



## Antibiotic activity of *Plectranthus ornatus* Codd., a Traditional Medicinal Plant

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### ABSTRACT

The dichloromethane extract of *Plectranthus ornatus* Codd., a traditional medicinal plant, showed antibiotic activity with minimum inhibitory concentration (MIC) values of 0.4 mg.mL<sup>-1</sup> and 100 percent of biofilm inhibition against *Staphylococcus aureus* strains isolated from animals with mastitis infections. Based on these antibacterial activities, in addition to ethnopharmacological reports from healing men and farmers in Brazil, an herbal soap was produced from this active extract and was tested both *in vitro* and *in vivo*. *In vivo* assays conducted on these herbal soaps led to results similar to those previously conducted with the active extract. These results indicated the great potential of this plant for use as an excipient by preparing herbal antibacterial soaps as an alternative veterinary medicine aimed at controlling bovine mastitis infections on small Brazilian farms.

**Key words:** bovine mastitis, herbal soaps, *Plectranthus ornatus*, *Staphylococcus aureus*.

### INTRODUCTION

*Plectranthus ornatus* Codd. (family Lamiaceae), is an ornamental and traditional medicinal plant, popularly known in Brazil as “Boldinho” (Lukhoba et al. 2006). It is African native plant which was brought to the Americas by the Portuguese. Ethnopharmacological studies have recommended the use of this plant to treat digestive problems. However, *P. ornatus* leaves have been used by healing men and farmers, in some regions of Brazil, as an antibiotic for the treatment of skin infections

(Brasileiro et al. 2006, Diaz et al. 2010). Ethno veterinary practices using *P. ornatus* leaves have become very common as a viable and alternative treatment method, as these leaves are safe, easy to obtain, and inexpensive, they can be found on small farms, and they represent a less aggressive method to heal animals. Most farmers' approaches are based on empirical knowledge, having achieved significant results in cattle (Marinho et al. 2007). Therefore, some farmers in Brazil have reported the use of this plant to treat bovine mastitis, an inflammation of the mammary gland, which is characterized by physical, chemical, and bacteriological changes in the milk caused by

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*Staphylococcus aureus*. This bacterium produces biofilms, a group of cells that adhere to a surface, and is frequently embedded within a self-produced matrix of an extracellular polymeric substance that causes a dramatic decrease in its susceptibility to the antimicrobial agents. This formation is considered an important virulence factor that is frequently associated with clinical infections (Otto 2008).

The genus *Plectranthus* is rich in diterpenes and triterpenes. This class of compounds had shown antimicrobial activity (Wellsow et al. 2006, Stavri et al. 2009). The ability of this genus to produce antimicrobial metabolites has led to several phytochemical studies around the world through the isolation of several antimicrobial diterpenes, such as plectronatin A and two labdane derivatives, as well as plectronatins B and C (Rijo et al. 2014). The biological activities of the compounds from the genus *Plectranthus*, associated with their Ethnopharmacological use reported by healing men and farmers, aroused our interest in developing an herbal soap from this plant that can specifically be used by milkers on small farms to wash their hands and milking utensils.

## MATERIALS AND METHODS

### PLANT MATERIAL AND EXTRACTION

Samples of *P. ornatus* Codd. were grown in a greenhouse at the Horticultural Department of the Federal University of Viçosa (UFV), Minas Gerais, Brazil. The plants were propagated by cuttings rooted in pre-commercial substrate (Plantmax®). The plants were harvested 150 days after transplantation and growth. The plants were cut close to the ground, and the aerial parts (leaves) were dried at 40°C for 20 days in an air circulation oven, after which they were grinded into a fine powder. A voucher (39644) was deposited in the herbarium of the Department of Botany of UFV. Forty grams of powder were extracted using petrol, dichloromethane, and ethanol (400 mL) for 1 h at

room temperature, applying the ultrasound method for 10 days for each solvent. The solvents were removed under vacuum at 40°C and stored at 4°C.

### MICROORGANISMS TESTING

The bacterial resistant strains (3828, 4075, 4125, and 4158) were isolated from animals with mastitis infections, which were kindly provided by Embrapa Dairy Cattle - Laboratory of Milk Microbiology (Juiz de Fora, Minas Gerais, Brazil), and purchased from the American Type Culture Collection (ATCC, 29213). Bacteria were routinely cultured in brain heart infusion (BHI) at 37°C for 18 h prior to experiments, and cell concentration was adjusted to  $10^6$  CFU.mL<sup>-1</sup> by optical density at 600 nm. Stock cultures were maintained in BHI containing glycerol at -80°C.

### ANTIBACTERIAL SUSCEPTIBILITY TESTING

Hole-plate diffusion assay was initially performed to test the antibacterial activity of the crude extracts. To perform the assay, bacteria were cultivated overnight and a suspension containing  $10^6$  CFU.mL<sup>-1</sup> was spread on plates containing Müller-Hinton agar (Himedia®). Holes (5) of approximately 5 x 3 mm were made in the agar and were filled with 30 µL of the extract stock solution (50 mg.mL<sup>-1</sup>). After incubation at 37°C for 24 h, inhibition zones were measured in millimeters and compared to the controls. The controls were prepared with 30 µL of DMSO (negative control) and 5 mg.mL<sup>-1</sup> of ciclopirox olamine (Uci-Farma®). This antibiotic was used as the positive control due to its antibacterial properties (Jue et al. 1985). Inhibition zones greater than 7 mm were considered positive (Nascimento et al. 2000). Student's t-test ( $p < 0.05$ ) was performed to compare the results of the inhibition zones obtained from the extracts with the positive control. Tests were performed twice in triplicate.

#### MINIMAL INHIBITORY CONCENTRATION (MIC) ASSAY

Extract activity on bacterial growth was determined by applying the microdilution method (CLSI 2009). The microorganisms were initially cultured in Petri dishes containing BHI agar (Himedia®), which were incubated for 24 hours at 37°C. Subsequently, the isolated colonies were subcultured in Mueller-Hinton broth (Himedia®), which was incubated at 37°C for 180 rpm until the culture reached the exponential phase. These colonies were then diluted to an optical density corresponding to 0.5 in the McFarland standard scale ( $OD_{620} = 0.10$ ). Microplate holes were filled with 100 µL of Mueller-Hinton broth (Himedia®) extracts containing concentrations ranging from 0.1 mg.mL<sup>-1</sup> to 10 mg.mL<sup>-1</sup> and 10<sup>6</sup> CFU.mL<sup>-1</sup> bacterial suspensions. Whereas the DMSO could be bactericidal a control of microbial growth in this solvent was done with 100 µL of bacterial suspension and 100 µL of Müller-Hinton broth with DMSO at the highest concentration used in the preparation of the extract. After 24 h at 37°C, 4 µL of *p*-iodonitrotetrazolium (INT, I8377, Sigma®) was added to each well, and the plate was incubated for an additional 2 h at 37°C. A change in the color of the medium from yellow to pink-violet was used as an indication of bacterial growth. The minimal inhibitory concentration of the antibiotics was determined by the same procedure, with concentrations ranging from 0.1 µg.mL<sup>-1</sup> a 500 µg.mL<sup>-1</sup>.

#### BIOFILM INHIBITORY CONCENTRATION (BIC) ASSAY

Bacterial suspensions were inoculated on microplates containing 180 µL of BHI with different concentrations of the active extracts (MIC, 1/2 MIC, 1/4 MIC, 1/8 MIC, and 1/16 MIC) until reaching the final concentration of 10<sup>6</sup> CFU.mL<sup>-1</sup>. After, these concentrations were incubated at 37°C for 24 h. The supernatant was withdrawn, and the

wells were washed three times with 0.85% saline solution. The remaining bacterial mass was dried at 37°C for 15 min and stained with 200 µL of crystal violet 0.1% for 30 min. Wells were rewashed and dried as previously described, followed by the addition of 300 µL of ethanol and the measurement of absorbance at 630 nm. This test was performed twice in triplicate.

#### CHEMICAL PROFILING

##### *GC-MS analysis*

GC-MS analysis was carried out on a QP2010 Ultra Shimadzu system, employing the following conditions: column fused silica capillary column Rtx-5MS (30 m; 0.25 mm ID; 0.25 film µm). Helium (99.999%) was used as a carrier gas at a constant flow of 1 mL/min, and an injection volume of 1.0 µL was employed (split ratio of 10:1) at an injector temperature of 290°C and an ion source temperature of 200°C. The oven temperature was programmed at 80°C for 5 minutes, then increased to 285°C at a 4°C rate/min and kept at this temperature for 40 minutes. Mass spectra were taken at 70 eV, a scan interval of 0.5 s, and fragments from 35 to 700 Da. The MS transfer line temperature was 290°C.

##### *Identification of phytochemicals*

The compounds were identified using the Wiley 7 library database, together with the National Institute of Standards and Technology (NIST) library. The name, molecular weight, molecular formula, and area under peak of the test materials' components were ascertained.

##### *Production of the herbal soap*

The dichloromethane extract of *P. ornatus* (250 mg) was incorporated into a soap, which was formulated according to the BR 1005633-5 patent (Diaz and Pizziolo 2012). Later, the semi-solid mixture was poured into a mold and allowed to solidify. Soap

without extract was also produced to be used as a reference product.

#### ANTIBACTERIAL ASSAY OF THE HERBAL SOAP

##### *In vitro*

The agar-dilution method was employed in an *in vitro* evaluation. The herbal soap (1.0 g) was dissolved in distilled water (50 mL) to obtain a 2% suspension. The suspension was vigorously shaken to dissolve the soap, to disperse the foam, and to homogenize the suspension. Next, 1.0 mL of the soap solution was added to 20 mL of sterile molten culture media in Petri-dishes and allowed to set. One-hundred  $\mu\text{L}$  of suspension containing  $10^6$  CFU.mL<sup>-1</sup> of a resistant 4075 *S. aureus* strain was then streaked on the plates. After incubation at 37°C for 24 h, inhibition zones were compared to the control to observe the presence or absence of microbial growth.

##### *In vivo*

Gloves contaminated with *S. aureus* from animals with mastitis infection were used to perform the *in vivo* evaluation (*topical test performed according to our institutional ethics, protocol number 773.182*). The herbal soap (1.0 g) was dissolved in distilled water (100 mL) to obtain a 1% suspension. This suspension was then vigorously shaken to dissolve the soap, to disperse the foam, and to homogenize it. After, the gloves (12 pairs, 6 for each control soap and herbal soap treatment) were immersed in these solutions for 30 minutes. Before being immersed in the soap, the gloves that the milkers had used to milk the cow's udder, which had been contaminated with *S. aureus*, were swabbed, and the sample was placed in bottles with sterile normal saline solution. After being immersed in both the herbal and control soaps, the gloves were again swabbed, and the samples were placed in separate bottles with normal sterile saline solution. Aliquots

from the respective treatments were cultured on an agar plate at 37°C for 24 h to observe the presence or absence of microbial growth.

#### RESULTS AND DISCUSSION

According the results observed in the assays, no differences were observed between the two crude extracts of the plant in relation to the antimicrobial activity of the solvent used in this study. The petrol and dichloromethane crude extracts were active, but the dichloromethane was slightly more active than the petrol extract (Table I).

By analyzing the MIC values obtained for the petrol and dichloromethane crude extracts (Table II), it could be concluded that, these values are lower than some values previously found in extract with antimicrobial activity (Duarte et al. 2004, Virtuoso et al. 2005). Based on the MIC values, extracts can have strong (0.05 to 0.5 mg.mL<sup>-1</sup>), moderate (0.6 to 1.5 mg.mL<sup>-1</sup>), or weak activity (> 1.5 mg.mL<sup>-1</sup>) (Aligiannis et al. 2001). Using these criteria, the extracts of *P. ornatus* can be considered to be between the strong and moderate inhibitors for the strains used in this study. However, when compared to the positive control, the MIC values were still low.

A biofilm is a group of cells that adhere to any surface and are frequently embedded within a self-produced matrix of an extracellular polymeric substance. This wrapping makes the microorganism highly resistant to antibiotics and difficult to treat. *Staphylococcus* spp. is known as a commensal agent that can be found on the skin and mucosal surfaces and is the most frequent cause of infections associated with biofilms (Otto 2008). In the present study, the active extracts were evaluated for anti-biofilm activity in subinhibitory concentrations (2 x MIC; MIC; ½ MIC; ¼ MIC; ⅛ MIC and 1/16 MIC) to evaluate Biofilm Inhibitory Concentration (BIC) on pre-formed biofilms. The

TABLE I  
*Staphylococcus aureus* inhibition zones of extracts from *Plectranthus ornatus* leaves (mm).

Crude extracts	<i>S. aureus</i>							
	ATCC29213	3828	4075	4125	4158	Inhibition zones $\pm$ SD	<i>p</i> value t test (0.05)	<i>p</i> value t test (0.05)
Petrol	14.3 $\pm$ 1.24	14.8 $\pm$ 1.10	13.0 $\pm$ 0.80	14.1 $\pm$ 0.80	15.8 $\pm$ 0.90	0.001	0.003	0.005
Dichloromethane	15.7 $\pm$ 1.24	17.7 $\pm$ 2.10	15.7 $\pm$ 0.94	15.1 $\pm$ 1.24	17.9 $\pm$ 1.20	0.001	0.003	0.001
Ciclopirox olamine <sup>a</sup>	25.0 $\pm$ 0.10	23.0 $\pm$ 0.10	23.0 $\pm$ 0.47	25.0 $\pm$ 0.45	25.0 $\pm$ 0.50	0.003	0.001	0.001
DMSO <sup>b</sup>	0.00 $\pm$ nd	0.00 $\pm$ nd	0.00 $\pm$ nd	0.00 $\pm$ nd	0.00 $\pm$ nd	nd	nd	nd

<sup>a</sup>Positive control; <sup>b</sup>Negative control; \* Inhibition zones are the mean including border (7 mm) diameter  $\pm$  standard deviation.

BIC values were between 2 x MIC (Supra MIC), MIC, and ½ MIC (Sub MIC) (Table III).

The results showed that active extracts are able to inhibit the formation of biofilms. Concentrations corresponding to the Supra MIC and MIC of dichloromethane extracts were enough to inhibit approximately 100% of biofilms formed by *S. aureus* ATCC 29313, 3828, 4075, and 4158. According to table III, the best BIC value (100% inhibition), obtained for the dichloromethane extract against *S. aureus*, can indicate that this extract inhibit bacterial film formation in the initial phase of adhesion and formation of biofilms. This value was similar to those found for antibiotic substances reported in prior literature (Nostro et al. 2007). The same was observed for Supra MIC and MIC of the crude petrol extract on *S. aureus* 3828 and ATCC 29313. Concentrations corresponding to Sub MIC of both extracts were enough to inhibit approximately 30-80% of the biofilms formed by *S. aureus* ATCC 29313, 3828, 4075, 4128, and 4158. MIC and BIC results justify the choice of the dichloromethane extract as an antiseptic agent in the form of an herbal soap.

The *in vitro* results demonstrated that the herbal soap obtained from a dichloromethane crude extract reduces the bacterial load to 89  $\pm$  3.0 CFU (Figure 1 a, b). These results are in agreement with those observed by small farmers.

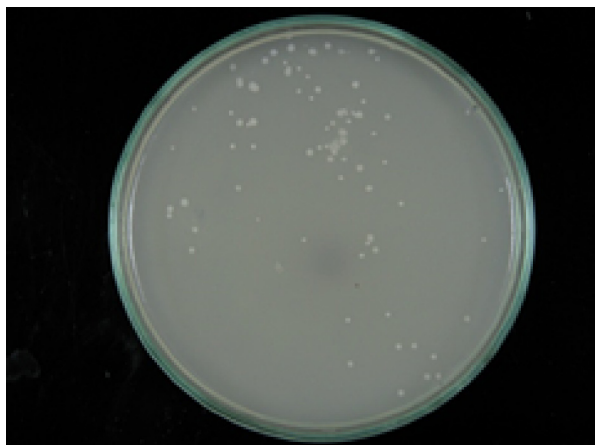
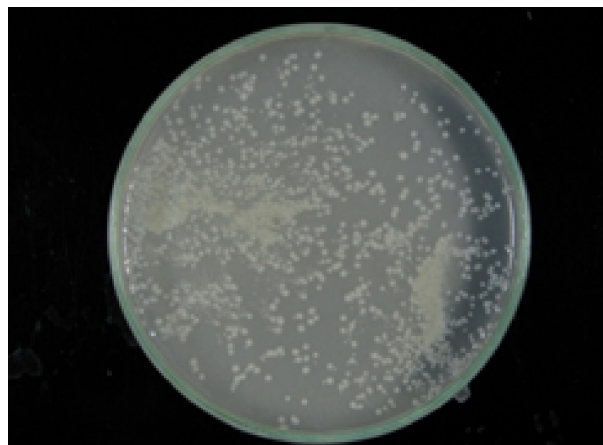
According to the results observed in the *in vivo* evaluation (Figure 2 a), microbial growth was observed in the Petri dishes after immersing the milker's gloves in the control soap (Figure 2 b). By contrast, in figure 2 (c) microbial growth was not observed in the Petri dishes after immersing the milker's gloves in the 1 % suspension of herbal soaps with an active extract of *P. ornatus* for 30 min.

The chemical constituents in the petrol and dichloromethane extracts of the aerial parts of *P. ornatus* belong to different chemical groups, according to the GC/MS analysis of the plant extracts.

**TABLE II**  
MIC values (mg.mL<sup>-1</sup>) of extracts from *Plectranthus ornatus* leaves against *Staphylococcus aureus* strains.

Crude extracts	<i>S. aureus</i> MIC (mg.mL <sup>-1</sup> )				
	ATCC29213	3828	4075	4125	4158
Petrol	0.6	0.4	0.5	0.6	0.6
Dichloromethane	0.6	0.5	0.7	0.6	0.5
Ciclopirox olamine <sup>a</sup>	0.05	0.05	0.05	0.05	0.05

<sup>a</sup>Positive control.



**a** - Control (soap without dichloromethane extract of *P. ornatus*).

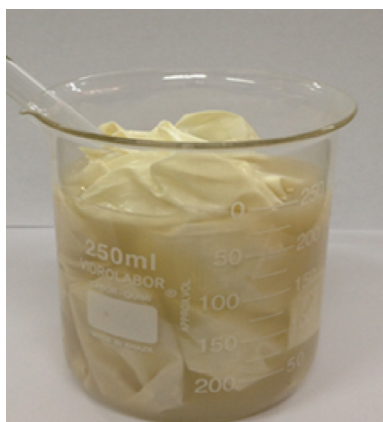
**b** - Herbal soap with dichloromethane extract of *P. ornatus* (89±3.0 CFU).

**Figure 1** - *In vitro* antibacterial activities of herbal soap produced with the active extract of *P. ornatus*. Tests were performed in triplicate.

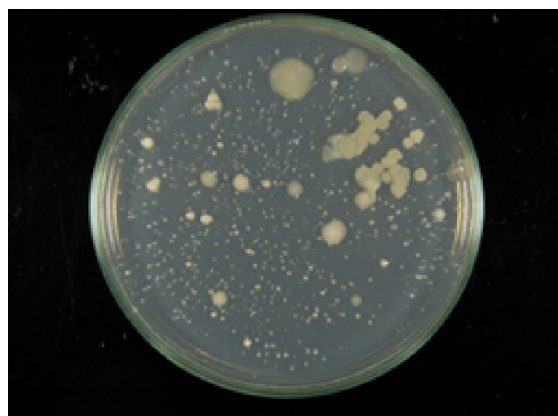
**TABLE III**  
BIC values obtained from the actives extracts of *Plectranthus ornatus* on *Staphylococcus aureus* strains.

Crude extracts	MIC Concentration	<i>S. aureus</i> % of inhibition <sup>b</sup>				
		ATCC29213	3828	4075	4125	4158
Petrol	Supra MIC*	100	100	70	80	50
	MIC**	100	100	50	50	50
	Sub MIC***	50	50	50	30	50
Dichloromethane	Supra MIC*	100	100	100	100	100
	MIC**	100	100	100	50	100
	Sub MIC***	50	50	80	50	50
Ciclopirox olamine <sup>a</sup>		100	100	100	100	100

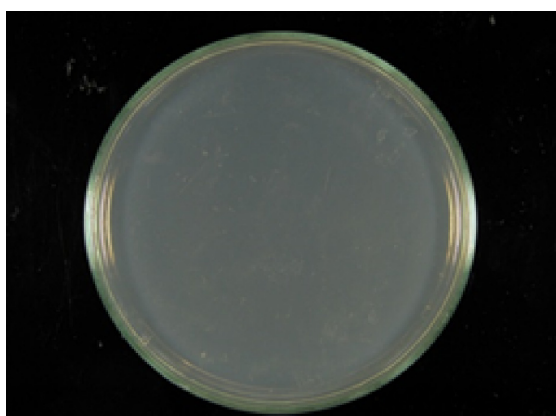
<sup>a</sup>Positive control; <sup>b</sup>The values of biofilms BIC obtained were between 2xMIC\*, MIC\*\*, and ½ MIC\*\*\*.



**a** - Milker's gloves immersing in the soap solution.



**b** - Result immersing the milker's gloves in the soup control (without active extract).



**c** - Result after the milker's gloves had been immersed in the herbal soap (with active extract).

**Figure 2** - *In vivo* antibacterial activities of the herbal soap with the active extract of *P. ornatus* in the milkers' gloves.

This analysis revealed the presence of compounds like diterpenes, triterpenes, and flavonoids, which are known to exhibit antimicrobial activities that inhibit bacterial growth (Table IV). Therefore, we can assume that the antimicrobial activity observed in crude extracts may well be associated with these types of compounds (Figure 3) (Rijo et al. 2011, Roberto et al. 2007).

### CONCLUSIONS

According to the results obtained in this study, the herbal soap from *P. ornatus* can be used as an antiseptic agent in pre and post-dipping without drawbacks of disinfectants formulated based on iodine or sodium hypochlorite. These can also be used as adjuvant, such as disinfectants for disease

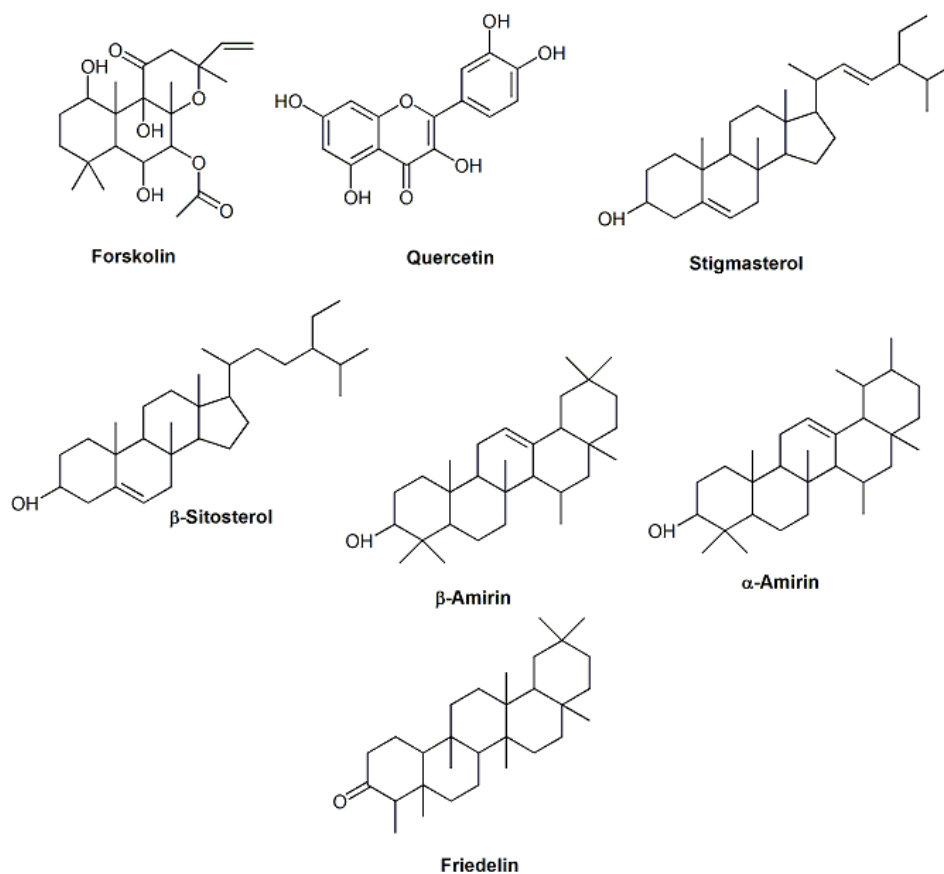
control. These herbal soaps demonstrated a high level of inhibition against *S. aureus* from cows' udders and indicates the potential of this plant as an excipient in the production of antiseptic soaps to fight bovine mastitis infections, especially on small farms. Our results validate the use of this plant by small farms to control this disease.

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**TABLE IV**  
**Main compounds identified by GC-MS in dichloromethane extract of *P. ornatus* leaves that present previously antibacterial activity.**

No.	RT (min)	Name of the compound	Molecular Formula	MW	Peak area (%)	References
1	16.08	Forskolin	C <sub>22</sub> H <sub>34</sub> O <sub>7</sub>	410	80	Rijo et al. 2011
2	17.91	Quercetin	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	302	76	Walker et al. 2009, Manríquez-Torres et al. 2007, Mattana et al. 2010
3	19.09	Stigmasterol	C <sub>29</sub> H <sub>48</sub> O	412	86	Edilu et al. 2015, Manríquez-Torres et al. 2007, Yinusa et al. 2014, Gowdu Viswanathan et al. 2012
4	19.70	β-sitosterol	C <sub>29</sub> H <sub>50</sub> O	414	82	Edilu et al. 2015, Manríquez-Torres et al. 2007
5	20.22	β-amirin	C <sub>30</sub> H <sub>50</sub> O	426	84	Mattana et al. 2010
6	20.77	α-amirin	C <sub>30</sub> H <sub>50</sub> O	426	85	Mattana et al. 2010
7	22.48	Friedelin	C <sub>30</sub> H <sub>50</sub> O	426	83	Gowdu Viswanathan et al. 2012, Mokoka et al. 2013, Annan et al. 2009



**Figure 3** - Compounds identified by GC-MS in dichloromethane extract of *P. ornatus* leaves.



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