GLOBAL JOURNAL OF BIOLOGY, AGRICULTURE & HEALTH SCIENCES
(*Published By*: Global Institute for Research & Education)
www.gifre.org

Polyphenol-Rich Food Colourant G8000™ Inhibits Gut Microorganisms *in vitro* and Increase Number of Bowel Movements in Humans

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Abstract

G8000 is a natural polyphenol rich food colourant obtained from grape juice. We investigated the antimicrobial potential of G8000 in different gut microorganisms *in vitro* and assess G8000 effects in the intestinal transit of healthy volunteers. Antimicrobial activity of G8000 was tested by agar diffusion and minimal inhibitory concentration (MIC) assay. Additionally, 15 healthy individuals consumed G8000 daily for 28 days and the number of bowel movements was assessed. The results of the microbial growth in agar showed antimicrobial activity of G8000 against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but not against *Escherichia coli* or *Candida albicans*. As for the MIC assay, G8000 was able to inhibit all tested microorganisms at different concentrations. The number of daily bowel movements increased from 0.81±0,47 in day 0 to 1.31±0,47 after 28 days of G8000 intake, in average (p=0,02). We conclude that G8000 demonstrated specific antimicrobial activity with possible association with increased bowel movement.

Keywords:-Natural food colourant, grape, polyphenols, antimicrobial activity, gut microorganisms

I. Introduction

Medicinal plants are sources of phytochemicals which are able to initiate different biological activities including antimicrobial action. Polyphenols are amongst the most studied group of bioactive compounds found in vegetables and fruits. The typical polyphenol structure, with several hydroxyl groups on aromatic rings, has been identified in higher plants, and several hundreds are found in foodstuffs (Tomás-Barberán & Andrés-Lacueva, 2012). Fruits, especially those with red and blue colors, are the most important sources of phenolic compounds in the diet. Generally classified in phenolic acids, flavonoids, stilbenes, and lignans, phenolic compounds are often used in the food industry as natural colorants and antioxidants. As nutraceutics, polyphenols exert several effects such as anticarcinogenic activity, reduction of the atherosclerotic plaque formation, reduction of the smooth muscle cells proliferation in blood vessels, decrease of postprandial LDL oxidation and inhibition of endothelin (with vasodilatory effect), among others (Flamini et al, 2013; Gorelik et al, 2013; Gollücke et al, 2013a; Gollücke et al, 2013b; Landete, 2012; Moura et al, 2012).

In a recent review, Duda-Chodak et al, 2015, mentioned the limited number of published studies investigating the influence of polyphenols on gut microbial. Cueva et al, 2013 demonstrated the antibacterial activity of wine phenolics against Gram-negative bacteria and Oliveira et al, 2013, showed the potential of grape pomace against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Earlier, Brown et al, 2009 observed only moderate effectiveness of grape skins against *Helicobacter pylori*.

G8000 is a natural food colourant obtained by nanofiltration of *Vitis labrusca* grape juice (mostly of Concord variety), with subsequent concentration at 68° Brix by evaporation. The product is purple viscous syrup highly soluble in water, with applications in the food industry. Its major chemical composition was assessed by our group in 2011 and 2015 using ESI-MS (Electrospray Ionization - Mass Espectrometry). G8000 contains mainly sugars (monossacharides), organic acids (mainly malic and tartaric acids), phenolic acids (mostly p-coumaroyl tartaric acid and caffeoyl-L-tartaric acid), and anthocyanins (mainly malvidin-3-O-glucoside, peonidin-3-O-glucoside and petunidin-3-O-glucoside) (Aguiar Jr. et al, 2011, Peres et al, 2015). Earlier we have demonstrated that G8000 was able to exert important biological activities in both animals and humans (Aguiar Jr. et al, 2011, Moura et al, 2011; Paiotti et al, 2013, Pires et al, 2013, de Jesus et al, 2014, Marchi et al., 2014, Peres et al, 2015).

Aiming at providing new data concerning polyphenols on gut microbiota, the objective of this investigation was to test the antimicrobial potential of G8000 in different gut microorganisms *in vitro* and to assess its effect in the intestinal transit in healthy volunteers.

II. Methods

II.1 Antimicrobial effect *in vitro*

To investigate the antimicrobial efficacy of G8000, the agar well diffusion assay was performed against three bacteria: *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 14948), *Pseudomonas aeroginosa* (ATCC 27853) and the fungus *Candida albicans* (ATCC 5314). The method was performed as proposed by the CLSI (2008).

The nutrient agar culture broth (10021307 VAL: 19/09/2017 LAB: Kasvi LOT) was used for the growth of the microorganisms, while G8000 acted as the inhibitory agent. 8.4g of nutrient agar culture was diluted to 300ml distilled water and sterilized in autoclave for 15 minutes at 121°C. At room temperature, G8000 at 1%, 1.5%, 2% or 2.5% were added to the culture broth. Microorganisms were grown in BHI (Brain Heart Infusion) for 24 hours at 37°C then seeded immediately on the petri plates by depletion technique in a sterile laminar flow hood. The seeded plates were incubated for 24 hours at 37°C.

After 24 hours it was observed growth in all plates. Then, new samples were prepared by adding the additive inhibitory portions per ml: per 100 ml of broth was placed 1 ml of inhibiting additive, which resulted in initial samples of 300 ml of broth to 3 ml of additive. Since there was growth in all strains tested, was added, in ascending order, 1.5 ml of the additive to each sample which led to concentrations of 300 ml / 4.5 ml, 300ml / 6ml and eventually 300ml / 7,5ml and the method was followed to each sample.

II. 2 Minimum inhibitory concentration (MIC)

All assays were performed in triplicate using strains from the American Type Culture Collection (ATCC): *Staphylococcus aureus* (29213) *Escherichia coli* (14948), *Pseudomonas aeruginosa* (27853) and *Candida albicans* (5314). For bacteria, the determination of the minimum inhibitory concentration of the G8000 colourant was performed according to the broth dilution method (microtechnique) proposed by the CLSI (2008). Bottom microplates were used in a "U" sterilized with 96 holes (TPP®, USA). Each well received the inoculums, the liquid culture medium Brain Heart Infusion (BHI) and crude G8000 solutions at final concentrations ranging from 0.004 to 40 mg / ml, with a final volume of 100 uL. The inoculums were adjusted for each microorganism at a concentration of 5 × colony forming units per 105 mL (CFU / mL), under the rules of "Clinical and Laboratory Standards Institute" (CLSI). Shortly after micropipetting, plates were capped and incubated at 37°C for 24 h without agitation. After the incubation period, 30 uL of resazurin 0.02% was added in a sterile aqueous solution to each well. After 30 minutes of reincubation, reading was performed. Resazurin facilitates the observation of microbial presence, since absence of blue color indicates microbial growth, whereas red color indicates the presence of viable cells in growth. Thus it was possible to determine the lowest concentration of the agent (G8000) capable of inhibiting the growth of the assessed bacteria. For pure substances (Vancomycin and Gentamicin), which were also used as positive controls, the tested concentrations ranged from 0.0000115 to 0.0059 mg/mL, comprising 100 uL of the final volume. The BHI liquid culture medium was used as negative control.

For *Candida albicans*, microplates were used with 96 holes, each hole received the inoculum, the culture medium and the G8000 solution diluted to the final volume of 200µL in each well (CLSI, 2008). A solution of amphotericin B at concentrations ranging from 0.000031 to 0.016 mg/ml was used as a positive control. Two standard strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) from the "American Type Culture Collection" (ATCC) were used as technique quality controls.

Additionally, analytic controls of broth sterility (with RPMI 1640 medium -Gibco) and inoculums control (at $7.5x10^2$ CFU/mL) were performed. Shortly after micropipetting of RPMI culture medium and controls, the microplates were capped and incubated at 37°C for 24 hours without agitation. After the incubation period, as an optional procedure in this method, 30µL of resazurin (Sigma, ST. Louis, MO, USA) in 0.02% sterile aqueous solution were added to each well followed by a 4 hours reincubation. The blue color resazurin (7-hydroxy-3H-phenoxazine-3-one-10-oxide) oxidizes in the presence of resofurine viable cells, a red coloring substance, facilitating the observation of microbial growth. Using this method it was possible to determine the lowest concentration of G8000 capable of inhibiting the microbial growth.

II. 3 Assessment of daily bowel movements

A total of 15 healthy subjects, 9 men and 6 women, 23.7±4.5 years old were offered 70 g of G8000™ (Golden Sucos, Farroupilha-RS, Brazil) daily for 28 days. The dose is based on the amount of polyphenols measured in the product in a preliminary study (Peres et al., 2015). The dose provided an individual intake of 3g polyphenol/day, equivalent to 1.5 L of Concord grape juice. G8000 intake occurred during one of the main daily meals and subjects were asked not to alter their eating habits and daily physical activity during the experiment. Subjects were informed on the procedures used in the study and gave their written consent. The study was performed in accordance with the ethical standards of the Declaration of Helsinki and was also approved by the Ethics Committee at Universidade Federal de São Paulo, São Paulo-SP, Brazil. On day 0 and day 28, volunteers answered a questionnaire about their daily number of bowel movements The answers obtained on day 0 and day 28 were then compared applying the Student´s t-test (IBM SPSS software pack version 1.0 - IBM Corp.; Armonk-NY, USA). A $p<0.05$ was considered for statistical significance.

III. Results

The microbial growth in nutrient agar culture showed that at 1% G8000 was unable to promote inhibition of growth for all tested species. However, at 2% and 2.5%, the product inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. No inhibiting activity was observed against *Escherichia coli* or *Candida albicans* in all tested concentrations. The results of the MIC experiment are shown in table 1. G8000 was able to inhibit the growth of all tested microbials at different concentrations. The most efficient inhibition occurred against *Pseudomonas aeroginosa* at 5 mg/ml, while the actions against *Staphylococcus aureus* and *Candida albicans* required 10 mg/ml of G8000. *Escherichia coli* growth was inhibited at 30 mg/ml. Positive controls gentamicin and vancomycin were more efficient inhibitors in terms of concentration than G8000. Nevertheless, G8000 was able to inhibit the growth of all tested microbials, whereas gentamicin and vancomycin were specific inhibitors of only one or two microbials. Amphotericin B was not able to inhibit the growth of any tested species.

Regarding the evaluation conducted in humans, figure 1 summarizes the average results of 15 volunteers. On day 0, the individuals declared that the number of daily bowel movements occurred once a day or once very two days with an average of 0.81±0,47 daily movements. After 28 days of G8000 consumption, the number of daily bowel movements

raised to one to two times a day, with an average of 1.31±0,47 daily movements. The increase was statistically significant at p=0.02.

IV. Discussion

It has been well established that polyphenols exert physiological effects in human health. Several studies have demonstrated that phenolic compounds have antioxidant and anticarcinogenic activities, exert protective effects on the human cardiovascular system, and act as coadjutants in obesity, metabolic syndromes and diabetes (Gollücke et al, 2013). However, the antimicrobial effects of polyphenols are still not clear and more data on the subject are needed.

Earlier, Simpson et al, 2013 observed that infusions prepared using the most abundant polyphenols in rooibos showed antimicrobial activity against three species of bacteria: Gram-positive *Staphylococus epidermidis* and *Staphylococcus aureus* and Gram-negative *Escherichia coli*. Polyphenol-rich fractions from *Sida alba* L. (Malvaceae) showed significant effects against ten bacteria strains (Gram-negative and Gram-positive), specially *Enterococcus faecalis* (Konaté et al, 2012). Effects of methanolic, ethylacetate (EtOAc) and hexanic fractions of five Cameroonian medicinal plants were studied against 10 pathogenic microorganisms of the urogenital and gastrointestinal tracts. The results showed that all tested plant extracts were active against infections caused by resistant agents (Assob, 2011). Phenolic extracts from grape skin from 14 grape varieties showed antimicrobial activity against Gram-positive (*Staphylococcus aureus, Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli O157:H7, Salmonella Infantis, Campylobacter coli*) (Katalinic et al, 2010). Conversely, polyphenolic compounds widely found in olive mill wastewater were not effective against Gram-positive (*Streptococcus pyogenes and Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). Only hydroxytyrosol at 400 µg/mL caused complete growth inhibition of the four strains while gallic acid at 200 and 400 µg/mL was effective against *S. aureus* and *S. pyogenes* (Tafesh et al, 2011).

In respect to grape products and grape polyphenols, reports of antimicrobial activity were also found. A extract of polyphenols obtained from wine residue (seeds, skin and pomace) from New Zealand Pinot Noir were effective against *Staphylococcus aureus* and *Candida albicans*. Extracts from Pinot Meunier were less effective (Cheng et al, 2012). Quercetin and resveratrol from muscadine grape skin extract showed strong anti-*Helicobacter pylori* activity *in vitro* independent of pH, according to Brown & Jiang, 2011. Sugita-Konishi et al. (2001) reported reduction of *Salmonella enteritidis* and *Escherichia coli* growth in red and white wines within 30 minutes.

It is clear that alcohol enhances antimicrobial activity in wine. Therefore, the supposed microbial inhibition exerted by non-fermented grape juice was also investigated. Two strains of *E. coli* O157:H7 (ATCC 43895 and ATCC 43894) survived in Chardonnay grape juice (Just & Daeschel, 2003). Peng et al. (2008) reported that gram-negative bacteria such as *E. coli* O157:H7 and *Pseudomonas aeruginosa* were more resistant on grape seed extracts than gram-positive bacteria. Rhodes et al. (2006) reported inhibition of *L. monocytogenes* in grape juice within 10 min but no effect against *E. coli* after 60 min. Conversely, fresh and processed red muscadine grape juice (*Vitis rotundifolia*) and polar fractions containing malic, tartaric and tannic acids showed strong antimicrobial activity against *E coli* O157:H7 (Kim et al, 2009).

The present study tested the antimicrobial activity of G8000 against three bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli*) and fungus (*Candida albicans*) present in the intestinal microbiota. G8000 exerted antimicrobial activity against Gram-negative *Staphylococcus aureus* and against *Pseudomonas aeruginosa*, but not against *Escherichia coli* or *Candida albicans*, in the agar well diffusion assay. When using the MIC method G8000 was able to inhibit the growth of all tested microbials at different concentrations: *Pseudomonas aeruginosa* at 5 mg/ml, *Staphylococcus aureus* and *Candida albicans* at 10 mg/ml and *Escherichia coli* 30 mg/ml. Compared to the controls, G8000 was less effective in terms of concentration and more efficient in terms of range. The product was able to inhibit all tested species, while the positive controls gentamicin and vancomycin inhibited only specific strains. G8000 is a product from grape juice with high sugar and water contents (43.4 and 35.7%, respectively) (Peres et al, 2015). That fact might explain the need of a higher concentration. Additionally, in our study, the inhibition capacity varied according to the method used.

Our results confirm partially the findings by Cheng et al (2012) and Oliveira (2013): grape compounds showed effectiveness against *Staphylococcus aureus*. Likewise, Ranjitha et al (2014) reported antimicrobial activity of grape pomace, considered a waste of wine industries, against *Staphylococcus aureus* but less against *E. Coli*. Grape pomace powders (GPP) and grape pomace extracts (GPE) of five different Turkish grape varieties completely inhibited *S.aureus*, but *E coli* was less sensitive. (Sagdic et al, 2012). As a whole, the reports indicate that grape products are more efficient inhibitors of both *Staphylococus aureus* and *Pseudomonas aeruginosa*, but less efficient against *E. coli*, which are in agreement with our findings. There are very few data on anti-candida activity attributed to grape products. Our results demonstrated the inhibition of *Candida albicans* growth, in accordance with the results of Simonetti et al (2014), reporting anti-candida activity of grape seed extracts (GSE) of *Vitis vinifera*.

The mechanisms involved with grape products ability to inhibit certain microbials are yet not established. Duda-Chodak (2015) reviewed recent findings on the matter and reported that the inhibition capacity is class-specific and dependable on polyphenol's structure, probably as a result of the 4-carbonyl group in the C ring of the flavonoid skeleton. Earlier, the author suggested that the presence of this group is critical for the inhibitory activity of flavonols and flavanone aglycones. Flavonoid aglycones (at doses 4–250 µg/ml), but not their glycosides, were able to inhibit growth of some intestinal bacteria (Duda-Chodak, 2012).

To the best of our knowledge there are very few studies associating grape products and the intestinal transit. The purpose of our human assessment was to investigate if the microbial inhibiting effects *in vitro* could promote effects *in vivo*. Huang et al (2012) showed that a water-soluble carbohydrate concentrate (WSCC) from grapes shortened gastrointestinal transit time and diminish the exposure of intestinal mucosa to toxic ammonia and other detrimental compounds. In that case, the use of a fiber concentrate, and not the whole fruit, explains the results. In our findings, the number of daily bowel movements increased from less than once a day to once or twice a day. That finding is not explainable by the amount of fiber intake alone: the amount of fiber content in G8000 is 1.4%, according to the manufacturer, representing 2.5% of the Adequate Intake (AI) of an adult male (USDA, 2005). That small increment in

daily fiber consumption is not sufficient to explain the increase in intestinal transit time. Our hypothesis is that the modification of the gut microbiota promoted by G8000 may have induced changes in bowel movements. According to Bustos et al., 2012, flavonols have demonstrated the ability to modulate the gut microbiota by affecting the adhesion of bacteria to intestinal cells. Anthocyanins (the most prevalent polyphenol in grapes) significantly enhanced the growth of *Bifidobacterium* spp. and *Lactobacillus–Enterococcus* spp., suggesting that anthocyanins and their metabolites may exert a positive effect on the intestinal bacterial population (Hildalgo et al., 2012). Further studies on the association of grape polyphenols consumption and increase in number of bowel movements are necessary in order to advance in this argument.

V. Conclusion

The present study demonstrated that G8000, a natural food colourant from grapes has antimicrobial activity against the bacteria and fungus *in vitro*. The consumption of 70g/day of G8000 was able to increase the number of bowel movements in healthy volunteers, demonstrating an effect *in vivo*.

The authors declare no conflict of interest

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Annexure

Table 1. Results of the Minimum Inhibitory Concentration (MIC) assay using G8000 and positive controls gentamicin, vancomycin and amphotericin B against three strains of bacteria (*Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*) and fungus *Candida albicans*.

	G8000 (mg/mL)	Gentamicine (mg/mL)	Vancomycin (mg/mL)	Amphotericin B (mg/mL)
Candida albicans ATCC 5314	10	-	-	-
Staphylococcus aureus ATCC 29213	10	-	0,001475	-
Escherichia coli ATCC 14948	30	0,0007375	-	-
Pseudomonas aeruginosa ATCC 27853	5	0,0003688	-	-
*Candida krusei** ATCC 6258	-	-	-	0,002
*Candida parapsilosis** ATCC 22019	-	-	-	0,001

Performed in triplicate. **Candida Krusei* and *Candida parapsilosis* are quality control strains for the antifungal assay.

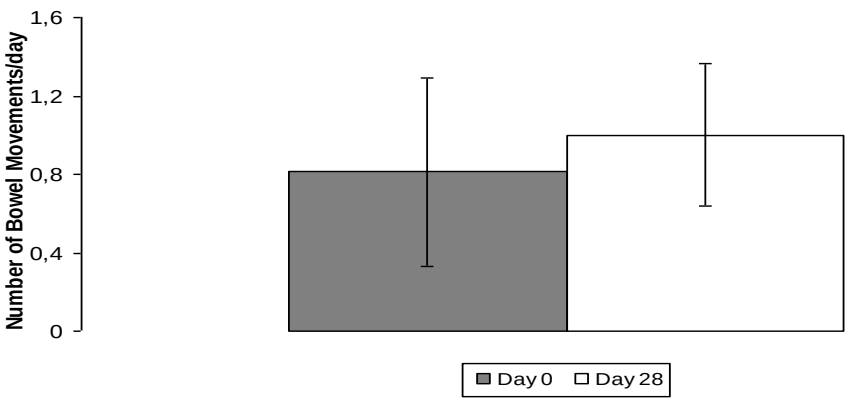

Figure 1. Number of bowel movements before (day 0) and after (day 28) consumption of 70g of G8000. Average of 15 individuals.