

Chemical Composition, Antimicrobial and Antioxidant Activities of the Essential Oil of *Bursera graveolens* (Burseraceae) From Perú.

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ABSTRACT

Palo santo oil is an important component of the traditional Peruvian medicine and is known to have antibacterial properties. This essential oil is extracted from fallen branches of the tree *B. graveolens*. To validate some of its properties, the antimicrobial and antioxidant activities of palo santo oil obtained from northern Peru were first examined. Based on the measurements of the diameter of growth inhibition (Agar Disc Diffusion method), antimicrobial activities ranging from moderate to high were revealed against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella choleraesuis* and *Candida albicans*. This study confirms that the essential oil of *B. graveolens* demonstrates significant antimicrobial properties. The antioxidant activity was determined by using 2,2-diphenyl-1-picrylhydrazil (DPPH), the result, $IC_{50} = 545.25 \mu\text{g/mL}$, showed a weak antioxidant activity. The total phenolic content was found to be $5.71 \pm 0.16 \text{ mg/100 g}$ using the Folin – Ciocalteu method. About the composition of this essential oil, it was submitted to hydrodistillation and analysis by gas chromatographic equipped with a flame ionization detector (FID). Twenty-six compounds were identified, representing 67.1 % of the total oil. The most abundant compound was α -Terpinene (31.57%). Thus, different types of monoterpenes were the predominant constituents of the essential oil.

Keywords: *Bursera graveolens*, Antimicrobial Activity, Antioxidant Activity, Total Phenolic, Terpinene.

INTRODUCTION

The study of the chemical components of essential oils from aromatic and medicinal plants is proving to have antioxidant, antibacterial, antiviral, antimycotic, anti-inflammatory, and anti-tumor properties (Zaouali *et al*). The *Bursera graveolens* better known as palo santo or holy branch is a tree located in the dry forests on the north coast of Perú and they extend by the departments of Piura, Tumbes, Lambayeque and small portions of Cajamarca and La Libertad, and grow in generally sandy soils. This tree is known in traditional Peruvian medicine for helping ease symptoms of flu and asthma, dermal conditions, in addition also as anti-inflammatory, soothing and relaxing proper-

ties. The holy stick, of fragrant wood, is used as incense in the religious processions of the coast and mountain of this country. Anti-inflammatory properties has been found in extracts of the bark of this plant (Manzano *et al*) and references to the oil chemical composition is poor. Studies of essential oil of stems and leaves of palo santo tree showed a large content of limonene (Leyva, *et al*). Based on a previous study of the composition and characteristics of this plant, the aim of this study was to evaluate the antioxidant and antimicrobial activities of *B. graveolens*.

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MATERIALS AND METHODS

All the research was made in the Laboratories of physical-chemistry and microbiology of La Molina Calidad Total Laboratorios – UNALM, which is in Lima, the capital of Perú.

Plant material

Samples of dry wood logs were collected, in June 2011, belonging to Province of Department of Piura in Perú. This plant was used, cut and ground.

Extraction of essential oil

In order to observe if the method of extraction of essential oil influenced the antimicrobial activity, the ground wood were submitted to two methods of extraction: steam distillation and hydro-distillation type Clevenger (Bruneton, 1991), both of methods took 3 h. Oils extractions were obtained by separation from the aqueous phase, dried with anhydrous sodium sulphate, filtered and stored in dark vials at 4°C for later analysis. Solutions of 150 µL of oil in 100 mL of n-hexane were prepared before GC.

Chromatographic analysis

GC analysis was carried out according to using for essential oils. Varian 450 gas chromatograph equipped with a flame ionization detector and attached to supelco-wax TML 10 column (30 m x 0.53 mm) was used. Two micro-liter of the sample, split 1:20 (dissolved in hexane as 150 µL of oil in 100 mL) was injected into the system. The constituents were identified by comparing their relative retention time with those of authentic compounds injected in the same conditions.

Antimicrobial activity

The antimicrobial activity of essential oils of *B. graveolens* (obtained by steam distillation and hydro-distillation type Clevenger methods) was determined through the agar disc diffusion.

Microbial strains

The antimicrobial activity was tested using *B. graveolens* oil against *taphilococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes wild*, *Clostridium perfringes* ATCC 13124, *Escherichia coli* ATCC 25992, *Salmonella choleraesuis* ATCC 14028 and yeast *Candida albicans wild*. Bacterial strains were cultured by 24 h at 36 ± 1°C in nutrient broth.

Disc diffusion method

The antimicrobial activity of oil was determined through the agar disc diffusion method. All tests were performed in twelve repetitions. The disc diffusion

method was made according to Sacchetti *et al.* (2005). Tryptic soy agar (TSA) was distributed into sterilized Petri dishes with a diameter of 9 cm (15 mL) and Trypticase Soy Broth (TSB) was sterilized in autoclave at 121 °C × 15 min. For the yeast, Caldo Sabourud was used. Biochemical and confirmatory tests of the microorganisms were carried out before each use. The microorganisms were inoculated in TSA agar medium at 45° C. The inoculum of bacterial strains used was 1x10⁸ CFU/mL which was poured in TSA. The filter paper (6 mm in diameter, Whatman N ° 4) were individually impregnated with 4 µl of *Bursera graveolens* essential oil and then placed onto the agar plates. Before incubation, all Petri dishes were kept in the refrigerator (4°C) for 2 h and incubated after at 36 ± 1° C for 24 hr for bacteria growth. After incubation the diameter (mm) of the inhibition zones of the growth of microorganisms were measured including the diameter of discs. The activity was also evaluated according to Duraffourd C *et al.* (1986): Null (-), if it was less than or equal to 8 mm; Limit sensitivity (sensitive = +) from 9 to 14 mm; (very sensitive = ++) from 15 to 19 mm and highly sensitive (S.S = +++) if it was equal to or greater than 20 mm. Oxytetracycline (gram-positive bacteria), chloramphenicol (gram-negative bacteria), fluconazole (yeast) served as a positive control.

Antioxidant Activity

The antioxidant activity was assessed by 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Sigma-Aldrich brand. This method developed by Brand-Williams *et al.* (1995), is that the DPPH free radical, is highly reactive due to has a free electron, (violet blue color with methanol) which is decolorized to pale yellow color by reaction of an antioxidant substance. Both a methanolic solutions of DPPH (20 µg/mL) and essential oil of *Bursera graveolens* (800 µg/mL) were prepared. Neutralization reactions were made to these methanolic solutions, a Constance volume of DPPH radical and volumens of essential oil a different concentrations. The mixtures were then shaken and allowed to stand at room temperature in the dark. After 30 min, the decrease in absorbance a 517 nm was measure against a blank (methanol solution) by using a Shimadzu Spectrophotometer model UV-160 A. The standard antioxidant substance used as reference control was Ascorbic Acid, Fisher brand. Essential oil of *B. graveolens* providing 50 % inhibition (IC₅₀) was calculated from the plot of absorbance against essential oil concentration. Test were carried out in triplicate.

Assay for total phenolics

Total phenolic constituents of essential oil of *Bursera graveolens* was determined by literature methods involving

Folin-Ciocalteu's phenol reagent and gallic acid standard. Absorbance values were measured at 760 nm. The same procedure was repeated for all the standard gallic acid solutions (1 – 1000 $\mu\text{L}/0.1\text{ mL}$).

Total phenols of the essential oil, as gallic acid equivalent, was determined by using the absorbance value of the essential oil measured at 760 nm. Gest was carried out in triplicate and gallic acid equivalent value was reported as mean \pm SD of triplicate.

Statistical analysis

The results of antioxidant and antimicrobial activities were stated in mean \pm standard deviation using Minitab 17th edition statistical software. The variations of different inhibition zones by each microorganism were made by two methods (steam distillation and hydro-distillation type Clevenger) for each microorganism. All of them were tested by a variance analysis (Anova procedure) at $p < 0.05$. The significance of differences between means was determined by Tukey at $p < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

Dry wood logs were used, cut and ground. The essential oil was obtained by two methods of extraction: steam distillation and hydro distillation type Clevenger. Each of them took 3 h and both of them extractions were obtained by separation from the aqueous phase, dried with anhydrous sodium sulphate and filtered. The essential oil was stored at 4 °C protected from light, for later analysis.

The best yield, was obtained with hydro distillation by the Clevenger method. It was 3.59 % v/w. The yield by the steam distillation method was 1.63%. on the basis of the weight of dried plant material.

Twenty-six compounds consisting up to of 67.1 % of the essential oil were identified by GC (Table 1). The plant essential oil consisted of α -Terpinene (31.57%), isocaryophyllene (6.61%), piperitone (5.61%), β -trans-ocimene (4.93%), 6-allyl-o-cresol (4.63 %), 1-tetradecen 3.27%, durenol (2.36 %), linalol (1.53%), 3-octanol (1.1%) germacren (0.92 %). Thus, different types of monoterpenes were the predominant constituents of the essential oil. This essential oil presents as a majority component to Monoterpene (α -terpene), followed by a sesquiterpene (isocaryophyllene) and aliphatic alcohol (3-octanol) also be present.

These results differ totally from the components that are obtained from the leaves and stems of this same plant with limonene as a priority component (Manzano, P *et al.* 2009). These differences are mainly due to the con-

ditions of climate, temperature and geographical area. An analysis of constituents groups of essential oil of *B. graveolens*, showed oil possesses 37.5% of monoterpene hydrocarbons, 15.28% of oxygenated monoterpenes, and 8.55% of sesquiterpene hydrocarbons. Have not presented oxygenated sesquiterpenes. The α -Terpinene is the largest amount, following of Ocimene. Studies by Fernandez (2008) showed that β -Trans ocimene presented antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis*.

Thymol, although present in small quantities, is a phenolic compound well known for its antioxidant potential present in essential oils of plants (Aeschbach *et al.*, 1994; Baratta, Deans, Biondi, & Ruberto, 1998; Lagouri, Blekas, Tsimidou, Kokkini, & Boskou, 1993. Antimicrobial effect also is reported (Castro Luna, 2008). *B. graveolens* essential oil may have mutagenic properties due to the presence of epoxy compounds. This group is very unstable and compounds are acting under a system of mutual collaboration or synergism (Laouri *et al.* 1993).

Amount of total phenolic

Based on the absorbance value of *B. graveolens* essential oil reacting with Folin – Ciocalteu phenol reagent and compared with the absorbance values of standard solutions of gallic acid, total phenolics content of the essential oil was estimated as 5,71 mg /100 g. This value indicates that each 100 grams of essential oil contains phenolic compounds equivalent to about 5,71 mg of pure gallic acid.

Antioxidant Activity

The results are present in Table 2. DPPH radical scavenging activity by the Ascorbic Acid standard and *Bursera graveolens* essential oil were expressed as IC₅₀ values. The concentrations of analytes required for the conversion of the DPPH radicals by more stable molecule required a high concentration of essential oil of *Bursera graveolens* IC₅₀ = 545.25 $\mu\text{g}/\text{mL}$ against ascorbic acid IC₅₀ = 1.47 $\mu\text{g}/\text{mL}$. This large value of concentration required by oil as an antioxidant is in accordance with low phenolic compounds content which was reflected in its Folin-Ciocalteu test. Although the antioxidant metabolites in plants are studied mainly based on their polyphenolic structures, there is a literature that reports that antioxidant activity is also found in essential oils whose components are mainly terpenoids and terpenoids. The composition of the essential oil of palo santo does not predominate the oxygenated monoterpenes to which the antioxidant power is attributed to them. Choi *et al.* (2000) report that linalool has a good antioxidant capacity against the radical DPPH, however, this mole-

cule is present in the essential oil of *B. graveolens* in very low percentage, only 1.53%. B-myrcene, β -pinene and limonene also show anti-radical activity against DPPH between 8.8 and 16.5% uptake; in *B. graveolens* essential oil these substances are present in a low percentage of 0.02, 0.28 and 0.19% respectively.

Cuentas R, et al (2008) indicate that it is important to emphasize that the antioxidant activity is not only given by the sum of the antioxidant capacities of each component, it also depends on the microenvironment in which the compound is found, being able to interact with each other, producing synergistic or inhibitory effects.

Antimicrobial activity

The antimicrobial (anti-bactericidal and antifungal) activity of the essential oil *Bursera graveolens* obtained by steam distillation and Clevenger was determined by the Agar Diffusion Disc method against a panel of 7 microorganisms and their potency were assessed qualitatively and quantitatively by the present or absence and measuring of inhibition zones, zone diameters. The results are given in Table 3.

These results show that the pure essential oil of *B. graveolens* has substantially antimicrobial activity against all bacteria and yeast evaluated. Likewise, the antibiotics chloramphenicol, oxytetracycline and fluconazole were sensitive to gram positive bacteria, gram negative bacteria and fungi respectively.

The values of the microbial growth inhibition halos shown in Table 3 show slightly greater growth of inhibition halo by obtaining the essential oil by the Clevenger method than by the method of steam trapping except for the strain *Candida albicans* showing a greater result of the inhibition halo (17.0 ± 1.7 mm) with the steam trapping method. The anaerobic bacterium *C. perfringens*, according to Duraffourd would be classified as extremely sensitive (+++) to the oil, with a growth of the zone of inhibition corresponding to 24.3 ± 1.5 mm. *C. albicans* with a halo of inhibition of 14.6 ± 1.8 mm and *B. cereus* with 13.3 ± 1.3 mm correspond to a sensitivity classified as very sensitive (++). The action of the essential oil was moderate with a sensitivity (+) in gram positive strains, *S. aureus* and *L. monocytogenes* (9.80 ± 1.3 and 10.4 ± 1.1 mm respectively) and gram negative strains, *S. choleraesuis* (9.9 ± 0.9 mm). The antimicrobial activity of the essential oil *B. graveolens* is highlighted, by the two methods: vapor drag and Clevenger. The values obtained are higher than the antimicrobial activity of other essential oils. Tepe, B. (2005), reported that *C. albicans* was sensitive to the essential oil *Salvia tomentosa* but with a halo of inhibition of microbial growth equal to 12.5 mm. In the case of the bacteria studied, the

selected microorganisms are both morphologically and physiologically different then the values obtained are representative of the antibacterial activity that *Bursera graveolens* oil can present. In general, the antimicrobial activity of *B. graveolens* essential oil was more pronounced against gram positive bacteria than gram negative bacteria, a fact previously observed with other essential oils of other plant species (Nostro A, et al), (Outara B, et al). This generally higher resistance between gram negative bacteria has been attributed to the presence of their outer phospholipid membranes, almost impermeable to the lipophilic compounds present in the microorganism.

a Inhibition zone in diameter (mm) around the impregnated discs.

b Antibiotics : CL, chloramphenicol; OX, oxytetracycline; FL, fluconazole

c NA (not applicable)

The results show that the essential oil has an inhibitory activity against *C. perfringens* higher than those reported by other oils. In addition, *C. perfringens* were found to have antimicrobial activity against extracts of *Allium sativum* (garlic), *Coriandrum sativum* (coriander), *Eugenia caryophyllata* (clavode olor), *Origanum vulgare* (Oregano), *Rosmarinus officinalis* (Rosemary) and *Thymus vulgaris* (thyme), reporting inhibition halos of 19.4, 15.2, 14.9, 0, 14.4 and 0 mm respectively. Other studies on essential oils of *Origanum vulgare* and *Thymus vulgaris*, if they produced an inhibitory effect on *C. perfringens*; And also on other enteric bacteria such as *E. coli*, *P. aeruginosa*, *S. aureus*, *B. cereus*, *L. monocytogenes*, and *C. botulinum*, producing inhibition halos ranging from 7 to 24 mm.

The inhibitory effect of essential oils has been well studied and is related to its composition. Substances such as thymol, carvacrol, linalool, cinnamic aldehyde, allicin and eugenol have proven inhibitory capacity. Its lipophilic action has the ability to pass cell membranes, break down polysaccharides, fatty acids and lipids, permeabilizing the cell membrane; Which leads to the loss of ions, to the collapse of the proton pump and to the decrease in ATP, which inevitably leads to cell death (Mattisek et al). The essential oil of *B. graveolens* does not present any of these components representatively however there is remarkable antimicrobial activity.

No reports have been found of the resistance of the strains to the essential oil under study but the inhibitory activity must be produced by synergistic effects between the components of the oil obtaining a significant activity and in some cases greater than other oils studied. Thus Candan F. (2003) reported antimicrobial activity in essential oil of *Achillea millefolium subsp.* *Afan millefolium*

Table 1: Chemical composition of essential oil of *B. graveolens* (%)

N°	T.R. , min	Compound	Composition (%)
14	4,26	β-Pinene	0.03
15	4,68	β-Pinene (↑S)	0.28
16	5,18	β-Mircene	0.02
18	5,79	α – Terpinene	31.57
20	6,14	Limonene	0.19
21	6,86	β – Felandrene	0.48
22	7,30	β - trans Ocimene	4.93
25	10,34	Octyl acetate	0.08
29	12,22	3-Octanel(Amileticrabinol)	1.1
36	15,03	2S-trans-mentone	0.51
37	15,47	Copaene	0.42
38	15,67	2R-Cis-Mentone	0.02
43	16,6	Germacrene D	0.92
44	16,86	Linalool	1.53
49	18,45	Isocaryophillene	6.61
57	20,66	α-Caryophillene	0.46
62	22,07	1-tetradecene	3.27
64	22,31	Germacrene B	0.14
65	22,57	Piperitone	5.61
66	22,72	Epóxide	1.35
74	27,43	Melonal	0.22
75	28,83	Epóxide	0.22
76	29,52	6-Ally-O-cresol	4.63
81	38,05	Thymol	0.47
83	39,86	Durenol	2.36

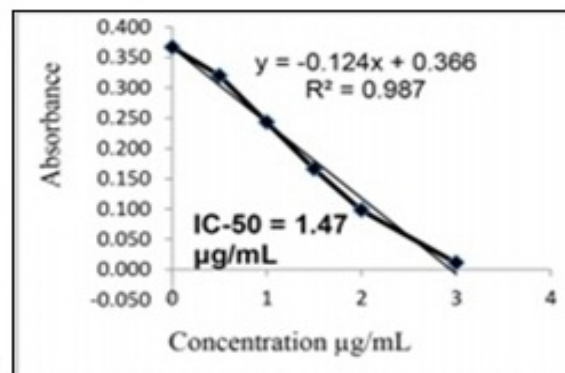
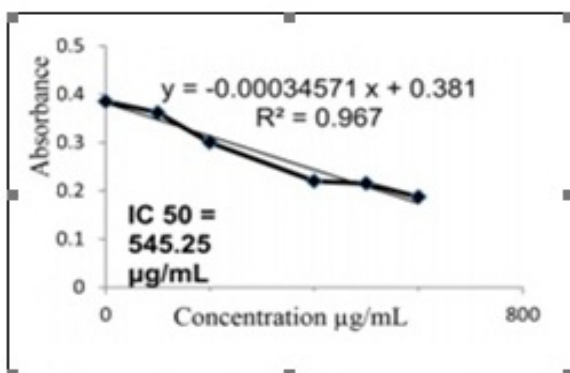
Table 2: Inhibition concentrations of Ascorbic Acid standard and sample of *B. graveolens* essential oil against DPPH

Table 3: Antimicrobial activity of *Bursera graveolens* essential oil

Microorganism	Type	Essential oil obtained by Clevenger (4 µL/disco) DD ^a	Essential oil obtained by steam distillation (4 µL/disco) DD	CL ^b	OX	FL
Gram - Positive						
<i>S. aureus</i>	ATCC 25923	9.8 ± 1.3	9.3 ± 0.9	NA ^c	17.8 ± 1.5	NA
<i>B. cereus</i>	ATCC 11778	13.3 ± 1.3	12.8 ± 0.9	NA	27.2 ± 1.1	NA
<i>L. monocytogenes</i>	Wild	10.4 ± 1.1	8.1 ± 0.8	NA	21.4 ± 2.4	NA
<i>C. perfringens</i>	ATCC 13124	24.3 ± 1.5	15.8 ± 0.9	NA	36.5 ± 2.0	NA
Gram - Negative						
<i>E. coli</i>	ATCC 25922	10.2 ± 0.9	9.4 ± 0.9	20.6 ± 1.0	NA	NA
<i>S. choleraeuis</i>	ATCC 14028	9.9 ± 0.9	9.8 ± 1.2	22.0 ± 1.6	NA	NA
Fungi						
<i>C. albicans</i>	Wild	14.6 ± 1.8	17.0 ± 1.7	NA	NA	18.3 ± 2.6

against *S. aureus*, *B. cereus*, *E. coli* and *C. perfringens* with microbial growth inhibition halos equal to 8, 10, 0 and 12 mm respectively, values lower than those reported by palo santo of 9.8, 13.3, 10.2 and 24.3 mm (Table 3). A recent study by Benites J *et al.* determined the antibacterial activity of essential oil in leaves and stems of *Senecio atacamensis* Phil of Chile against *S. aureus* and *E. coli* reporting for the former a HICM = 10.5 ± 0.7 mm, similar to that reported in the essential oil of palo santo (HICM = 11.0 ± 0.5 mm) whereas for the latter it did not report antibacterial activity, whereas in the essential oil of *B. graveolens* an HICM was obtained = 10.0 ± 0.8 mm. The essential oil of *Senecio atacamensis* Phil presented among its main components the α -terpinene, α -phellandrene and p-cymene while the palo santo essential oil reported α -terpinene as its main component.

Essential oils are very complex mixtures containing a wide variety of components. Thus, it can be argued that the antimicrobial effect is observed as a result of the activity of the active compounds, as well as the possible synergistic effects between the minor components that accompany it. The mechanism of action of terpenes is not fully understood, but is speculated to involve membrane disruption of lipophilic compounds (Cowan, 1999).

CONCLUSION

The essential oil of *B. graveolens* presents a strong antimicrobial activity against all the microorganisms studied. Especially against *Clostridium perfringens* with a diameter of inhibition equal to 24.3 mm and against the yeast *Candida albicans* with an inhibition diameter

equal to 17 mm. The oxidant activity is low with an IC₅₀ of 545.25 µg / mL.

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CONFLICT OF INTEREST

None

ABBREVIATION USED

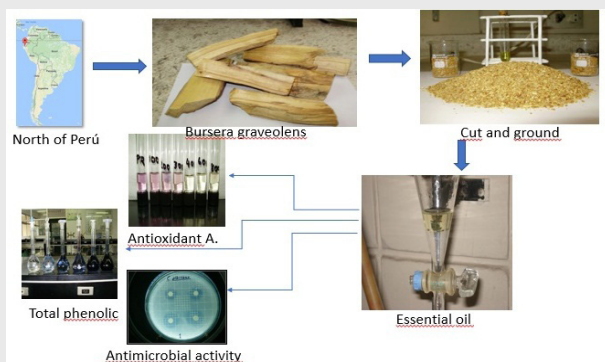
TSA: Trypticase soy agar; TSB: Trypticase soy broth; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; HICM: zone of inhibition of microbial growth; IC₅₀: half maximal inhibitory concentration; CL: chloramphenicol; OX: oxytetracycline; FL: fluconazole; FID: flame ionization detector.

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PICTORIAL ABSTRACT



SUMMARY

- Essential oil of *Bursera graveolens* or better known as palo santo from Perú obtained by two methods of extraction, presents a strong antimicrobial activity against a battery of seven microorganisms evaluated, while a weak antioxidant activity and a low total phenolics were recognized.
- The results of antimicrobial activity were stated using Minitab statistical software and Anova procedure.

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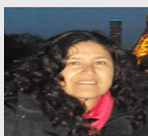
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