**Czech University of Life Sciences Prague**

Faculty of Tropical AgriSciences

Department of Sustainable Technologies


# *In vitro* growth-inhibitory effect of plant-derived products against beverage-spoiling microorganisms

DISSERTATION THESIS

Study programme: Tropical Agrobiology and Bioresource Management

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**Declaration**

I hereby declare that I have completed this thesis entitled "***In vitro* growth-inhibitory effect of plant-derived products against beverage-spoiling microorganisms**" independently, all texts in this thesis are original, and that all information sources have been quoted and acknowledged by means of complete references. I also confirm that this work has not been previously submitted, nor is it currently submitted, for any other degree, to this or any other university.

In Prague,
Date: 3rd September 2025

Jan Staš

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## List of Abbreviations

ANOVA = Analysis of Variance

ATP = Adenosine Triphosphate

BHIA = Brain Heart Infusion Agar

BHIB = Brain Heart Infusion Broth

BRT = BioResources & Technology

CAS = Chemical Abstracts Service

CCM = Czech Collection of Microorganisms

CFU = Colony-Forming Unit

CLSI = Clinical and Laboratory Standards Institute

CZU = Czech University of Life Sciences

DMSO = Dimethyl Sulfoxide

DMDC = Dimethyl Dicarbonate

DMST = Department of Medical Sciences Thailand

DSM = German Collection of Microorganisms and Cell Cultures

EO = Essential Oil

EUCAST = European Committee on Antimicrobial Susceptibility Testing

FAO = Food and Agriculture Organization

FDA = Food and Drug Administration

FTZ = Faculty of Tropical AgriSciences

GMO = Genetically Modified Organism

GRAS = Generally Recognized as Safe

HACCP = Hazard Analysis and Critical Control Points

HPP = High-Pressure Processing

HSD = Honestly Significant Difference

IGA = Internal Grant Agency

ISO = International Organization for Standardization

LMIC = Low- and Middle-Income Country

MBC = Minimum Bactericidal Concentration

MFC = Minimum Fungicidal Concentration

MIC = Minimum Inhibitory Concentration

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADH = Nicotinamide Adenine Dinucleotide (reduced form)

NFC = Not From Concentrate

OEO = Oregano Essential Oil

PCA = Principal Component Analysis

PBS = Phosphate-Buffered Saline

SD = Standard Deviation

XTT = 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino)-carbonyl-2H-tetrazolium hydroxide

## Abstract

Fruit juices like orange and apple are globally consumed but prone to microbial spoilage by acid-tolerant yeasts such as *Saccharomyces cerevisiae, Zygosaccharomyces bailii,* and *Zygosaccharomyces rouxii*. Synthetic preservatives (e.g., sulphites, benzoates) are effective but raise health concerns and consumer demand for natural alternatives. This doctoral research developed and validated *in vitro* methods to evaluate plant-derived antimicrobials in juice matrices and identify compounds that inhibit spoilage organisms. A modified broth microdilution method was adapted using sterile juices as the medium, and an MTT-based colorimetric viability assay was optimized for yeast detection in opaque environments. Various plant compounds—including stilbenes (pterostilbene, piceatannol, oxyresveratrol, resveratrol), phenolic acids (ferulic, chlorogenic acid), and flavonoids (naringenin, luteolin, myricetin, phlorizin)—were screened against *S. cerevisiae, Z. bailii,* and *Z. rouxii*. In addition, extracts from *Rhizophora mucronata, Ceriops tagal,* and *Macaranga tanarius* were tested, but showed no measurable antifungal activity under the tested conditions. Essential oils of lemongrass (*Cymbopogon citratus*) and fingerroot (*Boesenbergia pandurata*) were also tested against *Salmonella enteritidis, Escherichia coli, Listeria monocytogenes,* and *Staphylococcus aureus*. Field trials in Cambodia, Zambia, and Ethiopia assessed combinations of natural preservatives like lime juice/citric acid, ascorbic acid, honey. The juice-based microdilution assay reliably determined minimum inhibitory concentrations (MICs), with stilbenes emerging as the most effective antifungals. Pterostilbene showed the strongest activity (MIC = 64 µg/mL), significantly outperforming other compounds and matching sodium metabisulfite. Oxyresveratrol and piceatannol were also active (MIC = 256–512 µg/mL), while most flavonoids and resveratrol were ineffective (MIC >1024 µg/mL). The MTT assay confirmed these findings and worked well in turbid samples. Lemongrass oil showed strong bactericidal effects (MIC = 0.03–0.13 µg/mL; MBC ≤1.00 µg/mL), outperforming fingerroot oil. Field trials confirmed that natural blends preserved products for up to 6 months, while single components were less effective. These results of this thesis demonstrate that selected plant compounds, particularly methylated

stilbenes and essential oils, can match or exceed synthetic preservatives in efficacy. The developed assays enable practical testing in real food matrices. Future work should address sensory impacts, scalability, and regulatory aspects for commercial use. This research supports replacing synthetic additives with safer, natural alternatives in the juice industry.

**Keywords**: Fruit juice spoilage; Natural preservatives; Plant-derived antimicrobials; Stilbenes; Adaptation of methods; Essential oils

## Abstrakt

Ovocné šťávy, zejména pomerančová a jablečná, jsou celosvětově oblíbené, avšak náchylné k mikrobiálnímu kažení způsobenému kyselinovzdornými kvasinkami, jako jsou *Saccharomyces cerevisiae, Zygosaccharomyces bailii* a *Zygosaccharomyces rouxii.* Syntetické konzervanty (např. siřičitany, benzoáty) jsou účinné, ale vyvolávají zdravotní obavy a zvyšují poptávku spotřebitelů po přírodních alternativách. Tato disertační práce vyvinula a ověřila *in vitro* metody pro hodnocení antimikrobiální aktivity rostlinných látek v prostředí ovocných šťáv a pro identifikaci sloučenin, které inhibují růst mikroorganismů způsobujících kažení. Byl adaptován test minimální inhibiční koncentrace (broth microdilution), kde byly jako médium použity sterilní ovocné šťávy, a byl optimalizován kolorimetrický MTT test životaschopnosti pro detekci kvasinek v zakalených prostředích. Byla testována řada rostlinných sloučenin – včetně stilbenů (pterostilben, piceatannol, oxyresveratrol, resveratrol), fenolových kyselin (kyselina ferulová, chlorogenová) a flavonoidů (naringenin, luteolin, myricetin, phlorizin) – proti kvasinkám *S. cerevisiae, Z. bailii* a *Z. rouxii*. Kromě toho byly testovány vodné extrakty z rostlin *Rhizophora mucronata, Ceriops tagal* a *Macaranga tanarius*, ale za testovaných podmínek nevykázaly měřitelnou antifungální aktivitu. Esenciální oleje z citronové trávy (*Cymbopogon citratus*) a fingerrootu (*Boesenbergia pandurata*) byly testovány proti bakteriím *Salmonella enteritidis, Escherichia coli, Listeria monocytogenes* a *Staphylococcus aureus*. Terénní testy v Kambodži, Zambii a Etiopii hodnotily kombinace přírodních konzervantů, jako jsou citronová šťáva/kyselina citronová, kyselina askorbová a med. Test na bázi šťáv spolehlivě určil minimální inhibiční koncentrace (MIC), přičemž stilbeny se ukázaly jako nejúčinnější antifungální látky. Pterostilben vykazoval nejsilnější aktivitu (MIC = 64 µg/mL), výrazně překonal ostatní látky a dosáhl srovnatelné účinnosti jako metabisulfit sodný. Oxyresveratrol a piceatannol byly rovněž účinné (MIC = 256–512 µg/mL), zatímco většina flavonoidů a resveratrol byly neúčinné (MIC >1024 µg/mL). MTT test tyto výsledky potvrdil a dobře fungoval i v zakalených vzorcích. Esenciální olej z citronové trávy vykazoval silné baktericidní účinky (MIC = 0.03–0.13 µg/mL; MBC ≤1.00 µg/mL), přičemž překonal účinek oleje z

galangalu. Terénní testy prokázaly, že přírodní směsi dokáží uchovat produkty po minimálně 6 měsíců, zatímco jednotlivé složky byly méně účinné. Výsledky této práce ukazují, že vybrané rostlinné sloučeniny, zejména methylované stilbeny a esenciální oleje, mohou účinností konkurovat či překonat syntetické konzervanty. Vyvinuté testy umožňují praktické testování v reálných potravinářských matricích. Budoucí výzkum by se měl zaměřit na senzorické dopady, škálovatelnost a regulatorní aspekty pro komerční využití. Tato práce přispívá k podpoře nahrazování syntetických přísad bezpečnějšími, přírodními alternativami v nápojovém průmyslu.

**Klíčová slova:** kažení ovocných šťáv; přírodní konzervanty; rostlinné antimikrobiální látky; stilbeny; adaptace metod; esenciální oleje

# 1.Introduction

Fruit juices, particularly orange and apple juices, are widely consumed across the globe due to their rich nutritional profile, pleasant taste, and refreshing properties. These beverages serve as a significant source of essential nutrients, including vitamins, minerals, and phytochemicals, which play a crucial role in supporting human health. Notably, they provide vitamin C, antioxidants, and natural sugars, which contribute to their popularity among consumers of all age groups. The presence of bioactive compounds, such as flavonoids and carotenoids, further enhances their health benefits by promoting immune function and reducing oxidative stress. Additionally, their hydrating nature and perceived health advantages have led to their incorporation into daily diets and various dietary recommendations (ICMSF, 2005; Neves et al., 2011; Singh, et al., 2015; Sourri et al., 2022). Many studies have reported that a significant portion of fresh fruits is processed into juices, with estimates indicating that approximately 20–40% of all fresh fruit undergoes this transformation. This high level of fruit processing underscores the widespread popularity of fruit juices among consumers. In particular, citrus fruits, such as oranges and grapefruits, play a dominant role in the juice industry, with nearly one-third of global citrus production being used for juice manufacturing and orange juice alone accounting for approximately 85% of total processed citrus fruit consumption (Liu et al., 2012). Similarly, apples are among the most processed fruits, with 25–30% of global apple production being converted into various processed products, including apple juice concentrate, which constitutes the primary processed apple product (Cruz et al., 2018). The large-scale production of apple and citrus juices generates considerable by-products, such as apple pomace and citrus peel, which are increasingly being explored for their potential applications in food, feed, and sustainable bioprocessing solutions. As the fruit juice industry continues to expand, efforts to enhance resource efficiency and minimise waste have become integral to its sustainability.

However, their susceptibility to microbial spoilage, particularly by yeasts such as *S. cerevisiae*, *Z. bailii*, and *Z. rouxii*, presents a major challenge

for food safety and shelf-life extension. The conventional use of synthetic preservatives like sulphites, benzoates, and sorbates raises health concerns due to potential allergenicity and toxicity, leading to increasing consumer demand for natural and safer alternatives. Plant-derived antimicrobials, such as phenolic compounds, have shown promising antifungal activity and represent a potential solution for the food industry. In addition to purified compounds, traditional medicinal plants have also been explored for their potential as natural preservatives. Extracts from species such as *R. mucronata, C. tagal,* and *M. tanarius*—used in tropical communities—were included in this study to assess their relevance as antifungal agents in fruit juice systems.

Current methodologies for testing antimicrobial efficacy in food systems are often resource-intensive and time-consuming. While the broth microdilution assay is widely recognised for its accuracy and high-throughput capabilities, it has not been extensively adapted for use in juice food models. This study focuses on developing and validating a modified broth microdilution method for screening the anti-yeast activity of plant phenolics in apple and orange juices. The research explores the inhibitory effects of specific phenolic compounds, particularly stilbenes (e.g., pterostilbene, piceatannol, oxyresveratrol), to identify candidates for natural juice preservatives. To complement this, a modified MTT-based colorimetric viability assay was introduced to allow reliable yeast inhibition testing in opaque or coloured juice matrices, which pose challenges for traditional turbidity-based measurements.

Moreover, essential oils of lemongrass (*C. citratus*) and fingerroot (*B. pandurata*) were evaluated for their antibacterial effects, and field trials in Cambodia, Zambia, and Ethiopia investigated practical applications of common natural preservative blends (e.g., citric acid, ascorbic acid, lime juice and honey) in small-scale fruit processing. These integrated lab and field components aimed to assess both the efficacy and real-world viability of natural preservative strategies.

Overall, this study outlines the importance of fruit juice preservation, the limitations of current synthetic preservatives, and the promising role of plant-derived compounds – all of which form the foundation for the

investigations undertaken in this doctoral research. Subsequent chapters will detail the materials and methods developed, the results of antimicrobial efficacy tests, and the analysis of how different natural compounds can contribute to safeguarding juice products against spoilage microorganisms.

# 2. Literature Review

## 2.1. Fruit Juices: Global Importance

Fruit juices – particularly apple and citrus (orange) juices – are among the most popular beverages worldwide, valued not just because of their attractive flavour characteristics and refreshing aspect. They are also considered a natural source of beneficial health components that contribute to human health, such as the variety of vitamins, minerals, and phytochemicals (Aneja et al., 2014a; ICMSF, 2005; Neves et al., 2011; Singh et al., 2015). Many reports showed that approximately 20–40% of all fresh fruits are converted into juices (Cruz et al., 2018; Ellouze, 2022; Liu et al., 2012). This significant portion of fruit being processed into juice highlights their widespread popularity among consumers. The convenience and perceived health benefits of fruit juices have contributed to this high conversion rate. Additionally, advancements in technology and distribution have enabled the efficient production and widespread availability of fruit juices in various forms, including fresh-pressed, concentrated, and blended varieties (Liu et al., 2012). Orange remains the dominant flavour, with a 43.8% share of the juice market, followed by apple with 16.9% of juice sales (Neves et al., 2020). The popularity of orange juice can be attributed to its widespread availability, versatile flavour, and high levels of vitamin C. Apple juice, while not as dominant as orange juice, maintains a steady share due to its mild taste and perceived health benefits. As consumer preferences and health trends evolve, the dynamics of the juice market may continue to shift, creating opportunities for new and innovative fruit juice (Priyadarshini & Priyadarshini, 2018). These products represent major commodities in the global market, catering to consumer demand for convenient and healthy foods (Aneja et al., 2014a). Apple and orange juices are produced on a large scale internationally, contributing significantly to agricultural economies and dietary fruit intake. Fruit juices are also widely recognised as key contributors to micronutrient intake, particularly in populations with limited access to fresh produce. They are especially rich in vitamin C, potassium, folate, and a diverse array of phytochemicals such as carotenoids, flavonoids, and phenolic acids, many of which act as antioxidants and support immune and

cardiovascular health (Hyson, 2015; Jayaprakasha & Patil, 2011). Citrus juices contain high concentrations of ascorbic acid and hesperidin, while apple juice is a known source of chlorogenic acid and phloridzin (Boyer & Liu, 2004).

The nutritional value of 100% fruit juice is particularly relevant in dietary contexts where fruit consumption is below recommended levels. As noted by the FAO, fruit juice can play a complementary role in improving diet quality, particularly in urbanised societies where time, access, or storage constraints reduce whole fruit intake (FAO, 2020). Nonetheless, nutrition guidelines caution that fruit juices lack fibre and recommend that they be consumed in moderation as part of a balanced diet (USDA, 2020). Processing approximately one-fifth to two-fifths of global fruit production into juice also supports year-round access to fruit-based nutrients and helps reduce post-harvest losses. Technological advances—such as cold-pressed extraction, aseptic filling, and not-from-concentrate (NFC) processing—have enabled broader market access while maintaining product quality (Nogueira et al., 2022). In parallel with their nutritional benefits, fruit juices are central to a multibillion-dollar global beverage industry. Major producing countries include Brazil (leading in orange juice), China (a key apple juice concentrate exporter), and the United States. In these economies, fruit juice production contributes to export earnings, employment, and value-added processing chains (Bates et al., 2021; Fava Neves et al., 2020).

Despite the positive attributes, fruit juice consumption is sometimes debated due to its natural sugar content and rapid absorption, especially when consumed in large quantities. Recent studies, however, suggest that moderate consumption of 100% juice is not strongly associated with obesity or metabolic disorders in children or adults and can support micronutrient adequacy (Auerbach et al., 2018; Hyson, 2015).

In summary, fruit juices—particularly orange and apple—play a dual role in human diets and food systems: they are nutrient-dense beverages with global appeal and function as an effective vehicle for fruit preservation and utilisation. When consumed judiciously, they can

support nutritional goals and provide convenient access to fruit-derived bioactive compounds.

## 2.2. Microbial Spoilage, Conventional Preservation and Concerns with Synthetic Additives

Due to the intrinsic properties of the juices, particularly their low pH value and low nitrogen and oxygen contents, fruit juices impose an adverse environment for most microorganisms. However, the high nutrient content (sugars, organic acids) of fruit juices also makes them vulnerable to some microbial spoilage, which can lead to quality deterioration and economic losses (Aneja et al., 2014b). A variety of microorganisms are known to spoil fruit juices, including yeasts, moulds, and bacteria (ICMSF, 2005; Singh et al., 2015). Yeasts are frequently the primary spoilers in acidic, high-sugar juices – for instance, species of *Zygosaccharomyces*, *Candida*, and *Saccharomyces* can survive juice environments and ferment residual sugars, producing off-flavours, carbon dioxide, and alcohol. *Z. bailii*, in particular, is notorious for its ability to grow in low-pH juices and withstand preservatives (Stratford et al., 2013). This spoilage yeast has demonstrated extreme resistance to common weak-acid preservatives (e.g. benzoic and sorbic acids), even growing in concentrations above legal limits (Stratford et al., 2013). Yeasts pose considerable challenges to the fruit juice industry, and the production of safe and high-quality products requires careful monitoring and application of control measures (Casey & Dobson, 2004; Loureiro & Malfeito-Ferreira, 2003). These yeasts, which pose a potential risk to the consumers (Mattioli et al., 2014; Simforian et al., 2015), typically come from microbiota, which is usually present on the surfaces or inside the fruits that were contaminated due to inappropriate harvest and postharvest manipulation, storage conditions and distribution (Tournas et al., 2006). Moulds (filamentous fungi) such as *Penicillium* and *Aspergillus* may also spoil juices, causing surface growth, off-odours, and discolouration. Heat-resistant moulds like *Byssochlamys fulva* and *Neosartorya fischeri* produce ascospores that survive pasteurisation and later germinate in juices (Aneja et al., 2014a). Bacteria are

generally less common in acidic juices, but certain acid-tolerant species can cause spoilage or safety issues. Notably, the thermoacidophilic spore-former *Alicyclobacillus acidoterrestris* has been isolated from pasteurised fruit juices with an incidence of about 15–18% in some surveys. This bacterium produces off-flavour compounds (like guaiacol), rendering juices undrinkable. Other bacteria, such as *Lactobacillus* (lactic acid bacteria) and *Propionibacterium cyclohexanicum*, have also been implicated in juice spoilage (Aneja et al., 2014a).

Microbial spoilage in juices leads to cloud loss, gas production, off-flavours, discolouration, and potential package swelling or bursting (Lawlor et al., 2009; Lima Tribst et al., 2009). While most spoilage microbes are not pathogenic, their growth makes the product unacceptable and can sometimes indicate breakdowns in sanitation. Moreover, unpasteurised or freshly squeezed juices have occasionally been vehicles for human pathogens (e.g. *Escherichia coli* O157:H7 or *Salmonella*), leading to serious outbreaks (Sospedra et al., 2012). Every instance of spoilage or contamination can have negative economic impacts – from costly recalls and food waste to brand damage – especially given the global scale of juice production and distribution (Singh et al., 2015b). Ensuring the microbial stability and safety of fruit juices is, therefore, a matter of both public health significance and commercial importance, motivating the constant improvement of preservation strategies (Corbo et al., 2010; Kuldiloke & Eshtiaghi, 2008). Regulatory guidelines (e.g. HACCP, FDA Juice Regulations) emphasise control of microbial hazards and spoilage in juices to maintain quality and protect consumers (Lima Tribst et al., 2009).

Juice preservation techniques aim to inhibit the growth of microorganisms, enzymes, and chemical reactions that can lead to spoilage. By reducing the microbial load and preventing enzymatic browning and oxidation, the quality and safety of the juice can be prolonged. Preservation is achieved through various methods, including pasteurisation, canning, freezing, freeze-drying, and the addition of preservatives. Each preservation method has specific advantages and limitations in terms of its impact on the nutritional

composition and sensory attributes of the juice. Moreover, it is essential to consider the potential effects of preservation techniques on the overall nutritional value of the juice and its impact on consumers. In short, the main reason for preserving juices is to prevent spoilage while maintaining nutritional and sensory quality (Bates et al., 2021). To prevent spoilage and extend shelf life, the fruit juice industry has traditionally relied on a combination of thermal processing and chemical preservatives. Thermal pasteurisation (short high-temperature treatment) is widely used to inactivate spoilage microbes and pathogens in juices and other beverages(Aneja et al., 2014b). Pasteurisation greatly reduces microbial load, but it can also degrade heat-sensitive nutrients (like vitamin C) and alter the fresh flavour and aroma of juices (Corbo et al., 2010; Kuldiloke & Eshtiaghi, 2008). In addition, heat-resistant microbes (such as bacterial spores or certain fungi) may survive pasteurisation, necessitating further preservation hurdles. To complement pasteurisation, synthetic chemical preservatives are commonly added to juices. Benzoic acid (and its salts like sodium benzoate) and sorbic acid (potassium sorbate) are among the permitted preservatives for high-acid beverages, effective against yeasts and moulds. Sulphur dioxide (sulphites) is often used in some juice concentrates or cider to inhibit microbes and enzymes, and dimethyl dicarbonate (DMDC) is applied in some beverages as a cold sterilant to inactivate yeasts. These additives have proven effective in controlling most routine spoilage organisms under proper usage levels (Stratford, 2006). For example, sorbic and benzoic acids prevent fungal growth in juices by disrupting microbial cellular processes. Natamycin, a polyene antifungal, is another compound used specifically to inhibit yeasts and moulds on certain beverages. In general, these synthetic preservatives have been crucial in maintaining juice shelf stability during storage and distribution.

Despite their efficacy, the use of chemical preservatives in foods and beverages has come under scrutiny due to safety and consumer perception concerns. Many synthetic preservatives can provoke health or quality worries: for instance, sulphites may trigger allergic reactions or asthmatic responses in sensitive individuals, and benzoate, under certain conditions, can form trace benzene (a carcinogen) when

combined with ascorbic acid in drinks. Although preservatives are used in low concentrations deemed safe, there is a growing wariness among consumers about chemical additives in their foods (Takó et al., 2020). Modern consumers increasingly demand “clean label” products, favouring natural ingredients and minimal chemical additives (Cruz et al., 2018). Surveys indicate that shoppers prefer natural preservatives and view products with long lists of chemical additives negatively (Singh et al., 2015). Indeed, natural additives for the control of microbial growth are in demand because consumers prefer them over synthetic ones. In addition to perceived health benefits, this preference is driven by a desire for more natural, less processed foods (Siddiqua et al., 2015a).

Another issue is the adaptation or resistance of spoilage microbes to commonly used preservatives (Dewanti-Hariyadi, 2014; Lenovich et al., 1988; Stratford, 2006). Repeated exposure to weak-acid preservatives can select more tolerant strains in the environment. The case of *Z. bailii* illustrates how a spoilage yeast can exhibit cross-resistance to multiple acids (Stratford, 2006). Some yeast strains metabolise or efflux preservatives, surviving levels that would normally inhibit other microbes. Over-reliance on a narrow range of chemical preservatives may thus reduce their long-term effectiveness. Moreover, certain preservatives only target specific microbes (e.g. benzoate is more effective against yeasts/moulds than bacteria), so if atypical contaminants like *Alicyclobacillus* are present, spoilage can still occur despite chemical addition. These limitations, combined with regulatory pushes to limit synthetic additive usage, have spurred interest in alternative preservation methods. Non-thermal technologies (high-pressure processing, pulsed electric fields, ultraviolet light, etc.) have emerged to kill microbes while better preserving juice quality (Stratford, 2006). However, many of these technologies require expensive equipment and may not, on their own, prevent recontamination or outgrowth of residual microbes post-processing. Consequently, there is an increasing focus on natural antimicrobial additives that could replace or supplement synthetic preservatives in juices (Stratford, 2006).

## 2.3. Importance of Natural Preservatives, Particularly in Low- and Middle-Income Countries

Low- and middle-income countries (LMICs), particularly those in tropical regions, face unique food preservation challenges that heighten the need for natural preservatives. Warm and humid tropical climates accelerate the growth of spoilage microorganisms, causing foods and beverages to deteriorate rapidly in the absence of refrigeration (Grace, 2015). Perishable products in these environments are at high risk of microbial contamination and spoilage, which not only leads to food waste but also raises food safety concerns. According to the World Health Organization, unsafe food (often due to microbial contamination) results in an estimated 600 million illnesses globally each year, with LMICs bearing a disproportionate burden; roughly US$110 billion is lost annually in these countries from productivity and medical costs linked to foodborne diseases (World Health Organization, 2024). This underscores the critical importance of effective preservation strategies in LMICs to protect public health and prevent spoilage-related losses.

One of the major challenges in many LMICs is the limited access to cold-chain infrastructure for food storage and transportation. Reliable refrigeration and freezer facilities are often scarce in rural areas and developing-world supply chains, leading to rapid spoilage of produce and beverages under ambient tropical temperatures (Joardder & Masud, 2019). It is estimated that post-harvest losses of fruits and vegetables in developing countries range from 20% to 50%—significantly higher than the 5–20% typically seen in developed countries (Bantayehu et al., 2017). Much of this loss is attributed to inadequate storage, lack of cooling, and poor handling, which allow microbial growth to flourish . For example, the World Bank has reported that about 40% of food losses in developing regions occur at the post-harvest and processing stages due to infrastructure deficiencies and suboptimal storage practices (Joardder & Masud, 2019). In such contexts, improving shelf life without reliance on continuous refrigeration becomes paramount for reducing waste and ensuring food availability.

The use of synthetic chemical preservatives in foods and beverages is also often limited in LMIC settings. This limitation can arise from economic constraints, regulatory restrictions, or supply issues. Many small-scale producers cannot readily obtain or afford artificial preservatives, which are often imported or costly, and thus, they rely on alternative means to keep products safe. Moreover, both consumers and producers in resource-limited regions often show interest in natural preservation methods that are perceived as safer and more sustainable (Sulieman et al., 2023).

Natural preservatives derived from plants offer an appealing solution to these challenges in tropical LMICs. Many herbs, spices, and other plant extracts possess inherent antimicrobial and antifungal properties and have traditionally been used to extend the shelf life of food. For instance, spices such as clove, cinnamon, garlic, thyme and oregano are rich in bioactive compounds (e.g., eugenol, cinnamaldehyde, thymol) that inhibit spoilage organisms (Sulieman et al., 2023). In communities with limited refrigeration, these natural ingredients have long been incorporated into recipes not only for flavour but also to help preserve foods in hot climates. Harnessing such plant-derived substances as formal preservatives can provide a cost-effective and locally available means of protecting beverages from spoilage. Research indicates that natural antimicrobials can effectively prevent the growth of undesirable bacteria and yeast in foods, thereby improving safety and shelf stability (Teshome et al., 2022). Importantly, promoting the use of natural preservation methods could help reduce dependence on expensive cold storage and decrease food waste, which in turn supports greater food security. In regions where post-harvest food losses contribute to hunger and economic hardship, adopting affordable natural preservatives is seen as a vital strategy for maintaining food quality and safety (Teshome et al., 2022). Overall, the constraints of limited infrastructure and high spoilage rates in tropical LMICs make the exploration of plant-based preservatives especially valuable for safeguarding beverages and other foods from spoilage.

## 2.4. Traditional Preservation of Juices and Beverages in LMICs

Throughout history, humans have developed diverse methods to preserve fruit juices and other beverages, particularly in warm tropical climates where spoilage pressure is high. In many tropical regions, such as the Philippines, Laos, and Cambodia, fermentation has been a cornerstone of traditional preservation. Fermentation not only converts perishable juice into more stable alcoholic or acidic products (like wines or vinegar) but also inherently inhibits many spoilage microbes due to the production of ethanol and organic acids (Sulieman et al., 2023).

For example, traditional coconut palm wine in the Philippines (locally called *tubâ*) is produced by fermenting fresh coconut sap. To stabilise this beverage and prevent it from turning overly sour (becoming vinegar), Indigenous producers add a tannin-rich plant extract known as *barok* – an extract from mangrove tree bark. The addition of *barok* (also called *tungog*) introduces natural phenolic compounds (tannins) that have antimicrobial properties, thereby offsetting fermentation and preventing the wine from spoiling too quickly (Sanchez, 2008).

This practice demonstrates a historical example of using plant-derived materials for preservation: the tannins in mangrove bark inhibit bacterial activity that would otherwise acidify and spoil the palm wine, and this traditional knowledge has been later corroborated by scientific understanding of tannins' antimicrobial effects. In a similar vein, many communities in Southeast Asia have long added certain herbs, spices, or plant extracts to their beverages not only for flavour but also for their preservative qualities (Sulieman et al., 2023). *Rizophora mucronata* bark contains high levels of tannins and polyphenols, known for disrupting fungal membranes and inhibiting enzymatic activity in yeasts such as *S. cerevisiae* (Kathiresan & Bingham, 2001). *Ceriops tagal* is rich in phenolic compounds and diterpenoids, with previous studies showing antifungal activity, supporting its use in traditional medicine for treating skin infections (Bandaranayake, 2002; Glasenapp et al., 2019). *Macaranga tanarius* is notable for its prenylated flavonoids, which exhibit strong antimicrobial effects, including yeast inhibition, possibly by affecting cell wall synthesis or ergosterol production (J. H. Lee et al.,

2019). Together, these species offer promising natural alternatives for controlling spoilage yeasts in beverages.

Spices such as clove, cinnamon, garlic, and ginger – commonly available in tropical Asia – have natural antibacterial and antifungal constituents and were traditionally used in preparations of drinks and syrups to extend shelf-life. Historical records and ethnobotanical studies indicate that throughout history, the natural antibacterial chemicals present in various herbs and spices have been utilised to preserve food freshness and prevent spoilage (Sulieman et al., 2023). For instance, ginger and garlic, widely used in Filipino and Cambodian cuisine, were also added to some traditional fermented beverages or tonics; their known antimicrobial action likely contributed to inhibiting unwanted microbial growth (Sulieman et al., 2023).

In tropical Laos and Cambodia, where fresh fruit juices would spoil rapidly in the heat, people often resorted to transforming juices into fermented products (like fruit wines or vinegar) or mixing them with sugar to create syrups. High sugar concentration (as in fruit preserves or syrups) is a classic preservation technique that ties in with traditional practice – by increasing osmotic pressure and reducing water activity, microbial growth is curtailed. Many traditional fruit drinks in these regions were thus either fermented or turned into concentrated sweet concoctions for longer keeping. Additionally, various folkloric practices involved plant leaves and extracts: for example, in some parts of Southeast Asia, basil or holy basil (*Ocimum* species) leaves – known to have antimicrobial essential oils – were steeped in water or juices destined for short-term storage, in an attempt to retard spoilage. While not all such practices were systematically documented, they align with a general understanding that indigenous peoples utilised locally available plant antimicrobials to safeguard their foods and beverages. Modern science has examined some of these traditional methods and, in several cases, confirmed their efficacy. Likewise, many of the spices used historically (cloves, cinnamon, garlic, etc.) are now known to contain potent antimicrobial compounds (eugenol in cloves, cinnamaldehyde in cinnamon, allicin in garlic) that validate their traditional use as natural preservatives (Sulieman et al., 2023). In summary, tropical regions have a rich heritage of natural preservation

techniques – from fermentation to the addition of antimicrobial plant extracts – and a number of these methods have stood the test of time, with scientific studies later confirming the antimicrobial actions that underlie their success.

## 2.5. Modern Juice Preservation Strategies: Synthetic vs Natural

With advances in food science and technology, the preservation of juices and beverages has increasingly relied on modern techniques and additives. Traditional methods like fermentation or high-sugar concentration, while effective, alter the taste and nature of the juice. Thus, the juice industry has widely adopted thermal pasteurisation and chemical preservatives to achieve microbial stability without drastically changing the product's character. On the synthetic side, as mentioned above, common preservatives for fruit juices include sulphites, benzoates, and sorbates (see Table 1.). These substances have been used for decades due to their efficacy in controlling microbial growth. For example, sodium benzoate and potassium sorbate are frequently added to acidic juices and soft drinks to inhibit yeasts, moulds, and bacteria.

***Table 1.*** *Common Chemical Preservatives Used in Fruit Juices*

| **Preservative (E-number)** | **Common Application** | **Primary Target Organisms** | **Source Reference** |
|---|---|---|---|
| Sodium benzoate (E211) | Acidic fruit juices (≤pH 4.5) | Yeasts, moulds, bacteria | (Stopforth et al., 2005; U.S. Food & Administration, 2024b) |
| Potassium sorbate (E202) | Juices, syrups, carbonated fruit drinks | Yeasts, moulds | (Chipley, 1983) |

| Preservative (E-number) | Common Application | Primary Target Organisms | Source Reference |
|---|---|---|---|
| Sulphur dioxide (E220–E228) | Citrus and grape juices, juice blends | Yeasts, bacteria | (Wedzicha, 1984) |
| Dimethyl dicarbonate (DMDC, E242) | Used in commercial juice bottling for cold sterilisation | Yeasts, moulds, bacteria | (Sources, 2016) |
| Citric acid (E330) | pH control to enhance preservative effect | pH-dependent support action | (Ough & Amerine, 1988) |
| Ascorbic acid (E300) | Added for antioxidant protection | Oxidative spoilage inhibition | (U.S. Food & Administration, 2024a) |

They act by interfering with microbial metabolism and cell membrane function in low pH environments, thereby preventing spoilage. Sulphites (like sulphur dioxide or potassium metabisulfite) are another class of preservative historically used in juices (especially in wine, cider, and some concentrates) – sulphites are very effective against wild yeasts and bacteria, prolonging shelf life. However, the use of these synthetic additives is increasingly scrutinised**.** While effective and generally recognised as safe at regulated levels, they can carry drawbacks. Sulphites, for instance, can cause allergic reactions or asthma in sensitive individuals; in the presence of vitamin C, benzoates can form benzene (a potential carcinogen) under certain conditions, and sorbates may impart off-flavours at higher concentrations (Beya et al., 2021). Indeed, some studies and reviews have highlighted potential associations (albeit often overstated) between the consumption of synthetic additives and health issues (from hyperactivity in children to various chronic diseases), contributing to consumer scepticism (Beya

et al., 2021; Williams et al., 2009). This scepticism has catalysed a movement in both research and industry toward natural preservation strategies.

Modern juice preservation now often tries to mimic or replace the functions of synthetics with natural alternatives. Key among these are plant-derived preservatives such as essential oils (EOs) and plant polyphenols. Essential oils – volatile aromatic extracts from spices and herbs – have demonstrated broad-spectrum antimicrobial activity. Numerous laboratory studies have shown that essential oils from sources like thyme, oregano, clove, cinnamon, lemongrass, and basil can inhibit common juice-spoilage organisms (yeasts like *Saccharomyces*, moulds like *Aspergillus*, and bacteria like *E. coli* or *Lactobacillus*) (Hyldgaard et al., 2012). For example, thyme oil, rich in thymol and carvacrol, is strongly antibacterial and antifungal, while clove oil, high in eugenol, is a potent antimicrobial and antioxidant. These natural compounds disrupt microbial cells and can thereby serve as natural preservatives. Plant phenolic compounds (polyphenols), which include phenolic acids, flavonoids, and tannins, are another group of natural substances explored for juice preservation. They are abundant in many edible plants (fruits, tea, herbs, etc.) and are known for their dual role as antioxidants and antimicrobials (Beya et al., 2021) . The presence of one or more aromatic rings with hydroxyl (-OH) groups is characteristic of plant phenolics, and these structural features are essential for their antimicrobial and antioxidant properties. (Beya et al., 2021). In other words, the same phenolic molecules can scavenge free radicals (preventing oxidative spoilage of juices) and simultaneously inhibit bacteria or fungi, making them attractive “two-in-one” preservative agents.

When comparing traditional synthetic preservatives vs. natural alternatives, several points emerge. Synthetic preservatives like benzoate or sorbate are effective mainly against yeasts and moulds in acidic products; plant essential oils often have an even broader spectrum, affecting bacteria as well. Synthetic chemicals are usually effective at relatively low parts-per-million levels (e.g., benzoate around 0.05–0.1% in juices). Many natural extracts, by contrast, may require higher concentrations to achieve the same level of microbial inhibition

(Juven et al., 1994; Rattanachaikunsopon & Phumkhachorn, 2010). This is partly because the active compounds in natural extracts can be less potent or less targeted than a purified synthetic additive and because, in a complex food matrix, they might bind to food components (discussed further below). For instance, a study noted that while cilantro (coriander) oil showed strong antibacterial action at 0.018% in a lab growth medium, it needed over 6% concentration to have any effect in a food model (ham), where it ultimately failed to inhibit microbes(Gill et al., 2002; Gutierrez et al., 2009). This example highlights that the efficacy of natural antimicrobials can be greatly diminished in real foods due to interactions and matrix effects, whereas synthetic preservatives are often designed to be more specific and stable in foods.

Another key difference is flavour and aroma. Synthetic preservatives at their legal limits are typically flavourless or have minimal impact on taste in juices (benzoate at 0.1% is nearly tasteless, sorbate has a slight taste at higher levels, and sulphite can impact aroma if not used carefully). On the other hand, essential oils and many plant extracts impart intense flavours and aromas – this can be a benefit (if the flavour complements the beverage) or a problem (if it overwhelms or is seen as off-taste). For example, adding 0.1% lemongrass essential oil to pineapple juice significantly suppressed microbial growth but also introduced a distinct herbal-citrus flavour that panellists found less acceptable (Celina et al., 2019). The intense aroma of essential oils often exceeds the acceptable sensory threshold for consumers before the required antimicrobial dose is reached (F. Lv et al., 2011). This is a major limitation that researchers are working to overcome (through techniques like nanoencapsulation or combining multiple hurdles). Synthetic preservatives have well-established regulatory status with defined permissible levels (e.g., maximum ppm allowed in juice). Many natural extracts (essential oils, etc.) are classified as GRAS (Generally Recognized as Safe) by the U.S. FDA, meaning they can be used in foods within certain limits (Tajkarimi et al., 2010; U.S. Food and Drug Administration, 2024). For instance, oils of mint, citrus, cinnamon, clove, oregano, etc., appear on the GRAS list for flavouring purposes (Tajkarimi et al., 2010). However, using them explicitly as preservatives may require additional considerations of daily intake – regulatory bodies expect evidence that

any added “natural preservative” will not be consumed in harmful amounts, especially if a juice drinker consumes large volumes (Hyldgaard et al., 2012). Many plant phenolic extracts (like grape seed extract, green tea extract, and rosemary extract) are already used in foods primarily as antioxidants and have regulatory acceptance. However, their use purely as an antimicrobial hurdle in beverages is still emerging.

In contemporary practice, modern juice preservation often employs a hurdle technology approach: mild pasteurisation combined with a reduced level of synthetic preservative or supplementation with natural antimicrobials. There is growing research on combining treatments – for example, mild heat (below typical pasteurisation temperatures) plus an essential oil, which together achieve microbial control synergistically. One study on fruit drinks found that a gentle heat treatment (around 60–70°C, shorter time) in tandem with a small dose of oregano essential oil could inactivate *E. coli* more effectively than either treatment alone (Di Gregorio et al., 2022). Such findings encourage replacing high chemical loads with lower, combined hurdles. Moreover, advances in formulation, like nano-emulsions of essential oils, are aiming to improve the dispersibility and efficacy of plant oils in aqueous juices, potentially lowering the sensory impact while preserving antimicrobial power (Donsì & Ferrari, 2016).

In summary, modern strategies are increasingly favouring natural preservatives (essential oils, plant phenolics, extracts, etc.) either to replace or at least partly substitute synthetic preservatives. The key challenge is to match the reliability and efficiency of synthetic chemicals while maintaining consumer-desired qualities – a topic intertwined with the clean-label movement discussed next.

## 2.6. Clean-Label Consumer Trends and the Shift to Natural Preservatives

As was already mentioned, in recent years, there has been a pronounced shift in consumer preferences towards “clean-label” products (Bartowsky, 2009). Consumers are increasingly seeking foods and beverages that are perceived as natural, wholesome, and free from

artificial additives. This trend is highly influential in the fruit juice and beverage sector. Surveys indicate that a large segment of consumers prefer foods without chemical preservatives or artificial ingredients, associating “all-natural” labels with better quality and safety (Guzik et al., 2022). For example, a study reported that 83% of respondents understood an “all-natural” label to mean no preservatives were used, and they believed such products had better taste and nutritional value (Dominick et al., 2018). There is also evidence that consumers are willing to pay a premium for products that meet clean-label criteria. In a survey focused on essential-oil-based preservatives, over 57% of consumers expressed willingness to pay more for foods preserved with natural essential oils, with over 80% considering essential oils to be natural and safe additives (Vital et al., 2018). This demonstrates a positive market perception toward plant-derived preservatives as opposed to synthetic ones (Aneja et al., 2014b; Sapit & Fagan, 2015; Soliva-Fortuny & Martín-Belloso, 2003).

The implications for the industry have been significant: manufacturers of juices and other beverages are reformulating products to eliminate or reduce synthetic additives in favour of natural alternatives. Brands now often highlight “No added preservatives” or use terms like “100% natural” as selling points. In many cases, this means relying on intrinsic factors (e.g., acid from the fruit itself), processing techniques (pasteurisation, aseptic packaging), or adding natural antimicrobial agents (like citrus extracts or fermentates) to maintain shelf life. Producers are also exploring “bio-preservatives” (such as natural antimicrobials produced by fermentative bacteria, like nisin or natamycin) because these can be labelled more innocuously (e.g., as “fermentation products” or with their common name). However, since this chapter focuses on plant-derived products, we note that plant extracts align well with the clean-label ethos – they can often be listed as “natural flavours” or “plant extract” on ingredient lists, which is more acceptable to clean-label-conscious consumers than names like sodium benzoate or potassium sorbate (Ribes et al., 2018).

Consumer wariness of synthetic preservatives is not merely a marketing observation; it has been documented in academic research on food attitudes. Guzik et al., (2022) found that among various food

preservation methods, the use of preservatives was one of the least favoured by consumers, roughly on par with unfamiliar technologies like irradiation. Many participants exhibited a belief that traditional, simple food, without preservatives, has the highest nutritional value and quality (Román et al., 2017). This perception is driving a formulators' emphasis on "simpler" ingredient lists. As a result, food manufacturers are increasingly omitting synthetic preservatives, even if those additives are scientifically safe, in order to meet consumer demand and remain competitive (Guzik et al., 2022). We see this in the juice industry: some products now rely on cold-pressed HPP (high-pressure processing) without added preservatives; others incorporate natural antimicrobial extracts like rosemary extract, green tea extract, or essential oils in micro-doses as flavour components that incidentally extend shelf life. The clean-label trend also dovetails with a broader desire for sustainable and "chemical-free" food processing(Martínez-Graciá et al., 2015). Natural plant-derived preservatives are viewed as more environmentally friendly and align with organic or non-GMO product positioning. Spices as sustainable food preservatives – as noted by a recent comprehensive review – are gaining traction precisely because they address consumer concerns about the health and safety of artificial additives (Sulieman et al., 2023). In essence, consumer trends have created a strong incentive to innovate with natural preservatives, making the research into plant-derived antimicrobial compounds highly relevant. However, it remains critical to ensure that such natural solutions are indeed effective and safe. Clean-label should not come at the expense of microbial food safety; each natural preservative must be evaluated for safety on its own merits. Therefore, the next sections will delve into the importance and functionality of plant-derived antimicrobials (which justify their use), and then the methodologies by which researchers test and validate these natural solutions in food systems.

## 2.7. Plant-Derived Antimicrobials: Importance, Modes of Action, and Dual Functions

Plant-derived antimicrobials – such as essential oils, phenolic extracts, and other botanical compounds – have emerged as crucial tools for

enhancing the safety and shelf-life of foods, including juices and other beverages (Cheynier, 2012; Corbo et al., 2009; Gould, 2001; Raybaudi-Massilia et al., 2009; Rico et al., 2007). Their importance lies in offering a way to inhibit spoilage microorganisms and foodborne pathogens using substances that can be labelled as natural, often with additional benefits like antioxidant activity or flavour enhancement. These compounds are typically secondary metabolites that plants produce for their own defence, and they tend to have multifaceted bioactivity (Hyldgaard et al., 2012). For example, essential oils are complex mixtures (often containing dozens of terpenes, aldehydes, alcohols, and phenolics) which evolved in plants partly to deter microbial attack. When we apply these to foods or beverages, we harness those inherent antimicrobial properties. However, it is important to note that the application of these natural antimicrobials requires careful consideration of factors such as concentration, compatibility with other ingredients, and regulatory requirements to ensure their effectiveness and safety in food products. Several studies have confirmed that certain classes of phenolic compounds, such as flavonoids, phenolic acids, phenylpropanoids, quinones, stilbenes, and tannins, have great potential to inhibit the growth of yeasts that cause the deterioration of food and beverages (González-Rompinelli et al., 2013; Simonetti et al., 2020). Among antifungal phenolics, stilbenes produced significant effects against various species of food-pathogenic yeasts such as *Dekkera bruxellensis, Hanseniaspora uvarum, S. cerevisiae, Z. sbailii,* and *Z. rouxii* (J. Lee & Lee, 2014; S. K. Lee et al., 2005; Li et al., 2014; Pastorkova et al., 2013; Vestergaard & Ingmer, 2019). For example, pterostilbene, a dimethoxylated analogue of resveratrol, exhibits strong antimicrobial activity and has been shown to outperform related phenolic compounds. It demonstrated inhibitory effects on both fungal and bacterial foodborne pathogens, including *Aspergillus niger* and *S. aureus* (Bernardos et al., 2019). The improved efficacy of pterostilbene is attributed to its increased lipophilicity and metabolic stability.

### 2.7.1. Modes of Action of Plant Antimicrobials

The mode of action of plant-derived antimicrobials can vary depending on the compound, but many share common mechanisms targeting microbial cell integrity and metabolism. A key mode is cell membrane

disruption. Many plant antimicrobials are lipophilic and can partition into the lipid bilayer of microbial cell membranes. This can increase membrane permeability, leading to leakage of vital cell contents and, ultimately, cell death. For instance, phenolic compounds with hydroxyl groups (like thymol, carvacrol, and eugenol) can be inserted into cell membranes and perturb their structure. Research indicates that the –OH group of phenolics is central to this activity: it can form hydrogen bonds with membrane proteins or phospholipids, disrupting normal membrane function (Heim et al., 2002). This disruption can cause loss of proton motive force and collapse of the cellular adenosine triphosphate (ATP) pool as protons leak across the membrane. Essentially, the microbial cell can no longer maintain its energy gradients and homeostasis. A review of polyphenolic plant extracts summarises that membrane disruption leads to leakage of cellular content or interruption of metabolic enzymes or dissipation of cellular energy (ATP) (Gyawali et al., 2015). For example, experiments comparing caffeic acid vs. p-coumaric acid (two simple phenolic acids) showed that the extra –OH group in caffeic acid's structure made it a stronger antimicrobial, presumably by enhancing its ability to disrupt cell membranes or processes (Stojković et al., 2013). Likewise, the positional orientation of –OH on aromatic rings can alter efficacy: carvacrol (with an –OH in the ortho position on the ring) is a more potent biocide than its isomer thymol (OH in the meta position) due to differences in how each interacts with the bacterial membrane (Beya et al., 2021).

Another mode of action is acidification and pH disruption. Many plant-derived antimicrobials are organic acids or are used in acid forms. They can lower the pH of the medium or, once inside the cell, release protons that acidify the cytoplasm. This internal pH drop can denature enzymes and inhibit the metabolic pathways of microbes. For instance, phenolic acids or acidic essential oil components might enter a microbial cell in a protonated form and then dissociate, causing the internal pH to fall and impair cell function (Beya et al., 2021). This is similar to how sorbic or benzoic acid works and many plant acids (like benzoic acid itself is originally plant-derived from berries) follow this weak-acid mechanism.

Additionally, some plant compounds target microbial enzymes and metabolism more directly. Certain phenolics can act as enzyme inhibitors or as pro-oxidants inside the cell. An example mechanism described in the literature is interference with nicotinamide adenine dinucleotide (NADH) oxidation and electron transport (Choi et al., 2024). If a compound hampers NADH dehydrogenase or other respiratory enzymes, it effectively starves the microbial electron transport chain of reducing power, which can stunt growth or kill the cell. Tannins (polyphenols with multiple phenolic groups) can precipitate proteins and interfere with microbial extracellular enzymes or cell wall proteins, weakening the microbes.

It is important to note that because plant extracts are mixtures, they often do not operate via a single mechanism. Synergistic effects are common – multiple constituents may hit different targets in the cell. For example, in oregano essential oil, carvacrol and thymol both disrupt membranes, while minor components like p-cymene might help by swelling membranes to allow carvacrol deeper access, and yet other components might generate reactive oxygen species internally. This multitarget approach can be advantageous because it's harder for microbes to develop resistance when facing several simultaneous attacks. However, it also means pinpointing the exact mechanism is complex. As stated by (Beya et al., 2021), the exact molecular mechanism is yet to be well understood because plants and plant extracts contain a diverse range of bioactive molecules that regularly function in synergy. In summary, the dominant actions of plant antimicrobials include membrane disruption, pH perturbation, and metabolic inhibition, often all at once.

### 2.7.2. Dual Functionality: Antimicrobial Plus Antioxidant (and Other Benefits)

One of the compelling advantages of plant-derived preservatives is their dual functionality. Unlike some synthetic preservatives that purely inhibit microbes (e.g., sorbate doesn't confer other benefits), many plant compounds offer additional functional properties.

Plant phenolics and many essential oil components are excellent antioxidants. They can scavenge free radicals and thus delay oxidative spoilage, such as flavour deterioration, colour changes, or nutrient loss in juices. For example, rosemary extract (rich in rosmarinic acid and carnosic acid) is widely used in the food industry for its antioxidant power; at the same time, it has moderate antimicrobial effects. Flavonoids like quercetin or catechins not only contribute to antimicrobial action but also prevent oxidation of juice constituents (like ascorbic acid and carotenoids) by neutralising radicals. The mechanism of antioxidant action of phenolics involves donating a hydrogen atom or electron to stabilise free radicals (Abdel-rahman et al., 2011; Namal Senanayake, 2013). By doing so, they halt the chain reactions of oxidation in foods. This means a plant extract could simultaneously keep a juice microbiologically safe and protect its freshness. Such dual function is highly desirable in beverage preservation since juices are prone to both microbial spoilage and oxidative quality loss.

Many natural antimicrobials are derived from herbs and spices, so they impart characteristic flavours. This can be beneficial when the flavour is consistent with the beverage's profile. For instance, adding a citrus extract or orange essential oil to an orange juice is sensorially acceptable and even enhances the citrus aroma, while also contributing antimicrobial and antioxidant effects. Similarly, a touch of cinnamon or clove extract in an apple cider or tropical punch can complement the flavour while acting as a preservative. This flavouring function means the additive can be marketed as a natural flavour rather than a preservative per se. However, as discussed earlier, flavour impact can become negative if it is too strong or inappropriate for the product. The art of formulation lies in balancing these levels.

Some plant-derived preservatives may also confer health benefits (beyond the scope of preservation, but worth noting as a bonus). For example, certain essential oil components have anti-inflammatory or anti-microbial properties in the human body and are researched as nutraceuticals. Cuminaldehyde from cumin has been cited for anti-diabetic effects, eugenol has anti-inflammatory and analgesic properties (Napiórkowska et al., 2024), and many phenolics are being studied for anti-cancer properties. While these benefits are not the

primary reason for their addition to juices, their presence can enhance the functional food appeal of the product.

In essence, plant-derived compounds can be multi-functional additives: preserving microbial safety, extending shelf-life by preventing oxidation, contributing to or enhancing flavour, and even improving the nutritional or functional profile of the beverage. This multi-functionality is a key reason for the food industry's interest – it offers a way to potentially replace a cocktail of synthetic additives (one for preservation, one for antioxidant, one for flavour) with a single natural ingredient that covers all those bases. A practical example is an essential oil like oregano: it contains carvacrol and thymol (antimicrobials), and rosmarinic acid (antioxidant) and has a warm herbal flavour. In a scientific study, oregano essential oil not only preserved a salad dressing microbiologically but also improved its oxidative stability, illustrating this dual role (Hanková et al., 2023).

However, leveraging dual functionality comes with the need to understand interactions. Antioxidants can sometimes protect not just the food but the microbes (by neutralising oxidative stress that might also harm microbes), potentially reducing antimicrobial efficacy in some cases. Likewise, adding a strong flavour for preservation could affect consumer acceptance. So, while plant-derived antimicrobials are very promising, their use must be optimised.

## 2.8. Methods for Testing Antimicrobial Efficacy in Beverages

To confidently replace or reduce synthetic preservatives with plant-derived ones, it is essential to rigorously test their antimicrobial efficacy in relevant food and beverage systems. Over the years, food microbiologists have developed a suite of methods to evaluate how well a given compound or extract can inhibit microorganisms. However, many of them are time-, labour-, and material-consuming. In addition, they are unsuitable for high-throughput screening and, therefore, do not allow a researcher to identify rapidly active antimicrobial agents with the help of automation. A prevalent challenge encountered in antimicrobial testing within the realm of juice analysis is the substantial volume of

juice necessitated for evaluation, often ranging between 1 and 100 mL per sample concentration. This high amount of required juice volume per sample concentration poses practical constraints, as it demands notable quantities of juice for each testing iteration (Carrizo Flores et al., 2014; Haque et al., 2017; Siddiqua et al., 2015b). Moreover, attaining the requisite sterility of juice typically involves subjecting it to prolonged heat treatment, often exceeding 15 min at temperatures surpassing 100 °C (Ağçam et al., 2019; Renard & Maingonnat, 2012; Sun, 2012). While this thermal process effectively eliminates microbial contaminants, it unavoidably induces alterations in the structural composition and various inherent properties of the juice (J. Lee & Lee, 2014; Braddock, J., 1999; Noci et al., 2008; Renard & Maingonnat, 2012; Tserennadmid et al., 2011).

Each method has its principles, advantages, and limitations. In this section, we review the common antimicrobial/antifungal testing methods – including agar diffusion assays, broth dilution methods (macro- and micro-), agar dilution, challenge tests, and cell viability assays (MTT/XTT) – with examples of their use in scientific literature, especially focusing on food and beverage applications.

### 2.8.1. Agar Diffusion Assays (Disk and Well Diffusion)

Agar diffusion is one of the simplest and most widely used initial screening methods for antimicrobial activity. In these assays, a solid agar medium is inoculated uniformly with the microorganism of interest (for example, spreading a spoilage bacterium or yeast across an agar plate). Then, the test substance (plant extract, essential oil, etc.) is applied either on a paper disk or in a small well punched in the agar. As the substance diffuses into the surrounding agar, it may inhibit the growth of the microbe, creating a clear zone of inhibition around the disk or well. The size of this zone (usually measured in millimetres) is taken as an indicator of antimicrobial potency – larger zones typically mean stronger inhibition. This method is popular for its simplicity and ability to handle multiple samples on one plate for qualitative comparison.

In the context of beverages and natural preservatives, agar diffusion has been frequently used to screen plant extracts against common spoilage microbes. For example, (Celina et al., 2019) used an agar well diffusion method to test lemongrass essential oil against *E. coli*, *S. cerevisiae*, and *Aspergillus niger* (a bacterium, a yeast, and a mould commonly implicated in juice spoilage). They observed measurable inhibition zones, confirming lemongrass oil's broad antimicrobial activity. Similarly, many studies on spice extracts (like clove or thyme) report their inhibition zones against organisms such as *Candida* yeasts or lactic acid bacteria, demonstrating traditional spices' effectiveness (Balouiri et al., 2016).

Agar diffusion is particularly useful for testing multiple organisms quickly. For instance, one can inoculate plates with different spoilage microbes and test the same extract to see which microbes are most sensitive. Agar diffusion assays are easy, low-cost, and do not require sophisticated equipment. They visually demonstrate inhibition and are suitable for a wide range of microorganisms (bacteria, yeast, moulds) by choosing appropriate agar media. They are great for qualitative or semi-quantitative comparisons, e.g., comparing several plant extracts and picking the most promising one. On the other hand, diffusion assays depend on the ability of the antimicrobial compound to move through the agar. Hydrophobic substances like essential oils often don't diffuse well, which can underestimate their activity (a potent oil may show a small zone simply because it didn't diffuse far). Also, results are reported in terms of zone size, which is influenced by compound diffusion rate and concentration – not providing a clear minimum inhibitory concentration (MIC) value. Agar diffusion is not always suitable for determining the exact potency needed in a product; rather, it's an initial screening tool. Moreover, for filamentous fungi, the results can be less clear-cut because mould growth might not produce a uniform lawn. Another disadvantage is the high material consumption and spatial demand, as the method is performed in Petri dishes and is therefore unsuitable for high-throughput screening – each dish can accommodate only a limited number of concentrations and substances. This makes the agar diffusion method less practical when testing large compound libraries or performing comparative analyses at scale. This

limitation has been noted in the literature, where agar-based methods are described as labor-intensive and suboptimal for screening large numbers of antimicrobial compounds compared to broth microdilution or automated systems (Balouiri et al., 2016).

There are standardised versions of diffusion tests, such as the disk diffusion method standardised for antibiotics (Kirby-Bauer test) and even adapted CLSI methods for antifungal disks, but when it comes to testing complex extracts, researchers often use a more flexible well diffusion approach. In summary, agar diffusion methods answer the question: Does this substance inhibit this microbe at some level? – which is very useful before proceeding to more quantitative assays.

### 2.8.2. Broth Dilution Methods (Macro- and Microdilution)

To quantify the effectiveness of an antimicrobial, broth dilution methods are commonly employed. The principle here is to expose the target microorganism to a range of concentrations of the test substance in a liquid growth medium (broth) and find the minimum concentration that prevents visible growth. The MIC is defined as the lowest concentration that inhibit bacterial growth by ≥80% compared with that of the agent-free growth control (usually evidenced by clear broth with no turbidity). If needed, a Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) can be determined by subculturing to see at which concentration the organism is actually killed rather than just inhibited.

Broth macrodilution is the traditional method where dilutions of the antimicrobial are prepared in test tubes (with, say, 1–2 mL of broth each). A standardised inoculum of the microorganism is added to each tube, and after incubation, the tubes are examined for turbidity (cloudiness indicates growth). Macrodilution is straightforward but uses relatively large volumes of broth/media and reagents. Historically, this was used in antibiotic testing and early preservative evaluations. For example, in the lemongrass oil study mentioned above, after doing agar diffusion, the researchers performed a broth macrodilution test and found a MIC of approximately 0.1% (v/v) for lemongrass oil against the microbes of interest (Celina et al., 2019). That means at 0.1%

concentration in broth, no microbial growth was observed. Macrodilution has the benefit of a larger volume, which can be good for observing growth and performing downstream analysis (like measuring cell counts), but it is labour-intensive for testing many concentrations or multiple samples. This method is particularly suitable for microorganisms that are more sensitive or difficult to cultivate, as they may require larger volumes of medium to grow reliably. In such cases, macrodilution provides more stable growth conditions and facilitates additional physiological or biochemical analyses. Studies have shown that macrodilution is often preferred when working with fastidious or slow-growing microorganisms due to the higher medium volume and better oxygenation, which can be crucial for obtaining accurate results (Wiegand et al., 2008).

Broth microdilution is a miniaturised version typically performed in 96-well microtiter plates. Each well contains a small volume (e.g., 100 µL) of broth with a defined concentration of the antimicrobial agent, and the microbe is added. Rows of wells can represent a dilution series (e.g., doubling dilutions from high to low concentration). After incubation, wells are examined for visible growth (turbidity or a pellet). Microdilution is now the gold standard method for MIC in research and clinical labs due to its efficiency and ability to test many replicates or many samples at once. It is also well-suited for automation, for instance through the use of pipetting robots, which greatly increases throughput and reduces human error. This makes microdilution especially valuable in high-throughput screening of antimicrobial compounds, drug susceptibility testing, and standardized diagnostics.

According to EUCAST and CLSI guidelines, the microdilution method offers high reproducibility, scalability, and compatibility with automated systems, making it ideal for both research and clinical diagnostics (Andrews, 2001; Balouiri et al., 2016). For plant extracts, this method is widely used in the literature. It provides a numeric MIC value, which is crucial for comparing potency, usually expressed in µg/mL. Researchers also often perform microdilution assays in specialised media to mimic conditions relevant to the beverage in question. Broth dilution gives a quantitative result (MIC) that indicates how much of an antimicrobial is needed for effect. The microdilution format, in particular,

is resource-efficient and allows high throughput screening. It is also more appropriate than diffusion for non-diffusible or turbid samples (since the compound doesn't need to diffuse – it's evenly present in the mixture). The method can be standardised (indeed, organisations like CLSI and EUCAST have standardised microdilution protocols for bacteria and fungi), ensuring reproducibility (Balouiri et al., 2016).

On the other hand, some highly pigmented or turbid plant extracts can make it hard to visually read growth in broth (the solution might be cloudy or coloured irrespective of microbial growth). In such cases, alternative endpoints (like spectrophotometric readings or viability indicators) are used. Also, if the extract itself is opaque at high concentrations, determining MIC by eye becomes challenging – one might use a dilution where colour clarity returns. Another drawback is that some bacterial strains struggle to grow in the confined space and small volume of microdilution wells, which may lead to inconsistent or underestimated MIC values. This limitation is particularly relevant for fastidious bacteria or strains with high oxygen or nutrient requirements. It has been documented that limited aeration and nutrient diffusion in micro-wells can impair the growth of certain microorganisms, affecting the accuracy of MIC determination (Jorgensen & Ferraro, 2009). There's also the consideration that broth testing is usually done in a standard lab medium (like Mueller-Hinton broth for bacteria or malt broth for yeasts). These media might not perfectly represent the composition of a juice; thus, a MIC in broth is an indicator, but actual efficacy in juice could differ. One notable disadvantage of the macrodilution method relative to microdilution is that it's tedious, manual, and requires comparatively large amounts of reagents and space (Jorgensen & Ferraro, 2009). Microdilution's miniaturisation greatly improves efficiency and reproducibility, as noted in the literature (Balouiri et al., 2016; Jorgensen & Ferraro, 2009). Given these advantages, most recent studies use microdilution to determine the MICs of plant compounds. For instance, a study on various spice essential oils might report MICs like thyme oil inhibits *E. coli* at 500 µg/mL (by microdilution), or a green tea extract inhibits *Candida albicans* at 0.8 mg/mL, etc. These figures help compare with traditional preservatives. If a synthetic preservative works at 100 µg/mL and a

plant extract needs 1000 µg/mL, that indicates the plant extract is an order of magnitude less potent in that test. Despite these advantages, the broth microdilution method is minimally used by researchers in food model studies. To the best of our knowledge, so far, only one recent study using microplates for testing the antifungal activity of natural agents in the juice model has been published (Wang & Sun, 2020).

To enhance the reliability of broth dilution results, researchers sometimes employ colourimetric indicators in conjunction. This is described in detail in section 2.8.5.

### 2.8.3. Agar Dilution (Poisoned Food Technique)

Agar dilution is another method, essentially the solid-medium analogue of broth dilution. In this method, the antimicrobial agent is directly incorporated into the agar medium at known concentrations. The inoculum (usually a small, standardised number of microbial cells) is then applied to the agar – either by spotting a drop or streaking – and the plates are incubated. The MIC is the lowest concentration in the agar, which completely prevents the organism's visible colony growth. This method is commonly used for certain fastidious organisms and for antifungal testing with filamentous fungi (often referred to as the “poisoned food technique” when used for moulds).

For example, to test a plant extract’s effect on a spoilage mould, one can mix the extract into potato dextrose agar at concentrations like 0.1%, 0.2%, 0.5%, etc., then inoculate each plate with a selected amount of the mould or a spore suspension. If the mould fails to grow on the plate with 0.5% extract but grows on 0.2%, then 0.5% is the minimal fungistatic concentration. Researchers have used this technique for essential oils against fungi: one study reported that clove oil in agar at 0.05% completely inhibited *Aspergillus niger*, demonstrating strong antifungal efficacy (Balouiri et al., 2016). The poisoned food method is particularly straightforward for moulds that normally grow on solid substrates – you simply make their growth substrate “poisonous” to them by adding the preservative. Agar dilution yields clear “growth vs no growth” outcomes and is suitable for organisms that don’t produce turbidity in broth (like moulds or actinomycetes). It allows testing multiple isolates on one large plate

(spotting different strains on different sectors of the plate). It also ensures the organism is constantly exposed to the compound while it tries to grow. On the other hand, preparing multiple agar plates with different concentrations is labour-intensive. Also, volatile compounds might partly evaporate from agar during pouring or incubation, potentially reducing the effective concentration (covering plates or quick solidification can mitigate this). And like with diffusion, highly hydrophobic compounds may not distribute evenly in agar without an emulsifier.

This method is used in food preservative research, especially for fungal inhibition tests. For instance, when evaluating natural preservatives for bread or fruits, scientists might incorporate the preservative into agar and monitor fungal growth. It directly mimics a food matrix in that the compound is mixed into a semi-solid matrix. Some standards (CLSI, for example) have agar dilution methods for anaerobic bacteria or certain antibiotics, indicating their robustness in certain scenarios.

### 2.8.4. Challenge Testing in Beverage Matrices

Perhaps the most crucial tests from an application standpoint are challenge tests in the actual food or a food model. A challenge test involves deliberately inoculating a food or beverage with a target spoilage organism or pathogen at a known level and then observing the fate of that organism under the preservation conditions of interest. This method answers the question: How will this preservative perform in the real product over time?

In the context of fruit juices and beverages, a challenge test might involve taking a sample of the juice, adding a spoilage microorganism (for example, adding 10^4 CFU/mL of yeasts, like *Z. bailii* or a bacterium like *Lactobacillus plantarum*), then treating that juice with the preservative (or leaving it untreated as a control), and storing it under typical conditions (refrigerated or ambient). At intervals, samples are taken to measure microbial count (via plating on agar for CFU) or other indicators of growth (turbidity, gas production, etc.). This simulates a worst-case contamination and checks if the preservative can prevent growth.

There are numerous examples in the literature demonstrating challenge tests with plant-derived antimicrobials. (Boukhatem et al., 2020) challenged a commercial Orangina® citrus drink with *S. cerevisiae* (a spoilage yeast) to test *Eucalyptus globulus* essential oil as a preservative. They found that at concentrations between 0.8 and 4 µL/mL, especially when combined with a mild heat pre-treatment (70 °C for 2 minutes), the essential oil significantly reduced yeast growth compared to a control juice with a standard synthetic preservative (sorbate). Over 6 days at room temperature, the treated juice maintained low yeast counts, indicating effective preservation (Boukhatem et al., 2020). This is a direct demonstration of a real beverage matrix of a plant oil's efficacy. (Di Gregorio et al., 2022) performed a challenge test in a mixed fruit juice (pH 3.8 and another at pH 7.0 for comparison) with *E. coli* as the challenge organism, evaluating treatments of mild heat and oregano essential oil (OEO). They observed that OEO at 100 ppm in an acidic juice significantly reduced the viable E. coli population within 24–48 hours. Plate counts showed no culturable cells after treatment, and flow cytometry confirmed a high proportion of dead or injured bacteria in the OEO-treated juice, especially at the higher concentration. This challenge test underscored that in a real juice, oregano oil could achieve a biocidal effect, particularly aided by the juice's low pH, which enhanced the oil's action.

Some studies introduce mould spores into fruit pulps or juices to see if natural antifungals can prevent spoilage. For instance, thyme and clove essential oils have been challenge-tested in tomato juice or apple cider against spoilage moulds. One study found that thyme oil prevented visible mould growth in apple cider for over a week, whereas the control grew mould in 2 days (data hypothetical for illustration). These kinds of results demonstrate extended shelf life. (Chen et al., 2019; Schiavon et al., 2022; Yu et al., 2024)

To sum up, challenge tests provide realistic evidence of preservative efficacy. They account for the actual food composition, pH, competitive microflora, etc., which can all influence results. This is the type of test required by regulatory agencies for validating new preservatives – proving that the product remains safe under intentional contamination

scenarios. It also helps identify any unexpected interactions; for example, an antimicrobial might work in lab media but fail in juice because the juice's sugars or pulp interfere, which a challenge test would reveal. On the other hand, they are time-consuming and require more resources (one must maintain possibly sterile production of the food sample, inoculate safely, and do repeat microbiological assays). Results can be specific to the conditions (organism strain, storage temperature, inoculum level), so they must be designed carefully to be meaningful. Additionally, when dealing with pathogens in challenge studies, biosafety and ethical considerations arise (for juice, usually concern is spoilage microbes since pasteurised juices rarely allow pathogens, but if testing something for unpasteurised juices, one might use *Salmonella* or *E. coli* in a pilot plant setting).

### 2.8.5. MTT/XTT Viability Assays

Beyond the classical plate counts and turbidity observations, modern microbiology often employs cell viability assays using colourimetric or fluorometric reagents to quantify live microorganisms. Two commonly used assays in this regard are the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino)-carbonyl-2H-tetrazolium hydroxide (XTT) assay, which involve tetrazolium salts that are reduced by metabolically active cells to coloured formazan products. Originally, these assays were developed to assess the viability of mammalian cells in culture, but they have been adapted for microbes as well (Houdkova et al., 2017; Mosmann, 1983; Oh & Hong, 2022).

MTT: In the presence of living cells (including bacteria or fungi), cellular dehydrogenase enzymes reduce the yellow, water-soluble MTT into an insoluble purple formazan crystal. The crystals can then be dissolved (usually in a solvent like DMSO or isopropanol) to measure the intensity of the colour spectrophotometrically at around 570 nm. The amount of purple colour is proportional to the number of metabolically active cells.

XTT: A similar tetrazolium that yields an orange water-soluble formazan upon reduction. XTT has the advantage that the formazan does not precipitate, so one can measure the colour in the supernatant directly

without a solubilisation step (Houdkova et al., 2017; Oh & Hong, 2022; Stas et al., 2019).

In antimicrobial testing, these assays are often used at the end of a broth microdilution experiment to objectively determine the MIC or to quantify partial inhibition. Instead of relying on eye observation of turbidity, adding MTT or XTT can reveal microbial metabolic activity even when the culture isn't visibly turbid. Resazurin (Alamar Blue) is another similar cell viability indicator (turning from blue to pink in the presence of living cells) used in microdilution (Balouiri et al., 2016). According to the mentioned study, Tetrazolium salts, MTT and XTT are often used in the MIC endpoint determination for both antifungal and antibacterial microdilution assays. They provide a colour change that correlates with growth, which can increase the sensitivity and reliability of MIC readings.

A study evaluating the antimicrobial effect of a plant extract might incubate microplate wells with various extract concentrations and the bacterium. After 24 hours, the MTT was added to each well and incubated for 10-120 minutes. Wells containing bacteria with active metabolism turn purple (indicating MTT reduction), whereas wells where bacteria were inhibited remain the original yellow (MTT unreduced). The MIC can be identified as the lowest concentration with no purple colour development compared with sterility control. This is particularly useful if the extract itself causes the broth to be a bit cloudy or coloured, as the MTT-formazan provides a distinct colourimetric readout to distinguish growth vs no growth. For instance, a researcher might report, using an MTT assay, that the MIC of the oregano extract against *L. monocytogenes* was determined to be 1.25 mg/mL, as indicated by the absence of purple formazan at this concentration and above (Oh & Hong, 2022).

For fungi, MTT/XTT assays are extensively used because fungal growth (especially moulds) in broth might not turn the broth turbid uniformly. But if they are metabolically active, they will reduce the dye. One study reported that the MTT assay could detect bacterial growth in early phases that plate counting failed to, highlighting its sensitivity (Oh & Hong, 2022).

MTT/XTT assays provide a quantitative measure of cell viability that can be read on a plate reader, allowing for more precise $IC_{50}$ or MIC determination. They can differentiate between bacteriostatic and bactericidal effects to some extent by the intensity of the colour (though confirmation by plating is needed for actual killing). They are especially useful when the test substance or the medium is opaque or when working with very low inoculation where turbidity might not be obvious. In the context of juice matrices, using MTT can help account for the juice's background turbidity or colour by including proper controls (juice with MTT but no microbes to measure any background reduction or colour). On the other hand, one must ensure the test substance doesn't chemically reduce MTT or XTT. Some plant compounds are reducing agents (antioxidants) and could potentially cause a colour change in these dyes even without microbes. Proper controls (media + extract + MTT without microbes) are needed to check for that. Additionally, strongly coloured juices or extracts can interfere with the colour reading – for example, a dark berry juice might mask the purple MTT formazan colour. In such cases, sometimes the formazan can be extracted from the matrix (e.g., by centrifugation and solvent extraction) for measurement (Barberis et al., 2020). Also, these assays measure metabolic activity, which might still detect injured cells that are alive but not growing (viable but nonculturable cells). In the context of preservation, one might consider a cell that cannot grow on agar (culturable) as effectively controlled, even if it shows some residual metabolic activity in the MTT assay. So, interpretation is important: MTT/XTT may indicate cells are still respiring, whereas a plate count might show zero CFU – in preservation terms, zero CFU is what matters for spoilage. Some studies combine both, using plate counts and MTT staining to get a fuller picture of live, injured, and dead populations (Di Gregorio et al., 2022).

Despite these caveats, viability assays are a powerful complement to food preservative research. They have been adapted, for example, to quickly screen a variety of plant extracts in microplates for anti-yeast activity in juice-like conditions: researchers can inoculate each well with a yeast and different extract, add MTT after a set time, and read absorbances to see which extracts suppressed metabolic activity the

most. This high-throughput capability expedites the discovery of promising natural preservatives.

### 2.8.6. Specific Adaptations for Food Matrices (e.g., Fruit Juices)

When applying microdilution or MTT assays to real food matrices like juices, scientists often need to adapt protocols to account for the complexity of the material, for example, pre-treatment of juice. Juices can be centrifuged or filter-sterilized to remove pulp and any native microflora before spiking with a known microbe for tests. In the *juice challenge test* by Giorgi et al., they centrifuged a commercial juice to "reduce the non-specific background" before inoculation (Di Gregorio et al., 2022). This indicates removing solids and possibly some native compounds to make the test more controlled. When using an indicator like MTT in a juice, one must include a control of juice + MTT + preservative (no inoculum) to check if the juice or preservative alone causes any colour change. Similarly, because juices often have their own colour, when reading optical density or colourimetric results, those readings need to be blanked against a sample with juice but no indicator to avoid misconstruing juice's colour as microbial growth. In an opaque juice, visual clarity isn't a good indicator of microbial inhibition because the juice was never clear to begin with. Instead, plate counting is frequently used as the definitive measure in such cases: after incubation with the preservative, an aliquot of juice is diluted and plated on appropriate agar to count survivors. In mentioned study on Orangina, they diluted and plated juice samples at intervals to get CFU/mL and thus objectively quantify preservation efficacy (Boukhatem et al., 2020). This is a direct way to adapt the microdilution concept to a real juice – essentially performing the MIC test in the juice itself and checking growth by plating. If the juice has high-reducing sugar or ascorbic acid, it might reduce MTT non-biologically (since MTT is an oxidant). One way around this is to use alternate viability stains or methods (like ATP assays or flow cytometry) that are less affected by such compounds. Another adaptation could be using XTT instead of MTT because XTT's formazan stays in solution and can be separated from solids more easily by just taking the supernatant.

In summary, the repertoire of testing methods ranges from simple petri-dish assays to sophisticated cell viability measurements. In the literature on plant-derived preservatives, it's common to see a combination: an initial agar diffusion screening, followed by broth microdilution to get MIC, and finally, a challenge test in an actual food model to confirm practical efficacy (Mohd Israfi et al., 2022). This tiered approach builds evidence from basic to application. Each method's results reinforce the conclusions: for instance, a clove extract might show a large inhibition zone (diffusion), an MIC of 0.05% in broth, and then in a challenge test in fruit nectar, it might keep the product mould-free for X, days, whereas the control spoiled. Multiple peer-reviewed studies employ exactly this progression to evaluate natural antimicrobials.

In conclusion, the literature review of this dissertation has provided a comprehensive overview of the landscape of using plant-derived products to inhibit beverage spoilage microbes. We covered historical precedents for natural preservation, surveyed modern synthetic vs natural preservative strategies, discussed the consumer-driven shift towards natural "clean-label" solutions, highlighted the scientific rationale behind plant antimicrobials (their modes of action and multi-functional benefits), and reviewed the arsenal of methods used to test these compounds *in vitro* and in food models, citing multiple studies for illustration. This foundation sets the stage for the subsequent chapters, which will delve deeper into specific plant-derived antimicrobials, detailed experimental results in *in vitro* and real juice systems, and the potential integration of these natural solutions into industrial beverage preservation. The collective literature evidence underscores a strong potential for plant-derived preservatives to meet the dual demands of ensuring microbial safety and aligning with consumer preferences for natural ingredients. Moving forward, careful formulation and thorough testing (using the methods described) will be key to unlocking their full application in the beverage industry.

# 3. Objectives of the Research

This PhD research aimed to develop and validate innovative, practical, and high-throughput *in vitro* methods for assessing the antifungal efficacy of plant-derived compounds in fruit juice matrices, with the broader goal of identifying natural preservative alternatives suitable for both industrial use and implementation in resource-limited settings.

The specific objectives were:

1. **To develop and validate a modified broth microdilution method for testing anti-yeast compounds directly in fruit juice matrices.**
   This objective focused on adapting the standard broth microdilution technique for use with complex food systems, specifically apple and orange juices. The method was designed to be accurate, reproducible, and suitable for high-throughput screening in a 96-well microplate format without relying on artificial growth media. Validation included determining MIC values for various phenolic compounds and comparing their performance in different juice types.
2. **To compare the growth-inhibitory effects of selected plant-derived phenolic compounds against beverage-spoilage yeasts.**
   Using the validated juice-based microdilution method, a range of natural compounds—including stilbenes, phenolic acids, and flavonoids—was screened against *S. cerevisiae, Z. bailii,* and *Z. rouxii.* This objective aimed to identify compounds with consistent and potent antifungal activity under real juice conditions.
3. **To develop and apply a colourimetric MTT-based viability assay for evaluating antifungal activity in opaque juice matrices.**
   Recognising the limitations of turbidity-based methods in coloured or cloudy juices, this part of the research focused on optimising an MTT viability assay compatible with juice environments. The method was applied to quantify the anti-yeast

effect of selected phenolics, comparing its sensitivity and efficiency to conventional approaches.

4. **To assess the antibacterial activity of essential oils from lemongrass and fingerroot against foodborne pathogens.**

   The fourth objective examined the inhibitory and bactericidal effects of two essential oils in a broth-based system at different storage temperatures. It aimed to benchmark the efficacy of these traditional plant antimicrobials against known food pathogens and determine their potential applicability as natural preservatives in beverages.

5. **To investigate the antifungal activity of traditional Philippine plant extracts against beverage-spoilage yeasts.**

   This objective aimed to assess the inhibitory potential of ethanol-based extracts from *R. mucronata, C. tagal, and M. tanarius* (bark, leaf, and fruit materials) against *S. cerevisiae, Z. bailii,* and *Z. rouxii* using a modified broth microdilution method. The purpose was to evaluate the relevance of traditional medicinal plants as natural beverage preservatives.

6. **To evaluate the performance of natural preservation methods in real-world tropical food processing environments.**
   The final objective focused on applying simple, natural preservative combinations (e.g. citric acid, honey, lime juice) in small-scale processing of juices in Cambodia, Zambia, and Ethiopia. The goal was to assess their practical effectiveness, acceptability, and potential to replace synthetic additives in development project settings.
   *Relevance of Objective 6:* By validating natural preservative methods in juice-processing settings of less developed countries, the thesis ensured that it addressed not only laboratory efficacy but also practical feasibility in resource-limited contexts.

Overall, this study outlines the importance of fruit juice preservation, the limitations of current synthetic preservatives, and the promising role of

plant-derived compounds – all of which form the foundation for the investigations undertaken in this doctoral research. Subsequent chapters will detail the materials and methods developed, the results of antimicrobial efficacy tests, and the analysis of how different natural compounds can contribute to safeguarding juice products against spoilage microorganisms.

The selection of tested compounds was guided by documented antifungal efficacy, regulatory approval, and their relevance to natural juice preservation. Pterostilbene, a methoxylated analogue of resveratrol, has shown strong antifungal activity through mechanisms involving membrane disruption and reactive oxygen species generation (Mizuhara et al., 2023). Stilbenoids such as pterostilbene and oxyresveratrol are also highlighted in phytochemical surveys for their relevance in food preservation (Riviere et al., 2012). Phenolic acids like ferulic and gallic acid have demonstrated antimicrobial effects in acidic environments through inhibition of microbial enzymes and damage to membrane integrity (Gyawali & Ibrahim, 2014). Essential oils such as those from lemongrass and fingerroot were included due to their broad-spectrum antimicrobial activity and synergy with other preservation methods (Burt, 2004). Additionally, their effectiveness against juice-spoiling yeasts like *Zygosaccharomyces* spp. has been experimentally confirmed (Kačániová et al., 2020). Finally, mangrove bark extracts were selected for their ethnobotanical significance and emerging evidence of antifungal properties in tropical contexts.

# 4. Materials and Methods

## 4.1. Adaptation and Validation of a Modified Broth Microdilution Method for Screening the Anti-Yeast Activity of Plant Phenolics in Apple and Orange Juice Models

### 4.1.1. Tested Plant Phenolic Compounds and Control

Curcumin (purity >94%, CAS 458-37-7), chlorogenic acid (>95%, 327-97-9), eriodyctiol (>95%, 4049-38-1), ferulic acid (>99%, 537-98-4), luteolin (>98%, 491-70-3), myricetin (>96%, 529-44-2), naringenin (>99%, 67604-48-2), oxyresveratrol (>95%, 29700-22-9), phlorizin (>99%, 60-81-1), piceatannol (>98%, 10083-24-6), pterostilbene (>98%, 537-42-8), resveratrol (>99%, 501-36-0), and sodium metabisulfite (Control, >99%, 7681-57-4) were obtained from Sigma-Aldrich (Prague, Czech Republic). Dimethyl sulfoxide (DMSO) (Penta, Prague, Czech Republic) was used as a solvent for the compounds tested.

### 4.1.2. Yeast Strains and Growth Media

The anti-yeast activity was evaluated against *S. cerevisiae* (DSM 2548) purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and two *Zygosaccharomyces* species obtained from the Czech Collection of Microorganisms (Brno, Czech Republic), namely *Z. bailii* (CCM 8239) and *Z. rouxii* (CCM 8224). The sub-cultures were maintained on Sabouraud Dextrose agar slants (*S. cerevisiae*) and gelatine disks (*Z. bailii, Z. rouxii*) at 4 ◦C. The inocula of tested yeasts were grown in Sabouraud Dextrose broth (*S. cerevisiae*) and Glucose Yeast Peptone broth (*Z. bailii, Z. rouxii*) at 25 ◦C for 48 h. All the cultivation media were obtained from Oxoid (Basingstoke, UK).

### 4.1.3. Preparation of Juices

The juices were prepared by squeezing fresh orange (*Citrus sinensis*) and apple (*Malus domestica)* fruits, which were checked beforehand to exclude the rotten, cracked, or unripe ones and washed thoroughly several times to remove unwanted dirt. The fruits were obtained from the local market (Eso Potraviny, Prague, Czech Republic), washed with distilled water, and peeled with a sterile knife. To separate the fruit pulp, the juice was filtered through a kitchen iron sieve with a pore size of 0.5 mm and subsequently centrifuged at room temperature using a Minispin centrifuge (Eppendorf, Hamburg, Germany) at 120 rpm for 2 min. The liquid part was then transferred to sterile plastic Falcon™ 50 mL High-Clarity Conical Centrifuge Tubes (Gama Group, Ceske Budejovice, Czech Republic), stored at −25 ◦C and used within 5 days (Bonat Celli et al., 2016). Before the testing, the juice was sterilised by filtration through a Pragopor membrane nano filter (Pragochema, Uhrineves, Czech Republic) with a pore size of 0.23 µm in a glass vacuum filtration system (Sartorius, Gottingen, Germany). From this second filtration, sterile transparent juice was obtained and directly used. The pH of the juices measured by a CyberScan pH 510 (Eutech Instruments, Singapore) varied between 3.3 and 3.6 for apple juice and 3.5 and 3.7 for orange juice.

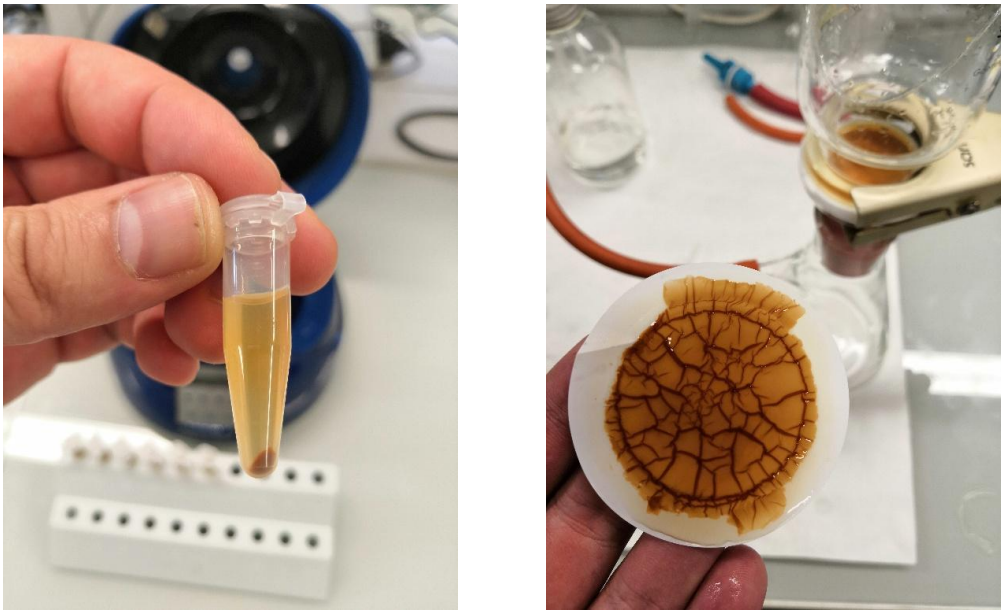


***Figure 1**. Sedimentation of fresh apple juice by Minispin centrifuge (left) and sterilisation of the apple juice by filtration through a Pragopor membrane nano filter (right).*

### 4.1.4. Anti-Yeast Assay

The anti-yeast activity was determined by the broth microdilution method performed in standard 96-well microtiter plates (Gama group, well volume = 400 µL) according to the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute CLSI, 2015), European Committee on Antimicrobial Susceptibility Testing (European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2021), Food and Drug Administration (Food and Drug Administration (FDA), 2009), and International Organization for Standardization (International Organization for Standardization (ISO), 2019), modified for the simulation of conditions of yeast growth in a juice matrix. Instead of broth, 100 µL of sterile juice was used as a medium in each well. Each sample of the tested compound was dissolved in DMSO, except for sodium metabisulfite, for which deionised water was used. All the samples were then diluted in sterile juices. Eight two-fold serially diluted concentrations of samples in a range of 8–1024 µg/mL were prepared and inoculated with 5 µL of yeast suspension with a concentration of $10^7$ CFU/mL. The inoculation was performed by

dispensing a single 5 µL drop into each well using a calibrated microliter syringe, ensuring precise and consistent delivery of the yeast suspension. This method aligns with standard practices for broth microdilution assays, as outlined by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI), 2017). The inoculated and non-inoculated juice wells were prepared as growth and purity controls, respectively. After the inoculation, microtiter plates were incubated at 25 °C for 24 h in the Peltier-cooled incubator IPP55 (Memmert, Buechenbach, Germany), and yeast growth was then measured spectrophotometrically as turbidity using a Cytation 3 Multimode Reader (BioTek Instruments, Winooski, VT, USA) at 405 nm (Cos et al., 2006). The MICs values were expressed as the lowest concentrations, showing at least a ≥80% reduction of microorganisms' growth compared to the compound-free growth control. The assay was performed as three independent experiments, each carried out in triplicate, and median/modal values were used for final MIC determination. According to the widely accepted standard in MIC testing, the mode and median were used for the final value calculation when the triplicate endpoints were within the two- and three-dilution range, respectively. Sodium metabisulfite was used as the positive reference control in a range of concentrations 8–1024 µg/mL. As a result of experiments performed without dissolved compounds, DMSO and distilled water did not inhibit the yeast growth of any strain at the tested concentrations (≤1%).

### 4.1.5. Statistical Analysis

The statistical analysis was conducted for each treatment, meaning that the microbial growth-inhibitory effect of all individual compounds on each of the yeasts was evaluated in both juices, taking the evaluated MIC in juices as the response variable. All data were analysed through a variance analysis (ANOVA—one way). Also, probability values of $p < 0.05$ were considered significant. The difference between the treatment means was estimated using multiple ranges of Tukey's HSD (honestly significant difference) with a 95% confidence interval test using the R software (version 4.3.2). At the same time, a principal component analysis (PCA) was carried out for interpreting results as it simplifies complex datasets. By transforming original variables into uncorrelated

components, PCA reveals underlying patterns that are not readily apparent in raw data. It condenses information, aiding in visualisation and identifying influential factors. Ordering components by variance explains which variables, like MIC, yeast strains, or juice types, contribute most to observed differences. This facilitates uncovering hidden structures, detecting outliers, making informed decisions, and enhancing data interpretation and analysis.

## 4.2. Adapted MTT Colorimetric Assay

### 4.2.1. Chemical compounds

All used compounds (Benzoic acid, citric acid, curcumin, gallic acid, potassium sorbate, pterostilbene, sodium metabisulfite, sorbic acid, tannic acid and dye thiazolyl blue tetrazolium bromide) were purchased from Sigma-Aldrich.

### 4.2.2. Juice sample preparation

The juice was prepared by squeezing the fresh orange fruit (*Citrus sinensis*) obtained from the local market in Prague. After that, the juice was filtrated through an iron sieve to separate the pulp and then through the membrane nano filter (Pragopor) with a pore size of 0.23 μm in a filtration machine (Sartorius). By this filtration, we obtained sterile juice.

### 4.2.3. Yeast and culture media

The standard strain of the *S. cerevisiae* was used (DMS 2548). The cultivation and assay media was Sabouraud (SA). Yeast and cultivation media were purchased from Oxoid (Basingstoke, UK).

### 4.2.4. Food matrix MTT assay

A modified MTT colourimetric assay was developed to evaluate the antifungal activity of selected natural compounds against *S. cerevisiae (*DSM 2548) in sterile orange juice. This approach adapts the conventional microdilution-based viability assay to realistic conditions found in fruit-based beverages.

As shown in Figure 2, the assay was performed in 96-well flat-bottom microplates (Greiner Bio-One). Each well was filled with 100 µL of sterile-filtered orange juice (prepared as described in Section 3.2.2). Test compounds were added to reach final concentrations ranging from 8 to 1024 µg/mL. The compounds tested included benzoic acid, citric acid, curcumin, gallic acid, potassium sorbate, pterostilbene, sodium metabisulfite, and tannic acid—all selected based on their reported antifungal activity and natural origin.

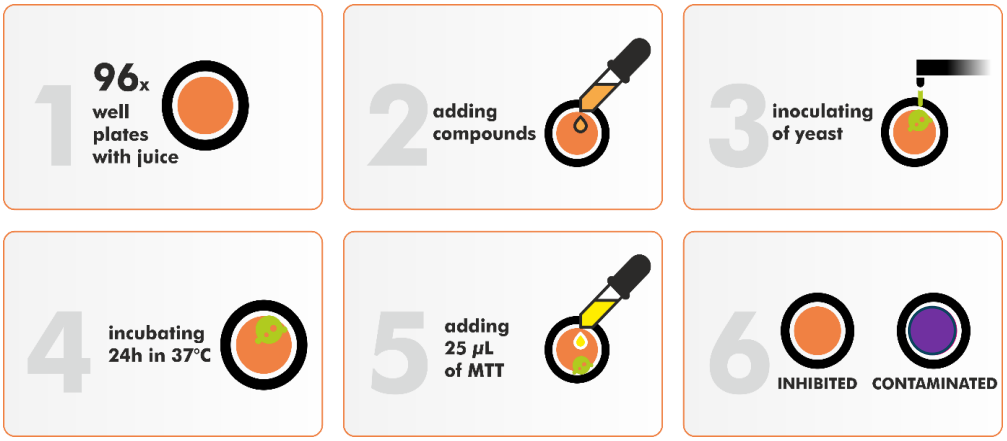



***Figure 2.*** *Scheme of Food matrix MTT assay.*

The plates were then inoculated with a suspension of *S. cerevisiae* ($10^7$ CFU/mL) using a 96-pin multi-blot replicator (National Institute of Public Health, Prague, CZ), which delivered consistent micro-droplets of inoculum into each well. This technique allowed for the efficient and uniform distribution of the yeast across the entire plate. The final yeast density per well was $5 \times 10^5$ CFU.

Plates were incubated at 37 ± 1 °C for 24 hours under static conditions. Following incubation, 25 µL of MTT solution (5 mg/mL in PBS) was added to each well. Plates were further incubated at 25 °C for 30 minutes to allow for colour development. Wells where yeast metabolism remained active turned purple (formazan formation), whereas inhibited cultures remained yellow-orange.

No solubilization step was performed, as the visible colour shift was sufficient for determining viability. Each plate included appropriate positive controls (juice + yeast, no compound) and negative controls (juice only).

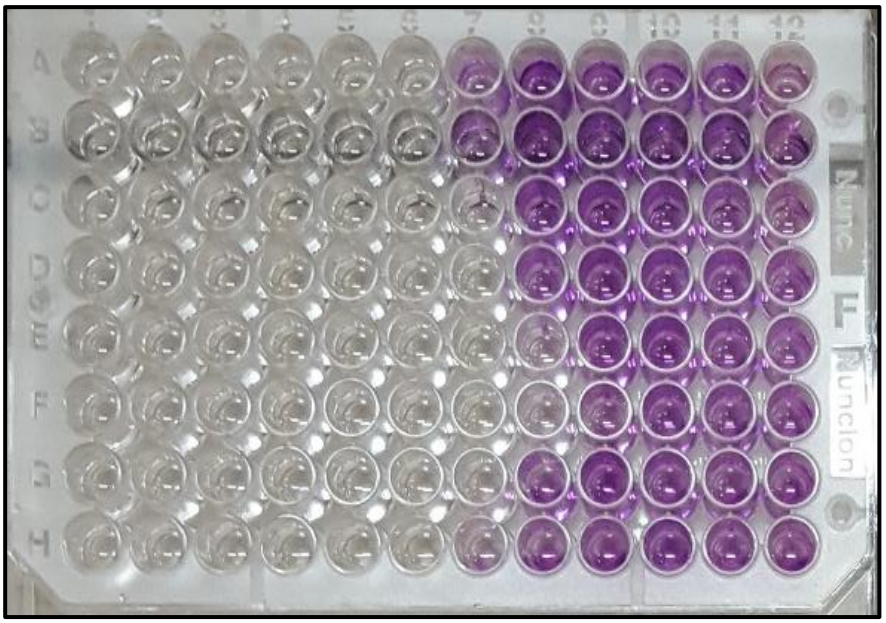



***Figure 3.*** *96-well microplates after MTT assay. Cleary visible purple colour indicates the contamination of microorganism.*

## 4.3. Antimicrobial efficacy of lemongrass (*Cymbopogon citratus*) and fingerroot (*Boesenbergia pandurata*) essential oils against foodborne pathogens

### 4.3.1. Essential Oils

The essential oils (EOs) of lemongrass (*Cymbopogon citratus*) and fingerroot (*Boesenbergia pandurata*) were used in this experiment. Both EOs were obtained from BOTANICESSENCE Essential Oils, Thailand. The EOs were certified by Ecocert SA (F32600).

### 4.3.2. Bacterial strains and culture conditions

Tested pathogenic bacteria were comprised of *S. enteritidis* (DMST) *E. coli* (DMST), *L. monocytogenes* (F2365), and *S. aureus* (DMST). These microorganisms were chosen as they are commonly associated with the spoilage of refrigerated foods, as an indicator of pathogenic microorganisms, or as spoilage microorganisms. These standard reference strains, obtained from the Department of Medical Sciences, Thailand, were provided by the Faculty of Agro-Industry, Prince of Songkla University (Hat Yai, Thailand). The stock cultures of bacterial strains were prepared overnight in brain heart infusion broth (BHIB) at 37°C, and then they were streaked on the brain heart infusion agar (BHIA) and incubated for 24 hours at 37°C. The cultures were kept under refrigerated conditions and were sub-cultured after every ten 10 days.

### 4.3.3. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The determination of MIC of the essential oil of lemongrass on the test bacterial strain was done using the broth dilution method, as explained by (Hammer et al., 1999) with different concentrations of oil. The cultures of the test strains were prepared by inoculating the test strain in a sterilised test tube containing 5 mL nutrient broth. The tubes were incubated overnight at (37±1) °C. The MIC was defined as the lowest concentration of the test compound to inhibit the growth of microorganisms, and the MBC was defined as the lowest concentration of the test compound to kill the microorganisms. The test tubes containing 5 mL of sterilised BHIB with 0.5% (v/v) tween-80 were cured with different concentrations of lemongrass oil ranging from 0.015% – 2.0% (v/v). BHIB with 0.5% (v/v) tween 80 without oil was used as positive growth control. An aliquot of bacterial suspension (100 µL) was added uniformly to each tube. The tubes were incubated at (37±1) °C for 24 hours. Turbidity was assessed visually, after the period of incubation. The lowest concentration at which no visible growth occurs in either culture tube was taken as the MIC (Hammer et al., 1999). Then, all tubes showing no increase in turbidity after 24 h were streaked on

nutrient agar plates to check the bacterial growth. Each trial was repeated twice.

#### 4.3.4. Microbial assay

The sterile tubes with BHBI (5 ml) were inoculated with 2 different essential oils at concentrations MIC, 2xMIC, and 4xMIC and with 0.5 % (v/v) of tween-80. Then 100 µL of active inoculums of each bacteria (approximately $10^6$ colony-forming units, CFU/mL) was added. Sampling for viable cells was carried out on days 0, 1, 3 and 5 at two different storage temperatures (4 °C and 25 °C). The viable plate counts were monitored as follows: 50 µL sample of each treatment was spread on the surface of BHIA, and the colonies were counted after incubation at 37 °C for 24 h. At each assay time, controls without essential oil were also tested.

### 4.4. *In Vitro* Growth-Inhibitory Effect of Plant Extracts Against Beverage-Spoiling Microorganisms

#### 4.4.1. Plant material

Samples of the plant material were collected in Philippines in June 2019 and authenticated by ethnobotanist prof. Ladislav Kokoska, Department of Crops Sciences and Agroforestry, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague (CZU). Specifically, bark of *C. tagal* was purchased in the local market in Baybay (Leyte), bark and leaves of *M. tanarius* were collected in campus of Visayas State University, Baybay (Leyte), and fruits of *R. mucronata* were collected in Igdarapdap (Guimaras). The voucher specimens of *M. tanarius* (no. 02557KBFRA) and *R. mucronata* (no. 02533KBFR4) were deposited in the herbarium of the Department of Botany and Plant Physiology of the Faculty of Agrobiology, Food and Natural Resources, CZU.


***Figure 4**. Collected samples of M. tanarius bark. Visayas State University, Baybay. 2019.*

### 4.4.2. Extracts preparation

In this assay, five plant-based extracts from three different Philippine plant species were used: *R. mucronata* (fruits), *C. tagal* (bark), and *M. tanarius* (bark, leaf sediment, and leaf supernatant). The selection of these plants was based on their traditional medicinal use and reported antifungal activity. Their ethnobotanical background and regional relevance supported their inclusion as candidates for natural food preservatives. Extracts of various parts of *R. mucronata, C. tagal,* and *M. tanarius* were prepared by grinding the dried material into fine powder using a mechanical grinder Grindomix mill (Retsch, Haan, Germany), and 15 g of dry matter was extracted in 450 ml 80% ethanol (Penta, Prague, Czechia) for 24 h at room temperature using a laboratory shaker (GFL3005, GFL, Burgwedel, Germany). Therefore, the drug extract ratio was 1:30. In case of *M. tanarius*, leaf extract, the resulting suspension was allowed to settle undisturbed, then separated into two fractions: the supernatant liquid (“leaf liquid”) and the settled particulate matter (“leaf sediment”). Both fractions were tested independently to evaluate any difference in antifungal efficacy between soluble and particulate components of the extract.

Extracts were prepared in the Laboratory of Ethnobotany and Ethnopharmacology, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague. Following the extraction, the crude mixtures were subjected to vacuum filtration using a Buchner funnel connected to a vacuum pump to separate the large debris. The filtrate was then concentrated using a rotary vacuum evaporator (R-200, Buchi Labortechnik, Flawil, Switzerland) in vacuo at 40°C under reduced pressure. According to the recommendations of Cos et al. (2006), the dried residue was finally diluted in 100% dimethylsulfoxide (DMSO) (Penta, Prague, Czechia) to obtain stock solutions with a final concentration of 51.2 mg/ml and stored at −20°C until their use.

### 4.4.3. Yeast Strains and Growth Media

The anti-yeast activity was evaluated against *S. cerevisiae* (DSM 2548) purchased from the German Collection of Microorganisms and Cell Cultures and two *Zygosaccharomyces* species obtained from the Czech Collection of Microorganisms, namely *Z. bailii* (CCM 8239) and *Z. rouxii* (CCM 8224). The sub-cultures were maintained on Sabouraud Dextrose agar slants (*S. cerevisiae*) and gelatine disks (*Z. bailii, Z. rouxii*) at 4 ◦C. The inocula of tested yeasts were grown in Sabouraud Dextrose broth (*S. cerevisiae*) and Glucose Yeast Peptone broth (*Z. bailii, Z. rouxii*) at 25 °C for 48 h. All the cultivation media were obtained from Oxoid.

### 4.4.4. Microbial assay

The anti-yeast activity was determined by the Modified Broth Microdilution Method as described in section 4.1.4.

## 4.5. Practical Application of Natural Preservatives in Low- and Middle-Income Countries

### 4.5.1. Field Trial Design and Processing Protocols

Field trials were conducted to evaluate the efficacy of natural preservative strategies in real production settings. This served as an extension of the core research, applying the most promising natural preservatives in small-scale juice processing units in Cambodia,

Zambia, and Ethiopia. Cambodia served as the primary trial site where protocols were first developed; identical methods were subsequently implemented in Zambia and Ethiopia.

### 4.5.2. Mango Juice Processing

All mangoes were sourced from local farms and processed at peak physiological ripeness. Mango juice was prepared by blending or lightly cooking fresh mango pulp with potable water and added sugar, typically in the range of 5–15% (w/v), depending on local preferences and the availability and price of sugar. Given the primarily qualitative nature of this field-based research, processing methods varied slightly across countries, reflecting differences in infrastructure, materials, and cultural practices.

Following juice preparation, the mixture was hot-filled into pre-sterilised 250 mL glass bottles. To retain heat-sensitive bioactive compounds, natural preservatives such as lime juice, honey, or ascorbic acid were added after cooking, once the juice temperature had dropped to approximately 60 °C. Glass bottles were pre-sterilised by boiling and, after filling, underwent a short boiling water treatment (10–15 minutes) to ensure proper sealing and enhance shelf stability, while aiming to preserve the functionality of the added natural preservatives.

***Figure 5.*** *Mango juice preparation using lime juice and honey, and sterilisation by boiling for 15 minutes. Arba Minch, Ethiopia, 2023.*

### 4.5.3. Preservative Treatments and Controls

Natural preservatives tested included combinations of lime juice, citric acid, ascorbic acid (vitamin C), and honey. Treatments were applied either individually or in combination (e.g., lime juice + honey, citric acid + honey). Each treatment batch was processed under identical conditions for comparative evaluation.

In Cambodia, reference control batches were also prepared using synthetic preservatives—0.1% w/v potassium sorbate for juices/jams and 0.1% w/w sodium metabisulfite for dried fruits. These chemicals were not available in Zambia or Ethiopia, where only natural preservative treatments were tested.

### 4.5.4. Monitoring and Evaluation

Juices were stored at ambient temperature (25–30 °C), protected from direct sunlight and excessive humidity. No refrigeration was used at any site to simulate typical rural storage conditions. Product stability and quality were monitored over a six-month period, with inspections on days 1, 5, 10, 30, 60 and 180. At each time point, samples were opened and examined for visual spoilage (e.g., mould, gas formation, colour change) and organoleptic changes (e.g., odour, taste). Microbial testing was qualitative, based on visible growth or spoilage. Colour changes (e.g., browning) were recorded visually. Sensory evaluations were conducted by small local panels (10 community members per site) using a basic hedonic scale (acceptable/unacceptable) with panellist subjective descriptions as well. Observations were documented in field logbooks, and the end of shelf life was recorded as the first clear sign of spoilage or unacceptable quality. In Cambodia, synthetic preservative control products were assessed in parallel to benchmark natural preservative efficacy.

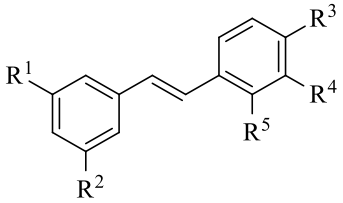
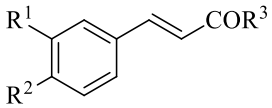
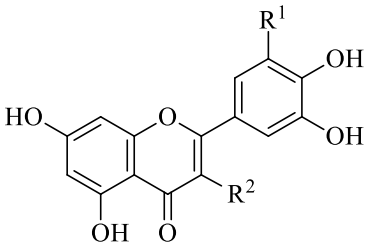
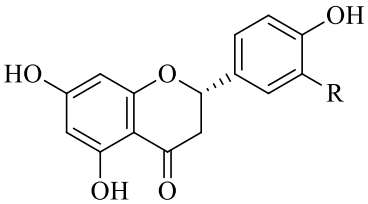
By including this component, the study bridges laboratory results with practical application, verifying in an applied experiment that simple natural additives (like citric acid, lime juice, and honey) can indeed prolong juice shelf-life in tropical, resource-limited environments.

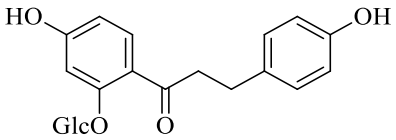
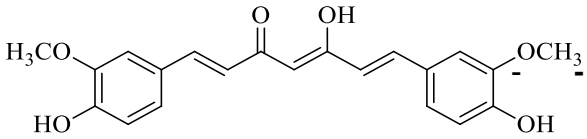
# 5. Results and Discussion

## 5.1. Adaptation and Validation of a Modified Broth Microdilution Method for Screening the Anti-Yeast Activity of Plant Phenolics in Apple and Orange Juice Models

Four of the twelve compounds tested in this study exhibited inhibitory activity against all three yeasts grown in both juices, with MIC values ranging from 32 to 1024 µg/mL. As can be seen in Table 2, the level of the inhibitory effect depended significantly on differences in the chemical structures of the tested compounds. Stilbenes with methoxy and hydroxy groups (pterostilbene) or four hydroxy groups (oxyresveratrol and piceatannol) and phenolic acid (ferulate) with methoxy and hydroxy groups produced the growth-inhibitory effect against all yeasts tested. Pterostilbene, a dimethylated analogue of resveratrol with methoxy groups at positions A-3, -5 and hydroxyl group on B-4′, demonstrated the most potent anti-yeast effect within all yeasts tested. Principally, the presence of methylated hydroxyphenyl groups in the pterostilbene structure is acknowledged to raise its biological activity (Chong et al., 2009; Pastorkova et al., 2013). Moreover, the results indicated that the higher number of hydroxyl groups in the compound structure strengthens its biological activity. This follows the evidence that the increased hydroxylation of phenolic compounds leads to higher toxicity to microorganisms (Evans & Marjorie, 2006; Pastorkova et al., 2013).

***Table 2**. Chemical structures and anti-yeast activity of natural compounds assayed in this study.*

| Chemical Structure | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | Name | Anti-Yeast Activity |
|---|---|---|---|---|---|---|---|
| **Stilbenes** | | | | | | | |
|  | OH | OH | OH | H | OH | oxyresesevera trol | Yes |
| | OH | OH | OH | OH | H | piceatannol | Yes |
| | $OCH_3$ | $OCH_3$ | OH | H | H | pterostilben e | Yes |
| | OH | OH | OH | H | H | resveratrol | No |
| **Phenolic acids** | | | | | | | |
|  | $OCH_3$ | OH | OH | - | - | ferulic acid | Yes |
| | OH | OH | quinic acid | - | - | chlorogenic acid | No |
| **Flavonoids** | | | | | | | |
|  | OH | H | - | - | - | luteolin | No |
| | H | OH | - | - | - | myricetin | No |
|  | OH | - | - | - | - | eriodictyol | No |
| | H | - | - | - | - | naringenin | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
|  | - | - | - | - | - | phloridzin | No |
| **Diarylheptanoids** | | | | | | | |
|  | - | - | - | - | - | curcumin [a] | No |

[a] Shown in the keto-enol form, which is more abundant in nature than the diketo form.

All the flavonoids and other tested compounds did not show any antifungal activity, which is contrary to the study (Pastorkova et al., 2013) in which the flavonoids inhibited the growth of *S. cerevisiae, Z. bailii,* and *Z. rouxii* at MICs varying from 256 to 512 µg/mL. Various results of yeast susceptibility testing can be caused by a modification of our assay, especially by the difference between the composition of the fruit juices and the standard growth medium, which corresponds with the study of (Xu et al., 2018), which observed that different compositions of growth media affected the anti-yeast activity of tested agents. Similarly, the study of (Xu et al., 2018) reported the effect of different growth media on the antimicrobial efficacy of compounds tested against *Candida* species. Among the individual compounds assayed, the greatest anti-yeast action was observed for pterostilbene in both orange and apple juices against all microorganisms tested, with MIC values ranging from 32 to 128 µg/mL, followed by piceatannol (MICs = 256–512 µg/mL) and oxyresveratrol (MICs = 512–1024 µg/mL).

***Table 3.****The growth-inhibitory effect of plant phenolic compounds active against yeasts in apple and orange juice food models.*

| Compound | Yeast/Juice Food Model/MIC [a] (µg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | ***Saccharomyces cerevisiae*** | | ***Zygosaccharomyces bailii*** | | ***Zygosaccharomyces rouxii*** | |
| | **Orange** | **Apple** | **Orange** | **Apple** | **Orange** | **Apple** |
| Ferulic acid | 1024 | 512 | 1024 | 512 | 512 | 512 |
| Oxyresveratrol | 512 | 512 | 512 | 512 | 512 | 1024 |
| Piceatannol | 512 | 512 | 512 | 256 | 256 | 512 |
| Pterostilbene | 32 | 32 | 32 | 64 | 32 | 128 |
| Sodium metabisulfite [b] | 512 | 1024 | >1024 | >1024 | 1024 | 512 |

The minimum inhibitory concentrations (MICs) are expressed as mode and median values of three independent experiments (each performed in triplicate), according to the standard approach for MIC determination when replicate endpoints fall within the two- and three-dilution range, respectively. [a] MIC: minimum inhibitory concentration. [b] Positive reference control.

These results are in accordance with several studies that proved the anti-yeast activity of these three stilbenes. For example, pterostilbene, piceatannol, and oxyresveratrol have a high growth-inhibitory effect against *C. tropicalis* (Kim & Lee, 2018; Li et al., 2014; Plumed-Ferrer et al., 2013). Moreover, these results were confirmed by the study, where a newly developed *in vitro* tetrazolium-based colourimetric assay using standard 96-well microtiter plates and MTT was used for the high-throughput screening of the anti-yeast activity of plant-derived preservative candidates in juice food models (Stas et al., 2019). Despite the fact that the anti-yeast activity of these compounds is known in the literature, to the best of our knowledge, this is the first report on their inhibitory effect on the growth of pathogenic yeasts in fruit juice. These

results (see Table 3) suggest that stilbenes could be used as perspective agents to control the growth of beverage-spoilage microorganisms. Nevertheless, further research should explore cost-effective and scalable solutions. Our study used only pure compounds (with a purity higher than 94%), which are more expensive than plant extracts with lower purity and less suitable for large-scale production (Davidson et al., 2015; Singh Tomar et al., 2015). Especially among the stilbenes, pterostilbene emerges as an especially promising candidate for beverage preservation. This compound, renowned for its antioxidant, anti-inflammatory, and anticarcinogenic properties (Remsberg et al., 2008; Rimando et al., 2002) naturally occurring in *Vitis vinifera* and *Vaccinium corymbosum* (Lin et al., 2009; Roupe et al., 2008), seems to be the most promising agent for not only extending shelf-life but also potentially enhancing the health-related aspects of preserved beverages. Piceatannol, a hydroxylated analogue of resveratrol naturally occurring in various plants such as *V. vinifera* and *Passiflora edulis*, is known for exhibiting strong antimicrobial and antioxidative activity as well as anticancer potential (Kukreja & Wadhwa, 2014; Nowak et al., 2013; Piotrowska et al., 2012). Furthermore, the results showed that pterostilbene's antimicrobial action is significantly higher compared to oxyresveratrol, another stilbene that naturally occurs in *V. vinifera* and *Morus alba* (Likhitwitayawuid, 2021). In addition to the stilbenes, ferulic acid was the only compound showing anti-yeast activity with MICs ranging from 512 to 1024 µg/mL. These values are slightly lower than in the study (Kimani et al., 2021) in which ferulic acid exhibited a MIC = 2000 µg/mL against *S. cerevisiae* in the malt-extract growth medium when assayed by the standard microplate method. Most microorganisms tested were more susceptible to active phenolics (MICs ≥32 µg/mL) than to sodium metabisulphite (MICs ≥512 µg/mL), which corresponds with previously published studies (Jackson, 2000; Pastorkova et al., 2013). The standard deviations of yeast-growth control wells were as follows: for apple juice, *S. cerevisiae* (SD = 0.088), *Z. bailii* (SD = 0.064), and *Z. rouxii* (SD = 0.038); and for orange juice, *S. cerevisiae* (SD = 0.125), *Z. bailii* (SD = 0.066), and *Z. rouxii* (SD = 0.069). These results indicate that the variations in individual yeast growth were minimal, ensuring that

the juice matrix provided a stable environment for consistent and reliable MIC testing.

Compared to other *in vitro* quantitative methods assaying antimicrobial agents in fruit juices using macrodilution in tubes and flasks (Ribes et al., 2019; Sagdic et al., 2011), microplate-based methods allow for the cost- and labour-effective high-throughput screening of antimicrobials in low volumes of growing matrices. As discussed in several studies, they are suitable for the simple and fast determination of the antimicrobial potential of beverage preservatives at different concentrations, while several agents may be assessed in one microplate (Balouiri et al., 2016; Gonzalez-Pastor et al., 2023; Michael et al., 2008; Sanchez Armengol et al., 2021; Zhang et al., 2023). Our study introduces a novel modified broth microdilution method that further enhances these advantages by being not only more cost- and labour-effective but also significantly more rapid than traditional methods. This method allows for the quicker determination of antimicrobial activity, making it an ideal tool for high-throughput screening. Recently, a published study by (Wang & Sun, 2020) used microplates for testing the antifungal activity of natural agents in apple juice sterilised at 100 °C for 30 min. Consistent with their results, our study shows that the susceptibility testing of food pathogenic yeasts to plant-derived compounds using a juice food model based on the modified broth microdilution method provides accurate and reproducible results. In addition, our experiments confirmed the suitability of juice sterilisation by membrane filtration. Heat treatment such as pasteurisation at high temperatures for a long time (80–100 °C, 10–30 min) can inevitably impact the sensory characteristics, nutritional integrity, and, specifically, the vitamin C content of the juice. Cumulatively, these effects contribute to a reduction in the overall quality of the product. This reduction in quality emphasises the need for alternative sterilisation methods that can maintain the organoleptic and nutritional properties of the juice while ensuring microbial safety (Deng et al., 2022; Mandha et al., 2023; Vikram et al., 2005).

Concerning the multiple-range test, it was seen that the inhibition levels presented by the pterostilbene treatment and sodium metabisulfite (control) are significantly different, and the same behaviour was seen

when comparing the inhibition levels of pterostilbene and ferulic acid. Both groups had a confidence level of under 95%; this indicates that when observing the MIC values of the other evaluated compounds, they did not display a statistical difference in their growth-inhibitory effect against the yeasts. The pterostilbene exhibited the lowest MIC values and demonstrated the least variability in the data. This suggests that the inhibitory effect of pterostilbene on the yeasts was suitable and consistently homogeneous. The consistent behaviour observed in the data indicates pterostilbene's more reliable and predictable inhibition performance than other compounds.

On the other hand, in the case of compounds studied in apple juice, a significant difference between the MIC values ($p = 0.0498$) between the treatments evaluated was also obtained. After evaluating the multiple-range values, a significant difference was recorded between pterostilbene and oxyresveratrol and between pterostilbene and sodium metabisulfite.

When examining the inhibitory effect of the compounds in apple juice, pterostilbene demonstrated analogous outcomes to those observed in orange juice. The achievement of a notably low MIC, coupled with minimal deviations concurrent with a high degree of uniformity, underscores its efficacy and homogeneous behaviour. The congruence in results between the two fruit juices suggests a consistent and robust inhibitory profile of pterostilbene. Its performance in apple juice mirrors the established trend noted in orange juice, reinforcing the compound's potential applicability in various juices. Non-significant comparisons are not displayed due to their lack of relevance.

Pterostilbene is the dimethylated analogue form of resveratrol; it has been reported that it may be more effective in preventing microbial growth (Li et al., 2014; Mattio et al., 2020). This seems to be due to pterostilbene's methoxy moiety, which plays a crucial role in its antifungal activity (Li et al., 2014). It has been reported that the method of action of this stilbene allows it to have non-ionic surfactant-like characteristics, which causes damage to the lipid bilayer, causing an imbalance in the permeability of the plasma membrane—in this case, of the yeast *S. cerevisiae* (Mizuhara, Inoue, Kurotaki, Matsumoto, et al.,

2023). This mechanism of action may also be applicable to the other two yeasts investigated in this study.

Figure 6 displays the outcome of the PCA for each treatment, where each axis (x–y) captures a percentage of the original data variability—the PC1 captured 90.17%, whereas the PC2 depicts 6.73%; therefore, both axes captured 96.9% of the total variance in the data.

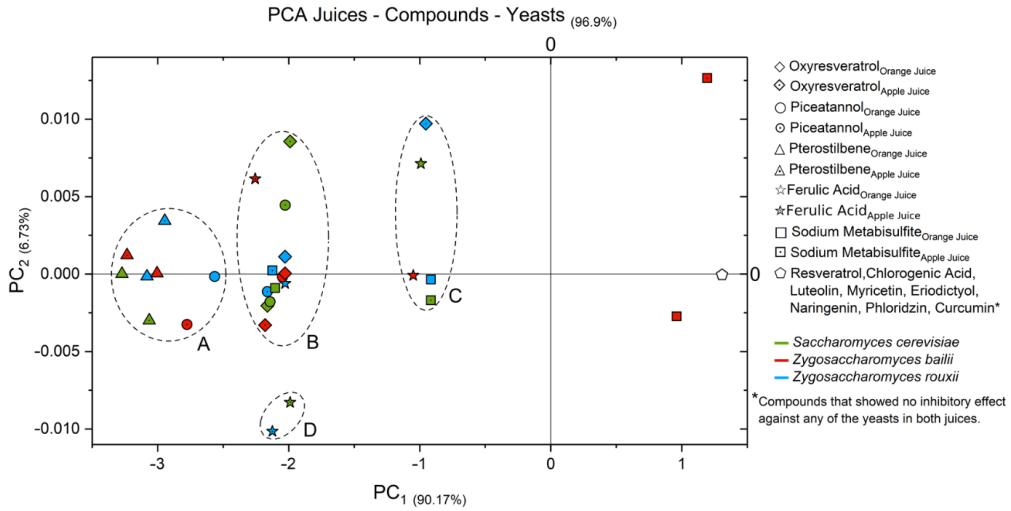



***Figure 6**. Principal component analysis (PCA) of the evaluated compounds and their yeast inhibitory behaviour per juice.*

Points that are close to each other exhibit similar yeast growth-inhibitory activity; however, four groups were identified and clustered in the plot: the first one (Group A) consists of pterostilbene, which belongs to the chemical structure of stilbenes. This compound exhibited a similar growth-inhibitory effect for the three evaluated yeasts in orange and apple juice, highlighting that this compound also showed the lowest MIC, disregarding the yeast and juice type. Group A also comprises piceatannol in orange and apple juice regarding the inhibition action against *Z. bailli* and *Z. rouxii*, respectively.

The second group (Group B) comprises oxyresveratrol in all tests except for *Z. rouxii*/apple juice; piceatannol for all scenarios except *Z. rouxii*/orange juice and *Z. bailii*/apple juice; ferulic acid for *Z. rouxii*/orange juice and *Z. bailii*/apple juice; and sodium metabisulfite for *S. cerevisiae*/orange juice and *Z. rouxii*/apple juice. Stilbenes and

phenolic acids represent Group B. Similar inhibitory effects were achieved for this group as the clustered components shared similar MIC values; nevertheless, the inhibition of the growth of all three yeasts was seen only in apple juice.

Group C, represented by oxyresveratrol for *Z. rouxii*/apple juice; ferulic acid for *S. cerevisiae*/orange juice and *Z. bailii*/orange juice; and sodium metabisulfite with very similar behaviour for *Z. rouxii*/orange juice and *S. cerevisiae*/apple juice, displayed a related inhibitory effect; however, the growth inhibition of the three yeasts was achieved only in orange juice for this group.

Lastly, a sub-cluster labelled D was discovered for ferulic acid inhibiting *S. cerevisiae* and *Z. bailii*, denoting a specific effect when controlling their growth in apple juice.

Finally, in this study, the compounds resveratrol and chlorogenic acid, all evaluated flavonoids, and diarylheptanoids, which lack anti-yeast activity, were located on axis 0 of the graph (pentagon).

## 5.2. Adapted MTT Colorimetric Assay Food Model

This experiment assessed various plant-derived compounds for inhibiting yeast in orange juice, using a modified MTT viability assay (see Figure 7). The MICs determined highlighted pterostilbene as the most effective antifungal agent among those tested (64 µg/mL). Pterostilbene showed lower MIC values in orange juice than other phenolics, indicating superior efficacy. For instance, if other compounds (e.g. benzoic acid) required higher concentrations to inhibit yeast growth, pterostilbene achieved the same or greater inhibition at a fraction of that concentration (as shown in Table 5). This finding is consistent with recent literature on pterostilbene's potent antifungal properties. Pterostilbene has been reported to exhibit strong fungicidal activity against a range of fungi due to its higher lipophilicity (Mizuhara, Inoue, Kurotaki, Matsumoto, et al., 2023).

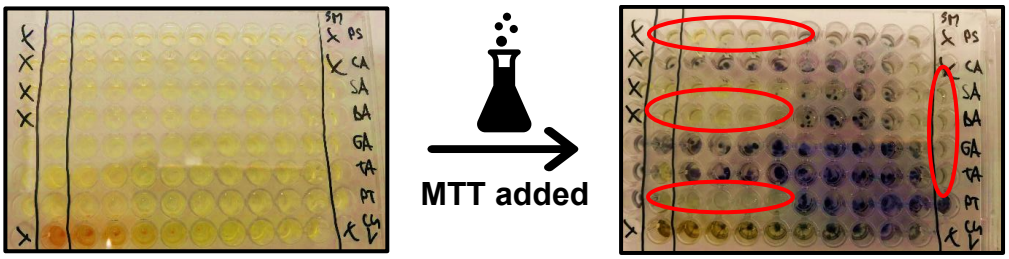



***Figure 7.*** *Visual outcome of the MTT assay showing colour change of contaminated wells after 30 minutes at room temperature. The purple formazan colour reflects microbial metabolic activity, while the absence of colour indicates inhibition. Rows correspond to different tested phenolic compounds and the sodium metabisulfite control.*

The two additional methoxy groups on pterostilbene's stilbene backbone make it hydrophobic, facilitating better penetration of microbial cell membranes and potentially disrupting cells more efficiently (Mizuhara, Inoue, Kurotaki, Matsumoto, et al., 2023). In *C. albicans* biofilm models, pterostilbene showed high efficacy (low MIC and even *in vivo* activity), whereas resveratrol was less active (Li et al., 2014).

***Table 4.*** *Anti-yeast activity of selected compounds against S. cerevisiae in apple juice.*

| Compound | MIC F (µg/mL) |
|---|---|
| Citric acid | **ND** |
| Potassium sorbate | **256** |
| Sorbic acid | **128** |
| Benzoic acid | **256** |
| Curcumin | **ND** |
| Gallic acid | **ND** |
| Tannic acid | **ND** |
| Pterostilbene | **64** |
| Sodium metabisulfite | **128** |

*MIC F = Final Minimal Inhibitory Concentration = mean of MIC 1, MIC 2, and MIC 3.*
*ND = Not Detected*

Our results parallel those trends, suggesting pterostilbene's chemical structure grants it a stronger antifungal punch in the acidic, sugar-rich environment of fruit juice. Other phenolic compounds tested (for example, if they included e.g. ferulic acid, vanillin, etc.) exhibited higher MICs, meaning they were less effective at stopping yeast growth. It is known that simple phenolic acids or flavonoids often have only fungistatic effects at concentrations feasible in foods (Letsiou et al., 2024). By contrast, pterostilbene's combination of antioxidant capacity and cell membrane injury to yeasts makes it a standout compound (Li et al., 2014). This suggests it could potentially serve as a natural preservative in high-sugar beverages, where robust anti-yeast activity is needed.

We employed an adapted MTT colourimetric assay to quantify yeast viability in orange juice, as opposed to relying solely on classic agar diffusion or broth turbidity methods. The MTT assay offered several distinct advantages in this complex food matrix. First, orange juice is opaque and strongly pigmented, which makes standard optical density measurements of microbial growth unreliable. In our preliminary trials, the turbidity and colour of the juice interfered with detecting moderate increases in yeast cell density. The MTT assay circumvents this by measuring metabolic activity: live yeasts reduce the MTT tetrazolium dye to a coloured formazan product, providing a clear quantitative readout (absorbance of the formazan) that correlates with viable cell count. This approach is more sensitive in opaque systems – indeed, MTT was able to detect early-stage yeast growth that plate counts or turbidity methods do not (Oh & Hong, 2022). Second, agar diffusion (e.g. a disk diffusion test) is poorly suited to hydrophobic or low-diffusivity compounds in a food matrix. Many phenolics do not diffuse well in agar, potentially underestimating their activity (Letsiou et al., 2024). In the same comparative study, only the most potent extract (rosemary) showed a clear inhibition zone on yeast agar, whereas other extracts appeared inactive in diffusion yet still slowed yeast growth in liquid culture. This underscores that the lack of an inhibition zone doesn't always mean a lack of activity – it could be solubility issues. The MTT microplate method keeps the compounds in direct contact with the yeast in liquid juice, more accurately reflecting their true efficacy. Third,

the MTT assay is relatively rapid and amenable to high-throughput screening. Moreover, we could ascertain the viability of yeast under each treatment by reading the colour intensity without the need to subculture or plate the samples (Esposito & Ismail, 2022). Traditional broth dilution MICs in a food matrix might require serial dilutions and plating on agar to determine if growth occurred (since turbidity is hard to read in juice), which is laborious and time-consuming. Our colourimetric method simplifies this process to a one-step assay in the juice itself. Moreover, because MTT specifically measures viable metabolism, it can distinguish fungistatic vs fungicidal effects in a single assay by comparing the colour development to uninoculated control. In summary, the adapted MTT assay provided a more sensitive, quantitative, and rapid assessment of antifungal efficacy in orange juice than conventional methods would. It effectively addressed the challenges posed by the juice matrix (opacity, native microbes, etc.), thus yielding reliable MIC data for each natural compound.

The strong performance of pterostilbene and the moderate efficacy of other compounds can be contextualised with recent studies on natural antifungals in fruit beverages. Our research has shown that certain plant phenolics can inhibit spoilage yeasts in juice systems. Our finding that pterostilbene is highly effective is supported by its known activity against spoilage fungi: pterostilbene inhibited *Aspergillus flavus* growth and aflatoxin production significantly at low concentrations in model food systems (Hu et al., 2023). Likewise, it has been used to control *Botrytis cinerea* (grey mould) on fruit postharvest, indicating broad antifungal utility (D. Xu et al., 2022). In the realm of beverages, other phenolic constituents like thymol (from thyme) or eugenol (from clove) have demonstrated the ability to reduce yeast counts in fruit juices when used at sufficient doses (Almeida et al., 2018). For instance, spearmint essential oil (rich in carvone, a phenolic terpene) at 3.75 µL/mL (~0.375%) significantly reduced populations of *Pichia anomala* and *S. cerevisiae* in pineapple juice within 48 h. Similarly, a combination of citrus extract and mild heat was reported to lower fungal spoilage in tropical fruit juices (Almeida et al., 2018).

These studies illustrate that plant-based antimicrobials can work in juice matrices, but often, the needed concentrations are relatively high, or

the treatment time is long. Against this backdrop, pterostilbene's low MIC in orange juice is especially noteworthy – it suggests a phenolic molecule can be effective at levels that might be organoleptically acceptable. The literature on phenolics in juices also highlights that efficacy can depend on the juice composition. Phenolics may react with ascorbic acid or sugars, potentially reducing their active form over time, and juice clarity can be affected by the precipitation of some phenolics. We did not observe significant haze formation with pterostilbene in orange juice during the test duration, an encouraging sign. If pterostilbene is to be used as a preservative, one must also consider its stability in juice (it is relatively stable compared to some polyphenols, due to its methoxylated structure) and its safety/regulatory status.

This novel colourimetric food matrix assay for the determination of the growth-inhibitory effect of plant compounds in orange juice using MTT seems to be more material and labour-saving than the conventional counting method. This method could be used in research of new eco-preservative development for shelf-life extension in fruit beverages, which could substitute commonly used additives.

To support dissemination of the research outcomes, a scientific poster based on the data presented in this chapter was prepared and presented at the International Conference on Advanced Production and Processing – ICAPP, Novi Sad, 10$^{th}$ – 11$^{th}$ October 2019. The full poster is included as Appendix B.

## 5.3. Antimicrobial efficacy of lemongrass (*Cymbopogon citratus*) and fingerroot (*Boesenbergia pandurata*) essential oils against foodborne pathogens

According to gas chromatography analysis provided by the manufacturer, the compositions of lemongrass EO was following: geranial (47.46%), neral (33.34%), geraniol (5.30%), citronellal (2.53%), myrcene (1.10%), linalool (1.07%), geranyl acetate (0.85%), and limonene (0.35%). The composition of fingerroot EO was as follows: β-ocimene (24.68%), 1,8-cineole (17.99%), camphor (17.94%),

guaniol (11.97%), camphene (5.98%), d-limonene (3.84%), methyl cinnamate (2.32%), l-linalool (2.03%), α-terpinenol (1.15%), and α-pinene (1.00%).

Lemongrass essential oil (EO) demonstrated stronger antibacterial activity than fingerroot EO against all four pathogens tested (*S. aureus, L. monocytogenes, E. coli, and S. Enteritidis*). All results are shown in Table 6 and Table 7. At refrigerated temperature (4 °C), MIC values ranged from 0.03–0.25% (v/v), roughly half the concentrations required at room temperature (25 °C), where MICs spanned 0.06–0.50%. In each condition, lemongrass EO yielded lower MIC and MBC than fingerroot EO (e.g. MIC often ≤0.125% for lemongrass vs. ≥0.25% for fingerroot). This indicates lemongrass's inherently higher potency.

***Table 5.*** *MIC and MBC of the lemongrass essential oil against the tested organisms.*

| **Tested organism** | MIC 4°C (µg/mL) | MBC 4°C (µg/mL) | MIC 25°C (µg/mL) | MBC 25°C (µg/mL) |
|---|---|---|---|---|
| *Listeria monocytogenes* (LM) | 0.03 | 0.13 | 0.13 | 0.50 |
| *Staphylococcus aureus* (SA) | 0.03 | 0.06 | 0.06 | 0.50 |
| *Escherichia coli* (EC) | 0.03 | 0.25 | 0.06 | 1.00 |
| *Salmonella montevideo* (SM) | 0.03 | 0.13 | 0.13 | 0.50 |

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration;

***Table 6.*** *MIC and MBC of the fingerroot essential oil against the tested organisms.*

| **Tested organism** | MIC 4°C (µg/mL) | MBC 4°C (µg/mL) | MIC 25°C (µg/mL) | MBC 25°C (µg/mL) |
|---|---|---|---|---|
| *Listeria monocytogenes* (LM) | 0.25 | 0.50 | 0.50 | 2.00 |
| *Staphylococcus aureus* (SA) | 0.06 | 0.50 | 0.50 | 1.00 |
| *Escherichia coli* (EC) | 0.13 | 0.50 | 0.50 | 1.00 |
| *Salmonella montevideo* (SM) | 0.13 | 1.00 | 0.50 | 0.50 |

MIC = Minimal Inhibitory Concentration; MBC = Minimal Bactericidal Concentration;

These findings align with other studies showing lemongrass's broad-spectrum efficacy. For instance, lemongrass EO inhibited *E. coli* with MIC ≈20 µg/mL (0.002%) in one report (Mukarram et al., 2021), and showed MIC ~0.08% against *S. aureus* in another (Gao et al., 2020) – values comparable to our observations. Fingerroot EO, while antimicrobial, was consistently less potent. Its higher MICs are likely due to compositional differences: lemongrass oil is rich in citral (geranial + neral), a known membrane-disruptive monoterpenoid, whereas fingerroot oil contains more modestly active terpenes (e.g. ocimene, camphor, cineole) (Phanthong et al., 2013). The dominance of citral in lemongrass oil is a key factor in its superior bactericidal efficiency. Our results corroborate recent findings that lemongrass and similar spice EOs have stronger inhibitory effects on foodborne bacteria than fingerroot and other Thai spice oils (Lertchirakarn & Muangrat, 2023). The EO treatments were markedly more effective at 4 °C than at 25 °C, as evidenced by the lower MIC/MBC values at refrigeration (the additional results are provided in Appendix A). This pattern likely arises from both microbial physiology and EO chemistry. Psychrotrophic growth of the pathogens is slowed at 4 °C, so a smaller amount of EO can maintain a bacteriostatic environment. In contrast, at ambient temperature, the bacteria proliferate faster, necessitating higher EO

concentrations to achieve the same inhibition. Additionally, higher temperatures may reduce the apparent potency of EOs due to increased volatility and compound loss. Indeed, raising the exposure temperature has been shown to diminish EO efficacy, presumably as active constituents evaporate or degrade (Lertchirakarn & Muangrat, 2023). For example, in the same study, oregano EO's antibacterial effect dropped at 70 °C because its volatile antimicrobials dissipated. In our 5-day assay, oils at 25 °C likely experienced greater evaporation and oxidative changes than at 4 °C, lessening their sustained activity. There may also be changes in bacterial membrane fluidity or stress responses with temperature, but the net result here was clearly a need for ~2× higher concentrations at 25 °C to achieve equivalent control as at 4 °C , as shown in Tables 6 and 7. Interestingly, some recent work has noted specific temperature–EO interactions: for instance, cinnamon EO outperformed mint EO against *Salmonella sp*. at 25–37 °C, but at 4 °C mint vapour was comparatively more effective (Vepštaitė-Monstavičė et al., 2023). Such results underscore that temperature can modulate both microbial susceptibility and EO bioavailability in complex ways, but it needs to be noted that we did not analyse the oils' composition after storage, so this observation remains speculative, because without specific compositional data, we interpret the temperature effect cautiously and recommend further analysis of EO stability under different storage conditions.

Despite promising *in vitro* results, neither EO achieved significant pathogen inhibition when applied in the meat matrix, which is beyond the scope of this thesis and thus will not be discussed here.

To harness essential oils as preservatives in actual foods, formulation innovations are required. One approach is nanoencapsulation of the EO in carriers such as lipid nanoparticles, emulsions, or biopolymer capsules. Encapsulation can increase EO stability (protecting against oxidation/evaporation), improve dispersion in the aqueous phase of foods, and provide controlled release of actives (Chouhan et al., 2017; Ojeda-Piedra et al., 2022). Recent studies show that encapsulated EOs exhibit enhanced antibacterial performance because the nano-carriers increase solubility and target delivery of the actives to microbial cells (Ojeda-Piedra et al., 2022). For instance, nano-emulsions of essential

oils were able to distribute in food systems more uniformly, reducing required effective doses and minimising flavour impact (Chouhan et al., 2017). Lalonde et al. (2019) note that emulsifiers or solvent-based delivery can prevent EO components from binding to food ingredients, thus preserving antimicrobial availability (Gurtler & Garner, 2022). In practice, emulsifying lemongrass EO into a fine oil-in-water emulsion or coating could help it diffuse through a food product and contact bacteria instead of sequestering in fat (Gurtler & Garner, 2022). Edible coatings containing nano-encapsulated EOs are being explored for meats and produce, showing improved log reductions of pathogens compared to the free EO application (Ali et al., 2020; Das et al., 2021). Another strategy is using of EO combinations which can exhibit synergistic effects. Blending lemongrass with complementary EOs (e.g. oregano, clove) might broaden the antimicrobial spectrum and allow lower amounts of each oil (reducing sensory load) while achieving additive or synergistic inhibition (Lertchirakarn & Muangrat, 2023). Finally, integrating EOs into active packaging (e.g. antimicrobial sachets or films) can continuously release small amounts of vapour-phase EO to suppress surface contamination (Lalonde et al., 2019). Overall, employing nanoencapsulation and tailored delivery systems could address the solubility and volatility issues, whereas combining hurdles (e.g. mild heating, acidic pH, or other natural antimicrobials) with EOs can yield a synergistic preservation effect *in situ*. Future studies should test such strategies – for example, embedding lemongrass EO in a chitosan nanoparticle suspension – to evaluate whether food safety and shelf-life improve without compromising flavour.

Lemongrass and fingerroot EOs produced bacteriostatic effects against *E. coli, L. monocytogenes, S. aureus* and *S. enteritidis in vitro*. From the present study, it is clear that lemongrass essential oil is more efficient than fingerroot essential oil against all tested organisms. Both EOs showed an inhibitory effect even in very low concentrations; hence, both provide a potential for their use as natural preservatives in food.

The results presented in this chapter were shared at the Tropentag 2018 conference held in Ghent, Belgium, in the form of a scientific poster. The poster is included as Appendix C. A summary was also published in the official conference proceedings (Baert et al., 2018).

## 5.4. *In Vitro* Growth-Inhibitory Effect of Plant Extracts Against Beverage-Spoiling Yeasts

None of the five extracts derived from the Philippine plant species exhibited measurable antifungal activity against *S. cerevisiae, Z. bailii,* or *Z. rouxii* in the assay. All tested extracts, including bark-derived and leaf-derived fractions from *M. tanarius*, failed to inhibit yeast growth at the tested concentrations. This outcome contrasts with earlier findings on these plants' antimicrobial properties. *R. mucronata* traditionally used in wound care, is rich in tannins and polyphenols—compounds known to damage yeast membranes and interfere with enzymes (Kathiresan & Bingham, 2001). *C. tagal* contains phenolics and diterpenoids with reported antifungal properties (Bandaranayake, 2002; Glasenapp et al., 2019), while *M. tanarius* has prenylated flavonoids with known antimicrobial activity, including ergosterol pathway inhibition in fungi (Lee et al., 2019).

Several factors may explain why these extracts failed to inhibit *S. cerevisiae*, *Z. bailii*, and *Z. rouxii*, despite their phytochemical richness. First, *Zygosaccharomyces spp*. are among the most resistant spoilage yeasts, particularly *Z. bailii*, which can survive preservatives like sorbate and benzoate well above regulatory limits (Stratford et al., 2013). Their stress tolerance and ability to metabolise inhibitory compounds likely protected them from moderate levels of plant-derived inhibitors.

Second, the extract preparation method employed in this study relied exclusively on ethanol as the extraction solvent. While ethanol is commonly used in phytochemical research for its ability to solubilise both hydrophilic and moderately lipophilic compounds, it may not have extracted the full spectrum of bioactive constituents, particularly highly non-polar compounds such as essential oils or certain diterpenoids. Nevertheless, compared to water-based methods, ethanol generally offers improved recovery of antifungal agents like flavonoids and polyphenols (Abeysinghe, 2010; Belhadj-Salah et al., 2022; Do et al., 2014; Sultana et al., 2009). The lack of activity observed here suggests that either the ethanol extract still lacked sufficient quantities of key antifungal compounds, or the compounds present were not bioavailable

or stable under the test conditions. Additionally, factors such as vacuum filtration and storage may have contributed to the oxidative degradation of sensitive components—e.g., prenylated flavonoids or tannins—potentially diminishing antifungal efficacy (Cosme et al., 2020; H.-W. Lv et al., 2023; Pasquet et al., 2024).

Third, the tested concentrations may not have reached the effective threshold required for inhibition, particularly in complex food systems. While MIC values for phenolic-rich extracts can fall within 0.1–2 mg/mL against bacteria, higher concentrations are often required for yeasts, particularly *Zygosaccharomyces* species (Stratford et al., 2013). The extracts used may simply have been too dilute or the bioavailability of active components too low.

Finally, although the microdilution method is standardised and widely used for antifungal testing, the liquid juice matrix may not fully reflect the environmental conditions in which these plant extracts are traditionally effective. For example, *R. mucronata* and *C. tagal* have shown antifungal effects in topical applications and on skin infections (Bandaranayake, 2002), but show limited efficacy in aqueous systems. It's possible their antifungal modes of action—such as protein binding or chelation—are more suited to surface-level or contact environments than planktonic yeast cells in juice.

Despite these negative findings, the experiment yields valuable insight. Notably, this is one of the first attempts to screen Philippine *Rhizophora*, *Ceriops*, and *Macaranga* species specifically against *Z. bailii* and *Z. rouxii*, yeasts known for their resistance to both traditional and natural preservatives. The data suggest that, while ethnobotanically promising, these extracts may not be viable as single-agent antifungal preservatives in beverages under the tested conditions, unless applied at higher concentrations or reformulated for enhanced efficacy. Nonetheless, it remains possible that they could be effective against other spoilage organisms or pathogens more common in locally produced beverages in the Philippines, which differ in composition and microbial profiles from the model systems used here. Future research should include chemical analysis (e.g., compound isolation via HPLC-MS) of these extracts to identify which compounds are actually present

after the extraction process, as this would clarify the discrepancy between traditional use, literature reports, and our negative findings. Such analyses, combined with further testing against region-specific microbial contaminants, may reveal more targeted applications for these natural resources.

## 5.5. Practical Application of Natural Preservatives in Low- and Middle-Income Countries

Because these field trials were part of on-the-ground development projects, the data collected were largely qualitative and focused on practical outcomes. Nevertheless, they provided valuable insights into the performance of natural preservatives under real tropical conditions. Across all three countries, juices treated with natural preservative combinations showed significantly better shelf-life than untreated ones, which typically spoiled within days/weeks in tropical heat. The most effective treatment was a combination of a natural acid (lime juice or citric acid) with honey, which consistently preserved the quality of juices for up to 12 months. In contrast, using only acid or honey alone resulted in visible deterioration over time. These results support the principle of "hurdle technology," where multiple mild preservation methods, like acidity and high sugar content, are combined to inhibit microbial growth and oxidation. The acid lowered pH and reduced browning, while honey contributed osmotic pressure and natural antimicrobial compounds such as hydrogen peroxide (Mandal & Mandal, 2011).

Together, a synergistic effect was observed: for example, mango juice with combined citric acid and honey remained unfermented and sensorily fresh (aroma unchanged, no gas production) throughout storage, whereas a batch with citric acid alone eventually developed a slight fermented odour after ~3 months (likely due to yeast activity that tolerates acidic but low-sugar conditions). The honey thus significantly boosted the antimicrobial stability in acidic environments, an observation supported by laboratory studies on honey's antibacterial efficacy in foods (Suhartatik et al., 2024), which demonstrated that adding honey to fruit juice can substantially inhibit the growth of bacteria and total yeast/moulds, improving microbiological quality. Our field

results reinforce this, showing that even under non-refrigerated, village-level storage, honey's presence helped suppress spoilage organisms.

When comparing the natural preservatives to synthetic preservatives (from the Cambodia reference batches), the natural solutions showed promising, albeit slightly variable, performance. In the Cambodia trials, juices treated with the standard chemical preservative, sodium metabisulfite (typically at 0.1–0.2% w/v), a known potent antioxidant and antimicrobial for juice produce, unsurprisingly showed no browning and no microbial growth throughout the test period – sulphite treatment is well-known to produce very stable juices by inhibiting both enzymes and fungi (Krokida et al., 2001). The lime + honey treated juices performed nearly as well: panellists noted that the colour of lime + honey mango juice was vibrant with minimal darkening (almost comparable to the sulphite treated juice), and no mould was seen in those samples. Another interesting sensory observation was that the juice with lime juice and honey had a more "fruity" aroma and a rich taste that local tasters preferred in blind comparisons, whereas the sorbate/sulphite-added juice was sometimes commented to have a slight aftertaste. This subjective feedback suggests the natural preservatives did not detract from juice quality and may even enhance flavour (honey adding a mellow sweetness and lime adding a fresh tang). It aligns with consumer acceptability findings in literature – for example, a study (Olaniran et al., 2020) on tropical juice preservation found that a juice blend treated with 1% lime juice was the most preferred by a sensory panel, scoring higher in taste acceptance than the same juice with conventional additives.

In our Ethiopian trials, focusing also on mango juice, the absence of any synthetic preservative option meant relying entirely on natural methods. The mango juice that received a combined citric acid (or lime juice) plus honey treatment fared the best: it showed no sign of fermentation (no bulging of bottles or fizzing upon opening) and retained a bright orange colour after 6 weeks. In contrast, an untreated mango juice (prepared as an experimental extreme case) spoiled in only two days at ambient storage (developing fermentative gas and off-odour), underscoring that some form of preservation is absolutely necessary for such conditions. Juice treated with only ascorbic acid

(vitamin C) at a high dose (approx. 0.1% w/v) maintained colour well initially but eventually fermented after about 30 days, indicating that acid alone (without enough overall acidity or other hurdles) was insufficient to check microbial growth long-term. This concentration is commonly used in juice processing to prevent enzymatic browning and oxidative colour changes, but its antimicrobial effect is limited unless combined with other preservation strategies (H. S. Lee & Coates, 1999). Meanwhile, the batches with honey (which also increases the total soluble solids of the juice) had better outcomes; honey alone delayed spoilage to ~4 weeks, and honey + acid extended it to several months (trial ended after 6 months). These qualitative findings are consistent with controlled studies showing that lowering pH and adding sugar can substantially prolong juice shelf-life. (Olaniran et al., 2020) reported that in a mixed fruit juice stored at ~27 °C, adding 2–4% lime juice effectively suppressed fungal growth and bacterial growth for 5 weeks, far outperforming unpreserved juice, which saw microbial counts skyrocket within 2 weeks. In our field context, we achieved comparable inhibitory effects with a combination of acid and honey, achieving shelf stability on the order of months rather than days.

Looking at the sensory and quality parameters, the natural preservative treatments generally preserved the colour, smell, and taste of the fruit products as well as (and sometimes better than) the synthetic preservatives. The inclusion of ascorbic and citric acids was particularly important for colour retention in products like dried fruits and juices that are prone to enzymatic browning. Even without lab instrumentation, it was evident that samples treated with an acidic dip remained more brightly coloured. For instance, Ethiopian mango juice with lime juice added retained a vibrant orange-yellow hue with minimal browning, while a comparable juice without acid turned a dull brown within a few days due to oxidation. This aligns with existing reports that citric or ascorbic acid pretreatment yields superior colour outcomes in dried and juice products (Nyangena et al., 2019).

Such positive sensory feedback is crucial for adoption – the goal was not only to prevent spoilage but also to ensure the preserved foods remain enjoyable to eat. In Zambia and Ethiopia, where panellists initially had no experience with chemical preservatives, the naturally

preserved juices were judged on their own merits and found to be of high quality. In Cambodia, where a comparison could be made, the community tasters indicated an equal or higher preference for the naturally preserved samples over the sulphite/sorbate-preserved ones, suggesting that the substitution of chemicals with lime and honey did not compromise consumer acceptance. This finding is encouraging for clean-label food production: it suggests that natural preservative systems can meet both safety and sensory criteria, a result echoed by other researchers who note that natural acids and sweeteners can preserve foods while maintaining or even enhancing taste (Olaniran et al., 2020).

From a food safety and shelf-life perspective, the trials demonstrated that these natural preservation methods can significantly extend the usability of fruit juices in tropical environments. In all three countries, the best-performing treatments (acid + honey) protected the products against visible spoilage for the full six-week period. This shelf stability at ambient temperature is quite remarkable for preservative-free or minimally processed fruit juices, which would ordinarily spoil in a matter of days in the absence of refrigeration (Olaniran et al., 2020). Despite the lack of detailed microbial counts, the absence of spoilage in preserved samples — even under tropical, non-sterile field conditions — suggests low microbial activity and validates the effectiveness of the interventions. Simple practices like sterilisation or canning, combined with natural preservatives such as lime juice and honey, proved effective in extending shelf life without refrigeration. These methods were adopted by local partners: in Cambodia, a women-led cooperative replaced sulfites with honey and lime in dried fruit production; in Zambia and Ethiopia, similar combinations were integrated into mango juice preservation. Follow-up confirmed continued use and reduced spoilage, demonstrating the practical value of these low-cost, locally available solutions. Overall, the trials showed that natural preservatives can be successfully applied in small-scale tropical juice processing, improving food safety and reducing post-harvest losses without relying on industrial additives or cold chains.

**Cambodia:** In the Cambodia trials, which served as the pilot implementation, other product types (dried fruits and jams) were also

tested with multiple preservative variants. The presence of synthetic preservative controls in this site allowed for a benchmark comparison. Results showed that the natural treatments – particularly lime juice combined with honey – could nearly match the efficacy of chemical preservatives in preventing spoilage over 6 months. The cooperative in Cambodia successfully integrated the best-performing natural method (lime or citric acid + honey) into their production. By the end of the trial, they favoured this method for routine use, noting that the naturally preserved dried fruits and jams had excellent quality. The learned lessons from Cambodia (e.g., optimal dip concentrations and handling practices) were documented as a protocol for transfer to other countries.

***Figure 8**. Preparation of mango juice, jams and compote. Kampong Speu, Cambodia. 2023.*

**Zambia:** Following the Cambodia pilot, Zambia implemented the preservative protocols focusing on dried fruits and jams (mango and banana were the primary fruits used, reflecting local availability). The

same combination of citric or lime juice with honey was applied based on the Cambodian results. Despite differences in local climate (Zambia's dry season temperatures were slightly lower at times than Cambodia's tropical climate), the natural preservatives performed well. No major spoilage issues were observed in the treated Zambian products during the monitoring period. Community taste-testers in Zambia, who had not previously used chemical additives, responded very favourably to the quality of the jams and dried fruits preserved with honey and citrus – many remarked that the products "tasted just like the fresh fruit," indicating minimal loss of flavour. The success in Zambia confirmed that the methodology was robust across different settings. The cooperative there continued using the techniques, especially the honey-citric dip for dried mango, which allowed them to store dried slices from the peak harvest and sell them gradually without spoilage.

***Figure 9.*** *Installation of basic solar dryers for mango and other fruit processing. These dryers were used for some of the experiments. Mongu, Zambia. 2022. Photo by Radim Kotrba.*

**Ethiopia:** In Ethiopia, the trial centred primarily on mango juice preservation, as the local partner's interest was in developing bottled juices from abundant mango gluts. Using the protocol refined in Cambodia, Ethiopian mango juice was pasteurised and then treated with lemon/lime juice and honey as natural preservatives. The ambient storage tests showed that this approach could keep the juice unspoiled for several weeks to a few months, a significant improvement over traditional practices where fresh juice would last only a day or two. Some small batches of dried mango and papaya were also tried in Ethiopia using citric acid and honey dips; although done on a limited scale, these, too, remained mould-free and retained good colour for the trial duration. The results in Ethiopia demonstrated the versatility of the natural preservative system for both high-moisture products (juices) and low-moisture products (dried fruit). The local women's group that participated was able to establish a micro-enterprise selling preserved mango juice in nearby towns, providing an incentive to maintain the preservative practice beyond the project timeline. They reported that consumers appreciated the fact that the juice contained only natural ingredients. This reflects a broader trend of public preference for "naturally preserved" foods, which our project was able to leverage in a practical way.

In conclusion, the practical application of plant-derived and natural preservatives in Cambodia, Zambia, and Ethiopia confirmed that such methods are not only scientifically sound but also operationally feasible in development project settings. The methods established in Cambodia could be successfully exported to other regions with minimal modification, and the results from all three countries consistently pointed to the effectiveness of a combined acid and honey treatment in preserving fruit juices and other products. These trials bridged the gap between *in vitro* laboratory findings and real-world usage, ultimately leading to improved food safety and reduced waste in the participating communities. The sustained adoption of the techniques by local cooperatives underscores the impact and relevance of the approach for tropical agro-processing initiatives aimed at clean-label and sustainable food preservation.

***Figure 10.*** *Workshop for local schools focused on the importance of fruit preservation and its role in human nutrition. Arba Minch, Ethiopia, 2023. Photo by the author.*

# 6. Conclusion

The results of a series of experiments with plant-derived phenolic compounds clearly confirmed the suitability and effectiveness of a newly developed modified broth microdilution method for assessing the anti-yeast activity of natural agents in fruit juices. Therefore, it can be concluded that this innovative assay can be used to filter the anti-yeast activity of juice preservative agents because it saves time, material, and labour compared to the methods usually used. This novel modified broth microdilution method is not only cost- and labour-effective but also rapid, significantly reducing the time required for antimicrobial testing. The modified assay demonstrated high reproducibility and robustness, making it suitable for high-throughput screening of natural preservatives in food systems. Among all compounds tested, four showed notable inhibitory activity against all yeasts tested in both orange and apple juice models. Pterostilbene emerged as the most effective compound, followed by oxyresveratrol, piceatannol, and ferulic acid. The level of inhibition depended significantly on differences in the chemical structures of tested compounds, whereas stilbenes produced the strongest anti-yeast effect. In summary, stilbenes, especially pterostilbene, were shown to be worthy of attention and promising agents for further investigation in the field of beverage additives. Notably, only the stilbene group consistently demonstrated homogeneity in its inhibitory activity against all three yeast strains, regardless of the juice type. However, more detailed research on their technological and organoleptic properties is necessary before any practical application.

To support core objectives, the research also included exploratory evaluations of essential oils and further refinements of a modified MTT viability assay. Lemongrass and fingerroot essential oils showed strong antibacterial activity *in vitro*, especially under refrigerated conditions, although their practical use in real food systems remains limited by volatility and sensory constraints. Meanwhile, the MTT assay was successfully adapted for use in turbid juice matrices and provided a reliable, rapid method for quantifying yeast viability, further strengthening the methodological toolkit developed in this study.

Although exploratory in nature, field trials in Cambodia, Zambia, and Ethiopia illustrated the potential for using simple natural preservative combinations (e.g., honey, lime juice, citric acid) in low-resource food processing environments. While these were not controlled scientific experiments, they validated the practical relevance of the laboratory findings and highlighted the importance of adaptable preservation strategies for small-scale producers.

In conclusion, this thesis provides a scientifically grounded, practically applicable framework for evaluating and promoting plant-derived antimicrobial agents as natural preservatives for fruit-based beverages. The modified broth microdilution method in particular offers a powerful, scalable tool for future preservative screening, enabling safer and more consumer-friendly alternatives to synthetic additives.

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- **Staš, J.,** Houdková, M., Banout, J., Duque-Dussán, E., Roubík, H., & Kokoska, L. (2024). *Adaptation and validation of a modified broth microdilution method for screening the anti-yeast activity of plant phenolics in apple and orange juice models. Life*, *14*(8), 938. https://doi.org/10.3390/life14080938
- Lotfy, H.R., **Staš, J.** & Roubík, H. Renewable energy powered membrane desalination — review of recent development. *Environ Sci Pollut Res* 29, 46552–46568 (2022). https://doi.org/10.1007/s11356-022-20480-y
    - Correction published July 2022: Lotfy, H.R., Staš, J. & Roubík, H. Correction to: Renewable energy powered membrane desalination — review of recent development. *Environ Sci Pollut Res* 29, 46569 (2022). https://doi.org/10.1007/s11356-022-20947-y

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- **Staš, J.,** Petrtýl, M., Verner, V., & Roubík, H. (2024, October). *Where sand meets water: The potential of extensive aquaculture in the Western Province of Zambia*. In *5TH INTERNATIONAL MULTIDISCIPLINARY CONFERENCE FOR YOUNG RESEARCHERS.* Prague (oral presentation)
- **Staš, J.,** Petrtýl, M., & Roubík, H. (2023, October). *Development of methodology for comparison of manure and biogas plants digestate (bio-slurry) for fishpond fertilising*. In *4th Multidisciplinary Conference for Young Researchers 2023.* Prague. (oral presentation)

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### Posters

- Duque Dussán, E., **Staš, J.,** & Banout, J. (2022, September). *Coffee bean drying shrinkage comparison by finite element simulations and real image processing*. Poster, available online.

- Rázková, Z., Duque Dussán, E., **Staš, J.**, & Banout, J. (2021, November). *Solar drying applications in conventional Vietnamese beef jerky*. In ELLS conference 2021. At Warsaw, Poland. Available online.
- Duque Dussán, E., Villada, A., **Staš, J.**, & Banout, J. (2021, November). *Coffee seed drying predictive finite element model*. Poster. Available online.
- **Staš, J**., Duque Dussán, E., Villada, A., & Banout, J. (2021, November). *Effects of different drying methods on organoleptic properties of traditional Vietnamese beef and buffalo jerky*. Poster presented at the 2nd Multidisciplinary Conference for Young Researchers – Sustainable Development Trends and Challenges Under COVID-19. Available online.
- **Staš, J.** Houdková, M., Banout, J., Kokoska, L. (2019, October). *Development of new in vitro colorimetric MTT assay for high-throughput screening of anti-yeast activity of plant-derived preservative candidates in an orange juice food model*. 1st International Conference on Advanced Production and Processing – ICAPP. http://dx.doi.org/10.13140/RG.2.2.22377.81763
- Vongkamjan, K., Banout, J., & **Staš, J.** (2018, September). *Antimicrobial efficacy of lemongrass (Cymbopogon citratus) and fingerroot (Boesenbergia pandurata) essential oils against foodborne pathogens*. Poster, at *Tropentag 2018*, Ghen, Belgium. Available online.

List of research and development projects

- **2025:** Vermicompost & Biochar Integration for Climate-Smart Farming (V-BIOCHAR). Cambodia
- **2024:** Vermiculture based Sustainable Organic Innovative Livelihoods (V-SOIL). Cambodia
- **2024:** Nutrient-rich Powder Production in Kampong Speu Province, Cambodia
- **2022-2023:** The Use of Mango Stones as a Source of Valuable Fats and a Renewable Energy, Cambodia. Project consultant, laboratory works.
- **2022-2024:** Integrated Farming III, Zambia. Project coordinator; Small-scale aquaculture; integrated farming systems.

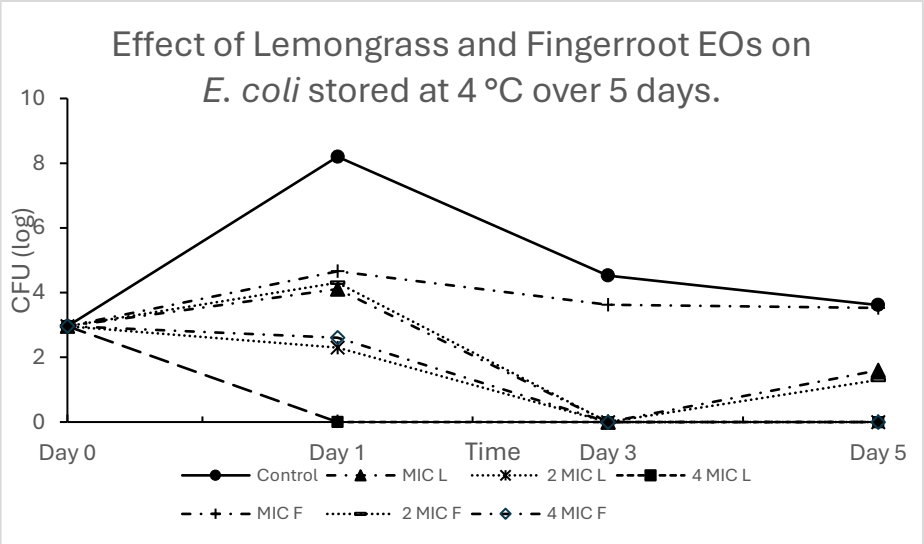
- **2021-2024:** Bio-plynovou technologií k vyšší odolnosti komunit v západní provincii Zambie
- **2020-2023:** Increasing productivity and supporting the development of mango, cassava and organic fertilizer value chains in Western Province, Zambia.
- **2020-2023:** Implementation of the fruit value chain for improved nutrition and efficient production in Arba Minch, GamoGofa, SNNPR, Ethiopia.
- **2020-2021:** Introducing the model of Hybrid Solar Driers for fruits, vegetables, herbs and NTFPs, Georgia. Project by UNDP. Expert on solar drying technologies.
- **2019-2023:** Integrated Farming II, Zambia.
- **2018-2021:** Agribusiness for life: Livelihood, Innovation, Food Security, and Empowerment (Zambia).
- **2014-2025:** Member of IGA project at FTA: "Natural products as preservatives in food processing".
- **2019:** PISAI (Participatory and Integrative Support for Agricultural Initiative).
- **2019:** Project SIMPLE ( Support of International Platform Merging Labour and Education).
- **2018-2019:** Strengthening of teaching, research and networking capacities at University of Barotseland in Mongu for agricultural development of West Province, Zambia.
- **2017-2018:** ALFABET program: 6-month internship, Prince of Songkla University, Thailand, research in the field of food processing.
- **2015-2016:** Support of scientific and research capabilities of teachers and students at Hue University of Agriculture and Forestry, Vietnam. Research workshops conducted.
- **2011-2013:** Renewable Energy Resources for Rural Areas in Thua-Thien Hue Province. Research workshops conducted.
- **2012-2013**: Capacity Building of Higher Agricultural Education in Cambodia

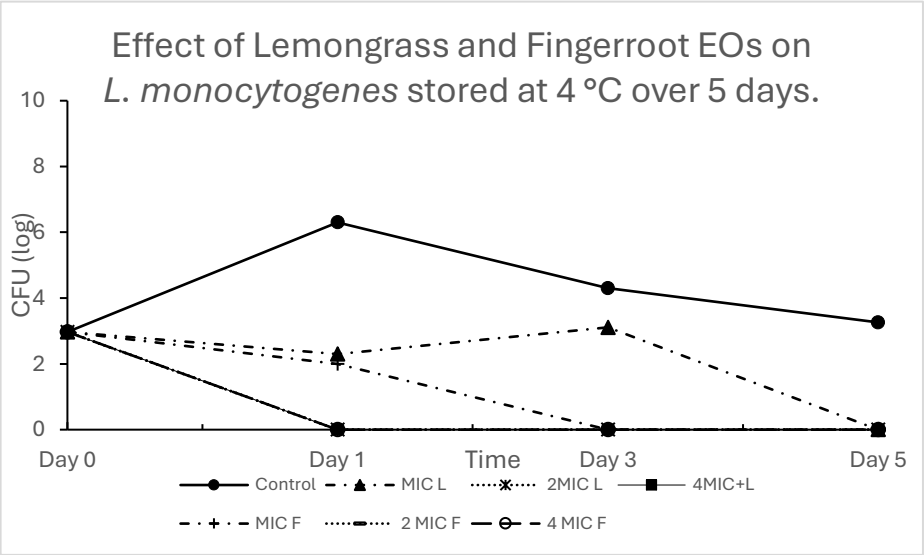
List of EU projects

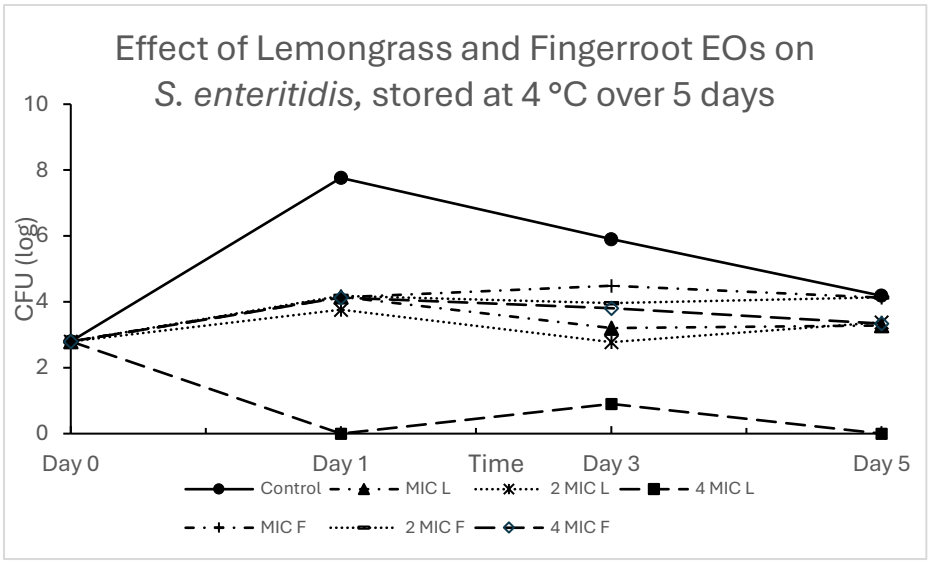
- SCORE: Structural Capacities for Tackling Wicked Problems Programme
- OSIRIS: Open Science to Increase Reproducibility In Science
- COMUNIDAD: Combined Use of EGNSS and Copernicus Data to Develop Innovative Downstream Services for the Users from Chile and Colombia
- UNICOM: Universities – Communities: strengthening cooperation
- BIO-CAPITAL**:** Mobilising investments for protecting and restoring biodiversity by harnessing innovative financial solutions and advanced geospatial analytics

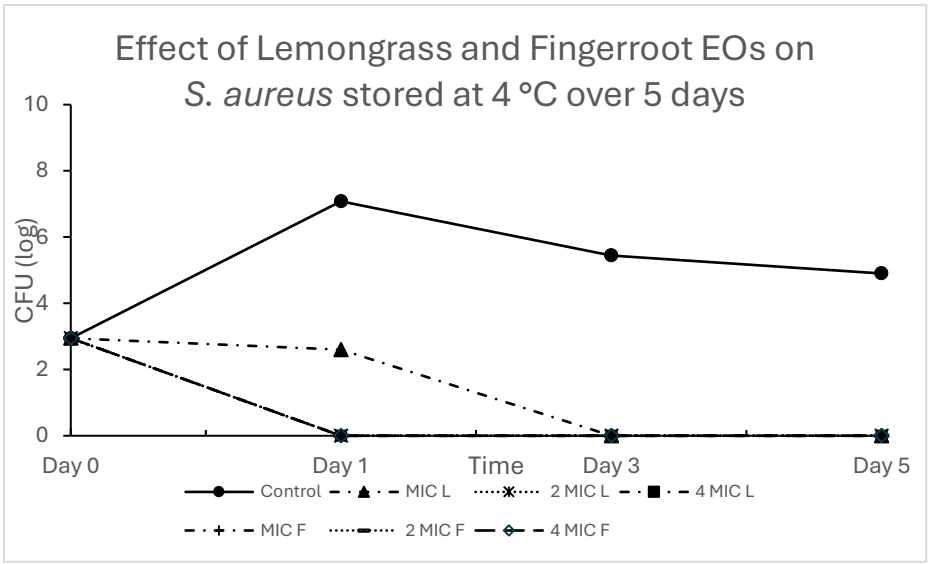
# 8. Appendices

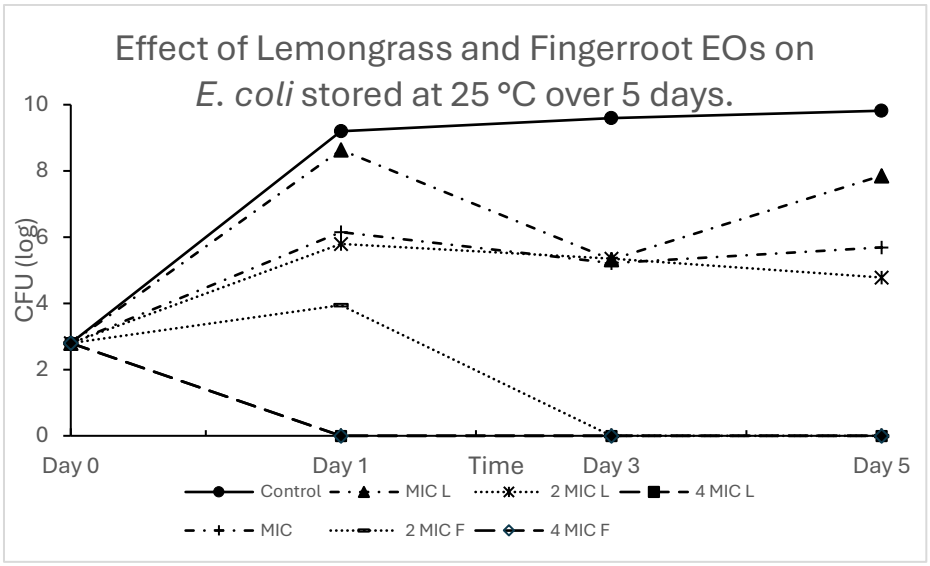
## 8.1. Appendix A. Additional results (Chapter Lemongrass and Fingerroot EOs against tested food-borne pathogens at different temperatures (4 °C and 25 °C) over the time

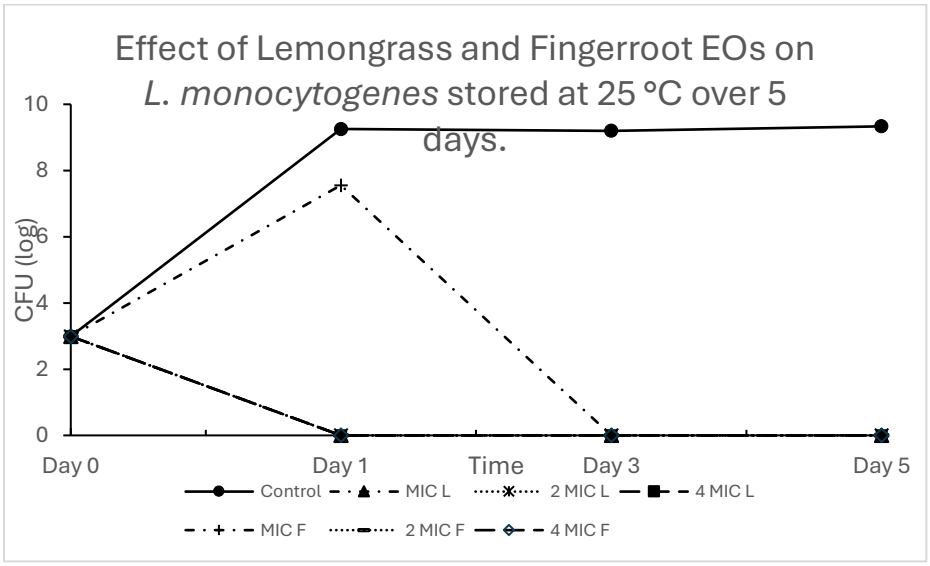


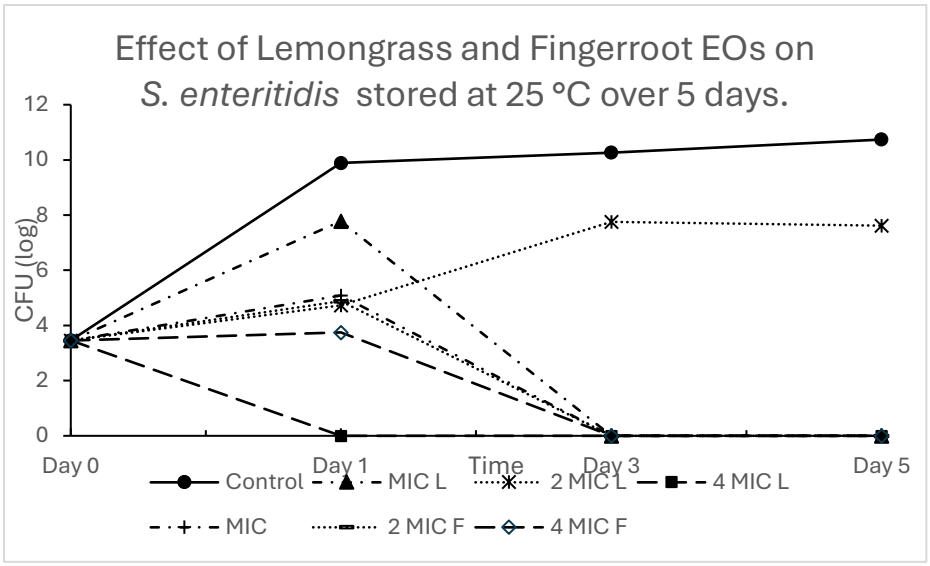



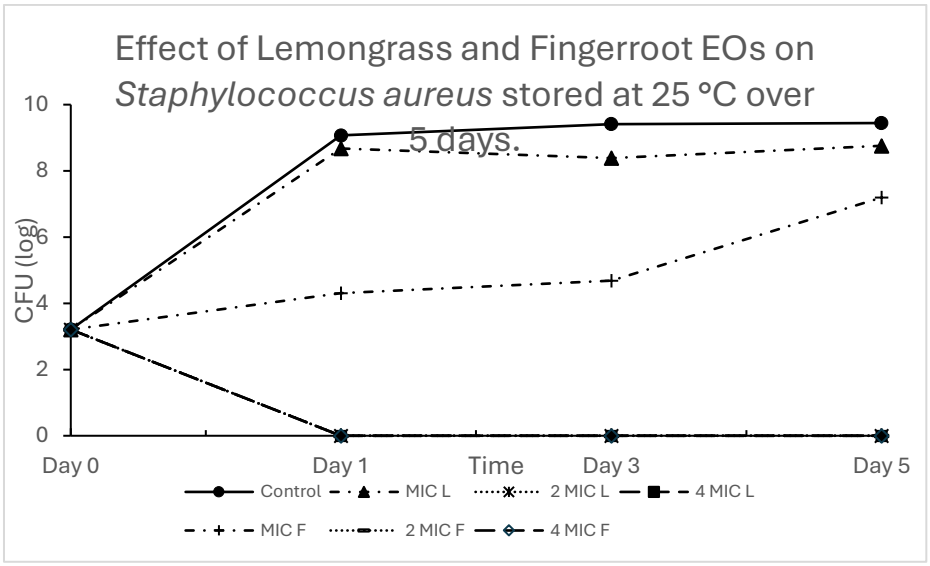

Effect of Lemongrass and Fingerroot EOs on
S. enteritidis, stored at 4 °C over 5 days
CFU (log)
10
8
6
4
2
0
Day 0
Day 1
Time
Day 3
Day 5
Control
MIC L
2 MIC L
4 MIC L
MIC F
2 MIC F
4 MIC F


Effect of Lemongrass and Fingerroot EOs on
S. aureus stored at 4 °C over 5 days
CFU (log)
10
8
6
4
2
0
Day 0
Day 1
Time
Day 3
Day 5
Control
MIC L
2 MIC L
4 MIC L
MIC F
2 MIC F
4 MIC F


Effect of Lemongrass and Fingerroot EOs on *E. coli* stored at 25 °C over 5 days.
CFU (log)
10
8
6
4
2
0
Day 0
Day 1
Day 3
Day 5
Time
Control
MIC L
2 MIC L
4 MIC L
MIC
2 MIC F
4 MIC F


Effect of Lemongrass and Fingerroot EOs on *L. monocytogenes* stored at 25 °C over 5 days.
CFU (log)
10
8
6
4
2
0
Day 0
Day 1
Day 3
Day 5
Time
Control
MIC L
2 MIC L
4 MIC L
MIC F
2 MIC F
4 MIC F


Effect of Lemongrass and Fingerroot EOs on S. enteritidis stored at 25 °C over 5 days.
CFU (log)
12
10
8
6
4
2
0
Day 0
Day 1
Day 3
Day 5
Time
Control
MIC L
2 MIC L
4 MIC L
MIC
2 MIC F
4 MIC F


Effect of Lemongrass and Fingerroot EOs on Staphylococcus aureus stored at 25 °C over 5 days.
CFU (log)
10
8
6
4
2
0
Day 0
Day 1
Day 3
Day 5
Time
Control
MIC L
2 MIC L
4 MIC L
MIC F
2 MIC F
4 MIC F

## 8.2. Appendix B. Scientific poster based on the chapter 5.2 was presented at the International Conference on Advanced Production and Processing, Novi Sad, 10th – 11th October 2019.

# Development of new *in vitro* colorimetric MTT assay for high-throughput screening of anti-yeast activity of plant-derived preservative candidates in an orange juice food model

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Czech University of LifeSciences Prague, Kamycka 129, 16500, Prague, stas@ftz.czu.cz

## Introduction

Yeasts are the usual contaminants in fruit juices and responsible for decreasing the quality and of such products. Preservatives are principally added to fruit juices to enhance their shelf life. However, aside from their advantages, some of the artificial preservatives may possess life-threatening side effects. Plant-derived compounds and their mixtures are perspective materials for the development of new preservative agents. For this reason, the antifungal activity of plant extracts and essential oils has been studied intensively, however the **conventionally used methods are time and material consuming**. Therefore, we **developed simple *in vitro* method (Food matrix MTT assay)** suitable for high-throughput screening of anti-yeast potential of plant-derived compounds in orange juices using standard 96-well microtiter plates and the tetrazolium-based colorimetric assay - MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide].

## Materials and Methods

### Juice sample preparation

The juice was prepared by squeezing the **fresh orange fruit** (*Citrus sinensis*) obtained from local market in Prague. After that, the juice was filtrated trough iron sieve to separate the pulp and then through the membrane nano filter (Pragopor) with pore size 0.23 μm in filtration machine (Sartorius). By this filtration, we obtained sterile juice.

### Chemicals

All used compounds (Benzoic acid, citric acid, curcumin, gallic acid, potassium sorbate, pterostilbene, sodium metabisulfite, sorbic acid, tannic acid and dye thiazolyl blue tetrazolium bromide) were purchased from Sigma-Aldrich (Prague, Czech Republic).

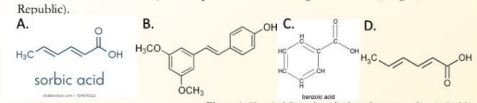


**Figure 1**: Chemical formulas of selected compounds – A. Sorbic Acid, B. Pterostilbene, C. Benzoic Acid, D. Potassium Sorbate[4].

### Yeast and culture media

Standard strain of the *Saccharomyces cerevisiae* was used (DMS 2548). The cultivation and assay media was Sabouraud (SA). Yeast and cultivation media were purchased from Oxoid (Basingstoke, UK).

### Food matrix MTT assay

MTT assay is well know method for assessing cell metabolic activity. It is reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color[1]. This method is normally used in **culture medium**, but in this study we applied it directly **to food sample–to an orange juice.**

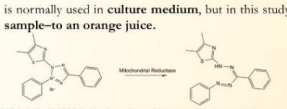


**Figure 2:** Enzymatic reduction of MTT to formazan[1].

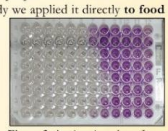

**Figure 3:** A microtiter plate after an MTT assay[2].


**Figure 4:** Scheme of Food matrix MTT assay.

## Results and discussion

The results of *in vitro* growth-inhibitory effect of plant-derived compounds against yeast *Saccharomyces cerevisiae* in orange juice, in liquid phase using the newly developed food matrix MTT assay method are summarized in **Table 1.** The lowest minimum inhibitory concentration (MIC) values were observed for Pterostilbene (64 μg/mL) followed by Sorbic acid with MIC 128 μg/mL.

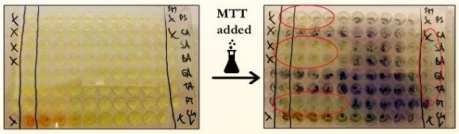


**Figure 5:** The change in colour of contaminated wells after 30 minutes in room temperature.

**Table 1**: Minimal Inhibitory Concentration (MIC) of selected compounds. MIC (F) = Final Minimal Inhibitory Concentration = mean of MIC (1); MIC (2) and MIC (3). ND = Not Detected

| Compounds | MIC (F) | MIC (1) | MIC(2) | MIC(3) |
|---|---|---|---|---|
| Citric acid | ND | ND | ND | ND |
| Potassium sorbate | 256 | 256 | 128 | 256 |
| Sorbic acid | 128 | 128 | 128 | 128 |
| Benzoic acid | 256 | 256 | 256 | 256 |
| Curcumin | ND | ND | ND | ND |
| Gallic acid | ND | ND | ND | ND |
| Tannic acid | ND | ND | ND | ND |
| Pterostilbene | 64 | 64 | 128 | 64 |
| Sodium metabisulfite | 128 | 128 | 128 | 256 |

### Comparison with conventially used methods

**Agar disk diffusion method** was not suitable for some extracts. The antimicrobial potency of different samples may not always be compared, mainly because of differences in physical properties, such as solubility, volatility and diffusion characteristics in agar. Compounds having a good diffusion coefficient and low antimicrobial activity may penetrate the agar medium even in small amounts.

**Dilution methods – broth microdilution/macrodilution**

Test samples that are not entirely soluble may interfere with turbidity readings, emphasizing the need for a negative control or sterility control, i.e. extract dissolved in blank medium without microorganisms, which leads to usage of higher number of materials[3]. This method is labor and time-consuming and difficult to use in clinical laboratories. Another problem yet to be resolved is the proper interpretation of trailing growth in broth dilution MIC tests with azole antifungal agents[3].

## Conclusion

This novel colorimetric food matrix assay for the determination of growth-inhibitory effect of plant compounds in orange juice using MTT seems to be materials and labor-saving than conventional counting method. This method could be used in research of new eco-preservatives development for shelf-life extension in fruit beverages, which could substitute commonly used additives.

## Acknowledgement

This research was financially supported by the Czech University of Life Sciences Prague (projects IGA 20195003 and 20195006).

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3. Scorzoni L. et. al. „The use of standard methodology for determination of antifungal Activity of natural products against medical yeasts. Brazilian Journal of Microbiology (2007) 38:391-397 ISSN 1517-8382.
4. Yang, W., Wu, Z., Huang, Z.Y. et al. J Food Sci Technol (2017) 54: 337. https://doi.org/10.1007/s13197-017-2754-x

Czech University of Life Sciences Prague
Faculty of Tropical AgriSciences

## 8.3. Appendix C. Scientific poster based on the chapter 5.3 was presented at the Tropentag Conference, Gent 2018.

# Antimicrobial efficacy of lemongrass (*Cymbopogon citratus*) and Fingerroot (*Boesenbergia pandurata*) essential oils against foodborne pathogens


**Jan Stas[1], Kitiya Vongkamjan[2], and Jan Banout[1]**

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[2] Department of Food Technology, Prince of Songkla University, Hat Yai, Songkla, 90112, Thailand

Email: **stas@ftz.czu.cz**

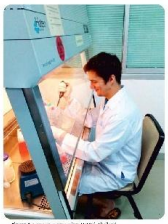

### Introduction

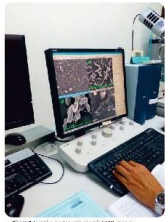
The consumption of ready-to-eat meat products such as sausages, meatloaf, dried meat and cakes is increasing mainly in developing countries (Heinz and Hautzinger, 2010). However, the risk of contamination with food-borne pathogens from poorly processed and stored meat products, such as *salmonellosis, Escherichia coli* and other pathogens pose a great health hazard that needs to be controlled. Presently, these pathogens are being controlled with the aid of synthetic or natural preservatives. However, concerns about the safety of chemical additives are on the rise in past recent years. As a consequence, consumers are progressively demanding the use of natural products as alternative for synthetic preservatives (Balciunas et al., 2013). Plants are a source of bioactive molecules and have been widely used both traditionally and commercially to increase the shelf-life and safety of foods (Sasidharan et al., 2008). Thus, this study investigates the potential of essential oils (EOs) of Lemongrass and Fingerroot as a natural preservative to control four common foodborne pathogens *in vitro*.

### Objectives

The main objective was to identify *in vitro* antimicrobial efficacy of two EOs against four different food-borne pathogens

Particular objectives were:
1.To determine the minimal inhibitory concentration (MIC) of EOs
2.To analyse *in vitro* inhibition over time in different conditions





### Materials and methods

**Essential Oils**

The EOs of Lemongrass (*Cymbopogon citratus*) and Fingerroot (*Boesenbergia pandurata*) were used in this work. Both EOs were obtained from BOTANICESSENCE Essential Oils, Thailand. The EOs were certified by Ecocert SA (F32600).

**Bacterial strains and culture conditions**

Tested pathogenic bacteria were comprised of *Salmonella enteritidis (DMST), Escherichia coli* (DMST), *Listeria monocytogenes* (F2365), and *Staphylococcus aureus* (DMST). These microorganisms were chosen as they are commonly associated with the spoilage of refrigerated foods. All species were supplied by the Faculty of Agro-Industry, Prince of Songkla University (Hat Yai, Thailand). The stock cultures of bacterial strains were prepared overnight in brain heart infusion broth (BHIB) at 37 °C and then they were streaked on the brain heart infusion agar (BHIA) and incubated for 24 hours at (37 °C). The cultures were kept under refrigerated conditions and were subcultured after every ten days.

Table 1. MIC and MBC of the lemongrass EO against tested organisms (%, v/v)

| Tested organism | MIC 4°C | MBC 4°C | MIC 25°C | MBC 25°C |
|---|---|---|---|---|
| Listeria Monocytogenes (LM) | 0.03 | 0.13 | 0.13 | 0.50 |
| Staphylococcus Aureus (SA) | 0.03 | 0.06 | 0.06 | 0.50 |
| Escherichia Coli (EC) | 0.03 | 0.25 | 0.06 | 1.00 |
| Salmonella Montevideo (SM) | 0.03 | 0.13 | 0.13 | 0.50 |

Table 2. MIC and MBC of the fingerroot EO against tested organisms (%, v/v)

| Tested organism | MIC 4°C | MBC 4°C | MIC 25°C | MBC 25°C |
|---|---|---|---|---|
| Listeria Monocytogenes (LM) | 0.25 | 0.50 | 0.50 | 2.00 |
| Staphylococcus Aureus (SA) | 0.06 | 0.50 | 0.50 | 1.00 |
| Escherichia Coli (EC) | 0.13 | 0.50 | 0.50 | 1.00 |
| Salmonella Montevideo (SM) | 0.13 | 1.00 | 0.50 | 0.50 |

**Microbial assay**

The broth macrodilution method was used to determine he MICs and MBCs of oils as explained by Hammer et al. The MIC was defined as the lowest concentration of the test compound to inhibit the growth of microorganisms and the MBC was defined as the lowest concentration of the test compound to kill the microorganisms.

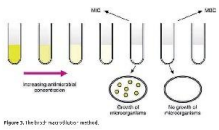



Figure 3. Broth macrodilution method.

**Inhibition over time**

The sterile tubes with BHI broth (5 ml) were inoculated with 2 different EOs at concentrations MIC, 2xMIC, 4xMIC and with 0.5 % (v/v) of tween-80. Then 100 µL of active inoculums of each bacteria ($10^6$ CFU/ml) was added. Sampling for viable cells were carried out at day 0, 1, 3 and 5, at two different storage temperatures (4 °C and 25 °C). The viable plate counts were monitored as follow: 50µL sample of each treatment was spread on the surface of BHIA and the colonies were counted after incubation at 37 °C for 24 h. At each assay time, controls without EOs were also tested.

### Results and discusion

Both EOs were found effective against all four tested organisms. Gram positive organisms (*S. aureus, L. monocytogenes*) showed similar sensitivity to EOs as gram negative organisms (*E. coli, S. enteretidis*). Similar observations were made by Onawunmi and Ongulana et al. The antibacterial activity was found progressively increasing with the increase in concentration of oil. On the other side, the antibacterial activity of both EOs was lower in the case of higher temperature (25°C) in all tested organism. As can be seen at table 1 and table 2, the MIC at 4°C varies from 0.03 to 0.25% and at 25°C from 0.06 to 0.50% respectively. Lemongrass essential oil showed higher efficacy against all tested organism in both temperatures.

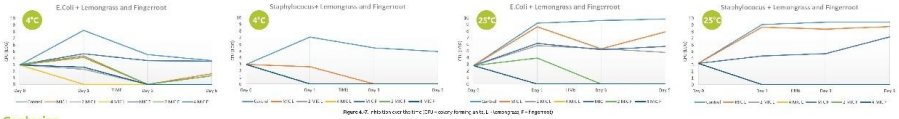



Figure 4-7. Inhibition over time (CFU = colony forming units, L = lemongrass, F = fingerroot)

### Conlusion

Lemongrass and fingerroot EOs produced bacteriostatic effect against *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella enteritidis in vitro*. From the present study it is clear that lemongrass EO is more efficient than fingerroot EO against all tested organisms. Both EOs showed inhibitory effect even on very low concentrations, hence both the spices provide a potential for their use as natural preservatives.

## 8.4. Appendix D. Shortened Curriculum vitae

### Work experience

**1/2023-current**

**Projects manager and Junior researcher at** BioResources & Technology

**1/2022–12/2023**

**Specialist in Project center**: Faculty of Tropical AgriSciences (FTA), Czech University of Life Sciences Prague (CZU). Main responsibilities: Assisting with writing the project proposals; Communication with project applicants; Preparation of documentation etc.

**10/2017–current**

**Ph.D. student/candidate**: Tropical Agrobiology and Bioresource Management, (FTA, CZU). Main responsibilities: Management of laboratory team; Research activities; Scheduling team meetings; writing minutes of the meetings; booking rooms, etc.

**2018–current**

**Referent for PR of the faculty and Webmaster** (FTA, CZU). Main responsibilities: Web mastering; Social networks management; Creation of the content for web and social networks (FB, IG, YTB,…); Communication with employees, institutions; Organization of online/physical meetings and events; Mails monitoring; Proof-reading of documents, etc.

**2019–current**

**Technical expert**: Holistic Solutions s.r.o., **an expert on integrated farming systems; solar drying, renewable energy, food processing, aquaculture.** Main responsibilities: Visits of abroad project localities; Management of the trips; Monitoring of work in progress; Arranging business trips, accommodation, and travel booking; collecting documents for claims reimbursement, etc.

**2014–2022**

**Laboratory assistant** (FTA, CZU): Food security, Renewable energy, Natural compounds in food preservation, Microbiology, Solar drying, Aquaculture systems, etc.

**2018–2021**

**Social Media Specialist** (FTA, CZU). Main responsibilities: Copywriting; Creating social media content; Organizing meetings with the PR team; communicating with other social media specialists etc.

**10/2017 – 4/2018**

**Doctoral researcher:** microbiology, natural compounds in food processing, food preservation methods. Faculty of Agro-Industry Prince of Songkla University, Hat Yai, Thailand

**10/2017 – 4/2018**

**Scientific Researcher: the use of solar dryer** for preparing traditional Vietnamese beef and buffalo jerky

Hue University of Agriculture and Forestry, Vietnam

*Seven years of experience with the implementation of development and research projects in tropical and less developed countries (Vietnam, Cambodia, Zambia, Ethiopia, Georgia). Teaching activities on tropical agriculture and sustainable development with a special focus on food processing technologies, food safety, solar drying, and aquaculture. Short-term and long-term internships on development and research projects. Assistance in organizing and facilitating summer schools (Cambodia, Thailand). Courses taught at FTA: Global Food Security, Renewable Energy for Food Processing.*

## Education

**10/2017–current**

**Doctoral degree**: Tropical Agrobiology and Bioresource Management (studied in English), FTA, CZU **Specialisation:** Food processing, solar drying, microbiology, aquaculture, sustainable development, use of natural compounds in food preservation
**Thesis:** "*In vitro* growth-inhibitory effect of plant-derived products against beverage-spoiling microorganisms"
**Internships**: Prince of Songkla University, Thailand (7 months), Chiang Mai University, Thailand (1 month), Big Terra Alpha s.r.o., Czech Republic (2 months)

**2015 – 2017**

**Master's degree (Ing.):** Sustainable Rural Development in Tropics and Subtropics (studied in English), FTA, CZU

**Specialisation**: Food processing, sustainable agriculture, solar drying, food quality, aquaculture, integrated farming, etc.

**Thesis:** "Using a solar dryer for preparation of traditional Vietnamese beef jerky"

**Internship:** Hue University of Agriculture and Forestry, Vietnam (5 months)

**2012 – 2015**

**Bachelor´s degree (Bc.):**

Sustainable Rural Development in Tropics and Subtropics (studied in Czech), FTA, CZU

**Specialization:** renewable energy sources, aquaculture, microbiology, solar drying, fish processing natural preservatives

**Thesis:** "Natural compounds used for reduction of enzymatic browning in food processing".

**Internship:** Royal University of Agriculture, Cambodia (2 months)

## Relevant memberships, courses and workshops

Member of BioResources & Technology - *The main long-term research interest of the Biogas Research Team is to reveal the current state, bottlenecks and perspectives of biogas plants in developing countries. The main objective of the currently solved research is to determine the real impact of small biogas plants in developing countries on the environment and to climate change and to contribute crucially to the current global debate on small biogas plants in developing countries.*

Member of Food Security Research Group - *The research is focused on various aspects of food security in developing countries. The most important part of the research is oriented on* ***food processing technology*** *and its applicability in developing countries in order to ensure sufficient production of good quality and healthy foods. The priority of our research is the preservation of food using economically viable techniques based on renewable energy sources (e.g.* ***solar drying, solar cooking****) as well as the use of natural compounds as food preservatives.*

Member of Capacity Building & Agricultural Development Team - *The essential goal of the team is capacity building and strengthening the skills of people to respond to the main international challenges addressed to the well-being of the next generations: agriculture, food safety and security, technologies in life sciences, balanced management of the natural resources, sustainable rural development, and adaptation to climate change.*

Member of Laboratory of Ethnobotany and Ethnopharmacology - *Mostly focused on the study of chemistry and bioactivity of underutilized (neglected) crops of tropical and subtropical origin. Species used by native peoples in traditional folk medicinal systems are plants of special interest.*

Member of Laboratory of Food Processing Technologies - *This laboratory focuses on the study of the technological aspects of* ***food processing****, with an emphasis on their applicability in the tropical areas of the developing world. A priority of our research is* ***food preservation by drying****, using simple and economically feasible techniques to support renewable energy sources (e.g. solar drying, solar cooking).*

Former member of BeFair at CZU - *organization of lectures, screenings, discussions and exhibitions with topics as fairtrade, development, globalization, sustainability, etc.*