FINAL DEGREE PROJECT OF BIOTECHNOLOGY

STUDIES ON THE INHIBITORY POTENTIAL OF ORCHID EXTRACTS AGAINST ACNE ASSOCIATED BACTERIA

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ABSTRACT

The orchid family is one of the largest families of the flowering plants around the world. They have an undoubtedly beauty, the reason why they have become an object of multibillion-dollar business. They have shown medicinal properties used for treatments of different diseases as skin, infectious diseases, problems concerning the digestive, respiratory and reproduction organs, the circulation, against tumours, for pain relief and fever.

The most commercially and economically important orchid is *Phalaenopsis* spp., however, their parts (leaves, stems, roots) are unused vegetal waste with high potential added value. They contain phenolics, flavonoids, and antioxidant activity that could be a potential source of natural antioxidants. The inhibitory activity may be linked to the presence of phytochemicals such as phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids and alkaloids in its extracts. In particular, tannic acid acts as an antimicrobial agent that precipitate proteins and prevent the development and growth of microorganism.

Acne is one of the most common skin diseases through the population which affects more often teenagers but also adults of both genders. It is a disorder of the sebaceous glands of the skin in which sebum secretion is excessive, microbial involvement is considered to be one of the main mechanisms contributing to the development of acne, in particular due to *Propionibacterium acnes, Staphylococcus epidermidis* and *Staphylococcus aureus.*

There are a number of antibiotics for acne treatment, but there is a problem with the development of bacteria resistance to antibiotics, so several plants have shown inhibition to these microorganisms as *Rosmarinus officinalis, Matricatia chamomilla, Syzygium aromaticum, Melaleuca alternifolia, Symplocos racemose*, among others.

The objective of this study is to check the potential inhibition of *Phalaenopsis* extracts against *Propionibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, the main microbial contributors to the development of acne. The results revealed that methanolic and ethanolic extracts have inhibitory activity against these microorganisms, being a potential source to develop treatments against acne associated bacteria to reduce the use of antibiotics and, therefore, the generation of antibiotic resistance in the bacteria.

Key words: extracts, orchids, acne, bacteria, inhibition.

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RESUMEN

Las orquídeas son la segunda familia de flores más grande en el mundo. Poseen una indudable belleza, razón por la que se han convertido en objeto de negocios multimillonarios, pero, además, han mostrado propiedades medicinales utilizadas en el tratamiento de varias enfermedades de la piel e infecciosas, problemas en los órganos digestivo, respiratorio y reproductivo, la circulación, antitumoral, alivio del dolor y la fiebre.

La orquídea con más importancia comercial y económica es *Phalaenopsis* spp., sin embargo, sus partes (hojas, tallo y raíces) son desechados como residuos vegetales, los cuales tienen un potencial valor añadido. Contienen fenoles, flavonoides, y actividad antioxidante que podrían ser una fuente potencial de antioxidantes naturales. Su actividad inhibitoria podría estar relacionada con la presencia de fitoquímicos como fenoles, taninos, flavonoides, saponinas, glucósidos, esteroides, terpenoides y alcaloides en sus extractos. En particular, el ácido tanínico como agente antimicrobiano precipita las proteínas previniendo el desarrollo y crecimiento de los microorganismos.

Por otra parte, el acné es una de las enfermedades más comunes de la piel, la cual afecta más a menudo a los adolescentes, pero también a adultos de ambos sexos. Se trata de un desorden en las glándulas sebáceas de la piel donde las secreciones son excesivas, la implicación microbiana es considerada uno de los mecanismos que contribuye el desarrollo del acné, en particular, *Propionibacterium acnes*, *Staphylococcus epidermidis* y *Staphylococcus aureus*.

Existen diferentes antibióticos para tratar el acné, pero, cada vez más, aparecen bacterias resistentes a estos antibióticos, sin embargo, un número elevado de plantas han mostrado inhibición contra estos microorganismos como *Rosmarinus officinalis*, *Matricatia chamomilla*, *Syzygium aromaticum*, *Melaleuca alternifolia*, *Symplocos racemose*, entre otras.

El objetivo de este estudio es comprobar el potencial inhibitorio de extractos de *Phalaenopsis* contra *Propionibacterium acnes*, *Staphylococcus aureus* y *Staphylococcus epidermidis*, los mayores contribuyentes en el desarrollo del acné. Los resultados revelaron que los extractos metanólicos y etanólicos tienen una actividad inhibitoria contra estos microorganismos, siendo una fuente potencial para el desarrollo de tratamientos contra las bacterias relacionadas con el acné, de modo que se reduzca el uso de los antibióticos y, por tanto, la generación de la resistencia de las bacterias a éstos.

Palabras clave: extractos, orquídea, acné, bacterias, inhibición.

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1. INTRODUCTION

Orchid family (Orchidaceae) is the second largest family of flowering plants with approximately 28,484 species with more than 850 genera (KewWCSP, 2017) increasing this diversity towards the tropic, being Colombia the greatest country in number of species in America (3,000 spp.) followed by Ecuador and Brazil (2,500 spp. each one).

The most represented genera of orchids are *Dendrobium* (183 species), *Bulbophyllum* (62 species), *Eria* (53 species), *Coelogyne* (43 species), *Vanda* (31 species), *Habenaria* (30 species), *Haemaria* (20 species), *Liparis* (20 species), and *Paphiopedilum* (19 species).

Orchids are the ornamental elite because of their complex flowers of exquisite beauty, reason why they became an object of multibillion-dollar business. Apart from their ornamental value, many orchids have apparent medicinal and glycosidal importance. However, the fact that orchids could plan an important role in herbal medicines is often overlooked. The history of orchids probably started with their use for medicinal purposes. Numerous orchid species have been and are being used in different countries for therapeutic properties. A considerable number of studies have been published on medicinal uses of orchids throughout the world (Hossain M. M., 2011).

More recent ethnopharmacological studies show that orchids are used in many parts of the world and in treatments of a number of diseases: skin, infectious diseases, problems concerning the digestive, respiratory and reproduction organs, the circulation, against tumours, for pain relief and fever.

The most commercially and economically important orchid is *Phalaenopsis* spp., but their plant parts are often left unused. Minh N. et al. (2016) determined the total phenolics, flavonoids, and antioxidant activity of ethanol extracts, and it is suggested that the root extracts could be a potential source of natural antioxidants.

2. BACTERIA ASSOCIATED WITH ACNE

The human microbiota diversity at the strain level and its association with human health and disease are largely unknow. However, many studies have shown that microbe related human diseases are often caused by certain strains of a species, rather than the entire species being pathogenic (Fitz-Gibbon S. et al., 2013).

Acne vulgaris, commonly called acne, is one of the most common skin diseases with a prevalence in up to 85% of teenagers and 11% of adults "(White, 1998)" (Fitz-Gibbon S. et al., 2013), peaking at 41% in 13-18-year-old girls and 35% in boys aged 17-20 year (Muna Jalal Ali et al., 2019). This disease is a disorder of the sebaceous glands of the skin in which sebum secretions is excessive. The glands become plugged and inflamed in the process. Acne also referred to as a pimple can be defined as a clinical condition brought about by the blockage of the pilosebaceous follicles of the face and the upper trunk. Frequently, acne is a transient problem observed more often during puberty, but some manifestations can last forever.

The ethology and pathogenesis of acne are still unclear, however microbial involvement is considered to be one of the main mechanisms of acne development. In particular, *Propionibacterium acnes* has been detected to be an important pathogenic factor. Antibiotic therapy against *P. acnes* has been a mainstay treatment for more than 30 years. However, despite decades of study, it is still not clear how *P. acnes* contributes to acne pathogenesis while being a major commensal of the normal skin flora. Whether *P. acnes* protects the human skin as a commensal bacterium or functions as a pathogenic factor in acne, or both, remains to be elucidated.

Propionibacterium acnes is a bacillus bacteria (Figure 2.1) with approximately 0.5-0.8 µm wide and 1.0-5.0 µm long; they do not have cilia or flagella neither a capsule around them, and its cell wall is made of a thick layer of peptidoglycan. Gram-positive, mesophilic being 37ºC its optimum temperature, catalase positive, indole positive, reduce nitrates to nitrites and anaerobic, however, some studies indicated that it is aerotolerant (López Beatriz, 2020).

The most used culture medium for *P. acnes* is blood agar where their crops originate white crops, round edges and raised surfaces, generally surrounded by a β-haemolysis halo (Avilés C., 2010) (Figure 2.2.).

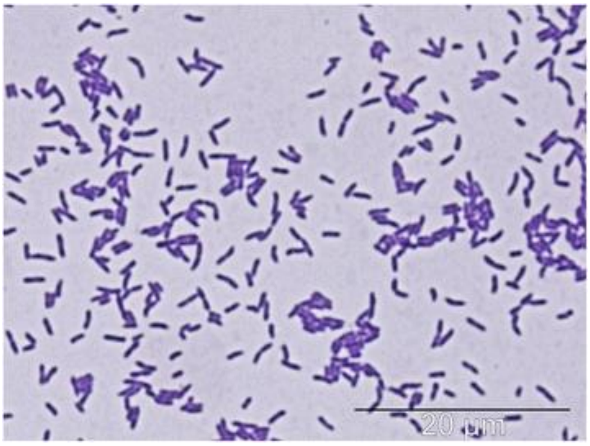

Figure 2.1. *Propionibacterium acnes* under the microscope. (Koskinen, P., Deptula, P., Smolander, O. *et al., 2015).*

Figure 2.2. *Propionibacterium acnes* on plate. Retrieved from https://www.flickr.com/photos/21997898@N04/9606798810.

As commented above, *P. acnes* is a dominant member of the resident microbiota of healthy human skin, and an exclusive bacteria inhabitant of normal human facial sebaceous follicles. Nevertheless, several *in vivo* and *in vitro* studies show that induces inflammatory responses in host keratinocytes, sebocytes, and monocytes and influences local cell growth and differentiation (Figure 2.3.). *P. acnes* is occasionally implicated in serious opportunistic infections such as endocarditis, osteomyelitis, and meningitis, and in severe, postsurgical infections after implantation of a foreign body.

Figure 2.3. *Propionibacterium* spp. on skin infection (Douglas I. J., 2018)

Due to hormonal influences, during puberty the sebaceous glands enlarge and the number of *P. acnes* rises from an almost undetectable level. It is not only becomes one of the dominant and ubiquitous members of the commensal skin microbiota and an exclusive inhabitant of healthy sebaceous follicles, but is also suspected to induce the inflammatory papules and pustules of acne, and may even provoke the initial hyper-keratinisation of the sebaceous duct starting the acne process (Lomholt and Kilian, 2010).

Staphylococci are also usually associated with skin and mucous membrane vertebrates. They can be found in bacteriological cultures of the nose and skin of healthy humans. Some of them are members of the normal skin and mucous membranes flora of humans; others cause suppuration, abscess, a variety of pyogenic infections and fatal septicaemia. In humans, two species are important, *Staphylococcus epidermidis*: a non-pigmented (Figure 2.4.), non-pathogenic form, usually found in the skin and mucous membrane, it does not present cilia or flagella, gram-positive, gamma-haemolytic, facultative aerobic, mesophilic being 37ºC its optimum and catalase positive (López Beatriz, 2020). And *Staphylococcus aureus*: a yellow pigmented form (Figure 2.4.) associated with pathological conditions such as boils, pimples and impetigo; gram-positive, possesses a capsule around it, facultative aerobic and mesophilic being 37ºC its optimum (Gil Marielsa, 2018).

Figure 2.4. *Staphylococcus aureus* (left) and *Staphylococcus epidermidis* (right) – colonies on blood agar. Retrieved from https://www.jfmed.uniba.sk/fileadmin/jlf/Pracoviska/ustav-mikrobiologie-a-imunologie/VLa/STAPHYLOCOCCI.pdf

The common organism related with pus formation in acne is *Staphylococcus aureus*. Its pimple pathogenicity has been attributed to virulence factors possessed by organisms. These virulence factors include coagulase, leukocidins, Protein A, hyaluronidase, hemolysin, lipase and a variety of toxins such as enterotoxin and dermonecrotin toxin. *S. aureus* is resistant to several antibiotics such as ampicillin, penicillin, erythromycin, streptomycin, cloxacillin, and cotrimoxazole. However, it is sensitive to gentamicin, tetracycline, amoxicillin, augmentin, chloramphenicol and sulfamethoxazole (Adejuwon O. et al., 2010).

In conclusion, *Staphylococcus epidermidis* is considered the major skin bacteria that causes the formation of acne and *Propionibacterium acnes* plays an essential role in the pathogenesis of acne. It is involved in the development of inflammatory acne activating complements and by its ability to metabolize sebaceous triglycerides into fatty acids, which chemotactically attract neutrophils. In addition, *Staphylococcus epidermidis* is usually involved in super facial infections within the sebaceous units.

3. THE USE OF PLANT EXTRACTS AGAINST ACNE ASSOCIATED BACTERIA

Several topical and systematic therapies are available for acne; antibiotics, comedolytic agents, and anti-inflammatory drugs are available as a topical therapy, whereas modern systematic cure includes antibiotics, hormones, zinc and laser treatment. Anti-acne drugs are still being primarily used in the treatment for more than 40 years. Various topical and oral drugs like Clindamycin, Salicylic acid, Isotretinoin, Erythromycin, Triclosan, Tetracycline, Minocycline, and Metronidazole are available in the market for acne treatment.

However, an excessive use of these drugs over a long time can lead to the rising resistance of bacteria to them. These drugs have limitations with respect to toxicity and side effects also such as skin dying, headache, nausea, etc. To overcome these limitations, there is a need for the development of effective, safe and low-cost anti-acne drugs. There has been reported that natural substances derived from plants showed activity against acne and has been used for various types of acne treatments (Vora J. et al., 2019).

Vora J. et al. (2019) demonstrated that methanolic extracts of seven plants showed activity against *P. acnes.* Extracts of *Rosmarinus officinalis* and *Matricaria chamomilla* showed highest inhibition zone against *P. acnes* (8 mm) and *Acacia nilotica* showed moderate inhibition zone against *P. acnes* (6 mm and 4 mm respectively).

That study also showed the potential activity of *R. officinalis* against *S. aureus* at lower concentrations with other pathogenic strains as *P. acnes* and *Kocuria sp. A. nilotica* is not effective against *Escherichia coli* and *S. aureus* but very efficient inhibiting *Kocuria* sp., *Bacillus subtilis* and *P. acnes* which are responsible for acne.

M. chamomilla has effective compounds with cytotoxic effects and antiproliferative as well as anti-inflammatory properties, reason why the extracts of this plant in contained in many cosmetic products. Also, there was antibacterial activity of this plant extracts against bacteria causing acne (Vora J. et al., 2019).

Camellia sinensis, Azadirachta indica and *Cassia acutifolia* acetone extracts have high mean of antibacterial susceptibility against bacterial isolates. Combinations of acetonic extracts have also high antibacterial susceptibility against bacteria isolates, but *Camelia sinensis* and *Azadirachta indica* combination was the best antibacterial agent candidate to treat acne vulgaris diseases (Muna Jalal Ali et al., 2019).

The antibacterial activity of *Rosmarinus officinalis* (Rosemary) essential oil against *P. acnes* was observed with atomic force microscopy (AFM), with this observation there was determined changes in morphology and size of *P. acnes* (Fu Y et al., 2007).

Cinnamomum zeylanicum, *Rosa centifolia* (rose), *Lavandula angustifolia,* and *Syzygium aromaticum* (clove) displayed antimicrobial activity against both *S. epidermidis* and *P. acnes*. Only the latter two are recommended in the aromatherapeutic literature for acne treatment. *Leptospermum scoparium* (manuka) showed remarkable activity for both *P. acnes* and *S. epidermidis*; however, Tween 80 was used as a solvent, which may overexaggerate the antimicrobial activity (Orchard A. and Van Vuuren S, 2017).

Another study also found *L. scoparium* to effectively inhibit *P. acnes. Origanum scabrum* and *Origanum vulgare* also notably inhibited *S. epidermidis* and *S. aureus.* Unfortunately, these oils were not studied against *P. acnes*. *Cymbopogon citratus* was shown to effectively inhibit *P. acnes*; however, no data was available against *S. epidermidis*.

Essential oils such as *Santalum album, Vetiveria zizanioides, Viola odorata* (violet), *Citrus aurantium* var. *amara* (petitgrain), and *Citrus bergamia* (bergamot) are a few that are recommended for acne treatment and other microbial infections in the aromatherapeutic literature that are yet to be investigated (Orchard A. and Van Vuuren S., 2017).

Some clinical studies have shown promising results: a four-week trial comparing *Ocimum gratissimum* oil with 10% benzoyl peroxide and a placebo was conducted and was aimed at reducing acne lesions in students. The 2% and 5% *O. gratissimum* oils in the hydrophilic cetomacrogol base were found to reduce acne lesions faster than standard therapy, and they were well tolerated. The 5% preparation caused skin irritation despite being highly effective. Overall, *O. gratissimum* oil showed excellent potential in the management of acne as it was effective as benzoyl peroxide, although it was less popular with patients due to the unpleasant odour "(Orafidiya L.O. et al., 2015)" (Orchard A. and Van Vuuren S., 2017).

Melaleuca alternifolia oil demonstrated *in vitro* antimicrobial and anti-inflammatory activity against *P. acnes* and *S. epidermidis* and is in fact the essential oil on which most clinical trials have been undertaken. I. B. Bassett et al. (1990) performed one of the first rigorous single-blinded randomised (RCT) controlled trials where 124 patients judged the efficacy of 5% *M. alternifolia* gel in comparison to 5% benzoyl peroxide lotion in the management of mild to moderate acne. Both treatments showed equal improvement in the acne lesions.

Other oil studies included a gel formulation containing acetic acid, *Citrus sinensis* (orange), and *Ocimum basilicum* (sweet basil) essential oils, which was tested in acne patients. The combination of these antimicrobial essential oils and the keratolytic agent resulted in a 75% improvement in the rate of acne lesion "(Matiz G. et al., 2012)" (Orchard A. and Van Vuuren S., 2017).

Kumar et al. (2007) showed that *Symplocos recemosa* could possibly act as a bactericidal agent to *P. acnes* and *Quercus infectoria* extract also showed good antimicrobial effects against this microorganism, but a high concentration was required to kill both, *Propionibacterium acnes* and *Staphylococcus epidermidis* as compared to the ethanolic extract of *Symplocos racemose* and *Berberis aristate* which showed antimicrobial properties against *Propionibacterium acnes.*

Phytochemical screening of *Symplocos racemose* extract showed positive results for the presence of alkaloids. Its strongest effect was against *P. acnes*, however, alkaloid and its derivates also have activities against *Staphylococcus aureus* and methicillin-resistant *S. aureus.*

It is possible that berberine an alkaloid present in *Symplocos racemose* may act in the same mechanism to inhibit *P. acnes* and *S. epidermidis.* Therefore, the active component of *Symplocos racemosa* extract could be of interest for further development as an alternative treatment for acne.

4. BIOCHEMICAL AND PHARMACEUTICAL PROPERTIES OF DIFFERENT ORCHIDS EXTRACTS

Due to the medicinal properties of some plants used as treatments for several diseases showed above and, in particular, with orchids. It is important to know which are the compounds that make these plants useful as medicines.

Many natural products including pigments, enzymes and bioactive components are soluble in water, which explains the highest yield of extract, while some of the solvents especially acetone are selective for tannins. Degrees of solubility are present in different solvents for diverse phytoconstituents.

Genus *Anoectochilus* (Orchidaceae), with more than 40 species, is widespread throughout the tropical regions. Several species of this genus are used in Chinese folk medicines, such as *A. formosanus* Hayata, *A. koshunensis* Hayata, and *A. roxburghii* Lindl. Of these plants, *A. formosanus* and *A. koshunensis* are distributed only in Taiwan Province (China) and Okinawa (Japan). *Anoectochilus roxburghii*, which is distributed on southern China, Japan, Sri Lanka, India, and Nepal, is also called “King Medicine” in China.

Dendrobium species (Orchidaceae), known as “Shihu” or “Huangcao” in China are widely distributed throughout Asia, Europe and Australia by more than 1,100 species. There are 74 species and 2 variations of *Dendrobium* plants found in China and about 30 species are used in traditional or folk medicine for antipyretic, eyes-benefiting, immunoregulatory purposes, etc.

In recent years, the amounts of wild *Dendrobium* have decreased extremely due to the aggravated environment and the excessive consumption because of its medicinal properties. Phenols comprising bibenzyl, phenanthrene and fluorenone are the main active components with over 60 structures identified. Pharmacological studies have demonstrated that some of them such as erianin and moscatilin displayed antitumor, anti-angiogenic, anti-platelet aggregation, anti-inflammation and immunoregulatory activities. Then, bibenzyl and phenanthrene are the most characteristics as chemical marker for genus *Dendrobium*, as the presence of over 40 compounds of those types were reported from this genus. Furthermore, fluorenone is special for this genus, and no other one was found to produce fluorenone up to date “(Chapman and Hall, 2005)” (Gutiérrez RM, 2010).

Water decoction of whole plant of *Anoectochilus formosanus* showed a potent tumour inhibitory activity in BALB/c mice after subcutaneous transplantation of CT-26 murine colon cancer cells. Water extract may activate murine responses, such as stimulating proliferation of lymphoid tissues and activating the phagocytosis of peritoneal macrophages against *Staphylococcus aureus*. Antitumor activity of *A. formosanus* may be associated with its potent immunostimulating effect “(Tseng et al., 2006)” (Gutiérrez RM, 2010).

Vanilla planifolia sheath is described as an antimicrobial agent, but it is not currently used in medicine for this purpose, although it may prolong the life of food products “(Fladby et al., 2004)”. *Vanilla pompona* was also used to flavour tobacco in Cuba. Further the antimicrobial effects of vanillin and vanillic acid isolated from *V. planifolia* were studied against several species and strains of *Listeria monocytogenes*, *Listeria innocua*, *Listeria grayi* and *Listeria seeligeri*. Mixtures of vanillin and vanillic acid exhibited additive inhibitory effects, particularly at lower pH “(Delaquis et al., 2005)” (Gutiérrez RM, 2010).

Another study investigated the mode of action of vanillin with regard to its antimicrobial activity against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua* was found that vanillin is primarily a membrane-active compound, resulting in dissipation of ion gradients and the inhibition of respiration "(Fitzgerald et al., 2004)" (Gutiérrez RM, 2010).

In a screening study of methylene chloride extract of *Galeola foliate* leaves and stem bark showed a broad spectrum antibacterial activity against 24 bacteria Gram-positive and Gram-negative. This extract was not active against moulds "(Khan et al., 2004)" (Gutiérrez RM, 2010).

As a part of a screening study, methanol extract of *Spiranthes mauritianum* leaves showed antibacterial activity against Gram-positive and have anti-inflammatory activity "(Matu and Van Staden, 2003)". In another study of herb extracts from Chinese medicinal plants there was found that *Bletilla striata* possesses antioxidant and antimicrobial capacity "(Luo et al., 2007)" (Gutiérrez RM, 2010).

Nervilia plicata is a medicinal orchid used by *Katunayka* tribe in Nilambur forests of Western Ghats region of Kerala for the treatment of urinary infections due to its antibacterial activity. The antibacterial substances may act as chemotherapeutic bactericidal and bacteriostatic agents against *E. coli*, *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia* and *Salmonella paratyphi* (Haridas et al., 2014).

Phalaenopsis spp. is the commercial and economic orchid most, however, some parts of the plant are often left unused, which can lead to waste management. Irimescu L.S. et al (2020) have studied the potential antimicrobial activity of methanolic extracts made of stems and roots of wastes, showing an inhibitory effect on pathogens like methicillin resistant *Staphylococcus aureus* and different *Candida* species.

These wastes which have an inhibitory activity that may be linked to the presence of phytochemicals such as phenols, tannins, flavonoids, saponins, glycosides, steroids, terpenoids and alkaloids in the orchid extracts. Tannins (tannic acid) are polyphenols, known as antimicrobial agents, that precipitate proteins and prevent the development and growth of microorganisms making nutritional protein unavailable to microbes. So, the growth of large numbers of fungi, yeasts, bacteria and viruses has been inhibited by tannins.

Then, the discovery of antibiotics in the early 20th century revolutionized medicine. Over the past few decades, the antimicrobials seemed to be a great promise of definitive victory in the battle against bacterial infections. However, due to natural mechanisms and frequent abuse of antibiotics, bacteria began to acquire resistance to many classes of antimicrobial drugs. Infections caused by multidrug-resistant strains are the main problem of modern medicine. Even the modification of the structure of known antibiotics does not guarantee the efficacy of their derivates and, in addition, is highly expensive. For this reason, many researchers has focused on antimicrobial agents of plant origin to use them for treatment of infections. It has been found that secondary plant metabolites can be effective against specific strains, sometimes even more than the already known antibiotics.

For instances, *Staphylococcus aureus* is classified as a member of the ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) which not only are responsible for a wide range of infection but also they are increasingly resistant to common antibacterial drugs "(Moloney, 2016)" (Rykaczewski M., et al. 2019).

Asseleih et al. (2015) made a study to obtain pharmacological and active compounds information about some orchids, obtaining the following results:

- *Epidendrum chlorocorymbos,* a strong anti-nociceptive effect was found in *Epidendrum mosenii* has as active metabolites 24-methylene-cicloartanol, dichloromethane extract, methanolic extract and phenanthrenes.

- *Habenaria floribunda,* with anti-oxidant activity, its active metabolites are habenarioal and the methanolic extract.
- *Oncidium ascendens,* biological activities such as inhibition of cancer cell lines proliferation and induction of apoptosis, its active metabolites are stilbenoids and 1,4-Beta-mannan.
- *Scaphyglottis fasciculata,* anti-inflammatory and anti-nociceptive, its active metabolites are lanostadienol derivative, gigantol, ciclobalanone, phenanthrenederivative and 3,4-dihydroxi-5,5'-dimethoxy bibenzyl.
- *Spiranthes eriophora,* anti-oxidant activity with ethanoic extract containing flavonoids and ferulic acid.
- *Stanhopea oculata*, anti-fatigue activity having as active metabolites hexanic and dichloromethane-methanol extract.
- *Vanilla planifolia* with biological activities including anti-oxidative, anti-inflammatory, anti-cancer, against cell stress, anti-microbial, anti-cholesterol, anti-nociceptive, anti-depressing and inhibition of acetyl cholinesterase. Its active metabolites are vanillin and hydro-ethanolic extract.

In the same way, Chinsamy et al. (2014) discovered that orchid extracts that displayed significant effects in anti-inflammatory, antioxidant and acetylcholinesterase (AChE) inhibitory assays may be potential natural plant product targets in the treatment of inflammatory and neurodegenerative disorders. Extracts include: *Ansellia Africana* ethanol (EtOH) root, *Bulbophillum scaberulum* dichlomethane (DCM) root, *Cyrtorchis arcuata* methanolic root, *E. hereroensis* DCM tuber, *E. petersii* DCM stem and *T. tridentata* DCM root extracts. The EtOH root extract of *B. scaberulum* exhibited the most potent selective inhibitory effect on 2-cyclooxygenase (COX-2). While the DCM tuber extract of *Eulophia hereroensis,* was the only extract to significantly inhibit both COX enzymes.

Preliminary tests suggest significantly higher levels of gallotannin content in *A. africana*, and *E. hereroensis* methanol root extracts. This may account for the significant anti-inflammatory activity. Similarly, the presence of condensed tannins in *E. hereroensis* root and *B. scaberulum* stem/root extracts may explain the observed anti-inflammatory effects.

Plant compounds such as flavonoids, naphthoquinones, alkylamides and phenolic phenyl-propane derivatives represent the usual compounds found in certain natural products that are responsible for COX inhibition. The presence of flavonoids in *B. scaberulum* and *Tridactyle tridentata* may explain the potent activity abserved in the anti-inflammatory and AChE inhibitory assays. The medicinal value of flavonoids includes anti-inflammatory, antifugal, antioxidant activities and wound healing.

The results of phytochemical test made by Sandrasagaran et al. (2014) show that bioactive compounds such as saponin, terpenoid, alkaloid, reducing sugar and flavonoid were present in most of the parts of *Dendrobium crumenatum.* Terpenoid and reducing sugar were absent in leaves extract. Flavanoid was absent in both stem and root extract. Cardiac glycosides were only detected in leaves whereas phlobatannins, anthraquinones and tannins were not detected in any of the plant extracts. Alkaloids have numerous functions and among them are most is analgesic, anti-inflammatory and antibacterial effects. The presence of terpenoid may contribute to analgesic and anti-inflammatory activity.

M. acuminata has 50 compounds in leaf extract and 43 compounds in stem extract which were grouped into seven major categories: fatty acids, sugars and glycosides, organic acids, phenolic acids, amino acids and amines, sterols, alcohols, and others. Among which linoleic acid and hexadecenoic acid gained much attention in cosmetic market due to their anti-inflammatory, acne reduction and moisture retention properties "(Mouad A.M. et al., 2004)". Also, methanolic leaf and stem extracts contain a wide array of α-hydroxy acids (AHA) which are used in many cosmetic formulations to treat acne scars (Bose B. et al., 2017).

Finally, plant extracts in organic solvent (methanol) provide more consistent antimicrobial activity. This can be explained in terms of the polarity of the compounds being extracted by the solvent and to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media. The methanolic extracts of stem, root and pseudo bulb were found to exhibit comparable antimicrobial activity to that of the standard antibiotics. The antimicrobial activity of leaf extract was generally low, whereas the antimicrobial activity of stem and root extracts were slightly better.

5. OBJECTIVES AND CONTEXT

Phalaenopsis spp. is known to be the most commercial and economic commonly used orchid. Nevertheless, some parts of the plant are usually left unused, even though they could have a high potential added value. Several reports analysed their potential content of phytochemical obtaining positive results (Minh T.N. et al., 2016).

In the cosmetic industry, these plants are evaluated for their potential anti-aging and skin depigmentation on Japanese female skin (Tadokoro T., et al 2010). Also, orchids are used for their therapeutic properties and they have been found in traditional medicine. It has been reported their use such tonic in hysteria, spasm, insanity and epilepsy, rheumatic treatments, tuberculosis, body aches, eczema, headache and fever, aphrodisiac and heart, respiratory, and nervous disorders (Bijaya, P., 2013).

The present study was performed on florists' waste of epiphytic orchids from the species *Phalaenopsis*. In this study we have tried to highlight that this plant has not only ornamental values, it has also important pharmaceutical values.

So, in this study it was tested the antimicrobial potential of methanolic and ethanolic extracts made of orchid waste, respectively from dried leaves, stems and roots of *Phalaenopsis* species against the bacteria strains that has been reported to be linked to acne development, *Staphylococcus aureus, Staphylococcus epidermidis* and *Propionibacterium acnes.*

The main purpose is to search for new antimicrobial agents in the field of ethnopharmacology, to isolate compounds with proven antimicrobial activity that can be used for an effective treatment of human diseases.

6. MATERIAL AND METHODS

6.1. Plant extracts

Phalaenopsis orchids have been harvested from Tria's Flower Shop, Greenhouse Băneasa, Romania. The plants have been cultivated under specific warehouses conditions and have been donated by the manufacturer.

The leaves, stems and roots of *Phalaenopsis* orchid species have been chopped in small pieces and shredded. In order to remove bark or other impurities which could have contaminated the final sample, the roots were washed with water. After cutting, the tissue was dried. The drying process was carried out in rooms with a constant temperature of 37° C for 7 days. For the preparation of the crude extract, the powdery vegetal material (leaves, roots and stems) was mixed in a stopper bottle (Erlenmeyer) with 80% methanol (ration 1/10) and placed in the microplate mixer at a controlled temperature for 60 minutes at 30° C, 150 rotations/ minute. The crude extracts obtained were then filtered through filter paper. Stems and roots were mixed with 70% ethanol (Nicolcioiu M.B. et al., 2017) following the same steps as with 80% methanol.

6.2. Tested microorganisms

To test the antimicrobial activity of *Phalaenopsis* extracts, four potentially pathogenic *Staphylococcus* spp. and *Propionibacterium acnes* (Table 6.2.1.) were selected. These pathogenic microorganisms were provided by the Faculty of Biotechnology U.S.A.M.V., Bucharest.

Table 6.2.1. Microorganisms used to test the *Phalaenopsis* antimicrobial activity**.** MRSA: methycilin resistant; MRSE: methycilin resistant; MSSE- methycilin sensible.

No.	Microorganisms	Origin
	Bacteria	
1.	*S. aureus* ATCC 6538	American Type Culture Collection
2.	*S. aureus* ATCC 43300 MRSA	American Type Culture Collection
3.	*S. epidermidis* ATCC 51625 MRSE	American Type Culture Collection
4.	*S. epidermidis* ATCC 12228 MSSE	American Type Culture Collection
5.	*P. acnes* ATCC 6919	American Type Culture Collection

6.3. Media

The culture media used for *Staphylococcus* spp. was media with mannitol (Table 6.3.1.) and for *Propionibacterium acnes* TSA (Tryptic Soy Agar) (Table 6.3.2.). Both media were autoclaved at 121° C for 15 minutes.

Table 6.3.1. Ingredients containing the mannitol media.

Ingredients	Amount (g)
Bacto-Brain Heart Infusion	5.0
Bacto-Typic Soy Agar	28.0

Mannitol, Difco	10.0
Bacto-Agar	4.0
Brom Cresol Purple	0.016

Table 6.3.2. Ingredients containing TSA (Tryptic Soy Agar) media.

Ingredients	**Amount (g/l)**
Casein peptone	15.0
Soy peptone	5.0
Sodium chloride	5.0
Agar	15.0

Final pH = 7.3 ± 0.2 at 25ºC.

6.4. Testing antimicrobial activity by spot method

To be able to measure the inhibition zones, it was used the drop-diffusion test method in order to determinate the antimicrobial activity (Diguta, C., et al. 2014). Strains in fresh culture were prepared by inoculation on liquid media and for a period of 24-48 hours, the microorganisms were cultivated at 37ºC.

According to the working method, the spread technique is used to inoculate pathogenic strains on the surface of the culture media distributed in Petri dishes.

First, *Staphylococcus* plates were prepared; 16 Petri dishes with mannitol medium, after got it dry 1 ml of microorganism was inoculated in each plate (4 plates for each microorganism). After 20 minutes, the microorganism has been established on the plate and then 5 µL of each sample was added as a spot into the Petri dish.

It has been separated ethanol extracts from methanol extracts, so 2 plates for each one by microorganism. As Control, in ethanol dishes 5 µL 70% ethanol solution and 5 µL antibiotic (Tetracycline 30 mcg) were used and in methanol dishes 5 µL 80% methanol solution and 5 µL antibiotic (Tetracycline 30 mcg).

Secondly, *Propionibacterium acnes* because of its anaerobiosis; a first layer of TSA was inoculated into Petri dishes following the same procedure as with *Staphylococcus*, but when the drops were dry, a second layer of TSA was added in order to get the anaerobiosis required. Also, these Petri dishes were close with paraffin.

The next step is to incubate 24-48 hours all the cultures at 37^{0}C. After that, the halo of inhibition has been determined measuring its diameter.

7. RESULTS AND DISCUSSION

After incubation, the inhibitory effect of *Phalaenopsis* extracts is indicated by a clear area (halo) around the extract spot. The diameter of each halo is showed in Table 7.1.

Table 7.1. Diameter (cm) of the inhibitory activity of *Phalaenopsis* extracts on *Staphylococcus* spp. and *Propionibacterium acnes* – 60 min extraction.

	***S. aureus* 43300**	***S. aureus* 6538**	***S. epidermidis* 12228**	***S. epidermidis* 51625**	***P. acnes* 6919**
ML	0	0.05	0.75	0.5	-
MS	0	0.04	0	0.5	-
MR	0	0	0.45	0.5	-
ES	0	0	0.9	0	-
ER	0	0.05	0.31	0,7	-
Methanol	0	0	0	0	-
Ethanol	0	0	0	0	-
Antibiotic	2.13	2.55	3.3	1.8	-

As a result of this preliminary study, it has been observed that MRSA (methicillin resistant) strain ATCC 43300 of *Staphylococcus aureus*, that there is no inhibition of any extracts (Figure 7.1.). However, in the same way, it should not be sensible to antibiotic, but it showed an inhibition halo, which may indicate that the stock suspension may have been unpurified.

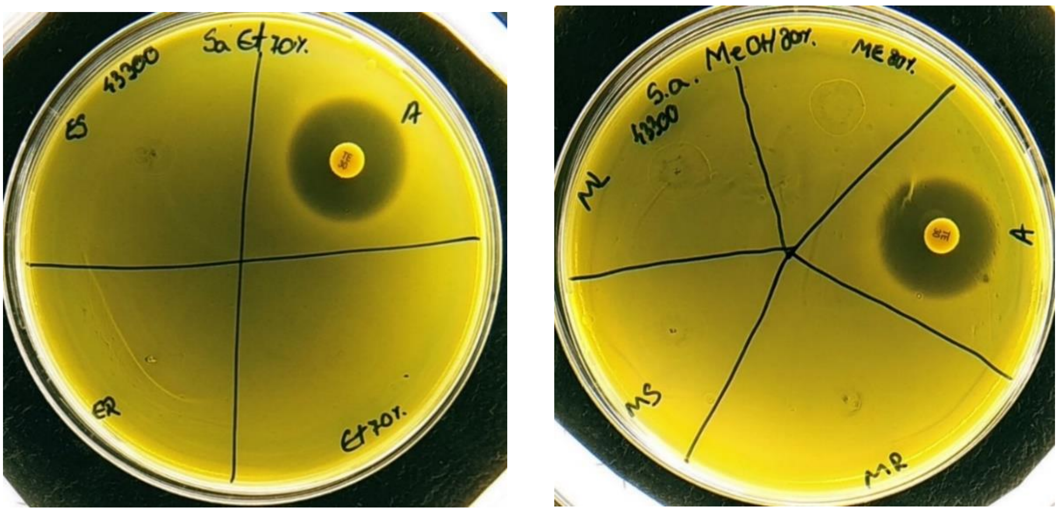

Figure 7.1. a (left) and b (right). *Staphylococcus aureus* ATCC 43300 MRSA on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis*. ES: ethanol stem, ER: ethanol root, Et 70%: 70% ethanol solution, ML: methanol leaves, MS: methanol stem, MR: methanol root, A: antibiotic, ME 80%: 80% methanol solution.

This may be also due to the fact that it was an old strain and might be lose their resistance to antibiotics. It has been observed that resistance to antibiotics require high energy consumption and some bacteria during a long period of time living without any threat, they change their metabolism adapting themselves to the environment, so they are able to lose their antibiotic resistance (Olivares Pacheco J. et al., 2017).

Instead, it has observed inhibition for *Staphylococcus aureus* ATCC 6538 in ethanol extracts of roots and in methanol extract of leaves and stems (Figure 7.2.).

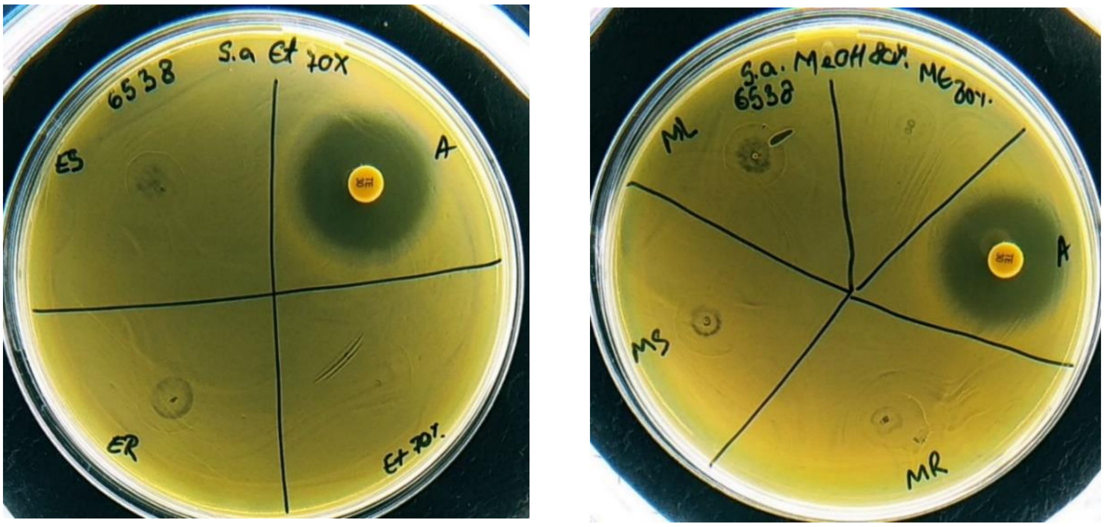

Figure 7.2. a (left) and b (right). *Staphylococcus aureus* ATCC 6538 on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis*. ES: ethanol leaves, ER: ethanol roots, Et 70%: 70% ethanol solution, A: antibiotic, ML: methanol leaves, MS: methanol stems, MR: methanol roots, ME 80%: 80% methanol solution.

For *Staphylococcus epidermidis* ATCC 51625 there are some inhibition in ethanol roots and methanol leaves, roots and stems (Figure 7.3.).

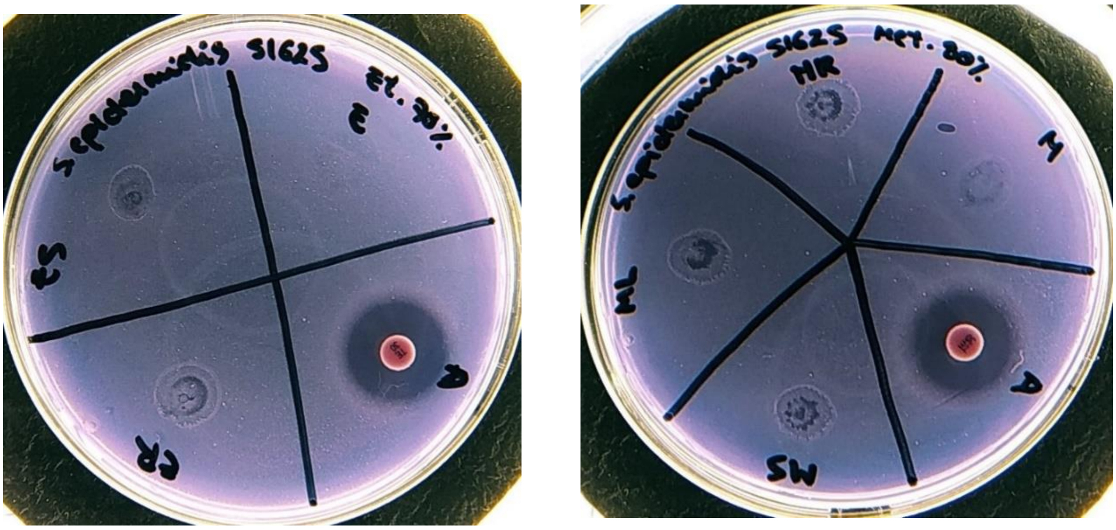

Figure 7.3. a (left) and b (right). *Staphylococcus epidermidis* ATCC 51625 MRSE on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis*. ES: ethanol stems, ER: ethanol roots, E: 70% ethanol solution, MR: methanol roots, ML: methanol leaves, MS: methanol stems, A: antibiotic and M: 80% methanol solution.

With *Staphylococcus epidermidis* ATCC 12228 plates, there was a contamination with *S. aureus* but it was considered also, because it could be interesting to see the behaviour of both microorganisms co-living and the inhibition level. Here, there are inhibition in ethanol roots and stems, and in methanol leaves and roots (Figure 7.4.).

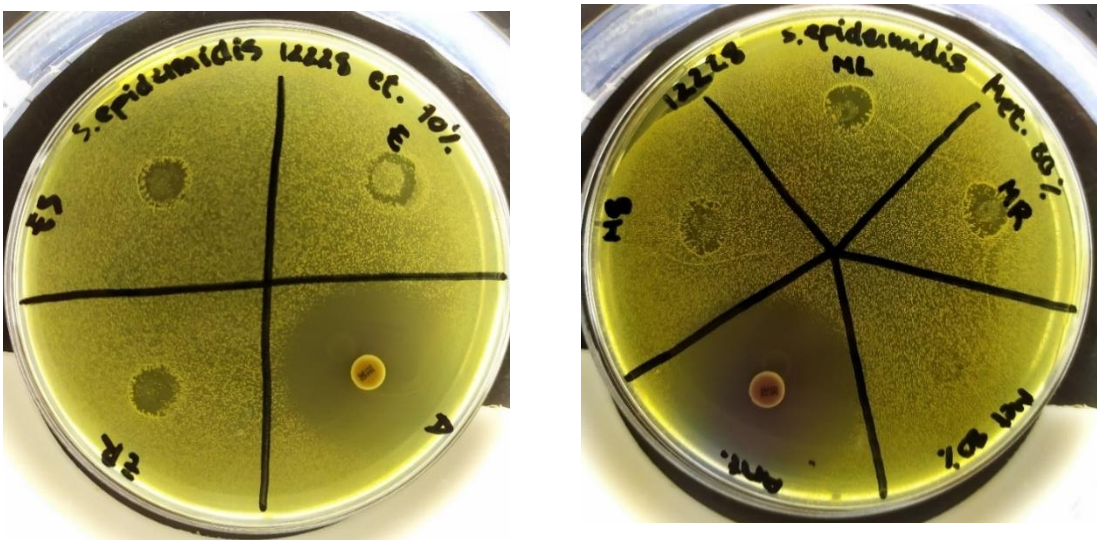

Figure 7.4. a (left) and b (right). *Staphylococcus epidermidis* ATCC 12228 MSSE on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis,* contaminated with *Staphylococcus aureus*. ES: ethanol stems, ER: ethanol roots, E: 70% ethanol solution, MR: methanol roots, ML: methanol leaves, MS: methanol stems, A/Ant: antibiotic, Met 80%: 80% methanol solution.

It was identified the contamination of *S. aureus*, because of the color of the colonies, they should not be yellow (Figure 2.4.) and the color of the antibiotic inhibition halo. *Propionibacterium acnes* did not grow, so there were any results.

Not satisfied with these results, the experiment was made again but for the extracts instead of 60 min of extraction, it was made an overnight extraction and, in this case, there was also ethanol extraction of leaves. *S. aureus* ATCC 43300 MRSA was replace for *S. aureus* ATCC 33592 MRSA. The diameter of each halo is showed in Table 7.2.

Table 7.2. Diameter (cm) of the inhibitory activity of *Phalaenopsis* extracts on *Staphylococcus* spp. and *Propionibacterium acnes* – overnight extraction.

	***S. aureus* 33592**	***S. aureus* 6538**	***S. epidermidis* 12228**	***S. epidermidis* 51625**	***P. acnes* 6919**
ML	0	0.25	0.4	0,4	-
MS	0	0	0.65	0,4	-
MR	0	0,2	0.2	0,4	-
ES	0	0	0.9	0.65	-
ER	0	0	1.15	0.6	-
EL	0	0.65	0.65	0.75	-
Methanol	0	0	0	0	-
Ethanol	0	0	0	0	-
Antibiotic	2.75	0.68	0	1.88	-

For *S. aureus* ATCC 33592 there is no inhibition for ethanol extracts neither for methanol extracts (Figure 7.5.).

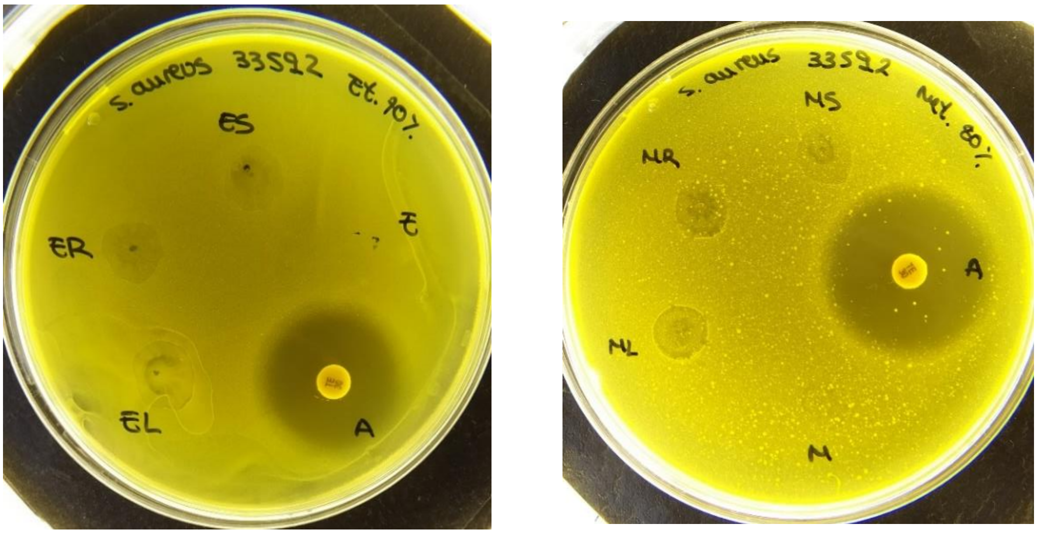

Figure 7.5. a (left) and b (right). *Staphylococcus aureus* ATCC 33592 MRSA on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis*. ES: ethanol stems, ER: ethanol roots, EL: ethanol leaves, A: antibiotic, E: 70% ethanol solution, MS: methanol stems, MR: methanol roots, ML: methanol leaves, M: 80% methanol solution.

Here, there is again the problem with the antibiotic because it is also a methicillin resistant strain, so an investigation of this event should be performed.

For *S. aureus* 6538 there is some inhibition in the case of ethanol leaves and in methanol roots and leaves (Figure 7.6.).

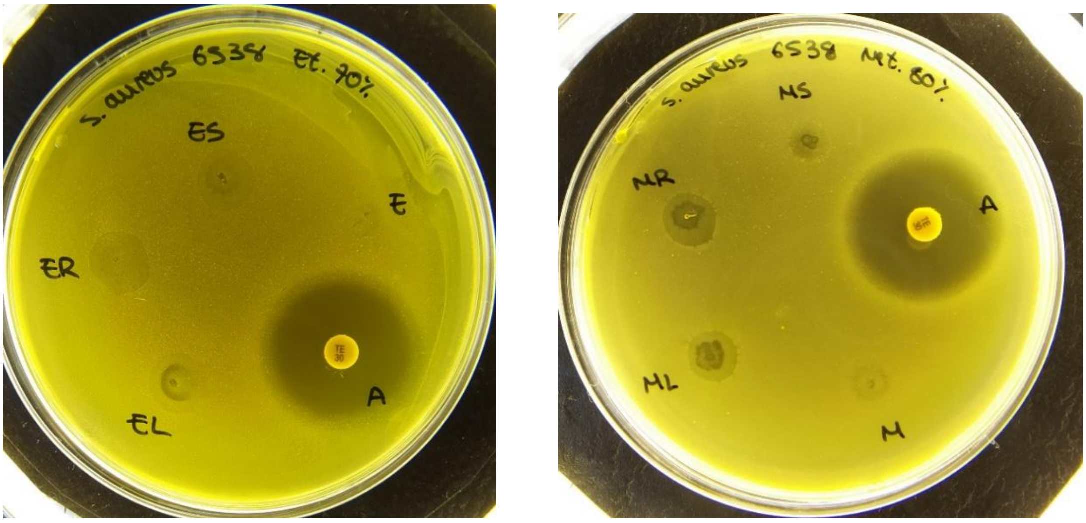

Figure 7.6. a (left) and b (right). *Staphylococcus aureus* ATCC 6538 on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis*. ES: ethanol stems, ER: ethanol roots, EL: ethanol leaves, A: antibiotic, E: 70% ethanol solution, MS: methanol stems, MR: methanol roots, ML: methanol leaves, M: 80% methanol solution.

S. epidermidis 12228 growth is inhibited by ethanol stems, roots and leaves extracts and by methanol stems, roots and leaves also (Figure 7.7.).

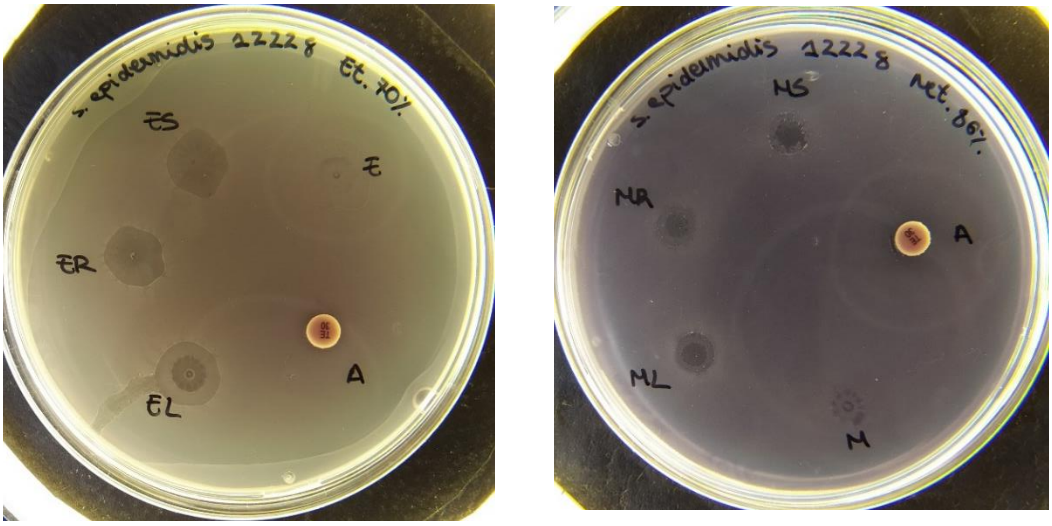

Figure 7.7. a (left) and b (right). *Staphylococcus epidermidis* ATCC 12228 MSSE on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis*. ES: ethanol stems, ER: ethanol roots, EL: ethanol leaves, A; antibiotic, E: 70% ethanol solution, MS: methanol stems, MR: methanol roots, ML: methanol leaves, M: 80% methanol solution.

In this case, this strain is MSSE but showed resistance to the antibiotic and susceptibility to the extracts.

For *S. epidermidis* 51625 there are some inhibition in ethanol stems, leaves and roots extracts and in methanol stems, roots and leaves extracts (Figure 7.8.).

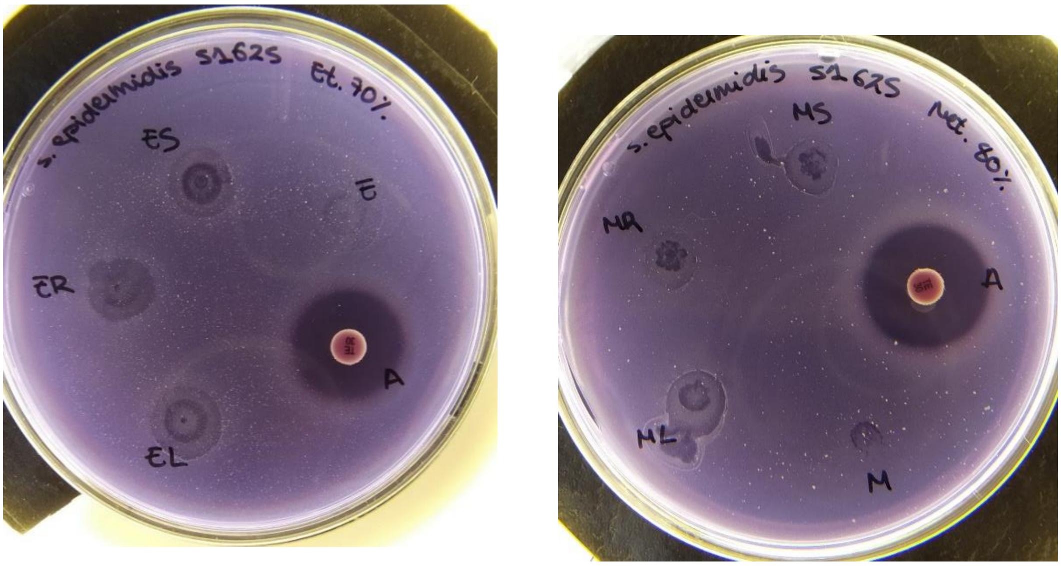

Figure 7.8. a (left) and b (right). *Staphylococcus epidermidis* ATCC 51625 MRSE on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis*. ES: ethanol stems, ER: ethanol roots, EL: ethanol leaves, A: antibiotic, E: 70% ethanol solution, MS: methanol stems, MR: methanol roots, ML: methanol leaves, M: 80% methanol solution.

With *P. acnes* we had problems again, it has grown but something with the media was wrong, it spent too much time to solidify even it seemed to be solid. It can be said because of the halo of the antibiotic, it is not well defined, and in methanol extracts there is a zone were the growth of *P. acnes* is minor. So, this zone could be a result of the mixture of roots and leaves extracts, showing a potential antimicrobial activity against *P. acnes* (Figure 7.9.).

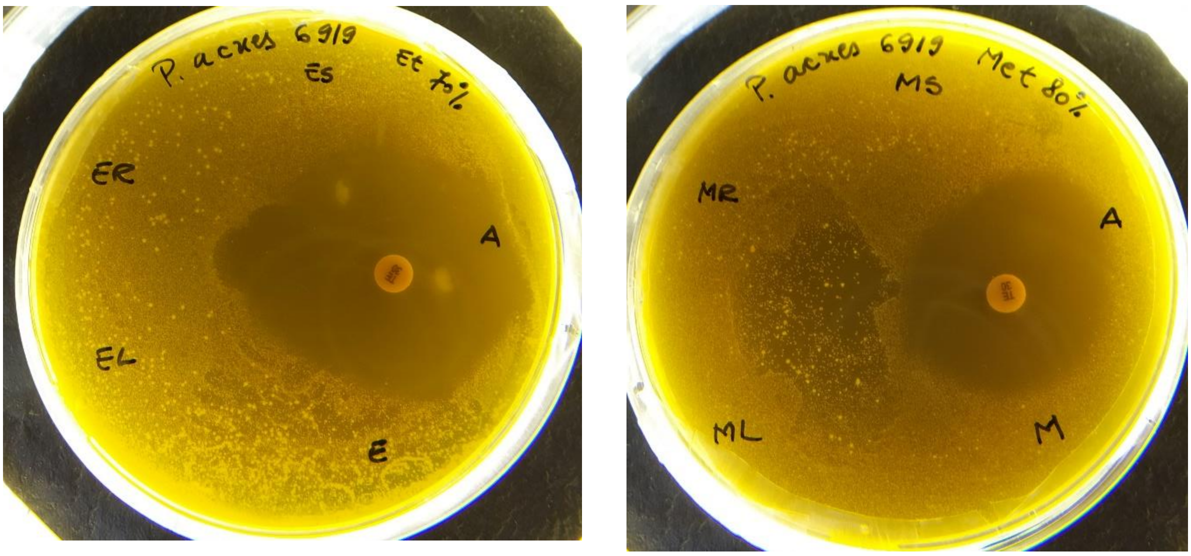

Figure 7.9. a (left) and b (right). *Propionibacterium acnes* ATCC 6919 on plate, tested with ethanol (a) and methanol (b) extracts of *Phalaenopsis*. ES: ethanol stems, ER: ethanol roots, EL: ethanol leaves, E: 70% ethanol solution, A: antibiotic, MS: methanol stems, MR: methanol roots, ML: methanol leaves, M: 80% methanol solution.

Anyway, this second try was useful to confirm that this procedure is effective in order to get the anaerobiosis environment desired for *P. acnes* growth.

An important finding is that the ethanolic and methanolic extracts made of stems, roots and leaves have an inhibition activity against the strain *S. epidermidis* 12228 which did not showed susceptibility to the antibiotic but did to extracts. This should be investigated because it should be methicillin sensible but does not show the halo of inhibition, in the same way, methicillin resistant strains do not show resistance to the antibiotic, so it must be figure out the reason of these events.

Nevertheless, it has been demonstrated that these plant extracts have some antimicrobial activity, but it is needed a larger sample to be able to confirm these results.

Also is noticeable the differences between 60 min of extraction versus an overnight extraction, the level of inhibition in slightly increased with the overnight extractions where the concentration may be higher. Such inhibitory activity may be linked to the presence of phytochemicals in the orchid extracts (Kumari R., et al, 2017).

The results may suggest a new valuable resource to replace the use of antibacterial drugs. Because of the abuse of antibiotics on a very large scale, as a panaceum, infections caused by multidrug-resistant strains are gaining resistance to antimicrobial drugs, due to their natural mechanisms, therefore antimicrobial agents of plant origin are needed to be use for the treatment of infections caused by multidrug-resistant strains. Considering that nowadays among the florists the orchids' waste, especially of *Phalaenopsis*, are registering increased quantities, their use to develop added-value pharmaceutical or cosmetics new products with antimicrobial activity can be a viable solution in an economy context.

8. CONCLUSION AND PERSPECTIVES

This was a preliminary study which has revealed that methanolic and ethanolic extracts made of stems, roots and leaves of *Phalaenopsis* orchids wastes have potential antimicrobial activity, with an inhibitory effect on pathogens associated with acne.

It is noteworthy that ethanolic and methanolic extracts, made of stems, roots and leaves have an inhibition activity against the strain *S. epidermidis* 12228 which did not showed susceptibility to the antibiotic.

Also is noticeable the differences between 60 min of extraction versus an overnight extraction, the level of inhibition in slightly increased with the overnight extractions where the concentration may be higher.

However, their degree of inhibition is not as effective as antibiotics. So, further investigation is needed to develop a treatment solution for the use of orchid extracts in anti-acne dermato-cosmetics. The following steps should be to taste the combination of these extracts and/or their combination with other plant extracts that showed potential inhibition against these pathogenic microorganisms. Also analyse the extracts in order to determine the compounds containing in them and see which are the most contributors to anti-bacterial activity.

In conclusion, for future investigation, more samples and microorganisms should be tested, the extracts that showed the highest antimicrobial activity should be tested increasing the concentration of such extracts and also try different combinations to obtain a highest level of inhibition.

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