THUJA OCCIDENTALIS EFFECT ON GRAM-POSITIVE BACTERIA

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Abstract. *In recent years, microbial biofilm resistance has increased, posing significant challenges for the medical community in terms of disease treatment and for the food industry in terms of contamination and loss of shelf life. The purpose of this work is to test the antimicrobial efficacy against Gram-positive bacteria represented by the following reference strains: Streptococcus pyogenes (ATCC 19615), Staphylococcus aureus (ATCC 25923), Listeria monocytogenes (ATCC 19114), Bacillus cereus (ATCC 10876) and Clostridium perfringens (ATCC 13124), as well as the MIC evaluation of three Thuja occidentalis (TO) extracts. In accordance with ISO 20776-1:2019, the evaluation was conducted by measuring the loss of microbial mass using spectrophotometry to determine the optical density (OD). As a result of our research, we can say that to extracts, especially at the first concentration tested cause an inhibiting effect on Gram-positive bacteria. The inhibitory effect on the Gram-positive bacteria is increasing: Cl.perfringens< S. pyogenes< S. aureus < B. cereus < L. monocitogenes. The demonstrated effect of TO2 recommends it as a potential future candidate in natural products with antimicrobial activity. Our findings could allow TO2 blends in many areas, such as products for bacterial dermatological treatments and the food industry, both areas being strongly affected by the increase in antibacterial resistance to standard, commercial antimicrobial products.*

Keywords*: Thuja occidentalis, bacteria, Gram-positive, antimicrobial.*

INTRODUCTION

Because of technological breakthroughs, plants' therapeutic characteristics have piqued many people's curiosity due to their low toxicity, pharmacological activity, and economic viability. Several studies have investigated the benefits of phytochemical compounds derived from plants and their effects on human health (CHOUHAN, 2017). Natural chemicals have recently sparked increased interest in the pharmaceutical industries for direct use or in combination with other molecules.

The massive use of antibiotics has resulted in the emergence of microbial resistance, which represents another problem affecting public health. (CARUNTU, S., 2020) This has led to a significant increase in demand for new antibiotics against these pathogens, and a new interest has emerged within the scientific community in using plant-based medicines with antimicrobial properties (OBISTIOIU, D. 2021). Plants and other natural sources can offer many complex and structurally diverse compounds, and plant extracts have been found to possess a range of properties, including antifungal, antibacterial, and antiviral properties. (ALEXA, E.2018, COCAN, I. 2018)

Thuja occidentalis (Cupressaceae), commonly planted as an ornamental tree in Europe, including Romania, is a coniferous tree native to Canada and North America. Originally from Europe, this tree can reach a height of 15-20 m and has a pyramidal shape with flattened branches and twigs in a single plane (NASER, B, 2005). It has small-scale-like leaves that are green all year, with the lower side having a brighter green due to the presence of resin glands. The seeds of Thuja occidentalis are small, green to brown, and found in 1-2 cm long coniferous pins. In traditional medicine, Thuja has been used to treat respiratory system disorders such as bronchial catarrh and urinary and reproductive system disorders like enuresis,

cystitis, amenorrhea, and autoimmune diseases and rheumatic conditions such as psoriasis. (ALVES, L.D.S, 2014)

MATERIAL AND METHODS

Plant material and extraction method

TO plants were taken from Liebling, Timis County, Romania (45°34′00″N 21°19′54″E). The extraction was carried out within the Physico-chemical Analysis Laboratory of the Interdisciplinary Research Platform of the University of Life Sciences Timisoara. 10 grams of TO (Thuja occidentalis smaragd TO 1, Thuja occidentalis golden smaragd TO 2, Thuja occidentalis fastigiata TO 3) were ground and extracted in the 1:10 ratio in an alcoholic mixture 70% (ethyl alcohol 96% Chimreactiv S.R.L, Romania). The extract was left to be stirred using the Hot Plate Stirrer magnetic agitator (IDL LMS-1003, IDL GMBH&CO, England) for 24 hours. Subsequently, the extract was filtered with filter paper, and the resulting mixture was again filtered using a syringe filter Whatman Uniflo 25mm 0.2 μm (Thermo Fisher Scientific Inc., France).

Microbiological method

The Gram-positive reference microbial strains (ATCC) used in this study were obtained from the culture collection of the Microbiology Laboratory of the Interdisciplinary Research Platform of the University of Life Sciences Timisoara.

TO samples were tested on the following reference strains: *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19114), *Bacillus cereus* (ATCC 10876) and *Clostridium perfringens* (ATCC 13124).

MIC is defined as the lowest test concentration, which does not cause any visible, detectable growth of microorganisms. Our previous research has described the method as a microbial mass loss by OD measurement by spectrophotometry according to ISO 20776-1:2019.

Microbiological method

A dilution of 10^{-3} of the fresh bacterial strain was used to perform the test, an inoculum equivalent to a standard of 0.5 McFarland. The bacterial strains were revived overnight in the Brain Heart Infusion (BHI) broth (Oxoid, CM1135) at 37 °C and subsequently switched to BHI Agar (Oxoid, CM1136) for 24 hours at 37 °C. The cultures were then diluted to an optical density (OD) of 0.5 McFarland standard (1.5×10^8CFU×mL) using BHI broth and evaluated with a McFarland densimeter (Grand-Bio, England). The dilutions were spotted at a volume of 100 μL in each well of the 96-well microdilution plate using a Calibra 852 digital multichannel pipette. The tested TO was added in the amount of 25 μL, 50 μL, 75 μL and 100 μL. Plates were covered and left for 24 hours at 37 °C. After 24 hours, the DO was measured at 540 nm using an ELISA reader (BIORAD PR 1100, Hercules, CA, USA). Triplicate tests were performed for all samples. Strain suspensions in BHI were used as a positive control.

The method of microdilution in broth is one of the most basic methods of testing antimicrobial susceptibility (CLSI, 2017). The technique involves testing double dilutions of

the antimicrobial agent analyzed in a liquid growth medium distributed in microtitre plates with 96 wells.

MIC is the lowest concentration of antimicrobial agent that inhibits growth. CLSI has standardized the broth microdilution method to test aerobically growing bacteria, yeasts and filamentous fungi. The EUCAST broth microdilution method is similar to that of CLSI (CLSI, 2017), with changes that typically refer to some test parameters, such as inoculum preparation, inoculum size and MIC reading.

The results are presented as bacterial growth rate (BGR%) rate and bacterial inhibition rate (BIR%), calculated rates using the formulas (1), (2):

$$BGR\% = \frac{OD_{SAMPLE}}{OD_{CONTROL}} \times 100\ (\%) \quad (1)$$

$$BIR\% = 100 - BGR\ (\%) \quad (2)$$

RESULTS AND DISCUSSIONS

The results are presented as values of the growth rate respectively of the inhibition rate and those obtained using the formulae given under the microbiological analysis method.

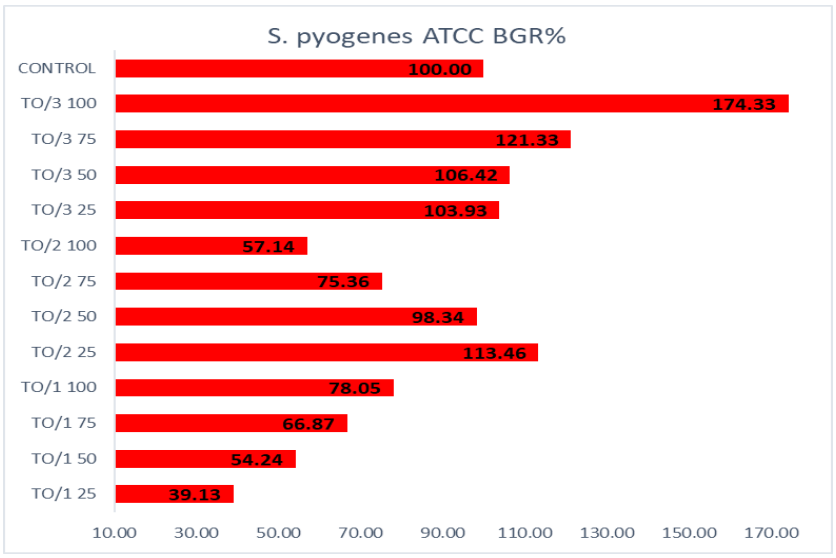

Figure 1. Graphic representation of the activity of TO extracts against *S. pyogenes* expressed as BGR%

Figure 1 shows the graphical representation of the activity of TO extracts against the strain of S. pyogenes expressed as BGR%. The bacterial growth rate in the first extract is positively correlated with the increase in concentration, which means that the extract had a potentiating effect against the strains of *Streptococcus pyogenes*. The difference is made by the TO 2 extract, which causes a decrease in the bacterial growth rate, meaning that the strain has a decreasing bacterial growth correlated with the increase in the concentration tested. TO 3 extract shows an increase in the bacterial growth rate, thus being positively correlated with the increase in concentration.

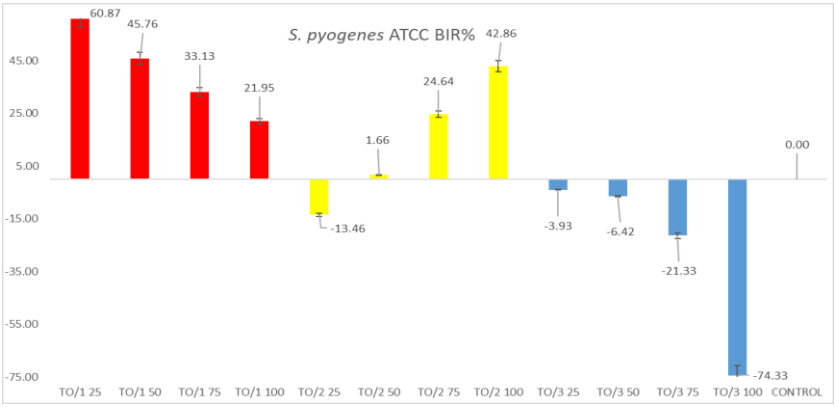

Figure 2. Graphic representation of the activity of TO extracts against *S. pyogenes* expressed as BIR%

Figure 2 is the bacterial inhibition rate of the TO extracts on *Streptococcus pyogenes*, expressed as a percentage rate expressed as a ratio of the OD value of different concentrations of the extract tested.

The antimicrobial activity of TO1 extract is negatively correlated with the increase in concentration; although the inhibition values are higher than the control one, the inhibition value decreases with the increase in concentration. If the BIR% value for the first concentration of 25 microliters is 60,87 %, the concentration of 100 microliters BIR % decreases to 21,95 %.

Of the 3 extracts tested, the effectiveness is demonstrated by extract 2 with an inhibition rate of 42,86 %. Extract TO1 has a potentiating effect, but the values tested are sufficient for the clear inhibition of the strain. In contrast, extract 3 proves a clear potentiating effect positively correlated with the increase in concentration, the inhibition values being all negative values that increase with the increase in the amount of extract tested. Our findings concerning the effectiveness of TO extracts against *S. pyogenes* are similar to the data presented by SAH, S. N, 2017 and SHIV, N. S., 2017.

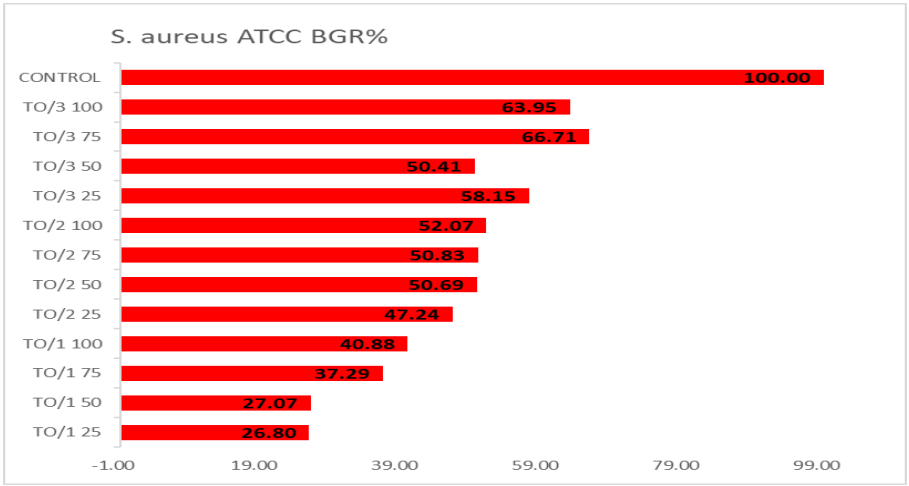

Figure 3. Graphic representation of the activity of TO extracts against *S. aureus* expressed as BGR%

Figure 3 shows the graphical representation of the activity of extracts against *S. aureus* expressed as bacterial growth rate, with the effectiveness of the tested extracts being reported as a percentage. The highest bacterial growth is identified in the TO3 extract, the low efficacy being proven only in the case of TO1 extract.

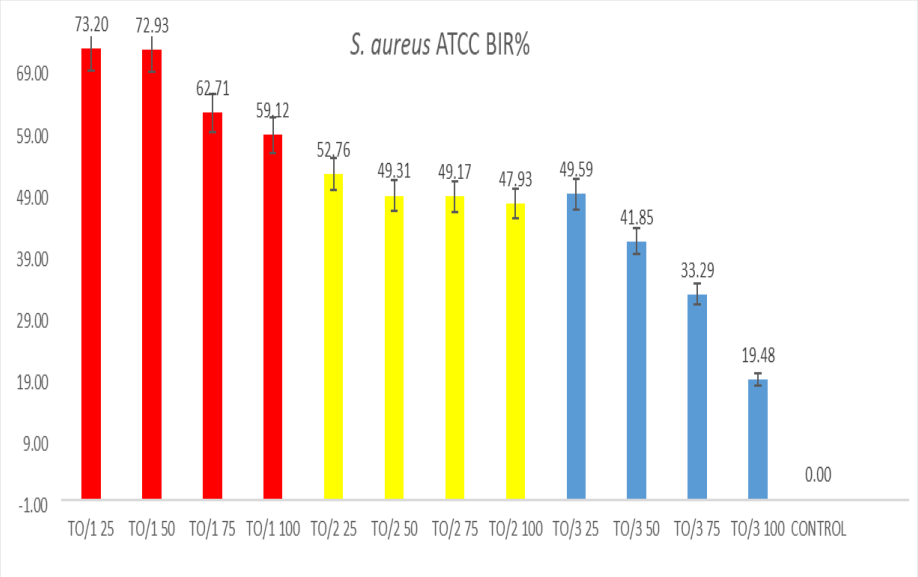

Figure 4. Graphic representation of the activity of TO extracts against *S. aureus* expressed as BIR%

The proven bacterial inhibition rate in the case of the *S. aureus* strain is shown in Figure 4 and shows the values obtained in the case of extract testing. These values prove that 1 extract was the most effective in terms of value, with an inhibition rate of 73,20 %. Although all the values obtained prove a good efficacy of the extracts against the staph strain, the results also prove a negative correlation with the increase in concentration, with the effect decreasing with the increase in the amount of extract tested. CARUNTU, S. 2020 presented similar findings to our results.

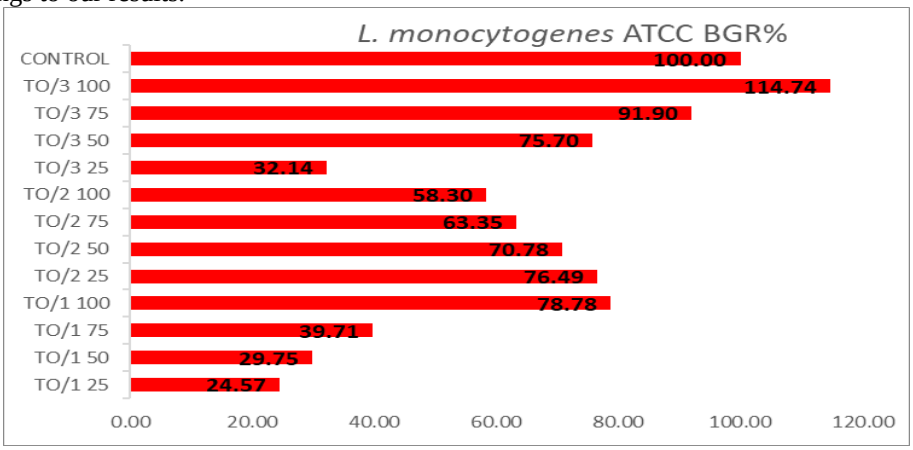

Figure 5. Graphic representation of the activity of TO extracts against *L. monocytogenes* expressed as BGR%

In the case of *L. monocytogenes* strain, the bacterial growth rate proves a high, stimulated increase in the case of TO 3 extract with values exceeding even the value obtained by the negative control. Thus, if in the case of control, the value of the growth rate is 100%, in the case of to 3 extract in the amount of 100 microliters, the value obtained is 114.74%.

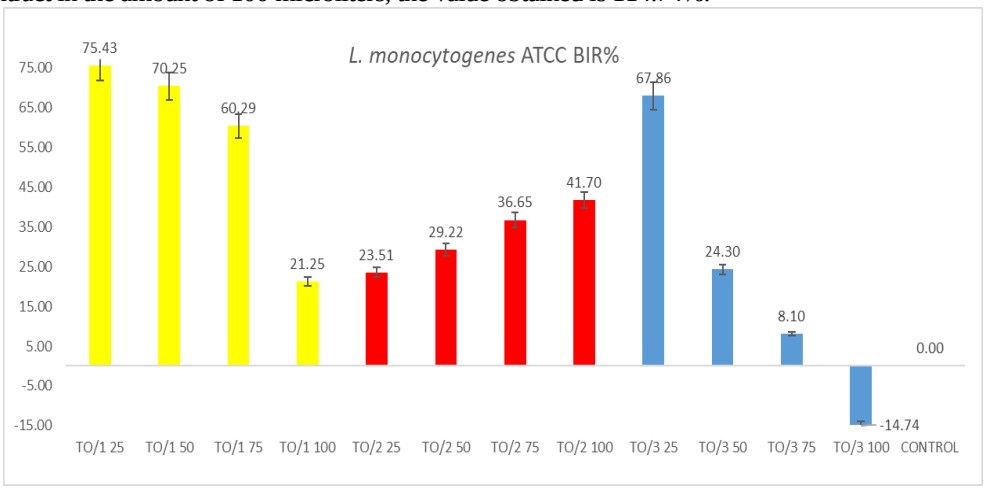

Figure 6. Graphic representation of the activity of TO extracts against *L. monocytogenes* expressed as BIR%

Figure 6 shows the graphical representation of the activity of TO extracts against the strain of *L. monocytogenes* expressed as a bacterial inhibition rate, a value expressed as a percentage. The graphic representation results that both the TO 1 extract and the TO 3 extract have an activity that exceeds the negative control but is negatively correlated with the increase in the concentration of the inhibition values, decreasing with the increase in the amount of extract tested. In contrast, in the case of TO 2 extract, the efficacy is in positive correlation with the increase in concentration so that the values obtained for the IRR% vary from 23,51 % in the case of the quantity of 25 microliters tested, and rises, in parallel with the increase in the amount tested, to 41,70 % in the case of the quantity of 100 microliters tested. JASUJA, N, 2017 demonstrated similar antibacterial effectiveness of the TO seeds against *L. monocitogenes*, with values of 20 ul extract tested.

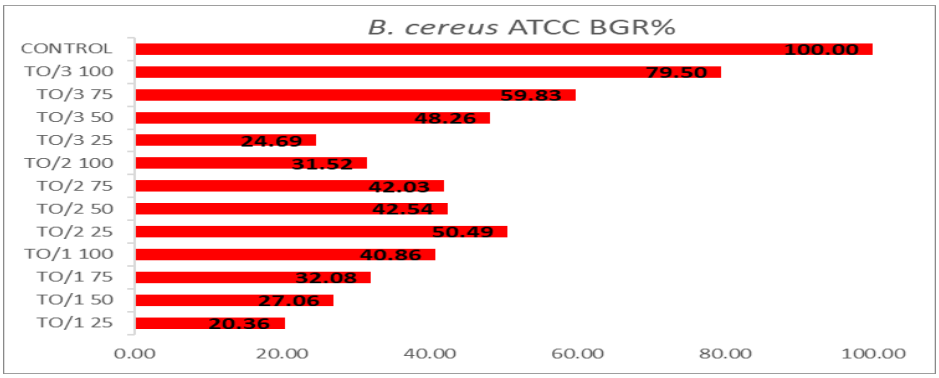

Figure 7. Graphic representation of the activity of TO extracts against *B. cereus* expressed as BGR%

The efficacy of extracts against *Bacillus cereus* strains is shown in Figure 7 as bacterial growth rate, the values obtained by the extracts tested being reported as a percentage of the value obtained in the case of positive control. The values obtained in the case of the bacterial growth rate show a sustained increase in the case of TO 3 extract with a value obtained of 79,50 % as opposed to the TO1 extract which obtained a growth process of 40,86 %.

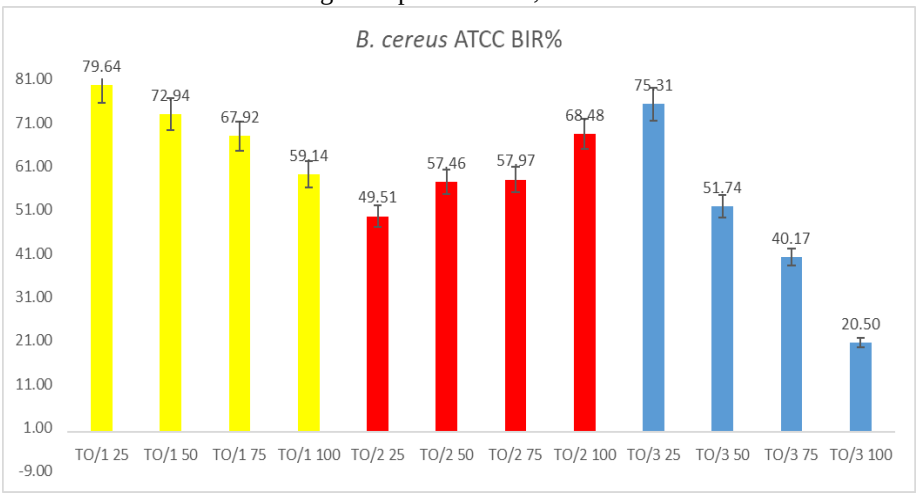

Figure 8. Graphic representation of the activity of TO extracts against *B. cereus* expressed as BIR%

Figure 8 shows the graphical representation of the activity of thuja extracts against *B. cereus*, with values obtained as bacterial inhibition rate. From the rates obtained, we can conclude that both to1 extract and TO 3 extract have antibacterial efficacy, but this effect decreases with increasing concentration. The difference is made by extract 2 which has an effectiveness positively correlated with the increase in concentration, the values obtained having a positive increase. IKRAM, M., E, 2017 and his collaborators demonstrated good antibacterial effectiveness of the TO seeds against *B. cereus*, with values of 20 ul extracts tested.

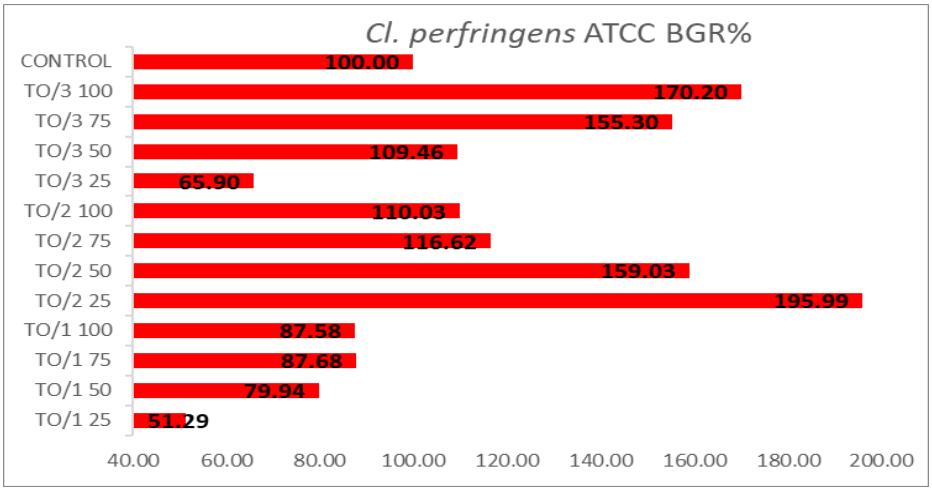

Figure 9. Graphic representation of the activity of TO extracts against *Cl. perfringens* expressed as BGR%

Figure 9 shows the bacterial growth values of the 3 extracts tested against the strain of *Clostridium perfringens*. The values obtained show a stimulated bacterial growth supported by extract 2 at the first test concentration, which exceeds the rate obtained in the case of the maximum tested concentration of extract 3. The same evolution of growth is presented by TEKADAY, D, 2020 and TSIRI, D., 2009 in their published research.

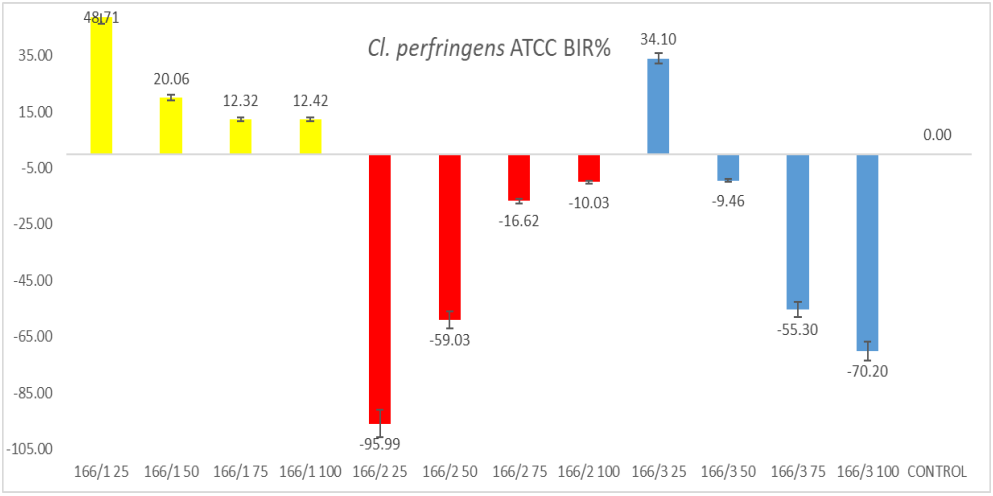

Figure 10. Graphic representation of the activity of to extracts against *Cl. perfirngens* expressed as BIR%

The bacterial inhibition rate calculated in the case of the strain of *Clostridium perfringens* proves an efficacy negatively correlated with the increase in concentration in the case of extract 1 and a low efficacy in the case of extract 2, which although it proves a positive correlation with the increase in concentration, the values obtained are negative, the concentration tested being insufficient for a positive result. In the case of extract 3 the first concentration tested gave a positive result the correlation being negative with the increase in concentration the following values obtained being decreasing as efficacy and increasing as negative values. KYOUNG, S, 2017 and NAKULESHWAR, D. J, 2013 published similar effectiveness of the TO plant against *Cl. perfringens*.

Table 1.

MIC values obtained in the case of TO extracts on atcc strains tested

	S. pyogenes	*S. aureus*	*l. monocyttogenes*	*B. cereus*	*Cl. perfringens*
TO 1	25µL	25µL	25µL	25µL	25µL
TO 1	50µL	50µL	50µL	50µL	50µL
TO 1	75µL	75µL	75µL	75µL	75µL
TO 1	100µL	100µL	100µL	100µL	100µL
TO 2	25µL	25µL	25µL	25µL	25µL
TO 2	50µL	50µL	50µL	50µL	50µL
TO 2	75µL	75µL	75µL	75µL	75µL
TO 2	100µL	100µL	100µL	100µL	100µL

TO 3	25µL	25µL	25µL	25µL	25µL
TO 3	50µL	50µL	50µL	50µL	50µL
TO 3	75µL	75µL	75µL	75µL	75µL
TO 3	100µL	100µL	100µL	100µL	100µL
Control	0	0	0	0	0

The light grey colour represents the samples in which the MIC was not found, and the subsequent concentrations showed a potentiating effect with negative BIR% values, and therefore, the effect decreased together with the concentration. The red colour highlights the samples where the MIC was determined and maintained and the yellow highlights the samples where the MIC was determined but the subsequent concentrations showed a potentiating effect maintained together with the increase in concentration.

CONCLUSIONS

As a result of our research, we can say that to extracts, especially at the first concentration tested cause an inhibiting effect on Gram-positive bacteria. The inhibitory effect on the Gram-positive bacteria is increasing: *Cl.perfringens< S. pyogenes< S. aureus < B. cereus < L. monocitogenes.*

The demonstrated effect of TO2 recommends it as a potential future candidate in natural products with antimicrobial activity. Our findings could allow TO2 blends in many areas, such as products for bacterial dermatological treatments and the food industry, both areas being strongly affected by the increase in antibacterial resistance to standard, commercial antimicrobial products.

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