International Scholars Journals

African Journal of Dentistry ISSN 3216-0216 Vol. 7 (4), pp. 001-005, April, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

An *in vitro* preliminary study on the growth inhibition of oral microflora by snake venom

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Accepted12 January, 2019

Oral health in most Brazilian municipalities is still a big challenge, particularly with regards to universalization, the equity of care and the high cost involved in restorative therapy. The demand to discover new natural products with antibacterial activity for the prevention of dental diseases, and perhaps with less health and financial impacts, would be very important to obtain an effective way to control the formation of a biofilm pathogenic and dental caries. The objective of this work is to study the feasibility of the use of the biotechnology venom, native from different snakes, to inhibit the growth of *Streptococcus mutans*, which is the principal agent involved in dental caries. Our results showed that the venom of snakes *Bothrops moojeni* and *Bothrops jararacussu* inhibited the growth of *S. mutans* and the component responsible for the inhibition appears to be hydrogen peroxide. Although, it is still not fully conclusive, the tests already carried out show that snake venom is an important tool used to inhibit the growth of pathogens, especially those involved in caries diseases.

Key words: Snake venom, caries, *Streptococcus mutans*, *Bothrops moojeni*, *Bothrops jararacussu*.

INTRODUCTION

Dental caries is one of the most common infectious diseases that afflict human beings and there is a tendency that it is not treated in many underdeveloped areas, mainly if we consider that, in rare cases, the patient only gets to relieve the pain with the tooth extrac-tion (Ajdic et al., 2002). As such, this picture configures dental caries as one of the main problems of oral health (Mattos-Graner et al., 1998; Ramos-Gomez et al., 2002; Gomes et al., 2004; Klein et al., 2004).

Even with the progress of the prophylactic solutions in relation to the disease, children between five and nine years old in the United States of America had at least a caries lesion in their teeth. The authors affirm that the percentile has increased by 84.7% among adults more than eighteen years old and approximately 50% for the population aged 75 years, all presenting at least a caries lesion in the dental root. More than ⅔ of the Mexico-American population, with the exception of Hispanic but including the Afro-American ones, had caries that were not treated. In some countries, the dental caries took endemic proportions; for example, in China, ¾ of the children's population aged five years presented signifi-cant evidences of the lesion. About 25% of the three years old children presented a lot of caries lesions that were developing, and in many states, these lesions were detected in children less than 18 months. This high degree of lesions affects the economy directly, in terms of the introduction of flour in precocious ages (Smith, 2002).

Some authors consider that *Streptococcus mutans* (Gibbons and van Houte, 1977a; Loesche and Straffon, 1979; Loesche, 1986), as well as the serologic group of *Streptococcus sobrinus* (Klein et al., 2004; Smith, 2002; Gibbons and van Houte, 1977a; Loesche and Straffon, 1979; Loesche, 1986; Lamont et al., 1991) are the largest responsible etiological agents for the dental caries in humans. Longitudinal studies confirm the relationship among the prevalence of the assay of *S. mutans* in the dental biofilm and the development of the decays (Carlsson et al., 1975; Masuda et al., 1979). The noxious effects of the dental caries do not affect only the teeth, although there are uncountable problems humans can go through after the caries are installed and not treated (infectious chronic process). One of the most important

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Table 1. Snakes' taxonomies and their antibacterial effects.

Family	Gender and species	Antibacterial Effects*				
		Escherichia *coli*	**Aeromonas *hydrophila***	**Staphylococcus *aureus***	**Pseudomonas *aeruoginosa***	**Bacillus *subitilis***
Elapidae	*Acanthophis antarcticus*	-	-	-	-	+
Elapidae	*Hoplocephalus stephensi*	#	#	#	#	#
Elapidae	*Naja melanoleuca*	-	-	-	-	-
Elapidae	*Naja mossambica*	-	+	+	-	+
Elapidae	*Notechis ater niger*	#	#	#	#	#
Elapidae	*Notechis scutatus*	-	+	+	+	+
Elapidae	*Oxyuranus microlepidotus*	#	#	#	#	#
Elapidae	*Oxyuranus scutellatus*	#	#	#	#	#
Elapidae	*Pseudechis colletti*	#	#	#	#	#
Elapidae	*Pseudechis australis*	+	+	+	-	+
Elapidae	*Pseudechis guttatus*	#	#	#	#	#
Elapidae	*Pseudechis porphyriacus*	#	#	#	#	#
Elapidae	*Pseudonaja textilis*	#	#	#	#	#
Viperidae	*Bothrops moojeni*	#	#	#	#	#
Viperidae	*Bothrops jararacussu*	#	#	#	#	#
Viperidae	*Agkistrodon bilineatus*	#	#	#	#	#

*, based on the bacteria tested by Stiles et al. (1991); +, show antibacterial effects; -, do not show antibacterial effects; #, do not use venom.

problems is the bacterial endocarditic, responsible for 45 to 80% of the cases that involve natural valves (Durack, 1995; Paik et al., 2003). The bactericidal action of the venoms in crude form had already been seen[15] in the venom of several species. As such, it is possible to make a correlation between these venoms and those used by us. However, Table 1 shows the taxonomies and bactericidal action of venoms.

L-amino acid oxidase (L-amino acid: O2 oxidoredutase, EC 1.4.3.2) is the component responsible for the yellow color of some snake venom, because it shows two soft forms of FAD for enzyme mol as group prosthetics. The native enzyme is a glycoprotein, dimmer of molecular weight of approximately 130 KDa, constituted by two subunits of molecular weight with approximately 70 KDa each and interlinked in a non covalent way (Jimenez-Porras, 1970). However, this enzyme catalyzes the oxidation disseminative of L-amino acids, producing the corresponding keto acid, hydrogen peroxide and ammonia (Tan and Saifuddin, 1991; Pessatti et al., 1995; Karthikeyan et al., 2004), as described in the following reaction:

$$LAO + H_2O \rightarrow \text{keto acid} + NH_3 + H_2O_2$$

MATERIALS AND METHODS

Snakes' venoms were supplied by Dr. Peter J. Mirtschin (Venom Supplies Pty Ltd.), via the Butantan Institute and CEVAP (Center of Studies of Venoms and Venous Animals), in crystallized form. The names of the snakes used for the extraction of the venom are shown in Table 2.

Bacterium assay of *S. mutans* (ATCC 25175) was cultivated in stuff at 37°C with liberation of constant CO_2 and in anaerobes, in tubes of rehearsals containing 15 mL of liquid middle BHI (Brain Heart Infusion) (Difco® Detroit - Michigan), from where a bracket of 100 µL was removed every 30 min and the optical density was verified by absorbance in a filter of 595 nm to determine the logarithmic curve and to find the LOG phase (growth). Later, the assay was cultivated in tubes of rehearsals containing 5 mL of liquid middle BHI and this content was sown in Petri plates, containing 15 mL of solid middle BHI/Agar, in order to homogenize the content. This process was accomplished to just remove the isolated colony of the bacterium (CFU). In a retreat of the isolated colony (CFU), the cultivation was remade in another test tube following the same procedure described previously. The incubation was made for a period of 26 h and the concentration of bacteria (CFU/mL^{-1}) was certainly done for the reading of 200 µL of the middle BHI in 96 well plates reader through a filter of 595 nm with a result of approximately 2,63 × 106 CFU/mL^{-1}.

To observe the presence of the antibacterial activity of the 16 snake venoms on the samples of *S. mutans*, 16 Petri plates were prepared with half of the solid cultivation of the Mueller Hinton/Agar (15 mL) and then, in each of the plates, a concentrate containing 200 µL of the liquid middle BHI with the cultivation of an isolated colony of *S. mutans* (as seen in the foregoing) was added. After five disks, an absorbent of 6 mm diameter was inserted halfway in each Petri plate with 15 µL of each snake's venom (2 mg/mL). After 26 h, observations were made on the disks containing the samples to check for the presence or absence of halos of inhibition on bacterial growth.

RESULTS AND DISCUSSION

The observation of the antibacterial effects in plates showed that just the venom of *B. moojeni* (3) and *B. jararacussu* (2) snakes presented a halo of inhibition of growth of the *S. mutans*, as it can be observed in Figure 1. The difference between a poisonous substance and a

Table 2. Snakes' taxonomies and their popular name.

Family	Gender and species	Popular name
Elapidae	*Acanthophis antarcticus*	Common death adder
Elapidae	*Hoplocephalus stephensi*	Stephen´s banded snake
Elapidae	*Naja melanoleuca*	Black cobra
Elapidae	*Naja mossambica*	Spitting cobra
Elapidae	*Notechis ater niger*	Peninsula tiger
Elapidae	*Notechis scutatus*	Mainland tiger
Elapidae	*Oxyuranus microlepidotus*	Inland taipan
Elapidae	*Oxyuranus scutellatus*	Coastal taipan
Elapidae	*Pseudechis colletti*	Collett´s snake
Elapidae	*Pseudechis australis*	King brown
Elapidae	*Pseudechis guttatus*	Spotted black
Elapidae	*Pseudechis porphyriacus*	Red bellied black snake
Elapidae	*Pseudonaja textilis*	Eastern brown snake
Viperidae	*Bothrops moojeni*	Caissaca
Viperidae	*Bothrops jararacussu*	Jararaca
Viperidae	*Agkistrodon bilineatus*	Tropical moccasin

pharmaceutical substance, or even a nutritional substance, is the administered or accumulated dose in the body, but in general, a poison is mortal in certain doses without any therapeutic function. Two examples of poisonous substances are flour and iodine. Both are extremely poisonous, but they have therapeutic applications in low doses, in that iodine is indispensable and flour is a good drug obstacle for decays.

The observation of the antibacterial effects in plates showed that just the venom of *B. moojeni* (3) and *B. jararacussu* (2) snakes presented a halo of inhibition of growth of the *S. mutans*, corroborating the results of Stiles et al. (1991). In their research, while studying 30 types of snake venoms of different species, in the Elapidae and Viperidae families, against gram-positive and gram-negatives bacteria, they observed that the inhibition halos were larger for *S. aureus* (gram-positive) in the family of the snakes (Viperidae), when compared to the ones produced by the venom of Elapidae snake. On the other hand, our results showed that the venom of *Naja mossambica*, *Notechis scutatus scutatus*, *Acanthophis antarcticus* and *Pseudechis australis* did not present an inhibition of the halo for the bacterium gram-positive *S. mutans*, thereby diverging the reports presented by Stiles et al. (1991) that the inhibition power for all the aforementioned serpents was observed, when incubated with *S. aureus*. This difference in the results can possibly be explained by the treatment of different bacteria, although the mechanisms of defense of the gram-positive bacteria are identical. Other factors that can compete for the divergence of the results are the origin and storage form, or venom solubilization.

In this study, the venom of 14 snakes of Elapidae and 8 snakes of Viperidae family, as well as the venom of bee and scorpion (Perumal et al., 2007) were studied using similar protocols of Stiles et al. (1991) for bacteria *S. aureus* (gram-positive), *P. aeruginosa*, *E. coli*, *P. mirabilis*, *P. vulgaris* and *E. aererogenes* (gram-negative). The researchers' group incubated the bacterium gram-positive *S. aureus* with the 6 snake venoms of the Elapidae family: *Acanthophis antarcticus*, *Pseudechis australis*, *Pseudechis colletti*, *Pseudechis guttata*, *Pseudechis porphyriacus* and *Pseudonaja textills*, which were also used in our experiments. Different from the results presented in this work, inhibition halos were observed for this snake venom. Additionally, a probable explanation for the differences among the results, for the gram-positive bacteria assay, is that the variability in the venom composition and concentration in a given species differ in many factors, such as: age, sex and geographical origin (Chippaux et al., 1991).

According to reports (Meier, 1990), venoms are complex mixtures, constituted mainly by proteins, among which the L-amino acids (LAO) are detached. To L-amino acid oxidization, a flavoenzyme catalyzes the oxidative deamination of the substratum L-amino acid in a keto acid with production of ammonia and hydrogen peroxide. LAO is the only FAD-dependent oxidization present in the snake venom and its toxicity possibly involves the generation of hydrogen peroxide formed through the reverse-oxidation of the transient reduction of the co-factor flavones by the molecule of oxygen (Stiles et al., 1991).

Hydrogen peroxide has known bactericidal action (Rutala, 1990) acting directly in the lipidic membranes of the bacteria. The use of the enzyme catalase decomposes hydrogen peroxide in water and oxygen, thereby annulling its bactericidal action. Due to the fact that it has the highest turnover number (kcat) known in enzymes, a catalase molecule can catalyze the decomposition of

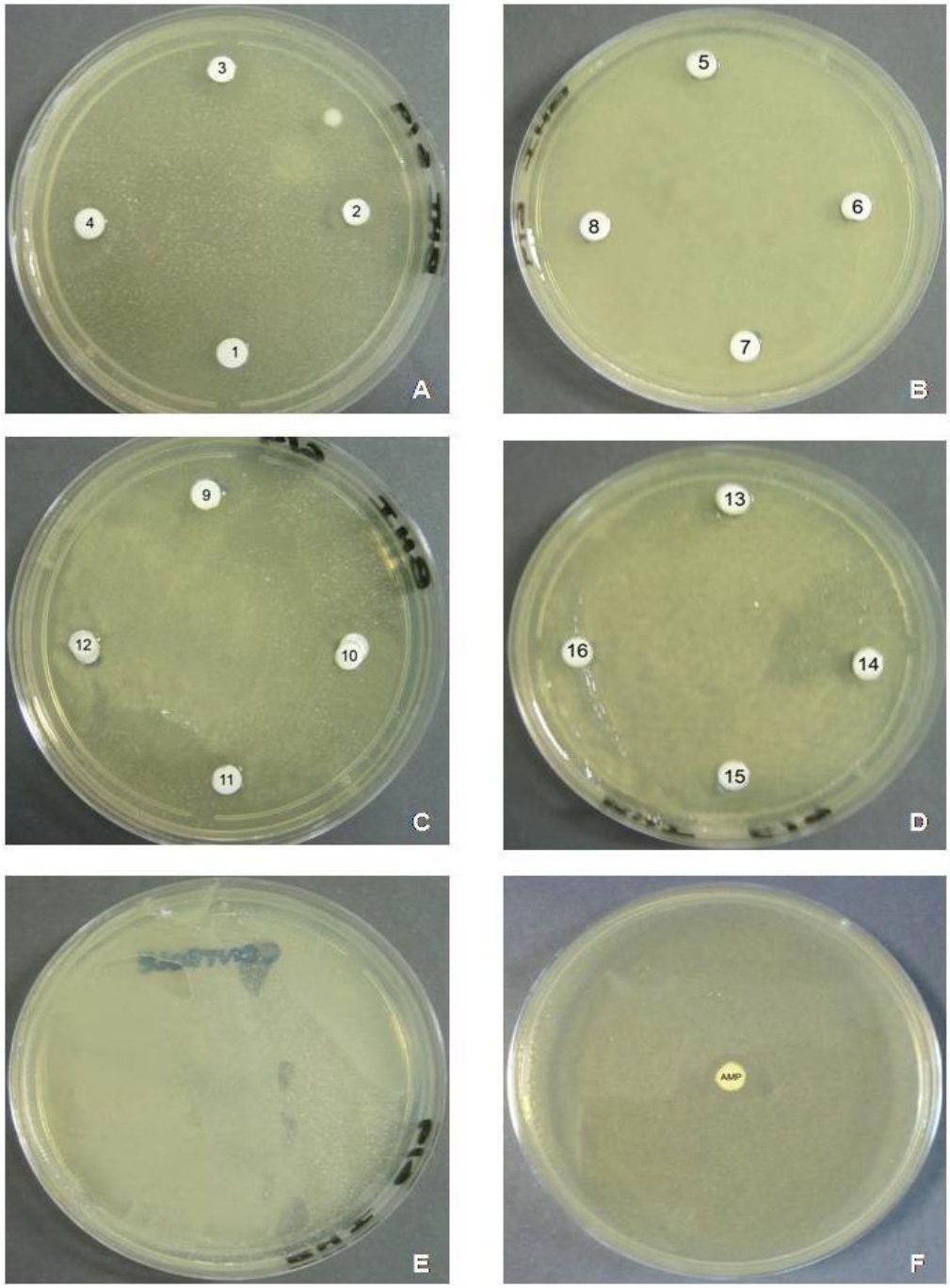

Figure 1. Bacterium assay in solid middle BHI/Agar with crude snake venom. **A** - (1) *Acanthophis antarcticus* (2) *Bothrops jararacussu* (3) de *Bothrops moojeni* e (4) *Agkistrodon bilineatus*; **B** - (5) *Hoplocephalus stephensi,* (6) *Naja melanoleuca,* (7) *Naja mossambica,* (8) *Notechis ater níger*; **C** - (9) *Notechis scutatus,* (10) *Oxyuranus microlepidotus,* (11) *Oxyuranus scutelatus,* (12) *Pseudechis australis*; **D** - (13) *Pseudechis colletti,* (14) *Pseudechis guttata,* (15) *Pseudechis porphyriacus,* (16) *Pseudonaja textills;* **E** - Control; **F** - Control with Ampicilin.

about 40,000.000 hydrogen peroxide molecules a second (Nelson, 2005); thus, turning it into an important enzyme for the desintoxication of this substance.

The enzymatic degradation of the phospholipids with the action in the membrane can be one of the important factors in the bactericidal properties of the animal venom, and is involved in a synergic action between the antimicrobial peptides and the venoms' enzymes (Perumal et al., 2007). Thus, the antibacterial effects were efficient in the selection of venom with a capacity of inhibiting the

growth of *S. mutans* and the poisons of the serpents of the gender Bothrops. However, *B. moojeni* and *B. jararacussu* were the only ones, among the appraised ones, that showed this capacity.

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