EXTRACTION AND ISOLATION OF BIOACTIVE COMPOUND FROM PIPER BETLE LEAVES AND ANTIMICROBIAL EFFICACY OF THE EMULSION

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ABSTRACT

Piper betle (daun sirih) extract and essential oil were reported to have multifunctional biological effects including medicinal and antimicrobial functions. The main objectives of this study were to extract bioactive compounds from the dried *Piper betle* leaves in order to determine the bioactive compounds from the extract, formulate the *Piper betle* emulsion, investigate the antibacterial activity and acute systemic toxicity of the *Piper betle* emulsion. The extraction of *Piper betl*e leaves was prepared by using three extraction methods namely Soxhlet extraction method, supercritical carbon dioxide (SCO_2) extraction method and probe type ultrasound - assisted extraction method. The probe type ultrasound - assisted extraction was found to be significant in improving extraction efficiency, reduce the consumption of extraction time and easy to be used. The extraction of *Piper betle* leaves using probe type ultrasound - assisted can achieve a high yield in a very short time (15 minutes) as compared to the Soxhlet extraction method (1440 minutes) and SCO_2 extraction method (120 minutes). A total of 72 bioactive compounds from probe type ultrasound - assisted extraction and 84 bioactive compounds from SCO_2 extraction were isolated and identified from the dried leaves of *Piper betle* by using gas chromatography - mass spectrometry (GC - MS) method. The presence of various bioactive compounds from *Piper betle* leaves justifies this plant contains medicinal properties and could be a promising source for potential antimicrobial agents. In this study, *Piper betle* emulsion was formulated using *Piper betle* essential oil, Tween 80, and water by using 20 kHz probe - type ultrasonic processor. Ultrasonic emulsification of *Piper betle* essential oil, Tween 80 and water was carried out and the effects of different concentration of surfactants (2.5 % w/v and 5 % w/v), various emulsification duration (30 minutes, 60 minutes and 90 minutes) and ultrasound intensity on the droplets size of emulsion has been studied. All of the parameters have significant effects on the diameter of droplets of *Piper betle* emulsions. It was observed that at 30 minutes of sonication time with surfactant concentration of 2.5 % w/v in 200 mL of water, a stable *Piper betle* emulsion with a mean diameter of droplet approximately 250 nm could be formed. The antibacterial activity of formulated emulsion was evaluated *in - vitro* and the minimal inhibitory concentration (MIC) was determined with the used of macrodilution method. The bacterial killing kinetic was also evaluated for this formulated emulsion. The MIC of the *Piper betle* emulsion for *Streptococcus mutans* was 2.5 mg/ml and *Staphylococcus aureus* was 5 mg/ml. The time kill kinetic study revealed that the formulated emulsion could act as microbiostatic agents. Meanwhile, the area under the curve for *Piper betle* emulsion against *Streptococcus mutans* and *Staphylococcus aureus* revealed that the number of bacterial cells were significantly ($p < 0.05$) reduced when compared to the control sample. This result proposed that, the formulated oil-in-water *Piper betle* emulsion has the potential to be used as an antibacterial agent against *Streptococcus mutans* and *Staphylococcus aureus* such as in the treatment of various oral diseases. Considering the pharmacological relevance of *Piper betle* leaves, the acute systemic toxic effects of the *Piper betle* emulsion were investigated. The overall findings of this study indicate that the *Piper betle* emulsion is non-toxic at the dose of 50 mL/kg body weight and can be considered safe for traditional and medicinal uses. From this study, the 20 kHz probe - type ultrasonic processor can be used to do the faster extraction of *Piper betle* leaves and form the oil-in-water *Piper betle* emulsion. The formulated *Piper betle* emulsion with mean diameter of 250 nm is confirmed non-toxic and can inhibit the growth of *Streptococcus mutans* and *Staphylococcus aureus*.

ABSTRAK

Ekstrak *Piper betle* (daun sirih) dan minyak pati daun sirih telah dilaporkan mempunyai pelbagai fungsi kesan biologi termasuklah fungsi perubatan dan antimikrob. Objektif utama kajian ini adalah untuk mengekstrak sebatian bioaktif daripada daun sirih dan mengenalpasti sebatian bioaktif daripada ekstrak, menghasilkan emulsi daun sirih, mengkaji aktiviti antibakteria dan kesan toksik sistem akut emulsi daun sirih. Pengekstrakan daun sirih dilakukan melalui tiga kaedah pengekstrakan iaitu kaedah pengekstrakan Soxhlet, kaedah pengekstrakan superkritikal karbon dioksida (SCO_2) dan kaedah pengekstrakan kuar ultrabunyi. Kaedah pengekstrakan kuar ultabunyi telah terbukti dapat meningkatkan kecekapan, mengurangkan masa pengekstrakan yang diperlukan dan juga mudah untuk dikendalikan semasa pengestrakkan dijalankan. Pengekstrakan daun sirih dengan menggunakan kaedah pengekstrakan kuar ultrabunyi, hasil yang lebih banyak dapat diperoleh dalam jangka masa yang pendek (15 minit) berbanding dengan kaedah pengekstrakan Soxhlet (1440 minit) dan kaedah pengekstrakan SCO_2 (120 minit). Sejumlah 72 Sebatian bioaktif yang diperoleh melalui kaedah pengekstrakan kuar ultrabunyi dan 84 sebatian bioaktif yang diperoleh melalui kaedah pengekstrakan SCO_2 telah diasingkan daripada daun sirih dan dikenalpasti melalui kromatografi gas spektrometri jisim (GC-MS). GC-MS menjustifikasikan daun sirih sebagai tumbuhan mengandungi sifat perubatan dan berpotensi menjadikan ia sebagai sumber agen antimikrob kerana daun sirih menunjukkan pelbagai sebatian bioaktif. Dalam kajian ini, emulsi daun sirih telah diformulasikan dengan menggunakan minyak pati daun sirih, Tween 80 dan air melalui kaedah pengekstrakan kuar ultrabunyi 20kHz. Pengemulsian ultrabunyi terhadap minyak pati daun sirih, Tween 80 dan air telah dijalankan dan kesan daripada kepekatan surfaktan yang berbeza (2.5 % w/v dan 5 % w/v), jangka masa pengemulsian yang berbeza (30 minit, 60 minit dan 90 minit) dan intensiti ultrabunyi ke atas saiz titisan yang diperoleh daripada emulsi tersebut telah dikaji. Setiap parameter yang dikaji mempunyai kesan yang signifikan terhadap saiz atau diameter titisan emulsi daun sirih. Ia juga dapat dilihat bahawa emulsi daun sirih yang stabil dengan purata diameter titisan pada 250 nm dapat dihasilkan dengan menggunakan kepekatan surfaktan pada 2.5 % dalam masa sonikasi 30 minit. Fungsi antimikrob emulsi daun sirih ini telah dinilai secara *in - vitro* dan kaedah pencairan makco digunakan untuk menilai kepekatan minima perencatan (MIC) bagi emulsi daun sirih. Kinetik perencatan bakteria juga digunakan untuk menilai kesan perencatan terhadap emulsi daun sirih. MIC bagi emulsi daun sirih terhadap *Streptococcus mutans* adalah sebanyak 2.5 mg/ml dan *Staphylococcus aureus* adalah sebanyak 5 mg/ml. Kajian masa kinetik perencatan menunjukkan bahawa emulsi daun sirih berpotensi untuk dijadikan sebagai agen mikrobiostatik. Dalam kawasan di bawah lengkung graf masa-perencatan menunjukkan bilangan sel *Streptococcus mutans* dan *Staphylococcus aureus* berkurang secara signifikan ($p < 0.05$) apabila dibandingan dengan sampel kawalan. Keputusan kajian ini menunjukkan bahawa emulsi daun sirih ini berpotensi untuk sebagai agen antibakteria terhadap *Streptococcus mutans* dan *Staphylococcus aureus* dalam rawatan terhadap pelbagai penyakit di dalam mulut. Memandangkan daun sirih mempunyai kepentingan farmakologi, kesan toksik sistem akut emulsi daun sirih dikaji. Penemuan keseluruhan kajian ini menunjukkan bahawa emulsi daun sirih adalah tidak toksik pada dos 50 mL/kg berat badan dan boleh dianggap selamat untuk kegunaan tradisional dan perubatan. Dalam kajian ini, kaedah pengekstrakan kuar ultrabunyi 20 kHz digunakan bagi mempercepatkan pengekstrakan daun sirih dan memformulasikan emulsi daun sirih. Formulasi emulsi daun sirih dengan diameter purata 250 nm adalah disahkan tidak toksik dan boleh merencatkan pertumbuhan *Streptococcus mutans* dan *Staphylococcus aureus.*

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LIST OF ABBREVATIONS

ANOVA	-	Analysis of Variance
AUC	-	Area Under Curve
ATP	-	Adenosine Tri Phosphate
BHI	-	Brain Heart Infusion
CER	-	Constant Extraction Rate
CHX	-	Chlorhexidine
DNA	-	Deoxyribonucleic Acid
FRIM	-	Forest Research Institute Malaysia
GC – MS	-	Gas Chromatography Mass Spectrometry
GRAS	-	Generally Recognized As Safe
Hr	-	Hour(s)
HLB	-	Hydrophilic - Lipophilic Balance
HPH	-	High Pressure Valve Homogenization
Log	-	Logarithm (10)
MARDI	-	Malaysia Agriculture Research and Development Institute
MIC	-	Minimal Inhibitory Concentration
Min	-	minute
O / W	-	Oil - In - Water
O / W / O	-	Oil - In - Water - In - Oil
PDI	-	Polydispersity Index
PIC	-	Phase Inversion Composition
PIT	-	Phase Inversion Temperature
R&D	-	Research and Development
SE	-	Spontaneous Emulsification
SEM	-	Scanning Electron Microscopy
SFE	-	Supercritical Fluid Extraction
TEM	-	Transmission Electron Microscopy
t – test	-	Student's T – test
UAE	-	Ultrasound - Assisted Extraction
v / w	-	Volume / Weight

WHO	-	World Health Organisation
W / O	-	Water - In - Oil
W / O / W	-	Water - In - Oil - In - Water

LIST OF SYMBOLS

CFU / ml	-	Colony Forming Unit / Milliliter
Cm^2 / s	-	Square Centimetre / Second
CO_2	-	Carbon Dioxide
g	-	Gram
g / cm^3	-	Gram / Cubic Centimetre
Hz	-	Hertz
K	-	Kelvin
KHz	-	Kilohertz
ml	-	Milliliter
MPa	-	Megapascal
Nm	-	Nanometer
SCO_2	-	Supercritical carbon dioxide Extraction
W / cm^2	-	Watt / square centimetre
µL	-	Microliter
µm	-	Micrometer
%	-	Percentage
ºC	-	Degrees Celsius

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CHAPTER 1

INTRODUCTION

1.1 Overview

Plants are one of the most significant sources for the production of natural products available nowadays. The plants with bioactive compounds are consider as medicinal or herbal plants. These bioactive compounds can easily be found and identifiable in various parts of the plants such as in the leaves, stems, flowers and even in fruits. The used of medicinal or herbal plants for the purpose of treatment has been reported since thousand years ago. Apart from being used in medical or treatment purpose, the medicinal herbs or plants are also being used as a part of flavouring, aromatherapy and Ayurveda which are common among Asia countries. Even dental and oral health products nowadays such as mouth freshener contained medical plants or herbs as they contain the important bioactive compounds.

There are few methods that can be used in the preparation of herbs. Traditionally, the methods may include the following : infusion (hot teas), decoction (boiled teas), tincture and cold soaking (Tan *et al.*, 2020). For the method infusion, it normally applies only to flowers or leaves, where they will need to be brewed with hot water for a few minutes. The next method will be decoctions in which the seeds, barks or roots of the herbs will be boiled with hot water. Duration taken for this method is much longer than infusion method. Cold soaking is a traditional method that involves only mixing a solvent with herb at room temperature. Beside the above mentioned methods, some of the traditional medical practitioners will dry the herbs under the hot and bright sun and then, mix the herbs with hot bath or even powdered the herb to become capsule, tablet and pills to be consumed (Wang *et al.*, 2012).

There are also traditional medical practitioners that believe that some herbal or medicinal plants can be helpful to cure certain diseases if they are being used appropriately. For this purpose, they usually prepare the crude plant extracts together rather than isolating them out as one single plant compound as all the bioactive compounds can work together to produce therapeutic effect. Some herbs can be very effective to humans' body systems when consumed in small quantity. However, some herbs can be poisonous and toxic to humans' body systems when consumed in a large quantity. For the same plant, the traditional medical practitioner may need to know the different preparation methods for different treatment conditions. For an example, preparing a hot ginger tea (infusion) can be helpful with treating insomnia or even relieve stress, while a different methods such as boiling ginger with alcohol can be used to treat various fungal and bacterial infections (Anosike *et al.,* 2009).

In different parts of the world, various uses of medicinal or herbal plants to meet the health care needs are very common nowadays such as in oral health care. In some parts of the developing countries, 70 % to 95 % of the populations are still depending on the traditional medicines as their primary cares while about 80 % of the world's populations are still relying on herbal remedies because they are unable to afford the expensive western drugs (Ahmad and Othman, 2013). With the long history of herbal medicines usage of more than 3000 years, they have made a better public acceptance nowadays. For those medicinal plants that are non - toxic and non - poisonous, they are safe to be used or consumed and are much cheaper than chemical drugs in terms of pricing. In terms of the availability or continuous supply of medicinal plants, this is also not a main concern as those medicinal or herbal plants are renewable sources as they are easy to grow and can be found anywhere within the rainforest. In return, this can help to make the cost to become a lot cheaper. Moreover, the processing of medical or herbal plants and production of health products are considered to be environment friendly and by products are bio - degradable with no major contribution to earth pollution.

Generally, bioactive compounds can be divided into three main categories that are: (1) terpenes and terpenoids; (2) alkaloids; and (3) phenolic compounds. The specific functions of many bioactive compounds may still require a lot of research and discovery in order to better understand. However, many studies and research have shown that some of the bioactive compounds have the functions of anti-oxidant and antimicrobial activities, antifungal, anti-inflammatory, anticancer and antimalarial (Ha *et al*., 2020). These medicinal values of the herbal plants are due to the presence of various bioactive chemical compounds such as alkaloids, fatty acids, tannins, flavonoids and phenolic compounds. Therefore, the plants with the medicinal values have to be investigated in depth in order to know their properties and efficacy better.

Plants synthesize phytochemical compounds such as phenolic compound, essential oil, terpenoids and others as these compounds help to protect the plants from ultraviolet (UV) light damage, oxidation, bacteria, fungi, insects and animals (Ogunnupebi *et al*., 2020). The phytochemical compounds also can be used to protect from any bacteria infection and to inhibit bacterial growth through with the used of different mechanisms. Other bioactive compounds will have different protection mechanisms to bacteria too. However, the most important thing is that the mechanism is workable and can help to protect the herbal or medicinal plants from different diseases.

Due to the values, different functions and various bioactive compounds from the herbal plants, phytochemical studies in developing various techniques for extraction are important. Many researchers have conducted extraction by using multiple different methods. The conventional techniques of medicinal plants extraction include maceration, hydro - distillation and soxhlet extraction. The ultrasound assisted extraction, enzyme - assisted extraction, microwave - assisted extraction, pulsed electric field assisted extraction, supercritical fluid extraction and pressurized liquid extraction are considered as non-conventional techniques (Azmir *et al*., 2013).

Plant-derived compounds are mostly secondary metabolites. Secondary metabolites refer to bioactive compounds produced via other metabolic pathways, with no direct involvement in the normal growth of a plant. The main role of secondary metabolite is relatively straightforward which is for the defence of the plant against microorganisms such as bacteria, fungi, and viruses. There are numerous examples of secondary compounds isolated from plants that have been effective as antimicrobial agents. Major groups of compounds that are responsible for antimicrobial activity from include essential oils, phenolics, flavonoids, terpenoids, and alkaloids (Guerriero *et* *al*., 2018). An example of an alkaloid is berberine. Berberine is the main antibacterial compound extracted from rhizoma Coptidis and cortex Phellodendri. Resveratrol is a phenolic compound extracted from grapes and Itadori plants. Resveratrol act as bacteriostatic agent on Gram-positive and Gram-negative bacteria. Essential oils are natural mixtures of hydrocarbons (terpenes), oxygen-containing (alcohols, aldehydes, ketones, carboxylic acids, esthers, lactones) and other sulphur-containing organic compounds. Essential oil from cinnamon, thyme, rosemary, lemongrass and ginger can directly use as preservation agent into food. According to World Bank report, the global herbal medicinal market was valued at USD 71.19 billion in 2016 and is expected to exhibit profitable growth over the forecast period until 2024 (Hexa Research, 2017). However, it is estimated only around 10% of the herbal plant derived have been examined for antimicrobial properties.

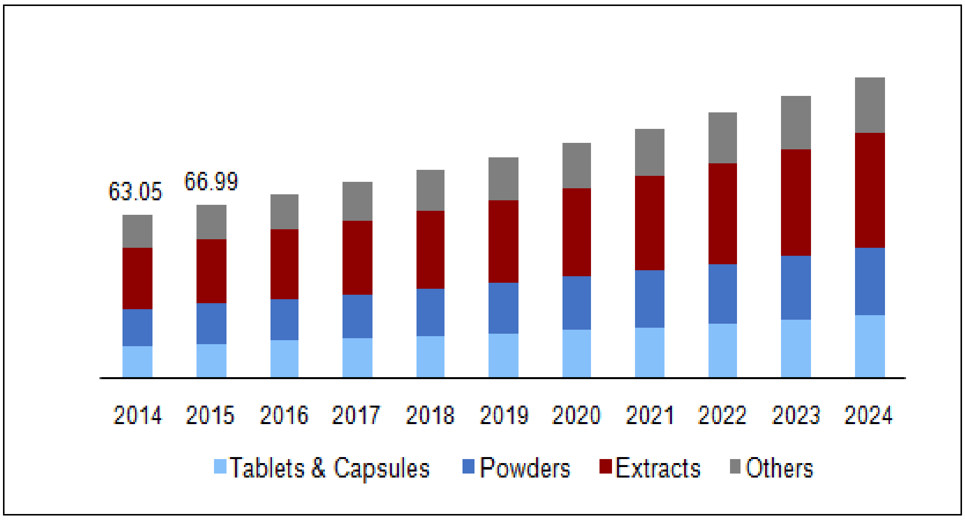

Figure 1.1 Global herbal medicine market revenue, by product, 2014 - 2024 (USD Billion) (Hexa Research, 2017)

Piper betle plant is a tropical herbal plant. It is a native plant located at central and eastern part of Peninsular Malaysia. Piper betel plant is believed to be very useful in treating various diseases such as asthma, skin diseases, halitosis, anti-allergic, anti-malaria, cuts and injuries (Patra *et al*., 2016). In my research, *Piper betle* is selected because of its traditional usage in treating the antibacterial infection. This is because *Piper betle* is one of the valuable medicinal plants with a lot of medicinal values and purposes. This study can be used to provide some new insights on the scientific basis of the traditional use of the *Piper betle* plant. The *Piper betle* plant contains a lot of secondary bioactive compounds. Various properties contained in its leaves can be used for antioxidant, antifungal, antidiabetic, anti-inflammatory, antimicrobial, chemopreventive and antifertility purposes (Bandaranayake *et al*., 2018).

Soxhlet extraction method easy to use and still being applied in almost every analytical laboratory. The Soxhlet extraction method is the main reference when comparing performance of different extraction methods. The equipment of Soxhlet extraction method is inexpensive and no filtration is required for the extraction after the leaching step. The solvent using in Soxhlet extraction system is recyclable and repeatedly in contact with the sample in order to displace the transfer equilibrium.

Supercritical fluid extraction (SFE) is green extraction method of bioactive compounds from different plant materials. When using the SFE, the system does not use any toxic solvents and the product obtained from SFE is better in quality, less degradation of bioactive compounds and can obtain higher yield. The selection of supercritical fluid is very important in the extraction process. The carbon dioxide (CO_2) is the most frequently used supercritical fluid solvent and can solubilize lipophilic substance (Pieczykolan *et al*., 2019). The carbon dioxide is not harmful to humans' health and does not pollute the environment.

The use of ultrasound technology (clean and green extraction method) can significantly increase the efficiency of the system and will obtained the extraction yield in higher amount from the extraction process. The ultrasound extraction method is easily obtained higher purity of the final product and the system can make the solvent greater penetration into plant cell with shorter processing time. For the shorter processing time, ultrasound extraction able to reduce the use of water, energy and the consumption of solvent during the bioactive compounds extraction process (Khadhraoui *et al*., 2021).

1.2 Problem Statement

In recent years, the use of natural plants to make drugs in order to control the microbial infections has become very popular throughout the world. The *Piper betle* plant is selected in this study is to investigate the potential of antimicrobial activity due to its ability in treating microbial infections. The bioactive compounds extracted from *Piper betle* leaves can provide an important information for the treatment of various microbial infections. Due to the high costs and side effects to human health in the formation of the synthetic drugs, extraction of bioactive compounds from *Piper betle* leaves to treat microbial infection can be a new alternative to replace the conventional drugs. This is because of the *Piper betle* leaves are much cheaper than conventional drugs, easy to obtain and contain variety of bioactive compounds needed (Nayaka *et al*., 2021).

The selection of the appropriate extraction method is very important in the discovery of the antimicrobial compounds. The conventional method used in the extraction of bioactive compounds from *Piper betle* plant is generally a time consuming process and the solvent used in the extraction process can be harmful to humans and environments. Moreover, some of the conventional extraction methods being applied are not suitable to be used to extract the thermo-sensitive compounds due to high chances of degrading the targeted compounds. Thus, to overcome the problem of conventional extractions applied, green technology such as probe type ultrasound - assisted extraction method and supercritical CO_2 (SCO_2) method are used in the extraction and isolation of valuable compounds from *Piper betle* plant. Both of these methods perform better than conventional methods as they consume less time, safe to humans and environments, cost effective, nontoxic and great recovery of bioactive compounds from plant. Supercritical fluid extraction that use carbon dioxide as a solvent has provided an excellent alternative to replace the non-environment friendly chemical solvents used in the conventional extraction methods. Even though both green technology methods are good, probe type ultrasound - assisted extraction method is more preferable in this study as compared to using SCO_2 extraction method because of lower yield and soxhlet extraction method due to need longer time to completed the *Piper betle* leaves extraction (Zhang *et al*., 2018).

One of the problem for most bioactive compound found in *Piper betle* leaves such as essential oils has poor water solubility. Therefore, formulating an oil-in water emulsion is one of the most promising strategies to overcome the poor water solubility problem. The formulated emulsion based delivery system can increase the water solubility of hydrophobic bioactive compounds and can also increase the absorption / diffusion rate of the targeted compounds. This *Piper betle* emulsion delivery system can help in the protection of the bioactive compounds from light, temperature, pH and oxidation. With this, the formulated emulsion can directly improve the bio - availability of *Piper betle* extract in many applications.

Piper betle leaves is popularly consumed as a mouth fresheners and can use as an oral care product. One of the main problems found for this medicinal plants is lack of toxicological evidence to increase the human confidence in their safety uses, particularly for use in the development of pharmaceutical products. The finding from this study can provides important information and data on toxicological properties of *Piper betle* emulsion formed from probe type sonication method and increase the confident use in development of new product. Therefore, this research was aimed to evaluate the acute systemic toxicity study of *Piper betle* emulsion in animal model by using swiss albino mice for the acute systemic toxicity study of *Piper betle* emulsion.

1.3 Objectives

General Objective

This study aims for extraction, identification of bioactive compounds, formulate of emulsion, investigate the antibacterial activity and acute systemic toxicity of *Piper betle* leaves.

Specific Objectives

(a) To investigate the best extraction method among soxhlet extraction method, supercritical CO_2 extraction method and probe type ultrasound - assisted extraction method in order to obtain bioactive compounds from *Piper betle* plant.

(b) To determine the isolated bioactive compounds of *Piper betle* leaves using Gas Chromatography Mass Spectrometry.

(c) To prepare oil in water emulsion from *Piper betle* extracts by using probe-type ultrasound and determining the structure and morphology of the *Piper betle* emulsion droplets.

(d) To evaluate the antimicrobial activity *of Piper betle* emulsion against *Staphylococcus aureus* and *Streptococcus mutans* and investigate acute systemic toxicity of the *Piper betle* emulsion.

1.4 Scope of Study

Raw material used in the study is the *Piper betle* leaves. The extraction of *Piper betle* leaves using Soxhlet extraction method, supercritical carbon dioxide (CO_2) extraction method and probe type ultrasound - assisted extraction method. After review the extraction yield, the simple, safe and good extraction method was chosen for phytochemical evaluation.

The extraction of *Piper betle* leaves consists of complex mixture of bioactive compound. The extract of *Piper betle* leaves was analysis by using Gas chromatography mass spectrometry (GC/MS). After review the GC/MS result, Probe type ultrasound - assisted extraction method is chosen to form the Oil-in-Water (O/W) emulsion because ultrasound processer is a device easy to operate, widely used for the formulating stable emulsions with small droplets diameter and frequently reported the produced emulsion can enhance antimicrobial activity. This study will focus on the parameter of *Piper betle* emulsion – effect of sample to surfactants ratio, droplets size, polydispersity index, and ultrasonic power intensity. The High Resolution Transmission Electron Microscopy (TEM, JEOL, JEM-2100) with a working voltage of 200 kV is use to determine the structure and morphology of the *Piper betle* emulsion.

The antimicrobial activity of *Piper betle* emulsion was evaluated by using minimum inhibitory concentration (MIC) and time-kill kinetics test. *Staphylococcus aureus* and *Streptococcus mutans*, were used in this study. MIC of the *Piper betle* emulsion was determined by the macrodilution technique. Time-kill kinetics tests were compared with the normal growth curve for each bacterial. The data was statistically analyzed by using student t - test. A p value of less than 0.05 was considered statistically significant.

The safety of the herbal plants is very importance. Therefore, this research also evaluates the acute systemic toxicity study of *Piper betle* emulsion in animal model. Selected of 10 healthy Swiss albino mice for weighing about 21-24 g used for the study. The control group only give water whereas the treated groups received a single dose of 50 mL/kg body weight of the oil in water *Piper betle* emulsion in toxicity study. The toxicity symptoms and mortality of the mice were observed for 4, 24, 48, and 72 hours after dosing.

1.5 Significance and Original Contributions of This Study

Plants serve an important role and purpose in every human life. They act as food and medicine for human beings. Most of the bioactive compounds found in medicinal plant can be used for therapeutic purpose and synthesis of useful drugs. The *Piper betle* plant is selected in this study because of its ability in treating the microbial infections and there are still a lot of bioactive compounds from *Piper betle* are largely unexplored. Furthermore, this study also aims to provide scientific basis regarding traditional use of the *Piper betle* plant.

Selection of appropriate extraction method of bioactive compounds from *Piper betle* leaves is very important. The probe type ultrasound assisted extraction method used in this study is the first extraction method applied to *Piper betle* leaves. This extraction method is a green method, as it uses water as a solvent. This extraction method is simpler as compared to other conventional methods. In addition, the cost of this novel extraction process is significantly less expensive than the conventional extraction processes. The utilization of the probe type ultrasound assisted extraction can also increase the yield of *Piper betle* leaves with less extraction time spent (Sridhar *et al*., 2021).

Emulsions based delivery system has shown multiple benefits in encapsulating hydrophobic bioactive compound. Challenges for reducing antimicrobial effectiveness of hydrophobic compound from *Piper betle* can be solved by forming emulsion solution. Low frequency, high power ultrasound is used for the *Piper betle* emulsion formation. Ultrasonic emulsification using probe type ultrasound has been successfully applied in the formation of oil in water *Piper betle* emulsion. The formation of *Piper betle* emulsion with particle size less than 250 nm is very novel and has not been published before. This novel emulsion delivery system can increase the antimicrobial efficacy and reduce the concentration of extract for the *Piper betle* leaves. The *Piper betle* emulsion solution from this research can be used for antimicrobial drug development as the solution is natural and environment friendly.

The finding from acute systemic toxicity study provides important information and data on toxicological properties of *Piper betle* emulsion. The result can increase the confidence in safe uses, particularly for use in the development of pharmaceutical products using *Piper betle* emulsion.

REFERENCES

Abbas, Shabbar, Khizar Hayat, Eric Karangwa, Mohanad Bashari, and Xiaoming Zhang. (2013). An Overview of Ultrasound-Assisted Food-Grade Nanoemulsions. *Food Engineering Reviews* 5 (3): 139–57.

Abismaïl, B., Canselier, J. P., Wilhelm, A. M., Delmas, H., and Gourdon, C. (1999). Emulsification by Ultrasound: Drop Size Distribution and Stability. *Ultrasonics Sonochemistry* 6 (1–2): 75–83.

Abubakar A.R., and Haque M. (2020). Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes, *J Pharm Bioallied Sci.*,12(1): 1–10.

Ahmad, Saari, and Noraini Othman. (2013). Strategic Planning , Issues , Prospects and the Future of the Malaysian Herbal Industry. *International Journal of Academic Research in Accounting, Finance and Management Sciences* 3 (4): 91–102.

Al-Adhroey, Abdulelah H., Zurainee M. N., Hesham M. A., Adel A. A., and Rohela M. (2011). Antimalarial Activity of Methanolic Leaf Extract of Piper Betle L. *Molecules* 16 (1): 107–18.

Altemimi A., Lakhssassi N., Baharlouei A., Watson D.G., and Lightfoot D.A. (2017). Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts, *Plants (Basel)*, 6 (4), pp 42.

Amalia, Husnun, Ratna Sitompul, Johan Hutauruk, and Abdul Mun. (2009). Effectiveness of Piper Betle Leaf Infusion as a Palpebral Skin Antiseptic. *Universa Medicina* 28 (2): 83–91.

Anon. (2012). “British Pharmacopoeia.” *Volume V*. London: The Stationery Office.

Anosike, C A, Obidoa,O., Ezeanyika,L.U.S., and Nwuba, M.M. (2009). Anti-Inflammatory and Anti-Ulcerogenic Activity of the Ethanol Extract of Ginger (Zingiber Officinale). *African Journal of Biochemistry Research (AJBR)* 3 (12): 379–84.

Anton, Nicolas, and Thierry, F. V. (2009). The Universality of Low-Energy Nano-Emulsification. *International Journal of Pharmaceutics* 377 (1–2): 142–47.

Appiah Theresa,Yaw Duah Boakye and Christian Agyare (2017). Antimicrobial Activities and Time-Kill Kinetics of Extracts of Selected Ghanaian Mushrooms, E*vidence-Based Complementary and Alternative Medicine,* Vol. 2017, 15.

Arambewela, L. S. R., Arawwawala, L.D.A.M. and Ratnasooriya, W.D. (2005). Antidiabetic Activities of Aqueous and Ethanolic Extracts of Piper Betle Leaves in Rats. *Journal of Ethnopharmacology* 102 (2): 239–45.

Arana V., Medina J., Esseiva P., Pazos D., and Wist J. (2016). Classification of Coffee Beans by GC-C-IRMS, GC-MS, and 1H-NMR, *Journal of Analytical Methods In Chemistry*, 2016 (11).

Aresta, Antonella, and Carlo Zambonin. (2016). Simultaneous Determination of Salicylic, 3-Methyl Salicylic, 4-Methyl Salicylic, Acetylsalicylic and Benzoic Acids in Fruit, Vegetables and Derived Beverages by SPME-LC-UV/DAD. *Journal of Pharmaceutical and Biomedical Analysis* 121: 63–68.

Aswathanarayan J.B. and Vittal R.R. (2019). Nanoemulsions and Their Potential Applications in Food Industry, *Frontiers in Sustainable Food Systems*, 3(95).

Azmi N.A.N., Elgharbawy A.A.M., Motlagh S.R., Samsudin N., and Salleh H.M. (2019). Nanoemulsions: Factory for Food, Pharmaceutical and Cosmetics, *Journal Processes*, 7, 617.

Azmir, J., I. S M Zaidul, M. M. Rahman, K. M. Sharif, A. Mohamed, F. Sahena, M. H A Jahurul, K. Ghafoor, N. A N Norulaini, and A. K M Omar. (2013). Techniques for Extraction of Bioactive Compounds from Plant Materials: A Review. *Journal of Food Engineering*.

Azahar N.I., Mokhtar M.N., and Arifin M.A. (2020). Piper betle: A Review On Its Bioactive Compounds, Pharmacological Properties, and Extraction Process, *IOP Conf. Series: Materials Science and Engineering* 991 (012044).

Bai, L., and McClements, D.J. (2016). Development of Microfluidization Methods for Efficient Production of Concentrated Nanoemulsions: Comparison of Single- and Dual-Channel Microfluidizers. *Journal of Colloid and Interface Science* 466: 206–12.

Barkat Ali Khan. (2011). Basics of Pharmaceutical Emulsions: A Review. *African Journal of Pharmacy and Pharmacology* 5 (25): 2715–25.

Begam, K. M. F., Ravichandran, P., and Manimekalai, V., (2018). Phytochemical Analysis of Some Selected Varieties of Piper Betle L, *International Journal of Current Pharmaceutical Research,* vol. 10, no. 2, pp. 89-93.

Benencia, F, and Courreges, M.C. (2000). In Vitro and in Vivo Activity of Eugenol on Human Herpesvirus. *Phytotherapy Research* 14: 495–500.

Bera Bijoy, Khazal Rama & Schroën Karin (2021). Coalescence Dynamics in Oil-in-water Emulsions At Elevated Temperatures, *Scientific Reports,* volume 11, Article number: 10990.

Bhalerao, Satish A, Deepa R V, Rohan V G, Nikhil C T, Yatin Y R, Vinodkumar S D, and Ashwin T. (2013). Phytochemistry, Pharmacological Profile and Therapeutic Uses of Piper Betle Linn. – An Overview. *Journal of Pharmacognosy and Phytochemistry Phytochemistry,* 1 (2): 10–19.

Bissa, Sharad, Dimple S., and Bohra, A. (2007). Traditions in Oral Hygiene: Chewing of Betel (Piper Betle L.) Leaves. *Current Science* 92 (1): 26–28.

Bouaziz, Amira, Dhekra Mhalla, Imen Zouari, Lobna Jlaiel, Slim Tounsi, Raoudha Jarraya, and Mohamed Trigui. (2016). Antibacterial and Antioxidant Activities of Hammada Scoparia Extracts and Its Major Purified Alkaloids.” *South African Journal of Botany* 105: 89–96.

Bridgers, E. N., Mari S. C., and Van D.T. (2010). Extraction of Anthocyanins from Industrial Purple-Fleshed Sweetpotatoes and Enzymatic Hydrolysis of Residues for Fermentable Sugars. *Industrial Crops and Products* 32 (3): 613–20.

Brunner, Gerd. (2005). Supercritical Fluids: Technology and Application to Food Processing. *Journal of Food Engineering* 67 (1–2): 21–33.

Burguera, José Luis, and Marcela Burguera. (2012). Analytical Applications of Emulsions and Microemulsions. *Talanta* 96: 11–20.

Caburian, Adeltrudes B, and Marina O. O. (2010). Characterization and Evaluation of Antimicrobial Activity of the Essential Oil from the Leaves of Piper Betle L . *E-International Scientific Research Journal* 2 (1): 2–13.

Chakraborty, Devjani, and Barkha Shah. (2011). Antimicrobial, Antioxidative and Antihemolytic of Piper Betel Leaf Extracts. *International Journal of Pharmacy and Pharmaceutical Sciences* 3 (3): 192–99.

Chanda S, Parekh J, Vaghasiya Y, Dave R, Baravalia Y, Nair R. Medicinal plants-from traditional use to toxicity assessment: A Review. *International Journal of Pharmaceutical Sciences and Research.* 2015; 6:2652.

Chemat, Farid, Maryline A.V., and Giancarlo Cravotto. (2012). Green Extraction of Natural Products: Concept and Principles. *International Journal of Molecular Sciences* 13 (7): 8615–27.

Chemat, Farid, Zill-E-Huma, and Muhammed K.K. (2011a). Applications of Ultrasound in Food Technology: Processing, Preservation and Extraction. *Ultrasonics Sonochemistry* 18 (4): 813–35.

Chikezie P.C., Ibegbulem C.O., Mbagwu F.N. Medicinal potentials and toxicity concerns of bioactive principles. Medicinal & Aromatic Plants. 2015;4:202.

Ciron, Chr Ian E., Vivian L. G., Alan L. K., and Mark A. E. A. (2011). Effect of Microfluidization of Heat-Treated Milk on Rheology and Sensory Properties of Reduced Fat Yoghurt. *Food Hydrocolloids* 25 (6): 1470–76.

Consoli, Larissa, Guilherme D.F.F., Rosiane L.D.C., and Míriam D.H. (2017). High Solids Emulsions Produced by Ultrasound as a Function of Energy Density. *Ultrasonics Sonochemistry* 38: 772–82.

Costa, A.L.R., Andresa G., Cristiane C.P.D.A, and Rosiane L.C. (2017). Emulsifier Functionality and Process Engineering: Progress and Challenges. *Food Hydrocolloids* 68: 69–80.

Cowan, Marjorie Murphy. (1999). Plant Products As Antimicrobial Agents. *Clinical Microbiology Reviews* 12 (4): 564–82.

Cravotto, Giancarlo, Luisa Boffa, Stefano Mantegna, Patrizia Perego, Milvio Avogadro, and Pedro Cintas. (2008). Improved Extraction of Vegetable Oils Under High-Intensity Ultrasound and/or Microwaves. *Ultrasonics Sonochemistry* 15 (5): 898–902.

Dasgupta, Nabasree, and Bratati De. (2004). Antioxidant Activity of Piper Betle L. Leaf Extract in Vitro." *Food Chemistry* 88 (2): 219–24.

Datta, A., Ghoshdastidar, S., & Singh, M. (2011). Antimicrobial Property of Piper betel Leaf against Clinical Isolates of Bacteria. *International Journal of Pharma Sciences and Research*, 2(3), 104–109.

Davis, H. T. (1994). Factors Determining Emulsion Type: Hydrophile-Lipophile Balance and Beyond. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 91: 9–24.

Deshpande, S N, and D G Kadam. (2013). GCMS Analysis and Antibacterial Activity of Piper Betle (Linn) Leaves Against Streptococcus Mutans. *Asian Journal of Pharmaceutical AND Clinical Research* 6 (suppl 5): 99–101.

Dubey, Rajesh R., and Rajesh H. Parikh. (2004). Two-Stage Optimization Process for Formulation of Chitosan Microspheres. *AAPS PharmSciTech* 5 (1): 20–28.

Doris Kumadoh and Kwabena Ofori-Kwakye (2017). Dosage Forms of Herbal Medicinal Products and Their Stability Considerations – An Overview, *JCR* 4(4): 1-8.

Elamathi M., (2016). Invitro Micropropagation of Piper betle L., *International Journal of Current Microbiology and Applied Sciences Special Issue*-3: 44-49.

Eloff, J. N. (1998). Which Extractant Should Be Used for the Screening and Isolation of Antimicrobial Components from Plants? *Journal of Ethnopharmacology* 60 (1): 1–8.

Ganguly, Sudipto, Soumyaditya Mula, Subrata Chattopadhyay, and Mitali Chatterjee. (2007). An Ethanol Extract of Piper Betle Linn. Mediates Its Anti-Inflammatory Activity via down-Regulation of Nitric Oxide. *The Journal of Pharmacy and Pharmacology* 59 (5): 711–18.

Garcia-Marquez, E., Higuera-Ciapara I., and Espinosa-Andrews H. (2017). Design of Fish Oil-in-Water Nanoemulsion by Microfluidization. *Innovative Food Science and Emerging Technologies* 40: 87–91.

Garg, S. C., and Rajshree Jain. (2005). Biological Activity of the Essential Oil of Piper Betle L. *Journal of Essential Oil Research* 4 (6): 601–6.

Gilani, Anwar H., Nauman A., Irfan M. K., Zeeshan A. R., and Nadia K. A. 2000. The Presence of Cholinomimetic and Calcium Channel Antagonist Constituents in Piper Betle Linn. *Phytotherapy Research* 14 (6): 436–42.

Goindi, Shishu, Amanpreet Kaur, Randeep Kaur, Atin Kalra, and Pratibha Chauhan. (2016). *19 - Nanoemulsions: An Emerging Technology in the Food Industry*. *Emulsions*. Elsevier Inc.

Grant Thamkaew, Ingegerd Sjöholm and Federico Gómez, (2021). A review of drying methods for improving the quality of dried herbs, Critical Reviews in Food Science and Nutrition, 61(11), 1763–1786.

Guan, Yunjing, Linyan Zhou, Jinfeng Bi, Jianyong Yi, Xuan Liu, Qinqin Chen, Xinye Wu, and Mo Zhou. (2016). Change of Microbial and Quality Attributes of Mango Juice Treated by High Pressure Homogenization Combined with Moderate Inlet Temperatures during Storage. *Innovative Food Science and Emerging Technologies* 36: 320–29.

Gupta, A., Eral, H. B., Hatton, T. A., & Doyle, P. S. (2016). Nanoemulsions: Formation, properties and applications. Soft Matter, 12(11), 2826–2841.

Guha, P. (2006). Betel Leaf : The Neglected Green Gold of India. *Journal of Human Ecology* 19 (6): 87–93.

Gupta, Shweta, Rajesh Kesarla, and Abdelwahab Omri. (2013). Formulation Strategies to Improve the Bioavailability of Poorly Absorbed Drugs with Special Emphasis on Self-Emulsifying Systems. *ISRN Pharmaceutics* 2013: 1–16.

Gyawali, Rabin, and Salam A. I. (2014). Natural Products as Antimicrobial Agents. *Food Control* 46: 412–29.

Hakansson, Andreas, Laszlo Fuchs, Fredrik Innings, Johan Revstedt, Christian Trägårdh, and Björn Bergenståhl. (2011). High Resolution Experimental Measurement of Turbulent Flow Field in a High Pressure Homogenizer Model and Its Implications on Turbulent Drop Fragmentation. *Chemical Engineering Science* 66 (8): 1790–1801.

Hashtjin, Adel Mirmajidi, and Soleiman Abbasi. (2015). Optimization of Ultrasonic Emulsification Conditions for the Production of Orange Peel Essential Oil Nanoemulsions. *Journal of Food Science and Technology* 52 (5): 2679–89.

He Z., Wang M., Li H., and Wen C. (2019). GC-MS-based Fecal Metabolomics Reveals Gender-attributed Fecal Signatures In Ankylosing Spondylitis, Scientific Reports, 9 (3872).

Hyldgaard, Morten, Tina Mygind, and Rikke Louise Meyer. (2012). Essential Oils in Food Preservation: Mode of Action, Synergies, and Interactions with Food Matrix Components. *Frontiers in Microbiology* 3 (JAN): 1–24.

Innings, Fredrik, Laszlo Fuchs, and Christian Trägårdh. (2011). Theoretical and Experimental Analyses of Drop Deformation and Break-up in a Scale Model of a High-Pressure Homogenizer. *Journal of Food Engineering* 103 (1): 21–28.

Jacques, R. A., J. G. Santos, C. Dariva, J. Vladimir Oliveira, and E. B. Caramão. (2007). GC/MS Characterization of Mate Tea Leaves Extracts Obtained from High-Pressure CO2extraction. *Journal of Supercritical Fluids* 40 (3): 354–59.

Jam Neda, Hajimohammadi Reza, Gharbani Parvin and Mehrizad Ali (2021). Evaluation of Antibacterial Activity of Aqueous, Ethanolic and Methanolic Extracts of Areca Nut Fruit on Selected Bacteria, *BioMed Research International*, Volume 2021(8).

Jane Buckle PhD, RN, in Clinical Aromatherapy (Third Edition), 2015.

Jafari, Seid Mahdi, Elham Assadpoor, Yinghe He, and Bhesh Bhandari. (2008). Re-Coalescence of Emulsion Droplets during High-Energy Emulsification. *Food Hydrocolloids* 22 (7): 1191–1202.

Jafari, Seid Mahdi, Yinghe He, and Bhesh Bhandari. (2007). Production of Sub-Micron Emulsions by Ultrasound and Microfluidization Techniques. *Journal of Food Engineering* 82 (4): 478–88.

Jeevan K.S.P., Garlapati V.K., Dash A., Scholz P., Banerjee R., (2017). Sustainable Green Solvents and Techniques for Lipid Extraction from Microalgae: A Review, *Algal Research*, 138-147.

Jenkins, C., Evans, S., Hawrelak, J., and Wohlmuth, H. (2009). Herbal Medicines Prepared by Traditional and Contemporary Methods – a Comparative Study. *Planta Medica* 75: 5.

Juttulapa, Maneerat, Suchada Piriyaprasarth, Hirofumi Takeuchi, and Pornsak Sriamornsak. (2017). Effect of High-Pressure Homogenization on Stability of Emulsions Containing Zein and Pectin. *Asian Journal of Pharmaceutical Sciences* 12 (1): 21–27.

Kaderides, K., Goula, A. M., & Adamopoulos, K. G. (2015). A Process For Turning Pomegranate Peels Into A Valuable Food Ingredient Using Ultrasound-Assisted Extraction and Encapsulation. *Innovative Food Science and Emerging Technologies*, 31, 204–215.

Kale S.N. and Deore S.L. (2017). Emulsion Micro Emulsion and Nano Emulsion: A Review, *Systematic Reviews in Pharmacy*, 8(1):39-47.

Kalemba, D. and Kunicka, A. (2003). Antibacterial and Antifungal Properties of Essential Oils. *Current Medicinal Chemistry* 10 (10): 813–29.

Kaltsa, O., I. Gatsi, S. Y., and Mandala, I. (2014). Influence of Ultrasonication Parameters on Physical Characteristics of Olive Oil Model Emulsions Containing Xanthan. *Food and Bioprocess Technology* 7 (7): 2038–49.

Kawaguchi, M. (2016). Silicone Oil Emulsions Stabilized By Polymers and Solid Particles. *Advances in Colloid and Interface Science*, 233, 186–199.

Khan, Jahir Alam. (2011). Evaluation of Antibacterial Properties of Extracts of Piper Betel Leaf. *Journal of Pharmaceutical and Biomedical Sciences* 11 (11): 1–3.

Khaw Kooi-Yeong, Marie-Odile Parat, Paul Nicholas Shaw, and James Robert Falconer (2017). Solvent Supercritical Fluid Technologies to Extract Bioactive Compounds from Natural Sources: A Review, *Molecules*, 22(7), 1186.

Khoddami, Ali, Meredith, A.W., and Thomas H R. (2013). Techniques for Analysis of Plant Phenolic Compounds. *Molecules (Basel, Switzerland)* 18 (2): 2328–75.

Komaiko, Jennifer, and McClements, D.J. (2015). Low-Energy Formation of Edible Nanoemulsions by Spontaneous Emulsification: Factors Influencing Particle Size. *Journal of Food Engineering* 146: 122–28.

Komaiko, Jennifer S., and David Julian Mcclements. (2016). "Formation of Food-Grade Nanoemulsions Using Low-Energy Preparation Methods: A Review of Available Methods." *Comprehensive Reviews in Food Science and Food Safety* 15 (2): 331–52.

Kowalski, Radosław, Grazyna Kowalska, Jerzy Jamroz, Agnieszka Nawrocka, and Damian Metyk. (2015). Effect of the Ultrasound-Assisted Preliminary Maceration on the Efficiency of the Essential Oil Distillation from Selected Herbal Raw Materials. *Ultrasonics Sonochemistry* 24: 214–20.

Kumar Amardeep, Ahmad Faizan and Zaidi sadaf (2019). Importance of Bioactive Compounds Present in Plant Productsand Their Extraction: A Review, *Agricultural Reviews* 40(4):249-260.

Kunle Okaiyeto and Oluwafemi O. Oguntibeju (2021). African Herbal Medicines: Adverse Effects and Cytotoxic Potentials with Different Therapeutic Applications, *International Journal of Environmental Research and Public Health* 18(11), 5988.

Kuroiwa, T., Kobayashi, I., Chuah, A. M., Nakajima, M., & Ichikawa, S. (2015). Formulation and stabilization of nano-/microdispersion systems using naturally occurring edible polyelectrolytes by electrostatic deposition and complexation. *Advances in Colloid and Interface Science*, 226, 86–100.

Lang, Qingyong, and Cm Wai. (2001). Supercritical Fluid Extraction in Herbal and Natural Product Studies—a Practical Review. *Talanta* 53: 771–82.

Lee, Laura L., Nima Niknafs, Robin D. Hancocks, and Ian T. Norton. (2013). Emulsification: Mechanistic Understanding. *Trends in Food Science and Technology* 31 (1): 72–78.

Leong, Thomas, Muthupandian Ashokkumar, and Kentish Sandra. (2011). The Fundamentals of Power Ultrasound - A Review. *Acoustics Australia* 39 (2): 54–63.

Liang, Rong, Shiqi Xu, Charles F. S., Yue Li, Fang Zhong, and Qingrong Huang. (2012). Physical and Antimicrobial Properties of Peppermint Oil Nanoemulsions. *Journal of Agricultural and Food Chemistry* 60 (30): 7548–55.

Lin, Y. T., Y. I. Kwon, R. G. Labbe, and Shetty, K. (2005). Inhibition of Helicobacter Pylori and Associated Urease by Oregano and Cranberry Phytochemical Synergies. *Applied and Environmental Microbiology* 71 (12): 8558–64.

Liu, Chung J., Chiu L. Chen, Kuo W. Chang, Cheng Hsin Chu, and Tsung Y. Liu. (2000). Safrole in Betel Quid May Be A Risk Factor for Hepatocellular Carcinoma: Case Report. *CMAJ*.

Liu Hua, Ruixu Zhan, Liangjian Wen, Zhenyu Zhong (2021). The Extraction of Natural Essential Oils and Terpenoids From Plants By Supercritical Fluid, *E3S Web of Conferences*, 271, 04018.

Luque de Castro, M. D., and García-Ayuso, L.E. (1998). Soxhlet Extraction of Solid Materials: An Outdated Technique With A Promising Innovative Future. *Analytica Chimica Acta*.

Luque de Castro, M. D., and Priego-Capote, F. (2010). Soxhlet Extraction: Past and Present Panacea. *Journal of Chromatography A*.

Lv, Guojun, Fumin Wang, Wangfeng Cai, Hang Li, and Xubin Zhang. (2014). Influences of Addition of Hydrophilic Surfactants on the W/O Emulsions Stabilized by Lipophilic Surfactants. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 457 (1): 441–48.

Mahmoudi, Souhila, Mustapha Khali, Abderahim Benkhaled, Karima Benamirouche, and Imen Baiti. (2016). Phenolic and Flavonoid Contents, Antioxidant and Antimicrobial Activities of Leaf Extracts from Ten Algerian Ficus Carica L. Varieties. *Asian Pacific Journal of Tropical Biomedicine* 6 (3): 239–45.

Manley A., Adrian L. Collins, Adrian Joynes, Per-Erik Mellander and Phil Jordan, (2020). Comparing Extraction Methods for Biomarker Steroid Characterisation from Soil and Slurry, Water, Air & Soil Pollution, 231(10):524.

Mao, L., & Miao, S. (2015). Structuring Food Emulsions to Improve Nutrient Delivery During Digestion. *Food Engineering Reviews*, 7(4), 439–451.

McClements, D. J., and E. A. Decker. (2000). Lipid Oxidation in Oil-in-Water Emulsions: Impact of Molecular Environment on Chemical Reactions in Heterogeneous Food Systems. *Journal of Food Science* 65 (8): 1270–82.

McClements, D. J., and Jafari, S. M. (2018). Improving emulsion formation, stability and performance using mixed emulsifiers: A review. Advances in Colloid and Interface Science.

McClements, David Julian. (2005). Emulsions Stability. In *Food Emulsions Principles, Practices, and Techniques*, 269–340.

McClements, David Julian, and Cansu E. G. (2016). Natural Emulsifiers — Biosurfactants, Phospholipids, Biopolymers, and Colloidal Particles: Molecular and Physicochemical Basis of Functional Performance. *Advances in Colloid and Interface Science* 234: 3–26.

McClements, David Julian, and Yan Li. (2010). Structured Emulsion-Based Delivery Systems: Controlling the Digestion and Release of Lipophilic Food Components. *Advances in Colloid and Interface Science* 159 (2): 213–28.

Mittal, V. & Matsko, N.B. (2012). Analytical Imaging Techniques for Soft Matter Characterization. New York, NY, USA: Springer Science & Business Media.

Mohanapriya S., and Sridevi J. (2020). Quantification of nutrient and phytoconstituent in fresh and dehydrated Piper betel Leaf, *International Journal of Applied Research*, 6(7): 01-04

Nalina, T., and Rahim Z. (2007). The Crude Aqueous Extract of Piper Betle L. and Its Antibacterial Effect towards Streptococcus Mutans. *American Journal of Biochemistry and Biotechnology* 3 (1): 10–15.

Nayaka N.M.D.M.W., Sasadara M.M., Sanjaya D., Yuda P.E.S.K., Dewi N.L.K.A.A., Cahyaningsih E., Hartati R. (2021). Piper betle (L): Recent Review of Antibacterial and Antifungal Properties, Safety Profiles, and Commercial Applications, *Molecules*, 26(8):2321.

Ncube, N S, Afolayan A. J., and Okoh A. I. (2008). Assessment Techniques of Antimicrobial Properties of Natural Compounds of Plant Origin : Current Methods and Future Trends. *African Journal of Biotechnology* 7 (12): 1797–1806.

Nguyen Lam Thi Truc, Nguyen Thuong Thi, Nguyen Hoa Ngoc, Bui Quynh Thi Phuong (2020). Simultaneous Determination of Active Compounds in Piper betle Linn. Leaf Extract and Effect of Extracting Solvents on bioactivity, Engineering Report, 2(10).

Nollet M., Boulghobra H., Calligaro E. and Rodier J.D. (2019). An efficient method to determine the Hydrophile-Lipophile Balance of surfactants using the phase inversion temperature deviation of CiEj/n-octane/water emulsions, *International Journal of Cosmetic Science*, 41, 99–108.

Pa N.F.C., Hong T.J., Edrus S.S.O.A. and Talib S.K.A. (2019). The Effects of Soxhlet and Power Ultrasonic-Assisted Soxhlet Extraction on the Yield of Terpenoids Extract from Jasminum sambac, *International Journal of Recent Technology and Engineering*, 8(2S4).

Palombo, Enzo A. (2011). Traditional Medicinal Plant Extracts and Natural Products with Activity against Oral Bacteria: Potential Application in the Prevention and Treatment of Oral Diseases. *Evidence-Based Complementary and Alternative Medicine* 2011: 15 pp.

Panda Debabrata and Sivakumar Manickam (2019). Cavitation Technology—The Future of Greener Extraction Method: A Review on the Extraction of Natural Products and Process Intensification Mechanism and Perspectives, *Applied Sciences*, 9, 766.

Pandey, Amita, and Shalini Tripathi. (2014). Concept of Standardization , Extraction and Pre Phytochemical Screening Strategies for Herbal Drug. *Journal of Pharmacognosy and Phytochemistry* 2 (5): 115–19.

Paranjpe, Rutugandha, Sushma R. G., Lakshminarayana N., Arpana Sagwal, Ghazia Asif, Anjali Pandey, and Ritu Aneja. (2013). Piper Betel Leaf Extract: Anticancer Benefits and Bio-Guided Fractionation to Identify Active Principles for Prostate Cancer Management. *Carcinogenesis* 34 (7): 1558–66.

Perazzo, A., V. Preziosi, and S. Guido. (2015). Phase Inversion Emulsification: Current Understanding and Applications. *Advances in Colloid and Interface Science* 222: 581–99.

Pereira, Camila G., and M. Angela A. Meireles. (2010). Supercritical Fluid Extraction of Bioactive Compounds: Fundamentals, Applications and Economic Perspectives. *Food and Bioprocess Technology* 3 (3): 340–72.

Parhi Rabinarayan and Suresh Padilama (2013). Supercritical Fluid Technology: A Review, *Journal of Advanced Pharmaceutical Science and Technology,* Volume No: 1 Issue No: 1, 13-36.

Pico, Yolanda. (2013). Ultrasound-Assisted Extraction for Food and Environmental Samples. *Trends in Analytical Chemistry* 43: 84–99.

Pin, K. Y., Chuah, A. L., Rashih, A. A., Mazura, M. P., Fadzureena, J., Vimala, S., & Rasadah, M. A. (2010). Antioxidant and Anti-inflammatory Activities of Extracts of Betel Leaves (Piper betle) From Solvents With Different Polarities. *Journal of Tropical Forest Science*, 22(4), 448–455.

Piorkowski, D. T., & McClements, D. J. (2014). Beverage Emulsions: Recent Developments In Formulation, Production and Applications. *Food Hydrocolloids*, 42, 5–41.

Prieto, Cristina, and Lourdes Calvo. (2013). Performance of the Biocompatible Surfactant Tween 80, for the Formation of Microemulsions Suitable for New Pharmaceutical Processing. *Journal of Applied Chemistry* 2013: 1–10.

Punuri, Jayasekhar, Pragya S., Saranya S., Ranjan T., and Utpal B. (2012). Piper Betle-Mediated Green Synthesis of Biocompatible Gold Nanoparticles. *International Nano Letters* 2 (1): 18.

Preziosi Valentina, Antonio Perazzo, Sergio Caserta, Giovanna Tomaiuolo and Stefano Guido (2013). Phase Inversion Emulsification, *Chemical Engineering Transactions,* 32, 1585-1590.

Rahman M.M. and Lamsal B.P. (2021). Ultrasound-assisted Extraction and Modification of Plant-based Proteins: Impact on Physicochemical, Functional, and Nutritional Properties, Comprehensive Reviews in Food Science and Food Safety, 20 (2).

Sahoo Swayam Sourav, Smita Shukla, Subhangankar Nandy and Sahoo Himanshu Bhusan (2012). Synthesis of Novel Coumarin Derivatives and Its Biological Evaluations, *European Journal of Experimental Biology*, 2 (4):899-908.

Raya S.A., Saaid I.M., Ahmed A.A., and Umar A.A. (2020). A critical review of development and demulsification mechanisms of crude oil emulsion in the petroleum industry, *Journal of Petroleum Exploration and Production Technology*, volume 10, 1711–1728.

Rad H., Sabet J., and Varaminian F. (2019). Study of solubility in supercritical fluids: Thermodynamic concepts and measurement methods - A review, *Brazilian Journal of Chemical Engineering*, 36(4), 1367-1392.

Ramji, Niranjan, Nivedita R., Ritu I., and Chandrasekaran S. (2002). Phenolic Antibacterials from Piper Betle in the Prevention of Halitosis. *Journal of Ethnopharmacology* 83 (1–2): 149–52.

Razak, Fathilah A., and Zubaidah H.A.B. (2003). The Anti-Adherence Effect of Piper Betle and Psidium Guajava Extracts on the Adhesion of Early Settlers in Dental Plaque to Saliva-Coated Glass Surfaces. *Journal of Oral Science* 45 (4): 201–6.

Ricaurte, Leidy, María D. J. P., Anamaria M., and María X. Q.. (2016). Production of High-Oleic Palm Oil Nanoemulsions by High-Shear Homogenization (Microfluidization). *Innovative Food Science and Emerging Technologies* 35: 75–85.

Roldán-Gutiérrez, Jannat M., J. Ruiz-Jiménez, and M. D. Luque de Castro. (2008). Ultrasound-Assisted Dynamic Extraction of Valuable Compounds from Aromatic Plants and Flowers as Compared with Steam Distillation and Superheated Liquid Extraction. *Talanta* 75 (5): 1369–75.

Rovetto, Laura J., and Niccolo V. Aieta. (2017). Supercritical Carbon Dioxide Extraction of Cannabinoids from Cannabis Sativa L. Plant Material. *The Journal of Supercritical Fluids* G-MODEL (SUPFLU-3883): 12.

Rui, P F F, Teresa A P Rocha-santos, and Armando C Duarte. (2016). Supercritical Fluid Extraction of Bioactive Compounds. *Trends in Analytical Chemistry* 76: 40–51.

Said, P.P., Rama C. P., and Birendra N. R.. (2014). A Green Separation of Lagenaria Siceraria Seed Oil. *Industrial Crops and Products* 52: 796–800.

Sakinah Depi, Rusdi and Misfadhila Sestry (2020). Review of Traditional Use, Phytochemical and Pharmacological Activity of Piper betle L., *Journal of Health Sciences and Research* Vol.5, Issue: 3.

Saleh, I. A., Vinatoru, M., Mason, T. J., Abdel-Azim, N. S., Aboutabl, E. A., & Hammouda, F. M. (2016). A Possible General Mechanism For Ultrasound-assisted Extraction (UAE) Suggested From The Results of UAE of Chlorogenic Acid From Cynara Scolymus L. (artichoke) Leaves. *Ultrasonics Sonochemistry*, 31, 330–336.

Sanli D., Bozbag S.E., and Erkey C. (2012). Synthesis of Nanostructured Materials Using Supercritical CO2: Part I. Physical transformations, *Journal of Materials Science* 47(7):2995-3025.

Sarkar, Madhumita, Paramita Gangopadhyay, Bidyut B., Kausiki C., Julie B., Purnima A., and Asima C. (2000). The Reversible Antifertility Effect of Piper Betle Linn. on Swiss Albino Male Mice. *Contraception* 62 (5): 271–74.

Saxena, Mohit, Naveen K. Khare, Priyanka S., Kodakandla V. S., and Santosh K. Srivastava. (2014). Antimicrobial Activity and Chemical Composition of Leaf Oil in Two Varieties of Piper Betle from Northern Plains of India. *Journal of Scientific and Industrial Research* 73 (2): 95–99.

Sengupta R., and Banik J.k., (2013). A Review On Betel Leaf (PAN), International Journal of Pharmaceutical Sciences and Research, Vol. 4(12): 4519-4524.

Schläger, Sabrina, and Birgit Dräger. (2016). Exploiting Plant Alkaloids. *Current Opinion in Biotechnology* 37: 155–64.

Scognamiglio, J., L. Jones, C. S. Letizia, and A. M. Api. (2012). Fragrance Material Review on 2-Heptylcyclopentanone. *Food and Chemical Toxicology*.

Shanti Bhushan Mishra, Essentials of Herbal Drug Technology: A Guide of Standardization Quality Control, 2019, chapter 9, page 182.

Shahavi, Mohammad Hassan, Morteza Hosseini, Mohsen Jahanshahi, Rikke Louise Meyer, and Ghasem Najafpour Darzi. (2015). Evaluation of Critical Parameters for Preparation of Stable Clove Oil Nanoemulsion. *Arabian Journal of Chemistry*, 1–6.

Sharma, Bansal, M., Visht, S., Sharma, P., & Kulkarni, G. (2010). Nanoemulsion: A new concept of delivery system. Chronicles of Young Scientists, 1(2), 2.

Shirsath, S. R., S. H. Sonawane, and P. R. Gogate. (2012a). Intensification of Extraction of Natural Products Using Ultrasonic Irradiations-A Review of Current Status. *Chemical Engineering and Processing: Process Intensification* 53: 10–23.

Sicaire, Anne G., Maryline A. V., Frédéric F., Patrick C., Sylvain T., and Farid C.. (2016). Ultrasound Induced Green Solvent Extraction of Oil from Oleaginous Seeds. *Ultrasonics Sonochemistry* 31: 319–29.

Silva, E. K., Gomes, M. T. M. S., Hubinger, M. D., Cunha, R. L., & Meireles, M. A. A. (2015). Ultrasound-Assisted Formation of Annatto Seed Oil Emulsions Stabilized by Biopolymers. Food Hydrocolloids, 47, 1–13.

Silva, Eric Keven, M. Thereza MG Rosa, and M. Angela A Meireles. (2015). Ultrasound-Assisted Formation of Emulsions Stabilized by Biopolymers. *Current Opinion in Food Science* 5: 50–59.

Silva, Pedro S., Sergey Zhdanov, Victor M. Starov, and Richard G. Holdich. (2017). Spontaneous Emulsification of Water in Oil at Appreciable Interfacial Tensions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 521: 141–46.

Singh, Yuvraj, Jaya Gopal Meher, Kavit Raval, Farooq Ali Khan, Mohini Chaurasia, Nitin K. Jain, and Manish K. Chourasia. (2017). Nanoemulsion: Concepts, Development and Applications in Drug Delivery. *Journal of Controlled Release* 252: 28–49.

Smith, A. J., D. Robertson, M. K. Tang, M. S. Jackson, D. MacKenzie, and J. Bagg. (2003). Staphylococcus Aureus in the Oral Cavity: A Three-Year Retrospective Analysis of Clinical Laboratory Data. *British Dental Journal* 195 (12): 701–3.

Smith, Roger M. (2003). Before the Injection--Modern Methods of Sample Preparation for Separation Techniques. *Journal of Chromatography. A* 1000 (1–2): 3–27.

Sonam Chouhan, Kanika Sharma, and Sanjay Guleria (2017). Antimicrobial Activity of Some Essential Oils—Present Status and Future Perspectives, Medicines (Basel), 4(3): 58.

Soria, Ana Cristina, and Mar Villamiel. (2010). Effect of Ultrasound on the Technological Properties and Bioactivity of Food: A Review. *Trends in Food Science and Technology* 21 (7): 323–31.

Sovová, Helena. (2012). Steps of Supercritical Fluid Extraction of Natural Products and Their Characteristic Times. *Journal of Supercritical Fluids* 66: 73–79.

Speranza, Paula, Ana Paula Badan Ribeiro, Rosiane Lopes Cunha, Juliana Alves Macedo, and Gabriela Alves Macedo. (2015). Influence of Emulsion Droplet Size on Antimicrobial Activity of Interesterified Amazonian Oils. *LWT - Food Science and Technology* 60 (1): 207–12.

Struzycka, Izabela. (2014). The Oral Microbiome in Dental Caries. *Polish Journal of Microbiology* 63 (2): 127–35.

Sukor N., Jusoh R., Rahim S.A., and Kamarudin N., (2018). Ultrasound Assisted Methods for Enhanced Extraction of Phenolic Acids from Quercus Infectoria Galls, Materials Today: Proceedings 5(10):21990-21999.

Taha A., Ahmed E., Ismaiel A., Ashokkumar M., Xu X., Pan S., and Hu H. (2020). Ultrasonic emulsification: An overview on the preparation of different emulsifiers-stabilized emulsions, Trends in Food Science and Technology, 105 (363-377).

Tam, Vincent H., Amy N. Schilling, and Michael Nikolaou. (2005). Modelling Time-Kill Studies to Discern the Pharmacodynamics of Meropenem. *Journal of Antimicrobial Chemotherapy* 55 (5): 699–706.

Tan N.F.M.S., Fauzi N.A.M. and Razak A.H.A. (2020). Qualitative Phytochemical Analysis and Antibacterial Potential of Chromolena Odorata Leaves as affected by Soxhlet and Maceration Extraction, *Journal of Advanced Industrial Technology and Application,* 1 (2), 38-48.

Tiwari, Brijesh K. (2015a). Ultrasound: A Clean, Green Extraction Technology. *Trends in Analytical Chemistry* 71: 100–109.

Truong, Dieu-Hien, Nguyen, Dinh Hieu, Ta, Nhat Thuy Anh, Bui, Anh Vo, Do, Tuong Ha, Nguyen, Hoang Chinh (2019). Evaluation of the Use of Different Solvents for Phytochemical Constituents, Antioxidants, and In Vitro Anti-Inflammatory Activities of Severinia buxifolia, *Journal of food quality*, Volume 2019.

Valle, Demetrio L., Esperanza C. Cabrera, Juliana Janet M. Puzon, and Windell L. Rivera. (2016). Antimicrobial Activities of Methanol, Ethanol and Supercritical CO2 Extracts of Philippine Piper Betle L. on Clinical Isolates of Gram Positive and Gram Negative Bacteria with Transferable Multiple Drug Resistance. *PLoS ONE* 11 (1): 1–14.

Venkadeswaran, Karuppasamy, Arumugam R. M., Thangaraj A., Vasanthakumar V., Mahalingam S., Ramalingam A., Philip A. Thomas, and Pitchairaj G. (2014). Antihypercholesterolemic and Antioxidative Potential of an Extract of the Plant, Piper Betle, and Its Active Constituent, Eugenol, in Triton WR-1339-Induced Hypercholesterolemia in Experimental Rats. *Evidence-Based Complementary and Alternative Medicine* 2014.

Vinatoru, M, T J Mason, and I Calinescu. (2017). Ultrasonically Assisted Extraction (UAE) and Microwave Assisted Extraction (MAE) of Functional Compounds from Plant Materials. *Trends in Analytical Chemistry* 97: 159–78.

Walsh, S E, J Y Maillard, A D Russel, C E Catrenich, A L Charbonneau, and R G Bartolo. (2003). Activity and Mechanism of Action of Selected Biocidal Agents on Gram -Positive and -Negative Bacteria. *Journal of Applied Microbiology* 94: 240–47.

Wang, Shengpeng, Yangyang H., Wen T., Xu W., Ruie C., Jiliang C., Meiwan C., and Yitao W. (2012). Compatibility Art of Traditional Chinese Medicine: From the Perspective of Herb Pairs. *Journal of Ethnopharmacology*.

Wang, Zheng, Marcos A. Neves, Hiroko Isoda, and Mitsutoshi Nakajima. (2016). Preparation and Characterization of Micro/Nano-Emulsions Containing Functional Food Components. *Japan Journal of Food Engineering* 16 (4): 263–77.

Yao Ye,YuePan and ShiqingLiu (2020). Power ultrasound and its applications: A state-of-the-art review, *Ultrasonics Sonochemistry*, 62 (104722).

Yong, S.X.M., Song C.P., and Choo W.S. (2021). Impact of High-Pressure Homogenization on the Extractability and Stability of Phytochemicals, *Frontiers in Sustainable Food Systems*, 4(593259).

Young, S. C., Chau J. W., Jing J. L., Pei L. P., Jui L. H., and Fen P. C.. (2007). Protection Effect of Piper Betel Leaf Extract against Carbon Tetrachloride-Induced Liver Fibrosis in Rats. *Archives of Toxicology* 81 (1): 45–55.

Zhang, Q.W., Lin L.G., and Ye W.C. (2018). Techniques for Extraction and Isolation of Natural Products: A Comprehensive Review, Chinese Medicine, 13(20).

Zheng, C. J., Jung-Sung Y., Tae-Gyu L., Hee-Young C., Young-Ho K., and Won-Gon K. (2005). Fatty Acid Synthesis Is a Target for Antibacterial Activity of Unsaturated Fatty Acids. *FEBS Letters* 579 (23): 5157–62.

Zulqarnain, Muhammad Ayoub, Mohd Hizami Mohd Yusoff, Muhammad Hamza Nazir, Imtisal Zahid, Mariam Ameen, Farooq Sher, Dita Floresyona and Eduardus Budi Nursanto (2021). A Comprehensive Review on Oil Extraction and Biodiesel Production Technologies, Sustainability, 13, 788.

Zygler, A., M. Słomińska, and J. Namieśnik. (2012). Soxhlet Extraction and New Developments Such as Soxtec. In *Comprehensive Sampling and Sample Preparation,* 2:65–82.