Aquatic plants of South Africa for pharmaceutical and cosmeceutical usage

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by

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The publication of this report emanates from a project entitled *Aquatic Plants of South Africa for Pharmaceutical and Cosmeceutical Usage* (WRC Project No. K8/1089).

Executive summary

Background

The study of the medicinal properties of indigenous South African aquatic plants is a relatively understudied field. In South Africa traditional medicine is the preferred form of primary health care for around 70% of its population. Each year 19 500 tons of medicinal plants are used in the treatment of illnesses affecting South Africans and while terrestrial ecosystems are abundant and rich in diversity, the lack of ethnobotanical studies done on wetland ecosystems have impacted the amount of potential resources that South Africans could be acquiring. Wetlands support a great diversity of plant species and some of those plant species have been traditionally used by communities as a source of medicine, food and building materials, proving that these plants can be applied to a variety of aspects in everyday life.

Rationale

A literature review of thirty four aquatic plants indigenous to South Africa revealed that seventeen of the aquatic plants had no reported traditional uses or biological testing, however, the other seventeen had been previously tested for their antimicrobial activity against human pathogens; they had also been documented to be used by different cultures for traditional medicine. Five aquatic species (*Commelina benghalensis, Equisetum ramossisimum, Mentha longifolia, Typha capensis* and *Zantedeschia aethiopica*) were chosen based on their traditional uses and very few to no biological testing reported in publications. Ethanolic extracts were prepared and tested for various *in vitro* antibacterial and enzyme inhibitory activities which are associated with common human ailments. *Propionibacterium acnes* is involved in the pathogenesis of acne vulagris while *Prevotella intermedia* and *Streptococcus mutans* are oral pathogens involved in the onset of periodontitis and dental caries, respectively. Tyrosinase is the rate limiting enzyme involved in melanin production and may cause skin hyperpigmentation when overproduced. Through this study, the research question, do indigenous South African aquatic plants have the potential to be effective alternative treatment for skin hyperpigmentation, acne and periodontal disease, was answered.

Objectives

Aim 1: Determine the enzymatic inhibitory activity of the selected indigenous South African aquatic plants against tyrosinase (the rate-limiting enzyme involved in the production of melanin).

Aim 2: Investigate the interaction between a tyrosinase inhibiting plant extract in a 1:1 combination with kojic acid.

Aim 3: Determine the antibacterial activity of the selected indigenous South African aquatic plants against *Propionibacterium acnes*.

Aim 4: Investigating the antibacterial activity of the selected indigenous South African aquatic plant extracts against *Streptococcus mutans* and *Prevotella intermedia*, two bacteria involved in the onset of periodontal diseases.

Aim 5: Investigate if the efficacy of the plant extracts can be increased in combination with peppermint essential oil.

Methodology

Ethanolic extracts of the five selected aquatic plant species were prepared and tested for their *in vitro* antibacterial activity using the microdilution broth assay. PrestoBlue was used as a growth reagent to detect actively metabolic bacterial cells. The enzyme inhibitory activity of the selected aquatic plants was tested using a colorimetric assay.

Results and discussion

Typha capensis exhibited a minimum inhibitory concentration (MIC) of 250 µg/ml against *P. acnes* while *Mentha longifolia* inhibited 50% of tyrosinase enzyme (IC_{50}) at 53.63 µg/ml. Combinational studies with the plant samples and peppermint essential oil for antimicrobial activity suggests that essential oils have a greater activity of inhibiting Gram-positive oral pathogens. Combinational studies suggest that together with the known drug (kojic acid) the IC_{50} of *M. longifolia* reduced significantly making it a possible alternative treatment for skin hyperpigmentation.

Conclusions and Further Research

Although this study focused on a small scale screening of only a few selected aquatic species based on their traditional uses, there is much promise for expanding the search to more aquatic plant types, families and species. Future prospects include additional combinational studies of *T. capensis* with current acne treatments (tetracycline) to assess the interaction between the two samples in combination for a more effective treatment or antibacterial agent against *P. acnes*.

The screening of more aquatic plants from various aquatic plant families and species will be considered in order to get a better understanding of which plant families may one day be used in pharmaceutical or cosmetic products. The search of medicinal aquatic plants will also expand beyond the riparian zone of plant collection and include species which are known as floating or submerged aquatic plants. Depending on their association with the water source in aquatic habitats, different classes of aquatic plants have been known to produce different types of chemical compounds which are known to be medicinal. By exploring the medicinal potential of different types of aquatic plants, an indication of which types produce more compounds with antibacterial or enzyme inhibitory activity could be given.

Future work also includes, testing the activity of aquatic plants for their potential to act as antioxidants (scavenging of free radicals as possible anti-aging and cancer treatments), antibacterial agents (against *Mycobacterium tuberculosis*, the bacteria involved in the pathogenesis of tuberculosis) and cytotoxicity (evaluates the safety of the plant samples) on different cell lines for potential treatments for cancer studies. Further testing of the aquatic plants against these common human ailments would provide a broader opportunity to identify an aquatic plant that could be incorporated into a product.

Although this study has focused mainly on the beneficial effects that medicinally important aquatic plants will have on the cosmetic and pharmaceutical industry, the impact that this project has on the ecology and conservation of South African wetlands can also be an advantage. In almost every publication about wetlands, the authors emphasise the importance of wetland conservation. Ecologically and economically the present study highlights the significant role that aquatic plants and ecosystems play in nature, something that communities often have trouble identifying. Communities are quick to replace wetlands with agricultural, urban or industrial lands, displacing the plants and reducing the natural abundance of resources. Finding medicinal uses for aquatic plants in both acne and skin

hyperpigmentation treatments, provides a reason for communities to protect their wetlands and riparian ecosystems which are home to possible alternative treatment options that could drastically improve the lives of patients with these common human ailments. The cultivation and upkeep of wetlands supplying the medicinal resources could provide many jobs for communities within the immediate area of a wetland.

Involving the community THPs in a workshop has helped identify the need of medicinally important plants within South African communities in terms of increasing collaborations between scientific research groups and traditional healers. The comments received from the survey indicated that subsequent workshops need to be organized in future. Through this project collaborations between the Water Research Commission, the University of Pretoria and the THP community will help create jobs for the cultivation and maintenance of medicinally aquatic South African plant species.

The potential of aquatic plants is very promising and this present research has only outlined a small percentage of the opportunities for the incorporation of aquatic plants into botanical based pharmaceuticals and cosmetics.

Acknowledgements

We would like to thank the Water Research Commission, University of Pretoria and National Research Foundation, South Africa.

University of Pretoria's garden curator, Mr. Jason Sampson, for making the plants available for the present study, is greatly acknowledged.

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1. Aquatic plants and their medicinal properties

Aquatic plants are found all over the world in ecosystems ranging from swamps and rivers to wetlands and ocean shores. They occur in any areas where the land is wet and have crucial roles in the functioning of the environment. Their biological importance include energy fixation for other organisms through photosynthesis and supplying oxygen. They are incredibly diverse with regards to their structural adaptations, distribution and secondary metabolite production which help them survive within flooded environments (Cronk & Fennessy, 2001).

Aquatic plants can be described as plants which are associated with an environment abundant in water and vegetation. This wet habitat may range from open water, to regions where the soil is only seasonally water-logged. The aquatic plants may be found either in, on or under the water with specific adaptations allowing them to survive in each situation (Tomlinson, 1983). These unique adaptations are developed in aquatic plants through evolution as they are believed to be phylogenetic descendants of terrestrial plants (Bornette & Puijalon, 2009).

1.1. Types of aquatic plants

Different types of aquatic plants exist according to their adaptations to the water-filled environment (Figure 1). These include, emergent, submerged, floating and riparian plants.

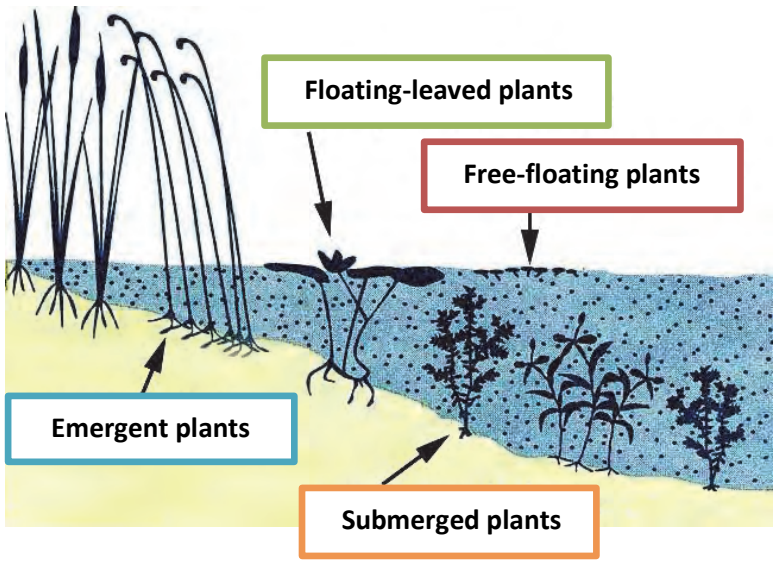


Figure 1: The different types of aquatic plants found in nature (Krischik et al., 1997)

1.1.1. Emergent plants

These aquatic plants are rooted within the soil with their basal structure usually beneath the water surface while their photosynthetic parts; including their leaves, stems and reproductive organs, are above the water surface (Cronk & Fennessy, 2001). Their aerial leaves are very similar in structure and function to those typically seen in terrestrial environments (Arber, 2010).

1.1.2. Submerged plants

Submerged plants have their entire structure submerged below the water surface where they spend their entire life cycle (Cronk & Fennessy, 2001). These types of aquatic plants have special leaf and stem adaptations to allow them to move with the water current without damage. Adaptations include soft stems lacking lignin as well as highly divided or elongated leaves that are very thin, allowing increased flexibility (Taiz & Zeiger, 2010).

1.1.3. Floating plants

There are two types of floating plants. Plants whose entire structure floats on the water are known as "free-floating plants". Their roots are not attached to any substrate but are instead hanging free within the water. Aquatic plant species that have large circular leaves that float on top of the water surface while their roots are attached to a substrate are known as "floating-leaved plants" (Cronk & Fennessy, 2001).

In both cases, their leaves are very broad, firm and leathery but flexible enough to withstand damage that may be caused by waves occurring in the water (Arber, 2010).

1.1.4. Riparian plants

Riparian plants grow along the banks of rivers, streams and lakes, in a narrow strip of land that borders the water source. These plants are distinct from terrestrial plants as they grow in soils that are water-rich or water-logged (Freitag, 2014).

The different types of aquatic plants produce a variety of secondary metabolites depending on their association with the water. Aquatic plants produce secondary metabolites that could potentially be used for their pharmacological properties as described below.

1.2. Potential medicinal properties

The difference between a normal plant and a medicinal plant is within its ability to produce secondary plant metabolites known as phytochemicals. While terrestrial plants have been extensively studied, the knowledge of the medicinal properties of aquatic plants has remained a relatively understudied field. There could be many reasons as to why this is so, ranging from the abundance of terrestrial plants already available, to the concern of wetlands being rapidly destroyed and destructed (Macaskill, 2010). However, due to the large abundance and variety of plant species available and relatively easy cultivation processes, this field holds much promise for the future and opens up many new opportunities for collaborations between the pharmaceutical industry and that of agriculture.

Like all plants, aquatic plants are sessile organisms and thus rely on many adaptations in order to survive various stresses including, water pollution, herbivory, microorganism interaction and environmental cues (Taiz & Zeiger, 2010). While aquatic plants vary in their structure and development, they also differ in the types of plant secondary metabolites they produce depending on their association with the water.

Many aquatic plants share their environment with not only other plants but microorganisms and wildlife as well. Like all plants, they have the ability to produce phytochemicals to help survive, grow and compete in such habitats. Phytochemicals according to history and drug development have been proven to be the medicinally important constituents of plants. These phytochemicals are used to treat human ailments due to their ability to initiate physiological effects (Bhowmik et al., 2013). Many studies have been conducted to investigate the bioactive compounds present within aquatic plants yet most of them rely on the plant-environment interactions in order to be produced.

According to Smolders et al. (2000), phenolic compounds within plant species are the most common secondary metabolites produced and have been investigated in various aquatic plant-herbivore interactions. It was discovered that in general, most submerged plants had less phenolics than emergent or floating-leaved types. This is due to the fact that while on the

surface of the water or completely outside of the water the plant is more susceptible to attack and thus requires more phenolics than those that live below the water surface. Another plausible reason is that due to the lower ultraviolet light exposure, submerged plants do not require much protection that phenolics provide against light stress (Smolders et al., 2000).

When deterring herbivores, chemical compounds, although less visible than morphological adaptations play an equally important role in plant defence. Compounds from the medicinally important family, coumarins, have been found in many plant families helping to deter feeders due to their bitter taste. They have also been found in many aquatic plant families including Cyperaceae, Araceae, Juncaceae and Poaceae (Figure 2) (Keddy, 2010).

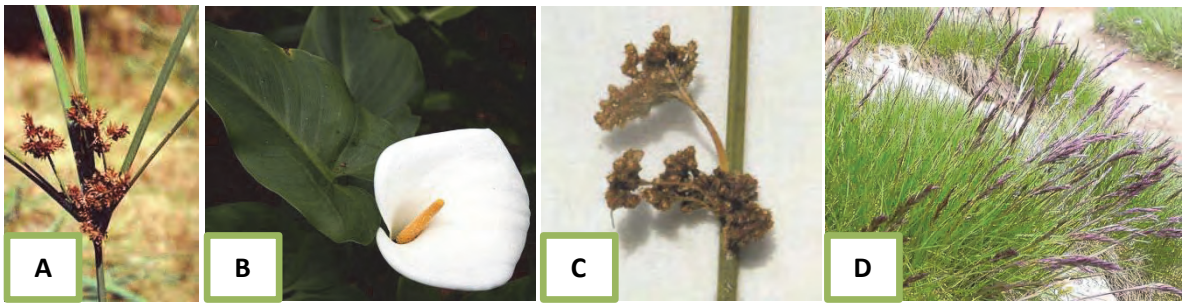


Figure 2: Aquatic examples of A) Cyperaceae (*Cyperus javanicus*), B) Araceae (*Zantedeschia aethiopica*), C) Juncaceae (*Juncus effusus*) and D) Poaceae (*Festuca alpina*) (Wallis, 2008; Storch, 2008; Gerber et al., 2004; Lautaret, 2000)

A study conducted by McClure (1970) highlighted the important role which secondary metabolites play in adapting aquatic plants to water-filled environments. Flavonoids were noted to have a variety of functions within plants but mainly aided growing plants during physiological stress and provided photo-protection through antioxidant activity (Agati et al., 2012; Huang et al., 2015). Flavonoids were seen to be the most prominent secondary metabolite class in free-floating aquatic species while both flavonoids and phenols were found in high concentrations in emergent species. Alkaloids were the highest in floating-leaved species such as Nymphaeaceae while, terpenoids were found to be more commonly present in plants that thrived in water-logged soils (riparian), both compound classes are important anti-herbivore compounds (Keddy, 2010). In a recent publication by Choi et al. (2002), it was found that some species of submerged plants, especially from the Haloragaceae

family had very high concentrations of hydrolysable tannins within their leaves that made up approximately 8-20% of their dry mass.

It can, therefore, be observed that freshwater plants have been reported to produce many structurally diverse, yet novel bioactive compounds that aid in chemical defence. Compounds include, antibiotics, alkaloids, mycotoxins and phenolic compounds which are all considered to be valuable sources of pharmaceutical compounds for the production of modern herbal remedies and drugs (Ramesh et al., 2013).

2. Literature Review

The following list of aquatic plants was adapted from the "Easy Identification of Aquatic Plants" book (Gerber et al., 2004). A literature review on these plants suggested that they have the potential to be used as alternative treatments in the pharmaceutical and cosmeceutical industry as seventeen of these aquatic plants were reported for traditional usage or had been tested for antimicrobial studies *in vitro* (underlined).

1. *Azolla pinnata* var. *africana*
2. *Aponogeton distachyos*
3. *Nymphaea nouchali* var. *coerulea*
4. *Nymphoides thunbergiana*
5. *Lagarosiphon muscoides*
6. *Lagarosiphon major*
7. *Potamogeton schweinfurthii*
8. *Potamogeton thunbergii*
9. *Utricularia stellaris*
10. *Cyclosorus interruptus*
11. *Floscopa glomerata*
12. *Ludwigia adscendens*
13. *Limosella maior*
14. *Marsilea* sp.
15. *Persicaria senegalensis*
16. *Plantago longissima*
17. *Carex austro-africana*
18. *Cladium mariscus*
19. *Cyperus dives*
20. *Cyperus marginatus*
21. *Cyperus sexangularius*
22. *Eleocharis acutangula*
23. *Juncus effuses*
24. *Juncus lomatophyllus*
25. *Phragmites mauritianum*
26. *Prionium serratum*
27. *Schoenoplectus brachyceras*
28. *Schoenoplectus paludicola*
29. *Typha capensis*
30. *Zantedeschia aethiopica*
31. *Gunnera perpensa*
32. *Equisetum ramosissimum*
33. *Mentha longifolia*
34. *Commelina benghalensis*

3. Selection and collection of aquatic plants

The following five indigenous aquatic plant species and their relevant plant parts were selected and collected (Table 1). The plants were also added to the H.G.W.J. Schweickerdt Herbarium (PRU) database and given associated accession numbers.

Table 1: The plant species and relevant plant parts selected and collected for the present study and their associated PRU numbers

Plant	Part collected	PRU numbers
Commelina benghalensis (L.)	Whole plant including flowers	121874
Equisetum ramosissimum Desf.	Whole plant	121875
Mentha longifolia (L.) Hudson	Leaves and stems	121876
Typha capensis (Rohrb.) N.E.Br	Lowers stems and roots	121877
Zantedeschia aethiopica (L.) Spreng	Leaves	121878

With the use of plant materials among different cultures having an impact on their associated treatments, the plants chosen for the present study were identified according to known traditional uses. They were also researched to ensure that they are indeed aquatic plants (emergent, submerged, floating or riparian) that are indigenous to South Africa. Plants were selected due to their availability and abundance on the University of Pretoria Hatfield Campus and also for their rapid regeneration and growth making the project sustainable over a period of time. The description, distribution and traditional usage of each plant selected for the present study are as follows,

3.1. *Commelina benghalensis* (L.)

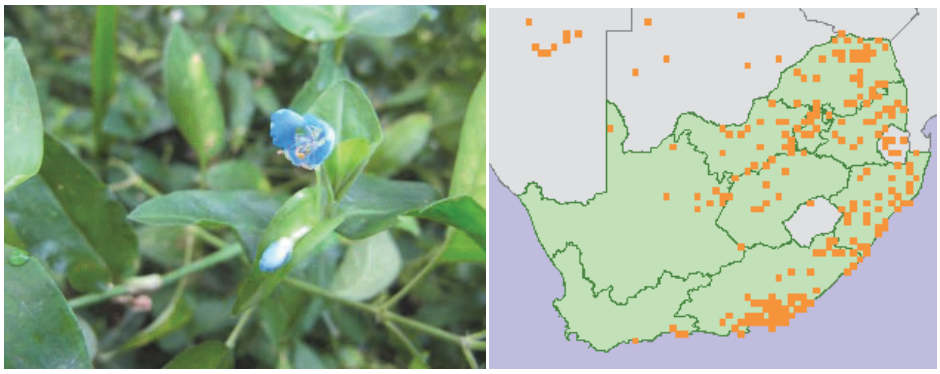


Figure 3: *Commelina benghalensis* with its characteristic blue flower (Photo taken by K. Szuman) and its distribution in southern Africa (Foden & Potter, 2005)

Commelina benghalensis differs from other *Commelina* species as it has blue flowers unlike *C. africana*, which has distinct yellow flowers. *C. benghalensis* is an annual or perennial herb that grows abundantly in all soil types and pH levels, however, it grows best in highly fertilised, moist or water-logged soils. This plant is native to South Africa and is considered a non-invasive species, however, in other parts of the world, *C. benghalensis* is considered as one of the world's worst weeds, affecting 25 crops in 29 countries (Global Invasive Species Database, 2008).

3.1.1. Traditional uses

C. benghalensis is used by different cultural groups for traditional healing in both India and Africa. The whole plant has been used medicinally to treat sore throats, burns, irritation of the eyes and stomach and infantile thrush. During famine periods in India and the Philippines, the leaves and stems of the weed were chopped and cooked as a nutrional source for people and made into feed for livestock. In southern Africa, the plant was found to combat infertility (van der Burg, 2004).

3.2. *Equisetum ramosissimum* Desf.

Figure 4: The apex and stems of *Equisetum ramosissimum* (Municipality of Sitia, 2013) and its distribution in southern Africa (Foden & Potter, 2005)

Equisetum ramosissimum is an annual perennial fern that has a wide distribution across all of South Africa, occurring mostly along rivers and streams. The plant is easily grown in ordinary soil found in gardens as long as the soil is kept moist (South African National Biodiversity Institute, 2015). The rhizome of the plant lies below the ground (subterranean) while the stem lies erect above the ground. Branches emerge in whorls at each node along the length of the stem (Roux, 2003).

3.2.1. Traditional uses

E. ramosissimum is a sweet yet slightly bitter tasting plant that cleanses the liver and clears eyesight by reducing the reddening and swelling of the eye as well as the pterygium of the cornea. It can be made into a decoction by adding 15-30g of dried plant material to boiling water as a treatment for diarrhoea and jaunditic hepatitis. In China, the whole plant is used to treat wounds and ulcers by making a decoction, while in India it is used as a cooling medicine for gonorrhoea and has for many centuries been used as a treatment for skin wounds (Mannan et al., 2008; Jain & Srivastava, 2005). In South Africa, infertile women drink a decoction made from the rhizome to aid in fertilization. A paste made from the branches and leaves can be applied to areas to treat fractured or dislocated bones. *E. ramosissimum* is also known to possess diuretic, haemostatic, antifungal and antiviral properties (Banjamin & Manickam, 2007). The Zulu communities have been reported to use the sap from the plant to reduce pain and heal wounds of toothaches and tooth extractions (Gerstner, 1939).

3.3. *Mentha longifolia* (L.) Hudson

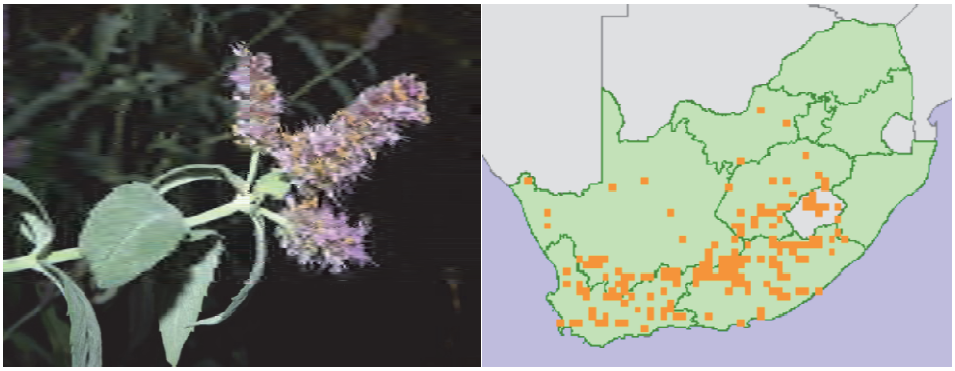


Figure 5: Flowering *M. Longifolia* (Perez, 2010) and its distribution in southern Africa (Foden & Potter, 2005)

Mentha longifolia is a mint species that is indigenous to South Africa. It is commonly found in marshes and along the banks of streams and rivers as they are water lovers and thrive in damp or wet environments. *M. longifolia* has lanceolate leaves (narrow and long with a sharp point) that are stalk-less and usually vary in colour from grey to dark green. It has small flowers that are grouped in a long spike at the tip of the stem, ranging in colours from white to mauve during summer months. It is a fast-growing perennial herb that creeps via an underground rootstock with a strong distinct mint smell (van der Walt, 2004).

3.3.1. Traditional uses

M. longifolia has been traditionally used internally as a treatment for colic, menstrual disorders, indigestion, flatulence, pulmonary infections and congestion, headaches, fever, coughs, colds and urinary tract infections. Externally, *M. longifolia*, is used to relieve swelling and treat sores and minor wounds of the skin. The leaves and stems can be added to boiling water to release a vapour that can be inhaled to relieve nasal and bronchial congestion (van der Walt, 2004).

3.4. *Typha capensis* (Rohrb.) N.E.Br

Figure 6: Characteristic spike of *Typha capensis* (Wursten, 2013) and its distribution in southern Africa (Gerber et al., 2004)

Typha capensis is a perennial, leafy aquatic plant that has a distinct velvety-brown (female) or yellow (male) flower-spike. *T. capensis* is regarded as an indigenous South African aquatic plant as it is associated with most freshwater bodies including marshes, streams, riverbanks, dams and lakes around South Africa in all provinces. The muddy substrate found around water bodies helps the plant to anchor its roots firmly into the ground. Even though it has a cosmopolitan appearance in aquatic ecosystems, it is regarded as a pest due to its ability to spread very fast as its tiny fluffy seeds are easily dispersed by the wind (Voigt & Porter, 2007; van Ginkel et al., 2011).

3.4.1. Traditional uses

Various plant parts were used by communities for everyday activities before they were considered for medicine. The narrow leaves are used as thatch for mats and baskets while the seeds are used as pillow stuffing. In most cases, the rhizomes or roots of *T. capensis* are used in traditional medicine. A decoction of the rhizomes is used to treat venereal diseases, bleeding, diarrhoea, swelling and urinary problems. During labour the decoction can either be taken orally or applied externally to promote the removal of the placenta and strengthen uterine contractions to ensure an easy delivery. *T. capensis* is also taken to promote fertility in women, enhance male potency and libido and improve circulation (Watt & Breyer-Brandwijk, 1962; Hutchings et al., 1996; Voigt & Porter, 2007; van Ginkel et al., 2011).

3.5. *Zantedeschia aethiopica* (L.) Spreng

Figure 7: Flowers, leaves and white spathe of *Zantedeschia aethiopica* (Storch, 2008) and its distribution in South Africa (Foden, 2010)

Zantedeschia aethiopica is commonly found along the edges of streams and ponds. It is a fast growing aquatic plant that thrives in very rich, well-drained soils. The genus, *Zantedeschia* is restricted to the African continent, making *Z. aethiopica* indigenous to South Africa. It is a perennial herb with fleshy rhizomes and stems. It has large evergreen leaves that are shaped like an arrow head that increase in size when grown in shade. The leaves are able to discharge excess water through their stomata in a process known as guttation, preventing water-logging and allowing them to grow in wet environments. *Z. aethiopica* has a very distinct white "flower" (spathe), however, the actual flower exists on the spadix (central column) which holds many tiny flowers arranged in a spiral pattern (Aubrey & Reynolds, 2001).

3.5.1. Traditional uses

The washed leaves of *Z. aethiopica* is heated and used as a dressing for wounds, boils, minor burns, insect bite and sores. Patients suffering from gout or rheumatism also use the warmed leaves as a poultice to reduce the pain. Traditional communities located in the Cape, powder the rhizome of *Z. aethiopica* and use it as a poultice for inflamed wounds. The plant can be boiled and eaten by mixing it with honey or syrup as a treatment for asthma, bronchitis or gargled for the relief of sore throats. The plant must be boiled or cooked in some way as the raw plant material causes swelling of the throat due to the presence of microscopic calcium oxalate crystals (Watt & Breyer-Brandwijk, 1962; Roberts, 1990; Rood, 2008; Wink & van Wyk, 2008).

4. Preparation of extracts

Different methods of extraction are utilised in phytopharmacy and involves the process of separating the medicinally active compounds from the inactive components within plant tissues by using a specific solvent. The resulting extract is ready for use as a medicinal agent in the form a dry extract. In the present study, the method of extract was an adaption of the maceration technique of extraction.

Maceration process of extraction: pieces or powdered form of the plant material together with the solvent (ratio of 1:20 herb/liquid) is placed into a container and left to stand for three days at room temperature with frequent shaking. This is to allow the soluble matter to completely dissolve, indicating complete extraction of the plant material. After three days (not longer as fungal contaminations can occur), the mixture is strained and the remaining solid pieces are squeezed to remove any excess liquids. The combined liquids are then filtered using a filter (Handa et al., 2008; Bimakr, 2010; Pandey & Tripathi, 2014).

The ethanolic extracts of the selected plant species were prepared in the following manner (Figure 8).

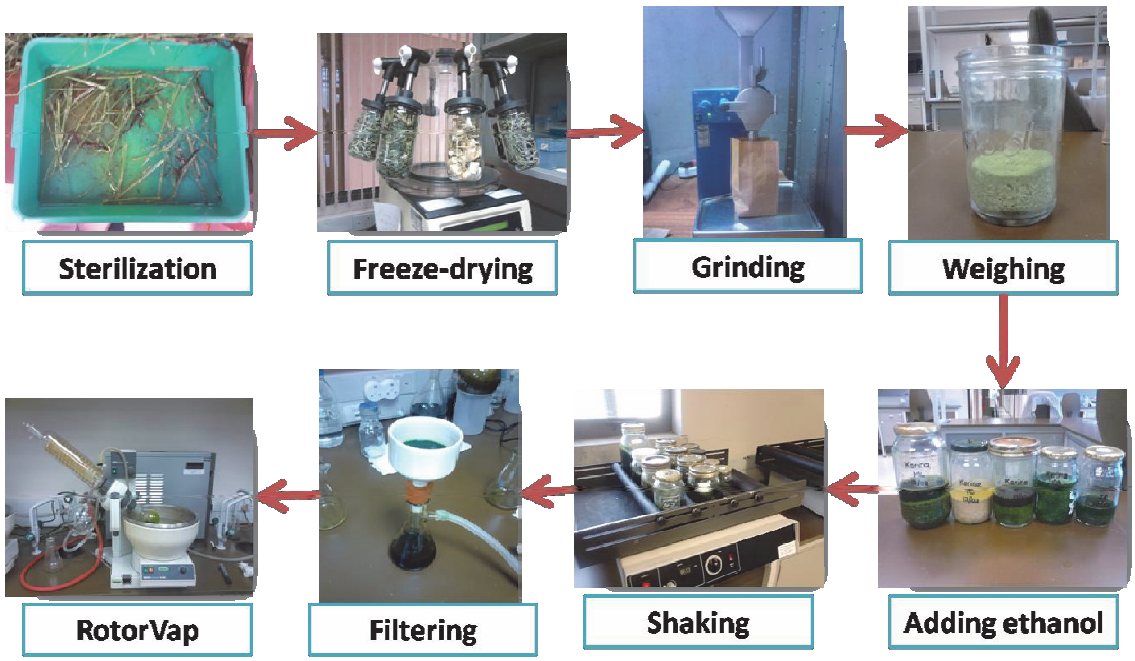


Figure 8: Process of extraction (Photos taken by K. Szuman)

The collected plant material was washed with distilled water to remove any insects, soil or surface contaminants. The plants were frozen at -80°C for 3 days to prepare the plant material for freeze drying. The freeze dryer was used to remove all water from the plant material to prevent fungal contamination that would usually occur if the plant was air dried. The dried plant material was ground to a fine powder using a 2 mm IKA grinder (MF 10.1 Head 2870900). The ground plant material was weighed to allow for a 1:5 ratio of plant material and 100% ethanol so as to not over saturate the medicinal compounds that may be present in the plant material. The plant material containing ethanol was placed on the shaker (Labcon Shaker 3086U) and left to shake for 7 days. The ethanolic extraction liquid was separated from the ground plant material using a filter (Whatmann N0.1 filter paper) and vacuum pump. The ethanol was evaporated from the sample to produce a concentrated extract using a rotary vapour (BUCHI Rotavapor B-480) apparatus. The resulting extract was placed in the -4°C freezer to be used for subsequent experiments. An extract is prepared prior to *in vitro* testing as the amount of extract can be easily weighed or measured to allow for accurate quantification without being influenced by residual ethanol.

The method of extraction used for the present study as explained above was efficient and reliable in order to obtain ethanolic plant extracts for *Commelina benghalensis*, *Equisetum ramosissimum*, *Mentha longifolia*, *Typha capensis* and *Zantedeschia aethiopica* that was then used in subsequent bioassays.

5. Objectives and research questions

Do indigenous South African aquatic plants have the potential to be effective alternative treatments for,

- Skin hyperpigmentation
- Acne
- Periodontal diseases

6. Efficacy of material for skin hyperpigmentation

6.1. Introduction

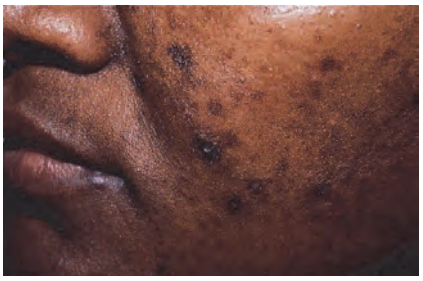


Figure 9: Hidden inflammation in a darker-skinned acne patient which produces prominent post-inflammatory hyperpigmented spots (White, 1998)

The diversity of skin colour across the globe ranges from very pale skin to very dark, but across this array of colours there is a desire for uniform and even skin tone (Ortonne & Bissett, 2008). Problems with the pigmentary system include the overproduction of melanin resulting in hyperpigmentation (darker patches of skin) disorders which is the result of either post-inflammatory, solar lentigos or melasma.

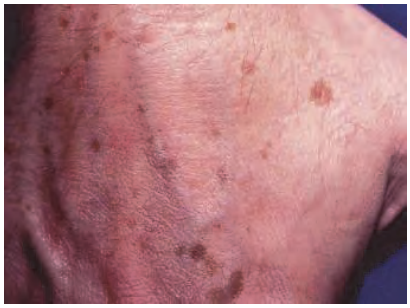


Figure 10: Solar lentigos on the hand (Ortonne et al., 2006)

Acne lesions, ingrown hairs, scratches and insect bites are among the common activities that can cause the skin to become inflamed resulting in post-inflammatory hyperpigmentation (PIH). Pigmentary disorders result due to the production of reactive oxygen species which stimulates melanin-producing cells (Figure 9) (Ortonne & Bissett, 2008).

Solar lentigos are hyperpigmented spots on the skin better known as age spots and are a result of chronic exposure of the skin to ultraviolet (UV) light resulting in a permanent genetic increase in the mRNA of melanogenesis-related proteins (Figure 10) (Ortonne & Bissett, 2008).

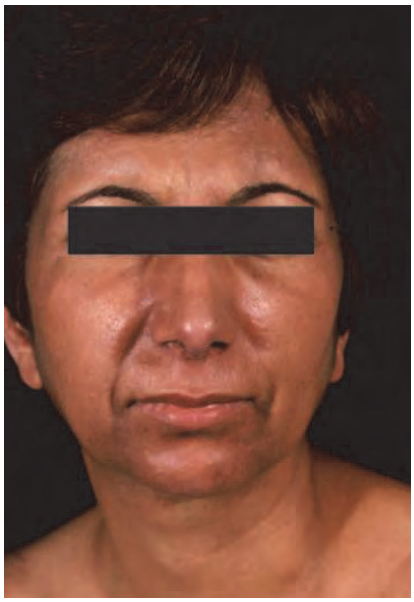


Figure 11: Melasma on the face (Ingber, 2009)

Melasma presents itself as symmetrical lesions on the facial skin and is a result of sun exposure, hormone therapy, pregnancy and the use of some drugs and medications (Figure 11) (Pearl & Grimes, 1995).

The enzyme involved in all cases of skin hyperpigmentation is tyrosinase.

6.1.1. Tyrosinase

Tyrosinase is a copper containing glycoprotein that functions as the rate limiting enzyme within the pathway of melanogenesis. Since tyrosinase is the first enzyme in this process, by targeting its degradation or inhibition, there could be a drastic decline in the abundance of melanin resulting in more evened skin tone hence it is the common target to alleviate cutaneous hyperpigmentation (Ando et al., 2007).

6.1.2. Current treatments

The desire for lighter skin and evened skin tone has resulted in many treatments that are currently available. Traditional depigmenting agents including hydroquinone, corticosteroids and kojic acid although highly effective, have many adverse side effects and safety issues ranging from ochronosis, atrophy, carcinogenesis and other local or systemic effects. The emergence of such issues has provided an opportunity for researchers to develop new products to address the pigmentation problems with less side effects, thus the search for novel natural and botanical extracts has begun (Zhu & Gao, 2008).

6.2. Aim

To determine the enzymatic inhibitory activity of the selected indigenous South African aquatic plants against tyrosinase (the rate-limiting enzyme involved in the production of melanin).

To determine the interaction between a tyrosinase inhibiting plant extract in combination with kojic acid.

6.3. Materials and methods

6.3.1. Materials

Analytical grade chemicals, substrate (L-tyrosine), enzyme (mushroom tyrosinase) and positive control (kojic acid) were purchased from Merck (Pty) Ltd and Sigma-Aldrich (Johannesburg, SA).

6.3.2. Methods

The anti-tyrosinase (enzyme inhibition) assay was performed according to the method described by Mapunya et al. (2011), with few modifications. The selected five aquatic plant extracts were dissolved in 100 µl dimethyl sulfoxide (DMSO) to 20 mg/ml stock solution. The stock solution was diluted with 50 mM potassium phosphate buffer (pH6.5) to 600 µg/ml in a 24-well plate. In 96-well microtitre plates placed on ice, 70 µl of varying concentrations of each plant sample was added to 30 µl of tyrosinase enzyme (333 units/ml in phosphate buffer pH 6.5) in triplicate. After 5 minutes of incubation on ice, 110 µl of substrate (2 mM L-tyrosine) was added to all wells. The final concentrations of each plant extract and positive control (kojic acid) was 200-1.5 µg/ml. The optical density of each microtitre plate was measured over a period of 30 minutes at a wavelength of 492nm using BIO-TEK power Wave XS multi-well plate reader (KC Junior). The 50% inhibitory concentration (IC_{50}) was determined by analysing the resulting data using the software GraphPad Prism 4.

The combinational study was performed in the same manner however, a ratio of 1:1 of plant extract and kojic acid was used.

6.4. Results and discussion

6.4.1. Results

6.4.1.1. Anti-tyrosinase activity of plant extracts

In the presence of a tyrosinase inhibitor the substrate L-tyrosine cannot be oxidised to dopaquinone. The inhibition of tyrosinase prevents the melanogensis pathway from producing melanin thus the solution will not turn a brown colour but rather remain colourless. This colour change is observed through the enzyme inhibitory activity of kojic acid (positive control) and *Mentha longifolia* (Figure 12). In the tested plant samples, it was observed that the inhibition of tyrosinase was only seen through the activity of *M. longifolia.*

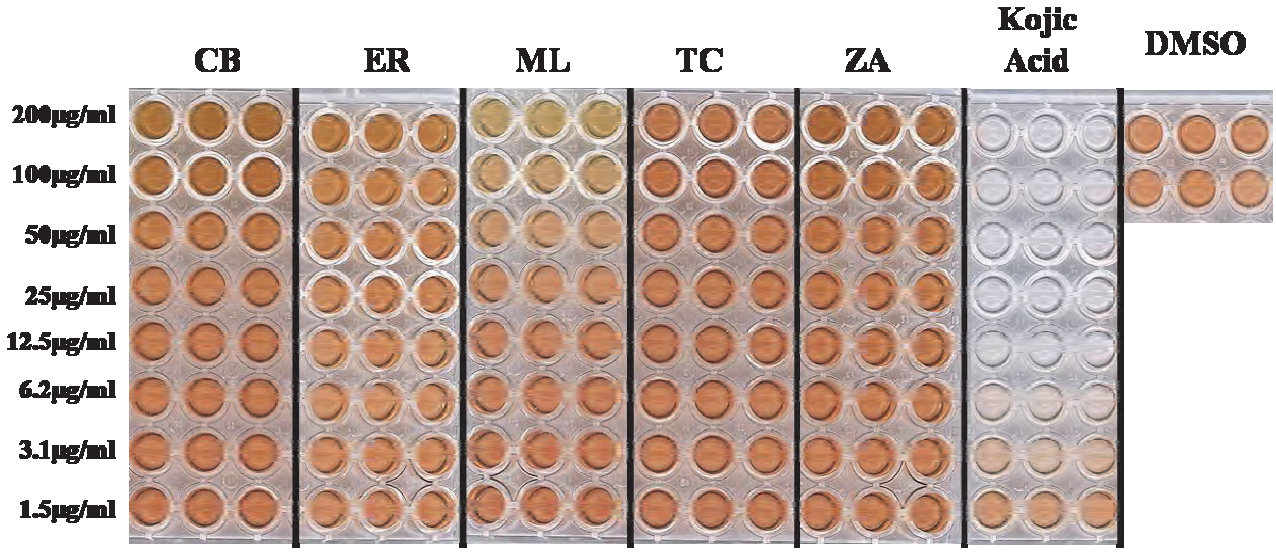


Figure 12: Results observed after 30 minute incubation of the anti-tyrosinase (enzyme inhibition) activity of the aquatic plant extratcs (CB: *Commelina benghalensis*; ER: *Equisetum ramosissimum*; ML: *Mentha longifolia*; TC: *Typha capensis*; ZA: *Zantedeschia aethiopica*) and controls (Kojic acid: positive control; DMSO (dimethyl sulfoxide: negative control)).

The positive control (kojic acid) completely inhibited tyrosinase at higher concentrations as the result is a colourless solution. The negative control, containing no tyrosinase inhibitor turned brown after incubation as the tyrosinase was not degraded or inhibited indicating that our reagents in the experiment had no contamination and the experimental procedure was carried out correctly.

The remaining four plant extracts (*Commelina benghalensis, Equisetum ramosissimum, Typha capensis* and *Zantedeschia aethipica*), did not show any significant colour change. Compared to the negative control, they were of similar brown indicating that no inhibition of tyrosinase occurred, thus melanin was still being produced. Hence, these plant extracts showed no inhibition at the tested concentrations.

The graphs represented in Figures 13 and 14 showed the percentage inhibition concentration of both the active plant extract (*M. longifolia*) and positive control (kojic acid) that was calculated from the absorbance values using an ELISA plate reader.

M. longifolia showed some tyrosinase inhibiting activity at the highest concentrations as seen in Figure 13. The highest percentage inhibition is 75% at 200 µg/ml of the plant extract and the lowest percentage inhibition is 5% at 3.1 µg/ml.

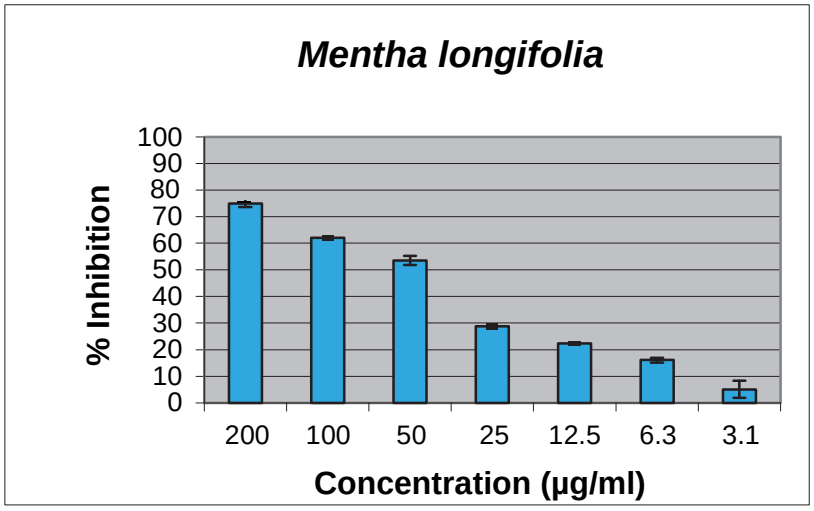


Figure 13: Graph representing the percentage tyrosinase inhibition of *Mentha longifolia* at the various concentrations (µg/ml)

Kojic acid showed tyrosinase inhibiting activity at all concentrations as seen in Figure 14. The highest percentage inhibition is 100% at 25 µg/ml of the sample and the lowest percentage inhibition is 25% at 1.6 µg/ml.

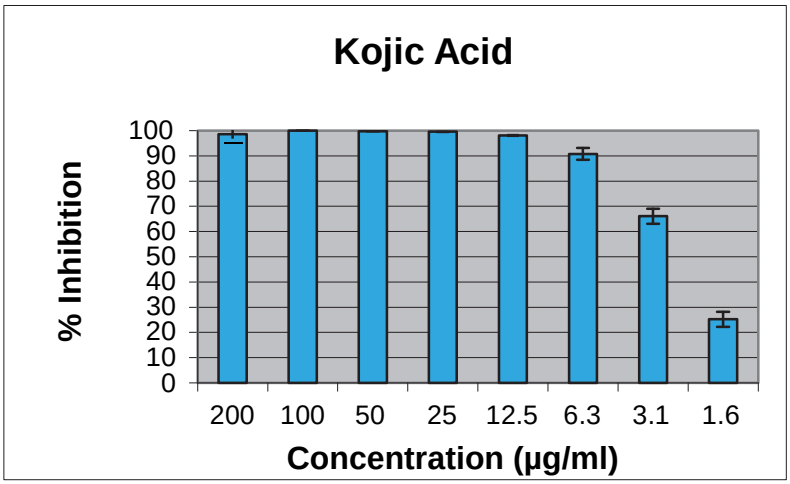


Figure 14: Graph representing the percentage tyrosinase inhibition of kojic acid (positive control) at the various concentrations (µg/ml)

Table 2 shows a comparison between the results obtained from the GraphPad Prism 4 software. The IC_{50} values of the samples indicate the concentration of the sample required to inhibit 50% of the tyrosinase enzyme together with the standard deviation, since the experiment was conducted in triplicate, the values given are an average.

Table 2: The IC_{50} values (µg/ml) of anti-tyrosinase activity of selected aquatic plant extracts

Sample	R^2	IC_{50} (µg/ml) ± Standard Deviation
Commelina benghalensis (L.)	-	>200
Equisetum ramosissimum Desf.	-	>200
Mentha longifolia (L.) Hudson	0.9446	53.63 ± 1.350
Typha capensis (Rohrb.) N.E.Br	-	>200
Zantedeschia aethiopica (L.) Spreng	-	>200
Kojic acid	0.9855	3.03 ± 0.006

The IC_{50} value of 3.03 µg/ml obtained for kojic acid is an acceptable value for a tyrosinase inhibitor. The IC_{50} values obtained for the plant extracts (*C. benghalensis, E. ramosissimum, T. capensis* and *Z. aethipica*) were higher than the tested concentrations. By analysing the plates using the software, it was easier to evaluate why no colour change was observed as an extract concentration of more than 200 µg/ml would be required to inhibit tyrosinase activity.

The only plant sample that showed an IC_{50} value within the tested concentration range was that of *M. longifolia*.

The R^2 of kojic acid and *M. longifolia* were calculated using the GraphPad Prism 4 analysis software. This value indicates how well the IC_{50} value correlates with the data. For the results to be accurate the R^2 value has to be as close to 1 as possible. According to Table 1, it is noted that the R^2 values for both kojic acid and *M. longifolia* are greater than 0.9, indicating the results obtained from this assay are accurate. No R^2 values were recorded for the other aquatic plant samples (*C. benghalensis, E. ramosissimum, T. capensis* and *Z. aethipica*) as their IC_{50} values did not show any significant inhibition of tyrosinase.

6.4.1.2. Combinational studies

To determine the sum fractional inhibitory concentration (∑FIC) index of the plant sample (*M. longifolia*) in a 1:1 combination with kojic acid against tyrosinase, the IC_{50} of each sample was determined alone (from previous experiments) as well as the IC_{50} of the combination (Figure 15). The ∑FIC index gives an indication of the interaction between the two samples (de Rapper et al., 2012).

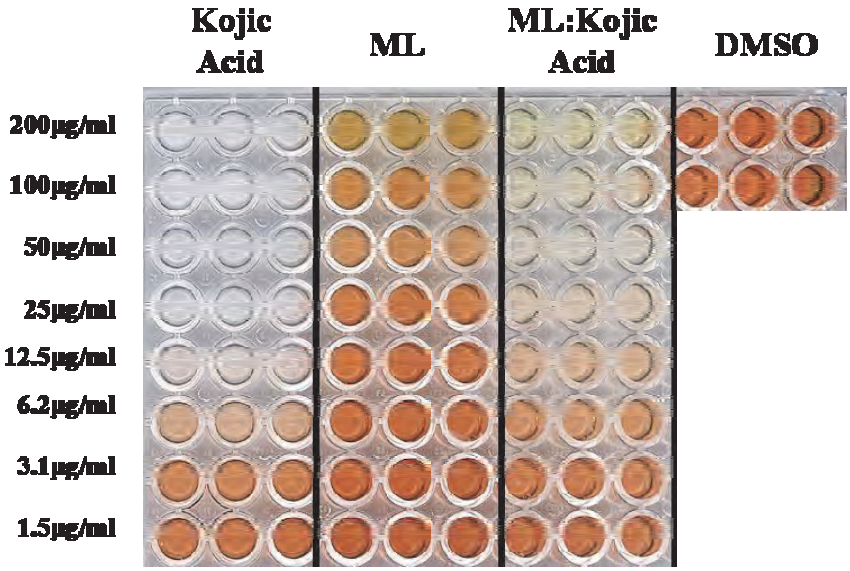


Figure 15: Results observed after 30 minute incubation of the anti-tyrosinase (enzyme inhibition) activity of the positive control (Kojic Acid), aquatic plant sample (ML: *Mentha longifolia*), combination of aquatic plant sample and positive control in a 1:1 ratio (ML:Kojic Acid) and negative control (DMSO: dimethyl sulfoxide)

In combination, *M. longifolia* and kojic acid showed tyrosinase inhibiting activity at all tested concentrations as seen in Figure 16. The highest percentage inhibition is 100% at 200 µg/ml of the tested samples in combination and the lowest percentage inhibition is 45% at 1.6 µg/ml.

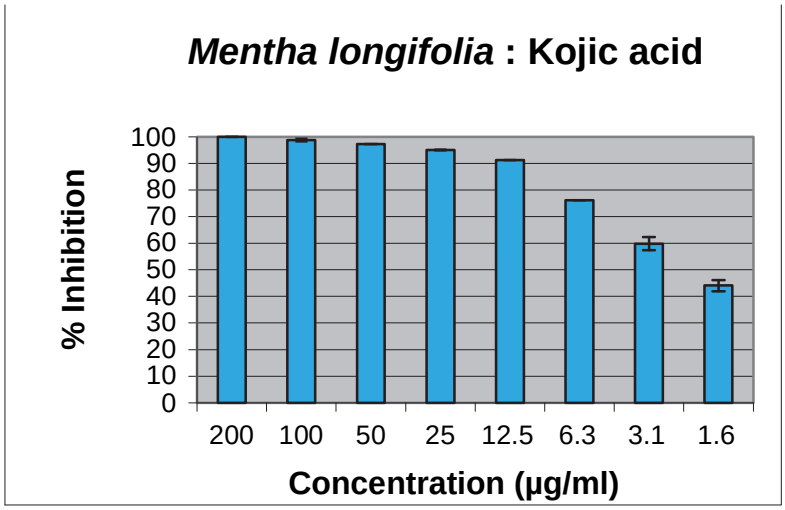


Figure 16: Graph representing the percentage tyrosinase inhibition of *Mentha longifolia* in combination with Kojic Acid at a ratio of 1:1 at the various concentrations (µg/ml)

Table 3 depicts a comparison between the results obtained from the GraphPad Prism 4 software. The IC_{50} values of the samples indicate the concentration of the sample required to inhibit 50% of the tyrosinase enzyme together with the standard deviation, since the experiment was conducted in triplicate, the values given are an average.

Table 3: The IC_{50} values (µg/ml) of anti-tyrosinase activity of the tested samples during combinational studies

Sample	R^2	IC_{50} (µg/ml) ± Standard Deviation
Mentha longifolia (L.) Hudson	0.9446	53.630 ± 1.350
Mentha longifolia and Kojic acid (ratio of 1:1)	0.9707	2.812 ± 0.027
Kojic acid	0.9855	3.030 ± 0.006

The FIC index was determined for each sample using the equation outlined in van Vuuren and Viljoen (2011) for combinational studies done in a 1:1 ratio (Equation 1). The resulting values are given in Table 4.

Equation 1: The sum fractional inhibitory concentration (∑FIC) index equation used to calculate the interaction between two samples (a and b) in a 1:1 ratio (van Vuuren & Viljoen, 2011)

$$\text{FIC}_{(a)} = \frac{\text{IC}_{50(a \text{ in combination with } b)}}{\text{IC}_{50(a \text{ individually})}}$$

$$\text{FIC}_{(b)} = \frac{\text{IC}_{50(b \text{ in combination with } a)}}{\text{IC}_{50(b \text{ individually})}}$$

$$\sum \text{FIC} = \text{FIC}_{(a)} + \text{FIC}_{(b)}$$

Table 4: The calculated fractional inhibitory concentration (FIC) index for each sample and the sum (∑FIC) in combination

Sample	FIC index
Mentha longifolia (L.) Hudson	0.052
Kojic acid	0.928
Mentha longifolia and Kojic acid (ratio of 1:1)	∑=0.980

6.4.2. Discussion

Mentha species (*M. spicata, M. pulegium* and *M. rotundifolia*) investigated in Algeria for their tyrosinase inhibitory ability were discovered to have IC_{50} values that ranged between 108 ± 20 µg/ml to 286 ± 45 µg/ml (Fatiha et al., 2015). When compared with the results obtained in the present study the IC_{50} of the tyrosinase inhibiting activity of *M. longifolia* is significantly lower (53.63 ± 1.350 µg/ml) than its species counterparts. The difference in tyrosinase inhibitory activity may be due to the variation in chemical constituents and concentrations present in within each species.

Fatiha et al. (2015) identified the total phenolic and flavonoid content within each *Mentha* species. Being part of the same genus, *M. longifolia* has a similar total phenolic and flavonoid content as the other *Mentha* plants. A phytochemical analysis study on the chemical constituents of *M. longifolia* revealed that mainly phenolic acids and flavonoids were present in the ethanolic extract of the *M. longifolia* (Akroum et al., 2009).

According to Hussain et al. (2010), the major chemical constituents of *M. longifolia* include piperitenone oxide, piperitenone and germacrene D (Figure 17). These compounds are classified as an oxide, monoterpene ketone and sesquiterpene, respectively (Božović et al., 2015).

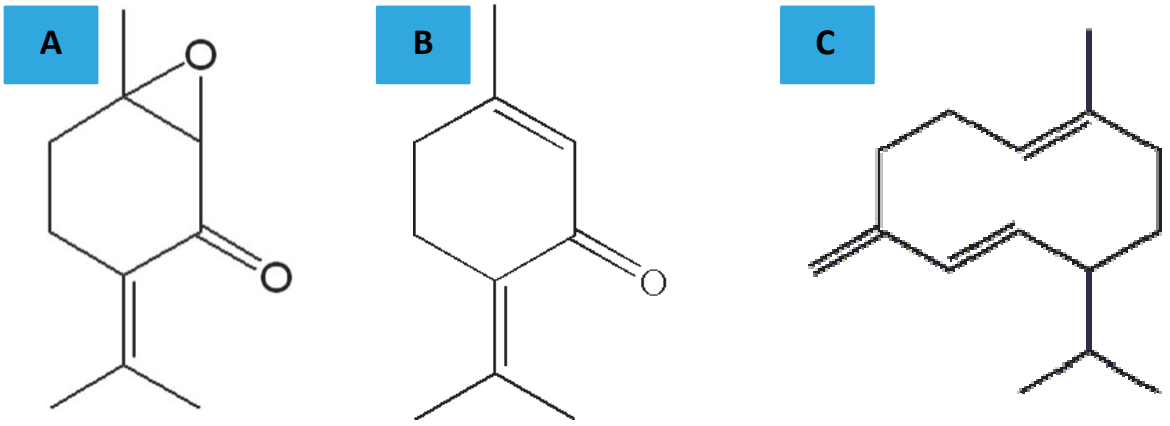


Figure 17: The chemical structure of (A) piperitenone oxide (B) piperitenone and (C) germacrene D, the main constituents of *Mentha longifolia* (Božović et al., 2015)

According to Mimica-Dukic and Bozin (2008), the pharmacological and biological effects of *Mentha* species is due to the presence of volatile oxygenated monoterpenes which give each *Mentha* plant its characteristic mint taste and aroma. These compounds are however, classified as non-polar, lipophillic (dissolvable in fats, oils, lipids and non-polar substances) constituents that have been found to be present in very low concentrations within the leaves and thus play a very small role in the medically beneficial effects of *Mentha* species. So although the main constituents of *Mentha* include, piperitenone oxide, piperitenone and germacrene D, these compounds alone do not confer medicinal value to *Mentha* species.

Thus focus has shifted to the more polar compounds (phenolics and flavonoids) being the medicinally valuable compounds in *Mentha* species due to their high concentrations (Mimica-Dukic & Bozin, 2008).

Previous studies suggest that the reason for the *Mentha* species ability to inhibit tyrosinase to be dependent on either,

(i) the ability of the hydroxyl groups found on the phenolic compounds to change the conformation of the enzyme through hydrogen bonding or

(ii) through its ability to scavenge free radicals which are formed during the reaction catalysed by tyrosinase (Fatiha et al., 2015).

It was also discovered that a high flavonoid content may also aid in the ability for the plant species to inhibit mushroom tyrosinase, as this class of compounds successfully chelates the copper ions present at the active site of the tyrosinase enzyme, making it inactive (Seo et al., 2003; Khazaeli et al., 2009). Due to the structural conformation of the flavonoid class of compounds, they are also able to inhibit the activity of tyrosinase by acting as cofactors or substrates of tyrosinase, reducing the number of active tyrosinase molecules present for L-tyrosine binding (Fu et al., 2005).

Mentha species form part of the Lamiaceae plant family which are considered as aromatic plants. Aromatic plants produce active compounds in the form of essential oils which are comprised mainly of polyphenols exerting pharmacological effects on living organisms (Khazaeli et al., 2009). The main chemical constituents of the essential oil of *M. longifolia* include, menthol, menthone and pulegone (Mikaili et al., 2013). These compounds differ from those present in the ethanolic extracts as essential oil extraction of plants is carried out in a different method which produces a different range of chemical constituents. The essential oils of *Mentha* species have been studied for many years as the aromatic menthol has been found to have many potential medical health benefits. Previous studies on the ability of the mint essential oils to inhibit the tyrosinase enzyme concluded that when these compounds are isolated on their own and tested for their ability to inhibit tyrosinase, results were inconclusive and the compounds exerted no effect. However, in combination as found in the essential oil, these compounds exert a synergistic effect on each other. This indicates that the complexity of the essential oil composition allows for various chemical species to interact together to synergistically inhibit tyrosinase (Fiocco et al., 2011). Although essential oils are the preferred form of cosmetics as they are easily absorbed by the skin, their hydrophobic

nature reduces the efficiency of the plant's activity (Edris, 2007). At a concentration of 240 µg/mL the peppermint essential oil of *M. x piperita* inhibited 50% of the tyrosinase enzyme while the maximal inhibitory effect was only 67% of tyrosinase (Fiocco et al., 2011). This indicates that although more accepted in terms of cosmetic use, the essential oil of *Mentha* species is less effective in inhibiting tyrosinase than ethanolic extracts. The variations in these results are due to the method of extraction as different types of compounds are extracted which may exert a different spectrum of activity.

In combination, it was found that the ∑FIC index of *M. longifolia* and kojic acid in a 1:1 ratio was 0.980. This value indicates the interaction that the two samples have together against the activity of tyrosinase. Depending on the value of the ∑FIC index, the samples can either work in synergy, antagonistically, additively or have no difference (Table 5) (van Vuuren & Viljoen, 2011; de Rapper et al., 2012).

Table 5: Interpreting the ∑FIC index of a combinational study between two samples in a 1:1 ratio (de Rapper et al., 2012)

∑FIC value	Interaction
∑FIC ≤ 0.50	Synergistic
∑FIC > 4.00	Antagonistic
1.00 > ∑FIC > 0.50	Additive
1.00 < ∑FIC ≤ 4.00	Indifferent

According to the calculated ∑FIC value of the two samples in combination (0.98), *M. longifolia* and kojic acid have an additive effect on one another. An additive effect is described when two substances used in combination have a total effect that is equal to the sum of each sample's individual effect (Jia et al., 2009). This effect differs from synergy which is the result of the interaction between the two substances which ultimately increase the efficacy of the combination while antagonistic interactions reduce the efficacy (Golan et al., 2011).

By assessing the interaction between two samples in combination, it allows one to determine what effects a combined treatment will have in terms of reducing potential adverse effects.

Kojic acid is known to produce contact dermatitis (swelling, reddened and itchy patches of the skin) due to prolonged use (García-Gavín et al., 2010). Kojic acid accumulates overtime and causes the allergic reaction known as contact dermatitis in susceptible individuals. By combining the plant sample (*M. longufolia*) with kojic acid it will reduce the amount of kojic acid required for each treatment while still being effective due to the additive interaction between the two samples. A lower concentration of kojic acid results in a less accumulation over time which ultimately leads to a reduced appearance of adverse effects and contact dermatitis in susceptible individuals.

6.5. Conclusion

From the results, it can be concluded that the dry ethanol extract of *M. longifolia* has the ability to inhibit tyrosinase activity with an IC_{50} of 53.63 ± 1.350. Although further studies should be done to assess the exact chemical constituents present in the South African variety of *M. longifolia* and whether it differs from those described in published work from other countries. This variation study would help in identifying the active constituents that are responsible for inhibiting tyrosinase and their possible mechanism of action. However, with the current results, it can be suggested that the reason for the activity of *M. longifolia* against tyrosinase is due to its high phenolic and flavonoid content and according to literature these compound classes interact with the enzyme in a variety of ways ultimately leading to its inhibition.

Combinational studies also suggest that, in combination *M. longifolia* and kojic acid have an additive effect ($\sum$=0.980) which allows one to reduce the amount of kojic acid required in current treatments ultimately reducing the risk of adverse effects accumulating over time while still being an effective treatment for skin hyperpigmentation.

7. Efficacy of plant material against bacterium associated with acne

7.1. Introduction

One of the most common human diseases of the skin is acne vulgaris caused by *Propionibacterium acnes*, effecting 85% of teenagers and 11% of adults (White, 1998; Fitz-Gibbon et al., 2013). Acne is only found in humans and is a condition that uniquely affects the pilosebaceous unit (hair follicles) of the face, back and chest which are associated with oil glands (Williams et al., 2012). It can regress spontaneously after puberty however, factors that promote its development include, increased sebum production, ductal cornification (open and closed comedones), alterations in the microbial flora and inflammation (Jappe, 2003; Bhatia & Maisonneuve, 2004; Degitz et al., 2007). These factors cause the appearance of excessive grease on the skin (seborrhoea), non-inflammatory lesions (open and closed comedones), inflammatory lesions (papules and pustules) and scarring that can range in severity (Williams et al., 2012).

7.1.1. Current treatments

Systemic antibiotics administered for the treatment of acne vulgaris have both antimicrobial and anti-inflammatory properties as they have the ability to reduce the population of *P. acnes* within follicles resulting in the inhibition of bacterial-induced inflammatory cytokine production (Haider & Shaw, 2004).

One of the most commonly prescribed and used antibiotic in the treatment for acne vulgaris is tetracycline. Tetracycline reduces the leukocyte chemotaxis as well as bacterial lipase activity (Haider & Shaw, 2004). Tetracyclines are effective antibiotics as their mechanism of action primarily relies on the compound binding to the bacterial ribosomes, preventing the possibility of the amino acyl-tRNA from binding to the acceptor site, inevitably inhibiting protein synthesis (Griffon et al., 2011). This ultimately reduces the population of *P. acnes* and decreases the production of sebum free fatty acids and extracellular lipases (Sapadin & Fleischmajer, 2006). However, the emergence of resistant strains of *P. acnes* has resulted in tetracycline treatments ineffective.

7.2. Aim

To determine the antibacterial activity of the selected indigenous South African aquatic plants against *Propionibacterium acnes*.

7.3. Materials and methods

7.3.1. Materials

Analytical grade chemicals were purchased from Merck (Pty) Ltd and Sigma-Aldrich (Johannesburg, SA). Materials required for the growth of the bacteria including mouse brain and heart infused (BHI) agar and broth as well as anaerocult jars and anaerocult A strips were purchased from Merck (Pty) Ltd. The bacterial strain, *Propionibacterium acnes* (ATCC 6919) was purchased from Anatech Analytical Technologies (Johannesburg, SA). The cell viability agent, PrestoBlue was purchased from Life Technologies (Johannesburg, SA).

7.3.2. Methods

7.3.2.1. Antimicrobial assay

The antibacterial activity of the five selected aquatic plant ethanol extracts was investigated using the microdilution assay described by Eloff (1998) with slight modifications. Pure cultures of *Propionibacterium acnes* (ATCC 6919) were obtained by streaking a Kwik-Stick on sterile brain and heart infusion (BHI) agar and incubating at 37°C. Subcultures were made 72 hours prior to experiment. The selected plant extracts were dissolved in 10% DMSO (dimethyl sulfoxide) to a concentration of 2 mg/ml. Seven-fold serial dilutions of 100 µl of each plant extract were prepared in sterile 96-well microtitre plates in triplicates. *P. acnes* subcultures were then inoculated in BHI broth to a density of $1.5x10^8$ colony forming units (CFU) per ml (CFU/ml) which corresponds to a 0.5 McFarland Standard. Inoculated BHI broth containing the bacterial suspension (100 µl) was added to the plates. Positive and negative controls included tetracycline (0.2 mg/ml) and 10% DMSO, respectively. After 72

hour incubation at 37°C in anaerobic conditions, 20 µl PrestoBlue was added. After 1 hour the MIC was determined by observing a colour change in the growth indicator.

7.4. Results and discussion

7.4.1. Results

The antibacterial activity of the ethanol extracts of the five aquatic plant extracts were recorded as respective minimum inhibitory concentration (MIC) values (Table 6).

Table 6: MIC (µg/ml) of the selected aquatic plant extracts against *P. acnes*

Sample	MIC (µg/ml)
Commelina benghalensis (L.)	>500
Equisetum ramosissimum Desf.	>500
Mentha longifolia (L.) Hudson	>500
Typha capensis (Rohrb.) N.E.Br	250
Zantedeschia aethiopica (L.) Spreng	>500
Tetracycline	3.13

PrestoBlue is a cell viability agent which is reduced in the presence of living cells from resazurin to resorufin. This reduction causes the colour change from blue (resazurin) to pink (resorufin). The pink fluorescent compound can then be qualitatively measured to determine the viability of the cells (Lall et al., 2013). In Figure 18.B, the colour of viable cells (*P. acnes*) compared to that of an absence of viable cells (broth) can be clearly observed. Using PrestoBlue, the MIC of a sample can then be determined as the lowest concentration at which no colour change is observed (Figure 18.A).

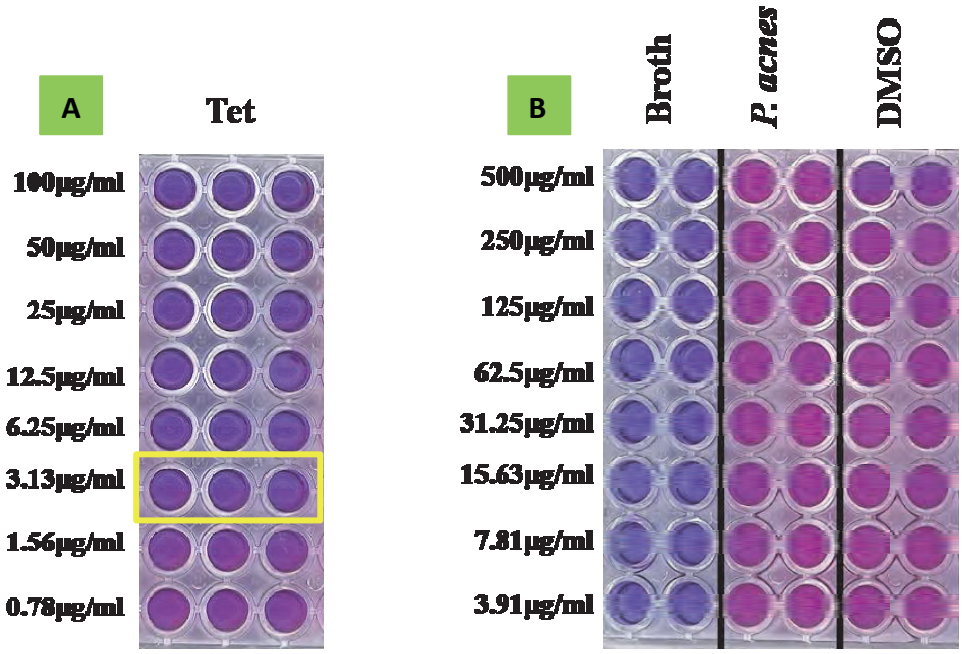


Figure 18: The observable MIC value (yellow block) after addition of PrestoBlue for the control plates. (A) Tet: (Tetracycline) positive control. (B) DMSO (dimethyl sulfoxide): negative control; *P. acnes* (*Propionibacterium acnes*).

The controls (broth, *P. acnes*, DMSO) showed that the experimental procedure was carried out correctly without contamination. The blue colour in the well labelled as broth indicated that there was no contamination in the BHI broth used during the experimental procedure. Pink in both the *P. acnes* and DMSO wells indicates that the bacteria grew and that the solvent used to dissolve the plant extract had no inhibitory activity against the proliferation of the bacteria, respectively.

The only aquatic plant extract which showed inhibition after the addition of PrestoBlue was *Typha capensis* (Figure 19). When compared to the activity of tetracycline (positive control), *T. capensis* was not a strong inhibitor of *P. acnes*, with an MIC of 250 µg/ml. The ethanol extracts of the other aquatic plant species however, were not effective. The pink colour in all wells showed that *Commelina benghalensis*, *Equisetum ramosissimum*, *Mentha longifolia* and *Zantedeschia aethipica* had no significant antibacterial activity against *P. acnes* at the tested concentrations.

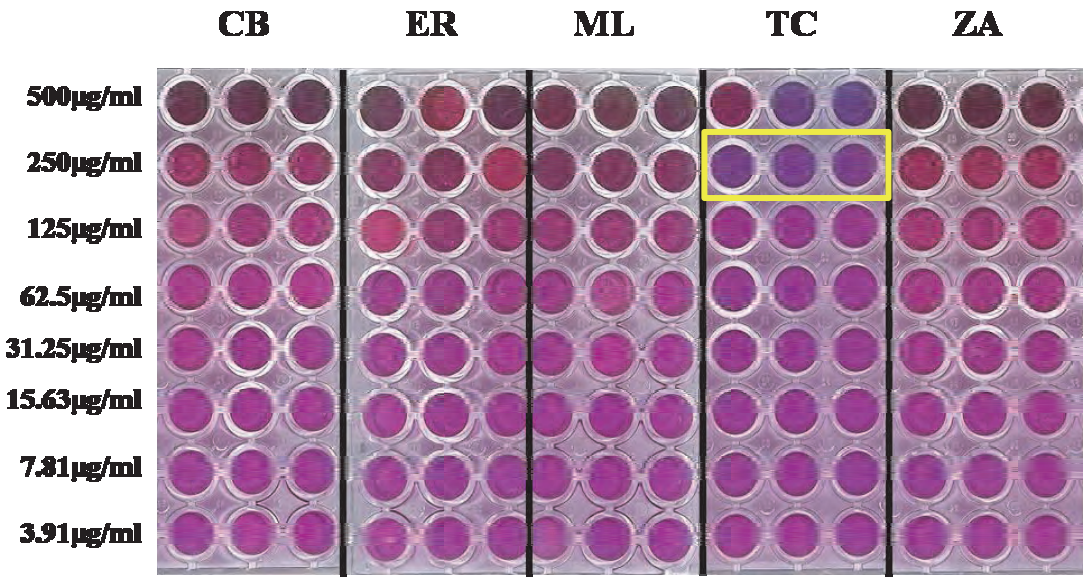


Figure 19: The observable MIC value (yellow block) after addition of PrestoBlue to the selected ethanol plant extracts. CB: *Commelina benghalensis*; ER: *Equisetum ramosissimum*; ML: *Mentha longifolia*; TC: *Typha capensis*; ZA: *Zantedeschia aethiopica*.

7.4.2. Discussion

T. capensis belongs to the Typhaceae family. According to a previous study, the methanolic extract of the rhizomes of *Typha latifolia* showed antibacterial activity against another skin bacterium, *Staphylococcus aerus* with an MIC of 500 µg/ml (Shukla & Mishra, 2013). The extract of the previous study is comparable to the present study as methanol extracts compounds of a similar polarity as ethanol. According to Londonkar et al. (2013), polar solvents (including ethanol and methanol) are able to extract more phytochemicals than the non-polar solvents. Extractable phytochemical constituents which can be obtained from polar solvents include tannins, flavonoids, alkaloids, phenols, steroids, saponins and aromatic compounds all of which have therapeutic value (Varghese et al., 2009; Londonkar et al., 2013). Antibacterial studies conducted on the methanolic extracts of *T. angustifolia* suggested that the ability for the plant genus to inhibit the growth of certain bacteria may be due to the presence of polar secondary metabolites (Varghese et al., 2009).

Although very few studies support the scientific validation of the medicinal properties of *T. capensis*, a recent study by Masoko et al. (2008) specifically investigated the biological activities of South African *T. capensis* varieties. Various solvents ranging in polarity were

used to make extracts of both the leaves and rhizomes of *T. capensis*. A preliminary phytochemical study revealed that both polar and non-polar components were present in each extract and the methanolic extract of the rhizomes had a better average activity against all tested bacteria (*Staphylococcus aerus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*) with an average MIC of 210 µg/ml (Masoko et al., 2008). This average MIC correlates with the results obtained in the present study of 250 µg/ml, however the same bacteria was not used in both studies.

The full phytochemical analysis of *T. capensis* is yet to be discovered but previous studies have reported that the rhizomes of a South African *T. capensis* yielded the novel compounds, typhaphthalide (benzyl phthalide) and typharin (isocoumarin), among flavones, phenolics and long chain hydrocarbons (Shode et al., 2002). The significance of this finding is that the two novel compounds typhaphthalide and typharin are classified as phenolic compounds. The extraction technique used in the present study utilised the polar solvent, ethanol, which has the ability to extract both polar and non-polar constituents present in plants. The ability for it to extract the novel and unique polar substances from the rhizomes of *T. capensis* could be why this was the only aquatic plant which was able to inhibit the growth of *P. acnes*. These two compounds are only found in *Typha* species and not in the other tested plant species, including *Commelina, Equisetum, Mentha* and *Zantedeschia* species. The compound, typhaphthalide is a phthalide, a naturally occurring class of lactones found within certain plant families including Typhaceae. Pthalides are of medicinal importance as they are the building blocks for a number of biologically active compounds. The backbone and overall structure of pthalides are used as an intermediate in the synthesis of tetracyclic antibiotics (Purohit Nalini & Poonam, 2012). Due to this correlation between the known class of compounds, the typhaphthalides, which are likely present within the polar extracts of *T. capensis* it can be suggested that the activity of *T. capensis* as an inhibitor of *P. acnes* proliferation, could be related to the presence of these compounds.

The results of previous studies testing the ability of *Typha* species to inhibit bacterial growth can be comparable to the results obtained in the present study. *S. aerus* and *P. acnes* are both Gram-positive skin colonising bacteria which are found predominantly within the sebaceous glands of the skin (Grice et al., 2009). The results of previous studies show that *Typha* species were able to inhibit the growth of *S. aureus* at an MIC of 500 µg/ml while the present study suggests that *T. capensis* was able to inhibit the growth of *P. acnes* at 250 µg/ml. It is well known that Gram-positive bacteria are significantly more susceptible to plant extracts

than Gram-negative bacteria (Masoko et al., 2008). This difference in susceptibility helps *T. capensis* to inhibit the Gram-positive *P. acnes* as Gram-negative bacteria make the penetration of hydrophobic substances nearly impossible due to the presence of a hydrophilic outer membrane which protects the target cell membrane from external substances (Al-Bayati, 2009). Since P. *acnes* is a Gram-positive bacteria it lacks this hydrophilic outer membrane leaving the target cells easily accessible to plant extracts.

7.5. Conclusion

From the results it can be seen that only one ethanol extract of an indigenous South African aquatic plant, *Typha capensis*, showed activity against the proliferation of *Propionbacterium acnes* (MIC = 250 μg/ml). Although further studies need to be considered to fully explore the antibacterial compounds that may be present in the rhizomes of *T. capensis*, literature suggests that one of the two unique phenolic compounds may have antibacterial activity. Typhapthalides are a type of pthalides exclusively found in *Typha* species. The structure of pthalides forms the backbone for the synthesis of medicinally important antibiotics including tetracycline. Due to the similarities between these structures, this study proposes that the ability of *T. capensis* to inhibit the growth of *P. acnes* is due to the presence of a structurally similar compound to that of tetracycline. The resulting MIC value is however higher than that of tetracycline, as the compound may be present in lower quantities within the rhizome.

8. Efficacy of plant material against oral pathogens

8.1. Introduction

Periodontal diseases are included in the list of major health problems that humans face, with dental caries and periodontitis being among the most important and preventable infectious diseases (Nguyen & Martin, 2008). Oral care influences the general quality of life and poor oral health can be linked to more serious conditions including coronary heart diseases (Dorn et al., 1999). The impact that oral care has on a person has indicated the need for treatments, however, with the increased resistance of the bacteria associated with the disease to antibiotics and the adverse effects that some antibacterial agents have in developing countries there is a need to develop alternative preventative options and treatments that are safe, effective and economical for everyday use. Many agents are commercially available but their side effects include vomiting, diarrhoea and staining of teeth, thus the search for alternative products continues and the discovery of natural phytochemicals isolated from plants used as traditional medicines has been greatly considered (Palombo, 2011).

Streptococcus mutans is a gram-positive, anaerobic bacterium that lives within the mouth and has the ability to metabolise a variety of carbohydrates resulting in an acidic environment that becomes the leading cause of tooth decay and dental caries (Figure 20) (Palombo, 2011).

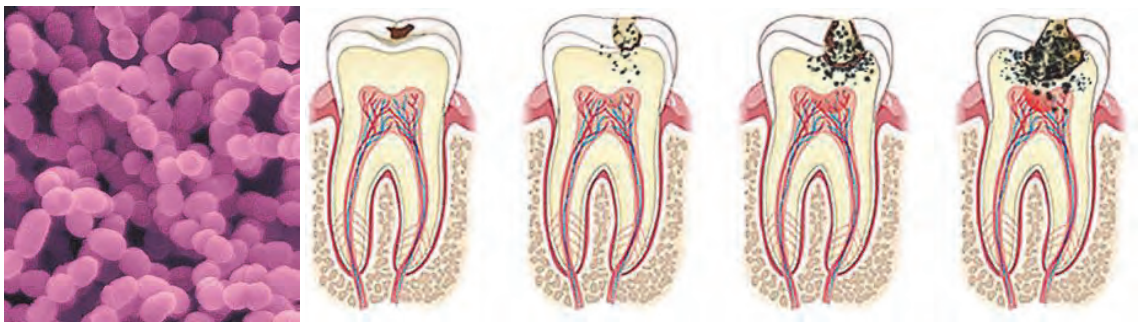


Figure 20: *Streptococcus mutans*, a Gram-positive oral pathogen and its role in breaking down tooth enamel leading to dental caries and tooth decay (Kunkel, 2006; Stop Cavity, 2015)

Prevotella intermedia is a black pigmented, anaerobic, Gram-negative oral pathogen (Dorn et al., 1998). This pathogen is found living within the periodontal pockets in between human

teeth where they co-exist with other oral microorganisms forming part of the human oral microbiota (Marcotte & Lavoie, 1998) however, when *P. intermedia* is found at higher bacterial populations than the other oral bacteria, periodontitis and acute necrotising ulcerative gingivitis are the symptomatic diseases associated (Figure 21) (Haffajee & Socransky, 1994).

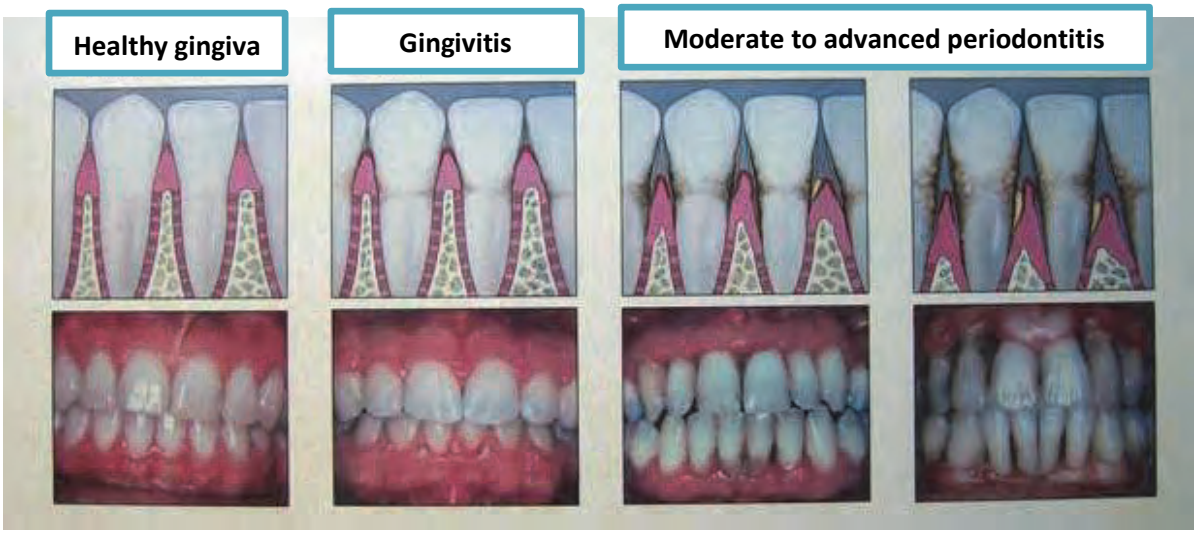


Figure 21: Different stages of periodontitis (Morley, 2012)

8.1.1. Current treatments

Fluoride has been proven to be one of the most effective measures against dental caries and the induction of fluoride into water systems has reduced the amount of dental caries cases over the years, however, the impact that fluoride has on human health poses a great risk in its use for the treatment. According to Bronckers et al. (2009), the excessive impact of fluoride during tooth development (in youngsters) causes enamel fluorosis. Enamel fluorsis disturbs the developmental process of teeth and results in enamel that is porous.

A range of antibiotics are used in the treatment of periodontitis including, penicillin, erythromycin, cephalosporin, clindamycin and tetracycline, however their uses are limited due to allergies, cost and resistance development (Montgomery & Kroeger, 1984).

Chorhexidine gluconate is currently used in many oral rinses available on the commercial market due to its antibacterial properties. It is a broad spectrum anti-microbial agent that is generally more effective than other anti-microbial compounds sold to patients for the treatment of various periodontal diseases (Henley-Smith et al., 2013).

Although effective in most cases, chlorhexidine gluconate has been reported to be inactivated by food and saliva. Adverse effects of the use of chlorhexidine gluconate include alterations of taste, mucosal irritation and stained teeth and tongue (Henley-Smith et al., 2013).

8.2. Aim

To investigate the antibacterial activity of the selected indigenous South African aquatic plant extracts against *Streptococcus mutans* and *Prevotella intermedia*, two bacteria involved in the onset of periodontal diseases.

To determine if the efficacy of the plant extracts can be increased in combination with peppermint essential oil.

8.3. Materials and method

8.3.1. Materials

Analytical grade chemicals were purchased from Merck (Pty) Ltd and Sigma-Aldrich (Johannesburg, SA). Materials required for the growth of the bacteria including mouse brain and heart infused agar and tryptone soy agar and their respective broth, anaerocult jars and anaerocult A strips were purchased from Merck (Pty) Ltd. The bacterial strains, *Streptococcus mutans* (ATCC 25175) and *Prevotella intemedia* (ATCC 25611) were purchased from Anatech Analytical Technologies (Johannesburg, SA). The cell viability agent, PrestoBlue was purchased from Life Technologies (Johannesburg, SA).

8.3.2. Method

8.3.2.1. Antimicrobial assay

The antibacterial activity of the five selected aquatic plant ethanol extracts was investigated using the microdilution assay described by Eloff (1998) with slight modifications. Pure cultures of *Streptococcus mutans* (ATCC 25175) and *Prevotella intemedia* (ATCC 25611) were obtained by streaking a bead on sterile brain and heart infusion (BHI) agar and tryptone soy (TS) nutrient agar, respectively. Both agars were supplemented with sucrose for additional nutrients. The plates were then incubated at 37°C. Subcultures were made 48 hours prior to experiment. The selected plant extracts were dissolved in 10% dimethyl sulfoxide (DMSO) and serially diluted (100 µl) in a 96-well plate across four wells. The final concentrations of the extracts ranged from 12.5-0.10 mg/ml while chlorhexidine gluconate (CHX, positive control) ranged from 12.5-3.8x10^{-4} mg/ml. *S. mutans* subcultures were then inoculated in BHI nutrient broth while *P. intermedia* subcultures were inoculated in TS nutrient broth, both to a density of 3x10^{8} colony forming units (CFU) per ml (CFU/ml) which corresponds to a 1 McFarland Standard. Inoculated broth containing the bacterial suspension (100 µl) was added to the plates. After 24 hour incubation at 37°C in anaerobic conditions, 20 µl PrestoBlue was added. After 1 hour the MIC was determined by observing a colour change in the growth indicator, as the lowest concentration that showed no bacterial growth.

The combinational studies were performed using the same procedure as described above, however, ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) of plant extract to peppermint essential oil were added as the 100 µl sample to the first row of the 96-well plate.

8.4. Results and discussion

8.4.1. Antibacterial activity of plant extracts

8.4.1.1. Activity against *Streptococcus mutans*

The antibacterial activity of the ethanol extracts of five aquatic plant extracts and the positive control were recorded as respective minimum inhibitory concentration (MIC) values (Table 7 and 8).

Table 7: MIC (mg/ml) of the selected aquatic plants extracts against *Streptococcus mutans*

Sample	MIC (mg/ml)
Commelina benghalensis (L.)	>12.5
Equisetum ramosissimum Desf.	>12.5
Mentha longifolia (L.) Hudson	>12.5
Typha capensis (Rohrb.) N.E.Br	>12.5
Zantedeschia aethiopica (L.) Spreng	>12.5
5% Chlorhexidine gluconate	0.061

8.4.1.2. Activity against *Prevotella intermedia*

Table 8: MIC (mg/ml) of the selected aquatic plants extracts against *Prevotella intermedia*

Sample	MIC (mg/ml)
Commelina benghalensis (L.)	>12.5
Equisetum ramosissimum Desf.	>12.5
Mentha longifolia (L.) Hudson	>12.5
Typha capensis (Rohrb.) N.E.Br	>12.5
Zantedeschia aethiopica (L.) Spreng	>12.5
5% Chlorhexidine gluconate	0.40

PrestoBlue is a resazurin based cell viability agent which is converted to resorufin through the reduction of actively metabolising cells. This reduction causes the colour change from

blue (resazurin) to pink (resorufin). The pink fluorescent compound can then be qualitatively measured to determine the viability of the cells (Lall et al., 2013). In Figure 23.B, the colour of viable cells (*P. intermedia*) compared to that of an absence of viable cells (broth) can be clearly seen, using PrestoBlue. The MIC of the samples can then be determined as the lowest concentration at which no colour change is observed (Figure 22).

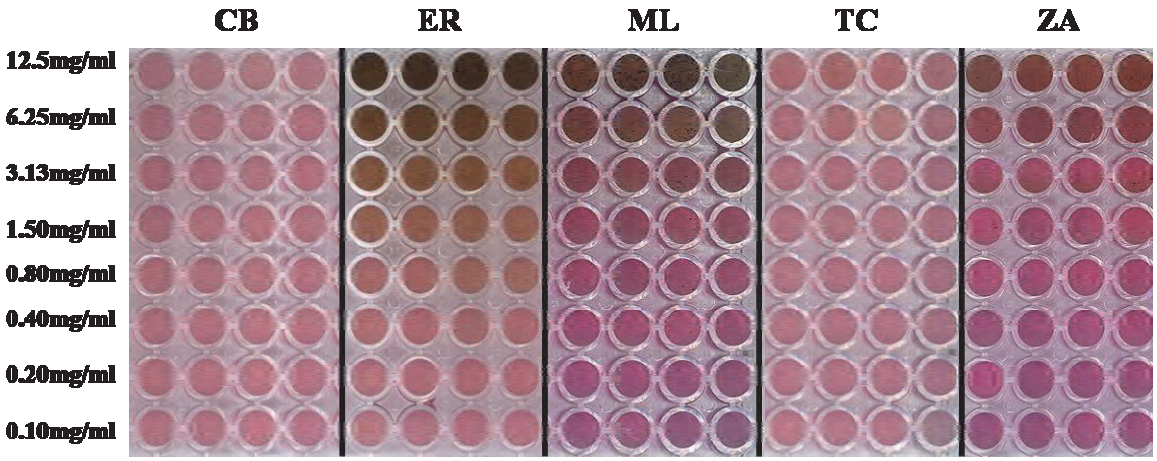


Figure 22: The observable MIC values after addition of PrestoBlue for the selected ethanol plant extracts with *P. intermedia*. CB: *Commelina benghalensis*; ER: *Equisetum ramosissimum*; ML: *Mentha longifolia*; TC: *Typha capensis*; ZA: *Zantedeschia aethiopica.*

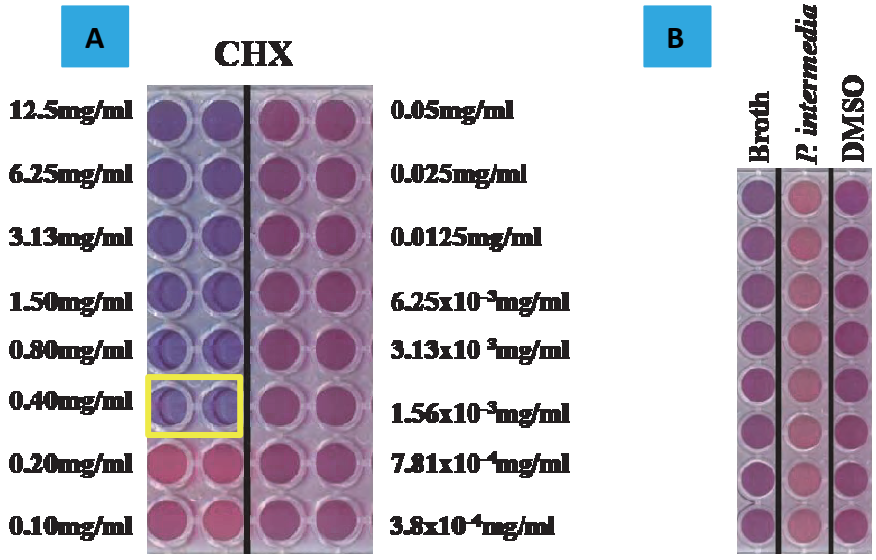


Figure 23: The observable MIC value (yellow block) after addition of PrestoBlue to the control plates for *Prevotella intermedia* containing (A) CHX: 5% Chlorhexidine gluconate (positive control); (B) Broth: Tryptone soy broth; DMSO: 10% dimethyl sulfoxide (negative control).

The controls (broth, *P. intermedia*, DMSO) showed that the experimental procedure was carried out correctly without contamination (Figure 23.B). The blue colour in wells labelled as broth indicated that there was no contamination in the broth used during the experimental procedure. Pink in both the *P. intermedia* and DMSO wells indicates that the bacteria grew and that the solvent used to dissolve the plant extract had no inhibitory activity to the proliferation of the bacteria, respectively.

The same observations were made for the *S. mutans* antibacterial assay after PrestoBlue was added.

8.4.1.3. Discussion

At the tested concentrations the plant extracts did not exhibit any significant antibacterial activity against the oral pathogens (Figure 22). According to literature, both *S. mutans* and *P. intermedia* are fairly resistant bacteria, thus the tested concentrations of plant extracts was not able to exhibit any antibacterial effect against these strains (Palombo, 2011). In publications of the activity of South African plants against oral pathogens, MICs were recorded at concentrations greater than 25 mg/ml (More et al., 2008).

Previous antimicrobial experiments, investigating the activity of *M. longifolia* against *S. mutans* used the microdilution method. The results obtained showed that the essential oil extract of *M. longifolia* exhibited an MIC of 15.6 µg/ml which is a significant value (Al-Bayati, 2009). The difference in activity observed between the present study and the aforementioned study is due to the different type of *M. longifolia* plant extract tested. In the present study dry ethanolic extracts of the whole plant were used however, in previous studies only the essential oils of the leaves were tested. Essential oils contain a much more concentrated supply of different groups of complex phytochemicals that may exhibit antibacterial activity when compared with an ethanol extract (Božović et al., 2015).

Antifungal studies on the efficacy of traditionally used South African plants have been performed to inhibit the growth of the oral fungal pathogen *Candida albicans* (Motsei et al., 2003). When comparing the results obtained in the two studies, one needs to consider the type of pathogens investigated. The pathogens are from different classes; with the present study focussing on bacterial pathogens while the previous study focused on fungal (yeast) pathogens. Both pathogens infect the oral cavity; *S. mutans* and *P. intermedia* are natural

7colonisers while *C. albicans* affects patients with suppressed immune systems due to HIV and AIDS as it is an opportunistic pathogen (Calderone & Fonzi, 2001). *C. albicans* like *S. mutans*, colonises the root canal eventually breaking it down. Both studies focussed on investigating the antimicrobial activity of South African plant extracts towards oral pathogens, thus making them comparable. Only one of the aquatic plants tested in the present study was evaluated for its antifungal activity, *Zantedeschia aethiopica*. Ethanolic extracts of the leaves of *Z. aethiopica* were used in both studies. The results concluded that *Z. aethiopica* had an MIC greater than 25 mg/ml (Motsei et al., 2003). This suggests that *Z. aethipica* did not exhibit any antifungal activity at the tested concentrations. The significance of this result indicates that oral pathogens are relatively difficult to inhibit, whether they are bacterial or fungal. The concentrations, at which the antimicrobial activity was tested, were not high enough to inhibit the growth and proliferation of these resistant oral pathogens. The persistence of these oral pathogens which colonise the root canals could be explained by their ability to produce biofilms and evade dental hygiene apparatus as they colonise within and between teeth (Botelho et al., 2007).

Commilina benghalensis, *Equisetum ramosissimum* and *Typha capensis* however, have not been reported in literature for any activity against oral pathogens at the time of this present study.

8.4.2. Combinational studies

The antibacterial results of the plant extracts in combination with peppermint essential oil are illustrated below for both *Streptococcus mutans* (Figure 24) and *Prevotella intermedia* (Figure 25).

8.4.2.1. Activity against *Streptococcus mutans*

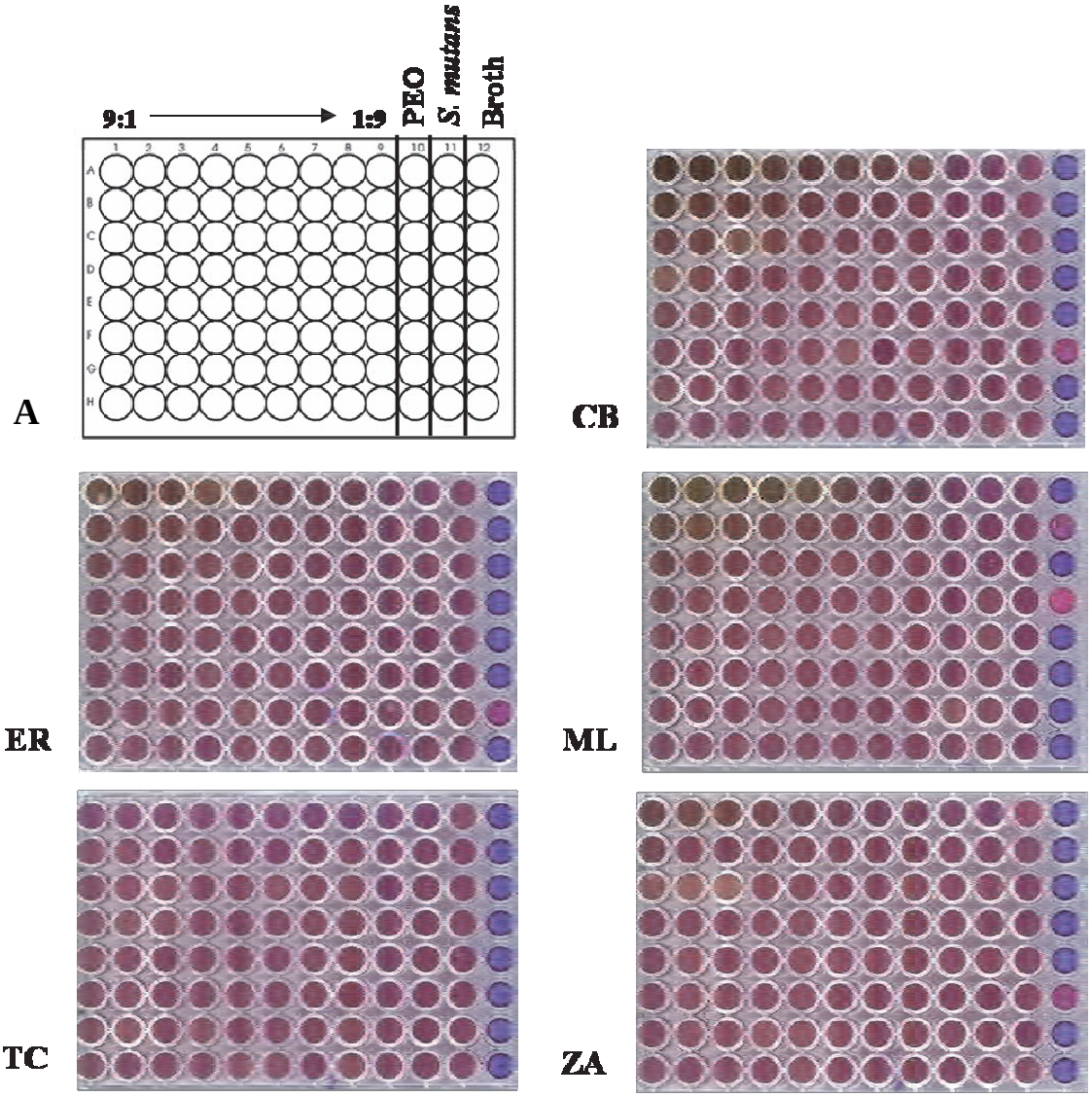


Figure 24: The resulting plates containing *Streptococcus mutans* after PrestoBlue was added. (A) Indicates the layout of the plate. The combinational studies included the interaction between the plant extracts (CB: *Commelina benghalensis*; ER: *Equisetum ramosissimum*; ML: *Mentha longifolia*; TC: *Typha capensis*; ZA: *Zantedeschia aethiopica*) and peppermint essential oil in varying ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9). The controls include, Broth: brain heart infusion (BHI) broth; Bacteria: *S. mutans*; PEO: peppermint essential oil.

8.4.2.2. Activity against *Prevotella intermedia*

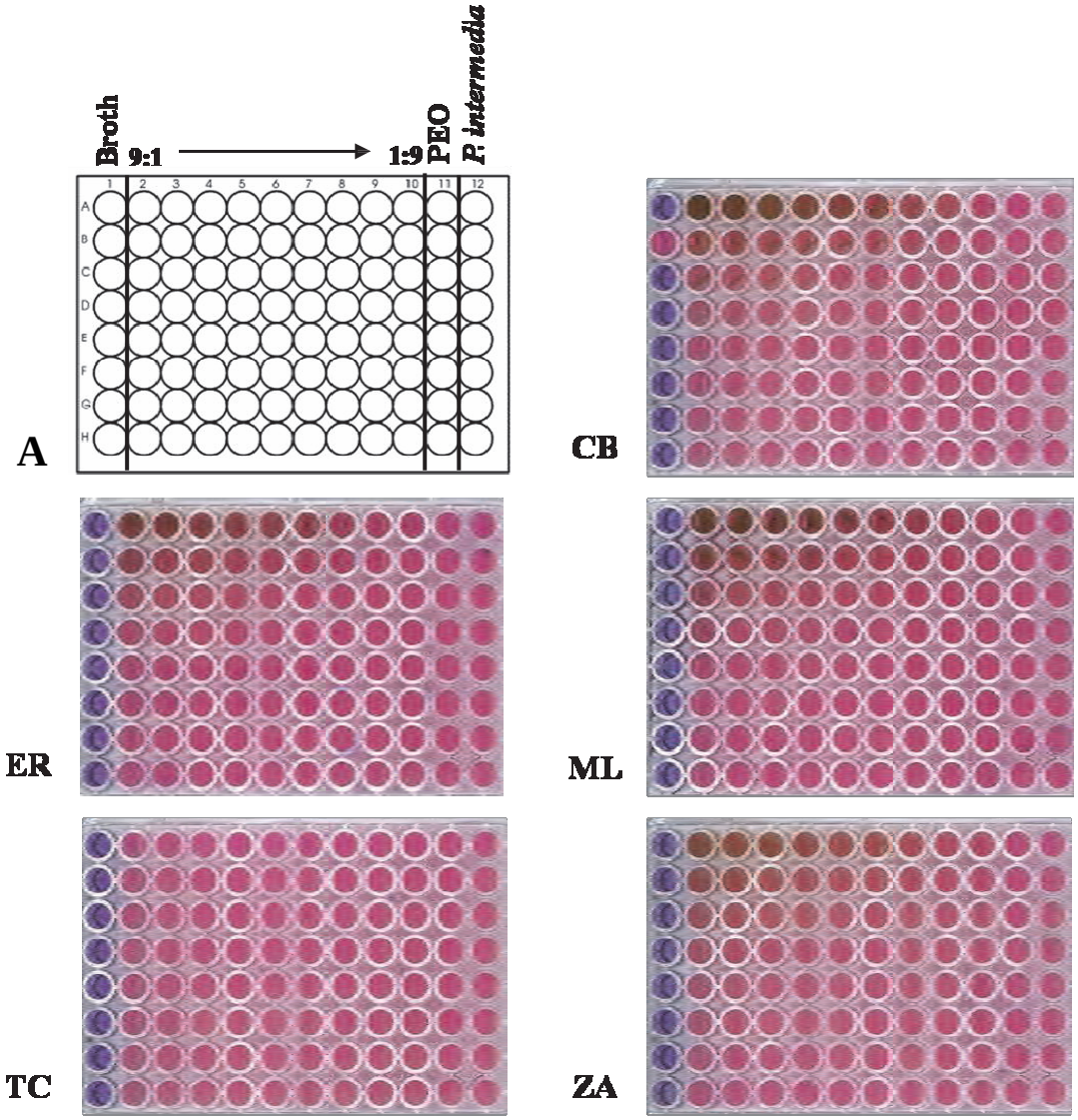


Figure 25: The resulting plates containing *Prevotella intermedia* after PrestoBlue was added. (A) Indicates the layout of the plate. The combinational studies included the interaction between the plant extracts (CB: *Commelina benghalensis*; ER: *Equisetum ramosissimum*; ML: *Mentha longifolia*; TC: *Typha capensis*; ZA: *Zantedeschia aethiopica*) and peppermint essential oil in varying ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9). The controls include, Broth: Tryptone soy (TS) broth; PEO: peppermint essential oil; Bacteria: *P. intermedia*

The results obtained for both antibacterial assays against each bacterium suggest that the experimental reagents had no contamination as the wells containing broth remained blue. The colour change of PrestoBlue to pink in the wells containing the bacterial strain indicates that the bacterial cells grew over the period of 48 hours. The efficiency of the plant extracts in combination with peppermint essential oil in varying ratios showed no inhibitory activity

against *Prevotella intermedia* as all the wells at all tested concentrations and ratios turned pink (indicating no inhibition due to the presence of living cells) (Figure 25). In combination with peppermint essential oil, the plant extracts showed some inhibitory activity against the growth of *Streptococcus mutans* (Figure 24). Although the MIC cannot be determined as the colour change is not as obvious, the purple intermediate colour in majority of the wells indicate that peppermint essential oil may have some inhibitory effect on the growth of *S. mutans*.

8.4.2.3. Discussion

According to the results obtained in the present study it can be noted that the combinational studies of the plant extracts together with the peppermint essential oil did not show any significant interactions. The ability for some of the higher concentrations of peppermint essential oil used in combination with the plant extracts to show slight inhibitory effects on the growth of *S. mutans* is due to the activity of peppermint essential oil on its own and not the tested combination. The reason for this is that the MIC cannot be easily determined; instead the lack of pink at all tested concentrations indicates that an added substance has some inhibitory activity on the growth of the bacteria. However, this interaction is absent in the combination studies performed on *P. intermedia* with peppermint essential oil.

According to previous studies on the antibacterial activity of essential oils, it has been reported that essential oils especially from *Mentha* species are a great source of novel antimicrobial compounds especially against those bacterial species classified as human pathogens. Like all essential oils, the extract of peppermint essential oil from *M. piperita* is obtained through distillation. This method of extraction allows essential oils which are known for their complex mix of compounds (including, terpenes, terpenoids, phenols, aliphatic and aromatic compounds) to be obtained (Božović et al., 2015). These compounds work in synergy with each other within the hydrophobic essential oil product giving a wide spectrum of pharmacological importance, including antibacterial effects.

Due to the hydrophobic nature of essential oils, it sometimes causes many problems when conducting an antibacterial microdilution assay. A hydrophobic substance resists being dissolved in water. In the present study, the peppermint essential oil was dissolved in acetone to reduce the hydrophobicity of the sample, however, the additional distilled water added to the peppermint essential oil and acetone mixture reduced the ability for the essential oil to be

fully dissolved in the 96-well plate (Božović et al., 2015). The water/acetone mixture reduces the acetone percentage, as a 100% acetone mixture could inhibit the growth of the bacteria. The inability to fully dissolve the test samples may be the reason for which the results of the combinational studies on *S. mutans* were obtained. The peppermint essential oil was only slightly effective in inhibiting the growth of *S. mutans* due to its poor solubility in the solvents providing a lower concentration of the peppermint essential oil to be available for antibacterial activity. Hence the reason for an overall slight inhibition across all plates. However, if a higher concentration was used, a better inhibition could have been observed.

The difference in activity of peppermint essential oil to inhibit each oral pathogen may be due to the morphological differences between *S. mutans* and *P. intermedia*. *S. mutans* is a Gram-positive bacterium while *P. intermedia* is a Gram-negative bacterium. Numerous studies investigating the ability of essential oils to exert an antibacterial effect on various types of bacteria have suggested that the structural differences between Gram-positive and Gram-negative bacteria may be the reason why some bacteria are more susceptible to essential oils than others.

Gram-negative bacteria are generally less affected by essential oils. Although they have a much thinner peptidoglycan layer compared to that of Gram-positive bacteria, they have an additional outer (lipopolysaccharide) membrane which is not present in Gram-positive bacteria (Figure 26) (Madigan et al., 2012). This additional outer layer is hydrophobic and surrounds the cell wall functioning as a preventative barrier to the diffusion of hydrophobic substances into the cytoplasm (Al-Bayati, 2009; Božović et al., 2015).

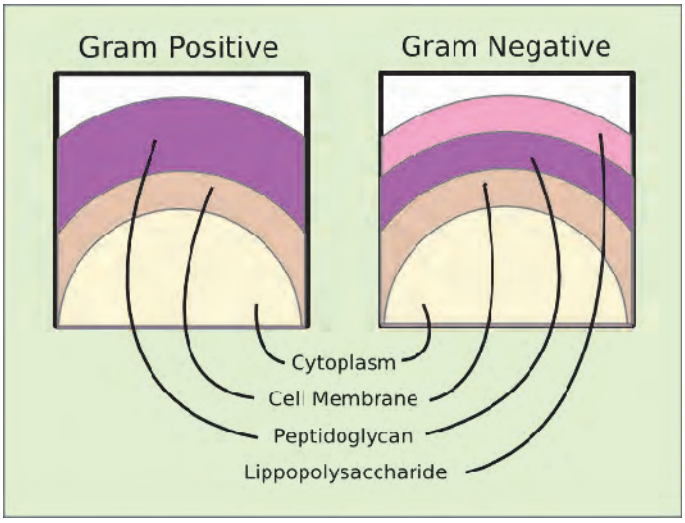


Figure 26: The differences in the cell wall composition of Gram-positive and Gram-negative bacteria (Ventenilla, 2014)

The presence of this additional layer in the Gram-negative, *P. intermedia*, prevents the diffusion of the hydrophobic peppermint essential oil into the target cell, ultimately protecting the bacteria from the antibacterial properties of peppermint essential oil. The ability for Gram-negative bacteria to resist the diffusion of essential oils into its cell has also been reported by Mann et al., (2000). *Pseudomonas aeruginosa* like *Prevotella intermedia* is a Gram-negative bacterium. Previous studies revealed that *Pseudomonas aeruginosa* was tolerant to the antibacterial activity of tea tree oil (*Melaleuca alternifolia*) (Carson & Riley, 1995). However, only recently has the ability for *Pseudomonas aeruginosa* to resist diffusion of hydrophobic tea tree oil components been demonstrated to be due to the presence and contribution of the additional outer membrane unique to Gram-negative bacteria. Other mechanisms have been postulated but evidence has suggested that such mechanisms are inadequate to ensure the survival of the cell when the lipopolysaccharide (outer membrane) barrier was removed (Mann et al., 2000).

8.5. Conclusion

From the results it can be concluded that the ethanol extracts of the five indigenous South African aquatic plants (*Commelina benghalensis*, *Equisetum ramossisimum*, *Mentha longifolia*, *Typha capensis* and *Zantedeschia aethiopica*) are not effective inhibitors of the proliferation of the tested oral pathogens (*Streptococcus mutans* and *Prevotella intermedia*). These bacterial species are evolved to produce biofilms to assist with creating the ideal anaerobic environment to allow their rapid proliferation within the oral cavity. The presence of these biofilms makes the bacteria extremely difficult to become susceptible to antibacterial agents when tested at lower concentrations.

Combinational studies suggest that in combination with peppermint essential oil the plant extracts did not improve their ability to inhibit the oral pathogens. It was however, noted that any slight inhibitory activity observed on the assays containing *S. mutans* were due to the ability for essential oils to penetrate the cell wall of Gram-positive bacteria. This observation was not seen in *P. intermedia* assays as it is a Gram-negative bacterium. The presence of an additional lipopolysaccharide layer prevents the diffusion of the hydrophobic antibacterial agents of peppermint essential oil into the cell.

9. Traditional healer practitioner workshop

A workshop was held in August at the University of Pretoria for the four traditional healers (and members of their practice or community) which Professor Lall and her students work with quite closely (Figure 28). The purpose of this workshop was to involve the traditional healer practitioners (THPs) and their community in the scientific research that is used to evaluate their plant-based healing formulas as well as expose them to the different research areas of cytotoxicity, antibacterial assays, plant extraction techniques and capsule preparation (Figure 27).

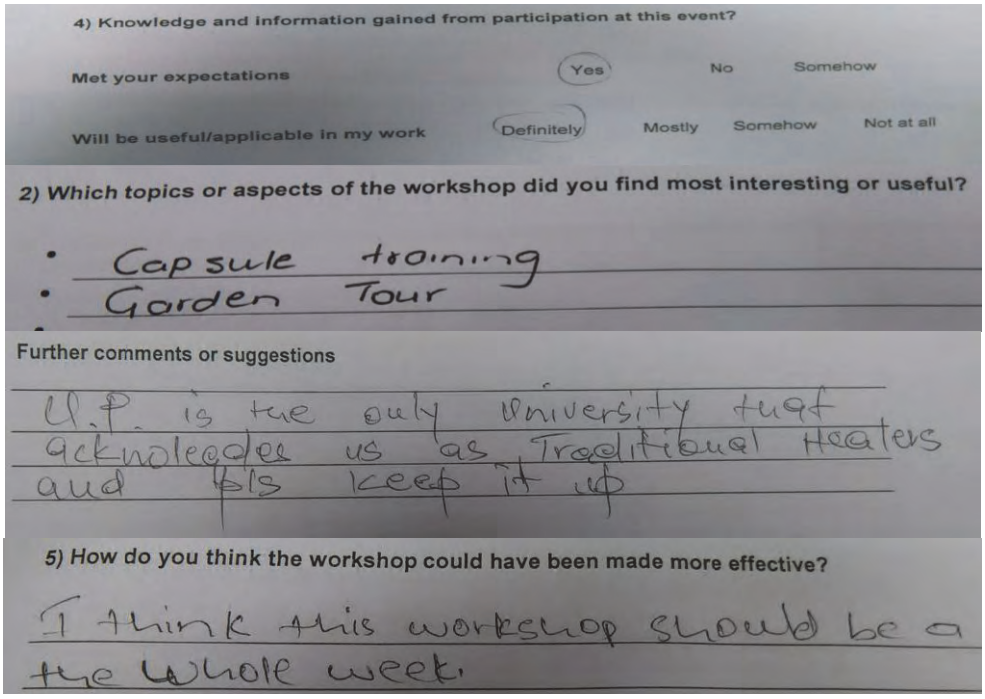
Figure 27: The techniques demonstrated and practiced at the THP workshop A) cell culture B) capsule preparation C) extract preparation D) antibacterial assay

The specific skills were chosen for the program to give the traditional healers an understanding of how the laboratory works and offer them new techniques that they could use in their traditional healing practices every day. The two day workshop was filled with many questions, sharing of knowledge and skills, and lots of fun.

Figure 28: The traditional healers and their members of the community which took part in the two day workshop

The workshop ended with a survey on the THPs opinions of the two days. Generally the answers received from the survey, showed that the workshop was a success. The THPs enjoyed all aspects of the demonstrations and practicals. They preferred the practical work (capsule making) over the demonstrations (antibacterial assays) as they enjoyed the hands on experience, however they learnt just as much from the demonstrations. The THPs were also very impressed that the University of Pretoria is the only institution to recognise the role that they play in society and communities.

Below are some scanned copies of the answers from the THP survey:



10.General conclusions and future prospects

Expanding the search for medicinal plant resources outside terrestrial ecosystems has provided some promise for using these plants as alternative treatments for common human ailments. A summary of the results obtained in the present study depicts how two of the aquatic plants have the potential to be used as treatment for skin hyperpigmentation and acne.

Table 9: Summary of results obtained, depicting the efficacy of the plant extracts against tested bacteria and enzymes

Plant extract	Tyrosinase IC_{50} (µg/ml)	Acne MIC (µg/ml)	*S. mutans* MIC (mg/ml)	*P. intermedia* MIC (mg/ml)
Commelina benghalensis (L.)	>200	>500	>25	>25
Equisetum ramosissimum Desf.	>200	>500	>25	>25
Mentha longifolia (L.) Hudson	53.630 ± 1.350	>500	>25	>25
Typha capensis (Rohrb.) N.E.Br	>200	250	>25	>25
Zantedeschia aethiopica (L.) Spreng	>200	>500	>25	>25
Positive control	Kojic acid 3.12 ± 0.006	Tetracycline 3.13	Chlorhexidine 0.061	Chlorhexidine 0.40

From the present study it can be concluded that there is potential for using aquatic plants which are indigenous to South Africa for pharmaceutical and cosmeceutical uses. Two of the selected plant species, *Typha capensis* and *Mentha longifolia* showed activity against *Propiobacterium acnes* (skin pathogen) with an MIC of 250 µg/ml and tyrosinase (enzyme involved in melanin production) with an IC_{50} of 53.630 µg/ml , respectively. Combinational studies of *M. longifolia* with kojic acid in a 1:1 ratio indicates that at a concentration of 100 µg/ml, *M. longifolia* had an additive effect ($\sum FIC = 0.980$) significantly reducing the IC_{50} of the two samples in combination to 2.812 ± 0.027. Using a plant sample that has an additive effect with a known drug minimises the adverse effects associated with the prolonged use of kojic acid as a reduced concentration will be required, while maintaining efficacy.

Although this study focused on a small scale screening of only a few selected aquatic species based on their traditional uses, there is much promise for expanding the search to more aquatic plant types, families and species. Future prospects include additional combinational

studies of *T. capensis* with current acne treatments (tetracycline) to assess the interaction between the two samples in combination for a more effective treatment or antibacterial agent against *P. acnes*.

The screening of more aquatic plants from various aquatic plant families and species will be considered in order to get a better understanding of which plant families may one day be used in pharmaceutical or cosmetic products. The search of medicinal aquatic plants will also expand beyond the riparian zone of plant collection and include species which are known as floating or submerged aquatic plants. Depending on their association with the water source in aquatic habitats, different classes of aquatic plants have been known to produce different types of chemical compounds which are known to be medicinal. Exploring the medicinal potential of different types of aquatic plants, could give an indication of which types produce more compounds with antibacterial or enzyme inhibitory activity.

Future work also includes, testing the activity of aquatic plants for their potential to act as antioxidants (scavenging of free radicals as possible anti-aging and cancer treatments), antibacterial agents (against *Mycobacterium tuberculosis*, the bacteria involved in the pathogenesis of tuberculosis) and cytotoxicity (evaluates the safety of the plant samples) on different cell lines for potential treatments for cancer studies. Further testing of the aquatic plants against these common human ailments would provide a broader opportunity to identify an aquatic plant that could be incorporated into a product.

Although this study has focused mainly on the beneficial effects that medicinally important aquatic plants will have on the cosmetic and pharmaceutical industry, the impact that this project has on the ecology and conservation of South African wetlands can also be an advantage. In almost every publication about wetlands, the authors emphasise the importance of wetland conservation. Ecologically and economically, the present study highlights the significant role that aquatic plants and ecosystems play in nature, something that communities often have trouble identifying. Communities are quick to replace wetlands with agricultural, urban or industrial lands, displacing the plants and reducing the natural abundance of resources. Finding medicinal uses for aquatic plants in both acne and skin hyperpigmentation treatments, provides a reason for communities to protect their wetlands and riparian ecosystems which are home to possible alternative treatment options that could drastically improve the lives of patients with these common human ailments. The cultivation

and upkeep of wetlands supplying the medicinal resources could provide many jobs for communities within the immediate area of a wetland.

Involving the community THPs in a workshop has helped identify the need of medicinally important plants within South African communities in terms of increasing collaborations between scientific research groups and traditional healers. The comments received from the survey indicated that subsequent workshops need to be organized in future. Through this project collaborations between the Water Research Commission, the University of Pretoria and the THP community will help create jobs for the cultivation and maintenance of medicinally aquatic South African plant species.

The potential of aquatic plants is very promising and this present research has only outlined a small percentage of the opportunities for the incorporation of aquatic plants into botanical based pharmaceuticals and cosmetics.

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