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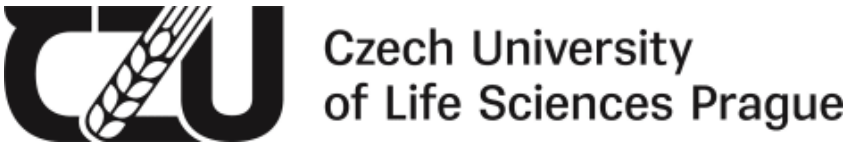

The influence of selected Cambodian plants bark extracts on ethanolic fermentation and contaminant microbial growth in beverages

MSc. thesis

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Declaration

I declare that the Diploma Thesis „The influence of selected Cambodian plants bark extracts on ethanolic fermentation and contaminant microbial growth in beverages“ is my own work and all the sources I cited in it are listed in the Bibliography.

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Summary

Natural palm sap has been known as a tasty and refreshing beverage in tropical countries. In Cambodia, it is traditionally preserved with the addition of plant barks with probable but unproven antimicrobial activity.

Ten ethanolic and aqueous extracts from five Cambodian plant barks were tested *in vitro* by the broth microdilution method. The results were determined as minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC).

Extracts of *Azadirachta indica* A. Juss., *Careya arborea* Roxb., *Hopea odorata* Roxb., *Hopea pierrei* Hance, and *Shorea roxburghii* G. Don were tested against pathogenic (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Candida albicans*) and ethanol producing microorganisms (*Hanseniaspora jakobsenii*, *Hanseniaspora uvarum*, *Leuconostoc palmae*, *Metschnikowia pulcherrima*, *Pantoea agglomerans*, *Saccharomyces cerevisiae*, *Zymobacter palmae* and *Zymomonas mobilis* subsp. *pomaceae)*. In addition to selected standard strains from ATCC and DSM collections, several wild yeasts and bacterial strains were isolated from fruits and determined with MALDI-TOF MS.

All plant extracts demonstrated certain degree of antimicrobial activity against some of the tested microorganisms (MIC range 64–1024 μg/ml). As the most active plant extract due to a broad range of inhibited microbes and lowest MIC values (64 μg/ml against *S. aureus* and *S. cerevisiae* DSM 70478) was the ethanolic extract of *H. odorata,* followed by *A. indica* and *C. arborea.* All three ethanolic extracts also showed bactericidal effects against most of the tested Gram-positive bacteria, *C. arborea* and *A. indica* even demonstrated bactericidal effect against Gram-negative *E. coli* (1024 μg/ml). On the other hand, the least efficient extracts were the aqueous ones obtained from *H. pierrei* and *S. roxburghii.*

A. indica and *C. arborea* ethanolic extracts affected the growth of yeast cells in a concentration-dependent manner, namely *C. albicans, H. uvarum, and S. cerevisiae*. Low concentrations of extracts (8-16 μg/ml) prevented cell death and increased their growth, while medium concentrations (32-128 μg/ml) were able to fully inhibit the growth of yeasts, and higher concentrations (256-1024 μg/ml) were predominantly not efficient for growth-inhibition. This hormetic effect of these extracts has not been previously described in the scientific literature and could be investigated more explicitly.

The trial fermentation test has been performed and three yeasts, namely *H. uvarum* T5, *M. pulcherrima* T9, *S. cerevisiae* DSM 70465 were chosen for the further experiments with Cambodian plant extracts. The yeasts were selected due to their good fermentative ability indicated by the highest values of produced ethanol detected by GC-FID.

The results of this thesis suggest all tested extracts as perspective material for further isolation and identification of active antimicrobial compounds that can be used as potential beverage preservatives.

Key words: *Azadirachta indica; Careya arborea; Hopea odorata; Hopea pierrei; Shorea roxburghii;* fermentation.

Souhrn

Palmová šťáva je v tropických zemích známá jako chutný a osvěžující nápoj. V Kambodži se palmová šťáva tradičně konzervuje přidáním kůr některých stromů s pravděpodobnou, ale neprokázanou antimikrobiální aktivitou.

V této diplomové práci bylo testováno deset ethanolových a vodných extraktů z kůr pěti kambodžských stromů pomocí bujónové mikrodiluční metody *in vitro*. Výsledky byly vyjádřeny jako minimální inhibiční koncentrace (MIC) a minimální baktericidní koncentrace (MBC).

Antimikrobiální aktivita extraktů z kůry *Azadirachta indica* A Juss., *Careya arborea* Roxb., *Hopea odorata* Roxb., *Hopea pierrei* Hance a *Shorea roxburghii* G. Don byla ověřena proti patogenním (*Escherichia coli, Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Candida albicans*) a fermentujícím mikroorganismům (*Hanseniaspora jakobsenii, Hanseniaspora uvarum, Leuconostoc palmae, Metschnikowia pulcherrima, Pantoea agglomerans, Saccharomyces cerevisiae, Zymobacter palmae* a *Zymomonas mobilis* subsp. *pomaceae*). Kromě vybraných standardních kmenů bakterií a kvasinek ze sbírek ATCC a DSM, bylo také izolováno několik divokých kvasinek a bakterií z povrchu různých druhů ovoce. Izoláty byly identifikovány pomocí MALDI-TOF MS.

Všechny rostlinné extrakty prokázaly určitý stupeň antimikrobiální aktivity proti některým z testovaných mikroorganismů (64–1024 μg/ml). Nejúčinnější byl ethanolový extrakt z *H. odorata,* který inhiboval růst největšího počtu testovaných mikroorganismů a vykazoval nejnižší hodnoty MIC (64 μg/ml proti *S. aureus* a *S. cerevisiae* DSM 70478). V řadě nejúčinnějších následovaly *A. indica* a *C. arborea*. Všechny tři ethanolové extrakty také vykazovaly baktericidní účinek proti většině testovaných grampozitivních bakterií, *C. arborea* a *A. indica* dokonce prokázaly baktericidní účinek proti gramnegativní *E. coli* (1024 μg / ml). Nejméně účinné byly extrakty z kůry *H. pierrei* a *S. roxburghii*.

Ethanolové extrakty z *A. indica* a *C. arborea* vykazovaly hormetický efekt na kvasinky *C. albicans, H. uvarum* a *S. cerevisiae*. Nízké koncentrace extraktů (8–16 μg/ml) růst kvasinek stimulovaly, zatímco střední koncentrace (32–128 μg/ml) plně inhibovaly jejich růst a vyšší koncentrace (256–1024 μg/ml) nebyly účinné pro inhibici růstu. Hormetický efekt těchto extraktů nebyl ve vědecké literatuře dříve popsán a mohl by být nadále zkoumán.

Mimo jiné, byl proveden zkušební kvasný test, na jehož základě byly vybrány tři kvasinky (*H. uvarum* T5, *M. pulcherrima* T9, *S. cerevisiae* DSM 70465) pro další výzkum zmíněných extraktů z kůr kambodžských stromů. Uvedené kvasinky vykazovaly nejvýraznější fermentační schopnosti s nejvyšší produkcí ethanolu, který byl měřen pomocí GC-FID.

Výsledky této práce naznačují, že všechny testované extrakty mají antimikrobiální aktivitu a mohly by být prozkoumány za účelem izolace a identifikace účinných antimikrobiálních složek, jež by mohly v budoucnu posloužit jako potenciální konzervační činidla pro nápoje.

Klíčová slova: *Azadirachta indica; Careya arborea; Hopea odorata; Hopea pierrei; Shorea roxburghii;* kvašení.

CONTENTS

1 Introduction

New nutritional developments in society have contributed to a change in the trends of the global beverage market. Consumers are getting more informed about the impact of diet on their health and well-being. Beverages are not consumed anymore only to provide refreshment and hydration, but also to help in preventing nutrition-related disorders (Tenge & Geiger 2001).

Thermal processing and the use of chemical preservatives have also been reduced to produce more "natural" products (Juvonen et al. 2011). Chemical preservatives are being replaced with plant-derived compounds exhibiting strong antimicrobial and antioxidant activity (Bouarab Chibane et al. 2019).

Natural palm sap has high nutritional and medicinal values, and it is popular for its taste and nutritive value. The quality of nutrition and flavour of palm sap are appealing features for consumers. The appearance and flavour of palm sap indicate its stage of fermentation (Hebbar et al. 2018).

Fermented palm wine or toddy produced from palm sap is indigenous to the tropical regions of Asia, Africa, and South America. Palm wine is used in traditional ceremonies and in folk medicines. Moreover, it is known for its antioxidant properties and health-promoting benefits (Djeni et al. 2020).

Fermentation is generally considered as a safe and acceptable preservation technology for improving the hygienic quality and safety of foods. However, it can occure spontaneously also in fresh juices as an undesirable side effect of natural contamination. Local Cambodian palm sellers are therefore adding plant barks to palm sap for extension of its shelf life by inhibiting the growth of microorganisms that occur naturally in the sap. *Azadirachta indica*, *Careya arborea*, *Hopea odorata*, *Shorea roxburghii* are typical plants used for the palm sap preservation due to their antimicrobial and antifungal activities that are also applied in the Cambodian ethnomedicine.

2 Purpose and hypothesis of the study

The aim of this work is to verify the antimicrobial activity of bark extracts of Cambodian plants, namely *Azadirachta indica*, *Careya arborea*, *Hopea odorata, Hopea pierrei*, *Shorea roxburghii* against selected species of microorganisms *in vitro* and to determine the effect on ethanol fermentation in selected beverages.

Even though the antimicrobial activity of *Azadirachta indica*, *Careya arborea* has previously been described in several studies, the antimicrobial effect of *Hopea pierrei*, *Hopea odorata,* and *Shorea roxburghii* is still poorly known.

Based on ethnobotanical and pharmacological data summarized in the literature review, it is possible to expect, that the bark extracts of these trees will show activity against some microorganisms. And due to the certain traditional methods of palm sap preservation with use of these plants, it is expected that their extracts will inhibit the ethanol fermentation process in beverages with similar properties.

3 Literature review

3.1 Beverages overview

More and more consumers decide to consume minimally processed products from natural ingredients in order to reduce the intake of chemical additives from food and to obtain more quality products with better nutritive and sensory characteristics.

Simple carbonated soft drinks still dominate the global beverage market. Functional beverages and bottled water are ones of the fastest growing sectors in beverage production. The energy drinks sector has had the largest volume growth in the last years (Steen & Ashurst 2006). The juice sector is currently demonstrating dynamic growth mainly due to a change in lifestyle and consumer preferences to use healthier "new age beverages" (Priyadarshini & Priyadarshini 2018).

These facts lead to more complex changes in composition. Consumers are looking for more natural products with the minimal content of additives and lower dosage of technological treatment. Manufacturers all over the world simultaneously are struggling with antimicrobial safety hurdles. Due to possible microbial contamination, the keeping of drinks' natural properties with a minimum number of additives and use of moderate preserving techniques is demanding.

The beverage industry tries to respond to consumer demands and find a way to decrease implementation of chemical additives by using natural antimicrobials.

3.1.1 Microbiological health risks associated with beverages

Numerous microorganisms are found in beverages as contaminants, but only some of them can cause spoilage. Microbiological spoilage leads to deterioration of the sensory quality and typically appears as off-flavours, odours, and visual changes in the product. As microbes differ in their growth requirements, different beverages support different spoilage microbes (Juvonen et al. 2011).

Any spoilage requires a certain critical cell number (105–106 cells/ml) and therefore microbial growth. In addition to direct spoilage mediated by viable cells, carry-over of microbial metabolites from raw materials and the production process can lead to indirect spoilage. Microbial contamination of raw materials can cause off-flavours, overfoaming, production failures and product spoilage even if no viable cells are left (Stratford 2006).

In beverage production, foodborne disease outbreaks and spoilage problems also occur. Most of them relate to pathogens, such as *Escherichia coli* O157:H7, *Salmonella* spp, *Cryptosporidium parvum* and spoilage microorganisms such as *Alicyclobacillus acidoterrestris* (Tribst et al. 2009). Coliform bacteria as an indicator of pathogens in water are one of the most relevant biological parameters for the beverage and mineral water industry (Syposs et al. 2005).

So-called specific spoilage microbes can grow even in products produced under good manufacturing practices. Some of them can generally develop inside the acidic and low oxygen conditions. In case of production failures, less specialised opportunistic species are often involved, as they are more common in the production environment (Hernández et al. 2018).

Despite the considerable efforts in minimizing microbial cross contamination in beverage production, the pathogenic microorganisms from environmental, animal, or human sources can easily enter the system during any of the manufacturing steps. These steps can include harvesting and raw

material production, the manufacturing process, storage, transportation, and even retailing and household handling (Ranadheera et al. 2020).

Large-scale preservation methods, such as canning and aseptic processing, are well developed for industrial scale systems but they are energy demanding, expensive and require infrastructure, which are often not available in rural and remote areas of low-income countries (Phinney et al. 2019).

Food spoilage is a growing challenge in all countries around the world. And there are many reasons for spoilage along the food and beverage production chains. According to Phinney et. al. (2019), in low-income countries more than 40% of spoilage occurs during the postharvest and processing phases, which can be attributed to small-scale production limitations, a lack of access to improved farming practices, underdeveloped preservation and processing industries and poor transport infrastructure.

Fermented foods and beverages play an important role in human health and remain the oldest prevalent means of food processing and preservation all over the world. They represent what is thus one of the most affordable and suitable methods to maintain hygiene condition and food quality and security in poor and underdeveloped countries (Anal 2019).

South-East Asia is well-known for traditionally fermented beverages produced at a small scale and following traditional procedures. Fermentation in developing Asian countries is currently done through the age-old methods with little or no concerns for quality and safety of the products.

One of the most popular Cambodian drinks remains a palm wine, that is a long-term tradition in this country. Palm sap used for its producing is always collected in an open environment and is therefore easily affected by it, resulting in variations in the microorganism community structure and metabolite profiles (Djeni et al. 2020). There are reports that the yeasts and bacteria originate from the gourd, palm tree, and tapping implements lead to forcing of fermentation processes and fast deterioration of the product quality (Chandrasekhar et al. 2012).

3.2 Palm sap

Palm sap is a transparent liquid, with a sugar content of 100-144 g/kg, and pH of 7.0-7.4 (Lasekan & Abbas 2010). The sap contains sugars such as sucrose, glucose, fructose, cellobiose, maltose, xylose, arabinose and galacturonic acid (Moraha & Robinson 2015). The fresh sap is rich source of phenolics, vitamins B and C, protein, essential elements (nitrogen, phosphorous, potassium, and magnesium), and micronutrients (zinc, iron, and copper). It is remarkable that the sap has been found with low glycemic index of 35 when compared to refined white sugars which have a glycemic index of 65.

Analysis of the volatile compounds of the thermally treated palm juice using headspace solid-phase microextraction method yielded 30 odour-active compounds. There were 17 pyrazine compounds, 7 furan derivatives, 4 aldehydes and 2 ketones. The typical flavour components of palm juice were identified as ethyl lactate, 3-hydroxy-2-pentanone, and ethylhexanoate. Higher temperatures of above 110 ^{o}C led to a nearly exponential increase in the formation of pyrazine compounds and the typical roasted nut aroma of palm sugar (Lasekan & Abbas 2010).

Various species of Palmae family, such as raphia palm *(Raphia hookeri*), date palm (*Phoenix sylvestris*), toddy palm (*Borassus flabellifer*), oil palm (*Elaeis guineensis*), and coconut palm (*Cocos nucifera*) are generally used for the palm sap and foods originate from palms production (Djeni et al.

2020). These include traditional fresh juices and fermented beverages such as toddy, wine, and arak, concentrated syrup such as honey, and brown sugar (jaggery powder) (Hebbar et al. 2018). These are all produced by tapping sap from different palm trees, including mentioned above.

Tapping involves the collection of palm juice from the unopened spadix of the palm tree. This process requires making a small incision in the bark about 15 cm from the top of the trunk. A bamboo pipe is inserted into the base to collect the sap into a clean gourd, which is tied around the tree to collect the sap. It should be consumed within 5-12 hours after collection (Chandrasekhar et al. 2012). The fresh palm juice is a sweet, clear, colorless solution and it serves as a good digestive agent (Lasekan & Abbas 2010).

Palm juice helps in regulating high blood pressure and sugar metabolism due to the presence of potassium. It acts as a potential antioxidant for overall production of immune system, respiratory system, cardiovascular system, and reduction of inflammation. It also aids in electrolyte balance and acid/alkaline ratio (Hebbar et al. 2018).

3.2.1 Products made from palm sap

3.2.1.1 Palm wine

The high sugar content of palm juice creates a perfect environment for yeast and bacterial growth. Fermentation starts soon after the sap is collected and within a couple of hours becomes high in alcohol. The natural fermentation processes allow production of toddy, more commonly known as a palm wine. If the fermentation processes are allowed to continue for more than a day, toddy starts turning into a vinegar (Chandrasekhar et al. 2012).

Palm wine is a collective name for a group of alcoholic beverages produced from the palm tree sap by natural fermentation (Djeni et al. 2020). Fermented sap is whitish and has a pH about 3.6 and alcohol contents of 3.3-4.0%, depending on the stage of fermentation at which the wine is consumed (Lasekan et al. 2007).

A liter of palm wine contains approximately 300 calories. It is rich in protein and micronutrients, such as vitamins A, C and K. The fermentation process helps to increase the levels of thiamin, riboflavin, pyridoxin and vitamin B12 (Chandrasekhar et al. 2012).

The aroma of palm wine is characteristic for the distinct alcoholic, moody, non-typical floral fruity notes (Lasekan et al. 2007). The analysis of palm wine by using GC-MS identified 82 components, including 47 esters, 9 alcohols, 5 acids, 6 carbonyls, 2 acetals, 4 terpenes and 9 hydrocarbons (Chandrasekhar et al. 2012). 41 of the chemical compounds were identified as odour active. Quantitation and odour activity values of 13 key odorants of palm wine revealed that earthy-smelling 3-isobutyl-2-methoxypyrazine, buttery-smelling acetoin, fruity ethylhexanoate, 3-methylbutylacetate and popcorn-smelling 2-acetyl-1-pyrroline were the most potent odorants of those quantified in the palm wine sample (Lasekan et al. 2007).

Palm wine is indigenous to the tropical regions where palms grow, such as Africa, Asia, and South America. As part of cultural heritage, palm wine is especially used in traditional namings and marriage ceremonies, traditional incantations and in folk medicines. Both government and health professionals have recognized the importance of palm wine in the treatment of malnutrition in some Africans regions. Moreover, palm wine is well known for its antioxidant properties and its health-promoting benefits that were demonstrated in rat models (Djeni et al. 2020).

3.2.1.2 Palm sugar

Palm sugars are used for making chocolate, cake, sweet soy sauce, food coating, ice cream, and typical Bengali sweets (Hebbar et al. 2018).

The process of palm sugar making is similar for all palm saps (*B. flabellifer, C. nucifera, P. sylvestris, Caryota urens*). The collected palm juice is filtered and poured into a large pan. Afterward, the sap is cooked for 3-4 hours until it becomes concentrated, brown, and highly viscoused. This hot brown sugar mass is poured down into a mould made of bamboo, wood, or coconut shell. The sugar is cooled down and after 1 hour is ready for packaging (Lasekan & Abbas 2010).

The results of earlier research studies have confirmed that palm sugar consist mainly of sugars (glucose, fructose, and sucrose) at amounts of 89.2% (Hebbar et al. 2018). The approximate nutritional values of palm sugar are followed: 0.35% of protein, 0.17% of fat (due to naturally presented ethers), 0.74% of mineral compounds such as calcium (0.06%), phosphorous (0.06%), and iron. Vitamins such as nicotinic acid (5.24 mg/100g), thiamin (24 mg/100 g), riboflavin (432 mg/kg), and ascorbic acid (11 mg/100 g) are also presented (Vengaiah et al. 2013).

Due to the presence of sugars, palm sap is subjected to the Maillard reaction and caramelization while heating. The use of an open pan evaporator leads to a higher sucrose inversion and hydroxymethylfurfural formation that is due to Maillard reaction decrease the concentration of glucose and fructose in the final product (Kurniawan et al. 2018). Maillard reaction final products, such as pyrazines, are released when palm sap is heated above 110 °C. The initial quality of palm sap, boiling temperature, and heating time affects the volatile components of palm sap.

Pyrazines, furans, ketones, fatty acids, and organic acids are constituent volatile components of palm sugar, and they are responsible for its sweet, toasty, and nutty caramel-like aroma (Hebbar et al. 2018).

3.2.2 Palms using in palm sap and palm wine production

Harvesting of local non-timber forest products plays a central role for human well-being, especially in rural communities across tropical developing countries as food, medicine, and income generation. Palms rank as one of the most economically important plant families across the tropics (Mogue Kamga et al. 2020).

3.2.2.1 *Borassus flabellifer* L.

The palmyra, or toddy palm (*Borassus flabellifer* L., Arecaceae) grows wild from the Persian Gulf to the Cambodian-Vietnamese border. It is commonly cultivated in India, South East Asia, Malaysia and occasionally in other warm regions including Hawaii and Florida (Morton 1988).

Borassus flabellifer is a tall tree capable of growing up to 30 m high. It has fan-shaped leaves that are 2-3 m long. The flowers are small, clustered spikes that are followed by brown large round fruits (Chandrasekhar et al. 2012).

In addition to the sweet sap from the inflorescence and the many products of the leaves, trunk and underground seedlings, a thin orange pulp coating the fibers of the mature fruit is consumed

fresh or dried as a paste. The large seeds, when immature, before the shell hardens, contain jelly-like kernels esteemed for food (Morton 1988).

The chemical constituents of *Borassus flabellifer* include gums, albuminoids, fats, steroidal glycosides, and carbohydrates like sucrose. The male inflorescence constitutes borassosides and dioscin, spirostane-type steroid saponins. The presence of high content of crude flavanoids, saponins and phenolic compounds relates to the antioxidant activity. The fresh pulp is reported to be rich in vitamins A and C. The fresh sap is a good source of vitamin B complex (Sandhya et al. 2010).

The plant has been used traditionally as a stimulant, antileprotic, diuretic and antiphlogistic. The fruits are stomachic, sedative, laxative, and aphrodisiac in nature useful in hyperdipsia, dyspepsia, flatulence, skin diseases, haemorrhages, fever, and general debility. The roots and juice of the plant are useful in inflammatory reactions. The ash obtained by burning the inflorescence is a good antacid antiperiodic, and is useful in heart burn, and splenomegaly.

Survey of literature revealed that the medicinal plant *Borassus flabellifer* Linn. has been used as antidiabetic, antidote, anti-inflammatory, wound healing, anthelmintic, analgesic, and antipyretic remedy (Pramod et al. 2013)

In Cambodia the tree is a national floral symbol (Angkorwat) (Chandrasekhar et al. 2012)

3.2.2.2 *Cocos nucifera* L.

The coconut (*Cocos nucifera*) is an important member of the family Arecaceae, the subfamily Cocoideae (Chandrasekhar et al. 2012). The plant is originally from South East Asia and the islands between the Indian and Pacific Oceans. From that region, coconut palm has been brought to India, Africa, and from there dispersed to the American continent and to other tropical regions all over the globe (Lima et al. 2015)

There are mainly two distinct groups of coconut i.e., tall and the dwarf. The tall varieties grow slow and bear fruits 6 to 10 years after planting, its copra, oil, and fiber are of good quality. This type is comparatively hardy and lives up to a ripe age of 80 to 120 years. It grows better on well drained loamy and clayey soil. A year-round warm and humid climate favors the growth.

The dwarf varieties are fast growing and bear early i.e., takes 4 to 5 years. The nuts are yellow, red, green, and orange colored. These are less hardy and require favorable climatic conditions and soil type for better yield (DebMandal & Mandal 2011).

The parts of *C. nucifera* fruit like coconut kernel and tender coconut water have numerous medicinal uses such as antibacterial, antifungal, antiviral, antiparasitic, antidermatophytic, antioxidant, hypoglycemic, hepatoprotective and immunostimulant properties (DebMandal & Mandal 2011).

Phytochemical studies of the coconut fiber (mesocarp) ethanolic extract revealed the presence of phenols, tannins, leucoanthocyanidins, flavonoids, triterpenes, steroids, and alkaloids, while a butanol extract contained triterpenes, saponins, and condensed tannins. Flavonoids are responsible for its antioxidant properties. Condensed tannins are reported to possess antihelminthic activity by binding to proteins present in the cuticle of nematodes, thus intensifying the physical and chemical damage in helminth. The lyophilized extract and fractions, as well as ethyl acetate extracts, from the *C. nucifera* fiber are rich in polyphenols, compounds such as catechins, epicatechins, tannins, and flavonoids. (Lima et al. 2015)

Coconut has multifarious utility. Coconut water and coconut kernel contain microminerals and nutrients, which are essential to human health, and hence coconut is used as food by people all around the globe, mainly in the tropical countries (Yong et al. 2009; DebMandal & Mandal 2011).

Coconut sap is the richest source of nutrients compared with sugar palm juice and date palm sap. Six vitamins, including vitamin C, B1, B3, B4, B2, and B10 were detected in coconut sap. Major minerals are Na, K, Mg, lower amount of Ca, Zn, and Cu is presented. Coconut sap also contains higher amounts of antioxidants, such as gallic acid, protocatechuic acid caffeic acid, p-coumaric acid, and galangin.

Coconut sap records a greater economic value when used as nonfermented and fermented drinks, alcoholic beverages, vinegar, and acetic acid, etc. It is also used as a raw material to produce coconut sugar (Asghar et al. 2020).

3.2.2.3 *Raphia* spp

Raphias are large palms reaching up to 30 m in height. Most species have above-ground trunks, which may be solitary or clustered, while two (*R. regalis; R. vinifera*) have very short or subterranean trunks. When the stem is above a ground, it is typically hidden by old leaf sheaths and curly or straight trunk fibers. The pinnate leaves are very long reaching in some cases up to 25-30 m. Generally, *Raphia* thriving in humid and swampy tropical regions of Africa, Central and Southern America (Mogue Kamga et al. 2020). Hydromorphic soils are quite suitable for the growth of these palms.

The *Raphia* palm after a period of vegetative growth produces flowers and fruits only once and dies. The flower is signalled by the simultaneous appearance in the crown of more than one expanded spear leaves. It is usually at this stage that the palm is tapped for sap or wine. *Raphia hookeri* is the highest yielder of palm sap or palm wine followed by *Raphia vinifera* (Obahiagbon 2009).

Sap is the most important resource and the derived palm wine is probably one of the most studied products of *Raphia*. The elements detectable in the sap of *Raphia hookeri* palm include Cl, Na, K, Ca, Mg, Fe, Cu, Mn, Zn, P, and N. Thiamine, ribofavin and ascorbic acid are also present in the sap of *Raphia* palms. The source of the vitamins was linked to the yeast, *Saccharomyces cerevisiae* presented in the sap, being one of the major agents of fermentation of the latter (Obahiagbon 2009).

Raphia species are commonly used for medicinal purposes including digestive, muscular-skeletal, circulatory, blood system problems, inflammations, mental pain, subcutaneous tissue, pregnancy and birth disorders, bruises and poisoning. Medicinal herbs are often added to palm wine to cure a wide range of illness such as malaria, measles, and jaundice, also as a prevention against diarrhea and headaches (Mogue Kamga et al. 2020)

Just under a hundred different *Raphia* uses are reported in scientific literature and span a considerable number of different categories, ranging from food and beverages, construction, tools, handicrafts, and energy supply through to medicinal, cultural, and ecological purposes (Mogue Kamga et al. 2020).

3.2.2.4 *Phoenix sylvestris* Roxb.

Phoenix sylvestris Roxb. is a 7.5-15 m tall dioecious tree. It is widely distributed in India, Pakistan, Myanmar, Nepal, Bhutan, Bangladesh, Mauritius, China, and Sri Lanka (Pankaj et al. 2018).

Tree has no root suckers; stems are clothed with remains of petiole bases. Leaves are greyish green, 0.96-4 m long. They are quite glabrous, pinnately divided into numerous leaflets (Hasan et al. 2010).

P. sylvestris has been considered as a traditional medicine to cure various ailments like abdominal complaints, fevers, loss of consciousness, constipation, and heart problems. Different parts of the plant exhibit diverse medicinal properties such as being antipyretic, cardiotonic, laxative, diuretic, and antioxidant. The central tender part of the plant is used in the treatment of gonorrhea. The root of the plant is used to treat toothache, nervous debility, and helminthiasis. The fruits are edible and considered as restorative and tonic. The kernels are made into a paste with the root of Achyranthes aspera, eaten with betel leaves as remedy for ague. The fresh unfermented sap is used as a laxative. Neera and toddy are known as refreshing sweet drinks and good source of vitamins (Hasan et al. 2010; Pankaj et al. 2018).

Besides a high amount of carbohydrates, sap also contains reducing sugar (3.95%), crude lipid (1.15%), crude protein (1.08%), crude fibre (0.18%) and ash (0.46%). In mineral composition K (80mg/100gm) was found to be the most predominant, followed by Na (18.23mg/100gm), Ca (4.76mg/100gm) and Mg (2.23mg/100gm). The sap is rich in vitamin B3 (12.3mg/100g) and vitamin C (12.75mg/100gm) (Salvi & Katewa 2012).

P. sylvestris is traditionally important and known for its nutritional values throughout the world. It is a rich source of carbohydrates, phenols, amino acids, flavonoids, tannins, alkaloids, terpenoids, dietary fibers, essential vitamins, and minerals (Pankaj et al. 2018).

3.3 Ethanol fermentation

Alcoholic fermentation is the anaerobic transformation of sugars, mainly glucose and fructose, into ethanol and carbon dioxide. This process is carried out by yeast and by some bacteria such as *Zymomonas mobilis*.

Alcoholic fermentation is a complex process. At the same time a lot of biochemical, chemical, and physicochemical processes take place, making it possible to turn the juice into wine. Besides ethanol, other compounds are produced throughout alcoholic fermentation such as higher alcohols, esters, glycerol, succinic acid, diacetyl, acetoin and 2,3-butanediol. Without the production of these other substances, wine would have little organoleptic interest (Zamora 2009).

3.3.1 Bacteria and yeast caused fermentation in palm sap

Nutritionally rich palm sap creates an ideal source for microbial survival, growth and as such can enhance the production of unwanted second metabolites of yeast and bacteria. The unfermented sap contains 10-16.5% w/v sugars (mainly sucrose) that is fermented to ethanol and other minor constituents by a complex mixture of wild yeasts and bacteria. The naturally fermented palm wine contains about 5-6% v/v ethanol (Theivendirarajah & Chrystopher 1987).

Yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) are the most reported microbial constituent in literature and the species *Saccharomyces cerevisiae* is normally the main organism of interest, because it is mainly responsible for converting the sugary sap to alcohol.

Palm wine drinkers know that the drink tastes differently at different stages of fermentation because of yeast fermentation and accumulation of organic acids especially acetic acid from fermentation by AAB as fermentation progresses each day.

It is commonly known that palm wine fermentation consists of an initial lactic acid fermentation initiated by LAB, a middle alcoholic fermentation initiated by yeasts, followed by a final acetic fermentation by AAB. The consensus among palm sap and palm wine investigators is that the nature of fermentation depends on the chemical composition of sap, type of palm tree and its geographical location (Nwaiwu et al. 2016).

3.3.1.1 Lactic acid bacteria

Lactic acid bacteria are microaerophilic, Gram-positive non-spore-forming bacilli or cocci. They are strictly fermentative, aero-tolerant, or anaerobic, aciduric or acidophilic and have complex nutritional requirements (e.g., for carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins).

LAB ferment sugars predominantly to lactate. Depending on the species and growth conditions, sugar catabolism can also lead to formation of ethanol, acetate, formate or succinate. Some strains produce diacetyl, which tastes and smells buttery, and is an unwanted metabolite in beverages (Hammes & Vogel 1995).

Lactic acid bacteria are responsible for the pH reduction through production of organic acids, which give a sour taste to the palm wine, and are also associated with the aroma, consistency, and colour of palm wine by the production of polysaccharides. Indeed, one of the broadly recognized advantages of LAB along with acetic acid bacteria in food fermentation is the inhibitory effect on foodborne pathogens, particularly on the members of Enterobacteriaceae, which could explain the very low relative occurrence (<1%) of the potential pathogenic members of Enterobacteriaceae in the palm wine samples studied, despite the poor hygienic production environment (Djeni et al. 2020).

Palm wine contains many lactic acid bacteria, including *Streptococcus, Pediococcus, Leuconostoc* and *Lactobacillus* spp. *Lactobacillus plantarum* and *Leuconostoc mesenteroides* are known as the dominant lactic acid bacteria in palm wine (Chandrasekhar et al. 2012).

At the genus level, *Lactobacillus* and *Leuconostoc* account for nearly 60% of the overall relative abundance in most of the palm wine samples studied from Côte d'Ivoire by Djeni et. al. (2020). As reported in several culture-based studies, *Lactobacillus* dominated the palm wine samples. *Lactobacillus johnsonii* and *Lactobacillus helveticus* which were well known for their probiotic potential were found with a high abundance in ron palm and oil palm wines. Whereas *Leuconostoc mesenteroides* was abundantly present in the raphia wine samples.

Further analysis showed Lactobacilliaceae as the key differentiating taxa of the palm wine samples studied from Côte d'Ivoire, with a high presence (56% relative abundance) in ron palm and oil palm wines than raphia palm wine (28% relative abundance) (Djeni et al. 2020).

Bacteria are numerous in the partly fermented palm sap and as the fermentation proceeds, yeasts overgrew bacteria, that can be attributed to the increasing concentration of alcohol and lowering of pH during fermentation (Ezeronye & Okerentugba 2000).

3.3.1.2 Yeasts

Yeasts are the most noteworthy gathering of smaller scale life forms related to the deterioration of beverages (Hernández et al. 2018).

Uncontrolled fermentation and the rest of yeast inoculum that comes mainly from unsterilized pots, previously used for the collection of sap creates a great variability in palm sap microflora (Theivendirarajah & Chrystopher 1987).

Palm wine yeasts isolated from freshly tapped palm wine from different palm trees are most often identified as *Schizosaccharomyces pombe, Saccharomyces cerevisae, Debaryomyces hansenii* and *Zygosaccharomyces rouxii* (Chandrasekhar et al. 2012).

Genetic screening of 1200 palm wine yeasts by Ezeronye and Okerentugba (2000) lead to the selection of fourteen isolates with various genetic and physiological properties. Nine of the isolates were identified as *Saccharomyces* sp., three as *Candida* sp., one as *Schizosaccharomyces* sp., and one as *Kluyveromyces* sp.

Isolation of yeasts *Schizosaccharomyces pornbe, Saccharomyces chevalieri* and *Saccharomycodes ludwigii* from *Borassus flabellifer* L. (palmyrah) toddy was previously reported. The presence of *Torulopsis* sp. in palmyrah toddy together with *Saccharomyces cerevisiae* and *S. marxianus* from spontaneously fermented palmyrah sap was detected (Ezeronye & Okerentugba 2000).

Asporogenous, non-fermentative yeasts such as *Kloeckera apiculata* can be predominant during the initial stages of alcoholic fermentation of palmyrah toddy and the sporogenous fermentation yeast types appeared towards the later stages of fermentation.

Genus *Saccharomyces* has numerical predominance and superior fermentative ability. *Saccharomyces* sp. has become adapted to growth in the special conditions of palm sap. The yeasts belonging to the genus *Saccharomyces* and some strains of *Schiz. pombe* can utilize ethanol when it is the sole carbon source available. Possibly, part of the ethanol produced is utilized by these yeasts towards the end of the fermentation process when the sugar concentration is low. Also, this fact may account for the numerical predominance of the *Saccharomyces yeasts* in palmyrah toddy samples (Theivendirarajah & Chrystopher 1987).

Saccharomyces cerevisiae isolates were reported to ferment glucose, sucrose and raffinose, but not lactose and trehalose. They assimilated mainly glucose, sucrose, maltose, trehalose and raffinose, but not arabinose, cellubiose, lactose, ribose, and xylose (Amoa-Awua et al. 2007).

Hanseniaspora sp. (9.28%) and *Hanseniaspora valbyensis* (11.92%) were the next two predominant yeast species present in raphia palm wine, while *Hanseniaspora guilliermondii* (8.0%) and *Saccharomycodes* (3.4%) were present in oil palm wine. Pairwise comparison showed a higher abundance of *Hanseniaspora* in raphia palm wine by Djeni et. al. (2020).

The other yeast species detected in low abundance in the palm wine samples from Côte d'Ivoire, notably *Hanseniaspora valbyensis*, *Hanseniaspora guilliermondii* and *Saccharomycodes* were consistent with previous culture-based reports. Whereas *Saccharomycodes ludwigii* and *Zygosaccharomyces bailii* notably predominated in the palm wine

from Cameroon. These non-*Saccharomyces* yeasts with specific flavour-active characteristics can contribute to the unique flavour of the different palm wine type (Djeni et al. 2020).

The development of aroma and flavour of the palm wine was related to the yeast's growth and metabolic activity. Most of the yeast strains product esters and higher alcohols. Depending on the strain of yeast, considerable variation is observed in the relative concentrations of these compounds (Theivendirarajah & Chrystopher 1987).

3.3.1.3 Acetic acid bacteria

AAB are aerobic Gram-negative short or coccoid motile or non-motile rods. They are widespread in nature, particularly in sugar and ethanol-enriched habitats. The most common spoiling AAB belong to the genera *Acetobacter* and *Gluconobacter*.

Acetic acid bacteria acquire energy from the oxidation of sugars, organic acids, sugar alcohols and alcohols with the production of acetic, gluconic, lactic and succinic acids, acetaldehyde, and ketone compounds. The end products depend on the species and growth conditions. AAB do not have amino acid requirements, and ammonia can serve as sole source of nitrogen. B vitamins may be needed in certain conditions (Juvonen et al. 2011).

AAB are acid tolerant bacteria. Most species grow at pH 3.6–3.8, and some even at pH 3.0. The optimum temperature for growth lies at 25–30°C (Chandrasekhar et al. 2012).

Even though AAB are essential for toddy fermentation, heavy load of them in fresh sap may suppress the growth of other essential microorganisms and production of metabolities necessary for taste and flavour. This results in the spoilage of the substrate at the initial stages of fermentation. The brew develops a vinegar flavour which becomes more pronounced as fermentation advances (Shamala & Sreekantiah 1988).

Various studies have reported the presence of AAB in the fermented palm sap. Among the Proteobacteria phylum, members of AAB namely *Acetobacter*, *Gluconoacetobacter* and *Gluconobacter* were presented in the palm wines of Côte d'Ivoire. The overall predominance of *Acetobacter* (with an average relative abundance of 21%) was reported. At the species level, *Acetobacter pasteurianus* was found to be prevalent (~18% relative abundance). *Acetobacter indonesiensis* was reported as the predominant AAB in 'Bandji' palm wine of *Borassus akeassii* from Burkina Faso and *Gluconacetobacter* also identified in the coconut palm wine of Mexico.

Although AAB have been previously identified as wine spoilage bacteria, their population in palm saps is often underestimated due to the lack of appropriate cultivation techniques (Djeni et al. 2020). As it was noted before, the main advantage of a significant population of AAB in palm saps is inhibition of foodborne pathogens' growth, especially Enterobacteriaceae, and increase of susceptibility to spoilage during storage.

3.3.1.4 Other bacteria

Zymomonas sp. is mainly responsible for ethanol and carbon dioxide production in palm wine fermentation (Shamala & Sreekantiah 1988). *Zymomonas mobillis* degrades sugar to pyruvate using the Entner-Doudoroff pathway. The pyruvate is then fermented to produce ethanol and carbon dioxide as the only products (Chandrasekhar et al. 2012). Bacterium *Zymomonas*, predominantly

present in the Nigerian palm wine was negligible (~1% relative abundance) in the palm wines of Côte d'Ivoire (Djeni et al. 2020).

Another study reveals the presence of *Bacillus* genus bacteria, including isolated *B. cereus* and *B. sphaericus*. The reason for the abundance of these species is not clear. Spores of all isolated bacteria were found in soil and those of *B. cereus* are mostly found as food contaminants. *B. cereus* and *B. firmus* are acid producers and hence they may make the palmyrah toddy unsuitable for human consumption by producing acids during the latter part of fermentation. Also, it has been reported that *B. cereus* food poisoning can be caused if very large amounts of this bacterium are consumed (Theivendirarajah & Chrystopher 1987).

3.4 Methods of palm sap preservation

Many preservation techniques have been developed for enhancing the shelf life of palm sap. However, they were experimented on the sap collected by a traditional method which is unhygienic and partially fermented (Hebbar et al. 2018). Below are described contemporary sap preservation techniques.

Thermal processes such as pasteurization and sterilization are usually applied to extend the shelf life of palm sap. In India, palm sap is pasteurized by heating at 90–95 °C for 3–7 min, and in Thailand, pasteurization is normally done by heating at 60–70 °C for 60 min (Naknean et al. 2015).

It has been reported that conventional thermal processing influenced the nutritional, color, and flavor characteristic of palm sap. The thermochemical reactions such as Maillard reaction, inversion reaction, and caramelization affected the quality. The time and temperature factors need to be optimized to decrease the thermal degradation of palm sap.

Heating at the temperature 52–54 °C for 12–15 min shows the reduction of microorganisms. Most of the yeasts in palm sap samples were killed by heating at 68° and 70 °C for 25–30 min. The pasteurization process had no significant effect on the concentration of riboflavin, thiamine, and ascorbic acid (Baliga & Ivy 1961).

Sterilization at 80 °C for 25 min and 90 °C for 20 min in Lanka Glass Co. bottles was most satisfactory for coconut sap preservation. The treated samples could be kept for 6 months without a change of flavor characteristics. A yellowish discoloration which darkened to a brownish tint after 6 months of storage was observed (Mohanadas 1974).

Natural preservatives or antimicrobial agents could also be used as a part of hurdle technology so that heating time and temperature could be reduced because of thermal degradation reduction (Hebbar et al. 2018).

Okafor (1975) studied the effect of preservatives (sorbic acid and sodium metabisulfite) and pasteurization on palm wine. He reported that palm sap could be more effectively preserved by pasteurization at 70 °C for 30 min than preservatives in reducing the microbial population. He also suggested that combination of sorbic acid (1%) and pasteurization treatment may prove useful for preserving the palm sap.

Sulfite, propionic acid, and benzoate were used for preservation of palm sap (Hebbar et al. 2018). Suppression of non-ethanol producing microorganisms was observed in coconut inflorescence sap by the addition of 200 mg L^{-1} sodium metabisulfite (Samarajeewa 1985).

Chitosan and nisin can be applied as food additives in packaging material to inhibit the growth of microorganism in food (Naknean et al. 2015). Nisin proved to inhibit the growth of gram-positive bacteria and their spore forms. Naknean (2015) exhibited the application of nisin for extension of shelf life of pasteurized sap. He reported that pasteurization at 75 °C for 10 min along with 30 IU/mL nisin and low-temperature storage at 4 °C achieved 10 weeks of shelf life of sap as against 2 weeks shelf life for control sample. Pasteurization with zero additives was found to extend the shelf life of *Raphia* palm sap without much change in taste (Dioha et al. 2010).

As an alternative to the heat treatment, a centrifugation method was proposed. Centrifuged palm sap-based beverages did not show change in flavor but were not accepted by consumers due to the lack of characteristic white color (Okafor 1975). A membrane technique developed by National Chemical Laboratory (NCL), Pune, claims to extend the shelf life of 45 days under refrigerated storage conditions (4–8 °C) by removing the microorganisms present in palm sap without compromise in nutritional quality (Hebbar et al. 2018).

Reported techniques are efiicient for the extension of palm sap shelf-life, but alternative methods applied in rural areas need another approach due to the lack of equipment and, in some cases, no access to the modern technologies.

3.4.1 Biological preservation with natural antimicrobials

Antimicrobials have been widely used to combat spoilage and pathogenic microorganisms in several areas that involve human and animal health, including the food chain (Lopez et al. 2018).

Natural antimicrobials have also been known and widely used in the rural areas. Some plants found a place in palm sap and palm wine preservation.

In India, to prevent fermentation of coconut inflorescence sap, the inner surface of the earthen pot is coated with lime. This practice prevents fermentation only to a certain extent. In Thailand, Kiam wood (*Cotylelolbium melanoxylon Pierse*) is added in the bamboo tube during the collecting process of palm sap to slow down the growth of the microorganisms (Hebbar et al. 2018).

Extracts from the bark of different trees such as *Saccoglottis gabonensis*, *Vernonia amygdalina*, *Euphobia* sp., *Nauclea* sp., and *Rubiacae* species were used for the preservation of palm sap. In Nigeria, the aqueous extract from the bark of *Sucoglottis gabonemis* is traditionally used to inhibit the growth of yeasts and bacteria in palm sap (Ogbulie et al. 2007). The bark of this tree is also added as a preservative to a palm wine. The alkaloid and phenolic compounds extracted into the wine showed the high antimicrobial effect (Odunfa, 1985). However, Okafor (1975) reported that it only inhibited the growth of *Sarcina lutea* at 10% concentration and no other organisms were affected by using this extract.

Different fermentation inhibitors such as *Vateria copallifera*, *Careya arborea*, *Azadirachta indica*, and lime were explored for the preservation of coconut sap. It was observed that lime and *Vateria copallifera* bark at optimized concentration of 3 g L^{-1} sap proved to inhibit fermentation of palm sap more effectively. The presence of significant lower concentrations of alcohol due to the addition of lime in the sap was claimed. The bark of *Careya arborea* and leaves of *Azadirachta indica* did not show efficient inhibition of palm sap fermentation. However, the number of yeast cells was conciderably reduced (Kapilan 2015).

3.5 Antimicrobial potential of plant extracts

Certain plants and their extracts used as flavouring agents are known to possess antimicrobial activity offering a potential alternative to synthetic preservatives. In modern food industries mild processes are applied to obtain safe products which have a natural or "green" image (Kotzekidou et al. 2008). As scientific research has progressed, scientists have discovered antimicrobial activity of many plants and started to explore active principles of secondary metabolites from botanicals. As a result of their experiments a huge number of plants all over the world have been found to have antimicrobial activity (Tahamtan & Sharada 2019).

The all-biochemical activities in a plant cell are called metabolism and their products are called metabolites (Rungsung et al. 2015). Primary metabolites include carbohydrates, proteins, lipids, and nucleic acids. These organic compounds are necessary for plant growth and development. Secondary metabolites include alkaloids, saponins, flavonoids, terpenoids, glycosides, tannins etc, that are required for interaction with other plants and microorganisms (Shakya 2016).

Fruits, flowers, and seeds are usually rich in secondary metabolites, especially in annual plants. In perennial plant species, high amounts of secondary metabolites are found in bulbs, roots, rhizomes and the bark of roots and stems (Ambardar & Aeri 2013).

Plant extracts may exhibit different modes of action against the bacterial strains, such as interference with the phospholipid bilayer of the cell membrane which has therefore a permeability increase and loss of cellular constituents, damage of the enzymes involved in the production of cellular energy and synthesis of structural components, and destruction or inactivation of genetic material (Kim et al. 1995). In general, the mechanism of action is based on the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Kotzekidou et al. 2008)

Less research has focused on higher plants although identified plant compounds such as berberine, emetine, quinine, and sanguinarine still find specialised uses (Fabry et al. 1998). The concentration of secondary metabolites in trees is generally higher in bark, heartwood, roots, branch bases and wound tissue (Kawamura et al. 2010). They serve as defence agents against invading microorganisms. (Fabry et al. 1998).

3.6 Characteristics of selected Cambodian plants

3.6.1 *Azadirachta indica* Ant. Juss.

3.6.1.1 Botanical characteristics and biotope

Azadirachta indica is an evergreen 2-6 m tall tree of family Meliaceae (Dy Phon 2000). It grows with semistraight to straight trunk, extensive branching, and thick bark up to 3 m (Saleem et al. 2018).

These trees can live for up to 200 years. The bark varies much in thickness according to age and parts of tree from where it is taken; the external surface is rough, fissured, and rusty-grey. The inner is laminated with a yellowish surface. Leaves are 7-8.5 cm long and 1.0-1.7 cm wide and they have a slightly yellowish green color. Trees are often covered in delicate flowers from May to August. White and fragrant flowers are arranged axillary, looking like drooping panicles which are

up to 25 cm long. *A. indica* generates a semi-sweet, olive-sized fruit at the age of 4 years. The seed inside is rich in oil with tremendous medicinal and botanical properties (Bijauliya et al. 2018).

A. indica is naturally occuring in Pakistan, India, Bangladesh, Sri Lanka, Burma, Malaysia, Thailand, Australia, and Indonesia (Saleem et al. 2018). In Cambodia, there are two varieties: *A. indica* with denticulated leaflets and *A. integrifolia* with non-denticulated leaflets, both trees have the same usage (Dy Phon 2000).

These trees prefer sunny areas or secondary forests, but they can grow in stony and dry soils under different climatic conditions up to an altitude of 700 m. They are abundant in those regions where rainfall, temperature varied from 450 to 1,200 mm, 0 to 49 ℃, respectively (Saleem et al. 2018).

3.6.1.2 Ethnomedicinal usage

Azadirachta indica A. Juss has potential biological properties and has proved to be effective against various bacterial, fungal infections, dental disorders, skin diseases, leprosy, syphilis, malaria and has antiseptic properties (Jeba Malar et al. 2020). It shows various pharmacological attributes such antiviral, anti-inflammatory, antifeedant pesticides, antiscabic, antiallergenic, analgesic, and nematicidal effects (Paritala et al. 2015).

Leaves extracts possess hypoglycemic, hypolipidemic, hepatoprotective, antifertility, and hypotensive activities. Traditionally they are used to treat eye problems, earache, boils, and skin infections (Joshi et al. 2011). Leaf juice is used as nasal drop to treat worm infestation in the nose. Steam inhalation of bark is useful in inflammation of throat. Leaves have been applied as poultice to relieve boils, their infusion is used as an antiseptic wash to promote the healing of wounds and ulcers (Hashmat et al. 2012). A decoction of neem's leaves helps to treat nose troubles, may be used as a gargle in stomatitis and problems with gums. It is known as a good remedy for rheumatism, anorexia, blood contaminations, and even GIT worms (Joshi et al. 2011; Saleem et al. 2018)

Seeds are known for their antihelmintic, antileprotic, and antipoisonous properties. Seed oil exhibited spermicidal, antipyretic, antiartheritic, diuretic, antimalarial, and hypoglycemic effects. They have been used in cosmetics, soaps, toothpaste, and pest repellents producing. Oils obtained from the fruits and seeds are considered as vegetable oils. Traditionally, seed pulp and oil are used in leprosy and intestinal worms' treatment by folk practitioners (Saleem et al. 2018).

Flowers of the tree are used as astringent and antihelmintic agents whereas its fruit possess antihemorrhodal activity (Paritala et al. 2015). Traditionally people used fruits to cure haemorrhoids, urinary infections, and leprosy (Biswas et al. 2002). The dried flower is taken orally for diabetes. Hot aqueous extract of the dried fruit is used for piles and externally for skin disease and ulcers treatment (Hashmat et al. 2012).

Twigs are therapeutically applied as a remedy for cough relief. They are used as a cure for haemorrhoids, diabetes, tumors, spermatorrhoea, pyrexia, and urinary tract obstacles (Biswas et al. 2002; Paritala et al. 2015).

The gum obtained from the stem may help to heal wounds, scabies and ulcers when applied to skin (Biswas et al. 2002). The gum poseses tonical and stimulant properties similarly as the bark hot aqueous extracts do (Saleem et al. 2018).

The bark is used as an analgetic, alternative and curative of fever (Biswas et al. 2002). Barks and twigs effectively help to cure worm infestation, intestinal infections, stomach ache, constipation,

and dental problems. Bark of *A. indica* is applied as a cure against vomiting; it is used in treatment of respiratory disorders, and even malaria (Saleem et al. 2018).

3.6.1.3 Main phytochemical constituents

Azadirachta indica has been identified as the potential medicinal plant. 135 novel compounds were determined from different parts of the tree. Nevertheless, not all of them have been studied for its properties. *A. indica* is highly rich in secondary metabolites, which are chemically diverse and structurally complex, including isoprenoids (azadirone, protomeliacins, nimbin, azadirachtin, limonoids, gedunin) and nonisoprenoids (proteins, sulphur compounds, carbohydrates, dihydrochalcones polyphenolics, and their glycosides) (Paritala et al. 2015; Jeba Malar et al. 2020).

A. indica, just as the other members of Meliaceae, had been studied for its chemicals, especially limonoids (Paritala et al. 2015). Over 300 limonoids have been isolated and identified. They are modified trierpenes having a 4,4,8-trimethyl-17-furanyl steroid skeleton. The term limonoids was derived from limonin, the first tetranortriterpenoid obtained from citrus bitter principles (Roy & Saraf 2006). Limonoids' ring structure and chemical oxidation state parameters have insecticidal, insect antifeedant, and medicinal effects to animals and humans such as antibacterial, viral, and antifungal properties (Paritala et al. 2015).

Limonoids' possible anticarcinogenic properties are being explored. Nimbolide is found in many parts of the neem plant and was first described in the leaves and flowers. Nimbolide is a triterpene that is widely being used in treating many diseases. Some preclinical studies prove the anti-cancer activity of nimbolide and have shown it has tumorigenesis and metastasis inhibitor effects through the inhibition of tumoral cells with modulating of heat shock proteins.

Epoxyazadiradione (EAD) is an important limonoid found in *A. indica* fruits and seeds with cytotoxic activity, with a high selective cytotoxicity for several leukemia cells. EAD can induce apoptotic cell death via death-receptor-mediated pathways.

Gedunin is a tetranortriterpenoid isolated from neem seed oil with a D-lactone ring. In Indian medicine, the active product gedunin has been administered for infectious diseases such as malaria, but recent studies have shown the potential anticancer efficiency of this product against tumor cells in the ovaries, colon, and prostate through regulation of important signaling pathways. Possible antimalarial activity of gendunin (1) derivatives is creating a special interest for scientists from tropical countries. It was also proved as a compound against tumor cells in the ovaries, colon, and prostate through regulation of important signaling pathways (Moga et al. 2018).

During previous research from various plant parts of Meliacea family, including *A. indica,* tetranortriterpenoids with a modified furan ring such as febrifugin (2), methyl angolensate (3), luteolin-7-O-glucoside (4) (Fig. 1), deoxyandirobin (5) (Fig. 2) were isolated. A triterpenoid of the class of limonoids, found in the tree's nodal callus got a name after it – azadirachtin (Paritala et al. 2015; Jeba Malar et al. 2020).

In the heartwood of *A. indica* were found tetranortriterpenoids febrifugin (2), andfebrinins A and B (6) together with the flavonoids naringenin (7), quercetin (8), myricetin (9) and dihydromyricetin (10) (Paritala et al. 2015) (Fig. 2).

Flavonoids genistein-7-O-glucoside known as a highly potential antioxidant,

and (–)-epicatechin were detected in the seed oil. Among the other compounds, linolenic, linoleic, oleic, palmitic, and stearic acid were found. Lupeol and sitosterol are also important constituents of seeds.

Quercetin-3-O-l-rhamnoside and 3-O-rutinoside with proved antiinflammatory and antiulcer activities were found in the *A. indica* leaves (Paritala et al. 2015).

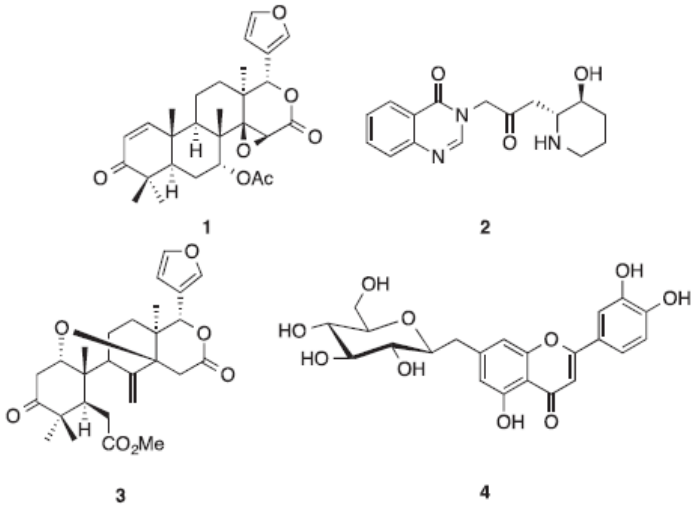


Figure 1. Chemical structures of selected limonoids (Paritala et al. 2015)

Fruits and leaves were reported to contain nimolicinol, bitter tetranortriterpenoid, that has potential pharmacological properties (Siddiqui et al. 1984).

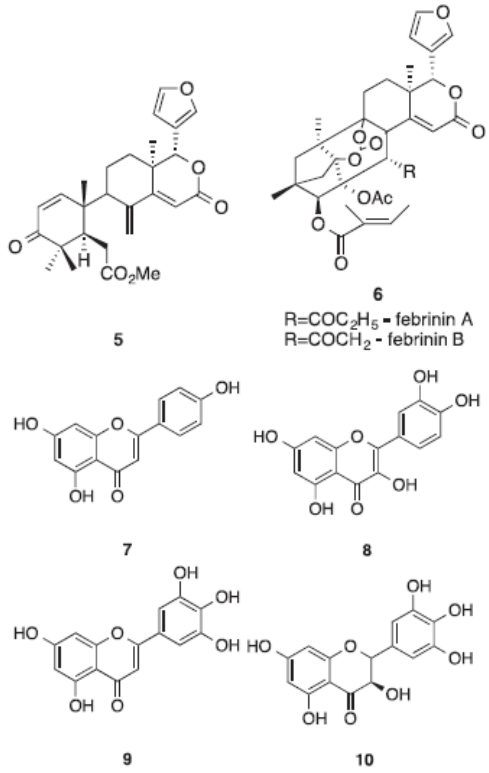


Figure 2. Chemical structures of selected limonoids (Paritala et al. 2015)

3.6.1.4 Antimicrobial activity of extracts

A. indica is a promising tree that has various biological properties and deals with multidrug resistance microbes (Paritala et al. 2015). It has antibacterial and potential anticancer activity that was reported by Moga et. all (2018).

Antibacterial activity of methanol and acetone flower extracts of *Azadirachta indica* by disk assay was tested against *Staphylococcus aureus, Listeria monocysgenes, Escherichia coli, Bacillus cereus* and *Salmonella infantis* by Alzoreky and Nakahara (2003). Buffered methanolic extract of *A. indica* has shown inhibition zones against *B. cereus* at lower doses (400 µg per disc). Acetone extracts were only active at double concentration. Authors mentioned the conclusion from another study (Longanga Otshudi et al. 2000) about the fact, that plants contained microbial inhibitors (i.e., flavonoids) soluble in aqueous methanol, and the flavonoid aglycones, may be more active than their glycosidic forms naturally present in plants (water solution) (Alzoreky & Nakahara 2003).

In the other study (Perumal Samy et al. 1998) was reported antibacterial activity of different crude extracts of seed kernel, seed coat and leaves of *Azadirachta indica*. Extracts were tested against *Escherichia coli, Pseudomonas aerogenes, Klebsiella aerogenes* and *Proteus vulgaris*. Only the seed kernel extracts showed significant antibacterial activity.

Antibacterial, antisecretory and antihemorrhagic activity of neem leaf extract was tested against multidrug-resistant *Vibrio cholerae* and reported significant antibacterial activity of the extracts (Thakurta et al. 2007).

The antibacterial activity of neem seed oils *in vitro* against fourteen strains of pathogenic bacteria using tube dilution technique was tested. More than 70% of selected organisms were inhibited at 125 µl/ml of the oil (Baswa et al. 2001).

Seed and leaf extracts of *Azadirachta indica* have shown the inhibitory activity on fungi such as *Candida albicans, C. tropicalis,* bacteria such as *Neisseria gonorrhoeae* and the multidrug resistant bacteria *Staphylococcus aureus* (Paritala et al. 2015)

The aqueous and alcoholic extracts of leaf, stem, and root of *A. indica* were effective against *E. coli, P. vulgaris, P. aeruginosa, S. aureus* and *B. subtilis*. The MIC values of the ethanolic extracts of the plant parts were lower than the respective aqueous extracts (Sharma et al. 2011)

Fabry et al. (1998) conducted an ethnopharmacological survey of *A. indica* stem bark and leaves against 105 different strains of bacteria. *A. indica* leaf extract had MIC and MBC values less than or equal to 8 mg/ml.

Antibacterial activity of *Azadirachta indica* stem bark was tested against pathogenic *Salmonella paratyphi* and *Salmonella typhi* using various solvent extracts. The *in vitro* antibacterial activity was performed by agar well diffusion method. The results were expressed as the average diameter of zone of inhibition of bacterial growth around the well. The ethanolic and methanolic extracts showed better antibacterial activity with zone of inhibition (20–25 mm) when compared with other tested extracts and standard antibiotic erythromycin (15 mcg) with zone of inhibition 13–14 mm (Al Akeel et al. 2017).

The bark extract of *A. indica* was screened for antibacterial activity by agar well diffusion assay technique against *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa,* and *Proteus mirabilis*. Neem's bark extract showed zone of clearance against *E. faecalis, P. aeruginosa,* and *P. mirabilis* at all concentrations (500-2000 µg/ml), whereas

antibacterial activity against *S. aureus* was observed only at higher concentration of >500 µg/mL (Singaravelu et al. 2019).

3.6.2 *Careya arborea* Roxb.

3.6.2.1 Botanical characteristics and biotop

Careya arborea Roxb. is an evergreen tree in Lecythidaceae family. It is usually a 7 to 15 m tall plant, with spreading branches and leaves up to 30 cm long and 7.5-15 cm wide (Dy Phon 2000). The bark has a dark gray color and exfoliates in thin strips. The tree has large white-pink or yellowish white flowers up to 9 cm across. Fruits are 6.3-7.5 cm in diameter, crowned with the calyx limb (Ambardar & Aeri 2013).

It is distributed in tropical regions like India, Sri Lanka, Malaysia, Thailand, Vietnam, Cambodia, and Laos in deciduous forests and grasslands (Gupta et al. 2019).

3.6.2.2 Ethnomedicinal usage

Careya arborea Roxb. is a significant medicinal plant known as 'kumbhiin' in Ayurveda. All parts of this tree have medicinal properties and have been used in traditional medicine of South Asia.

The plant is traditionally used in treatment of sores, inflammation, piles, cough and cold, toothache, wounds, colic, intestinal worms, hemorrhoids, spermatorrhoea, leukoderma, abscesses, ulcers, and smallpox (Gupta et al. 2019).

The stem bark is used in the treatment of tumors, skin diseases, bronchitis, and epileptic fits (Ambardar & Aeri 2013). It is commonly known as an effective remedy for diarrhoea and dysentery with bloody stool. According to Sambath Kumar review (2005) *Careya arborea* bark is also used for ear pain treatment and as antipyretic. The antioxidant and its *in vivo* hepatoprotective activity were also reported (Senthilkumar et al. 2007). In Kolli Hills of Tamilnadu, India local tribal people use the stem bark for the treatment of liver disorders.

There are mentions about using *Careya arborea* as an antidote to snake venom, a leech repellent, and fish poison in the scientific literature (Ambardar & Aeri 2013).

3.6.2.3 Main phytochemical constituents

Qualitative HPTLC phytochemical profile of *C. arborea* Roxb. bark, leaves, and seeds made by Gupta et al. (2019) shows the medicinal potential of the plant. The various phytochemicals present in bark, leaves and seeds of *C. arborea* namely alkaloids, anthracene derivatives, arbutin derivatives, bitter compounds, cardiac glycosides, coumarin derivatives, essential oils, flavonoids, lignans, pungent-tasting principles, saponins, triterpenes and valepotriates might be responsible for its pharmacological activities like antimicrobial, antioxidant, analgesic, antitumor, hepatoprotective, antidiarrhoeal, anticoagulant and diuretic.

Flowers of *C. arborea* contain triterpenoids, steroids, and tannins. Fruits have a high amount of phenolic compounds like a gallic acid, 3,4-dihydroxybenzoic acid, quercetin 3-O-glucopyranoside, kaempferol 3-O-glucopyranoside and qurcetin 3-O-(6-O-glucopyranosyl)-glucopyranoside isolated from methanol, n-hexane, ethyl acetate and dichloromethane extracts of *C. arborea* fruit (Khaliq

2016). Strong cytotoxic and antioxidant properties were determined due to high total phenol content present in the plant. The methanol and aqueous extracts possessed strong antioxidant activity against many oxidants in the *in vitro* antioxidant screening. The extracts exhibited potent cytotoxicity against cancerous RD, Hep-2 and HeLa cell lines and were found to be safe against the normal Vero cell line. (Ambardar & Aeri 2013).

Seeds of *C. arborea* are reported to possess starch, α-spinasterone, α-spinasterol and triterpenoids like desoxy barringtogenol C, barringtogenol C and barringtogenol D.

Acid hydrolysis of leaf ethanolic extract produced a triterpenoid lactone careyagenolide, maslinic acid and 2α-hydroxyursolic acid. A triterpenoid saponin arborenin and desacylescin III were isolated from methanolic extract. Other reported constituents are taraxerol, ellagic acid, n-hexacosanol, taraxerol acetate, quercitin and β-sitosterol, careaborin and tannins (Khaliq 2016).

Stem bark of *C. arborea* contains bioactive constituents including betulinic acid, betulin, and lupeol that have been reported as potential anti-inflammatory agents. Quercetin, known as a dietary-derived flavonoid, has an antioxidant activity (Begum et al. 2014).

Bark of *C. arborea* contains piperine, an alkaloid chemically known as 1-5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienylpiperidine that showed the significant central and peripheral analgesic activity on test mice. At oral doses of 10, 20 and 30 mg/kg body weight, piperine exhibited inhibition of acetic acid induced writhing in mice respectively (Ahmed et al. 2002).

3.6.2.4 Antimicrobial activity of extracts

Methanolic *C. arborea* extracts can be a potential source of natural antimicrobial and antioxidant agents (Kumar et al., 2006). Antibacterial activity of methanolic *C. arborea* bark extract against *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Vibrio cholera, Shigella dysenteriae, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumonia* and *Micrococcus luteus* has been reported. Methanolic extract of the leaf were effectively tested on *Staphylococcus aureus, Enterococcus faecalis, Citrobacter freundii, Acinetobacter baumannii, Escherichia coli, Enterobacter aerogenes, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa and Proteus vulgaris.*

Ethanolic and ethyl acetate fruit extracts have shown antibacterial activity against *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes* and *Staphylococcus epidermidis*. Leaf ethanolic extracts have revealed antibacterial activity against *Escherichia coli* isolated from patients with urinary tract infections. The antibacterial activity of ethanolic leaf extract against *Streptococcus pyogenes, Escherichia coli, Salmonella typhimurium, Bacillus cereus, Pseudomonas aeruginosa* and *Zymomonas mobilis* has been reported.

Antifungal activity of *C. arborea* methanolic bark extract against *Candida albicans, Aspergillus flavus, Aspergillus niger* and *Alternaria solani* was revealed (Khaliq 2016).

3.6.3 *Hopea odorata* Roxb.

3.6.3.1 Botanical characteristics and biotope

Hopea odorata Roxb. is an evergreen 20-35 m tall tree of family Dipterocarpaceae (Dy Phon 2000). The wood of *H. odorata* varies in color from a very pale yellow, or white to brown when first

cut and characteristically darkens to a brownish or yellowish-brown color after exposure to the air (Satiraphan et al. 2012).

The tree is characterized by a large crown growing to 45 m tall. Bole is straight, cylindrical, branchless wide to 25 m, with diameter of up to 4.5 m or more and prominent buttresses. The bark surface is scaly, grey to dark brown, yellow, or reddish inside. The leaves are ovate-lanceolate, 7-14 cm long and 3-7 cm wide. Yellowish white flowers are small with a sweet scent, very shortly pedicelled. Fruits are small, ovoid, rounded, 3-4 cm long (Orwa et al. 2009).

It is cultivated as an avenue and shade tree, but is widely spread in dense forests of Indochinese, Malay Peninsula, and Andaman Islands (Dy Phon 2000). *H. odorata* is usually occurring on deep rich soils, most commonly along the banks of streams and up to 600-m altitude (Orwa et al. 2009).

3.6.3.2 Ethnomedicinal usage

Different parts of *H. odorata* have been used as one of the most important traditional medicinal sources in the Indian subcontinent for long time. A plant commonly used as an antiinflammation remedy, also as treatment of yaws, and sores (Kabir et al. 2016). The wood has been used for treatment of blood disorders, fever, and as an expectorant. Its dried stem latex was ground and used for wound healing (Kabir et al. 2015). The stem bark of *H. odorata* has been traditionally used on Indian Andaman Islands to treat neck pains (Kabir et al. 2016). The tannin rich bark and leaves have been used for treating paralysis, haemorrhoids, diarrhea, and urinary incontinence (Prasad et al. 2008).

Bark of this plant is also a part of a remedy for the treatment of gums inflamations and incontinence (Dy Phon 2000). In Indochina, the bark is used as a masticatory (Orwa et al. 2009). A few years ago, Kabir et al (2015) found out that methanolic bark extract of *H. odorata* has selective *in vitro* cytotoxicity against HepG2 tumor cells. It can induce HepG2 cells into caspase-dependent apoptosis. Thus, *H. odorata* bark extract may have great potential as a therapeutic agent.

3.6.3.3 Phytochemical constituents

Phytochemical studies reported that the heartwood of *H. odorata* contains certain types of phenolic compounds, namely resveratrol oligomers like hopeaphenol, balanocarpol, ampelopsin H and hemlesyanol C (Scalbert et al. 2005). These polyphenols are reported useful as antioxidants, antibacterial, anti-inflammatory, antimutagens, scavengers of free radicals and therefore have implications in the prevention of pathologies such as cancer and cardiovascular disease (Kabir et al. 2015). Another study revealed that *H. odorata* may act as an antioxidant agent due to its H_2O_2 scavenging and thrombolytic activity (Kabir et al. 2016)

Flavonoids, namely quercetin, kaempferol, apigenin and quercetin 3-glucoside were found in the leaves (Satiraphan et al. 2012). A high amount of flavonoids in leaves was reported to exhibit moderate antibacterial activity (Kabir et al. 2015). Leaves of *H. odorata* also showed anthelmintic acitivity (Havsteen 2002).

Chromatographic fractionation of the n-hexane extract of *Hopea odorata* leaves led to the isolation of eight lupane triterpenes, including 3,30-dioxolup-20(29)-en-28-oic acid that was isolated for the first time from a natural source (Satiraphan et al. 2012).

The leaves, bark and wood were reported to contain 11, 13-15, and 10% tannin respectively (Orwa et al. 2009).

3.6.3.4 Antimicrobial activity of extracts

The antibacterial effect of *Hopea odorata* leaf extracts was analyzed in the disc diffusion technique. Methanol leaf extract showed the highest activity against *Staphylococcus. aureus, Bacillus subtilis, Salmonella typhi*, and *Shigella dysenteriae*. Ethanolic extract was active against *Bacillus megaterium* and *Escherichia coli.* Authors mentioned that a narrow zone of inhibition was found against *E. coli* compared to others activity (Kabir et al. 2015)

The fungal growth inhibitory activities of *H. odorata* heartwood and sapwood were tested and showed weak antifungal activities against *Gloeophyllum trabeum* and *Pycnoporus sanguineus* (Kawamura et al. 2010).

3.6.4 *Hopea pierrei* Hance

3.6.4.1 Botanical characteristics and biotope

Hopea pierrei Hance is a 15-30 m long evergreen tree with a spherical crown of Dipterocarpaceae family (Dy Phon 2000). The bole is sometimes straight but is often twisted and of poor shape; it is usually 50-80cm in diameter, occasionally up to 180 cm; and with stilt roots or 4-5 thin buttresses (Fern 2014). The dark grey leaves are eggshaped and coated with a white powder (Pumijumnong et al. 2019).

It is widely spreaded in the dense coastal forests of Cambodia, Laos, Vietnam, Thailand, and the Malay Peninsula (Dy Phon 2000). Populations in Cambodia are seriously reduced by past chemical warfare, exploitation, and habitat reduction (Fern 2014).

3.6.4.2 Traditional usage

The tree yields a high-quality timber and so is often harvested from the wild. Its timber is used for creating the constructions for items such as posts; beams, joists, rafters; flooring, sheathing and ceilings; it is also used for masts and spars; ship planking and decking; doors; moldings; pestle shafts in rice mills; carriage panels; furniture and cabinetwork; carpenter's sawframes. Because of its smooth straight grain and toughness, it should make excellent broom, rake, and hoe handles (Fern 2014). The smooth, light brown bark is used to make circles, ropes, and papers (Dy Phon 2000).

A pale-yellow resin obtained from the tree is used for torches and for caulking boats. Dried and powdered, it is thrown upon burning charcoal to give a representation of gunfire in theatrical performances (Fern 2014).

3.6.4.3 Ethnomedicinal usage

H. pierrei has local medicinal use. The bark contains tannins and is astringent (Fern 2014). Tannins are medicinally used as an antidiarrheal, haemostatic, and antihemorrhoidal remedy. They heal burns and stop bleeding. Tannins can form a protective layer over the exposed tissue and keep

the wound from infections. There are mentions in Ayurveda about the use of tannin bearing materials in tooth powders to strengthen gums (Minocha et al. 2015).

Any other mentions about ethnomedicinal or ethnofamacological properties of the tree were not found. However, the usage of *H. pierrei* in ethnomedicine may be like described above. The relation of plant to the same genus, as *Hopea odorata* may lead to the same conclusion. The lack of data about *H. pierrei* Hance leaves a wide field for further research.

3.6.5 *Shorea roxburghii* G. Don

3.6.5.1 Botanical characteristics and biotope

Shorea roxburghii is a semievergreen 20-25 m tall tree from Dipterocarpaceae family (Dy Phon 2000). It has straight, cylindrical bole up to 95 cm in diameter, with either small or no buttresses (Fern 2014).

The tree is widely distributed in Thailand, Cambodia, India, Laos, Malaysia, Myanmar, and Vietnam (Morikawa et al. 2012). It is present in teak and deciduous forests as well as in semievergreen forests and on limestone (Fern 2014).

3.6.5.2 Ethnomedicinal usage

Shorea roxburghii bark has been used for treatments of dysentery, diarrhea, and cholera in Indian folk medicine (Morikawa et al. 2012). The bark is also known as a masticatory. It is a good source of tannins when it is chewed with betel nuts. The plant's bark has been used for an astringent and as a preservative for traditional beverages in Thailand (Morikawa et al. 2012).

The dried flowers, combined with various other flowers, are used in the treatment of heart conditions and fevers. Young flowers are cooked as a source of vitamins (Fern 2014).

3.6.5.3 Phytochemical constituents

Shorea genus has been known to be a source of oligostilbenoids. These compounds from a family of polyphenols are known for their complexity of structures and biological activities such as cytotoxic, antibacterial, and antioxidant.

During the chemical study of *S. roxburghii* were revealed several stilbenoids, including resveratrol and its oligomers (Moriyama et al. 2016). Trans-resveratrol (3,5,40-trihydroxy-trans-stilbene) has been reported to have anti-aging properties and beneficial health effects in patients with cancer, cardiovascular, inflammatory, and central nervous system diseases (Morikawa et al. 2012).

From *S. roxburghii* methanolic bark extract were isolated 13 stilbenoids, namely (−)-hopeaphenol (1), (+)-isohopeaphenol (2), hemsleyanol D (3), (−)-ampelopsin H (4), vaticanols A (5), E (6), and G (7), (+)-α-viniferin (8), pauciflorol A (9), hopeafuran (10), (−)-balanocarpol (11), (−)-ampelopsin A (12), and trans-resveratrol 10-C-β-D-glucopyranoside (13), and two dihydroisocoumarins, phayomphenols A1 (14) and A2 (15) (Fig. 3).

The methanolic extract from the bark of *S. roxburghii* was found to inhibit plasma triglyceride elevation in olive oil treated mice, and to inhibit pancreatic lipase activity. For the most

part, these activities are primarily due to phayomphenols A1 (14) and A2 (15), that are called 3-acetyl-4-phenyl-3,4-dihydroisocoumarins (Fig. 3) (Ninomiya et al. 2017).

Hopeaphenol (1) isolated from a stem bark extract of *S. roxburghii* has potent antioxidant properties. It can be explained by its complex structure with many hydroxyl groups, which can donate hydrogen or electrons to free radicals and neutralize them. Hopeaphenol (1) has great potential for further use as an antioxidant in foods and pharmaceuticals (Subramanian et al. 2018)

Five known resveratrol oligomers, namely diptoindonesin D, hopeafuran (10), α-viniferan (8), hopeahainol A and hopeahainol C, were isolated from stem bark of *S. roxburghii*. Diptoindonesin D was reported to have an antibacterial activity (Sudto et al. 2019).

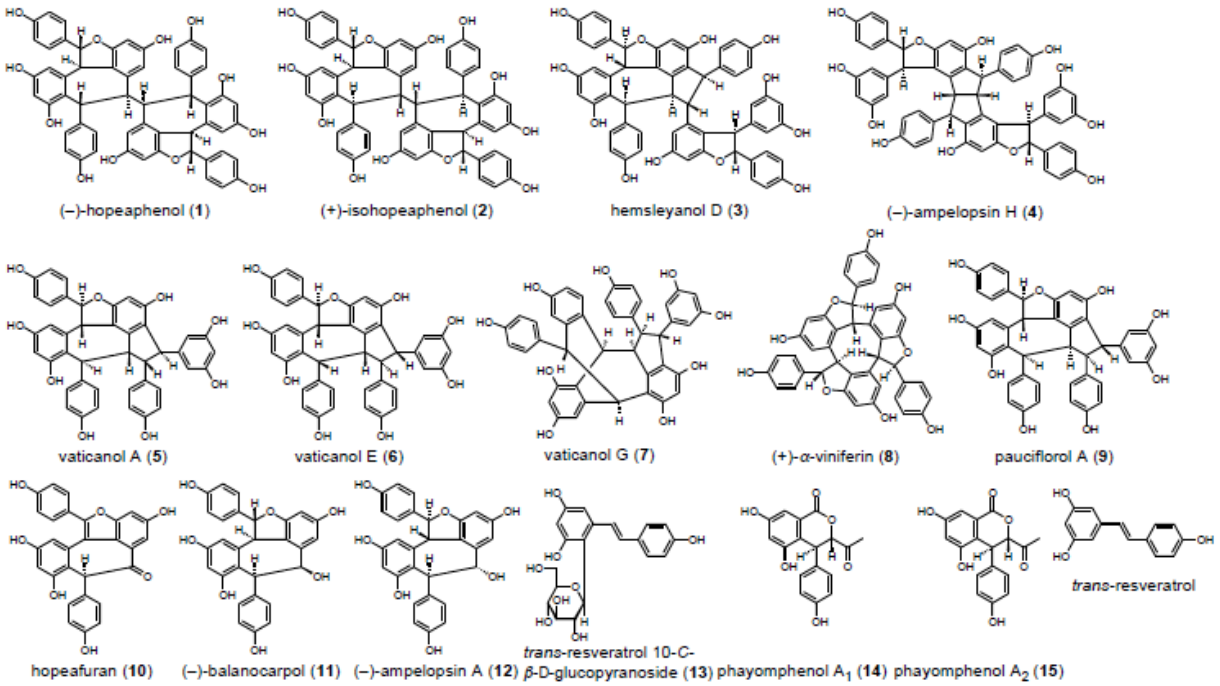


Figure 3. Stilbenoids and dihydroisocoumarins (1-15) from the bark of *Shorea roxburghii* (Ninomiya et al. 2017)

3.6.5.4 Antimicrobial activity of extracts

Diptoindonesin D isolated from stem bark of *S. roxburghii* was reported to possess significant minimum inhibitory concentrations against *Staphylococcus epidermis*, *Staphylococcus aureus* and *Bacillus cereus* (Sudto et al. 2019).

Ethyl acetate, ethanol, methanol, and aqueous extracts of *Shorea roxburghii* G.Don bark showed the inhibition of *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 19113, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19115, and *Vibrio parahaemolyticus* ATCC 17802 growth. Antibacterial activity was tested by disc diffusion method, MIC and MBC values were evaluated by broth dilution method. Only the aqueous extract was the exception and did not show antibacterial activity against *E. coli* ATCC 25922 and *S. typhimurium* ATCC 19113. The ethyl acetate extract showed the highest antibacterial activity against all the tested pathogens (Assavasirijinda 2010).

3.7 Selected methods for MIC and MBC evaluation *in vitro*

Testing of antimicrobial activity can be used for drug discovery, epidemiology, prediction of therapeutic outcome, and for *in vitro* investigation of extracts and pure drugs as potential antimicrobial agents (Balouiri et al. 2016).

From the range spectrum of antimicrobial susceptibility testing of extracts as potential antimicrobial agents and determination of their minimal inhibitory concentration (MIC) dilution methods were chosen for the experimental part of this work and described below.

Dilution methods allow estimation of the concentration of the tested antimicrobial agent in the agar or broth medium. They are considered as the most appropriate ones for MIC values determination. These methods approve to measure *in vitro* antimicrobial activity against bacteria and fungi in agar and broth with the same efficiency. MIC value is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the tested microorganism, expressed in mg/mL or mg/L (Pfaller et al. 2004).

For the procedure are needed two-fold dilutions of the agent with antimicrobial properties in a liquid growth medium dispensed in tubes with 2 mL volume for microdilution or with smaller volumes using 96-well microtitration plate for a microdilution method. Each tube or well is furtherly inoculated with a microbial inoculum in the same medium after standardization adjusted to 0.5 McFarland scale. Well-mixed tubes or microtitration plates are incubated under suitable condition for the tested microorganism (Cockerill 2012).

The major advantage of microdilution methods is miniature size that approves to minimize the amount of used reagents and storing place and reasonably increase reproducibility. There is also assistance in generating computerized reports if an automated reader is used. Reading devices may be helpful for the determination of MIC endpoint. They can facilitate reading and recording results with high ability to detect growth in every well (Jorgensen & Ferraro 2009).

Moreover, several colorimetric methods based on dye reagents have been developed and widely used. Amongst the dyes for MIC endpoint determination for antifungal and antibacterial microdilution assays are tetrazolium salts, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis {2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2H-tetrazolium-hydroxide} (XTT). Resazurin or the Alamar blue dye can also be used for this purpose as an effective growth indicator.

It is important to understand that many factors can affect the MIC results, including inoculum size, growth medium type, incubation period, and preparation method. Therefore, broth dilution has been standardized by CLSI and EUCAST (Jorgensen & Ferraro 2009; Balouiri et al. 2016).

The most common estimation of bactericidal or fungicidal activity is the determination of minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC). The MBC is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the final inoculum after incubation for 24 h under a standardized set of conditions. It can be determined after broth macrodilution or microdilution by subculturing a sample from wells or tubes, yielding a negative microbial growth after incubation on the surface of nonselective agar plates to determine the number of surviving cells (CFU/mL) after 24 h of incubation. The bactericidal endpoint (MBC) has been subjectively defined as the lowest concentration, at which 99.9% of the final inoculum is killed. MFC is also defined as the lowest concentration of the drug that yields 98%–99.9% killing effect as compared to the initial inoculum (Balouiri et al. 2016).

3.8 MALDI-TOF MS

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is nowadays one of the most useful phenotypic method for microbial identification. It is a reliable and accurate technique that can be used for identification of pathogens from blood samples or directly from patient samples, such as urine or blood. It has proven to be a simple and rapid tool for characterization and quantification of small and large biomolecules. MALDI-TOF MS gained the attention in molecular biology due to highly sensitive and fast bacteria, yeasts, molds, and viruses' identification. This technique allows detection of multiple microorganisms from biological origins, cells, and tissues (Marvin et al. 2003; Kailasa et al. 2020)

MALDI-TOF MS works on the principle of cocrystallized sample material ionization by short laser pulses. It is a very sensitive technique, so it is enough to use a small amount of microbial biomass for analysis. Whereupon a concentration of at least 10^5–10^7 CFU/ml is achieved, there are enough proteins to generate suitable profiles in MALDI-TOF MS (Correa-Martínez et al. 2019).

The sample is spotted onto a MALDI-TOF sample target with an appropriate matrix, then dried at room temperature. After the plate is placed into the MS and bombarded with a laser. It allows to create ions in a gas phase that pulsed into a vacuum flight tube, where accelerated ions time of flight is measured. Ionization occurs under high vacuum conditions ($1 \cdot 10^{-6}$ mbar) (Carbonnelle et al. 2011). The molecules ionization is caused by a strong ability of the organic matrix to absorb the laser energy (Kailasa et al. 2020).

The ionization allows separation of ions according to their molecular weight after migration in an electric field. Every molecule that was detected is characterized by the molecular mass (m), the charge (z), the ratio mass/charge (m/z), and the relative intensity of the signal.

Collected spectra are automatically compared with a reference databank containing a wide variety of isolates. The software compares the spectra and generates a score value based on the similarities between the analysed and stored data sets. This numerical value provides information about the validity of the identification. If a score is above 2.0, then it is a valid species level identification. Reliable genus level identification is provided with values between 2.0 and 1.7 (Sauer et al. 2008).

Spectral fingerprints vary between microorganisms: some peaks can be related with genus identification; others are specific to species and subspecies. Mass spectra can be different even if the species are the same. It depends on the growth conditions and selected extraction methods. Therefore, if growth conditions are well-controlled and samples are prepared up to the same standard, mass spectra are reproducible (Marvin et al. 2003).

MALDI-TOF MS is used in different fields as molecular biology, pharmaceutical research, and biotechnology. It provides highly accurate results of determination and identification of peptide sequences in biological samples. It is possible to detect a unique spectral fingerprint of the microorganism within minutes and identify not even different genera, but different strains from the same species (Carbonnelle et al. 2011). The size range for species level identification between 2 and 20 kDa is seemed to be the most stable and provides a strong signal to noise ratio. That size corresponds to ribosomal proteins molecular weight. RNA proteins ionize well, provide accurate spectra, and are only minimally affected by microbial growth conditions (Sauer et al. 2008).

3.9 GC-FID: Gas Chromatography with Flame Ionization Detection

The most employed techniques for the quantification of ethanol in a variety of products include densitometry, liquid chromatography with various detectors, and gas chromatography in conjunction with various detectors (Rollman et al. 2021).

The analysis of alcohol contained beverages benefit from chromatographic separation due to reduction of interferences and overlapping peaks. (Rollman et al. 2021).

During the chromatography analysis the components of a mixture are separated based on their differential interactions with two chemical or physical phases: a mobile phase and a stationary phase that is held in place by a supporting material.

During the operation of a FID, the carrier gas that is leaving the column is mixed with hydrogen, and the eluting compounds are burned by a flame that is surrounded by air and an oxygen-rich environment. Approximately one organic molecule in 10,000 results in the production of a gas-phase ion. These ions are detected by a collector electrode that is positioned above the flame. The magnitude of the current that is generated by these ions is related to the mass of carbon that was delivered to the detector. This signal can then be used for both the detection and quantification of organic compounds that are eluting from the column (Hage 2018).

The integration of the signals, which are collected by the detector in a measure related with the amount of each detected analyte creates a chromatogram. Besides, important information such as peak number/area/percentages, as well as their respective retention times, can be obtained from it (Vergilio et al. 2014).

4 Material and methods

Plant bark samples of *A. indica, C. arborea, H. odorata, H. pierrei, S. roxburghii* were collected in Cambodia in 2017 by Ing. Pavel Nový, Ph.D.

4.1 Chemicals

For the isolation of microorganisms from fruits were used: glycerol (Penta, Prague, Czech Republic) and Sabouraud Dextrose Agar (SDA) (Oxoid LTD., Basingstoke, United Kingdom). For their identification with MALDI-TOF MS were used: acetonitril (Sigma-Aldrich; Prague, Czech Republic), ethanol 70% (Penta, Prague, Czech Republic), formic acid (Sigma-Aldrich; Prague, Czech Republic).

The following media were used for the cultivation of microorganisms: Medium 10 (M10), Mueller-Hinton Agar (MHA), Mueller-Hinton broth (MHB), Sabouraud Dextrose Agar (SDA), M.R.S. Agar, Sabouraud Dextrose Broth (SDB), Standard Plate Count Agar (APHA), Yeast Mold Agar (YM), Yeast Mold Broth (YM) (all obtained from Oxoid, Basingstoke, United Kingdom).

Ethanol 70% (Penta, Prague, Czech Republic) and distilled water were used for bark extracts preparation. Dimethyl sulfoxid (DMSO) (VWR; Stříbrná Skalice, Czech Republic) was further used as an extract solvent.

Mueller-Hinton Agar (MHA), Mueller-Hinton Broth (MHB), Sabouraud Dextrose Broth (SDB), Sabouraud Dextrose Agar (SDA) (all obtained from Oxoid, Basingstoke, United Kingdom), and RPMI 1640 medium (Sigma-Aldrich; Prague, Czech Republic) were selected as culture media for antimicrobial activity tests.

Sodium chloride (Dorapis; Prague, Czech Republic), TrisBase (tris(hydrohymethyl)aminomethane) (Roth; Karlsruhe, Germany), Potassium chloride (KCl) (Penta; Prague, Czech Republic) and MOPS (VWR; Stříbrná Skalice, Czech Republic) were used as the buffering agents for media preparation. Hydrochloric acid (Lach-Ner; Neratovice, Czech Republic) and Sodium hydroxide (Lach-Ner; Neratovice, Czech Republic) were used to adjust the pH of the culture media.

Tioconazole (Sigma-Aldrich; Prague, Czech Republic) and Tetracycline (Sigma-Aldrich; Prague, Czech Republic) were selected as reference antibiotics. Thiazol blue tetrazolium bromide (MTT) (Sigma-Aldrich; Prague, Czech Republic) was used to evaluate antimicrobial activity.

4.2 Bark extracts preparation.

Although selected plants' barks are used by Cambodian natives as a natural preservative for palm saps, the ethanol has been chosen as an efficient solvent for the comparison with equivalent aqueous extracts antimicrobial activivity. The bark of *A. indica, C. arborea, H odorata, H. pierrei* and *S. roxburghii* was peeled from the stem using a sharp knife and chopped into pieces. Each dried bark sample was finely ground using laboratory grinder (IKA A11 basic). 10 g of ground plant material was extracted in 300 mL of 70% ethanol or distilled water for 24 h at room temperature using laboratory shaker (GFL 3006, Burgwedel, Germany). Ethanolic extracts were subsequently filtered, centrifuged (Hettich, Stockholm, Sweden) at 9,000 RPM for 5 minutes, filtered through membrane

microfilters (ø 0.45 µm) and concentrated using rotary evaporator (Heidolph, Schwabach, Germany), in vacuo at 40 °C. Aqueous extracts were subsequently concentrated in a freeze dryer.

Dried residues were finally diluted in 100% dimethylsulfoxide (DMSO) to obtain a stock solution, dissolved with use of a sonicator (Logicultrasonic PE, India) and stored at -20 °C until further use.

A. indica, C. arborea, H. odorata, H. pierrei ethanolic extracts were earlier prepared by Ing. Veronika Paťhová (Paťhová 2020) and were used for further testing of antimicrobial activity on other microorganisms in this MSc thesis.

The results of dried residue yields (%) of prepared extracts are shown in Table 1 and were calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Dry weight of extract (g)}}{\text{Dry weight of plant material (g)}} \cdot 100$$

Table 1. Yield (%) of tested extracts

Latin name [Family]	Extract solvent	Extract yield (%)
Shorea roxburghiana G. Don (Dipterocarpaceae)	ethanol	8.2
Hopea pierrei Hance (Dipterocarpaceae)	water	8.5
Hopea odorata Roxb. (Dipterocarpaceae)	water	11.8
Careya arborea Roxb. (Lecythidaceae)	water	7.0
Azadirachta indica A. Juss. (Meliaceae)	water	3.2
Shorea roxburghiana G. Don (Dipterocarpaceae)	water	4.2

4.3 Microorganisms and media

Antimicrobial activity was evaluated against 9 bacterial strains and 13 yeasts that were obtained from the American Type Culture Collection, the German Resource Centre for Biological Material (Braunschweig, Germany) and two of microbes were isolated directly from fruits (see chapter 4.5).

The sensitivity of tested bacteria to a standard antibiotic tetracycline (Sigma-Aldrich, Prague, Czech Republic) was checked as a positive control. In the case of yeast, tioconazole (Sigma-Aldrich, Prague, Czech Republic) was checked as a positive control. 96,5% ethanol was used as a dissolvent for tetracycline and DMSO was used as a dissolvent for tioconazole.

4.3.1 Bacteria

Bacterial strains were selected from both Gram-positive and Gram-negative bacteria. Following standard strains of the ATCC were used: *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphyloccocus aureus* ATCC 29213. Following standard strains of the DSM were used: *Leuconostoc palmae* DSM 21144, *Zymobacter palmae* DSM 10491, *Zymomonas mobilis* subsp. *pomaceae* DSM 3580. Two strains of *Pantoea agglomerans* isolated from plums (S1-b) and apples (J1) were also used for microdilution tests.

4.3.2 Yeasts

Standard yeasts of the DSM were used, namely *Hanseniaspora jakobsenii* DSM 26339, *Hanseniaspora uvarum* 26650, *Metschnikowia pulcherrima* DSM 70336, *Saccharomyces cerevisiae* DSM 70465, *Saccharomyces cerevisiae* 70478, *Saccharomyces cerevisiae* 70868. *Candida albicans* ATCC 10231 was a standard yeast strain of the ATCC.

In addition to standard strains, 6 yeast strains of *Hanseniaspora uvarum* and *Metschnikowia pulcherrima* isolated from various fruits (Table 2) were also tested.

4.4 Inoculum preparation

5 mL of pure sterile MHB were inoculated by 20 µL (2-3 drops) of microbial stock culture and incubated for 24 hours at constant temperature (37°C) in thermostat. For *Zymomonas mobilis* subsp. *pomaceae* DSM 3580 and for all the yeast strains SDB was used. Before testing, the overnight culture was suspended in 10 mL of pure broth and turbidity of suspension was adjusted to 0.5 McFarland using Densi-La-Meter II (Lachema, Brno, Czech Republic) units. This suspension was subsequently used to inoculate microtiter plates using multiblot replicator to reach the initial inoculum concentration of 5 x 10^5 CFU/mL and 2 x 10^3 CFU/mL for bacteria and yeasts, respectively.

4.5 Microorganisms' isolation and identification

Yeasts and bacteria on the fruits surface can cause the fermentation processes as primary contaminants. During the search for the natural microbiological contaminants of palm sap, it was decided to isolate microorganisms from the selected fruits: apple, blackberry, and grape. The microorganisms previously isolated by Ing. Veronika Páťhová were also furtherly tested (Paťhová 2020). The isolated microorganisms are listed in Table 2.

Table 2. Microorganisms isolated from fruits surface

Isolated microorganism	Code	Fruit	Specification
Hanseniaspora uvarum	OF1	Blackberry	Yeast
Hanseniaspora uvarum	VF1	Grape	Yeast
Pantoea agglomerans	J1	Apple	G- bacterium
Metschnikowia pulcherrima	V2	Cherry	Yeast
Hanseniaspora uvarum	T1	Wild cherry	Yeast
Hanseniaspora spp.	T5		Yeast
Metschnikowia spp	T9		Yeast
Pantoea agglomerans	S1-b	Plum	G- bacterium

H. uvarum strains OF1 and VF1 isolated by P. Cherepanova, remaining strains isolated by V. Paťhova

4.5.1 Isolation of microorganisms from selected fruits

Fruit pulps were squeezed into a beaker with sterilized physiological saline solution and properly stirred. Then a solution was diluted 10-fold and 300 µl of the solution were transferred to a Petri dish with SDA medium. The inoculated plates were incubated at 25 °C in a thermostat. Subsequently, the procedure was repeated until individual colonies were obtained. In the case of colonies with different morphological signs, the colonies were subcultured several times to obtain pure cultures.

Pure, well-developed colonies from freshly grown culture were transferred using a sterile loop into a microtube with 1 ml of solution (yeast: 50% glycerol solution in distilled water, bacteria: 25% glycerol solution in distilled water). The prepared solutions were vortexed and frozen for storage at -80 °C. Also, fresh colonies were transferred to 15 ml plastic tubes with MHB and henceforward were used for the antimicrobial assay.

4.5.2 Identification of isolated microorganisms with MALDI-TOF MS Biotyper

Bacteria and yeasts were identified by analysis of ribosomal proteins using MALDI-TOF MS (Bruker Daltonik GmbH, Leipzig, Germany) with automatic matrix movement. Samples' preparation and MALDI-TOF MS analyses were done according to the manufacturer's instructions.

The spectra were measured automatically using FlexControl software version 3.4. The data obtained from each isolate were imported into BioTyper software version 2.0 (Bruker Daltonik GmbH, Leipzig, Germany) and analyzed for the microorganisms' identification.

4.5.3 Samples' preparation: extraction with ethanol and formic acid

300 µl of distilled water was pipetted into a clean Eppendorf microtube (1.5 ml). Microorganism's fresh colony was transferred from the Petri dish into a microtube with distilled water and the solution was mixed with a micropipette. Then, 900 µl of ethanol was added, and the solution was mixed by pipetting and vortexing (1 min).

Then the solution was centrifuged at maximum speed for 2 minutes and the supernatant was outpoured. The sample was centrifuged again and the ethanol from the microtube's walls was released to the bottom of the Eppendorf microtube. The remaining ethanol was carefully removed by pipetting and the remaining pellet was allowed to dry for several minutes at room temperature.

40 µl of 70 % formic acid (prepared from 300 µl distilled water and 700 µl of 100 % formic acid) was added to the pellet. The solution was mixed by pipetting and then vortexing. Then, 40 µl of 100 % acetonitrile was pipetted into the solution and mixed again. As the next step, the sample was centrifuged (9,000 RPM, 2 minutes). 1 µl of the supernatant was added dropwise to a MALDI plate. After the supernatant had dried, 1 µl of MALDI matrix solution was added. After drying, the MALDI plate was ready for classification.

The plate was then inserted into MALDI-TOF MS and the analysis was performed.

4.6 Antimicrobial assay

4.6.1 Preparation of culture media

MHB and SDB were used for the bacteria testing, RPMI and SDB were used for the yeasts testing.

MHB for bacterial inoculants was prepared according to the manufacturer's instructions: 21 g of medium was dissolved in 1 L of distilled water and stirred on a magnetic stirrer. MHB buffer for microdilution tests was prepared as it was described below; with using a buffer instead of distilled water: 8 g of NaCl, 6,1 g of TrisBase and 0,2 g of KCl were dissolved in distilled water and properly stirred. Medium's pH was adjusted to 7.6 with HCl. Both solutions were sterilised in an autoclave at 121°C for 20 minutes.

SDB for yeasts inoculants was prepared according to the manufacturer's instructions: 30 g was dissolved in 1 L of water; the solution was stirred and sterilised in an autoclave at 121°C for 20 minutes.

RPMI medium was used for yeasts microdilution tests. 10.4 g of RPMI and 34.53 g of MOPS were dissolved in 1 L of distilled water. The pH was adjusted to 7.0 with NaOH. The prepared solution was filter-sterilized through a membrane filter (0.45 μm) using sterile vacuum filtration device and was transfused to a sterile glass bottle with septum.

MHA and SDA were prepared for Minimal Bactericidal Concentration (MBC) determination. Agars were prepared up to the manufaacturer's instructions and were stored in Petri dishes in refrigerator after the solidification.

4.6.2 Preparation of inoculum

For preparation of bacteria, 5 mL of pure Mueller-Hinton broth was inoculated by 25 μL of selected strain and stored for 24 hours at constant temperature (37°C) in a thermostat.

For yeasts preparation instead of Mueller-Hinton broth, Sabourad Dextrose broth was used. The samples were stored for 24 h in a thermostat with lower temperature (25°C).

Then, immediately before testing, prepared microorganisms were suspended in 10 mL of pure broth and turbidity of suspension was increased to 0.5 McFarland standard, using Densi-La-Meter II (Lachema, Brno, Czech Republic), that represented the concentration $5 \cdot 10^5$ CFU/ml for bacteria and $2 \cdot 10^3$ CFU/ml for yeasts.

4.6.3 Antibiotics preparation

Tetracycline that was dissolved in 96.5% ethanol was chosen as the antibiotic control for bacterial strains and tioconazole that was dissolved in DMSO was used as the control antifungal for yeasts. To assess the susceptibility of microorganisms to tetracycline and tioconazole, the range of concentrations of the antibiotics was dependent on the microorganisms' sensitivity.

The antibiotics were dissolved in the appropriate solvents at a concentration 100 times higher than the highest concentration tested. The solutions were then diluted 1:100 in culture medium to the desired concentration.

4.6.4 Broth microdilution method

All samples were tested in triplicate in three independent experiments. The results were evaluated as modus or median of three replicates/experiments and expressed as minimum inhibitory concentration (MIC).

The MICs were determined by standard broth microdilution method using 96-well microtiter plates. In each microplate, serial two-fold dilutions were performed in appropriate growth medium (100 µl) making concentrations ranging from 8 to 1,024 µg/mL by using a manual multichannel pipette.

100 µl of plant extract with concentration 2,048 µg/ml was pipetted into the first row, except the first – growth control and the last – purity control columns. A control antibiotic was always tested simultaneously in another microtiter plate. In three wells of the first row was pipetted 200 µl of antibiotic. Then, 100 µl was pipetted from the column containing the diluted plant extract or antibiotic to the second row, and after mixing the solutions in this column, 100 µl was pipetted again into the next row. This was continued until the end of the microtiter plate.

The inoculum was prepared and standardised as described above (chapter 4.6.2).

4.6.5 Determination of Minimum Inhibitory Concentration (MIC)

MICs were evaluated by visual assessment of bacterial growth after colouring with thiazolyl blue tetrazolium bromide dye (MTT) in concentration 600 µg/mL (Sigma-Aldrich, Prague, Czech Republic).

Coloring agent in volume of 20 µL was applied into wells on the microplates. The color had changed from yellow to blue due to presence of bacteria. The wells with lowest concentration that had no bacteria and stayed yellow were identified as the MIC.

Minimal fungicidal concentration was evaluated as the lowest concentration that inhibited any visual growth. The yeasts colonies were well grown and simply visually identified.

4.6.6 Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined as inhibition $\geq$ 99.99% CFU.

After MIC evaluation, 20 µl of the suspension was pipetted from the wells of a microplate with no microbial growth into a Petri dish with prepared agar (SDA – for yeasts, MHA – for bacteria). After the samples were dried, they were placed into an incubator at 35 ± 2 °C (for bacteria) and 25 ± 2 °C (for yeasts). After 24 hours of storing bacterial colonies growth was visually evaluated. Yeasts were checked after 48 hours of storing.

All tests were made in duplicate in three independent experiments.

4.7 Ethanol fermentation test

The ethanol fermentation tests were planned with pure apple and grape juices (dmBio, dm-drogerie markt GmbH + Co. KG, Karlsruhe, Germany) without additives and *Borassus flabellifer* palm sugar solution (sugar was acquired by Ing. Pavel Nový, PhD on the local market in Cambodia). Juices and sugar solution's pH and sugar content were measured at the laboratory temperature (20

°C) as shown in the Table 3. The concentration and pH of the sugar solution was adjusted based on literature data (Johnson 2011).

Table 3. Measured characteristics of juices and sugar solution

Tested solution	Sugar content (°Bx)	pH
Apple juice	11.9	3.9
Grape juice	16.9	3.3
Palm sugar	10.9	6.8

Based on the first trial single experiment with homemade apple juice (sugar content 21.7 g/100 mL, pH 3.8), ethanol producing yeasts (12) and bacteria (5) were tested (*Hansenula jakobsenii*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Pantoea agglomerans*, *Zymobacter palmae*, *Zymomonas mobilis* subsp. *pomaceae*, *Leuconostoc palmae*, *Saccharomyces cerevisiae*). Three yeasts with the highest ability to produce ethanol during the fermentation were chosen, namely *Hanseniaspora uvarum* T5, *Metschnikowia pulcherrima* T9, *Saccharomyces cerevisiae* DSM 70465 (see Table 4 in chapter 5).

Azadirachta indica and *Careya arborea* water bark extracts were chosen for the inhibition test of yeasts growth.

4.7.1 Preparation and standardization of samples and extracts

Ethanol fermentation test was made in triplicate in one experiment.

The stock solutions of *Azadirachta indica* and *Careya arborea* water bark extracts with a concentration of 512 µg/mL were prepared. 230.4 µg of dried extract (for apple, grape, and palm sugar juices) was weighed and dissolved in 4.5 ml of DMSO with further sonication for better dissolution. Furthermore 1.4 mL of extract was added to 138.6 mL sterilised apple juice, grape juice, and palm sugar solution. The stock juices were properly mixed, and 4995 µL of tested solution was pipetted to earlier prepared 10 mL plastic tubes with sterilised metal plugs.

All together 18 tubes with apple juice (6), grape juice (6), and palm sugar solution (6) were prepared. They were further used as controls of growth and purity, so 1% of DMSO was added to each tube.

All together 54 tubes were prepared for the fermentation tests (3 different yeasts were tested in 3 different media with adding of 2 different extracts in triplicate).

4.7.2 Preparation of inoculum

The inoculum was prepared and standardised as described above (chapter 4.6.2).

One mL of the standardized suspension was 10-fold diluted in sterilised apple juice, grape juice, or palm sugar solution, then 5 µL transerred to 4995 µL of juice or sugar solution to get initial inoculum concentration of 2×10^{-1} CFU/mL.

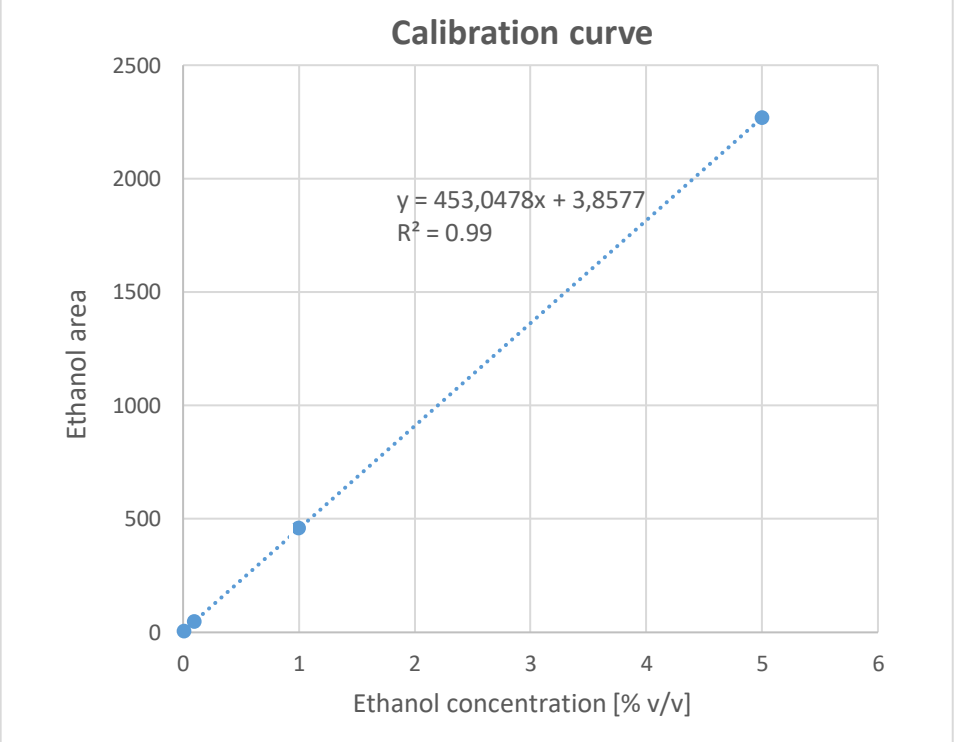
After inoculation, all tubes were closed with loose metallic plugs and were incubated at temperature of 30 °C for 24 h.

Sampling was made 3 times, after 6, 12 and 24 hours after the start of fermentation. 800 µL was taken from every tube and was transferred into an Eppendorf microtube. Samples were centrifigued at 5.000 RPM for 5 minutes. 500 µL of pure solution was removed into a glass vial without disturbing the cell pellet, and freezed at 20 °C until GC-FID analysis.

4.7.3 GC-FID method for the determination of ethanol

The ethanol content was determined by gas chromatography using an Agilent 7890A chromatograph, equipped with CP-WAX 57 CB (25 m x 0,32 mm x 1.2 µm) column (Agilent, Santa Clara, USA). The sample volumes of 0.3 µl were injected into the inlet preheated to 220°C in split mode at the split ratio of 1:50. Helium was used as a carrier gas at the flow rate of 10 ml/min. The temperature program started at 45°C for 0.75 min, then it was increased to 180°C at the gradient of 20°C/min and kept constant for 2.5 min; then the temperature was increased to 210°C (20°C/min) and kept constant for another 5.5 min. The detector was heated to 230°C. The ethanol content was quantified using external calibration (Figure 4).

Figure 4. Calibration curve for ethanol



5 Results

The results of growth inhibitory effect of aqueous and ethanolic extracts from bark of Cambodian plants against standard pathogenic microorganisms and fermenting microorganisms by the microdilution method are shown in Table 5-8. All 10 tested extracts showed antimicrobial activity against tested bacteria and yeasts, but MICs varied at concentration ranging from 64 to 1024 μg/ml.

5.1 MICs and MBCs of pathogenic microorganisms

Growth-inhibitory effects of extracts tested against selected pathogenic microorganisms are shown in Table 5 and 6. Tetracycline and tioconazole MICs values ranged from 0.25 to 8 μg/ml.

The first group of tested microorganisms was represented by 4 bacteria and 1 yeast. The most sensitive bacteria were *B. cereus* and *S. aureus* that had shown susceptibility to all tested extracts (MIC range 64-1024 μg/ml). The most resistant bacteria were *E. coli* and *P. aeruginosa* that were sensitive only to ethanolic extracts of *A. indica* and *C. arborea* (MIC range 512-1024 μg/ml)*;* and *A. indica*, *C. arborea,* and *H. odorata (*MIC range 512-1024 μg/ml), respectively. The only one tested pathogenic yeast was susceptible to all extracts tested except *S. roxburghii* in the range of 128-1024 μg/ml. Interestingly, that *C. albicans* demonstrated a hormetic effect at higher concentrations of *C. arborea* and *A. indica* ethanolic extracts (see chapter 6).

The most effective extracts that inhibited the growth of all tested pathogenic microorganisms were ethanolic extracts obtained from *C. arborea* (MIC range 256 to 1024 μg/ml) and *A. indica* (256 to 512 μg/ml), followed by *H. odorata* (64-512 μg/ml) which was not efficient against *E. coli, H. pierrei* (256-1024 μg/ml) which did not inhibit Gram-negative bacteria, and *S. roxburghii* (512-1024 μg/ml) which was not active against Gram-negative bacteria and the yeast.

Ethanolic extracts of *C. arborea* and *A. indica* had the broadest spectrum of antibacterial and antifungal activity as demonstrated in Table 6. The most sensitive microorganism was *S. aureus* (256 μg/ml), the most resistant against *C. arborea* and *A. indica* ethanolic extracts were Gram-negative bacteria (1024 μg/ml and 512 μg/ml, respectively), and also *B. cereus* (512 μg/ml) in a case of *A. indica.*

The lowest value of MIC in growth-inhibitory effect against *S. aureus* was 64 μg/ml for ethanolic extract of *H. odorata*. Noteworthy *H. odorata* inhibited *P. aeruginosa* growth at concentration 512 μg/ml.

All aqueous extracts were not effective enough to inhibit the growth of Gram-negative bacteria, but showed MICs in the range from 256 μg/ml to 1024 μg/ml. The least effective extract was *S. roxburghii* that could inhibit only Gram-positive bacteria (*B. cereus* and *S. aureus*) with MIC of 512-1024 μg/ml. *H. pierrei* successfully inhibited the growth of Gram-positive bacteria and the tested yeast. Interestingly that *H. pierrei* aqueous extract was more effective against *B. cereus* with MIC 256 μg/ml than an ethanolic extract that showed antimicrobial effect with MIC 1024 μg/ml, but in this case even MBC value was determined (1024 μg/ml). For *C. albicans* MIC of ethanolic extract was determined at 256 μg/ml, but for aqueous extract this value was 1024 μg/ml that described a reversed principle of activity for different microorganisms.

MBCs were observed only for *S. aureus* (512 μg/ml*), B. cereus* (1024 μg/ml) and *E. coli* (1024 μg/ml). Aqueous extracts of *C. arborea* and *A. indica* showed the highest MBCs against

S. aureus compared to other tested microorganisms in the whole experiment. MFC of *C. albicans* was not observed.

5.2 MICs and MBCs of fermenting microorganisms

MICs and MBCs of Cambodian plants extracts tested against selected fermenting microorganisms are demonstrated below in Table 7 and 8.

The MIC values of tioconazole used in yeast testing highly varied (0.063-8 μg/ml). Tetracycline used for bacteria testing showed MICs with the range from 0.5 to 2 μg/ml.

The second group of tested microorganisms was represented by 5 bacteria and 12 yeasts. The most sensitive bacterium was *L. palmae* that was not inhibited only by extracts of *H. pierrei*. Otherwise, all bacteria were susceptible to all ethanolic extracts. *P. agglomerans* S1-b was the most resistant and was not susceptible to aqueous extracts of *H. odorata*, *H. pierrei* and *S. roxburghii*, unlike *P. agglomerans* J1 that was not inhibited by *S. roxburghii* only. *Z. palmae* was also resistant to one aqueous *H. pierrei* extract only. *Z. mobilis* was not susceptible to any of two mentioned extracts. The most resistant yeasts were *H. jakobsenii, H uvarum* T1 and T5, *M. pulcherrima* T9 and V2, *S. cerevisiae* DSM 70465 (see Table 8) which were not inhibited by any tested extract. The most susceptible yeast against the ethanolic extract of *H. odorata* was *S. cerevisiae* DSM 70478 with MIC of 64 μg/ml. Interestingly that *H. uvarum* DSM 26650, *H. uvarum* OF1, *H. uvarum* VF1, *S. cerevisiae* DSM 70478, and *S. cerevisiae* DSM 70868 demonstrated a hormetic effect at higher concentrations of *C. arborea* and *A. indica* ethanolic extracts (see Table 9) that will be discussed in detail in chapter 6.

The ethanolic extract of *H. odorata* was the most efficient (MIC range 64 to 512 μg/ml), against 59% percent of fermenting microorganisms, followed by *H. pierrei* (128-1024 μg/ml) which inhibited the growth of 53% microbes. Both had the broadest spectrum of antimicrobial (256-512 μg/ml for *H. odorata* and 1024 μg/ml for *H. pierrei*) and antifungal activity with MFC range from 64 to 256 μg/ml and 128-256 μg/ml for *H. odorata* and *H. pierrei*, respectively (see Table 8)

The least efficient were aqueous extracts of *H. pierrei* and *S. roxburghii* as shown in Table 7. *S. roxburghii* aqueous extract was effective against only Gram-positive *L. palmae* and *Z. palmae* at MIC of 1024 μg/ml. Ethanolic *S. roxburghii* extract showed antibacterial activity against all tested fermenting bacteria at concentration 1024 μg/ml, but any antifungal activity was not observed for all *S. roxburghii* extracts even at the highest tested concentration (1024 μg/ml).

H. odorata water and ethanolic extracts had bactericidal activity against *L. palmae* and *Z. palmae* at concentration 1024 μg/ml; ethanolic extract showed the same activity also against *P. agglomerans* J1. MBCs were determined for all bacteria tested against *A. indica* and *C. arborea* as 1024 μg/ml. Ethanolic extract of *S. roxburghii* demonstrated MBC 1024 μg/ml. MFCs were not determined for any yeast.

5.3 Results of the trial ethanol fermentation test

The results of the first trial fermentation test are demonstrated in Table 4.

After one day fermentation at 25 °C, 3 yeasts, namely *H. uvarum* T5, *H. uvarum* T1, *M pulcherrima* T9 produced the highest amount of ethanol: 0.24% (v/v), 0.19% (v/v) and 0.1% (v/v), respectively (see Table 4). Other two yeasts, *H. uvarum* DSM 26650 and *S. cerevisiae* DSM 70465,

were less active and produced 0.09 and 0.05% (v/v) of ethanol, respectively. The best fermenting yeast genera was *Hanseniaspora*, succeeded by *Metschnikowia* and *Saccharomyces*. The least fermentatively active yeast was *S. cerevisiae* DSM 70868 with 0.003% (v/v) of ethanol. Notably, the wild yeasts isolated from the different fruit surfaces showed the highest values of detected ethanol.

Compared to yeasts, tested bacteria fermented the substrate much worse. *Z. mobilis* DSM 3580 demonstrated the highest amount of ethanol (0.005% (v/v)), followed by *Z. palmae* DSM 10491 and *P. agglomerans* S1-b with approximate 0.004% (v/v) of produced alcohol. The lowest value of detected ethanol was 0.0007% (v/v) that demonstrated *L. palmae.*

Table 4. Concentrations of ethanol produced by fermenting microorganisms detected with GC-FID

Microorganism	**Ethanol concentration (% v/v)**
H. jakobsenii	0,010
H. uvarum DSM 26650	0,091
H. uvarum T1	0,192
H. uvarum T5	0,236
H. uvarum VF1	0,005
L. palmae DSM 21144	0,001
M. pulcherrima DSM 70336	0,029
M. pulcherrima T9	0,102
M. pulcherrima V2	0,026
P. agglomerans J1	0,003
P. agglomerans Sb1	0,004
S. cerevisiae DSM 70465	0,053
S. cerevisiae DSM 70478	0,041
S. cerevisiae DSM 70868	0,002
Zb. palmae DSM 10491	0,004
Zm. mobilis DSM 3580	0,005

Table 5. *In vitro* growth-inhibitory effect of aqueous extracts from Cambodian plants against standard pathogenic microorganisms

Microorganism	*C. arborea*		*H. odorata*		*A. indica*		*H. pierrei*		*S. roxburghii*		Tetracycline	Tioconazole
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MIC
B. cereus	1024	-	256	-	512	-	256	-	512	-	0.5	n.t.
E. coli	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	1	n.t.
P. aeruginosa	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	8	n.t.
S. aureus	512	512	128	-	512	512	256	-	1024	-	0.25	n.t.
C. albicans	1024	-	512	-	512	-	1024	-	>1024	-	n.t.	0.063

MIC - Minimal Inhibitory Concentration (µg/ml); **MBC** - Minimal Bactericidal Concentration (µg/ml) (**MFC** - Minimal Fungicidal Concentration for *C. albicans* (µg/ml)); **n.t.** - not tested; **>1024 µg/ml** - MIC was not determined in the highest tested concentration.

Table 6. *In vitro* growth-inhibitory effect of ethanolic extracts from Cambodian plants against standard pathogenic microorganisms

Microorganism	*C. arborea*		*H. odorata*		*A. indica*		*H. pierrei*		*S. roxburghii*		Tetracycline	Tioconazole
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MIC
B. cereus	512	1024	256	-	512	-	1024	1024	1024	-	0.5	n.t.
E. coli	1024	1024	>1024	-	512	1024	>1024	-	>1024	-	1	n.t.
P. aeruginosa	1024	-	512	-	512	-	>1024	-	>1024	-	8	n.t.
S. aureus	256	512	64	1024	256	-	256	1024	512	-	0.25	n.t.
C. albicans	HE	-	128	-	HE	-	256	-	>1024	-	n.t.	0.063

MIC - Minimal Inhibitory Concentration (µg/ml); **MBC** - Minimal Bactericidal Concentration (µg/ml) (**MFC** - Minimal Fungicidal Concentration for *C. albicans* (µg/ml)); **n.t.** - not tested; **HE** - Hormetic Effect of an extract; **>1024 µg/ml** - MIC was not determined in the highest tested concentration

Table 7. *In vitro* growth-inhibitory effect of aqueous extracts from Cambodian plants against selected fermenting microorganisms

Microorganism	*C. arborea*		*H. odorata*		*A. indica*		*H. pierrei*		*S. roxburghii*		Tetracycline	Tioconazole
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MIC
L. palmae	1024	1024	512	1024	512	1024	>1024	-	1024	-	0.5	n.t.
P. agglomerans J1	1024	1024	1024	-	1024	1024	1024	-	>1024	-	0.5	n.t.
P. agglomerans S1-b	1024	1024	>1024	-	1024	1024	>1024	-	>1024	-	1	n.t.
Zmb. palmae	1024	1024	1024	-	512	1024	>1024	-	1024	-	2	n.t.
Z. mobilis	1024	1024	1024	1024	1024	1024	>1024	-	>1024	-	2	n.t.
H. jakobsenii	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	1
H. uvarum DSM 26650	1024	-	512	-	256	-	1024	-	>1024	-	n.t.	0.063
H. uvarum OF1	>1024	-	512	-	512	-	>1024	-	>1024	-	n.t.	0.125
H. uvarum T1	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.063
H. uvarum T5	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.5
H. uvarum VF1	>1024	-	512	-	1024	-	1024	-	>1024	-	n.t.	1
M. pulcherrima DSM 70336	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.063
M. pulcherrima T9	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.25
M. pulcherrima V2	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.5
S. cerevisiae DSM 70465	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	8
S. cerevisiae DSM 70478	>1024	-	1024	-	>1024	-	512	-	>1024	-	n.t.	0.125
S. cerevisiae DSM 70868	1024	-	1024	-	1024	-	>1024	-	>1024	-	n.t.	8

MIC - Minimal Inhibitory Concentration (µg/ml); **MBC** - Minimal Bactericidal Concentration (µg/ml) (**MFC** - Minimal Fungicidal Concentration for C. albicans (µg/ml)); **n.t.** - not tested; **>1024 µg/ml** - MIC was not determined in the highest tested concentration

Table 8. *In vitro* growth-inhibitory effect of ethanolic extracts from Cambodian plants against selected fermenting microorganisms

Microorganism	*C. arborea*		*H. odorata*		*A. indica*		*H. pierrei*		*S. roxburghii*		Tetracycline	Tioconazole
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MIC
L. palmae	512	1024	256	1024	256	1024	>1024	-	1024	-	0.5	n.t.
P. agglomerans J1	256	1024	256	1024	1024	1024	1024	-	1024	-	0.5	n.t.
P. agglomerans S1-b	512	1024	512	-	1024	1024	1024	-	1024	-	1	n.t.
Zmb. palmae	512	1024	512	-	512	1024	1024	-	1024	1024	2	n.t.
Z. mobilis	1024	1024	512	1024	512	1024	1024	-	1024	-	2	n.t.
H. jakobsenii	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	1
H. uvarum DSM 26650	HE	-	128	-	HE	-	256	-	>1024	-	n.t.	0.063
H. uvarum OF1	HE	-	256	-	HE	-	256	-	>1024	-	n.t.	0.125
H. uvarum T1	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.063
H. uvarum T5	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.5
H. uvarum VF1	HE	-	256	-	HE	-	256	-	>1024	-	n.t.	1
M. pulcherrima DSM 70336	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.063
M. pulcherrima T9	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.25
M. pulcherrima V2	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	0.5
S. cerevisiae DSM 70465	>1024	-	>1024	-	>1024	-	>1024	-	>1024	-	n.t.	8
S. cerevisiae DSM 70478	HE	-	64	-	HE	-	128	-	>1024	-	n.t.	0.125
S. cerevisiae DSM 70868	HE	-	256	-	HE	-	256	-	>1024	-	n.t.	8

MIC - Minimal Inhibitory Concentration (µg/ml); **MBC** - Minimal Bactericidal Concentration (µg/ml) (**MFC** - Minimal Fungicidal Concentration for C. albicans (µg/ml)); **HE** - Hormetic Effect of an extract; **n.t.** - not tested; >**1024 µg/ml** - MIC was not determined in the highest tested concentration

Table 9. *In vitro* hormetic effect of *A. indica* and *C. arborea* ethanolic extracts on the selected yeast's growth

Microorganism		*C. arborea*			*A. indica*		
		non-ihibiting concentrations range (µg/ml)		ihibiting concentrations range MICs (µg/ml)	non-ihibiting concentrations range (µg/ml)		ihibiting concentrations range MICs (µg/ml)
C. albicans	ATCC 10231	8-16	256-1024	32-128	8-16	256-1024	32-128
H. uvarum	DSM 26650	8-16	256-1024	32-128	8-16	256-1024	32-128
H. uvarum	VF1	8-16	256-1024	32-128	8-16	256-1024	32-128
H. uvarum	OF1	8-16	512-1024	32-256	8-32	512-1024	64-256
S. cerevisiae	DSM 70478	8	128-1024	16-64	8-16	256-1024	32-128
S. cerevisiae	DSM 70868	8-16	128-1024	32-64	8-32	256-1024	64-128

6 Discussion

Plant extracts have been used for many thousands of years in food preservation, pharmaceuticals, alternative medicine, and natural therapies. The beneficial antimicrobial effects of plant materials typically result from the secondary metabolites present in the plant. They are usually unique and taxonomically distinct. The antimicrobial effect is not always attributed to a single compound, sometimes it can be resulted from their combination (Joshi et al. 2011).

Plant-derived extracts due to their content of antimicrobial compounds possess potential as natural agents for food preservation (Kotzekidou et al. 2008). In the frame of this thesis, the antimicrobial effect of Cambodian plants bark extracts used as traditional palm sap preservatives has been confirmed *in vitro* by the microdilution method, but their effectiveness varied. The most efficient were extracts of *H. odorata, A. indica* and *C. arborea* that showed explicit growth-inhibitory activity against tested bacteria and yeasts.

Palm sap preservation

In Cambodia, bark from *A. indica, H. odorata, H. pierrei, S. roxbrghii,* and *C. arborea* are traditionally used for microbial growth inhibition in palm sap. Palm juice should be consumed within the first 24 hours after collecting to avoid degrading organoleptic properties. The antimicrobial activity of these barks slow down the fermentation process and thus prolongs the shelf life of palm sap by several hours.

Traditional using of plant-based extracts for palm sap preservations have been reported in several scientific articles and shortly summarized below.

In India, the inner surface of the earthen pot for coconut inflorescence sap collection is often coated with lime. In Thailand, Kiam wood (*Cotylelolbium melanoxylon Pierse*) is added in the bamboo tube during the collection of palm sap (Hebbar et al. 2018). The bark of *S. roxburghii* has been used as a preservative for traditional beverages in Thailand (Morikawa et al. 2012). These simple methods allow slowing down of the growth of the microorganisms by several hours.

Extracts from the bark of different trees such as *Saccoglottis gabonensis*, *Vernonia amygdalina, Euphobia* sp., *Nauclea* sp., and *Rubiacae* species were used for the preservation of palm sap. In Nigeria, the aqueous extract from the bark of *S. gabonemis* is traditionally used to inhibit the growth of yeasts and bacteria in palm sap (Ogbulie et al. 2007). The alkaloids and phenolic compounds were reported to show the high antimicrobial effect (Odunfa, 1985). However, Okafor (1975) reported that *S. gabonemis* bark only inhibited the growth of *Sarcina lutea* at 10% concentration and no other organisms were affected by using this extract.

Vateria copallifera, Careya arborea, Azadirachta indica are used for the preservation of coconut sap. Nevertheless, Kapilan (2015) reported that the bark of *Careya arborea* and leaves of *Azadirachta indica* did not show the efficient inhibition of palm sap fermentation, but the number of yeast cells was conciderably reduced

Hopea odorata

Ethanolic and aqueous extracts of *H. odorata* were efficient against 64% and 55% of all tested microorganisms, respectively. The extracts showed significant antibacterial (MIC range 256-1024 µg/ml) and even better antifungal (MIC range 64-512 µg/ml) activities.

MICs were determined for the ethanolic extract against *C. albicans* (128-512 µg/ml*)*, 3 strains of *H. uvarum* (64-256 µg/ml*)* and 2 strains of *S. cerevisiae* (128-256 µg/ml*)* (Table 7 and 8). The antifungal activity of *H. odorata* has been poorly explored. The only mention about the fungal growth inhibitory activities of its heartwood and sapwood was reported by Kawamura et al. (2010). *H. odorata* was able to unhibit the growth of *Gloeophyllum trabeum* and *Pycnoporus sanguineus.* In the frame of this thesis, the antifungal activity against *H. uvarum, H. jakobsenii, S. cerevisiae, M. pulcherrima* was tested for the first time.

The extracts were also first-time tested against following bacteria: *L. palmae, P. agglomerans, Z. palmae* and *Z. mobilis*. Results of the antimicrobial assay showed that *H. odorata* ethanolic and aqueous bark extracts were effective against *S. aureus* (MICs 64 and 128 µg/ml, respectively), *B. cereus* (MIC 256 µg/mL for both extracts) but were not efficient enough to inhibit the growth of *E. coli.* The antibacterial effect of *Hopea odorata* leaf extracts was analyzed in the disc diffusion technique by Kabir et al. (2015). Methanolic leaf extract showed the highest activity in concentration of 2 mg/ml against *S. aureus* and *Bacillus subtilis* that confirmed results of our experiment. However, the observed efficient MICs were lower in our case. Ethanolic extract was reported to be active against *Bacillus megaterium* and *E. coli* that could be relate to the higher tested concentration (Kabir et al. 2015). Authors mentioned that a narrow zone of inhibition was found against *E. coli* compared to other extracts activity.

Otherwise, all fermenting bacteria were inhibited (MICs range 256-1024 µg/ml), except *P. agglomerans* S1-b which was resistant against the aqueous extract. MBCs of *L. palmae, S. aureus, Z. mobilis, P. agglomerans* J1 were also observed.

Phytochemical studies reported that *H. odorata* contains certain types of phenolic compounds (Scalbert et al. 2005), and flavonoids (Satiraphan et al. 2012). Polyphenols were reported to have antioxidant and antibacterial properties (Kabir et al. 2015). Therefore, the significant efficiency of *H. odorata* extracts against bacteria can be related to the high content of polyphenols and flavonoids.

Careya arborea

Ethanolic and aqueous extracts of *C. arborea* were efficient against 41% and 46% of all tested microorganisms, respectively. Antibacterial activity of both extracts was observed in the MIC range from 256 to 1024 µg/ml. Antifungal activity was significantly worse: only *C. albicans*, *H. uvarum* DSM 26650 and *S. cerevisiae* DSM 70868 MICs were determined (1024 µg/ml).

Data about *C. arborea* extracts' antimicrobial activity against *L. palmae, P. agglomerans, Z. palmae, M. pulcherrima, H. jakobsenii, S. cerevisiae* and *H. uvarum* was not found, therefore it was tested for the first time in the frame of this thesis.

The antibacterial effect of *C. arborea* fruit extracts was analyzed by Kumar et. al. (2006) and Khaliq (2016), who reported antibacterial activity of methanolic, ethanolic and ethyl acetate *C. arborea* bark and leaf extract against *E. coli* and *P. aeruginosa.* These findings correspond with our antimicrobial assay results: MIC for *E. coli* and *P. aeruginosa* was determined as 1024 µg/ml. Gram-negative bacteria were expected to be less susceptible to the extracts than Gram-positive. This assumption is mainly due to differences in cell wall composition (Joshi et al. 2011). However, the efficiency of *C. arborea* extracts in our case was observed against both, but with less effective MICs values against Gram-positive bacteria (1024 µg/ml) for the ethanolic extract. The antibacterial activity of ethanolic leaf extract against *E. coli, P. aeruginosa, Z. mobilis*

and *B. cereus* has been reported in the study by Navya (2018). The results of the experiment also confirmed this fact: MICs were determined as 1024 µg/ml for *E. coli, P. aeruginosa, Z. mobilis* and as 512 µg/ml for *B. cereus*.

Kumar et. al. (2006) reported the antifungal activity of methanolic bark extract against *C. albicans* that corresponds with our results. Antifungal activity of *C. arborea* methanolic bark extract against *C. albicans* was previously revealed by Khaliq (2016). This finding was confirmed during antimicrobial assay tests not only against *C. albicans*, but also against *H. uvarum* and *S. cerevisiae* (MIC 1024 µg/ml). Notably, the ethanolic extract showed a hormetic effect (see Table 9) that is discussed below.

The ethyl acetate extracts showed a bacteriostatic effect as it was reported by Navya (2018). In our case, ethanolic extracts demonstrated MBCs in the range 512-1024 µg/ml.

Potential antioxidants quercetin (Begum et al. 2014) and piperine (Ahmed et al. 2002) were found in *C. arborea* bark. High antimicrobial activity of *C. arborea* bark can be caused by the bioactive constituents including alkaloids, flavonoids saponins, triterpenes, valepotriates etc., that were described in qualitative HPTLC phytochemical profile of this plant by Gupta et al. (2019) and may explain the inhibition of tested microorganisms.

Azadirachta indica

Ethanolic and aqueous extracts of *A. indica* showed effectivity against 41% and 50% of all tested microorganisms, respectively. The extracts showed significant antibacterial (MIC range 256-1024 µg/ml) and antifungal (MIC range 256-1024 µg/ml) activities. Notably, the aqueous extract was the most efficient against the fermenting yeasts: *H. uvarum* (256 µg/ml) and *S. cerevisiae* (128-256 µg/ml).

The antimicrobial activity of *A. indica* was tested for the first time against following microbes: *L. palmae, P. agglomerans, Z. palmae, H. jakobsenii, M. pulcherrima, S. cerevisiae* and *H. uvarum*.

MIC of the most susceptible pathogenic bacterium *S. aureus* in this experiment was determined as 512 µg/ml for aqueous extract and as 256 µg/ml for the ethanolic extract. According to Singaravelu et al. (2019), neem's bark extract demonstrated antibacterial activity against *S. aureus* only at higher concentration 1000-2000 µg/ml with use of agar well diffusion assay technique. The difference in results could be caused by the methods for antimicrobial assay. Antibacterial activity of methanol and acetone flower extracts of *A. indica* by disk assay was tested against *S. aureus, E. coli* and *B. cereus* by Alzoreky and Nakahara (2003). Buffered methanolic extract of *A. indica* has shown inhibition zones against *B. cereus* at lower doses (400 µg per disc). Acetone extracts were only active at double concentration. In our case, the MIC of *B. cereus* was 512 µg/ml for aqueous and ethanolic extracts.

The bark extract of *A. indica* was reported to have antibacterial activity against *P. aeruginosa, E. faecalis* and *P. mirabilis* in the MICs range 500-2000 µg/ml (Singaravelu et al. 2019), that corresponds with our results for E. coli and P. aeruginosa MIC of 512 µg/ml. Fabry et al. (1998) conducted an ethnopharmacological survey of *A. indica* stem bark and leaves against 105 different strains of bacteria and reported MICs values less than or equal to 8 mg/ml. All tested bacteria were successfully inhibited by *A. indica* extracts, except aqueous which was not effective against Gram-negative bacteria.

The antifungal activity of aqueous extract was observed against *C. albicans* and *H. uvarum* (256-1024 μg/ml), *S. cerevisiae* (1024 μg/ml) strains. El-Hawary et al. (2013) tested essential oils from the flowers and leaves of *A. indica* and reported the antifungal activity against *C. albicans*. Notably, *C. albicans*, *H. uvarum* DSM 26650, *H. uvarum* OF1, *H. uvarum* VF1, *S. cerevisiae* DSM 70478, and *S. cerevisiae* DSM 70868 demonstrated a hormetic effect in *C. arborea* and *A. indica* extracts, that will be discussed below.

MBCs were observed for *S. aureus* (512 μg/ml), *E. coli* (1024 μg/ml) and all fermenting bacteria (1024 μg/ml). Any MFCs were not observed.

A. indica is reported to be highly rich in secondary metabolites, including isoprenoids (azadirone, protomeliacins, nimbin, azadirachtin, limonoids, gedunin), dihydrochalcones polyphenolics and their glycosides (Paritala et al. 2015; Jeba Malar et al. 2020).

Over 300 limonoids have been isolated and identified from different plant parts of *A. indica*. The structure and chemical oxidation state parameters of limonoids relates to their antibacterial, antiviral, and antifungal properties (Paritala et al. 2015), though antifungal activity confirmation of *A. indica* was not found in the scientific articles.

The antimicrobial activity of plant-derived extracts is assigned to terpenoid and phenolic compounds which, due to their lipophilic character, accumulate in bacterial membranes causing energy depletion (Kotzekidou et al. 2008). In the frame of this thesis, *A. indica* extracts inhibited all tested bacteria (MICs range 256-1024 μg/ml), except Gram-negative, that could be related to the complexity of their membrane. The higher antibacterial activity of ethanolic extract can be related to the antimicrobial compounds and their active forms. *A. indica* contains microbial inhibitors (i.e., flavonoids), soluble in aqueous alcohol, so the flavonoid aglycones may be more active than their glycosidic forms naturally present in plant's water solutions (Alzoreky & Nakahara 2003)

Hopea pierrei

Ethanolic and aqueous extracts of *H. pierrei* showed effectivity against 55% and 31% of all tested microorganisms, respectively. The extracts showed significant antibacterial (MIC range 256-1024 μg/ml) and antifungal (MIC range 128-256 μg/ml) activities. Notably, the ethanolic extract was the second efficient after *H. odorata* due to the percentage of inhibited microorganisms.

In the frame of this thesis, *H. pierrei* extracts were tested for the first time. The relation of this plant to the same genus, as *Hopea odorata*, led to conclusion that it could have some similar pharmacological properties and potential antimicrobial activity. However, the lack of information about *H. pierrei* ethnopharmacological properties was found. Any reports about the antimicrobial activity of this tree were not found in the scientific databases.

H. pierrei was efficient against tested bacteria in a wide range of concentrations 256-1024 μg/ml. Gram-positive pathogenic bacteria (*B. cereus* and *S. aureus*) were inhibited (MIC 1024 μg/ml) and even bactericidal activity was observed (1024 μg/ml). Some strains of yeasts, especially *S. cerevisiae* and *H. uvarum*, showed suspectibility to ethanol and aqueous extracts in the range 128-1024 μg/ml. These findings left a broad field for further investigation of *H. pierrei* antimicrobial properties.

Shorea roxburghii

Ethanolic and aqueous extracts of *S. roxburghii* showed effectivity against 32% and 27% of all tested microorganisms, respectively. The extracts showed significant antibacterial (MIC range 256-1024 µg/ml) and antifungal (MIC range 128-256 µg/ml) activities.

The antimicrobial activity of *S. roxburghii* was tested for the first time against following microorganisms: *L. palmae, P. agglomerans, Z. palmae, Z. mobilis, H. jakobsenii, M. pulcherrima, S. cerevisiae* and *H. uvarum*.

Diptoindonesin D, isolated from stem bark of *S. roxburghii*, was reported to have the antibacterial activity against *S. aureus* and *B. cereus* (Sudto et al. 2019). This fact agreeds with our results: ethanolic and aqueous extracts of *S. roxburghii* bark suppressed the growth of *S. aureus* and *B. cereus* in the concentration range 512-1024 µg/ml. Assavasirijinda (2010) reported that ethanolic and aqueous extracts of *S. roxburghii* bark showed the growth inhibition of *S. aureus* ATCC 25923, *B. cereus* ATCC 11778, *E. coli* ATCC 25922. In our case, *E. coli* was not inhibited by the tested extracts. The aqueous extract of *S. roxburghii* did not show antibacterial activity against *E. coli* ATCC 25922, according to Assavasirijinda (2010).

Antifungal activity of *S. roxburghii* has not been previously reported, and any MICs for yeasts were not observed during the antimicrobial assay tests.

Bactericidal concentration was observed only in the case of ethanolic extract tested against *Z. palmae* (1024 µg/ml).

Shorea genus has been reported to have a large source of oligostilbenoids that are known for their antibacterial, and antioxidant properties (Moriyama et al. 2016). This fact can explain the antimicrobial activity of *S. roxburghii* against some tested microorganisms.

Hormetic effect of tested extracts on yeasts growth

Mild stress and hormesis are the most likely explanations for the beneficial effects of low doses of potentially toxic substances. The hormetic response is determined by the substance nature, physiological state of the organism, and specificity of downstream targets influenced (Semchyshyn 2020). In this respect, phytochemicals are considered as hormesis-providing compounds, that induce an adaptive stress response making organisms resistant not only to high (and normally harmful) doses of the same agent, but also against other stressors including oxidants, nutrients, and heat (Son et al. 2008).

In the previous scientific reports, the hormetic effects of plant-based extracts on yeast growth have been described (Bayliak et al. 2014; Semchyshyn 2020). The HE of plant-based ethanolic extracts on model immune cells was reported (Calabrese et al. 2019)

The analysis made by Calabrese et al. (2019) revealed that 70–80% of the ethanolic extracts induce pro- and anti-inflammatory response in hormetic concentration for immune cells. These findings demonstrate that a large proportion of herbal extracts display hormetic dose responses.

A wide variety of stressing agents have beneficial hormetic effects, however, the potential role of *A. indica* and *C. arborea* has not been previously investigated. The biphasic dependence, characterized by low-dose stimulation and high-dose repression of yeast colony growth, has been found for *C. albicans, H. uvarum* DSM 26650, *H. uvarum* OF1, *H. uvarum* VF1, *S. cerevisiae* DSM 70478, and *S. cerevisiae* DSM 70868.

A. indica and *C. arborea* ethanolic extracts affected the growth of yeast cells in concentration-dependent manner. Low concentrations of extracts (8-16 µg/ml) prevented cell death

and increased their growth, while medium concentrations (32-128 µg/ml) were able to fully inhibit the growth of yeasts, and higher concentrations (256-1024 µg/ml) mostly were not efficient for growth-inhibition.

It was supposed that at low concentrations *A. indica* and *C. arborea* may act as a mild stressor, inducing adaptive response, which leads to an increase in cellular resistance, and thereby increasing maintenance and yeast growth. Medium extract concentrations may be toxic inducing yeast cell death, what is an interesting observation, because HE is always connected with higher concentrations. However, in the context of this thesis' experimental part, it was observed that higher concentrations did not show inhibitory effect and MICs against tested yeasts were determined as >1024 µg/ml.

Bayliak et al. (2014) reported that the action of *R. rosea* extract on *S. cerevisiae* lifespan and stress resistance could involve hormetic mechanisms. This fact was explained as followed: *R. rosea* extract only at low concentrations (1 and 5 µl/ml) boosted the resistance of yeast cells against different stresses and provided life-prolonging effect, whereas at high concentrations (20 µl/ml) it had adverse effects on yeast stress resistance. These findings suggest that a shift towards stress resistance may be due to changes in gene expression. Results of this report support the utilization of yeast as a useful model to study *in vivo* molecular mechanisms responsible for health beneficial effects of natural phytochemicals (Bayliak et al. 2014) that can be applied in the pharmaceutical and food industries.

Taken together, our results suggest, that tested extracts of *A. indica*, *H. odorata, H. pierrei, S. roxbrghii,* and *C. arborea* barks showed the antimicrobial activity against pathogenic and fermenting bacteria and yeasts. Scientific investigation of those plants in future may help to understand better their potential and find a way to use them in food and beverage manufacturing technologies.

Ethanol fermentation test

The ethanol fermentation test showed that yeasts were more active ethanol producing microorganisms compared to bacteria. It is known that yeasts play a fundamental role in the alcoholic fermentation, and they are responsible for the conversion of sugars to ethanol and CO_2 (Tristezza et al. 2016). Maximum amount of ethanol found in fresh toddy sample was 3.4% (Shamala & Sreekantiah 1988), but in this trial test the apple juice was chosen as a substrate and all microorganisms were tested individually. As stated by Ayernor and Matthews (1971), the palm sap microbiological composition is complex and mostly influenced by the conditions of tapping and collection. Consequently, the trial fermentation test was maximally simplified, but the results were compared to palm sap microflora reported in the scientific articles.

The most active ethanol producing yeast in the apple juice substrate was *H. uvarum* (0.09-0.24% (v/v)). According to Djeni et. al. (2020), *Hanseniaspora* sp. is the next predominant yeast present in raphia palm wine after *Saccharomyces* sp., so the exploration of its fermentative abilities can be one of the keys leading to better understanding of palm sap fermentation.

Saccharomyces cerevisiae was able to produce 0.003-0.05% (v/v) of ethanol for one day fermentation. According to Theivendirarajah & Chrystopher (1987), *Saccharomyces* sp. are numerically predominated in palmyrah toddy samples. Their superior fermentative ability

and adaptation to growth in the different conditions make them a great model organism for palm sap investigation.

There were not found any mentions about the role of *M. pulcherrima* in palm sap fermentation, otherwise its fermentative ability is well known in wine production. Notably, non-*Saccharomyces* yeasts were reported to create specific flavour-active characteristics that can contribute to the unique flavour of the different palm wine type (Djeni et al. 2020), so supposedly the appearance of *M. pulcherrima* can relate to the creation of flavour compounds in palm wine.

Zymomonas mobilis was the most active fermenting bacterium (0.005% (v/v) of ethanol) in the frame of this experiment. This fact corresponds with findings of Shamala & Sreekantiah (1988) who reported that *Zymomonas* is mainly responsible for ethanol and carbon dioxide production in palm wine fermentation. Djeni et. al. (2020) also described *Zymomonas* as a predominantly present bacterium in the Nigerian palm wine.

This trial fermentation test was important for the selection of microorganisms for the further experiments with use of inhibiting Cambodian plant extracts. Three yeasts, namely *Hanseniaspora uvarum* T5, *Metschnikowia pulcherrima* T9, *Saccharomyces cerevisiae* DSM 70465 were chosen because of their good fermentative ability and the highest values of detected ethanol.

7 Conclusion

In the frame of this study, the antimicrobial activities of Cambodian plants traditionally used for palm sap preservation has been confirmed *in vitro* against a broad spectrum of microorganisms, such as pathogenic bacteria, palm natural contamination bacteria, and yeasts.

All ethanolic and aqueous extracts from *A. indica*, *C. arborea*, *H. odorata, H. pierrei,* and *S. roxburghii* barks showed certain antimicrobial activity potency. Considering the susceptibility of tested microbes, the most effective extracts were obtained from *H. odorata, A. indica* and *C. arborea*. The most active plant extract was ethanolic extract of *H. odorata* followed by extracts of *A. indica*, *C. arborea.*

A. indica and *C. arborea* ethanolic extracts showed the obvious hormetic effect against some yeasts that could be investigated more explicitly.

Although the fermentation inhibition experiments were not finished due to limited lab time during the last year, the obtained results proved that bark extracts of selected Cambodian plants may inhibit the broad spectrum of microorganisms, including yeasts and bacteria participating in the fermentation processes. These findings can be used by food and beverage producing industry, especially in tropical regions, as potential natural preservatives. The investigation of fermentation process and impact of Cambodian plant bark extracts on the microbial growth inhibition in model solutions would be desirable for further studies, whereas the aqueous extracts of *A. indica* and *C. arborea* seem to be the most promissing growth inhibiting agents for the fermentation tests.

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9 List of abbreviations and symbols

°Bx – degrees Brix, sugar content of an aqueous solution (1°Bx is 1 gram of sucrose in 100 grams of solution)

AAB – acetic acid bacteria

AOAC – the Association of Official Analytical Chemists

ATB – an antibiotic

ATCC – the American Type Culture Collection

CLSI – Clinical and Laboratory Standards Institute

CFU – a colony-forming unit

DMSO – dimethylsulfoxide

EUCAST – European Committee on Antimicrobial Susceptibility Testing

HE – plant extract showed hormetic effect

kDa – kilodalton

LAB – lactic acid bacteria

MBC – the Minimum Bactericidal Concentration

MHA – Mueller-Hinton Agar

MHB – Mueller-Hinton Broth

MIC – the Minimum Inhibitory Concentration

MTT – thiazolyl blue tetrazolium bromide dye

RPM – revolutions per minute

SDA – Sabouraud Dextrose Agar

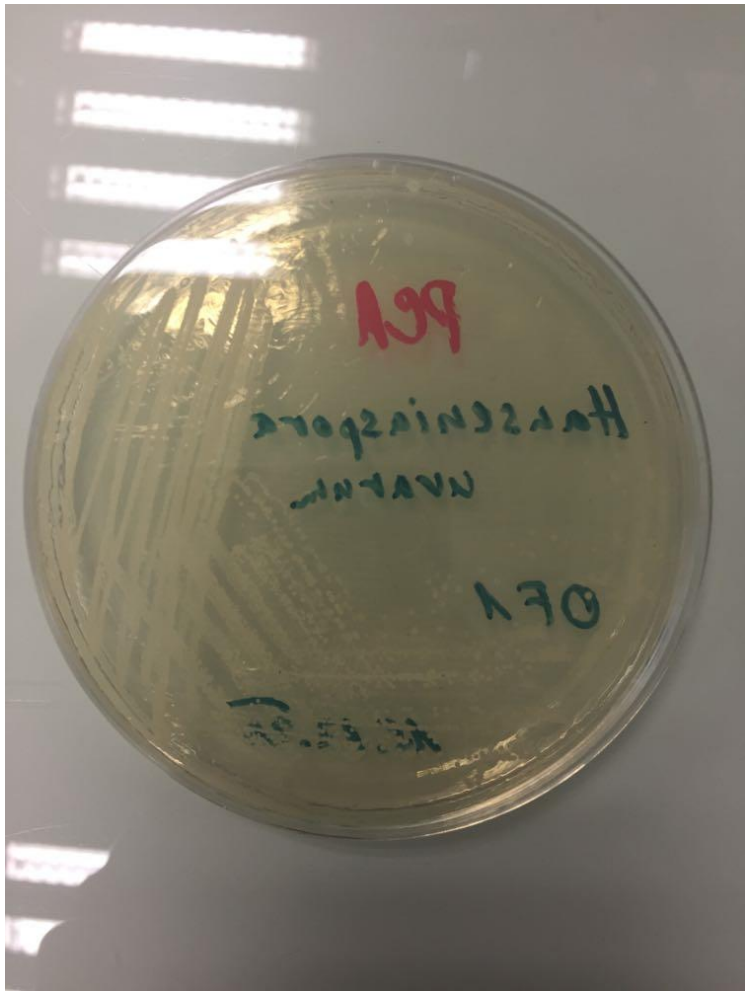
SDB – Sabouraud Dextrose Broth

Appendix

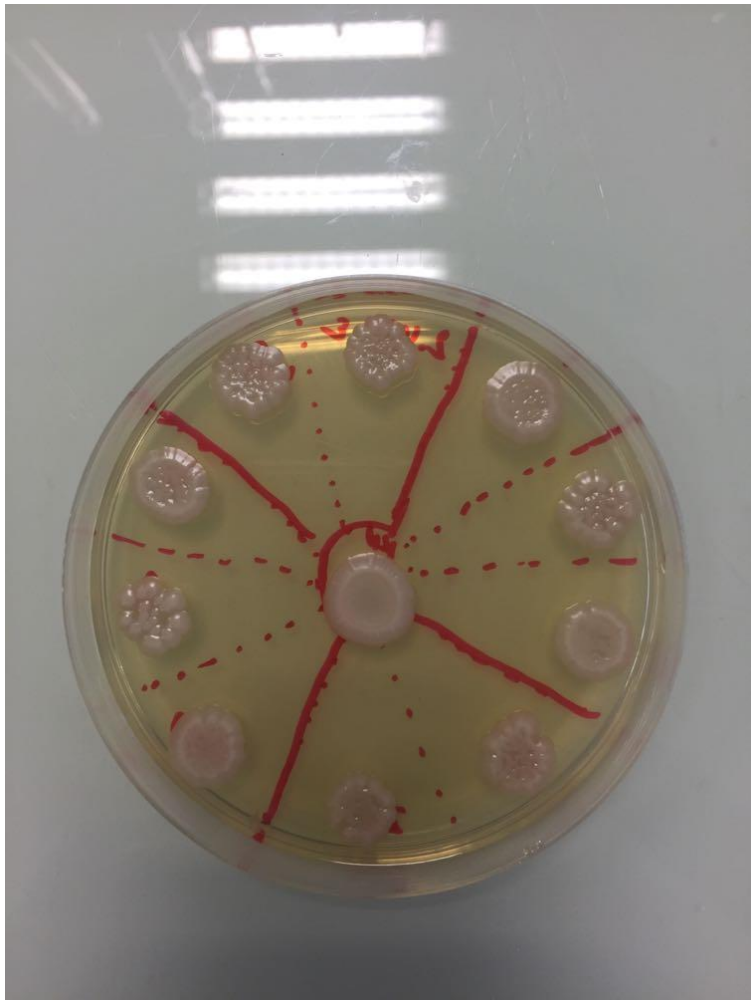
ANNEX I: A rotary evaporator (Heidolph, Schwabach, Germany)

ANNEX II: Lyophilised aqueous bark extracts of Cambodian plants

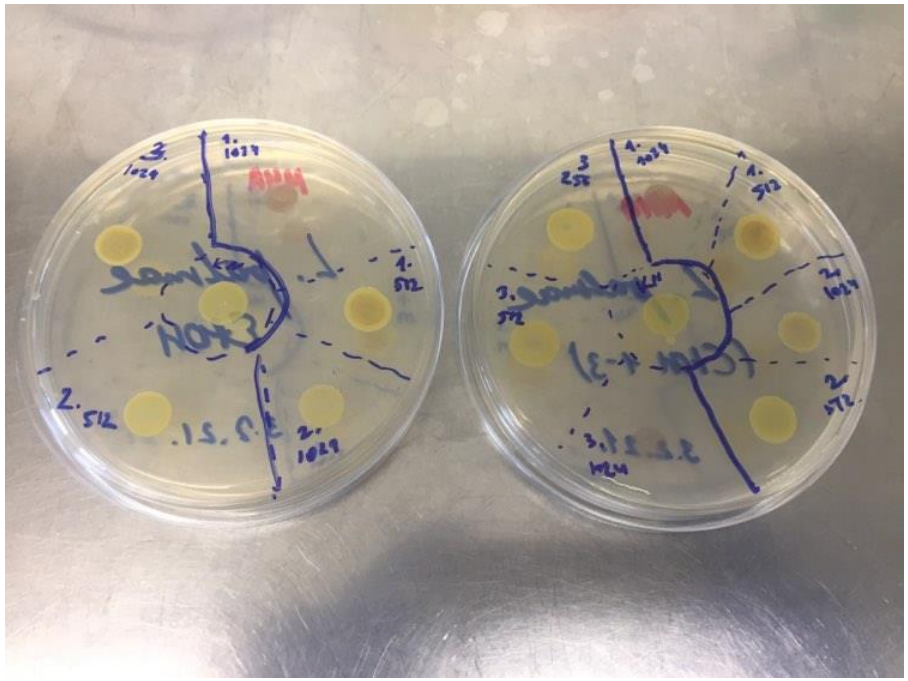
ANNEX III: *Hanseniaspora uvarum* OF1 on a Petri dish



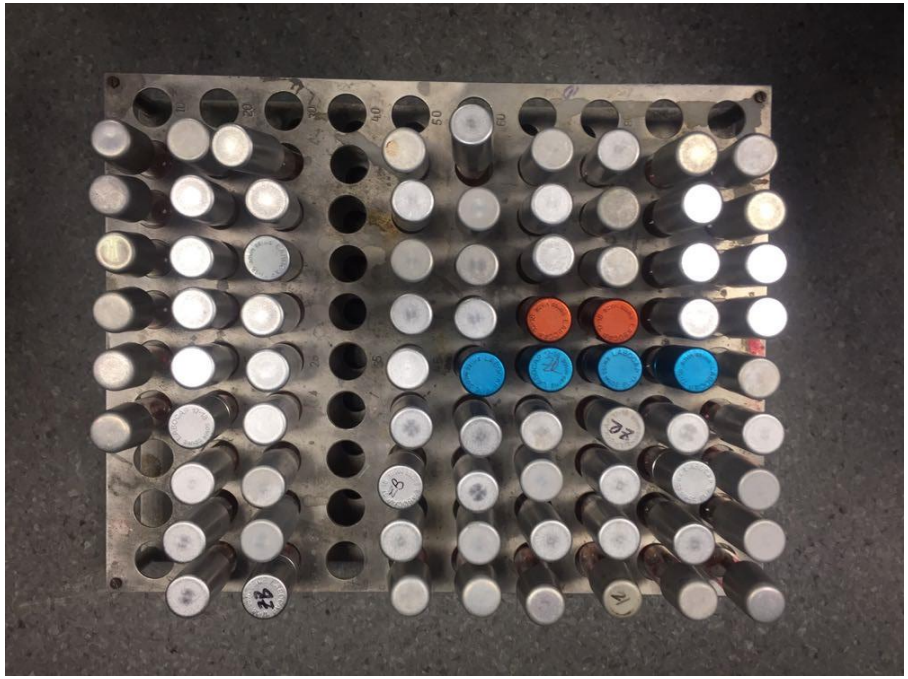
ANNEX IV: Colonies of *Hanseniaspora uvarum* DSM 26650 on a Petri dish (MFC determination)



ANNEX V: Colonies of *Leuconostoc palmae* DSM 21144 on a Petri dish (MBC determination)



ANNEX VI: Tubes with palm sugar solution, apple and grape juice prepared for the fermentation test of *Careaya arborea* and *Azadirachta indica*



ANNEX VII: A refractometer used for determination of sugar concentration

ANNEX VIII: The chromatogram of *Hanseniaspora uvarum* T5, *Saccharomyces cerevisiae* 70465 and *Metschnikowia pulcherrima* T9 obtained from the GC-FID measurement