2020). Determination of polyphenol contents in papaver rhoeas L. flowers extracts (soxhlet, maceration), antioxidant and antibacterial evaluation. Materials Today: Proceedings, 31, S183-S189. https://doi.org/10.1016/j.matpr.2020.08.082atsushima, A., Kuroki, Y., Nakajima, S., Kojima, H., Seki, T., & Ueyama, M. (2020). The incidence of toxic shock syndrome due to nosocomial methicillin-resistant staphylococcus aureus infection in burn patients: A four-year retrospective cohort study. *Burns*. https://doi.org/10.1016/j.burns.2020.07.005

Mondal, I. H., Rangan, L., & Uppaluri, R. V. (2019). Effect of oven and intermittent airflow assisted tray drying methods on nutritional parameters of few leafy and non-leafy vegetables of north-east India. *Heliyon*, *5*(11), e02934. https://doi.org/10.1016/j.heliyon.2019.e02934

Mustafa, N., Tanira, M., Dar, F., & Nsanze, H. (1999). Antimicrobial Activity of <I>Acacia nilotica</I> subspp. <I>nilotica</I> Fruit Extracts. Pharmacy and Pharmacology Communications, 5(9), 583-586. https://doi.org/10.1211/146080899128735306

Mutai, C., Bii, C., Vagias, C., Abatis, D., & Roussis, V. (2009). Antimicrobial activity of acacia mellifera extracts and lupane triterpenes. *Journal of Ethnopharmacology*, *123*(1), 143-148. https://doi.org/10.1016/j.jep.2009.02.007

Nakashima, H., Murakami, T., Yamamoto, N., Sakagami, H., Tanuma, S., Hatano, T., Yoshida, T., & Okuda, T. (1992). Inhibition of human immunodeficiency viral replication by tannins and related compounds. Antiviral Research, 18(1), 91-103. https://doi.org/10.1016/0166-3542(92)90008-s

Ng, Z. X., Yong, P. H., & Lim, S. Y. (2020). Customized drying treatments increased the extraction of phytochemicals and antioxidant activity from economically viable

medicinal plants. *Industrial Crops and Products*, *155*, 112815. https://doi.org/10.1016/j.indcrop.2020.112815

Ngo, T. V., Scarlett, C. J., Bowyer, M. C., Ngo, P. D., & Vuong, Q. V. (2017). Impact of Different Extraction Solvents on Bioactive Compounds and Antioxidant Capacity from the Root of Salacia chinensis L. Journal of Food Quality, 2017, 1-8. https://doi.org/10.1155/2017/9305047

Ott, E., Bange, F., Reichardt, C., Graf, K., Eckstein, M., Schwab, F., & Chaberny, I. (2010). Costs of nosocomial pneumonia caused by meticillin-resistant staphylococcus aureus. *Journal of Hospital Infection*, *76*(4), 300-303. https://doi.org/10.1016/j.jhin.2010.07.007

Pandey AK, Kumar S. (2013) Perspective on plant products as antimicrobial angents: A Reivew. Pharmacologia. 4, 469-480.

Papoutsis, K., Pristijono, P., Golding, J., Stathopoulos, C., Bowyer, M., Scarlett, C., & Vuong, Q., 2017. Effect of vacuum-drying, hot air-drying and freeze-drying on polyphenols and antioxidant capacity of lemon (*citrus limon*) pomace aqueous extracts. Int. J. Food Sci. Technol. 52 (4), 880-887.

Passos, M. D., Carvalho, A. R., Boeno, S. I., Virgens, L. D., Calixto, S. D., Ventura, T. L., Lassounskaia, E., Braz-Filho, R., & Vieira, I. J. (2019). Terpenoids isolated from Azadirachta indica roots and biological activities. *Revista Brasileira de Farmacognosia*, *29*(1), 40-45. https://doi.org/10.1016/j.bjp.2018.12.003

Qin, X., Sun, D., Ni, W., Chen, C., Hua, Y., He, L., & Liu, H. (2012). Steroidal saponins with antimicrobial activity from stems and leaves of Paris polyphylla Var. yunnanensis. *Steroids*, *77*(12), 1242-1248. https://doi.org/10.1016/j.steroids.2012.07.007

Rammohan, A., Bhaskar, B. V., Venkateswarlu, N., Rao, V. L., Gunasekar, D., & Zyryanov, G. V. (2019). Isolation of flavonoids from the flowers of Rhynchosia beddomei Baker as prominent antimicrobial agents and molecular docking. *Microbial Pathogenesis*, *136*, 103667. https://doi.org/10.1016/j.micpath.2019.103667

Rodríguez-Lucas, C., Fernández, J., Martínez-Sela, M., Álvarez-Vega, M., Moran, N., Garcia, A., Menendez, C., García-Prieto, E., & Rodríguez-Guardado, A. (2020). Pseudomonas aeruginosa nosocomial meningitis in neurosurgical patients with intraventricular catheters: Therapeutic approach and review of the literature. *Enfermedades infecciosas y microbiologia clinica (English ed.)*, *38*(2), 54-58. https://doi.org/10.1016/j.eimce.2019.04.019

Rosales, P. F., Bordin, G. S., Gower, A. E., & Moura, S. (2020). Indole alkaloids: 2012 until now, highlighting the new chemical structures and biological activities. *Fitoterapia*, *143*, 104558. https://doi.org/10.1016/j.fitote.2020.104558

Sadiq, M. B., Tharaphan, P., Chotivanich, K., Tarning, J., & Anal, A. K. (2017). In vitro antioxidant and antimalarial activities of leaves, pods and bark extracts of acacia nilotica (L.) del. *BMC Complementary and Alternative Medicine*, *17*(1). https://doi.org/10.1186/s12906-017-1878-x

Sarker, S. D., Latif, Z., & Gray, A. I., 2006. Natural products isolation. Totowa, NJ: Humana Press.

Sati, P., Dhyani, P., Bhatt, I. D., & Pandey, A. (2019). Ginkgo biloba flavonoid glycosides in antimicrobial perspective with reference to extraction method. *Journal of Traditional and Complementary Medicine*, *9*(1), 15-23. https://doi.org/10.1016/j.jtcme.2017.10.003

Seigler, D. (2003). Phytochemistry of acacia—sensu lato. *Biochemical Systematics and Ecology*, *31*(8), 845-873. https://doi.org/10.1016/s0305-1978(03)00082-6

Singh, R., Singh, S., Kumar, S., & Arora, S. (2007). Evaluation of antioxidant potential of Ethyl acetate extract/fractions of acacia auriculiformis A. Cunn. *Food and Chemical Toxicology*, *45*(7), 1216-1223. https://doi.org/10.1016/j.fct.2007.01.002

Sultana, B., Anwar, F., & Przybylski, R. (2007). Antioxidant activity of phenolic components present in barks of Azadirachta indica, Terminalia Arjuna, acacia nilotica, and Eugenia jambolana Lam. trees. *Food Chemistry*, *104*(3), 1106-1114. https://doi.org/10.1016/j.foodchem.2007.01.019

Takshak, S., & Agrawal, S. (2019). Defense potential of secondary metabolites in medicinal plants under UV-B stress. Journal of Photochemistry and Photobiology B: Biology, 193, 51-88. https://doi.org/10.1016/j.jphotobiol.2019.02.00

The World Health Organization. (2015). Global Action Plan on Antimicrobial Resistance.

Tung, Y., Wu, J., Kuo, Y., & Chang, S. (2007). Antioxidant activities of natural phenolic compounds from acacia confusa bark. *Bioresource Technology*, *98*(5), 1120-1123. https://doi.org/10.1016/j.biortech.2006.04.017

Tyagi P, Singh M, Kumari H, Kumari A. (2015) Bactericidal Activity of curcumin is associated with damaging of bacterial membrane. PLoS ONE. 20, e0121313.

Uzunuigbe, E., Osunsanmi, F. O., Masamba, P., A Mosa, R., Opoku, A. R., & Kappo, A. P. (2019). Phytochemical constituents and antioxidant activities of crude extracts from acacia Senegal leaf extracts. *Pharmacognosy Journal*, *11*(6s), 1409-1414. https://doi.org/10.5530/pj.2019.11.218

Vivas, R., Barbosa, A. A., Dolabela, S. S., & Jain, S. (2019). Multidrug-resistant bacteria and alternative methods to control them: An overview. *Microbial Drug Resistance*, *25*(6), 890-908. https://doi.org/10.1089/mdr.2018.0319

Vongsak, B., Sithisarn, P., Mangmool, S., Thongpraditchote, S., Wongkrajang, Y., & Gritsanapan, W. (2013). Maximizing total phenolics, total flavonoids contents and antioxidant activity of moringa oleifera leaf extract by the appropriate extraction method. *Industrial Crops and Products*, *44*, 566-571. https://doi.org/10.1016/j.indcrop.2012.09.021

Wang, X., Jin, X., Zhou, J., Zhu, R., Qiao, Y., Zhang, J., Li, Y., Zhang, C., Chen, W., Chang, W., & Lou, H. (2020). Terpenoids from the Chinese liverwort Heteroscyphus coalitus and their anti-virulence activity against candida albicans. *Phytochemistry*, *174*, 112324. https://doi.org/10.1016/j.phytochem.2020.112324

Way, M. L., Jones, J. E., Nichols, D. S., Dambergs, R. G., & Swarts, N. D. (2020). A comparison of laboratory analysis methods for total phenolic content of cider. *Beverages*, *6*(3), 55. https://doi.org/10.3390/beverages6030055

Werner, G., Strommenger, B., & Witte, W. (2008). Acquired vancomycin resistance in clinically relevant pathogens. *Future Microbiology*, *3*(5), 547-562. https://doi.org/10.2217/17460913.3.5.547

Willems, R. J., & Van Schaik, W. (2009). Transition ofEnterococcus faeciumfrom commensal organism to nosocomial pathogen. *Future Microbiology*, *4*(9), 1125-1135. https://doi.org/10.2217/fmb.09.82

Yusnawan, E. (2013). The effectiveness of polar and non polar fractions of ageratum conyzoides L. To control peanut rust disease and phytochemical screenings of secondary metabolites.

Jurnal Hama dan Penyakit Tumbuhan Tropika, 13(2), 159-166. https://doi.org/10.23960/j.hptt.213159-166

Zahin, M., Aqil, F., & Ahmad, I. (2009). THE IN VITRO ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF FOUR INDIAN MEDICINAL PLANTS. *International Journal of Pharmacy and Pharmaceutical Sciences*, *1*.

Zhang, Q., Lin, L., & Ye, W. (2018). Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Medicine*, *13*(1). https://doi.org/10.1186/s13020-018-0177-x

APPENDIX

Table 13: Tukey HSD Multiple Comparisons of Extracts on mZOI

Extract		Difference		Std. Error	Sig.	99% Confidence Interval	
						Lower Bound	Upper Bound
1	2	-0.006		0.0675	0.996	-0.219	0.208
	3	-0.408	**	0.0684	0.000	-0.624	-0.192
2	1	0.006		0.0675	0.996	-0.208	0.219
	3	-0.402	**	0.0684	0.000	-0.618	-0.186
3	1	0.408	**	0.0684	0.000	0.192	0.624
	2	0.402	**	0.0684	0.000	0.186	0.618

The error term is MSE = .046 from the SUA RCBD anova; Extract 1=acetone, 2=ethanol, 3=positive

** denotes significant difference at the 0.01 level.

Table 14: Adjusted Bonferroni Pairwise Comparisons for Extract-Bacteria Combination on mZOI

Extract	Bacteria	Bacteria	Difference		SE	p-value	99% Confidence Interval for Difference[b]	
							Lower Bound	Upper Bound
1	1	2	0.100		0.151	1.000	-0.452	0.653
		3	0.020		0.151	1.000	-0.533	0.572
		4	-0.044		0.151	1.000	-0.597	0.508
		5	-0.058		0.151	1.000	-0.611	0.494
	2	1	-0.100		0.151	1.000	-0.653	0.452
		3	-0.081		0.151	1.000	-0.633	0.472
		4	-0.145		0.151	1.000	-0.697	0.408
		5	-0.159		0.151	1.000	-0.711	0.394
	3	1	-0.020		0.151	1.000	-0.572	0.533
		2	0.081		0.151	1.000	-0.472	0.633
		4	-0.064		0.151	1.000	-0.616	0.489
		5	-0.078		0.151	1.000	-0.630	0.475
	4	1	0.044		0.151	1.000	-0.508	0.597
		2	0.145		0.151	1.000	-0.408	0.697
		3	0.064		0.151	1.000	-0.489	0.616
		5	-0.014		0.151	1.000	-0.567	0.539
	5	1	0.058		0.151	1.000	-0.494	0.611

		2	0.159		0.151	1.000	-0.394	0.711
		3	0.078		0.151	1.000	-0.475	0.630
		4	0.014		0.151	1.000	-0.539	0.567
2	1	2	-0.044		0.151	1.000	-0.596	0.509
		3	0.080		0.151	1.000	-0.472	0.633
		4	0.002		0.151	1.000	-0.550	0.555
		5	-0.086		0.151	1.000	-0.638	0.467
	2	1	0.044		0.151	1.000	-0.509	0.596
		3	0.124		0.151	1.000	-0.428	0.677
		4	0.046		0.151	1.000	-0.507	0.599
		5	-0.042		0.151	1.000	-0.594	0.511
	3	1	-0.080		0.151	1.000	-0.633	0.472
		2	-0.124		0.151	1.000	-0.677	0.428
		4	-0.078		0.151	1.000	-0.631	0.474
		5	-0.166		0.151	1.000	-0.719	0.387
	4	1	-0.002		0.151	1.000	-0.555	0.550
		2	-0.046		0.151	1.000	-0.599	0.507
		3	0.078		0.151	1.000	-0.474	0.631
		5	-0.088		0.151	1.000	-0.640	0.465
	5	1	0.086		0.151	1.000	-0.467	0.638
		2	0.042		0.151	1.000	-0.511	0.594
		3	0.166		0.151	1.000	-0.387	0.719
		4	0.088		0.151	1.000	-0.465	0.640
3	1	2	-0.441		0.151	0.067	-0.993	0.112
		3	0.123		0.151	1.000	-0.430	0.675
		4	0.003		0.151	1.000	-0.549	0.556
		5	-0.447		0.169	0.130	-1.064	0.171
	2	1	0.441		0.151	0.067	-0.112	0.993
		3	0.564	**	0.151	0.008	0.011	1.116
		4	0.444		0.151	0.063	-0.108	0.997
		5	-0.006		0.169	1.000	-0.623	0.612
	3	1	-0.123		0.151	1.000	-0.675	0.430
		2	-0.564	**	0.151	0.008	-1.116	-0.011
		4	-0.119		0.151	1.000	-0.672	0.433
		5	-0.569		0.169	0.021	-1.187	0.048
	4	1	-0.003		0.151	1.000	-0.556	0.549

		2	-0.444		0.151	0.063	-0.997	0.108
		3	0.119		0.151	1.000	-0.433	0.672
		5	-0.450		0.169	0.124	-1.068	0.168
	5	1	0.447		0.169	0.130	-0.171	1.064
		2	0.006		0.169	1.000	-0.612	0.623
		3	0.569		0.169	0.021	-0.048	1.187
		4	0.450		0.169	0.124	-0.168	1.068

** denotes significant difference at the 0.01 level.

Extract 1=acetone, 2=ethanol, 3=positive control

Bacteria 1=*S. aureus*, 2=*Y. enterocolitica*, 3=*E. faecalis*, 4=*P.aeruginosa*, 5=*P.alcalifaciens*

Table 15. Mean Comparison of Extraction Methods on Peroxidation Inhibition (Tukey and Least Squares Difference Test)

			Difference		SE	p-value	99% Confidence Interval	
							Lower Bound	Upper Bound
Tukey HSD	1 Ascorbic	2 BHT	2.382	***	0.306	0.000	1.092	3.672
		3 Negative	5.548	***	0.306	0.000	4.258	6.838
		4 Soxhlet	4.060	***	0.306	0.000	2.770	5.350
		5 Maceration	3.900	***	0.330	0.000	2.506	5.293
	2 BHT	1 Ascorbic	-2.382	***	0.306	0.000	-3.672	-1.092
		3 Negative	3.166	***	0.306	0.000	1.876	4.456
		4 Soxhlet	1.678	**	0.306	0.001	0.388	2.968
		5 Maceration	1.518	**	0.330	0.005	0.125	2.911
	3 Negative	1 Ascorbic	-5.548	***	0.306	0.000	-6.838	-4.258
		2 BHT	-3.166	***	0.306	0.000	-4.456	-1.876
		4 Soxhlet	-1.488	***	0.306	0.004	-2.778	-0.198
		5 Maceration	-1.648	***	0.330	0.003	-3.041	-0.255
	4 Soxhlet	1 Ascorbic	-4.060	***	0.306	0.000	-5.350	-2.770
		2 BHT	-1.678	***	0.306	0.001	-2.968	-0.388
		3 Negative Control	1.488	**	0.306	0.004	0.198	2.778
		5 Maceration	-0.160		0.330	0.987	-1.554	1.233
	5 Maceration	1 Ascorbic	-3.900	***	0.330	0.000	-5.293	-2.506
		2 BHT	-1.518	**	0.330	0.005	-2.911	-0.125

		3 Negative Control	1.648	**	0.330	0.003	0.255	3.041
		4 Soxhlet	0.160		0.330	0.987	-1.233	1.554

, * significance at the .01 and the .001 level, respectively.

Peroxidation inhibition; square root transformed

Table 16. Mean Comparison of Extraction Solvents on Peroxidation Inhibition (Tukey Test)

							99% Confidence Interval	
			Difference		SE	p-value	Lower Bound	Upper Bound
Tukey HSD	1 Ascorbic	BHT	2.382	***	0.368	0.000	0.862	3.902
		Negative	5.548	***	0.368	0.000	4.028	7.068
		Acetone	4.027	***	0.368	0.000	2.507	5.547
		Ethanol	3.227	***	0.368	0.000	1.706	4.746
	2 BHT	1	-2.382	***	0.368	0.000	-3.902	-0.862
		Negative	3.166	***	0.368	0.000	1.646	4.686
		Acetone	1.646	**	0.368	0.006	0.126	3.166
		Ethanol	0.845		0.368	0.212	-0.675	2.365
	3 Negative	Ascorbic	-5.548	***	0.368	0.000	-7.068	-4.028
		BHT	-3.166	***	0.368	0.000	-4.686	-1.646
		Acetone	-1.520	*	0.368	0.010	-3.040	0.000
		Ethanol	-2.321	***	0.368	0.000	-3.841	-0.801
	4 Acetone	1 Ascorbic	-4.027	***	0.368	0.000	-5.547	-2.507
		2 BHT	-1.646	***	0.368	0.006	-3.166	-0.126
		3 Negative	1.520	**	0.368	0.010	0.000	3.040
		5 Ethanol	-0.801		0.368	0.253	-2.321	0.719
	5 Ethanol	1 Ascorbic	-3.227	***	0.368	0.000	-4.746	-1.706
		2 BHT	-0.845		0.368	0.212	-2.365	0.675
		3 Negative	2.321	***	0.368	0.000	0.801	3.841
		4 Acetone	0.801		0.368	0.253	-0.719	2.321

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