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Research Article

CHEMICAL ANALYSIS OF BIOLOGICAL EFFECTS OF A HYDROALCOHOLIC EXTRACT PUNICA GRANATUM (POMEGRANATE)

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ABSTRACT

The use of medicinal plants has been encouraged by the World Health Organization because of its traditional use in crops and the benefits conferred its users. The development of antimicrobial drugs is important for the development of new sources for the therapeutic treatment of infectious diseases, since the characteristic of bacteria have become resistant to the action of antibiotics. The aim of this study was to evaluate the influence of an alcoholic extract of the bark of *Punica granatum* fruit as its possible genotoxicity, cytotoxicity and mutagenic action against the bacterium *Staphylococcus aureus*. To evaluate cytotoxicity, we employed the method of diffusion of nutrient agar disk, assessing the bacterial growth compared to the presence of impregnated disks of different volumes of extract and antibiotics. For the determination of genotoxicity and mutagenicity, plus extract were employed as genotoxic and mutagenic agents hydrogen peroxide and chloride stannous. The results showed that the extract has antibiotics like gentamicin, chloramphenicol, stannous chloride and hydrogen peroxide effect. The results suggest that the extract of *Punica granatum* probably has cytotoxic, genotoxic and mutagenic action, but there is a need for more specific analyzes to further elucidate the presence of the latter characteristic.

INTRODUTION

The culture of several people using plants as curative for various diseases (Rangel et al. 2001). The consumption of medicinal plants has increased dramatically, due to cultural and socioeconomic factors, since there are social classes with low financial power and have no access to allopathic medicine (BRAZIL, 2006).
Due to increased public interest in the use of these plants and their therapeutic potential, the scientific community has been searching for ways to obtain new herbal (Maciel MAM, Pinto, AC and JR Veiga, VF, 2002;. CALIXTO, 2005; MICHELIN et al., 2005; Noldin et al, 2006).

Herbal medicines are made from medicinal plants and/or its parts with known pharmacological characteristics. According Salvagnini and colleagues (2008), the World Health Organization has supported the use of medicinal plants, it is because of this use still be traditional among various peoples and by this practice bring many benefits to its users and curative factors. There are several auxiliaries, including the social and economic spheres, which work in advancing health through the use of medicinal plants. (Elisabetsky, 1991).

Hope to enter the medical industry characteristics that pertain to the healing potential of medicinal plants arising encouraged the research of scientists. However, incorrect utilization and consumption may allow toxic and/or action has the effect not of interest, this should occur due to lack of knowledge of these adverse effects. Therefore it is necessary first of all to know the plant, part of this being exploited, otherwise there can be side effects and even by their inappropriate use effects may occur (Pereira et al., 2004).

Among the effects of medicinal plants can be noted that micro-controller. The emergence of new drugs controlling microorganisms is a strong ally for population health and the current growth area for scientific technical area, in search of the appearance of new molecules with significant biological features such as antimicrobials. With this new reality, people have access to knowledge about medicinal plants from experiments, assisting those using natural sources such as pharmacy besides inducing the generation of new patents and / or processes (FERREIRA, FS1; SANTOS , SC1 *; Barros et al, 2011).

Additionally, another relevant to the development of antimicrobial herbal medicines reason is the fact that bacteria had the hallmark of becoming resistant to antibiotics. The emergence of new therapeutic aspects possible new alternative for the treatment of infectious diseases (Antunes et al., 2006).

A large number of medicinal plants have antimicrobial activity (YARNELL, 2002), and among them, *Punica granatum* has been used by people for this purpose (Werkman et al 2008) including the replacement of antibiotics as chloramphenicol and ampicillin in combating *Salmonella typhi* is the causative agent of typhoid fever (Perez and Anesini 1994).

In 2009, the Ministry of Health released the RENISUS (National List of Medicinal Plants of Interest to SUS) list, which is integral to *Punica granatum*, that this is a listing of currently 71 plant species of interest to the health care system with in order to encourage and support research in the field of herbal medicine in order to develop new drugs derived from medicinal plants that are safe and effective in combating diseases (PORTAL oF HEALTH, 2009).

The *Punica granatum*, known as pomegranate, is a shrub native to the middle east. This is highly branched and slightly spiny. Its composition is divided between seeds, corresponding to 50% of fruit weight, and transparent compartments containing a spongy tissue (rag). Popularly used to treat gastrointestinal disorders, loss of dental prophylaxis, relief of pain and other ear (Navarro et al. 1996). Studies show that pomegranate, because of its high amount of phenolic compounds can act as a preventive measure to oxidative processes, in other words, its composition is a great antioxidant (Lanski and Newman, 2007; JURENKA 2008).

Studies have reported that an extract of *Punica granatum* contains portions of Ellagic Acid and other ellagitannins. Such substances are able to induce vasodilatation, scavenging free radicals, besides presenting potential lipid-lowering, anti-inflammatory and anticancer (USTA et al.2013)

According NODA and colleagues (2002), the fruit contains anthocyanins (delphinidin, cyanidin and pelargonidin), quercetin, phenolic acids (caffeic, catequínico, chlorogenic, and ortho paracumárico, ellagic, gallic and quinic) and tannins (punicalagin). In addition to these substances, experiments argue that Punica granatum has flavonoids able to prevent the activity of cyclooxygenase and lipoxygenase enzymes oxidizing (F. A. JARDINI e J. MANCINI FILHO, 2007)

Thus, the aim of this study is to analyze the chemical, biological and toxicological properties of a hydro-alcoholic extract of *Punica granatum*

2. Materials and Methods.

The hydroalcoholic extract of Punica granatum was produced by EMBRAPA and given to our research group so that appropriate analyzes were performed.

The tests for cytotoxicity and genotoxicity of the extract were performed using the disc diffusion method. The bacterial strains used are of *Staphylococcus aureus* (*S. aureus* ATCC 8096) strain. The bacterial concentration was standardized using the range of 0.5 Mc Farland (Laborclin).

The cytotoxicity and genotoxicity tests were conducted in four steps:

Step 1: Removal of colonies and transfer to selective medium white medium (nutrient agar, Merck). Incubation was placed in a bacteriological incubator (Ps -101, scientific Solab) at 35 °C for 24h (maintenance of colonies).

Step 2: Withdrawal of colony maintenance, in addition saline (NaCl 0.9%) and turbidity compared to the equivalence scale with 0.5 MC Farland.

Step 3: Sowing the material solubilized in saline solution in plates (Petri dishes for up to 50mL) for the disk diffusion test.

Step 4: Performing the disk diffusion test.

The disc diffusion test was performed by depositing paper discs in sterile plates with different volumes in the extract at a concentration of 0,167g / ml, plus the use of antibiotics such as amoxicillin (500 mg / ml), chloramphenicol discs (30 mg / disc) discs ampicilin (10 mg / disc) discs gentamicin (10 mg / disk) and other substances such as H_2O_2 (hydrogen peroxide, 3 = volume%) and stannous chloride ($SnCl_2$ Vetec). For insertion of the material in the plates, automatic pipettes (Gopet 0,5-10µl II, and 20-200µl 100-1000µl) were used. All antibiotics employees are also encouraged to use the SUS through RENAME (National List of Essential Medicines).

Below is an outline of the test:

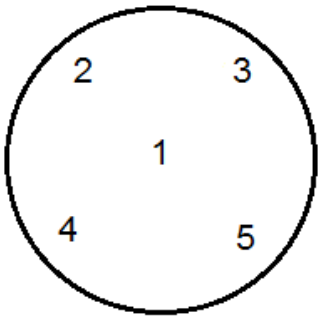

Plate 1 (duplicates A and B):

1- 24 µL NaCl (0.9%)

2 -24µL of diluted extract (0,167g / mL).

3- 12µL of diluted extract (0,167g / mL).

4- 24µL amoxicillin (50 mg / ml).

5-12µL of the diluted extract (0,167g / ml) + 12µL of amoxicillin (50 mg / ml)

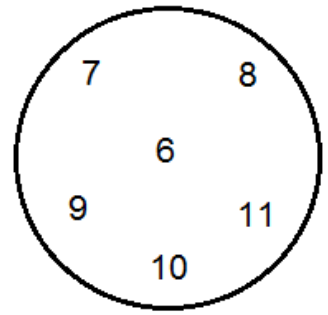

Plate 2 (duplicates A and B):
6 - Disc Chloramphenicol.
7- Disc Chloramphenicol + 12µL of diluted extract (0,167g / mL).
8- Disc Ampicilin.
9- Disk Ampicilin + 12µL of diluted extract (0,167g / mL).
10- Disc Gentamicin

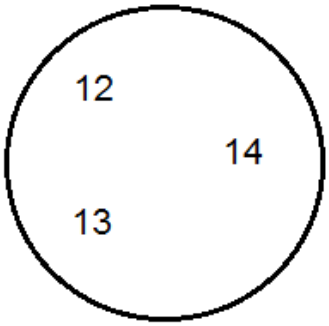

Plate 3 (duplicates A and B):
12-24µL of hydrogen peroxide (H_2O_2 volumes = 3%)
13-12µL of hydrogen peroxide (H_2O_2volumes = 3%)
14-12µL of hydrogen peroxide (H_2O_2 volumes = 3%) + 12µL of diluted extract (0,167g / mL).

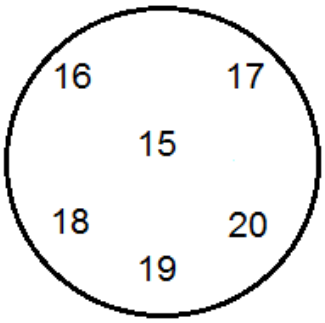

Plate 4 (duplicates A and B):
15-24 µL stannous chloride ($SnCl_2$) (5 mg / ml)
16-12µL of stannous chloride ($SnCl_2$) (5 mg / ml)
17-8µL diluted extract (0,167g / mL).
18- 8µL hydrogen peroxide ($H2O_2$ volumes = 3%)
19-8µL stannous chloride ($SnCl_2$) (5 mg / ml)
20-8µL stannous chloride ($SnCl_2$) (5 mg / ml) + 8µL of dilute extract (0,167g / ml) + 8µL of hydrogen peroxide (H2O2 volume = 3%).

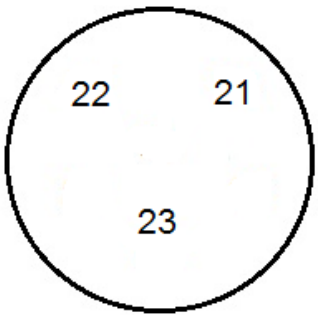

Plate 5 (duplicates A and B):
21-12µL of diluted extract (0,167g / ml) + 12µL $SnCl_2$ (5 mg / ml)
22-12µL of diluted extract (0,167g / ml) + 12µL of H_2O_2 (5 mg / ml)
23-12µL $SnCl_2$ (5 mg / ml) + 12µL of H_2O_2 (volume = 3%)

3. Results.

The disk diffusion tests with *S. aureus* (ATCC 8096) showed halos with the following dimensions:

Table 1. Results of Disk Diffusion (Duplicates Plate 1)

	Plate 1 A approximate values	**Plate 1 B approximate values**
1	No Halo	No Halo
2	Between 28 and 30 mm.	Between 28 and 30 mm.
3	Between 20 and 25 mm.	Between 20 and 25 mm.
4	>50mm	>50mm
5	>50mm	>50mm

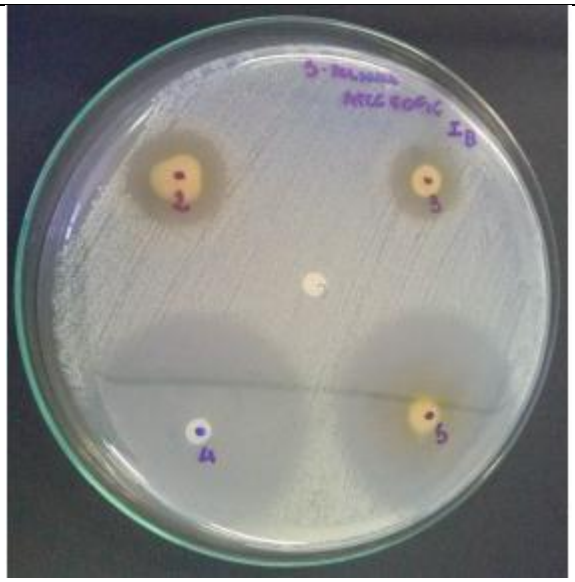

Figure 1. plate IB, 1- 24 ul NaCl (0.9%) ; 2- 24 ul diluted extract (0,167g / mL), 3- 12µL of diluted extract (0,167g / ml); 4 - 24µL of amoxicillin (50 mg / mL); 5- 12 ul amoxicillin (50 mg / mL + 12µL of diluid extract (0,167g / mL). Presence of "stain" on the discs concerning halos 2 and 3 Source: author.

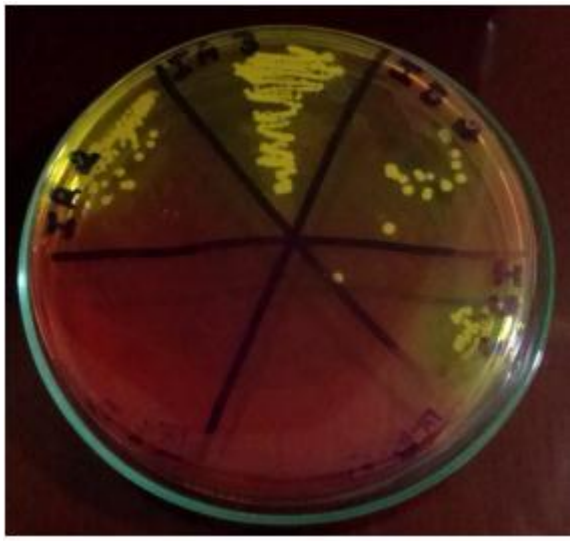

Figure 2. 18 Sowing the material bump the "stain" present in plates 1 and 2 in the middle salty mannitol (selective medium for S. aureus) strains resistant to investigation of the application of the extract. Source: author

Table 2. Results of Disk Diffusion (Duplicates Plate 2)

	Plate 2 A approximate values	**Plate 2 B approximate values**
6	Between 30 and32 mm	Between 30 and 32 mm
7	Between 30 and 32 mm	Between 30 and 32 mm
8	Between 35 and 40 mm	Between 35 and 40 mm
9	Between 40 and 45mm	Between 40 and 42mm
10	20 mm	20 mm
11	15 mm	15 mm

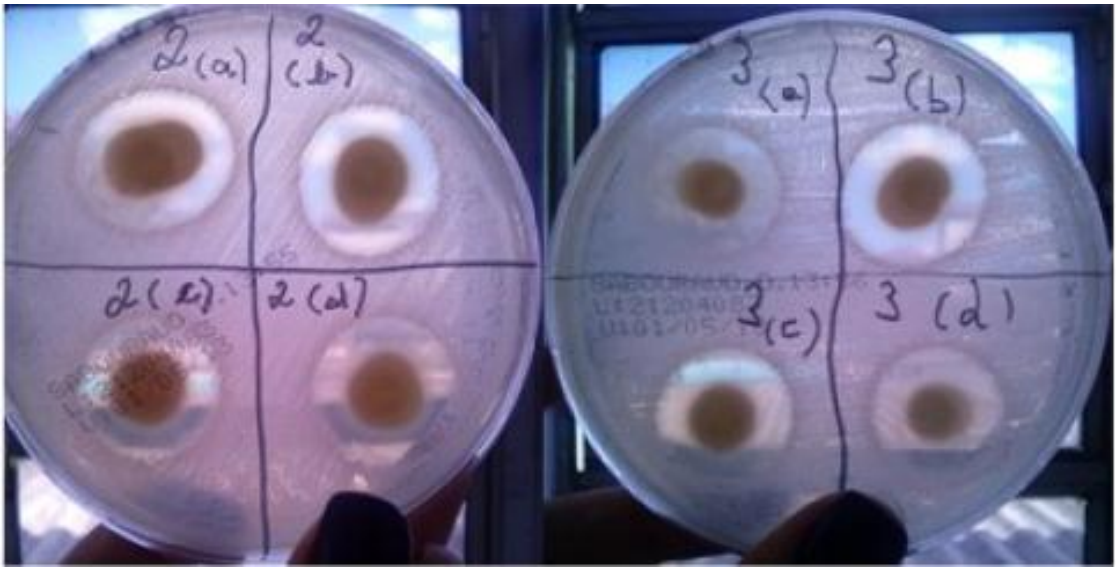

Figure 3. New disk diffusion only to the boards regarding guidelines of halos that grew in selective medium for S. aureus, material collide salty mannitol medium and seeded in white, showing that there was the same behavior. Source: author

The presence of spots in halos 1, 2 and 3 of the plate 1 led to speculation that it might exist resistant bacteria therein. To investigate this possibility, was performed one sowing of harvested material from spots in mannitol salt medium and growth was observed on selective medium.

Was made a new disk diffusion with the strains that grew in the middle mannitol in order to know if the bacteria would have the same behavior as the first broadcast. As a result, it was found that the bacteria followed the same orientation as the first test.

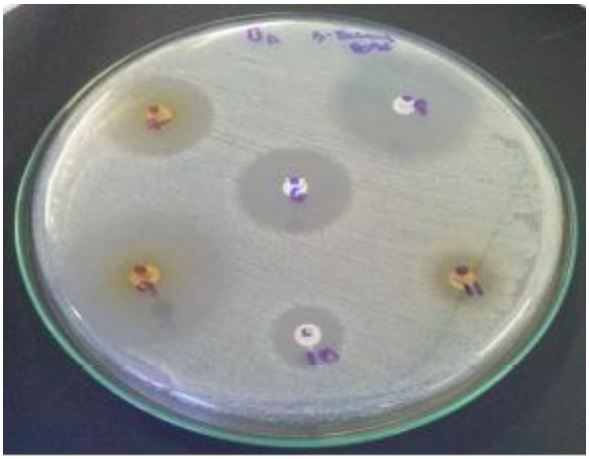

Figure 3. Plate IIA, 6- disk Chloramphenicol; 7-Chloramphenicol disc + 12µL of diluted extract (0,167 g / ml); 8-disk ampicillin, ampicillin; 9-disk + 12µL of diluted extract (0.16 g / ml); 10- disk gentamicin, 11-disc gentamicin+ 12µL of diluted extract (0,167g / mL) Source: author.

Table 3. Results of Disk Diffusion (Duplicates Plate 3)		
	Plate 3 A approximate values	**Plate 3 B approximate values**
12	>50mm	>50mm
13	50mm	Between 45 and 50 mm
14	>50mm	>50mm

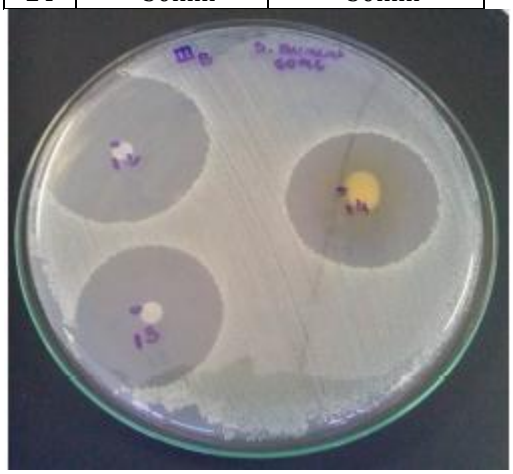

Figure 4. Plate IIIB. 12-24µL of hydrogen peroxide (volume = 3%), 13-12µL of hydrogen peroxide (volume = 3%), 14-12µL of hydrogen peroxide (volume = 3%) + 12µL of diluted extract . Source: author .

Table 4. Results of Disk Diffusion (Duplicates Plate 4)		
	Plate 4 A approximate values	**Plate 4 B approximate values**
15	Between 15 and 20 mm	Between 15 and 20 mm
16	Between 30 and 32 mm	Between 30 and 32 mm
17	Between 20 and 22mm	Between 20 and 22 mm
18	Between 45 and 50mm	Between 45 and 50mm
19	Between 10 e 12 mm	Between 10 and 12mm
20	Between 48 e 50 mm	Between 48 and 50mm

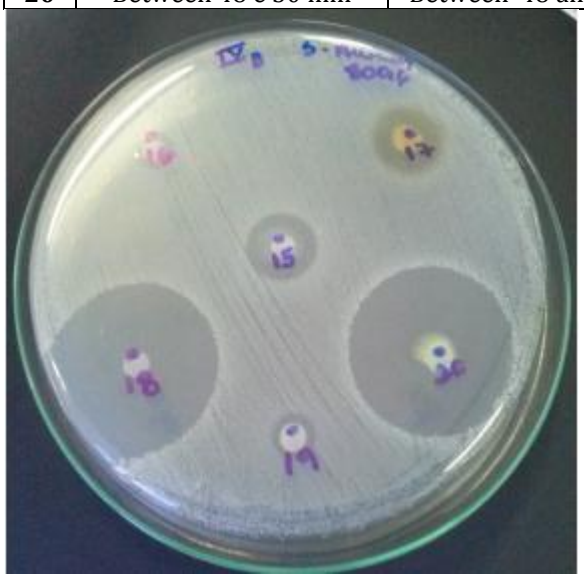

Figure 5. Plate IV B. 15- 24µL of stannous chloride(5mg / ml); 16- 12µL stannous chloride (5mg / ml) , 17- 8µL extract diluted (0,167g / ml); 18- 8µL hydrogen peroxide (% = 3 volumes) ; 19- 8µL stannous chloride (5mg / mL) 20- 8µL stannous chloride (5mg/mL) + 8µL of hydrogen peroxide + 8µL diluted extract (0,167g / ml) Source : author

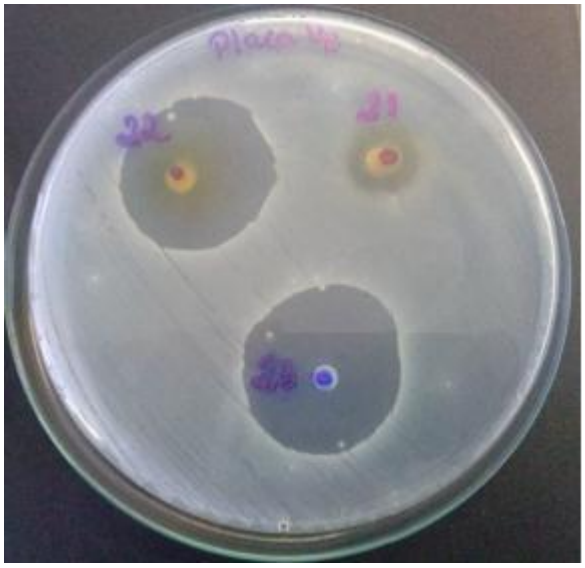

Figure 5. Plate VB. 21- 12µL of diluted (0,167g / mL) + 12µL of stannous chloride (5mg /mL); 22- 12µL of diluted(0,167g / mL) + 12µL of hydrogen peroxide (volume = extract extract 3%), 23- 12µL stannous chloride (5mg / mL) + 12µL of hydrogen peroxide (volume = 3 %). Source : author

Table 5. Results of Disk Difusion (Duplicates Plate 5)		
	Plate 5 A approximate values	**Plate 5 B approximate values**
21	20 mm	20 mm
22	40 mm	42 mm
22	40 mm	40 mm

3.4. Checking the Results of Statistical Analysis of Measure Halo:

There were no significant (p>0.05) changes seen from the following comparisons:

- 24 µL extract VS 12 µL extract → Action independent of dose.
- 24 µL extract VS Chloramphenicol.
- 12 µL extract VS Gentamicin
- 12 µL extract VS 24 µL SnCl2.
- 12µL of extract VS 12 µL extract + 12 µL SnCl2.
- 12 µL of extract + 12 µL of Amoxicillin VS 24 µL of Amoxicillin
- 24 µL of Amoxicillin vs 12 µL of extract + 12 µL of H_2O_2.
- 12 µL of extract + Ampicillin VS Ampicillin.
- 12µL of extract + 8µL of H_2O_2 VS 24µL of H_2O_2
- 8 µL of extract VS 24 µL of SnCl2
- 24µL of H_2O_2 VS 12µL of H_2O_2 VS 8 µL of H_2O_2 → Action independent of dose
- 24 µL of SnCl2 VS 12 µL of SnCl2 VS 8 µL of SnCl2 →Action independent of dose

There was considered significant changes (p<0.05) between the following comparisons:

- 12 µL of amoxicillin + 12 µL of extract <12 µL of extract + 12 µL of Chloramphenicol
- 12 µL of amoxicillin + 12 µL of extract <12 µL of extract + 12 µL of Gentamicin.
- Ampicillin + 12 µL of extract <Chloramphenicol.
- 12 µL of extract + Gentamicin <Chloramphenicol.
- 12 µL of H_2O_2 + 12 µL of extract <Chloramphenicol.
- 8 µL of extract <Ampicillin.
- 8 µL $SnCl_2$ <12 µL of $SnCl_2$ + 12 µL extract.
- 24 µL H_2O_2 <12 µL + 12 µL H_2O_2 extract.

Figure 6. Statistics of disk diffusion. A: 24 µl of NaCl (0.9%); B: 24µl of diluted extract; C: 12µl of the diluted extract; D: 24 µl of amoxicillin (50 mg / ml); E: 12µl of diluted extract + 12µl of amoxicillin; F: Disk Chloramphenicol; G: Disc Chloramphenicol + 12µl of diluted extract; H: Disc Ampicillin; I: Disc Ampicillin + 12µl of diluted extract; J: Disc Gentamicin; K: Disc Gentamicin + 12µl of diluted extract; L: 24µl of Hydrogen Peroxide (H2O2 = 3 volume%); M: 12µl of Hydrogen Peroxide (H_2O_2 volume = 3%); N: 12µl of Hydrogen Peroxide (H2O2 = 3 volume%) + 12µl of the diluted extract; O: 24 µl of stannous chloride (SnCl2) at 5 mg / ml; P: 12µl of stannous chloride (SnCl2) at 5 mg / mL; Q: 8µl extract diluted; R: 8µl of hydrogen peroxide (H_2O_2 volume = 3%); S: 8µl of stannous chloride (SnCl2) at 5 mg / mL; T: 8µl stannous chloride ($SnCl_2$) at 5 mg / ml + 8µl extract diluted+ 8µl of Hydrogen Peroxide (H_2O_2 volume = 3%); U: extract 12µl 12µl of diluted + $SnCl_2$; V: 12µl + 12µl of extract diluted H_2O_2 ,W: 12µl of $SnCl_2$ + 12µl of H_2O_2.

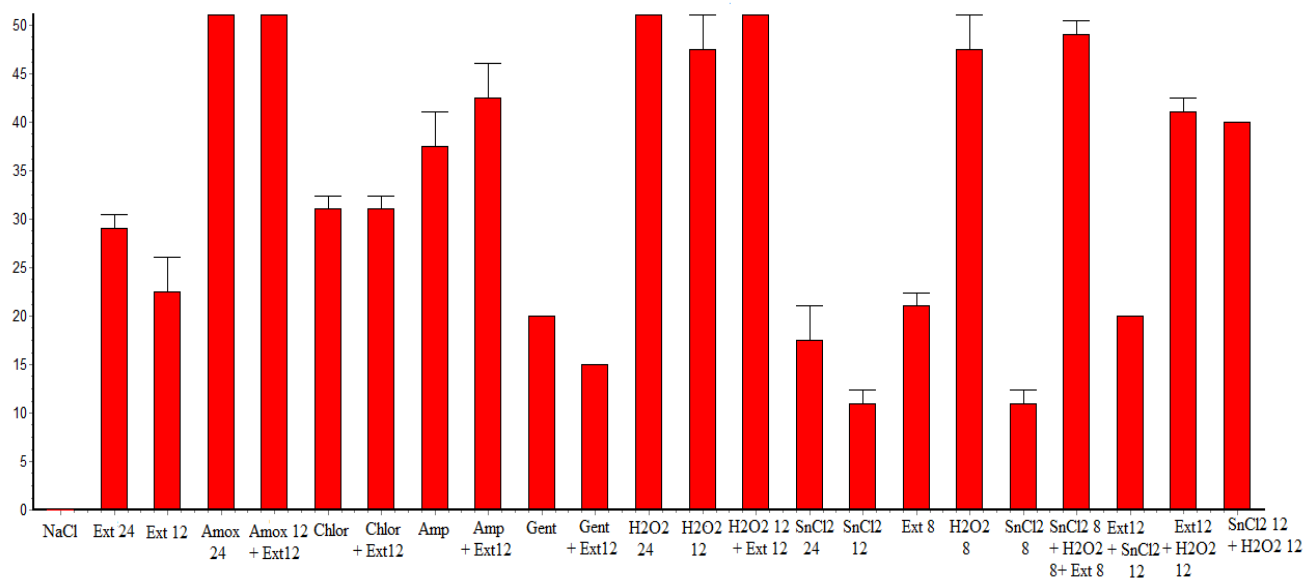

Figure 6. Statistics of disk diffusion:*Graphic representation of Diffusion Test Disk (Statistical Analysis)*

DISCUSSION

Through literature and this work, which results in the sensitivity of S. aureus to alcoholic extract Punica granatum independent of dose, it can be stated that this, as well as dyes derived from fruit, has antimicrobial activity (TRINITY et al, 2009; Werkman et al, 2008; RMR Cato et al, 2006), giving this cytotoxic potential.

Gentamicin and chloramphenicol act on bacterial protein synthesis (Rang, Dale, Ritter, 2001), it is suggested that the hydroalcoholic extract of pomegranate has a similar action to those due to similarity in the extent of halos assigned by statistical analysis beyond its best interacting with them when compared to antibiotics ampicillin and amoxicillin. Previous studies such as those reported by Canton et al (2010), and Adams et al (2013) show that there is a synergistic effect of the extract of *Baccharis dracunculifolia* DC and *Allium sativum*, respectively, gentamicin against S. aureus; Another study describes the possibility of using the extract of *Punica granatum* as an alternative to combat diseases in which the causative agent is resistant to the action chloramphenicol, as with *Salmonella tiphy* (Werkman, C. *et a*l, 2008 cited PEREZ, C and Anesini C .; 1994). One may suggest that the same mechanism can be used against *S.aureus,* as in the present study, the extract showed similar action to chloramphenicol.

The amoxicillin and ampicillin are antibiotics which inhibit cell wall synthesis (Rang, Dale, Ritter, 2001). In this paper, we realize there is a possibility that the extract be able to preserve the action of these antibiotics or small proportions of extract may have lower effects ampicillin, but not the same potentiates the action of these antibiotics nor interact so well when comparing with the chloramphenicol or gentamycin. Penicillins may have limited effect on the bacteria in question, given that *Staphylococcus aureus* is resistant to oxaciclina and meticiclina (Cato *et al*, 2006). Thus, it can be suggested that the relative limitation observed between the penicillins and the extract is not attributable to the effects of *Punica granatum*, but is related to the interaction of the antibiotic the microorganism.

Studies describe that *Punica granatum* contains a variety of phenolic compounds such as ellagic acid moieties and ellagitannins (USTA *et al*, 2013), anthocyanin, quercetin, phenolic acids, tannins (NODA *et al*, 2002) or that has flavonoids in its composition (Werkman, C. et al, 2008), which can giving it a potential antioxidant. In the present study, it was observed that pomegranate extract, depending on the proportions used, similar action has or is able to potentiate the effects of $SnCl_2$, which is a powerful reducing agent capable of inactivating *E. coli* strains, and through the generation of free radicals in vitro, can break bonds in the DNA plasmids (GIUSEPPE et al, 2007).

As with $SnCl_2$, the extract showed similar action on hydrogen peroxide (H2O2), which, like all detergents oxidants, has germicidal capacity arising from the production of nascent oxygen resulting from contact with catalase (enzymes present in blood and tissues), which makes it a great help in the sterilization of wounds from the mechanical action of this oxygen released (MORIYA T, Modena JLP, 2008). Furthermore, it is known that H_2O_2 is one of the components of the

Fenton reaction, which induces oxidative stress processes, through the production of free radicals (BARREIROS *et al*, 2006).

It is suggested that, in prokaryote cell model, the extract induces the oxidation, which could aid in the development of reactive oxygen species, which explains its bactericidal action previously tested by Trindade and colleagues (2009), which proves the antimicrobial activity of the tincture of peel of pomegranate front *S.aureus* describing the presence of halos with values between 8 and 13mm from the well through the method.

The *Punica granatum* has a cytotoxic effect, which is related to the sensitivity of *S.aureus* effect of the extract. It is speculated that this extract has genotoxic potential, which can be attributed to the good interaction and increase this leads to the action of H_2O_2 and $SnCl_2$ suggesting that the extract has the ability to induce the generation of reactive oxygen species and provide changes the bacterial genome. Such modifications in the DNA of the microorganism can also be linked to the possible action of the extract on ribosomal subunits in order to extract features, probably similar to the antibiotics gentamicin and chloramphenicol mechanism of action, which are able to inhibit protein synthesis bacteria by inhibition of ribosomal subunits coupling or through the production of aberrant proteins (Rang, Dale, Ritter, 2001). One may suggest that the extract has mutagenic action considering that possibly this is able to interfere with bacterial genes, however, so that this potential is better understood, it is necessary to incorporate more specific tests related to this feature .

CONCLUSION

From the results presented, it can be suggested that, against Staphylococcus aureus, hydro-alcoholic extract of Punica granatum possibly have cytotoxic, genotoxic, oxidative, inhibitory action of protein synthesis, enabling the incorporation of pomegranate extract as a potential alternative to treatment of staphylococcal diseases.

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