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Full Length Research Paper

Medicinal value of *Aptosimum albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.) to a small community living at Gochas, southern Namibia

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Traditional medicine is widely used, but its effectiveness is often questioned. Biofilm-producing bacteria and fungi are important in difficult-to-treat persistent and recurrent infections. The present study investigated the anti-biofilm properties of crude methanolic extracts from three medicinal plants used in Namibia, namely *Aptosimum albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.). Biofilm formation, inhibition and eradication were determined using microtiter plate assay. Extracts were tested against *Escherichia coli* ATCC 700928, *Staphylococcus aureus* ATCC 12600, *S. aureus* U3300, *Bacillus subtilis* ATCC 13933, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Pseudomonas aeruginosa* and *Candida albicans*. All isolates were strong biofilm producers. *A. albomarginatum* root extract moderately inhibited biofilm formation in *S. mutans* ATCC 25175 (60.0%), *E. coli* ATCC 700928 (51.6%) and *P. aeruginosa* (49.1%). *A. anthelmintica* twigs caused 58.4% biofilm inhibition in *C. albicans* and eradicated *S. aureus* U3300 biofilm by 74.8%. *D. schinzii* leaf extract inhibited *P. aeruginosa* biofilm by 67.3%, and in addition broke down *S. mutans* ATCC 25175 biofilm by 44.2%. These results validate the usefulness of the three plants as traditional medicine in some instances.

Key words: *Aptosimum albomarginatum*, *Albizia anthelmintica*, *Dicoma schinzii*, traditional medicine, anti-biofilm activity, flavonoids, saponins.

INTRODUCTION

All cultures across the globe have developed knowledge of local plants, enabling them to use these plants for medicinal purposes (Silvério and Lopes, 2012). Globally, many people still rely on such traditional medicine to remain healthy (van Wyk and Wink, 2015), because they often do not have access to modern medicine and

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antibiotic-based therapies (Silvério and Lopes, 2012).

African traditional medicine, which probably dates back to the origins of humankind, represents the most diverse of all medicinal systems, but is also the least systematized and most poorly documented of these systems. Traditional or folk medicine may be just as effective as conventional drugs, but its effectiveness is often questioned (van Wyk and Wink, 2015). Some strains of bacteria and fungi are able to form biofilms. Such biofilm producers are often responsible for difficult-to-treat persistent and recurrent infections. Hutcherson et al. (2015) define biofilms as dense, surface-attached communities of bacteria or fungi encased within a microbial-derived matrix that helps with colonization and survival. According to Mack et al. (2013) and Nazzari et al. (2014), this formation helps bacteria to withstand the host's natural immune defense mechanisms and to resist antibiotic treatment. In other words, as stated by Speziale and Geoghegan (2015), this is a survival strategy adapted by bacteria.

With drug-resistance being a never-ending problem, one should look into alternative treatment options, such as use of natural products, for example plant-derived products with antimicrobial and/or anti-biofilm activity. Such natural products may in some instances also be used as adjuvants together with antibiotics. Namibian people are using plants as natural medicine in the traditional setting to treat various illnesses, including biofilm-related infections. To rule out the possibility of placebo effects, such plants should be tested in the laboratory to validate their medicinal value.

The present study aimed to test crude methanolic extracts from three plants, namely, *Albizia albomarginatum* (Marloth and Engl.), *Albizia anthelmintica* (A. Rich Brongn.) and *Dicoma schinzii* (O. Hoffm.) for their anti-biofilm properties in seven bacterial strains and a fungus. Extracts were screened for phytochemicals that may contribute to these properties.

MATERIALS AND METHODS

Selection of traditional medicinal plants and collection of plant material

Plant material was collected from the veld at Gochas (Altitude: 1139m; GPS coordinates: 24°47'S, 18°49'E), located in the Karas Region, southern Namibia in the month of February 2015. Plants were selected based on indigenous knowledge of local people about their medicinal value in the traditional setting. Voucher specimens were prepared and submitted to the herbarium at the National Botanical Research Institute (NBRI) in Windhoek for scientific identification of the plants. The selected plants (Figure 1) as identified by the NBRI are the shrub *Aptosimum albomarginatum* (Marloth and Engl.), the tree *A. anthelmintica* (A. Rich Brongn.) and the shrub *D. schinzii* (O. Hoffm.).

Plant material/extracts used

Crude methanolic extracts were prepared at the Biomedical Research Laboratory, Biological Sciences Department at the University of Namibia. Plant parts used were *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* roots and leaves.

Preparation of plant material

A. albomarginatum roots, *A. anthelmintica* twigs, and *D. schinzii* roots and leaves were prepared for extractions in the laboratory. Plant material was washed with tap water, placed on towel paper on the benches and left to air-dry at room temperature for two weeks. Thereafter, the material was cut and crushed to finer pieces and blended to powder form using a Philips Problend 5 household blender. Blended material was sieved, weighed, put into labeled 50 ml Falcon centrifuge tubes and stored in the freezer at -20°C.

Preparation of crude extracts

To prepare crude methanolic extracts, 10 g of plant material from the different plant parts was added to 100 ml methanol. Flasks containing the extracts were parafilmed, placed in a cupboard and left to stand for three days (maceration) with occasional swirling. After three days, the extracts were gravitationally filtered through Whatman 110 mm filter papers. The extracts were rotary evaporated in round bottom flasks at reduced pressure (91mbar) and temperature (45°C) to evaporate the methanol, and to dry and concentrate them. To avoid thermal decomposition of compounds in the plant material, the temperature set for the rotary evaporator (Heidolph, Germany) did not exceed 45°C. The flasks were labeled, sealed with parafilm and kept at -86°C for a few hours. Thereafter, the frozen extracts were connected to a Christ Alpha 1-2 LD Plus freeze-dryer (Germany) for two to four days to further dry and concentrate them. Dried extracts were scraped off with a spatula, weighed and stored in labeled 50 ml centrifuge tubes, and kept at -86°C for further use. Yields of extracts were calculated using the formula used by Osungunna and Adedeji (2011):

Percentage yield = Quantity of dried extract (g) / Quantity of powdered sample (g) × 100.

Phytochemical screening for flavonoids, saponins and anthraquinones

Antimicrobial and anti-biofilm activity of plant extracts may be attributed to the presence of secondary metabolites such as flavonoids, saponins and anthraquinones. These compounds were screened for using the methods for qualitative chemical assays described by Farnsworth (1966), with minor modifications.

For screening of flavonoids, 0.5 g of powdered plant material was added to a conical flask and extracted with 15 ml water and methanol (Merck, Germany) mixture in the ratio 2:1. The mixture was left to stand at room temperature for 30 min after which it was filtered using Whatman 110 mm filter paper. Thereafter, some magnesium turnings were added to the filtrate, and concentrated hydrochloric acid (HCl) (Merck, Germany) was added dropwise. Appearance of a yellow color indicated the presence of flavonoids.

To screen for saponins, 0.5 g powdered plant material was mixed with 15 ml distilled water. The mixture was then heated in a water bath at 100°C for 30 min, and the filtrate was left to cool down to room temperature. It was then vigorously shaken in a test tube for 10 seconds and observed for formation of froth. Froth measured 2cm or higher that persisted for 10 minutes or more confirmed the presence of saponins.

To screen for anthraquinones, 0.5 g plant material was extracted with 10 ml ether-chloroform (Merck, Germany) in the ratio 1:1 for 15 min at room temperature. The mixture was filtered and 1 ml of the

Table 1. Screening for flavonoids, saponins and anthraquinones.

Variable	*A. albomarginatum* roots	*A. anthelmintica* twigs	*D. schinzii* roots	*D. schinzii* leaves
Flavonoids	+	-	-	-
Saponins	+++	++	-	+
Anthraquinones	-	-	-	-

Key: +++ High presence; ++ Moderate presence; + Low presence; - Absent.

filtrate was treated with 1 ml of 10% (w/v) sodium hydroxide (NaOH) (Sigma, USA) solution. Development of a red color indicated the presence of anthraquinones.

Biofilm assays

Microorganisms used

The following biofilm-producing strains were obtained from the University of Pretoria's Biochemistry Department:

Escherichia coli ATCC 700928, *Staphylococcus aureus* ATCC 12600, *S. aureus* U3300, *Bacillus subtilis* ATCC 13933, *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Pseudomonas aeruginosa* and *Candida albicans*. Strains were inoculated into Falcon centrifuge tubes containing 5 ml brain heart infusion broth (Merck, Germany) and grown to stationary phase at 37°C for 24 h.

Biofilm formation and inhibition

The methods of Christensen et al. (1985), Merritt et al. (2011), and Monte et al. (2014) were used for biofilm assays, with some modifications. To obtain a 0.5 McFarland turbidity standard of 1.5 x 10^8 CFU/ml, stationary-phase cultures were diluted 1:100 and 100µl of diluted culture was added to each of six 400 µl wells of a sterile flat-bottomed 96-well microtiter plate (Lasec, SA). Three of these six wells were each inoculated with 10 µl of crude methanolic extract dissolved in 100% Dimethyl sulfoxide (DMSO) just below minimal inhibitory concentration (MIC). MICs were determined by standard microbiological procedure, prior to this assay. Eight wells each contained 100 µl sterile brain heart infusion broth only as control. The plates were parafilmed at the lids to prevent them from drying out and incubated at 37°C for 24 h.

After incubation, planktonic cells were removed by placing the microtiter plate upside down on towel paper, and allowing for the paper to soak up any liquid. To remove remaining planktonic cells, each well was washed 3x by pipetting 400 µl triple distilled water into it, and inverting the plate onto towel paper. The biofilms in the wells were fixed by oven-drying the microtiter plates for 45 minutes at 60°C.

Wells were stained with 125 µl of 0.1% crystal violet, incubated for 15 min at room temperature and the crystal violet discarded. Excess stain was removed by washing (pipetting) 3x with 400 µl triple distilled water. Plates were air-dried for a few hours or overnight. Wells were de-stained with 200 µl of 33% glacial acetic acid (Merck, Germany) for 10 to 15 min. The contents of each well were briefly mixed by pipetting, and 125 µl was transferred to corresponding wells of a new clean microtiter plate. The optical densities (ODs) of stained biofilms were determined with a Multiskan Ascent plate reader (Thermo Labsystems, USA) at 595 nm. Readings from the broth control wells were averaged and subtracted from the test readings. Test readings were averaged and standard deviations calculated. Results for biofilm formation were interpreted using the classification by Christensen et al. (1985).

The equation I% = (1- (A_{595} of test / A_{595} of non-treated control) x 100) was used to calculate percentage inhibition (Kawsud et al. 2014). According to Manner et al. (2013), selection criteria (activity-based) for antimicrobials are as follows:

Highly active: ≥85% inhibition; moderately active: ≥40% inhibition; inactive: <40% inhibition.

Biofilm formation and eradication

Stationary-phase cultures were diluted 1:100, and 100µl of diluted culture was added to each of six 400 µl wells of a sterile flat-bottomed 96-well microtiter plate (Lasec, SA). Eight wells each contained 100 µl sterile brain heart infusion broth only as control. The plates were parafilmed at the lids to prevent them from drying out, and incubated at 37°C for 24 h. After incubation, planktonic cells were removed by decanting onto towel paper. To remove the remaining planktonic cells, each well was washed 3x by pipetting 400 µl triple distilled water into it and inverting the plate onto towel paper. Three wells with the grown biofilms were inoculated with 10 µl of extract (just below MIC) and 190 µl sterile triple distilled water. The remaining wells were filled with 200 µl water and plates were incubated at room temperature for another 24 h. After the second incubation and removal of liquid in the wells, the same steps for fixing, staining and de-staining were followed according to inhibition assay. The equation E% = (1- (A_{595} of test / A_{595} of non-treated control) x 100) (Kawsud et al., 2014; Teanpaisan et al., 2014) was used to calculate percentage eradication. The classification of activity for inhibition by Manner et al. (2013) was also used to interpret eradication results.

RESULTS AND DISCUSSION

Average percentage yields for *A. albomarginatum* root extract, *A. anthelmintica* twig extract, *D. schinzii* root extract and *D. schinzii* leaf extract were 12.5, 3.5, 6.1 and 7.3%, respectively. The plant material (roots, leaves and twigs) were used for qualitative detection of flavonoids, saponins and anthraquinones. According to Kamonwannasit et al. (2013) and Lee et al. (2016), these secondary metabolites can aid in a medicinal plant's antimicrobial properties, including anti-biofilm activity. The presence or absence of the compounds is indicated in Table 1. Flavonoids were detected only in *A. albomarginatum* roots. Saponins were present in *A. albomarginatum* roots, *A. anthelmintica* twigs and *D. schinzii* leaves. Anthraquinones were not detected.

Biofilm formation over 24 h is shown in Figure 2. All isolates were classified as strong biofilm formers, with *B. subtilis* ATCC 13933 being the strongest one. Single-

factor analysis of variance (ANOVA) in Microsoft Excel revealed that there was a significant difference in biofilm formation between the eight strains (P = 0.0002; F = 4.86; F crit = 2.18). A Tukey-Kramer Multiple Comparisons Procedure indicated that this difference was between *B. subtilis* ATCC 13933 and *S. sanguinis* ATCC 10556, *B. subtilis* ATCC 13933 and *E. coli* ATCC 700928, and *S. mutans* ATCC 25175 and *E. coli* ATCC 700928. The medicinal uses of the plants in this study, as well as anti-biofilm properties of their methanolic crude extract against seven bacterial strains and a fungus is discussed hereafter.

A. albomarginatum (Marloth and Engl.) as seen in Figure 1A is commonly known as "!Guxa" in Namibia (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015), also spelled "!Kuxa" (Sullivan, 1998) or "!Khuxa" (Huggins et al., 2013). The roots are pulverized, boiled as a tea and drunk to purify the blood and cleanse the uterus. Some believe that it can cure women who experience difficulty in conceiving. It also helps to relieve the symptoms of colds (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015).

Bacteria such as staphylococci may be associated with infection of the uterus, for example in the condition known as endometritis (inflammation of the endometrium). One form of this condition is known as bacteriotoxic endometritis, where it is caused by the toxins of bacteria rather than the presence of the pathogens themselves (Allen, 2004). Phytochemical screening revealed the presence of saponins in the roots (Table 1), and according to Wink and van Wyk (2008) saponins have anti-inflammatory effects. It is thus possible that the plant may be effective to treat conditions of the uterus, and saponins can play a role in its effectiveness.

According to Sullivan (1998), du Pisani, (1983) and Steyn (1981), Nama people shred the plant's roots and use it as a spice or coffee substitute. A decoction from the crushed root is drunk by the Nama to treat chest complaints, stomach disorders and coughs. Huggins et al. (2013) say that tea made from the plant is used to treat headaches, to induce vomiting and as a general body cleanser. Flavonoids were detected in the roots (Table 1). According to Wink and van Wyk (2008), flavonoids act as antioxidants and free-radical scavengers. Flavonoids may therefore be partly responsible for the plant's cleansing properties.

At a sub-MIC concentration of 0.625mg/ml, *A. albomarginatum* root extract caused 60.0% inhibition of biofilm formation in *S. mutans* ATCC 25175, and at 1.25 mg/ml it inhibited *E. coli* ATCC 700928 and *P. aeruginosa* biofilms by 51.6 and 49.1%, respectively. This is moderate inhibition, according to Manner et al. (2013). With inhibition below 40.0%, it was inactive against the fungus *C. albicans*, as well as the bacteria *S. aureus* U3300, *S. aureus* ATCC 12600, *S. sanguinis* ATCC 10556, and *B. subtilis* ATCC 13933, and could not eradicate pre-formed biofilms.

From these inhibition results, it is evident that *A. albomarginatum* roots may be used as traditional medicine to treat biofilm-related infections involving *S. mutans* ATCC 25175, *E. coli* ATCC 700928, and *P. aeruginosa*. The roots are not expected to be effective against biofilm infections caused by *C. albicans*, *S. aureus U3300*, *S. aureus* ATCC 12600, *S. sanguinis* ATCC 10556, and *B. subtilis* ATCC 13933.

A. anthelmintica (A. Rich Brongn.) seen in Figure 1B have many common names including "kersieblomboom", "worm-cure albizia" (Orwa et al., 2009; Hoffmann, 2014), "aruboom" and "oumahout" (Hoffmann, 2014). In traditional practice, the outer parts of the twigs are scraped off and the inner part is used as a chewing stick or toothbrush to clean the teeth and tongue (Coetzee, personal communication, February, 2015; A. Frederick, personal communication, February, 2015). Both bacteria and fungi may be associated with mouth infections, where they can be part of biofilm communities as dental plaque.

The bark, wood or root from *A. anthelmintica* is boiled and milk is added to treat an upset stomach or intestinal worms. Tea made from the roots and bark is drunk to treat malaria. The Samburu pastoralists in Kenya treat gonorrhea by boiling the roots, bark and leaves, mixing it with sheep fat and giving it as an enema. Otherwise the boiled bark and roots are consumed with milk (Sullivan, 1998; du Pisani, 1983; Fratkin, 1996). The stem bark is widely used as a purgative (Orwa et al., 2009). Consumption of this plant can also be hazardous. The seeds and bark contains alkaloids which have a toxic effect. Animal poisoning can occur in cattle and sheep; livestock can die from heart failure. Overdoses of the plant can also cause death in humans (Wink and van Wyk, 2008).

Twig extract (1.25 mg/ml) from *A. anthelmintica* showed inhibition of biofilm formation only in the fungus *C. albicans*, with moderate activity (58.4%). The twigs can thus be useful as a toothbrush to remove this fungus from the teeth and tongue. The extract eradicated biofilm also only in one strain – *S. aureus* U3300. The extract's activity may partly be due to saponins.

D. schinzii (O. Hoffm.) as seen n Figure 1C is also known as "Gu-!aru" (Coetzee, personal communication, February, 2015) or the "Kalahari fever bush" (Dugmore and van Wyk, 2008). The roots and leaves are pulverized, boiled as tea and drunk or used to steam yourself in the treatment of measles, chickenpox, the flu, colds and a blocked nose (Coetzee, personal communication, February, 2015). Unspecified parts are used to treat febrile convulsions in babies in the Kalahari, hence the name "Kalahari fever bush" (Sobiecki, 2002; Dugmore and van Wyk, 2008).

Measles, chickenpox, flu and colds are caused by viruses, but bacteria can be involved in congested nose

(A)

(B) (C)

Figure 1. *A. albomarginatum (A), Image credit:* southafricanplants.net; *A. anthelmintica (B), and D. schinzii (C)* growing in the veld at Gochas, Image credit: Sunette Walter

or sinus infections. There is an interesting folk tale behind the plant's traditional use in the Kalahari to treat febrile convulsions in babies. This tale known as "Dicoma's shadow" can be read in the book "Muthi and myths from the African bush" written by Dugmore and van Wyk (2008). van Wyk (2015) explains the story in short. It is said that if the shadow of the black shouldered kite (*Elanus caeruleus*) falls on a baby, the child will get sick, and this illness will be recognized by the spastic movements of the baby's arms, similar to the movements made by the bird's feathers when it is hanging over its prey. It is furthermore said that if the condition is not treated, the infant can develop feathers on its arms. An extract of the plant can be given both topically and internally, which will counteract the symptoms and cure the child. In the traditional African context, the symbol of the bird represents fever, since birds have a higher natural body temperature (40°C) compared to that of humans (37°C). "The condition of the bird" refers to fever. Referral to feathers on the arms is actually "the gooseflesh of fever" – one of the symptoms of febrile convulsions in infants.

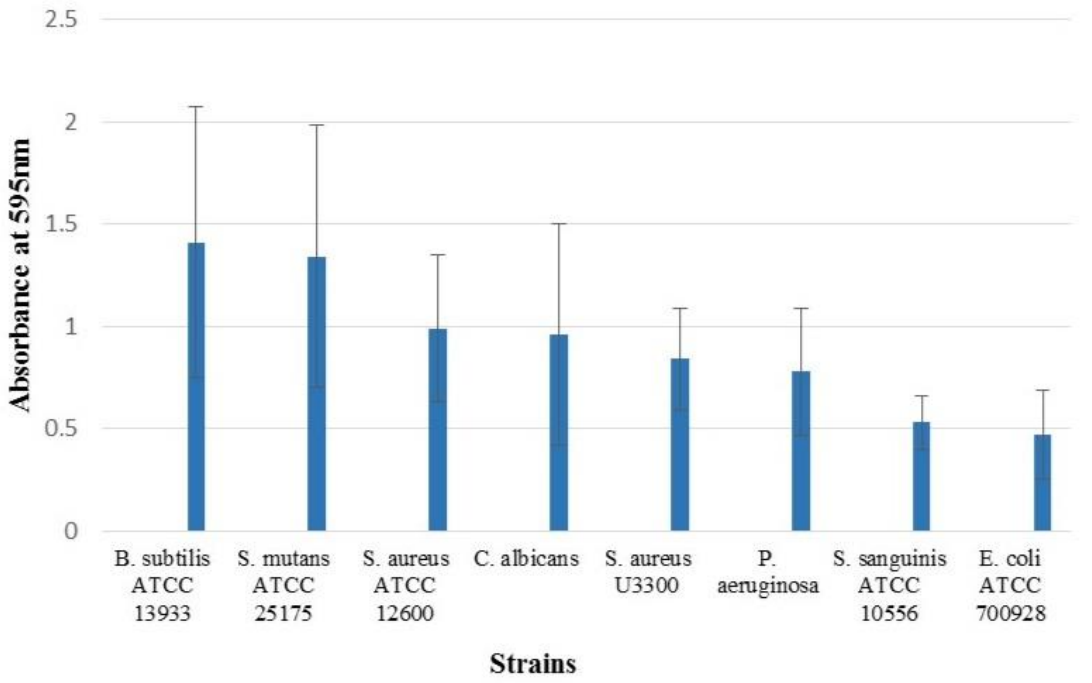

Figure 2. 24 h biofilm formation according to mean absorbance values at 595nm over eight microtiter plate assays. Formation was classified as strong/high with mean OD values > 0.240 (Christensen *et al*., 1985). Error bars indicate standard deviations. There was a statistically significant difference in biofilm formation between *B. subtilis* ATCC 13933 and *S. sanguinis* ATCC 10556, *B. subtilis* ATCC 13933 and *E. coli* ATCC 700928, and *S. mutans* ATCC 25175 and *E. coli* ATCC 7009298.

D. schinzii root extract was tested at a concentration of 1.25 mg/ml, and was unable to inhibit or eradicate biofilms. The plant's leaf extract (1.25mg/ml) however moderately inhibited biofilm formation in *P. aeruginosa* (67.3%) and is expected to help fight biofilm infections involving this bacterium. The extract eradicated the biofilm of *S. mutans* ATCC 25175 by 44.2%. Saponins in the leaves may be involved in biofilm eradication. *S. mutans* is normally not involved in the diseases mentioned by the locals at Gochas or in literature. The leaf extract may possibly be effective to treat other illnesses that are related to *S. mutans*, such as dental caries and endocarditis.

Conclusions

All strains in this study formed strong biofilms, with that of *B. subtilis* ATCC 13933 being the strongest. *A. albomarginatum* root extract moderately prevented biofilm formation in *S. mutans* ATCC 25175, *E. coli* ATCC 700928 and *P. aeruginosa,* and may therefore be effective as traditional medicine to treat biofilm infections involving these bacteria. *A. anthelmintica* twig extract inhibited *C. albicans* biofilm, and can thus be useful as a toothbrush or chewing stick to remove this fungus from the mouth. The twig extract may also be effective against biofilm infections involving the strain *S. aureus* U3300, as it was able to remove some of the bacterium's pre-formed biofilm. *D. schinzii* leaf extract moderately inhibited *P. aeruginosa* biofilm and moderately eradicated *S. mutans* ATCC 25175 biofilm. The leaves from this plant may thus be used in the traditional setting to treat biofilm infections related to these two strains. Anti-biofilm properties of the extracts under study may partly be attributed to the presence of flavonoids and saponins. The present work supports the use of the three medicinal plants in some instances.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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Development of emulsion containing watermelon extract for skin aging

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Watermelon [*Citrullus lanatus* (Thunb) Matsum and Nakai] is rich in vitamins C and B complex, and the red color of its pulp is due to the presence of lycopene. Lycopene is an antioxidant component and it is able to neutralize free radicals especially responsible for skin aging. Thus, a formulation containing lycopene extracted from watermelon was developed, and its physical-chemical stability and *in vitro* antioxidant activity were evaluated. For this, watermelon endocarp was separated and heated for pulp obtainment. Lycopene was extracted from watermelon pulp with acetone and ether successively, spectrometric quantified, and finally had its *in vitro* antioxidant activity determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The resulting extract was then incorporated in an emulsion, which was submitted to physical-chemical stability study at different conditions. Results show the presence of 38 µg of lycopene/g of watermelon pulp and 68.78% of DPPH inhibition, presenting antioxidant activity by in vitro assay. The developed formulation was stable when submitted to stress conditions and results point that it should be conserved in opaque packaging. Thus, it was possible to conclude that lycopene extract from watermelon pulp can be considered as a source of antioxidant component to be used in cosmetic formulation combating skin aging.

Key words: Antioxidant, cosmetic, 1,1-diphenyl-2-picrylhydrazyl (DPPH), lycopene, watermelon.

INTRODUCTION

A diet containing fruits and vegetables is recommended by health agencies and has been related to decreased diseases' risk due to its antioxidants components. One of those components are carotenoids, which are responsible for yellow, orange and red colors of vegetables and fruits such as tomato, papaya, guava, apricot and watermelon (Davis et al., 2006; Kong et al., 2010; Sies and Stahl, 1996). Watermelon [*Citrullus lanatus* (Thunb) Matsum and Nakai] originated from Africa and has been cultivated for over 5000 years. About

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4000 years ago, it was introduced in Egypt and Middle East, arriving in America in the 16th century (Edwards et al., 2003; Naz et al., 2013). It is a very popular fruit in Brazil, and its consumption is only smaller than the consumption of tomato, potato and onion (Andrade Junior et al., 2007).

Lycopene ($C_{40}H_{56}$), a liposoluble carotenoid present in watermelon pulp, has a symmetrical and acyclic structure, formed by carbon and hydrogen (Bramley, 2000; Schieber and Carle, 2005; van Breemen and Parkovic, 2008). Its structure has also 11 conjugated and two non-conjugated double bonds (Figure 1), conferring its antioxidant property (Sies and Stahl, 1996).

According to Andreassi et al. (2004), lycopene is able to neutralize reactive oxygen species such as singlet oxygen ($^1O_2$), which can be a very attractive property, once the human body's antioxidant enzymes (for example, superoxide dismutase, glutathione peroxidase and catalase) are not able to combat this radical. Singlet oxygen, generated by sunlight exposure, has been acknowledged to various oxidative stress effects on epidermis, such as thickening of epidermis, loss of firmness and reduction in elasticity leading to an acceleration of ageing process (Scotti and Velasco, 2003). Also, free radicals occurrence cause reduction of antioxidant substances in skin, and therefore the use of topical antioxidants may diminish or even avoid the skin ageing (Andreassi et al., 2004; Giacomoni, 2007; Isaac et al., 2008).

To provide satisfactory anti-aging action through antioxidant activity, the formulation should be stable and allow the effective release of active ingredient into the skin. According to ANVISA (Anvisa, 2004) and EMEA (Emea, 2003), stability study evaluates safety and efficacy of formulations, indicating product behavior when submitted to different environment conditions within a time frames. During the development of a new formulation, it is important to evaluate stability, prevent physical-chemical degradation and macroscopic instabilities (Anvisa, 2004; Cefali et al., 2015a).

Therefore, the aim of this study was to obtain lycopene from watermelon pulp, and to incorporate extracted lycopene in cosmetic formulations as a potential anti-aging agent.

MATERIALS AND METHODS

Watermelon, traditionally grown was purchased in a local market in Brazil. The emulsion was prepared using raw materials such as cetearyl alcohol, isopropyl palmitate, glycerin, carbomer, dimethicone, C12-20 acid PEG-8 ester, methylparaben, propylparaben, vitis vinifera seed oil, Butyrospermum parkii butter, ethylenediamine tetraacetic acid (EDTA) and butylated hydroxytoluene (BHT) supplied by galena (Sao Paulo, Brazil), mapric (Sao Paulo, Brazil), dinâmica (Sao Paulo, Brazil) and lubrizol (Sao Paulo, Brazil). Isopropanol PA, acetone PA and ether PA were supplied by Synth (Sao Paulo, Brazil) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) by Sigma-Aldrich (Sao Paulo, Brazil).

Obtaining of watermelon pulp

One natural watermelon was washed, halved and the endocarp (red part of fruit) without seeds was heated at 90°±5ºC to reduce water content (Rodriguez-Awaya, 2001).

Extraction of lycopene from watermelon pulp

Approximately, 50 g of dried watermelon pulp and 400 mL of acetone PA were mixed in magnetic shaker (Fisatom, Mod 753E, Sao Paulo, Brazil) for 1 h at 35±2ºC. This process was repeated three times to obtain higher concentration of lycopene. Supernatant was transferred to a separation funnel and 450 mL of ether PA was added to recover organic phase containing lycopene. This process was repeated three times. Organic phase was submitted to spectroscopy assay for determining lycopene presence and concentration in the extract (Rodriguez-Awaya, 2001).

Identification and quantification of lycopene

Lycopene content in the extract was analyzed by UV/VIS spectrophotometry (Thermo Scientific, Genesys, Sao Paulo, Brazil) with absorbance reading at 472 nm using ether as blank (Rodriguez-Awaya, 2001). Lycopene concentration was determined by Equation 1, where A is absorbance of extract, V is final volume of extract, A1 is molar absorption coefficient of lycopene and M is the mass of extract (Perkins-Veazie et al., 2001):

$$\text{Lycopene } \frac{\mu g}{g} = \frac{(A \times V \times 10^6)}{(A1 \times M \times 100)} \quad (1)$$

In vitro antioxidant activity assay of lycopene extract

Two and half milliliters of different extract concentrations (20, 40, 60, 80, 85, 90, 95 and 100%, v/v), diluted in ether, were placed in test tubes to which 2.5 mL of DPPH solution (0.004%, w/v in ethanol) was added, according to Cuendet et al. (1997) and Rufino et al. (2007). The assay was also performed for quercetin as antioxidant standard at concentrations of 0.25, 0.5, 1.0, 1.75 and 2.5 µg/mL and the inhibition percentage was calculated.

Development of an emulsion containing lycopene extract

An oil-in-water (O/W) emulsion was prepared according to Table 1, using the standard method (Prista et al., 1996). Lycopene extract obtained from watermelon was directly incorporated into the emulsion at a concentration of 5.0% using geometric dilution method.

Stability study of phytocosmetic

Phytocosmetic was evaluated according to stability assay recommended at stability guide of cosmetic products of ANVISA – Brazilian's National Health Surveillance Agency (Anvisa, 2004). For the study, emulsions were stored at different temperatures [5±4, 45±2, -5±2ºC and room temperature (27±2ºC) protected and exposed to indirect light]. Formulations were then submitted to macroscopic assessment (color, odor, appearance and phase separation) and pH tests over 15 consecutive days (preliminary stability test) and over 90 days for the accelerated stability test (assays performed on days 1, 7, 15, 30, 45, 60, 75 and 90). For freezing and thawing cycles, the phytocosmetic was subjected to

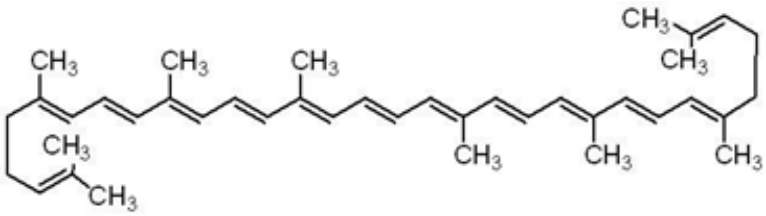

Figure 1. Structure formula of lycopene (Sies and Stahl, 1996).

Table 1. Composition of oil-in water (O/W) emulsion.

Components (INCI NAME)	Composition (%)
Cetostearyl alcohol	5.0
Isopropyl palmitate	1.5
Vitis vinifera seed oil	2.0
Butyrospermum parkii butter	1.5
Silicon oil	1.0
C12-20 acid PEG-8 ester	3.0
Metylparabene	0.5
Propylparabene	0.5
Butylated hydroxytoluene (BHT)	0.5
Carbomer (dispersion of 2.0%)	20.0
Glycerin	5.0
Sodium hydroxide solution	q.s pH 5.5-6.5
Aqua	q.s.p 100.0

alternating cycles of heat (45±2ºC) and freeze (–5±2ºC) temperatures for 24 h over 12 consecutive days. To be considered stable, results variation should not exceed 10% (Anvisa, 2004; Isaac et al., 2008). For pH measurement, a 10% (w/w) aqueous dispersion of samples was prepared in recently distilled water. The pH meter (Gehaka, model PG 1800, São Paulo, Brazil) electrode was directly inserted into the aqueous dispersion (Davis, 1997). A value compatible with skin pH (5.5 to 6.5) was deemed acceptable in the stability tests and variations in the results should not exceed 10%. Stability of the lycopene incorporated in formulation was also determined by spectroscopy at 472 nm. For this, 3 g of phytocosmetic was diluted in 30 mL of ether, filtered and submitted to spectroscopy analysis in the first day of stability study and after 30, 60 and 90 days stored to stress conditions (Cefali et al., 2015a).

In vitro antioxidant activity of phytocosmetic

Three grams of phycocosmetic diluted in 30 mL of ether was filtered, and 2.5 mL was transferred to tube with 2.5 mL of DPPH solution (0.004%, w/v in ethanol) and the inhibition percentage was determined (Cuendet et al., 1997; Rufino et al., 2007).

Statistical analysis

All assays were performed in triplicate. Then, statistical analysis was performed using ANOVA test ($P<0.05$) (Welch, 1951) for independent variables, using Origin 8.0 program.

RESULTS AND DISCUSSION

Obtaining of watermelon pulp

In general, using a watermelon unit (13.87 kg), it was possible to obtain 2.11 kg of pulp, reaching a yield of 15.25%. According to Leskovar et al. (2004) and Bruton et al. (2009), watermelon pulp consists of 93.0% water, 6.4% hydrocarbonates, 0.5% proteins, 0.3% fiber and 0.2% fat. Then, the small yield is related to the higher concentration of water, which was evaporated during the pulping process.

Lycopene extraction, identification and quantification

Extract showed red color and at 472 nm, presented 0.69±0.1 of absorbance, resulting in 38 µg of lycopene/g of fruit. This was similar to results found in literature. In their study, Nizu and Rodriguez-Amaya (2003) reported 35 and 36 µg/g of lycopene in watermelon cultivate in different states of Brazil, 73 µg/g in cherry, 53 to 69 µg/g in guava, 21 to 40 µg/g in papaya and 31 to 35 µg/g in tomato. In another study, Cefali et al. (2015b) found 46 µg/g in tomato.

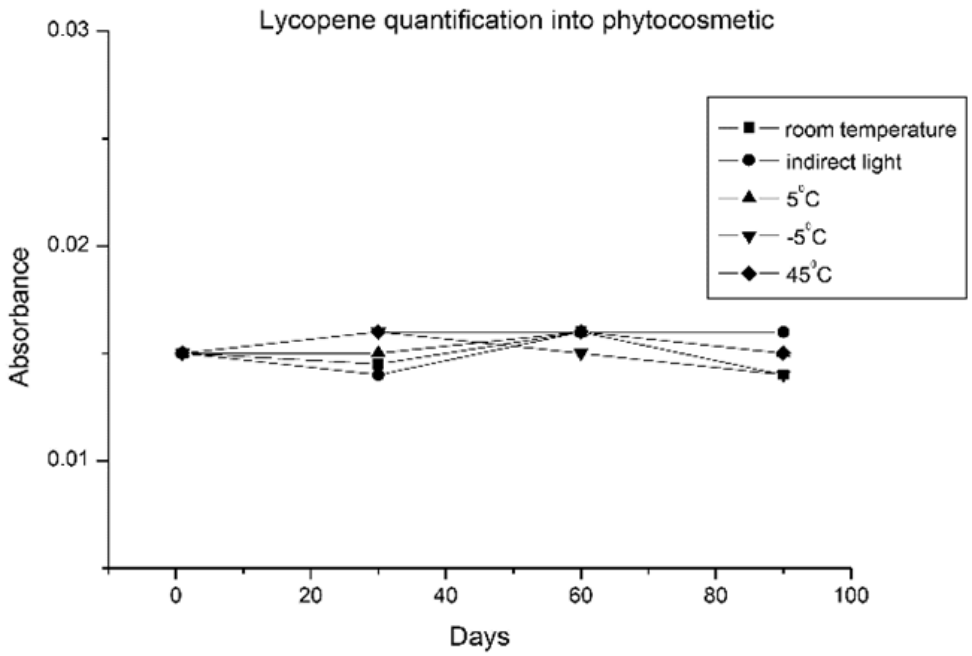

Figure 2. Lycopene quantification into phytocosmetic formulation.

Lycopene containing-emulsion development and stability study

Lycopene extract was incorporated in a shiny, odorless, white O/W emulsion. Final product was developed with moisturizing compounds, but focusing in a non-greasy feel after use. This formulation was subjected to three centrifuge cycles (3,000 rpm for 15 min) and was considered stable as it did not present phase separation, even after 24 h, therefore the developed formulation was used for lycopene extract incorporation. After extract incorporation, emulsion presented shiny appearance and yellowish coloration probably due to carotenoid presence (Sies and Stahl, 1996).

The phytocosmetic was submitted to stability study. Changes in color when stored at high temperature (45°C) and light exposure were observed. Phytocosmetic presented more intense coloration due to lycopene oxidation, which occurs because of its saturated structure (Bangalore et al., 2008; Fish et al., 2002; Sies and Stahl, 1996). This behavior was not observed when stored at other conditions and thus formulations were considered stable.

In the first 50 days of analysis, the phytocosmetic presented pH value around 6.52±0.26, which is compatible with skin pH. A standard deviation value of less than 0.05 under all studied conditions was observed, demonstrating formulation stability (Anvisa, 2004). For the accelerated stability test, the stability characteristics of the formulation remained under all stress conditions.

Assessment of pH and macroscopic characteristics was also performed in cosmetic without lycopene extract (blank formulation) and it was stable. The blank formulation presented pH value around 6.55±0.12 and pH had a standard deviation value of less than 0.05 when it was submitted to stress conditions. Therefore, it was possible to observe that the developed formulation was stable and lycopene extract did not significantly interfered in the stability parameters of formulation.

Lycopene concentration was also evaluated during stability study by spectroscopy. The absorbance values (around 0.015) had a deviation value of less than 0.05 during stability study (90 days) (Figure 2) and then, the formulation was considered a stable formulation to be used as an anti-aging product.

In vitro antioxidant activity of extract and phytocosmetic

According to Giacomoni (2007), cosmetic product containing antioxidants actives are commonly used to anti-aging combat and the use of vegetable compounds such as carotenoids are widely studied due to its antioxidant activity in many systems (Lademann et al., 2011). Therefore, lycopene extract and lycopene containing-formulation had their antioxidant activity evaluated using *in vitro* DPPH method. DPPH is a free radical and presents purple color when soluble in ethanol. In contact to antioxidant substances, DPPH is reduced to diphenyl-picrylhydrazyl, changing to a yellowish color, and decreasing its absorbance (Rufino et al., 2007). All

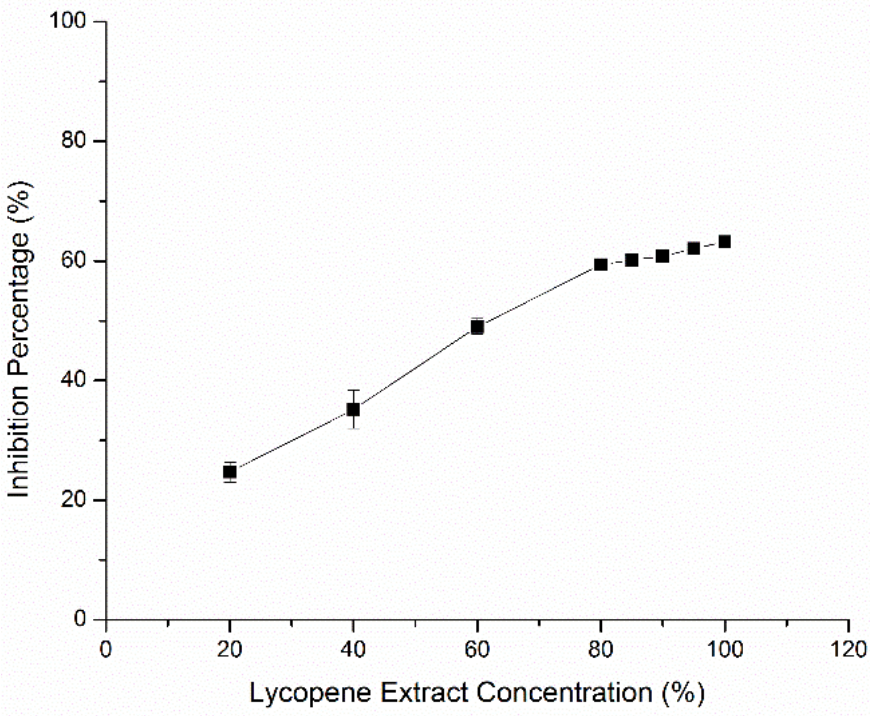

Figure 3. *In vitr*o antioxidant activity of lycopene extract in DPPH assay.

the extract containing lycopene solutions used (20, 40, 60, 80, 85, 90, 95 and 100%) presented translucent aspect and color change, macroscopically observed. From 80 to 100%, the samples presented yellow color, proving antioxidant activity of the extract. With spectroscopy absorbance, the inhibition percentage was calculated and presented in Figure 3. Fifty percent DPPH inhibition was achieved when using lycopene extracts concentration above 80% (v/v). The formulation also had its *in vitro* antioxidant activity and presented inhibition percentage equal to 30.50%, evidenced by a slight color change in the solution and an absorbance decrease (Shahzad et al., 2014). Although the formulation showed small inhibition percentage, once it contained only 5.0% of extract, it can be considered a promising anti-aging product because, according to Cefali et al. (2015a), lycopene showed an ability to be retained in epidermis when it's *in vitro* permeation was tested, and may reach a sufficient concentration to inhibit or avoid the action of free radicals.

Conclusion

The present study demonstrated that watermelon pulp contains lycopene, and this fruit can be considered a source of antioxidant component to be used in cosmetic formulation for skin aging combat. The developed formulation was stable when away from light in opaque packaging, avoiding oxidative degradation of carotenoid and browning of product.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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