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Assessment of *Salvia officinalis* (L.) hydroalcoholic extract for possible use in cosmetic formulation as inhibitor of pathogens in the skin

Charlene Silvestin Celi Garcia[1,*]; Mariana Roesch Ely[2]; Ronaldo Adelfo Wasum[2]; Bárbara Catarina de Antoni Zoppa[2]; Cláudia Wollheim[2]; Gilda Ângela Neves[3]; Valéria Weiss Angeli[2]; Kellen Cristhinia Borges de Souza[4]

[1]Academic of Course of Pharmacy at Universidade de Caxias do Sul.
[2]Teacher at Universidade de Caxias do Sul.
[3]Teacher at Universidade Federal do Rio de Janeiro
[4]Teacher at Universidade Federal de Ciências da Saúde de Porto Alegre

ABSTRACT

***Salvia officinalis* (L.), or common sage, is an aromatic herb that has been used in medicine and cooking since ancient times and has been investigated for the treatment of various diseases, especially infections and skin inflammation. We conducted phytochemical prospecting and quality control with hydroalcoholic extracts of dried sage, to identify active compounds in the plant. The aim was to assess antibacterial and antifungal activity against *Staphylococcus aureus, Streptococcus agalactiae, Candida albicans* and *Candida tropicalis*. Antimicrobial susceptibility was investigated *in vitro* by agar-overlay and well-diffusion techniques, in which disc and well were used. *Salvia officinalis* (L.) was not effective against *Streptococcus agalactiae, Candida albicans* or *Candida tropicalis*, but best results were observed for antibacterial activity against *Staphylococcus aureus*. Considering the results of the inhibition tests presented here, we suggest that cosmetic formulations containing *Salvia officinalis* (L.) could contribute to inhibitor of pathogens in the skin microbiota.**

Keywords: Hydroalcoholic extract of *Salvia officinalis* (L.). Phytochemical prospecting. Quality control. Bactericidal activity. Topical skin cleansing.

INTRODUCTION

Medicinal plants play an important role in the treatment of health. The use of herbal products, which are more economically viable than many drugs, appears to be an interesting alternative, helping to ease people's access to preventive care and treatment in cases of sensitivity to some of the commonly used drugs (Marinho & Araújo, 2007, Silva et al., 2006).

Corresponding Author: Charlene Silvestrin Celi Garcia - Rua Thomas Edson, 20, Apt. 701 - Centro - CEP.95180-000 - Farroupilha - RS - Brasil tel.: (54) 9987-4443 - e-mail:charlenescgarcia@hotmail.com

Several diseases that affect public health are of microbial origin, especially bacterial. Despite the variety of antimicrobial agents that act on a wide range of pathogenic microorganisms, there is an ongoing search for an ideal antimicrobial, exhibiting the broadest spectrum of action, combined with low toxicity, low cost and low drug resistance index (Alvarenga et al., 2007).

Salvia officinalis (L.) is a perennial shrubby herb belonging to the Lamiaceae family, originally from the East Mediterranean region, where it is common in the kitchen and in remedies. It is typically cultivated in temperate climatic areas where there is abundant sunlight. It is popularly known in Brazil as *sálvia, salva-comum, erva-santa, salva-mansa* and in English as sage. This plant is very widely used; the Greeks consumed it in the treatment of indigestion, and today it is used in popular medicine as a remedy for skin inflammations (Bruneton, 2001; Cunha, 2004). It is a source of essential oils and its constituents include cinerol, camphor, borneol, thujone, rosmarinic acid, flavonoids, tannins, saponins and estrogenic substances (Cunha, 2004; Viecelli & Cruz-Silva, 2009). It is not recommended to women who are pregnant or nursing, for it may stimulate uterine contractions and reduce milk secretion, and it should not be taken in cases of renal failure. It may cause sudden high blood pressure, bradycardia and toxicity to the central nervous system. It should neither be consumed in large quantities, nor over long periods of time (Bruneton, 2001; Simões et al., 2004).

Salvia officinalis (L.) is known for its antiseptic, wound-healing, antibacterial and antioxidant properties. Pereira et al. (2004) demonstrated that the essential oil from this plant had significant antimicrobial activity against 100 bacterial strains isolated from patients diagnosed with urinary tract infection. After 24 hours, the sage oil showed 100% efficacy when tested on *Klebsiella* and *Enterobacter* species, 96% on *Escherichia coli*, 83% against *Proteus mirabilis* and 75% against *Morganella morganii.*

Candida spp. cause a series of systemic infections in immunocompromised individuals (Sardi et al., 2011). They are normally found in the oral mucosa, gastrointestinal tract, vulvovaginal and epidermal regions, but may infect the system (Brooks et al., 2009). The essential oil of sage

has been used in pharmaceutical formulations, because it has a broad spectrum of antifungal activity, against dermatophytes, *Candida* sp. and filamentous fungi (Pinto et al., 2007). Thus, a glycolic extract of sage exhibited fungicidal capacity against 80% of *Candida albicans* strains (Molina et al., 2008).

Gram-positives cocci, such as *Staphylococcus aureus*, cause skin, urinary and respiratory infections; even *Streptococcus agalactiae*, which can cause meningitis and sepsis, leading infants to death, can be part of the human microbiota (Brooks et al., 2009). Studies have indicated that antibiotic sensitivity may be related to cell structural features, since Gram-positive bacteria were especially sensitive to extracts of plants from the Lamiaceae (Bara & Vanetti, 1998).

The objective of this study was to identify constituents of *Salvia officinalis* (L.) by phytochemical prospection, performing quality control tests on the dried plant and a hydroalcoholic extract of *Salvia officinalis* (L.) The *in vitro* activity of hydroalcoholic extract against bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*) and fungi (*Candida albicans* and *Candida tropicalis*) was investigated, suggesting that cosmetic formulations containing *Salvia officinalis* (L.) could contribute to inhibitor of pathogens in the skin microbiota.

MATERIAL AND METHODS

Salvia officinalis (L.) was collected in Farroupilha, Rio Grande do Sul, Brazil (Lon. $29^0$13'S by Lat. $51^0$21'O) and a specimen 37944 was deposited at the *Museu de Ciências Naturais* (*MUCS*), Universidade de Caxias do Sul (UCS). The plant was dried at 40-45°C in circulating air (Fabbe) for seven days and then ground in a Wiley mill and stored in amber bottles for further tests.

To prepare the hydroalcoholic extract of *Salvia officinalis* (L.) (1:10 w/v), the plant was ground, weighed and macerated in 80% ethanol (v/v) for nine days in an amber bottle. Next, the extract was filtered under reduced pressure and stored in amber bottles for further tests.

Phytochemical prospecting

Identification tests were carried out as described in the Permanent Commission of the Brazilian Pharmacopoeia (1988; 2000) for saponins, tannins, flavonoids, coumarins, alkaloids, quinones and methylxanthines. All tests were performed in triplicate.

Characterization of dried plant and the hydroalcoholic extract of *Salvia officinalis* (L.)

We ran quality control tests on the dried plant and the hydroalcoholic extract of *Salvia officinalis* (L.), to determine: relative density, % mass of dry residue, pH, presence of flavonoids - thin layer chromatography (TLC) with quercetin as standard, total flavonoids, mass loss during drying and foreign matter content (Brazilian Pharmacopoeia, 1988; 2000; Camargo, 2001). All tests were performed in triplicate.

Antimicrobial activity test

To assess antimicrobial activity of the hydroalcoholic extract of *Salvia officinalis* (L.), we used the agar disc and well diffusion tests, described in the Clinical and Laboratory Standards Institute (CLSI, 2005) and by the Permanent Commission of the Brazilian Pharmacopoeia (1988; 2000).

Preparation of inoculum

The microorganism *Staphylococcus aureus* was purchased (ATCC 29213), while the other microorganisms, *Streptococcus agalactiae*, *Candida albicans* and *Candida tropicalis*, were isolated from patients at the University Hospital at UCS.

The inoculum cultures were diluted in sterile saline solution to match their turbidity to 0.5 on the MacFarland scale, in order to obtain an approximate cell density of 1-2 x 10^8 CFU / mL. The plates were inoculated with a sterile swab (CLSI, 2005).

Agar diffusion tests

In the disc diffusion test, plates of culture medium (Mueller-Hinton, blood agar and Sabouraud) were inoculated uniformly with *Staphylococcus aureus*, *Streptococcus agalactiae*, *Candida albicans* and *Candida tropicalis*. Paper discs impregnated with 30 µL of hydroalcoholic extract of *Salvia officinalis* (L.) and control discs, impregnated with 30 µL of 80% ethanol (v/v), were allowed to dry, after were placed on the inoculated agar. The plates were incubated for 24 hours at 37 ± 1 °C and the inhibition zones were measured in millimeters.

In the (cylindrical) well diffusion test, 50 µL and 100 µL aliquots of *Salvia officinalis* (L.) extract were distributed in wells bored in plates of culture media (Mueller-Hinton and blood agar), previously inoculated with *Staphylococcus aureus* and *Streptococcus agalactiae*. Wells with 80 % ethanol (v/v) were used as positive control. For the test with *Candida albicans* and *Candida tropicalis,* 50 µL, 100 µL and 200 µL aliquots of *Salvia officinalis* (L.) extract were distributed in the wells in the inoculated test medium (Sabouraud) and 80% ethanol (v/v) was used as positive control. The plates were incubated for 24 hours at 37 ± 1 °C. After this, the inhibition zones, where present, were measured in millimeters. All tests were performed in triplicate.

RESULTS

Phytochemical prospecting

The results confirmed the presence of saponins, having a steroid or terpene hydrocarbon (lipophilic) part and a hydrophilic part of the molecule, which confer the property of reducing water surface tension, and hence detergent and emulsifying actions (Costa, 2001).

Salvia officinalis (L.) reacted positively with gelatin (forming insoluble compounds) and iron salts, indicating

the presence of tannins, which provide the astringency of this plant. The result was also satisfactory for the reaction of flavonoids with ferric chloride, indicating the presence of flavonols and flavones (Table I).

Table I. Results of phytochemical prospecting of dried plant of Salvia officinalis (L.)

Salvia officinalis (L.)	
Tannins – Reaction with gelatin	Positive
Tannins – Reaction with iron salts	Positive
Flavonoids – Reaction with alkali hydroxides	Negative
Flavonoids – Reaction with ferric chloride	Positive
Flavonoids – Reaction with oxalic/boric acids	Negative
Saponins	Positive
Derivatives of coumarin	Negative
Alkaloids – Dragendorff reaction	Negative
Alkaloids – Mayer reaction	Negative
Alkaloids –Bertrand reaction	Negative
Alkaloids – Bouchardat reaction	Negative
Methylxanthines	Negative
Quinones – Bornträger reaction (direct)	Negative
Quinones – Bornträger reaction with hydrolysis	Negative

Characterization of dry plant and hydroalcoholic extracts of *Salvia officinalis* (L.)

The beneficial effects of sage arise from numerous molecules of diverse chemical groups, including a range of phenolic compounds (Generalie et al., 2012). When quality control tests were carried out on dry *Salvia officinalis* (L.) and its hydroalcoholic extract, this study showed the presence of saponins, tannins and flavonoids. According to the limit for foreign matter given in the Brazilian Pharmacopoeia (1988; 2000), namely 1-2%, the plant analyzed was within the specifications; also, plants should have a moisture content of 8 to 14%. The assay for total flavonoids content was expressed as a percentage of quercetin; there are no literature data for comparison (Table II).

Table II. Results of quality control of dry plant and hydroalcoholic extract of *Salvia officinalis* (L.)

Assay	Dried sage	Extract of sage
TLC	Rf (sample): 0.60 ± 0.01 Rf (quercetin standard): 0.62 ± 0.01	Rf (sample): 0.60 ± 0.01 Rf (quercetin standard): 0.61 ± 0.01
Foreign matter	1.4 % ± 0.02	----------
Loss on drying	7.28 % ± 0.06	----------
pH	----------	6.25 ± 0.07
Dry residue	----------	0.964 % ± 0.09
Relative density	----------	0.856 ± 0.02
Total flavonoid content (determination)*	1.6 % 0.08	3.3% 0.05

*Total flavonoid content (determination) expressed as percentage of quercetin

Antimicrobial tests

The hydroalcoholic extract of *Salvia officinalis* (L.), when tested, did not form zones of inhibition against *Streptococcus agalactiae* or *Candida tropicalis*, in either of the methods used (agar diffusion from disc and well), indicating resistance or insensitivity to this extract.

The hydroalcoholic extract of *sage* was also tested against *Candida albicans* which showed zones of inhibition only in the well diffusion method, when 100 µL and 200 µL of plant extract were added. When 100 µL was added to the well, an inhibition zone of 18 mm was observed, and just 10 mm when the well contained 80% ethanol (v/v); the test was conducted in triplicate and only one plate showed an inhibition zone. With 200 µL, there was an inhibition zone of 40 mm around the well containing extract of *Salvia officinalis* (L.) and 20 mm around that containing 80% ethanol (v/v); the test was performed in triplicate and again only one sample showed an inhibition zone. These results show the insensitivity of *Candida albicans* to the hydroalcoholic extract of *Salvia officinalis* (L.)

Growth inhibition of *Staphylococcus aureus* by the hydroalcoholic extract of *Salvia officinalis* (L.) was demonstrated by both methods used and at all concentrations tested. The disc-diffusion method, with 30µL of extract in the paper disc, gave inhibition zones of 13, 12 and 13 mm (mean 12.6 mm ± 0.58); discs with 30µL of 80% ethanol (v/v) showed no inhibition zone.

The well-diffusion test, with 50 µL extract in the well, showed inhibition zones of 19, 18 and 19 mm (mean 18.6 mm ± 0.58), and when added 100 µL was added, inhibition zones of 21, 22 and 23 mm were formed (mean 22 mm ± 1). The wells to which 80 % ethanol (v/v) was added showed no inhibition zone.

Antibiotics oxacillin (1µg), gentamicin (10 µg), vancomycin (30µg), cefoxitin (30µg) and erythromycin (15µg) showed inhibition zones of, respectively, 13, 15, 15, 20 and 23 mm, according to the Clinical and Laboratory Standards Institute (CLSI, 2005). The well-diffusion test with 50 µL of extract showed a more pronounced inhibition zone than 1 µg oxacillin, 10µg gentamicin and 30µg vancomycin, and a smaller zone than 30µg cefoxitin and 15µg erythromycin. When tested at a concentration of 100 µL, the extract showed an inhibition zone larger than 1 µg oxacillin, 10 µg gentamicin, 30 µg vancomycin and 30 µg cefoxitin, being surpassed only by erythromycin 15 µg (Figure 1).

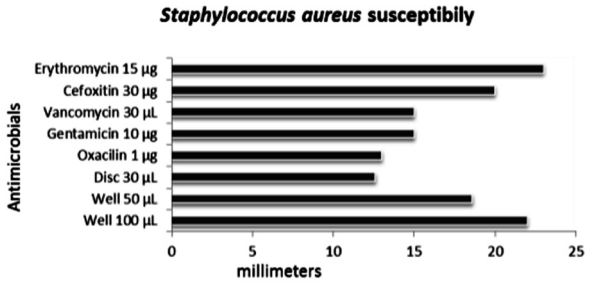

Figure 1: Assessment of the methods used, antimicrobial susceptibility testing by agar diffusion from disc and well. Average of inhibition zone of bacterial growth at various concentrations of the extract of Salvia officinalis (L.), compared with antibiotics according to information from Clinical and Laboratory Standards Institute (CLSI, 2005).

In the disc diffusion test, the disc was impregnated with 30 µL of extract, showing a smaller inhibition zone than antibiotics oxacillin (1 µg), gentamicin (10 µg), vancomycin (30 µg) , cefoxitin (30 µg) and erythromycin (15 µg) (Figure 1).

DISCUSSION

In this study, the antibacterial and antifungal activity of hydroalcoholic extract of sage against *Staphylococcus aureus, Streptococcus agalactiae, Candida albicans* and *Candida tropicalis* was assessed by *in vitro* tests with agar diffusion assays using paper discs and wells. Although the sage extract was not effective for most microorganisms investigated here, it showed antibacterial activity against *Staphylococcus aureus*.

Several factors may influence the bacterial resistance shown by *S. agalactiae* in this study. Apart from the genetic diversity recently reported for these bacteria (Corrêa et al., 2011), some proteins may contribute to differential adhesion processes and others relate to the presence of pili and capsule on cell surface, hiding molecular targets (Corrêa, 2009). It is hard to elucidate the mechanisms contributing to *S. agalactiae* resistance and how they interact with *Salvia officinalis* extracts. According to Bara & Vanetti (1998), sage shows activity against Gram-positives bacteria, but this trial failed to demonstrate any inhibition of *Streptococcus agalactiae*, classified as Lancefield group B (Lancefield, 1933).

The essential oil of sage is used in pharmaceutical formulations, being a broad-spectrum antifungal with activity against dermatophytes, *Candida* spp. and filamentous fungi (Pinto et al., 2007). The virulence of species of *Candida* is related to dimorphism, adherence, enzyme secretion, diversity of phenotypes and the variability of antigens and receptors that bind the complement system (Gilfillan et al., 1998). The secretion of proteinases is an important virulence factor for *Candida tropicalis* (Tamura et al., 2007). According to Colombo & Guimarães (2003), clinical isolates of this species are sensitive to amphotericin B (AmB) and, in most cases, to the triazoles, though occasional samples show resistance (Colombo & Guimarães, 2003). One of the factors responsible for drugs resistance of yeast is a decrease of ergosterol in the plasma membrane and / or changes in the composition of membrane lipids, or by changes in the composition of ergosterol / fungal membrane phospholipids, lowering the affinity of some antifungal drugs for the membrane (Boff, 2007).

There are some attributes of the cells of C. *albicans*, commonly called virulence factors, that confer the ability to produce disease. The most important factors triggering infection are: adherence to biological substrates and germ-tube formation, with the development of the filamentous form, phenotypic and genotypic variability, production of extracellular hydrolytic enzymes and toxins, antigenic variability, immunomodulation of host defense mechanisms and cell surface hydrophobicity (Álvares et al., 2007).

The presence of high concentrations of 1,8-cineol, thujone, camphor and monoterpenes afford *Salvia officinalis* (L.) antibacterial activity against both Gram-negatives and Gram-positives (Pereira et al., 2004; Delamare et al., 2007). Oleanolic acid, a constituent of *Salvia officinalis* (L.) extract, showed antibacterial activity to methicillin-resistant *Staphylococcus aureus* (Horiuchi et al., 2007).

The flavonoids, one of the components of sage that we identified by phytochemical prospecting and quality control tests of the extract of *Salvia officinalis* (L.), are polyphenolic structures that act as antimicrobials (antifungal, antibacterial and anti-virus), some being agents produced as phytoalexins in response to microbial attack; most of the flavonoids with this class of action are isoflavones, flavanones and flavones(?) (Silva, 2006). Compounds identified in the phytochemical prospecting and quality control testing of the extract may be significant inhibitors of the bacterium *Staphylococcus aureus*, which sensitize its cell membrane and alter the activity of calcium channels, causing increased permeability and release of vital intracellular constituents, as well as impairment of the enzymatic and cellular respiration (Celikel & Kavas, 2008; Porte & Godoy, 2001). According to Rocha (1994), cosmetics containing 1 % flavonoids already have microbicidal activity, but do not cause skin irritation (Rocha, 1994). Thus, formulations with hydroalcoholic extract of *Salvia officinalis* (L.) may have astringent, antimicrobial and anti-inflammatory properties, providing protection to skin that is susceptible to bacteria.

In this study, we also identified, by phytochemical prospecting and quality control tests, the presence of saponins and tannins in the extract of *Salvia officinalis* (L.). Saponins reduce the surface tension of water, and act as detergents, emulsifiers and antibacterials (Costa, 2001; Verdi et al., 2005). Tannins are phenolic compounds with antioxidant, anti-inflammatory and antibacterial activity against Gram-positives microorganisms (Pinho, et al., 2012). Thus, the compounds found in the *Salvia officinalis* (L.) extract may have contributed to the activity against *Staphylococcus aureus*.

However, our *in vitro* test results demonstrated that the hydroalcoholic extract had no inhibitory capacity against *Streptococcus agalactiae, Candida tropicalis* or *Candida albicans*, only showing antibacterial activity against *Staphylococcus aureus*. Considering the inhibition test results described here, we propose the inclusion of this extract in the formulation of a liquid soap to cleanse the skin. It is suggested that the formulation could be used in the hygienic treatment of skin with infective dermatitis, since *Staphylococcus aureus* is involved in these infections.

RESUMO

Avaliação do extrato hidroalcoólico de *Salvia officinalis* (L.) para possível uso em formulações cosméticas como inibidor de patógenos da pele

A *Salvia officinalis* (L.) é uma planta com uso difundido, utilizada no tratamento de diversas patologias, principalmente para infecções e inflamações cutâneas. Neste trabalho foi realizada prospecção fitoquímica e controle de qualidade com a planta seca e extrato hidroalcoólico para identificação dos compostos ativos da sálvia, tendo como finalidade comprovar sua atividade antibacteriana e antifúngica frente à *Staphylococcus aureus, Streptococcus agalactiae,*

***Candida albicans* e *Candida tropicalis*. Os métodos de escolha para avaliação *in vitro* foram ensaios de sensibilidade antimicrobiana por difusão em ágar com discos e cilindros. Dentre os ensaios realizados a sálvia não se mostrou efetiva para *Streptococcus agalactiae*, *Candida albicans* e *Candida tropicalis,* sendo o melhor resultado obtido com *Staphylococcus aureus*, em que se pode verificar-se atividade antibacteriana. Diante dos resultados obtidos, propôs-se uma formulação de sabonete líquido com extrato hidroalcoólico de *Salvia officinalis* (L.), para atuar na higiene da pele.**

Palavras-chave: Extrato hidroalcoólico de *Salvia officinalis* (L.). Prospecção fitoquímica. Controle de Qualidade. Atividade bactericida. Higienização tópica da pele.

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