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In vitro antimicrobial and antioxidant activity of aqueous and acetone extracts of *Syzygium grande* (Wight) Walp. leaves

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Abstract

The antimicrobial activity of aqueous and acetone extracts of *Syzygium grande* leaves was investigated on several microorganisms including bacteria, yeast and moulds using disc diffusion method. *In vitro*, both extracts showed significant antimicrobial activity against various species of bacteria. However, yeast and moulds species showed resistance against *in vitro* treatment with these extracts. The minimum inhibitory dosage (MID) values for the susceptible bacteria were in the range of 31.2-500µg/disc. Phytochemical analysis revealed the presence of saponins, reducing sugar, triterpenoids, tannins and flavonoids. These extracts were also evaluated for their antioxidant activity using 2, 2- diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. In the DPPH assay, acetone extract showed higher percentage of DPPH scavenging activity (79.65%) than aqueous extract (70.32%). The acetone extract also showed stronger reducing power activity (IC_{50} = 7.15µg/ml) compared to aqueous extract (IC_{50} = 12.84µg/ml). The antioxidant activity of these two extracts in both DPPH and FRAP assays was concentration dependent.

Keyword: Antimicrobial activity, Antioxidant activity, *Syzygium grande*, Myrtaceae.

Introduction

The rapid emergence and dissemination of multiple strains of antibiotic resistance microorganism to current antimicrobial agents has stimulated great interest in search for potential compounds from plants for therapeutic, medicinal, aromatic and aesthetic uses (Newman and Cragg, 2007; Kaur and Arora, 2009). Some plant extracts and phytochemical compounds like alkaloids, polyphenols, and essential oils are known to have antimicrobial properties and can be of great significance in therapeutic application against human pathogens, including bacteria, fungi and virus (Perez, 2003; Taquri *et al*., 2004; Ozturk and Ercisli, 2006; Okusa *et al*., 2007; Samoylenko *et al*., 2009). Many published reports have shown that not only the chemical derived from the plant has effect against a particular pathogen, but the antioxidant property of the plant extract also gives beneficial effect to human health (Puangpronpitag, and Sittiwet, 2009). Damaging free radicals and reactive oxygen species produced in normal and pathological cell metabolism can be formed when oxygen interact with certain molecules. This damage has been linked to heart disease, atherosclerosis, diabetes mellitus, arthritis, anemia, asthma, inflammation, neurodegenerative, aging processes and some cancers (Potterat, 1997). Antioxidants are known to have the ability to delay or prevent the oxidative chain reaction by scavenging free radicals and diminishing oxidative stress (Gerber *et al*., 2002; Durakova *et al*., 2010; Reuter *et al*., 2010). Plant-derived antioxidants like phenolic acids, polyphenols and flavonoids have been screened extensively in order to find new medicinal drugs which have efficient protection against various diseases related to oxidative stress and free radical-induced damage (Elita *et al*., 2012).

The genus *Syzygium* (Myrtaceae) comprises of about 1100 species in the tropics (Ayannar and Subas-Babu, 2012). They are trees or shrubs cultivated for ornaments in warm regions, which frequently produce edible fruits (Baily, 1949). The presence of diverse phytochemical compounds like flavonoids, tannins, triterpenoids and steroid have been reported from species of the genus *Syzygium* (Candy *et al*., 1968; Gottlieb *et al*., 1972; Nonaka *et al*., 1992; Slowing *et al*., 1994; Djipa *et al*., 2000; Manoharan *et al*., 2007; Ayannar and Subas-Babu, 2012). Moreover, biological activities of *Syzygium* species such as antibacterial, antifungal, antioxidant, anti-inflammatory, anti-nociceptive,

antivirus, and anticancer activity (Kurokawa *et al*., 1998; Djipa *et al*., 2000; Reynertson *et al*., 2005; Kumar *et al*., 2008; Tanko *et al*., 2008; Pinto *et al*., 2009; Aisha *et al.,* 2012) have been investigated. *Syzygium grande* (syn. *Eugenia grandis*) also known locally as Sea apple or Jambu Laut, is a common seashore tree. The tree is tall, growing to 30 m and has an irregular crown. The leaves are large, shiny, dark green, elliptic in shape and have a distinct down-turned tip. The flowers are oblong, large, white and fluffy (Manoharan, 2006). Its use in folk medicine is not known, but it is often planted along the roadsides to give shade. There are references to the use of the bark of *S. guineese* for the treatment of stomachache and diarrhea (Tsakala *et al*., 1996; Hamil *et al*., 2000; Oluwole *et al*., 2002) and the bark of *S. jambos* to relieve asthma and bronchitis (Lim, 2012). There is also a report of the use of clove oil of *S. aromaticum* to treat respiratory and digestive problems (Banerjee *et al*., 2006).

To our knowledge, there is only little information about the antimicrobial and antioxidant activity of *S. grande* and thus this plant species is being brought into focus in this study. The aim of this report is to evaluate antioxidant activity, antimicrobial activity and to screen for phytochemicals content in leaves of *Syzygium grande* from Myrtaceae family.

Materials and Methods

Chemical reagents: The chemical reagents such as DPPH [2, 2-diphenyl-1-picrylhydrazyl] and quercetin [3,3′,4′,5,7-pentahydroxy flavone] were purchased from Sigma-Aldrich (USA). Gallic acid was obtained from BHD Chemicals (England). Nutient Agar, Potato Dextrose Agar and Sabouraud Detrose Agar were from Lab M Limited (Lancashire, United Kingdom). All other chemicals used were of analytical grade.

Plant material: The leaves of *S. grande* were collected from Universiti Sains Malaysia Campus, Malaysia in June, 2011. The botanical identification was made by comparing with authentic herbarium specimen at the Herbarium, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The plant material was washed with water, shade dried and ground into fine powder using electric mill. The powdered material was kept in airtight container at 4 °C until used.

Plant preparation

Aqueous extract: Aqueous decoction (10%) was prepared. After filtration, solution was dried and subsequently weighed to yield a crude extract of 5.72g.

Acetone extract: Powdered leaves material was extracted after 24 h maceration by percolation with 80% acetone at room temperature. The leaves extract was dried and weighed to yield an extract of 4.36 g.

Phytochemical screening: The aqueous and acetone extracts of *S. grande* leaves were subjected to preliminary phytochemical screening of compounds which includes alkaloids, saponins, terpenoids and reducing sugar following the methodology of Harbone (1984). About 1 ml of the extract was diluted with 20 ml of distilled water and shaken in graduated cylinder for 15 minutes. Formation of 1 cm layer of foam showed the presence of saponins. To 1 ml of extract, 2 ml of trichloroacetic acid was added and the formation of yellow to red precipitate indicated the presence of terpenoids. For the detection of reducing sugar, 5 ml of Benedict's reagent into 1 ml of the extract was added and kept in boiling water bath. Red, yellow or green precipitate showed the presence of reducing sugar. The presence of alkaloids was determined first by adding 5 ml of the extract to 2 ml of hydrochloric acid. To this acidic medium, 1 ml of Dragendorff's reagent was added. The formation of orange or red precipitate showed the presence of alkaloids. Screening the presence of flavonoids and tannins was performed as described by Kumar *et al*. (2009) and Khan *et al*. (2011). About 5 ml of dilute ammonium solution was added to the portion of each extract followed by the addition of concentrated H_2SO_4. The appearance of yellow colour indicated the presence of flavonoids. The appearance of brownish green or blue black precipitate resulting from the addition of 0.1% ferric chloride indicated the presence of tannins.

Microbial Cultures: The microorganisms used in this study consisted of 6 strains of bacteria, 5 filamentous fungi and 1 yeast. *Staphylococcus aureus* (American Type Culture Collection, ATCC 12600), *Shigella boydii* (ATCC 9207), *Pseudomonas stutzeri* (ATCC 17588), *Bacillus spizizeni* (ATCC 6633) *Escherichia coli* O157: H7, *Aspergillus flavus*, *A. niger*, *A. terreus* and *A. fumigatus* were obtained from school of Biology at University Sains Malaysia, Malaysia. *Pseudomonas aeruginosa, Candida albicans* (clinical isolate) and *Penicillium expansum* were provided by the Department of Biology, Education College for pure sciences at University of Baghdad, Iraq. The bacterial strains were grown and maintained on Nutrient Agar (NA) slants, while yeast and fungi on Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar slants (PDA) respectively.

The inoculated agar slants were incubated at 37 °C for bacteria and yeast, and 30 °C for fungi.

Antimicrobial susceptibility test: Plant extracts were dissolved in Dimethylsulfoxide (DMSO) to a final concentration of 100 mg/ml. *In vitro* antimicrobial activity tests were then performed by disc diffusion method (Murray, 1995) with a slight modification in the volume and in the concentration of the extracts. About 0.1 ml of inoculum containing 10^8 CFU/ml of bacteria or yeast or 10^4 spore /ml of fungi was spread on Nutrient Agar, Sabouraud Dextrose Agar and Potato Dextrose Agar, respectively. Sterilized Whatman AA discs (6 mm in diameter) containing 20 µl of each extract (2000 µg/disc) were placed on the inoculated agar and incubated either at 30 °C for fungi, or at 37 °C overnight for bacteria and yeast. Discs prepared with only the corresponding volume of DMSO were used as control. The diameter of inhibition zones was expressed in millimeter. Each extract was tested against each microorganism in triplicate.

Determination of minimum inhibitory dosage (MID) of extracts: Minimum inhibitory dosage (MID), minimum dose per disc needed to inhibit growth of microorganism was carried out on the aqueous and acetone extracts of *S. grande* as described by Habsah *et al*. (2000). Serial dilutions of each extract from 15.6 – 2000 µg per disc were loaded onto filter paper discs according to disc diffusion method described above.

Antioxidant activity

DPPH radical method: The free radical scavenging ability of the aqueous and acetone leaves extracts of *S. grande* was conducted using method developed by Brand-Williams *et al*. (1995) with a slight modification. Briefly, serial dilutions of each extract (50 µl) was mixed with 150 µl 0f 300 mM ethanolic 2, 2-diphenyl-1-picrylhydrazyl solution in a 96 well micro-titer plate to give final concentrations of 1, 0.5 and 0.25 mg/ml. After 30 minutes incubation in the dark, at 37 °C, the decrease in absorbance was read at 515 nm using microplate reader spectrophotometer (Thermo Electron Corporation, Finland). DMSO was used as a negative control. Samples were measured in three replicate. DPPH radical inhibition percentage was calculated using the following equation:

Radical scavenging activity (%) = (control absorbance–sample absorbance) /control absorbance X 100.

Reducing power assay: The total antioxidant potency of plant extracts was conducted using iron (111) reduction method (Hinneburg *et al*., 2006). For each extract, 1 ml of different concentrations of plant extracts (5, 10, 15, 25, and 25µg/ml) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5ml of a 1% aqueous potassium ferricyanide [$K_3Fe(CN)_6$] solution. After incubation at 50 °C, 2.5ml of 10% trichloroacetic acid was added and the mixture was centrifuged at 3000 rpm for 10 min. Then, 2.5 ml of the upper layer solution was mixed with 2.5 ml of water and 0.5 ml of an aqueous $FeCL_3$ (0.1%) solution. Absorbance was read at 700nm and gallic acid was used as positive control. The iron (111) reducing activity determination was performed in triplicate. IC_{50} was obtained by graphically determination. A lower IC_{50} value indicates higher antioxidant activity.

Statistical analyses: All analyses were run in triplicate and results averaged. The results were processed using Microsoft Excel 2007 and Origin 6.0.

Results and Discussion

Phytochemical screening: The acetone and aqueous extracts prepared from *S. grande* were screened for their phytochemical analysis. Flavonoids, saponins, triterpenoids, tannins and reducing sugar were detected in both extracts with an exception being the absence of alkaloids (Table 1). The presence of saponins, flavonoids tannins and glycosides in some *Syzygium* species such as *S. cumini*, *S. samarangense* and *S. aromaticum* has been reported (Tanko, 2008; Borhade, 2012; Edema and Alaga, 2012). Contrasting with the findings of a previous study on a close related species (Shyamala Gori and Vasantha, 2012), namely *S. cumini,* alkaloids were not detected in the plant used in this study.

Antimicrobial activity: At a concentration of 100 mg/ml (2000µg/disc), each extract was tested against various microorganisms which consisted of 1yeast, 6 bacteria and 5 filamentous fungi. As shown in Table (2), the growth of yeast and fungal strains were not affected by these two extracts. The result seems to agree with previous report of Ajam (2011) that the methanolic extract of *S. grande* leaves showed some antibacterial activity against bacteria but not against fungi (*A. niger, A. flavus*, *Penicillium oxalicum* and *Cladosporium oxysporum*). This finding indicated that yeast and fungi would probably be more resistant to *S. grande* leaves extracts than bacteria. However, bacterial strains showed susceptibility towards the extracts. Both plant extracts inhibited the growth of *Staphylococcus aureus, Shigella boydii, Psuedomonas stutzeri*, *P. aeruginosa* and *Bacillus spizizeni* but were unable to inhibit the growth of *E. coli.*

Table (1): Class of Phytochemicals present in *Syzugium grande* leaf extracts.

Phytochemical compounds	Aqueous	Acetone Extract
Saponins	+	+
Alkaloids	-	-
Triterpenoids	+	+
Falvonoids	+	+
Tannins	+	+
Reducing sugar	+	+

Note: "+" = Present; "-"Absent

Minimum inhibitory dosage (MID) was carried out to evaluate the potency of the extracts in inhibiting the growth of bacteria. Comparison of the MID values of aqueous and acetone extracts (Table 3) showed that both extracts have similar activities against *B. spizizeni, S. boydii* and *P. stutzeri*. Difference was only observed with *S. aureus* and *P. aeruginosa* where the acetone extract was more active than the aqueous one. The strongest antibacterial activity of these two extracts was found against *B. spizizeni* with MID (31.2 µg/ml) while aqueous extract showed a lowest action as antibacterial agent against *S. aureus* with MID (500µg/ml).

Antioxidant activity: A stable free radical DPPH assay was used to evaluate the antioxidant activity of the extracts tested in a relatively short time. Hydrogen donating or radical scavenging properties of the examined plant extracts is based on its ability to change the colour of DPPH free radical from visible deep violet to yellow (Hinneburg *et al.*, 2006) which was quantitatively measured from the changes in absorbance spectro-photometrically at 517nm. This test is one of the known mechanisms by which antioxidants inhibit lipid oxidation (Barros *et a*l., 2007). The results obtained from this study showed that acetone crude extract of *S. grande* had higher inhibition activity than that of aqueous extract. Also, it was observed that the inhibition percentage of both extracts was concentration dependent (Figure 1). At a concentration of 1 mg/ml the DPPH inhibition percentages of acetone and aqueous extracts reached 79.65% and 70.32% respectively (Table 4). Inhibition of DPPH radicals above 50% is considered as significant for antioxidant properties of any compound (Sanches-Moreno *et a*l., 1998). Although the scavenging ability of both extracts were less than quercetin, the study showed that the extracts have the proton donating ability and could serve as a promising indicator of its potential antioxidant activity.

Table (2): Antimicrobial activity of the aqueous and acetone leaves extract of *Syzugium grande*

Test microorganism	Zone of inhibition	
	Aqueous extract	Acetone extract
Bacteria		
Escherichia coli O157: H7	-	-
Pseudomonas stutzeri ATCC 17588	++	++
Staphylococcus aureus ATCC 12600	+	+
Shigella boydii ATCC 9207	++	++
Bacillus spizizeni ATCC 6633	++	++
Pseudomoas aeruginosa	++	++
Fungi		
Aspergillus flavus	-	-
Aspergillus niger	-	-
Aspergillus terreus	-	-
Aspergillus fumigatus	-	-
Penicillium expansum	-	-
Yeast		
Candida albicans	-	-

Note: "-", no inhibition zone; "+", inhibition zone of < 10 mm diameter; "++", inhibition zones of > 10 mm diameter

Table (3) Minimum inhibitory dosage (µg/disc) of *Syzugium grande* extracts

Test microorganism	Aqueous extract	Acetone extract
Pseudomonas stutzeri ATCC 17588	125	125
Staphylococcus aureus ATCC 12600	500	250
Shigella boydii ATCC 9207	125	125
Bacillus spizizeni ATCC 6633	31.2	31.2
Pseudomoas aeruginosa	250	125

Note: All MID determinations were performed in triplicate, MID defined as the lowest dose for which no growth was observed around every tested disc.

The FRAP assay determined the ability of plant extracts to convert the ferric ion Fe^{3+} to Fe^{2+} (Gulcin *et al*., 2003) by donating an electron and, therefore, form Perl's blue which was monitored at wavelength of 700 nm. Figure (2) illustrates the reducing power of the plant extracts and reference drug at various concentrations. The increase in absorbance of the reaction mixture indicated the reducing power of the test samples.

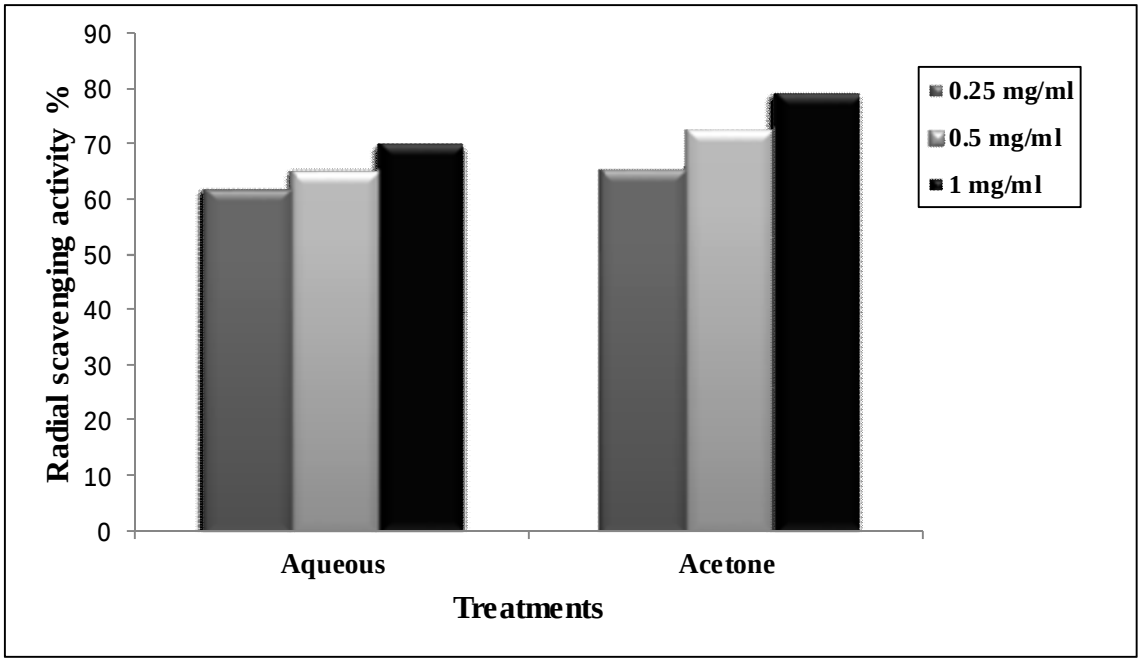

Figure (1): DPPH scavenging activity of *Syzugium grande* leaf extracts

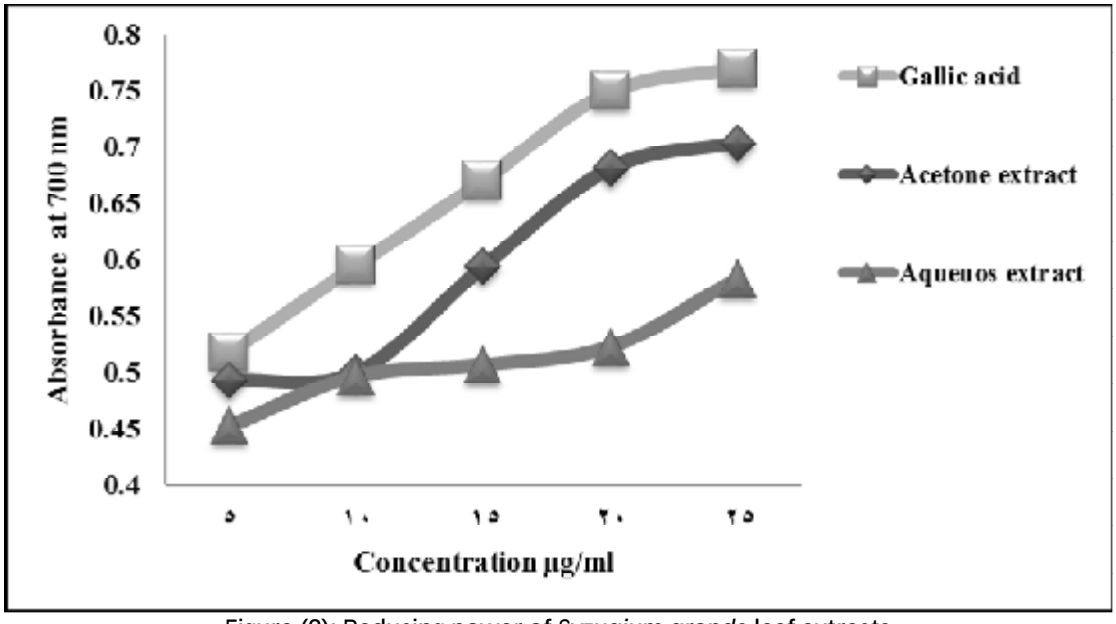

Figure (2): Reducing power of *Syzugium grande* leaf extracts

A higher absorbance value indicated a stronger reducing power. IC_{50} (inhibitory concentration) values (µg/ml) were calculated and indicated the concentration of sample at which the absorbance was 0.5 for reducing power (Table 4). Lowest IC_{50} indicated the highest antioxidant capacity. The result showed that the reducing power of the extracts increases with increasing concentration of the extracts (Figure 2) and this result is in agreement with finding of others (Oyedemi and Afolayan, 2011; Rameshkumar and Sivasudha, 2012). The IC_{50} values of iron reducing ability of aqueous extract, acetone extract and gallic acid were 12.84, 7.15 and 2.79µg/ml, respectively (Table 4). These data showed that these extracts displayed significant antioxidant activity (IC_{50}<15 µg/ml), but the reference substance was more potent than the *S. grande* extracts. It was reported that the reducing power might be attributed to the presence of reductones which are known to show antioxidant activity by breaking the free radical chain by donating a hydrogen atom (Shimada *et al*., 1992;

Elita *et al*., 2012). Xing *et al*. (2005) reported that reaction of reductones with certain precursors of peroxide prevent peroxide formation. It could be concluded from this study that the gallic acid and *S. grande* extracts are electron donors and could possibly reduce the oxidized intermediates of lipid peroxidation (Ordonez *et al*., 2006).

The current study found that the acetone extract of *S. grande* is more potent antioxidant than aqueous extract in both DPPH and FRAP methods. This finding suggests that compounds with antioxidant activity are found more in acetone extract than the aqueous one. However, the phytochemicals present in the *S. grande* extracts, which are responsible for these activities, need to be investigated. Saponins, triterpenoids, tannins, flavonoids and reducing sugars present in the plant extracts may be responsible for such activities. Several of such compounds are known to possess antimicrobial and antioxidant activity (Govindappa *et al.,* 2011). Some of these constituents mostly ellagitannin, castalagin, vescalagin and different kinds of pentacyclic triterpenoids, arjunolic acid, asiatic acid, friedelin and betulinic acid have been isolated from this plant (Nonaka *et al*., 1987; Manoharan, 2007). Djoukeng *et al*. (2005) reported the antibacterial activity of the triterpenoids of arjunolic acid and asiatic acid isolated from *S. guinneese* leaves extract. Recently, a survey of the biological activity of the Myrtales tannins revealed the antimicrobial and antioxidant effects of ellagitannis encountered in species of Myrtaceae (Yoshida *et al*., 2010). Hence, the activities observed in this study may be due to the presence of any of these compounds.

Table (4): Antioxidant activity of *Syzugium grande* leaf extracts.

Extract/References compounds	DPPH (%)	FRAP (IC_{50} µg/ml)
Aqueous extract	70.32	12.84
Acetone extract	79.65	7.15
Quercetin*	82.40	-
Gallic acid	-	2.79

Note: * The final concentration for quercetin was 25o µg/ml

In summary, our results clearly revealed that acetone and aqueous extracts from the leaves of *S. grande* possess significant antimicrobial and antioxidant activities. Phytochemical analysis showed the presence of different kinds of compounds such as saponins, tritepenoids, tannins and others. The extracts for this species can be considered as promising candidates for the development of an antimicrobial and antioxidant agents from natural sources. Further studies can be made to clarify the chemical nature of the bioactive principles responsible for these activities.

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