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Phytochemical Characterisation and Antibacterial Activity of Propolis on Cariogenic Microrganisms

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AMA and CNF conceived the project, designed the study, supervised the protocol and laboratory analyses and wrote the first draft of the manuscript. Author RT wrote the protocol, performed the laboratory and statistical analyses. Authors BK and ETF performed the laboratory and statistical analyses. Authors SN and CP drafted the manuscript and carried out literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Dental caries is also known as tooth decay is as a result of the softening of hard dental tissues from factors of multiple origins, with dental plaque bacteria being one of the most important factors. Due to the resistance of microorganisms to common antibiotics, modern medical sciences had been looking for new approaches in the treatment and prevention of oral diseases.
Objective: This study aimed to phytochemically characterise, evaluate the antibacterial activity of propolis on bacteria responsible for dental caries.
Methodology: This was an experimental study that took place in the Laboratory of Chemistry and Microbiology (Clinque Universities des Montagne (CUM)) of Bangangté between January to April 2018.

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Chemical screening of the aqueous and hydroethanolic extracts revealed the presence of secondary metabolites namely alkaloids, flavonoids, tannins, coumarins, saponins and terpenes, those being more abundant in the aqueous extract. The content of flavonoids was high. Macro-dilution was used to determine the minimum inhibitory concentrations (MIC) of propolis and Müller Hinton agar was used for obtaining the minimum bactericidal concentrations (MBC) and determination of inhibition diameters of the bacteria.

Results: The flavonoid content in the aqueous extract was estimated at 222.85 mg/ml and 18.77 mg/ml for the hydroalcoholic extracts. *S mutans and Lactobacillus* spp. were isolated from samples collected from caries affected teeth. The aqueous extract of propolis generally generates greater inhibition diameters than that of the hydroalcoholic extract on both lactobacillus and *Streptococcus mutans*, The MBC of the aqueous extract on *S. mutans* and *Lactobacillus* spp. was 50 mg/ml and the MIC was 25 mg/ml. The hydroalcoholic extract did not have MBC on both *S. mutans* and *Lactobacillus* spp. However, the MIC of this extract on both bacteria was 50 mg/ml. These antibacterial properties were not statically significant as that of gentamicin.

Conclusion: The evaluation of the antibacterial activity of our various extracts by determination of the MIC and MBC revealed that propolis was endowed with inhibitory properties vis-à-vis the growth of most bacterial strains tested: *Streptococcus mutans* and *Lactobacillus* spp. all 2 being gram-positive bacteria.

Keywords: Phytochemical characterization; antibacterial activity; propolis; S. mutans; Lactobacillus spp.

1. INTRODUCTION

The most common oral diseases that had been plaguing the human race for generation are dental caries and periodontitis. Dental caries are also known as tooth decay is as a result of the post-eruptive softening of hard dental tissues as a result of the convergence of factors of multiple origins with a dental plaque being one of the most important factors [1]. Dental plaque (soft deposits on the tooth surfaces) contains bacteria that are responsible for the fermentation of refined carbohydrates over a specific period of time resulting in the formation of acid. It is the acid produced by plaque bacteria that are responsible for the demineralization of hard dental tissues. Therefore teeth cleaning or brushing is an established oral hygiene modality which entails the removal of dental plaque, stains and other deposits from teeth to prevent dental caries and periodontal diseases [1]. According to the 2012 WHO report, caries affects 60-90% of schoolchildren in the world and almost 100% of adults [2].

In contemporary medicines, the prevention of dental caries is therefore based on medical intervention on already existing lesions and other preventive approaches focused on the improvement of hygiene and topical application of chemical substances that can make the enamel tissues cario-resistant [3,4,5]. The medical approach involves a surgical-restorative intervention on hard dental tissues which aims at arresting active carious lesions by treating already existing lesions like obturation of cavities, canal filling and extraction in the most severe cases. In the preventive approach, the use of fluoride, sealing of pits and fissures are applicable [3,4,5]. The surgical-restorative procedures used conventionally are traumatic and causes irreversible damage to dental hard tissues.

Both the surgical and the preventive approaches used by conventional medicines may present with side effects and some degree of toxicity [6]. However, alternative medicine offers a range of traditional pharmacopoeia products including propolis which are non-toxic.

Propolis is mainly composed of resin and vegetable balsam (50%), wax (30%), essential and aromatic oils (10%), pollen (5%), and various other substances including organic compounds and minerals (5%) [5,6]. It has been used in folk medicines in many regions of the world and has been reported to have various biological activities, such as antibacterial, anti-inflammatory, antitumor and immunomodulatory effects and its beneficial effects in medical sciences in the management of high blood pressure, alveolar bone regeneration, cancer of the prostate, and protection against dental diseases [7,8,9,10]. Apart from its medical properties, propolis serves as a functional food

since it can be used as food but offers additional physiological or psychological health benefits to traditional remediated and nutritional food. Besides, as a medication, it presents with little or no side effects.

Though the antibacterial approach is one of the best in the management of dental caries, this study was carried out to add to the already existing literature on the impact of propolis on the micro-organisms responsible for dental caries. Therefore this study was carried out to evaluate the antibacterial activity of propolis on the bacteria responsible for tooth decay.

2. METHODOLOGY

This was an *in-vitro* experimental study that took place at the biochemistry and Microbiology laboratories of Université des Montagne's, teaching hospital or Clinique Universitaire des Montagne's (CUM) of Bangangté-Cameroon between Januarys 2018 to April 2019.

The study was carried out on samples of carious lesions collected from 10 patients who presented with dental caries at the University de Montagne's dental clinic. The patients selected were adults who presented in the clinic with obvious dental cavities, diagnosed and confirmed dental caries by a dentist. Patients on antibiotics, antibacterial mouthwashes and other forms of alternative medicines were excluded from the study.

2.1 Study Materials

The dry propolis fragment used in our study was harvested in the savannah zone of the Western Region of Cameroon by beekeepers from this area. The propolis was dried at room temperature out of the sun for 3 weeks to preserve its chemical properties before it was grounded using an electric mill into a fine powder.

2.2 The Preparation of the Propolis Extracts

Hydro-ethanoic extract: Hydro-ethanoic extract of the propolis solution with was prepared by adding 150 g propolis powder to a solvent of 1350 ml alcohol using a 70\30 alcohol /water mixture. The mixture was left to macerate for 72 hours under constant magnetic stirring at 750 rpm and then filtered twice successively with Whatman paper #2. The resulting filtrate was clear and dark brown. It was stored in clean sealed bottles in a cool, dark place and protected from light.

Aqueous extract (water) of propolis: The aqueous extracts were obtained by boiling 300 g of propolis in two litres of water, filtered with Whatman filter paper No 2. The filtrate was kept in clean bottles, sealed and stored in a cool, dark and sheltered from light.

Phytochemical screening of propolis: Phytochemical screening chemical tests were carried out on hydro-ethanoic extract and aqueous extract of propolis using standard procedures to identify the constituents as described by Harborne, Sofowora and Trease [11,12,13].

Determination of total flavonoids: method by aluminium chloride: The method used was based on the principle of the direct colourimetric assay by aluminium chloride. Flavonoids have a free hydroxyl group in positron 5 capable of being highlighted, in the presence of aluminium chloride showing a yellowish complex by chelation of the Al^{3+} ion. The yellow colour produced is proportional to the number of flavonoids present in the extract.

2.3 Procedure

A calibration standard was prepared using tannic acid solutions of different concentrations from 0.03 to 0.3 g / l. 0.5 ml of the diluted solution was mixed with 1.5 ml of 95% ethanol is reacted with 0.1 ml of 10% aluminium chloride, followed by 0.1 ml of 1 M sodium acetate and 2.8 ml of distilled water and then left minutes in the dark. The absorbance of each solution was determined at 415 nm. Using the absorbance values obtained for the different quercetin solutions thus prepared, we plotted the calibration curve. The stock solution we used was a tannic acid stock solution at a concentration of 0.3 g/l. We carried out a dilution range of daughter solutions of various concentrations respectively 0.06 g / l, 0.12 g / l, 0.18 g / l, 0.24 g / l and 0.3 g / l.

2.4 Collection of Bacterial Samples

After explaining the study to the patients and obtaining their consent, the diseased tooth, was cleaned, dried and isolated with salivary cotton rolls. The sample was collected from; soft carious lesions using a sterile excavator. The sample was unloaded in transport tubes in the heart-brain broth.

In the laboratory, **t**he samples were first mixed by vortex and inoculated in culture media as follows; Fresh blood agar to isolate gram-positive cocci such as Streptococci; Chocolate agar to isolate gram-negative and positive cocci such *Actynomycetes* and *Lactobacillus*.

2.5 Identification of Bacteria by Gram Staining was carried out Using Standard Methods

Methods for assessing antibacterial activity propolis: Antibacterial activity was determined by the good diffusion method according to the NCCLS [14]. The test organisms were inoculated on Mueller Hinton agar plates and spread uniformly to form a lawn. Wells (5 mm diameter) were cut into the agar. The cut agar disks were carefully removed by the use of sterilized forceps. Into each well was introduced different concentrations (100, 200, 225, 250, 275, 300 mg/ml) of the extracts. Gentamicin was taken as a positive control. The plates were incubated at 37°C for 24 h. The zones of inhibition were then recorded.

Preparation of the culture medium: One thousand (1000) ml of Mueller Hinton Broth (MHB), Agar Chocolate Agar (MHA) and nutrient agar were respectively prepared by dissolving in distilled water, the powder previously made by the manufacturer as follows: 38 mg for MHA, 21 mg for MHB and 28 mg for nutrient agar. The solutions were then homogenized and sterilized by autoclaving at 121°C for 15 minutes and poured into petri dishes of 90 mm diameter. All this according to the recommendations of the manufacturing laboratory.

Preparation of the bacterial inoculums: The bacterial inoculum was prepared in 5 to 10 ml of sterile physiological saline from 24-hour pure cultures. The density of the suspension measured on a densitometer was adjusted to 0.5 Mac Farland (10^8CFU / ml). Allowed in agar to incubate for a few minutes then remove the supernatant using a Pasteur pipette. It was then applied on the discs on agar using a sterile dispenser or forceps and each antibiotic disc separated by at least 2 cm. The inoculated dishes were put in a jar and incubated in an oven at 37°C and an atmosphere enriched with 5 to 10% CO_2 for 18 to 24 hours.

2.6 Sensitivity Test

Determination of the minimum inhibitory concentration of propolis: Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of the propolis extract which inhibited the growth of the tested microorganisms. The minimum inhibitory concentration (MIC) of propolis was determined using the broth dilution method. A stock solution was prepared at 800mg/ml of each crude extract. Next, 2 ml of each extract at 800 mg/ml was introduced into the first tube of the dilution range. For this a series of tubes were prepared with broth to which various concentrations of propolis extracts were added viz., 0 mg/ml (negative control) ,0.78 mg/ml , 1.56 mg/ml, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 100 mg/ml, 200 mg/ml, 400 mg/ml. The antibiotic gentamicin was taken as a positive control (.08 µg/ml). Then, 15 µL of bacterial inoculum was added to each test tube and incubated at 37°C. The tests tubes were examined and MIC was determined. All sets were read visually and MIC values were recorded as the lowest concentration of propolis that had no visible turbidity (Fig. 1).

Determination of Minimum Bactericidal Concentrations (MBC): The minimum bactericidal concentration (MBC) is the minimum concentration corresponding to the lowest concentration of substance capable of killing more than 99.9% of bacterial inoculum (i.e less than 0.1% of survivors) after 18 to 24 hours of incubation at a temperature of 37°C. Thus, their determination is based on the subculture from the MIC on an agar medium [15].

2.7 Operating Mode

In each of the tubes lacking a bacterial pellet (unobserved visible growth) and the controls for the determination of the MIC were streaked on Mueller Hinton agar on Petri dishes. The petri dishes were incubated for 18 to 24 hours at 37°C. The MBC of each extract was deduced from the lowest concentration at which no culture was observed on Mueller Hinton agar (15). The operation was repeated three times and the mean value noted (Fig. 2).

Evaluation of the ratios MBC / MIC: These ratios make it possible to confirm the bacteriostatic or bactericidal character of a substance. When these ratios are greater than or equal to 4, the substance is said bacteriostatic; if these ratios are less than 4, the substance is considered bactericidal. If they are equal to 1, then it is called "absolute bactericidal" [15].

Measurement of inhibition diameters: The measurement of the inhibition diameters by the

solid medium diffusion method was performed from the MIC and MBC concentrations for each strain. This test was performed on each strain thrice.

The principle used is that of the antibiogram (culture and sensitivity). It consists of measuring the zones of growth inhibition of microorganisms generated by the extracts studied.

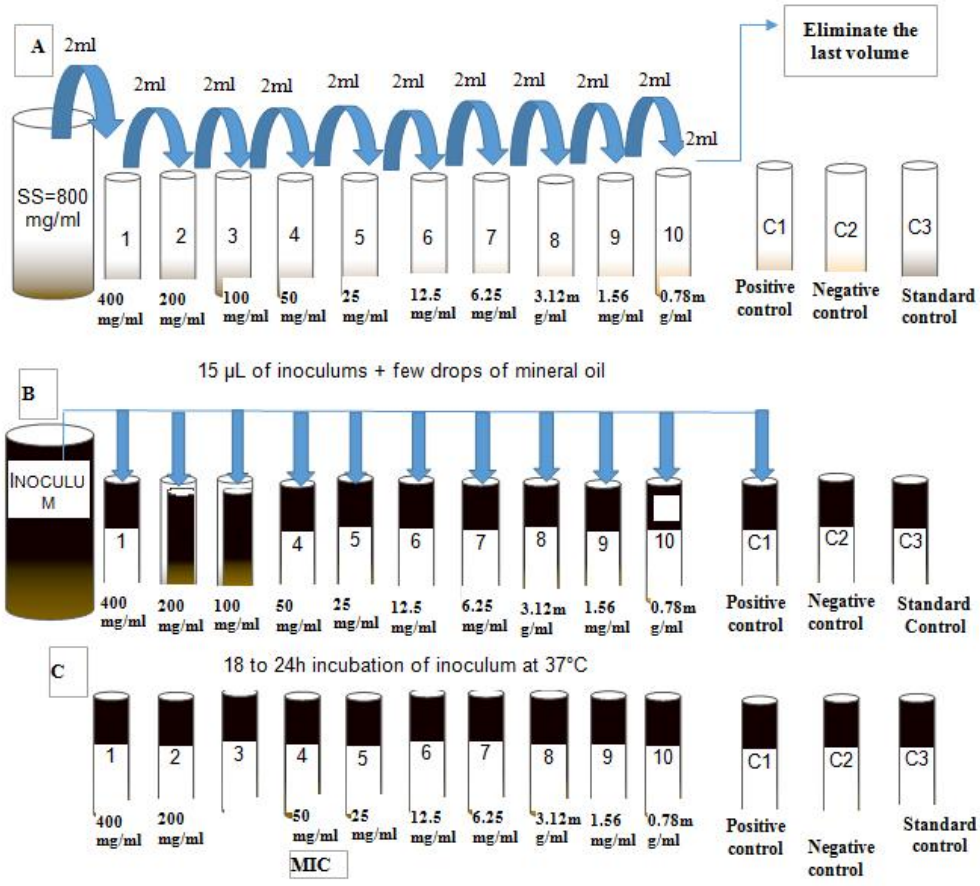

Fig. 1. Broth dilution method for determining MIC [15]

SS = Stock solution; NB: the control tubes consisted of; Positive control: broth + inoculum, Negative control: simple broth, Actual control: broth + extract

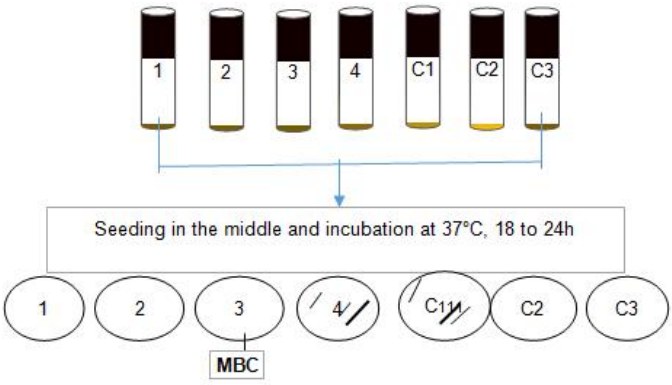

Fig. 2. Determination of MBC using culture Mueller Hinton agar on Petri dishes (1,2,3) and controls(C1, C2, C3)

C_1: broth + inoculum, C_2: simple broth, C_3: broth + extract

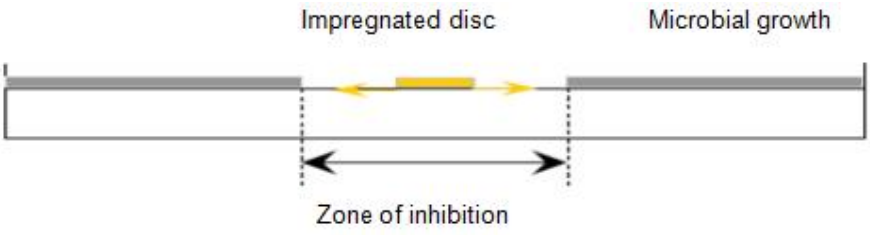

Fig. 3. Sensitivity test using impregnated amoxicillin disk on Mueller Hinton agar

2.8 Procedure

Sterile disc of 6mm in diameter cut from Whatman paper n°1, impregnated with 15µl of the different extracts of known concentration solutions (MICs and MBCs) were gently deposited on the surface of Mueller Hinton agar previously seeded by swabbing with bacterial inoculum. An Amoxocillin antibiotic disc (30µg) was deposited. Upon application, a disc of different extracts and amoxicillin diffuse from the disc in a uniform way in the agar. After 15 minutes at room temperature followed by incubation in the oven at 37°C for 18 to 24 hours, the disks were surrounded by circular zones of inhibition corresponding to an absence of culture. The negative control consisted of a disc impregnated with MHB (Fig. 3).

2.9 Reading

After incubation, the inhibition diameter is measured in millimetres using a graduated ruler, the diameter of each disk included. This test was repeated three times for each strain. According to Moreira et al, [16], sensitivity to different extracts was classified according to the diameter of the inhibition zones as follows:

- Not sensitive (-) or resistant: diameter < 8mm.
- Sensitive (+): diameter between 8 – 13.9 mm.
- Very sensitive (++): diameter between 14 to 19 mm.
- Extremely sensitive (+++): diameter > 19 mm.

2.10 Statistical Analysis

Data was captured into Microsoft excel spreadsheet, analyzed in SPSS and presented in the form of tables and figures. Bivariate analysis (t- test) was carried out to compare the inhibitory diameter of aqueous extracts of *S. mutans* and *Lactobacillus spp.* of the aqueous and hydroalcoholic extracts to that of gentamicin. Descriptive and inferential statistics such as unpaired t-test and ANOVA were employed to compare between the groups. $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

Extraction of propolis: The hydro-alcoholic extraction of propolis was obtained with a yield of 8.985% and the aqueous extracts with a yield of 4% yield calculated using the following formula (Table 1).

Chemical screening: The results of the chemical screening of the various propolis extracts show that there was a high abundance in the aqueous extracts of flavonoids, saponins, tannins, coumarins and quinines (Table 2).

Chemical analysis of the hydroalcoholic and aqueous extract of propolis revealed the presence of 6 metabolites namely coumarins, saponins, alkaloids, tannins, quinones and flavonoids. These different metabolites were more abundant in the aqueous extract than in the hydroalcoholic extract.

Determination of flavonoids: The equation of the obtained calibration line is $y = 1.0884x - 0.0175$.

It signifies that there was a strong correlation between optical density and concentration, with a coefficient of $R^2 = 0.9971$. This indicated that quercetin activity was concentration-dependent.

Identification of some cariogenic bacteria: Streptococcus mutans and *Lactobacillus* sp. were the bacteria isolated from the carious lesions.

3.1.1 Antimicrobial activity

Sensitivity test: The aqueous extract of propolis generally generates greater inhibition diameters than that of the hydroalcoholic extract on both *Lactobacillus* and *Streptococcus mutans*.

Table 1. Masses and yields of aqueous and hydroalcoholic extracts

Extracted types	Mass used (g)	Mass of the extract (g)	Yields (%)
Aqueous	300	12	4
Hydro Alcoholic	150	3	9

Table 2. Chemical components of propolis extracts

Metabolites secondary	Results	
	Hydro alcoholic extract	Aqueous extract
Flavonoids	+	++
Saponins	+	++
Tannins	+	++
Alkaloids	+	++
Coumarins	+	++
Quinones	+	++

Legend: (+) abundant, (++) very abundant

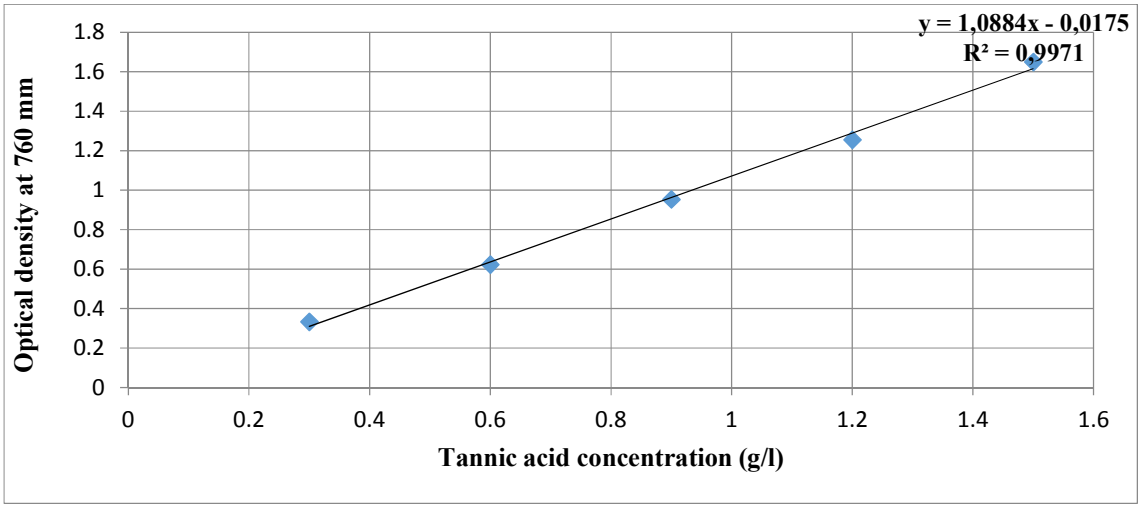

Fig. 4. Flavonoids calibration line

Table 3. Inhibition diameter of aqueous and hydro-alcoholic propolis extracts

microorganisms	Medium Ø of aqueous extracts 200 mg / ml	Ø average of hydro-alcoholic extracts 200 mg / ml
Streptococcus mutans	10 mm	4 mm
Lactobacilli spp.	18 mm	7.5 mm

MIC, MBC of the different extracts: The MBC of the aqueous extract on *S. mutans* and *lactobacillus spp* was 50 mg / ml and the MIC was 25 mg / ml. The hydroalcoholic extract did not have MBC on both *S. mutans* and *Lactobacillus spp* . However, the MIC of this extract on both bacteria was 50 mg / ml. (Table 4).

The MIC/MBC ratio of 2 was established for both *Lactobacillus spp.* and *S. mutans* about the aqueous extract. However, for the hydroalcoholic extract, no concentration showed a bactericidal effect on the germs from a single concentration of 50 mg / ml representing the MIC of *S. mutans* and L*actobacilli spp*.

Inhibition diameters of the different extracts: The diameter of the aqueous extract on *S. mutans* was 10 mm and on *Lactobacillus spp* was 11 mm. The hydroalcoholic extract had a diameter of 8 mm for both bacteria. For the

Table 4. MIC; MBC and MBC / MIC ratio for each of the microorganisms tested by the aqueous extract and the hydroalcoholic extract

Microorganisms	Aqueous extract			Hydro alcoholic extract		
	MBC	MIC	R	MBC	MIC	R
Streptococcus mutans	50	25	2	/	100	/
Lactobacillus spp.	50	25	2	/	100	/

Table 5. Inhibition diameters of extracts on bacteria corresponding to MICs

Microorganisms	Aqueous extract	Antibiotic gentamicin	Hydro alcoholic extract
S. mutans	10 mm	26 mm	8 mm
Lactobacillus spp.	11 mm	29 mm	8 mm

inhibitory diameter of the aqueous extracts on *S. mutans* was not statistically significant p= 0.14 as well as the hydroalcoholic extract p= 0.1, when compared to gentamycin. The inhibitory diameter of the aqueous extracts on *Lactobacillus spp* was not statistically significant p= 0.14 as well as the hydroalcoholic extract p= 0.2, when compared to gentamycin (Table 5).

3.2 Discussion

The current study carried out to phytochemically characterise and evaluate the antibacterial activity of propolis on 2 bacteria responsible for dental caries demonstrated that propolis had promising antimicrobial properties.

The secondary metabolites demonstrated in our study were (flavonoids, coumarins, saponins, terpenes and tannins) from both aqueous extracts and hydroalcoholic extracts was similar results obtained by Preeti et al. in India [17]. In the current study, coumarins, tannins, flavonoids, saponins and terpenes were more abundant in the aqueous extract than in the hydroalcoholic extract. Usman et al. in 2016 [18] reported the presence of the same metabolites Malasia, but their yields were more in hydroalcoholic extracts. This difference could be explained by the chemical composition of propolis which varies considerably according to geographical location, the local flora, plant specificities, season, time of collection, type of bees, concentration and nature of the solvents used for extraction [19,20].

Secondary metabolites are plant extracts responsible for their antimicrobial properties [21]. The most important pharmacologically active components in propolis were flavonoids (flavones, flavonols, flavonones), phenolics, and aromatics. Indeed, flavonoids are supposed to explain a large part of the biological activity in propolis [22]. The activity of propolis on *S. mutans* and *Latobacillus* spp could be due to secondary metabolites and flavonoids in particular [22,23].

Antimicrobial activity analysis of propolis extracts: In the current study, the MIC values (100 µg / ml) were identical for the 2 bacterial strains studied. The MICs of the hydroalcoholic extract was for *Streptococcus mutans* and for *Lactobacillus* sp. Grecka et al. (2019) also reported that the MIC values of propolis on the growth of Streptococcus mutans may vary between 32 and 256 µg/mL [24].

Another antibacterial property exhibited by propolis in the current study was its high inhibitory diameter against the bacterial strains used in the current study. The inhibition diameters of our extracts were respectively 8 mm for the hydroalcoholic extract on the 2 bacterial strains against 10 and 11 mm on *Lactobacillus* spp and *Streptoccocus mutans* for the acquous extract. A 10 year study on crude propolis extracts on hard dental tissues covered with plaque, isolated showed reductions in *Streptococcus m*utans counts and interference with their adhesion capacity and glucosyltransferase activity, which are considered major properties in the establishment of the cariogenic process [25,26]. A study on thirty children who performed the rinses over 1 hour after the rinse revealed a significant reduction in *Streptococcus mutans* count when compared to samples obtained in baseline [27].

Ozan et al. (2007) and Arslan et al. (2012) also found that propolis solutions are not as effective as chlorhexidine gluconate solutions in caries prevention; nevertheless, their anti-caries impact was statistically significant compared with a control group [28,29]. However, the study by Özan et al. showed, however, that propolis-

based solutions have a weaker cytotoxic effect on fibrobroblasts of the gum than chlorhexidine, which predisposes them to be used as an ingredient in mouthwashes [29].

Natural products offer a rich source of structurally diverse substances with a wide range of biological activities, which could be useful for the development of alternative or complementary anti-caries treatments. Due to its variable composition, it offers large possibilities of use both in the management and prevention control of caries.

The weakness of this study was that based on the capacity of our laboratory as we were able to isolate only 2 bacteria, A multispecies study might throw more insight on the impact of propolis on cariogenic bacteria. A study of the influence of propolis on the development of matured plaque would have given an insight into the different colonies of bacteria at a particular stage and the impact of propolis on them.

4. CONCLUSION

Our studies deminstrated that the propolis extracts contain secondary metabolites such as polyphenols, flavonoids, coumarins, tannins, saponins the alkaloids and the flavonoids thereof being most abundant in the aqueous extract in the hydroalcoholic extract.

The evaluation of the antibacterial activity of our various extracts by determination of the MIC and MBC revealed that propolis was endowed with inhibitory properties vis-à-vis the growth of most bacterial strains tested: *Streptococcus mutans* and *Lactobacillus* spp. all 2 being gram-positive bacteria.

The aqueous extract of propolis generally generates greater inhibition diameters than that of the hydro alcoholic extract on both *Lactobacillus* and *Streptococcus mutans*, The MBC of the aqueous extract on *S. mutans* and *Lactobacillus* spp was 50 mg / ml and the MIC was 25 mg / ml. The hydroalcoholic extract did not have MBC on both *S .mutans* and *Lactobacillus* spp . However, the MIC of this extract on both bacteria was 50 mg / ml. These antibacterial properties are not statically significant as that of gentamicin.

Also, this activity allowed us to highlight the bactericidal property of the aqueous extract concerning the hydroalcoholic extract which, although having a good yield, had only an essential inhibitory antibacterial activity even at high concentration. The variability of the chemical components and this resinous substance could explain its various properties in the medical and variable field.

5. RECOMMENDATIONS

Multispecies and toxicity studies should be carried and studies on the toxicity of propolis should be carried out.

The impact of propolis mouth rinse in plaque content and deposition should be studied.

CONSENT

As per international standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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