EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 1
31-44
Year: 2018

EVALUATION OF THE ANTIOXIDANT, ANTIMICROBIAL, CYTOTOXIC AND GENOTOXIC ACTIVITIES OF THE AQUEOUS EXTRACT OF CHALICES OF *HIBISCUS SABDARIFFA LINN*

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Article Received on 02/11/2017 **Article Revised on 23/11/2017** **Article Accepted on 14/12/2017**

ABSTRACT
Hibiscus sabdariffa L., which belongs to the family Malvaceae, is an important medicinal plant, originally from India, Sudan and Malaysia and popularly known in Brazil like "vinagreira", "azedinha", "quiabo azedo", "caruru-azedo", "caruru-da-guiné" e "quiabo-de-angola". The Malvaceae family, according to *in vivo*, *in vitro* and clinical tests, presents in its composition polyphenolic compounds and these auxiliaries in metabolic syndrome, obesity, dyslipidemias, and arterial hypertension, among others. The methodology consisted in obtaining an aqueous extract of the dried calyces of *Hibiscus sabdariffa* L. by traditional infusion method, 400.09 g of vegetal material processed in 2.0 L of distilled water at 80ºC for 1h and later lyophilization. The determination of percentage of total solids and moisture content of dry calyces, preliminary characterization of phytochemicals found in the extract (Mensah & Golomeke, 2015), antioxidant activity by the ABTS method (Re et al., 1999), profile and quantification of anthocyanins by HPLC (Gouvêa et al., 2012) and evaluation of the antimicrobial, cytotoxic and genotoxic activity by disc-diffusion method in nutrient agar against strain BW9091. The plant material used (dry calyces) had a mean value of total solids of 92.21g/ 100g and a mean humidity of 7.79%. The aqueous extract of the hibiscus calyces showed the phytochemicals flavonoids, saponins and coumarins, low antioxidant potential (2560µmol trolox/ 100 g dry basis) when compared to a literature and the presence of the anthocyanins delphinidin (unidentified) and cyanidin-3-glucoside at high concentrations, 0.524 mg/ mL and 0.225mg/ mL respectively. On the antibacterial, cytotoxic and genotoxic activity, it presented activity as monotherapy only in the volume of 24µL (50mg/ mL) and synergistic activity with bacterial cell wall inhibitory antibiotics (amoxicillin and ampicillin/ sulbactan) and hydrogen peroxide indicating its mechanism of action interaction with a cell membrane of *Escherichia coli* and be a special agent in cases of resistance to these antibiotics, presented by these bacterial.

KEYWORDS: Aqueous Extract. Antimicrobial Activity. *Hibiscus sabdariffa* l.

1. INTRODUCTION
In recent years the increase in the consumption of medicinal plants and derivatives has resulted in the expansion of the market of medicinal plants and herbal medicines as an alternative therapy (Veiga Junior et al., 2005). In Brazil, medicinal plants and their derivatives are among the main therapeutic resources of Therapeutic Medicine and Complementary and Alternative Medicine (BRASIL, 2006a).

World production of medicinal plants is a profitable, sustainable and growing area. This sector can present a great development opportunity for Brazil, as long as the norms of the National Health Surveillance Agency (WHO, 2011; PALHARES et al., 2015). In addition, this opportunity also allows for the study of biodiversity and the dissemination of traditional knowledge of science and technology institutions (HASENCLEVER et al., 2017).

According to the WHO, between 65% and 80% of the populations of developing countries use medicinal plants as medicines (WHO, 2011; PALHARES et al., 2015). It is estimated that currently 25% of the most recent drugs, in addition to 60% of antitumor drugs, are derived from substances derived from medicinal plants or synthetic

analogues or their derivatives (ZHANG et al., 2015). In 1999, WHO published monographs that contained a list of species with recognized medicinal benefits and the correct means of using them, since data on the constituents of some plants were poorly understood and the poorly regulated and monitored market (WHO, 1999). These monographs cover botanical, geographic, scientific and vulgar name, quality control tests, used parts of the plant, major chemical constituents, scientifically proven forms of use or popular medicine and end up discussing possible risks, adverse reactions and precautions in the species use of each section (WHO, 1999).

In Brazil, in addition to WHO recommendations, the National Agency of Sanitary Surveillance regulates the use of medicinal plants and herbal medicines. Anvisa has already published ordinances, decrees and resolutions aimed at the registration of medicinal plants (ANVISA, 2014). In addition, it has contributed to the integration of medicinal plants into the Unified Health System, in the scientific and technological development that proves safety and efficacy for a safe and rational access, in the recommendation and description of the main tests of quality control and toxicological analyzes, among others (OSHIRO et al., 2016).

In 2006, through the Ministry of Health, the National Policy for Medicines and Herbal Medicine was approved in Brazil (Decree No. 5.813, June 22, 2006), which established guidelines and priority lines for the development of actions aimed at guaranteeing safe access to the rational use of medicinal and phytotherapeutic plants, the development of technologies and innovations, the strengthening of productive chains and arrangements, the sustainable use of Brazilian biodiversity (BRASIL, 2006b).

A concern that accompanies the use of medicinal plants and their derivatives comes from the fact that their use is often associated with the concept of non-safety by the majority of the population (FONSECA, 2008; GHIZI & MEZZOMO, 2015). However, this concept does not reflect reality. Plants may interfere with organ function, dose-dependent toxic potential or interact with other medicinal products consumed together. Thus, it is essential the responsible, rational, safe and non-abusive use of the plants (CZELUSNIAK et al., 2012, ANVISA, 2014 and OSHIRO et al., 2016).

The discovery of secondary metabolites present in medicinal plants, as well as their mechanisms of biological action, has been one of the major challenges for chemistry, biochemistry and pharmacology (MARQUES, 2000). The development of new products of natural origin is also encouraged because it is estimated that of the 300,000 species that exist in the world only 15% have been evaluated to determine their pharmacological potential (DE LUCA et al., 2012, PALHARES et al., 2015).

Among the pharmacological properties sought in natural products is the antioxidant action. The evaluation of this activity has been an important issue considering its importance on human health, since free radicals and other oxidants are responsible for the premature aging and degenerative diseases associated 3with aging [cancer, cardiovascular diseases, diabetes, cataract, decline of the immune system and brain dysfunctions] (REID et al., 2005). The production of free radicals is controlled in living beings by several antioxidant compounds, which may have an endogenous origin or come from the diet and other sources (polyphenols (tannins, flavonoids, anthocyanidins, among others), tocopherols (vitamin E), ascorbic acid (vitamin C), selenium and carotenoids] (TSAI et al., 2005, LAGUERRE, LECOMTE and VILLENEUVE, 2007).

The food industry and cosmetics are also very interested in the study of lipid oxidation, due to the deterioration that oxidative damage can cause in their products (unpleasant odors and flavors, shortening the shelf life of products, decreased safety and nutritional quality , caused by the formation of potentially toxic compounds and, consequently, consumer rejection (TSAI et al., 2005).

In the last years, the challenge of the food and cosmetic industry has been the search for natural antioxidants (TSAI et al., 2005; LAGUERRE; LECOMTE; VILLENEUVE, 2007). However, despite the large amount of work developed, much of the existing potential remains unexplored (SENSES, 2002, LAGUERRE, LECOMTE, VILLENEUVE, 2007; RAMAKRISHNA & RAVISHANKAR, 2011; KENNEDY & WIGHTMAN, 2011).

According to research conducted in vivo, in vitro and clinical trials, the Malvaceae family presents polyphenolic compounds. The polyphenolic compounds help in the metabolic syndrome, obesity, dyslipidemias, arterial hypertension among others (DA-COSTA-ROCHA et al., 2014). They function as radical scavengers and sometimes as metal chelators (SHAHIDI et al., 1995), acting both in the initiation stage and in the propagation of the oxidative process. Phenolic compounds and some of their derivatives are therefore effective in preventing lipid oxidation (SOARES, 2002).
The specimen *Hibiscus sabdariffa L.*, belonging to the family Malvaceae, is an important medicinal plant, originating in India, Sudan and Malaysia, is popularly known as roselle, red sorrel, karkadeh (DA-COSTA-ROCHA et al. (Caruru-a-guiné) and "okra-de-angola" (MARTINS, 1990), in Brazil as "vinagreira", "azedinha", "sour okra". Many health-beneficial properties are attributed to hibiscus, they are: antioxidant action, they are used in the treatment of arterial hypertension, decrease of total lipids and cholesterol, for treatment of gastrointestinal and hepatoprotective disorder (MARTINS, 1990; FREITAS et al., 2013). The main constituents of *H. sabdariffa L.* are organic acids,

anthocyanins, polysaccharides and flavonoids. Several studies have identified delphinidin-3-sambubioside and cyanidin-3-sambubioside as the major anthocyanins present in extracts of the goblet of hibiscus and leaves. It is worth noting that although several researches have already been initiated regarding the *Hibiscus sabdariffa L*. specimen, there is still much to be explored in relation to its antimicrobial, cytotoxic and genotoxic activity, antioxidant action and as a natural source of secondary metabolites.

The objective of this work was to obtain an aqueous extract of the calyxes of *Hibiscus sabdariffa L.* (Synonymy: *Hibiscus cruentus Bertol*., *Hibiscus fraternus L*., *Hibiscus palmatilobus Baill*. and *Sabdariffa rubra Kostel*) and to analyze antioxidant, antimicrobial, genotoxicity and cytotoxicity activities of the extract obtained.

2. METHODOLOGY

The experiments were carried out in September of 2017 at the Laboratory of Chemical and Biological Analysis (LAQB) of UEZO (Foundation State University Center of the West Zone, Rio de Janeiro, Brazil.), at Plant 2 and in the Laboratory of Liquid Chromatography of EMBRAPA Agroindústria de Alimentos located in Guaratiba, Rio de Janeiro. The experimental procedures performed during the implementation of the proposal are described below.

2.1 Plant material

The plant material, 5kg of dehydrated hibiscus chalks from Cambodia, were donated by Interflora to the laboratory.

2.2 Aqueous extract

In the UEZO laboratory (LAQB), dried chalks of *Hibiscus sabdariffa L.* were processed for particle size reduction in the Mondial 400W Model L31 blender. A portion of the processed material was weighed (400.09 g) on a semi-analytical, Class II Bel Mark 2500 scale and then subjected to aqueous extraction with 2.0 L of distilled water heated in the microwave (Model MEF41 / series 11907921) at 80°C for 1.00 h.

The filtration was done manually with the aid of sterile gauze Hérika 15cm x 26cm (LOT: 0851). The extract was packed in amber bottles and subjected to refrigeration at -80°C for lyophilization. Lyophilization occurred for 8 days at a mean pressure of 74 µHg and 51°C.

2.3 Determination of total solids and the percentage of moisture

The determination of total solids was done with heavy-weighed (pre-weighed) and approximately 1 g of the processed chalices (weigth + sample was also weighed). The filters were left in a thermostated oven for 24 hours at 60ºC. After this time and observed the stabilization of the heavy mass at regular intervals (5 minutes) the value was noted and the total solids measurement was done according to the following equation:

((PF2-PF1) / A) * 100 = Total Solids g / 100g)

PF1 - weight of the filter weight (g); PF2 - mass of the filter weight + sample after oven drying (g); A - sample weight before oven drying (g).

2.4 Phytochemical analyzes

The methodology of the analysis of the phytochemicals present in the extract of the hibiscus flowers was based on Mensah & Golomeke (2015).

• Test for identification of Terpenoids and Steroids

A small portion of the lyophilized extract (10mg) was added to a test tube. Then, 1250 µl of acetic acid and 1250 µL of chloroform were added thereto. Thereafter, concentrated sulfuric acid (5 mL) was added slowly. The staining was observed. The coloration obtained was compared with that described by Mensah & Golomeke (2015). The appearance of red-violet color indicates the presence of terpenes, while a blue-green color indicates the presence of steroids.

• Assay for the identification of Flavonoids

A small portion of the lyophilized extract (10mg) was added to a test tube. Then, 1.5 mL of 50% methanol was added to the extract. The sample was heated. Metal magnesium was added to the tube and 5-6 drops of concentrated hydrochloric acid. The staining was observed. The coloration obtained was compared with that described by Mensah & Golomeke (2015). The appearance of red color indicates the presence of flavonoids, while an orange color indicates the presence of flavones.

• Test for the identification of Tannins

0.5 mL of the aqueous extract was added to a test tube. Then, 1-2 drops of a ferric chloride solution was added to the extract. The staining was observed. The coloration obtained was compared with that described by Mensah & Golomeke (2015). The appearance of blue color indicates the presence of gallic tannins and the greenish-black color the presence of condensed tannins (cathechics).

• Assay for the identification of Coumarin

500 µL of the aqueous extract was added to a test tube. Then, 750 µL of 10% NaOH was added to the extract. The coloration obtained was compared with that described by Mensah & Golomeke (2015). The appearance of yellow staining indicates the presence of coumarins.

• Assay for the identification of Saponin

500 µL of the aqueous extract was added to a test tube. Thereafter, 1 mL of sodium bicarbonate and 1% sodium bicarbonate were added to the extract. After stirring, the color obtained was compared to that described by Mensah & Golomeke (2015). The appearance of persistent foam is indicative of the presence of saponins.

• Test for the identification of glycosides

A few drops of acetic anhydrin were added to a small amount of the extract (10 mg) in a test tube. Then, 2-3 drops of concentrated sulfuric acid was added to the extract. The coloration obtained was compared with that described by Mensah & Golomeke (2015). The appearance of blue-green color shows the presence of glycosides.

2.5 Antioxidant activity

The analysis was carried out in Plant 2 of EMBRAPA Agroindústria de Alimentos located in Guaratiba - Rio de Janeiro and methodology based on Re and collaborators (1999).

The ABTS radical reduction method is one of the most widely used methods to measure antioxidant activity through the capture of the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS), which can be generated by a chemical reaction (manganese dioxide, potassium persulfate, ABAP); enzyme (peroxidase, myoglobin) or chemical electrochemistry (manganese dioxide, potassium persulfate, ABAP). With this methodology, one can measure the activity of compounds of hydrophilic and lipophilic nature (KUSKOSKI et al., 2005).

After preparation of the ABTS - [2,2'-azino-bis- (3-ethylbenzothiazoline-6-sulfonic acid)] radical - (7 mM - 0.03836 g of the ABTS reagent dissolved in 10 ml of deionized water), A solution of potassium persulfate (2.45 mM - 10 mL ABTS (Sigma Aldrich, USA) and 10 mL of persulfate mixed, homogenized and kept in an amber flask for at least 16 h protected from light) was prepared.

Approximately 0.1g of the lyophilized aqueous extract was weighed. 50 mL of methanol was added 50%. The vortex tube agitator solution was stirred for 10 seconds. The solution was allowed to stand at 1:00 h and sheltered from light.

Subsequently, the solution was centrifuged for 15 minutes at 20,000 RPM (revolutions per minute). The supernatant was collected and stored in a 25 mL amber flask.

To the residue was added 10 mL of 70% acetone. This new solution was subjected to the vortex type stirrer. The solution was allowed to stand and sheltered from light by 1:00 h. At the end of the period, the residue was subjected to a new centrifugation, under the same conditions mentioned above. The supernatant collected at this new extraction was deposited in the same 25 mL amber flask. The flask was then swollen with 5 mL of distilled water.

The ABTS + (previously prepared) radical was diluted in 95% ethanol until the absorbance was between 0.700 and 0.720, with the reaction white being 95% ethanol. 30 µL of the ethanol or test sample (results from previous extractions) + 3 mL of the diluted residue was added, waited 6 minutes for the reaction time and then the spectrophotometer readings were taken at the 734 nm wavelength. The test was performed in triplicate. Results were obtained with coefficient of variation (CV) below 10% (STRATIL et al., 2006; SURVESWARAN et al., 2007; PICCINELLI et al., 2004).

2.6 Profile and quantification of anthocyanins by HPLC

The analysis was performed in the Laboratory of Liquid Chromatography of EMBRAPA Agroindústria de Alimentos located in Guaratiba - Rio de Janeiro and methodology based on Gouvêa et al. (2012).

For analysis of 200 µL of the extract of the calyces of hibiscus, they were previously filtered in a 0.45 µM Millex PTFE filter. Subsequently, a dilution (1: 4) was made with 800 µL of the injection solution which is composed of methanol: 5% aqueous formic acid solution (10:90, v/ v).

Chromatographic analysis was performed on a Waters® Alliance High Performance Liquid Chromatograph Model 2690/5, Waters® Model 2996 Photodiode Array Detector (210 nm at 600 nm with quantification at 520 nm), reverse phase column (Thermo C18 BDS HYPERSIL, 100 x 4.6mm, 2.4µm) at 40°C in gradient elution mode of formic acid 5% in water (Phase A) and acetonitrile (Phase B) at a flow rate of 1.0 mL.min^{-1}, the volume of Injection: 20µL and the running time of 30 minutes.

Quantification was performed by external standardization and identification by comparison of retention time and UV/ VIS spectra of patterns previously isolated by the laboratory (GOUVÊA et al., 2014).

2.7 Antimicrobial, cytotoxicity and genotoxicity assessments: The analysis was carried out in the Laboratory of Chemistry and Biology of the UEZO with methodology developed in the disk-diffusion laboratory in nutrient agar.

The strain used was *Escherichia coli* BW 9091 (mutant strain for the xthA gene and its exonuclease III product). All material used was autoclaved at 120 ºC for 15 minutes. Initially, a quantity of TSB (Trypticase Soybeans) was placed in the test tube and then the strain of interest was incubated for 24 hours at 36-37 °C in a bacteriological incubator for reactivation of the strain that was stored at -18 °C with TSB nutrient broth + 10% glycerol. After checking the growth of the colonies, they were seeded with the aid of a bacteriological loop, plaque with nutrient agar and incubated for 24 hours at 36-37 ºC. Seeking to observe the formation of isolated colonies. The isolated colonies were resuspended in

NaCl (0.9 %) and turbidity of the saline suspension was verified according to the Mc Farland scale (0.5).
In the test of cytotoxicity and genotoxicity by diffusion in agarose gel, the surface of the disc in contact with cells of the bacterium form halos of inhibition of growth. The diameter of the halo corresponds to the bactericidal/ cytotoxic/ genotoxic activity of the tested product and its ability to diffuse into nutrient agar.

The culture medium used was nutrient agar solubilized in distilled water (28g/ L). Antibiotics amoxicillin 50mg/ mL, chloramphenicol (impregnated disc), norfloxacin (impregnated disc) and ampicillin/ sulbactan (impregnated disc) were used. Oxidants were also used as hydrogen peroxide (H_2O_2) 3% and stannous chloride ($SnCl_2$) 5mg/ mL, in addition to the lyophilized extract 50 mg/ mL. Plates with nutrient medium were seeded with the *E. coli* bacteria with the aid of swab. Bacteriological paper disks were used for $SnCl_2$ (5mg/ mL), H_2O_2 (3%), amoxicillin 50mg/ mL and lyophilized extract 50 mg/ mL (dilutions performed in 0.9% NaCl). Different volumes were impregnated in the disks (table 1).

Table 1 - Sequence of disks of the substances used in the microbiological test.

Disc Number	Substance / Concentration	Volume or Presentation
1	NaCl 0.9%	24 µL
2	Extract (50mg / mL)	24µL
3	Extract (50mg / mL)	12µL
4	Amoxicillin (50mg / mL)	12µL
5	Amoxicillin (50mg / mL) + Extract (50mg / mL)	12µL + 12µL
6	Norfloxacin	Impregnated disc
7	Norfloxacin + Extract (50mg / mL)	Impregnated Disc + 12µL
8	Amoxicillin (50mg / mL)	24µL
9	Chloramphenicol	Impregnated disc
10	Chloramphenicol + Extract (50mg / mL)	Impregnated Disk + 12µL
11	Ampicillin / Sulbactan	Impregnated Disc
12	Ampicillin / Sulbactan + Extract (50mg / mL)	Impregnated Disk + 12µL
13	Tin Chloride (5mg / mL)	24µL
14	Tin Chloride (5mg / mL)	12µL
15	Tin Chloride (5mg / mL)	8µL
16	Tin Chloride (5mg / mL) + Extract (50mg / mL)	12 µL + 12 µL
17	Extract (50mg / mL)	8µL
18	Extract (50mg / mL) + H2O2 (3%)	12µL + 12µL
19	H_2O_2 (3%) + Tin Chloride (5mg / mL)	12µL + 12µL
20	Extract (50mg / mL) + Tin Chloride (5mg / mL) + H2O2 (3%)	8µL + 8µL + 8µL
21	H_2O_2 (3%)	8 µL
22	H_2O_2 (3%)	12 µL
23	H_2O_2 (3%)	24 µL

The plates were then placed in the oven for 24 hours at 36-37 ° C. The whole test was carried out in triplicate and calculated to averages of the obtained halos. From the averages obtained were calculated the percentages of increase or decrease of the halos formed and expressed in graph.

3. RESULTS

The aqueous extract of the chalice of *H. sabdariffa L.* (783 mL) showed high viscosity, acid odor, wine-dark color and sour taste.

The percentage of yield (39.15%) was low because there were many losses during the manual filtration process, mainly due to the high viscosity of the material that made vacuum filtration impossible and the gauze that absorbed the amount of extract.

The mean value of total solids of the calyces of hibiscus was 92.21 g/ 100 g and the sample had a mean moisture content of 7.79 %. The analysis was performed in triplicate and all results obtained during the study were related to the dry mass (92.21 g).

Preliminary phytochemical screening according to the methodology of Mensah & Golomeke (2015) identified the presence of different classes of secondary metabolites (Table 2).

Table 2 - Preliminary phytochemical screening of the aqueous extract of hibiscus

Phytochemicals	Result
Terpenoids / Steroids	- / -
Flavonoids	+
Tannins	-
Cumarinas	+
Saponins	+
Glycosides	-

The antioxidant activity of the extract was evaluated by the ABTS methodology previously described in the methodology and the results of the obtained absorbances

were compared with the standard trolox curve (Figure 1). The analyzed sample presented total antioxidant activity of 2560 µmol of trolox/ 100 g of dry goblet.

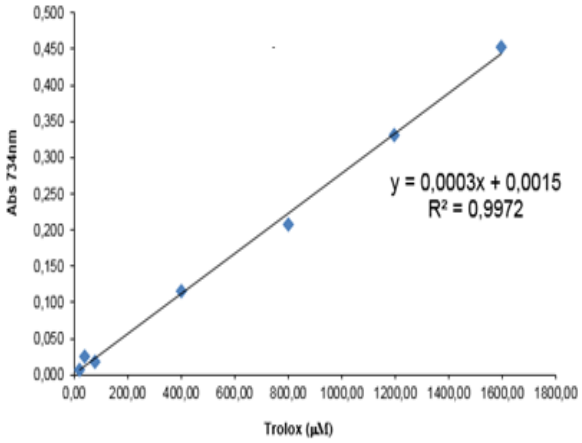

Figure 1: Trolox standard curve.

Through the chromatogram shown in Figure 2 it is possible to verify the ultraviolet profile of the aqueous extract of hibiscus.

The major peak 1 in the sample is a delphinidin confirmed by the UV / Vis spectrum. However, the retention time did not coincide with that of the laboratory standard (delphinidin-3-glycoside). It is possible that peak 1 is delphinidin-3-sambubioside, considering that the literature reports the presence of delphinidin-3-sambubioside in the hibiscus extract (CARVAJAL-ZARRABAL et al., 2012; DA-COSTA-ROCHA et al.., 2014). It is worth mentioning that delphinidin-3-sambubioside presents retention time prior to delphinidin-3-glycoside (SALAZAR-GONZÁLEZ et al., 2012). However, spectrometric analysis is necessary to confirm this data.

According to the UV / Vis spectrum data, the second major peak of the sample (Figure 2) is a confirmed cyanidin. The sample retention time coincided with the cyanidin-3-glycoside standard. It should be noted that this cyanidin has already been described in the species (CARVAJAL-ZARRABAL et al., 2012; DA-COSTA-ROCHA et al., 2014).

The calibration curve was performed with the cyanidin-3-glycoside standard. Based on this analysis, the concentrations of delphinidine (delphinidin-3-sambubioside) and cyanidin (cyanidin-3-glycoside) were respectively 0.524 mg/ mL and 0.225 mg/ mL.

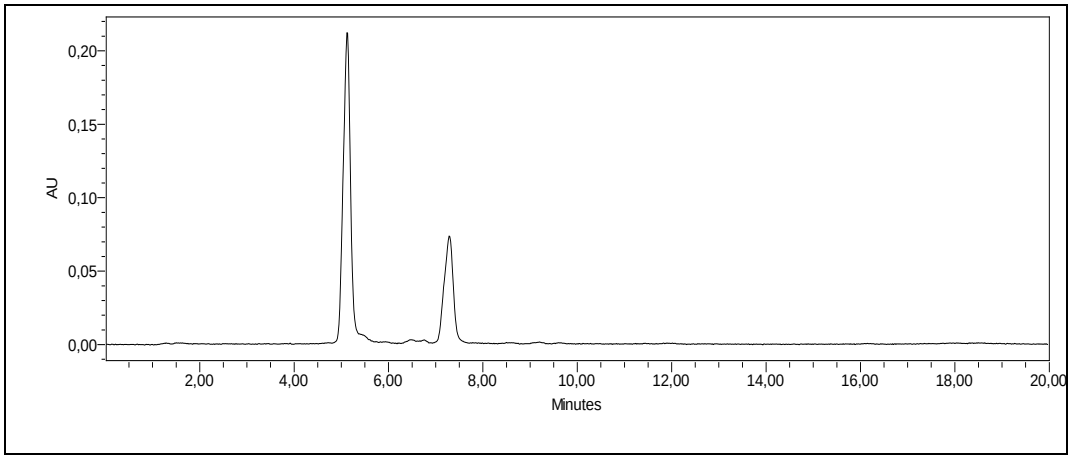

Figure 2: Chromatogram of aqueous extract of Hibiscus sabdariffa L. flowers.

The characteristic of peak 1 and 2 correspond to a delphinidin and a cyanidin, respectively at 520nm.

The antimicrobial activity, cytotoxicity and genotoxicity assays were described in Table 3. Analyzing the halos obtained, we can see the efficiency of the aqueous extract as bactericidal only in the volume of 24 µL.

As for the antioxidant action the results suggested influences on the oxidizing agents used: in the association SnCl2 12 µL and extract 12 µL reduced by 50% and in the case of hydrogen peroxide 12 µL, increased the diameter of the halo by 4.23%.

On the antibiotic amoxicillin concentration of 50 mg / mL in the volume of 12 µL, presented an increase of the average of the halos formed in the percentage of 11.75% and on the ampicillin / sulbactan demonstrated an increase of the average of the halos formed, percentage of 6,97 % when associated with the extract. There were no results for norfloxacin nor chloramphenicol.

Table 3: Inhibition Halos (mm) formed in the cytotoxicity and genotoxicity test strain BW 9091.

PLATES / DISCS	A	B	C	AVERAGE
I 1 NACL 0.9% 24 µL	0	0	0	0
I2 EXTRACT 24 µL	10	12	10	10.7
I3 EXTRACT 12 µL	0	0	0	0
I4 AMOXYCILLIN 12 µL	40	40	40	40
I5 AMOXYCILLIN 12 µL + EXTRACT 12 µL	44	44	46	44.7
II6 NORFLOXACIN (IMPREGNATED DISC)	24	24	22	23.3
II7 NORFLOXACINO + EXTRACT 12 µL	22	22	22	22
II8 AMOXYCILLIN 24 µL	48	50	50	9.3
II9 CLORANPHENICOL (IMPREGNATED DISC)	28	29	30	29
II10 CLORANPHENICOL + EXTRACT 12 µL	28	28	30	28.7
III11 AMPICILLIN / SULBACTAN (IMPREGNATED DISC)	28	30	28	28.7
III12 AMPICILLIN / SULBACTAN + EXTRACT 12 µL	32	30	30	30.7
III13 TIN CHLORIDE (5 MG) 24 µL	12	14	14	13.3
III14 TIN CHLORIDE (5 MG) 12 µL	10	10	10	10
III15 TIN CHLORIDE (5 MG) 8 µL	5	5	5	5
IV16 TIN CHLORIDE (5 MG) 12 µL + EXTRACT 12 µL	5	5	5	5
IV17 EXTRACT 8 µL	0	0	0	0
IV18 EXTRACT 12 µL + H_2O_2 12 µL	36	30	30	32
IV19 H_2O_2 12 µL + TIN CHLORIDE 12 µL	42	34	32	36
IV20 EXTRACT 8 µL + TIN CHLORIDE 8 µL + H_2O_2 8 µL	30	30	28	29.3
IV21 H_2O_2 24 µL	50	50	50	50
IV22 H_2O_2 12 µL	28	32	32	30.7
IV23 H_2O_2 8 µL	28	30	28	28.7

It is noteworthy that, after the addition of the extract (12 µL) over the stannous chloride (12 µL) (disc 16), the color of the extract was changed from wine to purple, and at the time of reading the extract lost its coloration, becoming transparent.

In relation to the antimicrobial, cytotoxic and genotoxic analysis it is important to highlight that the extract contributed with a 4.23 % increase in the inhibition halo when associated with H_2O_2; 11.25 % when associated with amoxicillin; 6.97 % when associated with ampicillin. The extract showed no activity when associated with the antibiotics chloramphenicol and norfloxacin; reduced the inhibition halo by 50 % when associated with stannous chloride.

4. DISCUSSION

Qualitative phytochemical tests did not detect the presence of tannins, glycosides and terpenoids/ steroids (Table 2). Our results presented differences when compared to the literature. Sekar et al. (2015), for example, found extracts of calyces and leaves of hibiscus, flavonoids, tannins, triterpenoids and fixed oils. The extracts were extracted by maceration with methanol at room temperature for 7 days under occasional stirring (SEKAR et al., 2015). Another important observation is that most of the studies reported use the decoction method rather than the infusion (used in this work), alcoholic or hydroalcoholic (MACIEL et al., 2012; JIMÉNEZ-FERRER et al., 2012; MENSAH & GOLOMEKE, et al., 2006).

Infusion/ decoction are traditional methods used in folk medicine for the production of "home-made tea," where the solvent used is water heated, as in the case of infusion, or boiling together with the plant material as in the case of decoction.

The presence of saponins, condensed tannins (stem), hydrolysable tannins (leaves), steroids and flavonoids (in both) and resin (only found in the stem) were found in the leaves and stem of Hibiscus sabdariffa L. Freitas and collaborators (2013). It is noteworthy that the literature cites that secondary plant metabolites can undergo qualitative and quantitative changes depending on environmental stimuli. Thus, light intensity, temperature, rainfall, soil nutrient availability, stage of development of plants, interactions between plants and between animals can influence the metabolic routes of the plants, causing different redirects of the compounds synthesis (GOBBO-NETO & Lopes, 2007).

Among the constituents phytochemicals present in the extract, the flavonoids stand out. These substances have several functions, among them, protection against the incidence of ultraviolet rays, protection against pathogenic microorganisms, antioxidant action, allopathic action and enzymatic inhibition (NIJVELDT, 2001; FREITAS et al., 2013). It also provides the pigmentation of flowers and significant beneficial effects on human health (WINKEL, 2004; BUTELLI et al., 2008; PATRA et al., 2013). Many studies have related the various biological activities attributed to hibiscus to human health (antioxidant and antimicrobial, for example), to the presence of phenolic compounds generally found in great quantity (CARVAJAL-ZARRABAL et al., 2012; DA-COSTA-ROCHA et al., 2014). This class of secondary metabolites (phenolic compounds) can be divided into: flavonoids

(anthocyanins, flavonols and their derivatives), phenolic acids (benzoic and cinnamic acids and their derivatives) and coumarins (KING & YOUNG, 1999; ANGELO & JORGE, 2007). Specifically in hibiscus extracts, hibiscitrin (hibiscetin-3-glucoside), sabdaritrine, gossipitrine, gossitrine and other glycosides of gossyptin, quercetin and luteolin, as well as chlorogenic acid, protocatechic acid, pelargonidic acid, eugenol and β (Roche et al., 2004).

With respect to the antioxidant activity the extract of the present work presented 2560 µmol of trolox/ 100 g of dry base, slightly below that described in the literature.

Salazar-González et al. (2012) found the following results in their extracts at concentrations of 0.1 g/ mL: hydroalcoholic extract [ethanol: water (50:50)] 8.035 µmol trolox/ 100 g, ethane extract 3.111 µmol trolox / 100 g and aqueous 6.276 µmol trolox/ 100g.

Fernández-Arroyo et al. (2011) found an antioxidant activity of 0.16 mmol of trolox / g extract in their aqueous extract of the calyxes of hibiscus at a concentration of 0.025 g/ mL. In the extract (70 % methanol / water with 0.1 % acetic acid solution) of the dried leaves of hibiscus (25 samples), Zhen and collators (2016) found an antioxidant activity ranging from 101.5 378 ± 17.5 to 152.5 ± 18.8 µmol trolox / g of dry leaf.

Based on the three studies, our extract even at higher concentrations presented activity below that described in the literature. The extraction methods, parts of the plant used and cultivation conditions may be some of the explanations, since in the work of Zhen et al. (2016), for example, another extraction methodology was used, the leaves were the studied plant material and, in addition, a very strict control of the conditions of cultivation of the species was made in order to guarantee the maximum yield with respect to the concentration of secondary metabolites of the plant.

Anthocyanins are derived from the flavonoid group and are present in the natural pigments of dried hibiscus calyces. Its color varies according to the pH (DA-COSTA-ROCHA et al., 2014). Sensitivity to pH is the main limiting factor that affects the color and chemical stability of the same. In acid solutions, anthocyanin is red, but with increasing pH the color intensity decreases. In alkaline solution, for example, it presents the blue color, however it is unstable (Mazza & Brouillard, 1987; Lopes et al., 2007). The biosynthesis of anthocyanins is regulated by multiple factors, especially by the presence of light, sugar and phytohormones that regulate expression of specific genes. The transcription factor MYBL2 has been proposed as a key regulator to induce the accumulation of anthocyanins under conditions of high luminosity and sugar. The expression of MYBL2 is induced under low light and stress-free conditions and the accumulated proteins interact with bHLH factors (GL3 / EGL3 / TT8) to form an inactive complex that suppresses the gene expression pathway and consequently the accumulation of anthocyanins. In contrast, high luminosity and or stress conditions suppresses the expression of MYBL2 and induces that of PAP1 and TT8. The result is the formation of an active MBW complex and the positive regulation of genes of the anthocyanin biosynthetic pathway (PATRA et al., 2013). After chlorophyll, anthocyanins are the most important group of pigments of vegetable origin (HARBORNE & GRAYER, 1988; LOPES et al., 2007). They are composed of the largest group of water-soluble pigments in the plant kingdom and are found in larger amounts in the angiosperms (Bridley & Timberlake, 1997; Lopes et al., 2007).

The functions performed by anthocyanins in plants are varied: antioxidants, light protection, defense mechanism and biological function. The bright and intense colors they produce play an important role in several plant reproductive mechanisms, such as pollination and seed dispersal (LOPES et al., 2007).

Regarding the color of the hibiscus extract, and previous description in the literature, it was expected that anthocyanins of the cyanidines and delfinidines class would be found. From the species, three anthocyanins were isolated: delphinidin-3-sambubioside, delphinidin-3-glycoside and cyanidin-3-glycoside from samples grown in Thailand and Trinidad (SHIBATA & FURUKAWA, 1969; DU & FRANCIS, 1973; DA-COSTA-ROCHA et al., 2014).

Many studies have also identified delphinidin-3-sambubioside and cyanidin-3-sambubioside as the main anthocyanins present in extracts (DEGENHARDT *et al*., 2000; ALARCON-AGUILAR *et al*., 2007; BELTRAN-DEBON *et al*., 2010; PENG *et al*., 2011; ALARCON-ALONSO *et al*., 2012; HERRANZ-LOPEZ et al., 2012; DA-COSTA-ROCHA et al., 2014).

Jiménez-Ferrer et al. (2012) found the anthocyanins delphinidin-3-sambubioside and cyanidin-3-sambubioside at the concentrations of 32.4 mg/ g and 19.9 mg/ g respectively in the aqueous extract of the hibiscus chalices. The results are superior and different from those found in this study, which were 2.62mg/ g for delphinidin, which did not coincide with the delphinidin-3-glycoside time (laboratory standard) we suggest that it may be delphinidin-3- sambubioside and 1.125mg/ g for cyanidin-3-glycoside. The only characteristic maintained would be the superiority in the concentration found of delphinidin in relation to cyanidin, since the time of extraction of our work was inferior to the one of them and the species of them coming from different locality (Mexico). However, Maciel et al. (2012) did not perform the analysis of the chromatographic profile of anthocyanins, only quantification of total anthocyanins by the differential pH method of the alcoholic extract of calyx and hibiscus fruits at the concentration of 0.4 g/ mL. This corresponds to twice the concentration used in

this work (0.2 g/ mL). They found the concentration of total anthocyanins of 85.9 mg/ 100 g for the calyces and 3.2 mg/ 100g for the seeded fruits (MACIEL et al., 2012). This result was much lower than that obtained in this study (262 mg / 100 g). It is noteworthy that this result corresponds only to data obtained from anthocyanin delphinidin, since for cyanidin-3-glycoside we obtained 112.5 mg/ 100 g. We believe that the differences in methodologies employed in the extraction or in the place of cultivation of the species [from this work come from Cambodia and Maciel and collaborators (2015) from southern Brazil]. As previously mentioned, the production of anthocyanins (GOBBO-NETO & LOPES, 2007) is regulated by several factors such as cultivation conditions and environmental factors (eg abiotic luminosity and stress) and southern Brazil is characterized by temperatures more enjoyable. Hibiscus is a plant that needs temperatures between 21-35ºC and annual precipitations between 800-1600 mm, an environment easily found in Cambodia that has high temperatures throughout the year and monsoons guarantee rainfall.

In another more recent work, Chou et al. (2016) used dry goblets purchased in Taiwan and made a 0.2 g/ mL aqueous extract at 4 °C for 24 h under stirring. They observed only the presence of delphinidin-3-sambubioside in the concentration of 4.84 µg/ mL. This result was much lower than that found in our study. Only for delphinidin (unidentified) and for cyanidin-3-glucoside respectively, 524 µg/ mL and 225 µg/ mL were detected. Although the concentrations of the extracts were the same, the methodologies used were different which may justify the differences found. In addition, our extraction was more efficient, even though it did not deplete the sample completely as observed so far, showing that the infusion method used by the population is more efficient in the extraction of compounds like the anthocyanins of the dry hibiscus chalices.

Prabhakaran et al. (2016) used a ethyl acetate fraction of the aqueous extract of the hibiscus flowers against bacterial strains (*Salmonella typhi*, *Escherichia coli*, *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus substilis* and *Lacto bacillus*) and observed a concentration of 50mg/ mL, similar to the concentration used in this study, 16mm inhibition halos for *Bacillus cereus* and 14mm for all other strains tested. Regarding the *E. coli* result, the values were higher than the one found in the present study. We found 10mm for strain BW9091 (knockout strain for the xthA gene and its product an exonuclease III).

The xthA protein represents 90% of the endonucleolytic activity found in *E. coli*, being an important member of the base breaking repair mechanism, the most important system capable of repairing the oxidative damage originated from several sources. Therefore, studying substances against these strains does not only contribute to the evaluation of the antibacterial activity, but, mainly, in the evaluation of the cytotoxic and genotoxic potential. In other words, in the ability to generate DNA damage from the production of reactive oxygen species since they do not have efficient repair mechanisms (DEMPLE et al., 1983; FRIEDBERG et al., 2006; MOTTA et al. al., 2010).

The differences in the sizes of halos formed (antibacterial activity) can be explained by the fractionation of the extract (not performed in our work) and the possibility of there being differences of chemical constituents with greater bactericidal potential. We can also consider the diluent used in the application on the disks. Our extract was diluted in 0.9% NaCl, used even as a negative control, and that of the researchers from India (PRABHAKARAN et al., 2016) was DMSO (dimethylsulfoxide), with only chloramphenicol (antibiotic inhibitor of protein synthesis) as positive control.

The use of solvents or emulsifying agents in tests to evaluate antimicrobial activity should be well selected, since introducing another substance together with the test substance of interest may lead to possible interactions both in bacterial growth and to accentuate or minimize the activity of the extract in question (NASCIMENTO et al., 2007). However, regardless of methodological differences, both studies are consistent with the intrinsic antimicrobial activity of hibiscus against *Escherichia coli* (PRABHAKARAN et al., 2016).

Mensah and Golomeke (2015) found an inhibition halo of 0.50 ± 0.74 mm and for the hydroalcoholic 25.00 ± 0.23 mm for the aqueous extract of the calyces of hibiscus against the *E. coli* strain, at the highest concentration tested (10mg/ mL). Sekar and collaborators (2015) found in their extract methanoic (leaves and fruits) in the highest concentration tested 1000µg/ mL, against the strain *E. coli*, 10 mm and 9 mm for leaf and fruit extracts respectively.

By evaluating the cytotoxic and genotoxic potential of the extract, we used the previously mentioned BW9091 bacteria and as positive controls $SnCl_2$ (stannous chloride), a tin salt that is a reducing agent capable of binding to the DNA and producing reactive oxygen species according to reaction Fenton (MOTTA et al., 2012) and H_2O_2 (hydrogen peroxide), a substance when isolated is innocuous, but capable of forming OH • radical with transition metals and often generates mutation in the DNA, as it can diffuse easily through the membranes Cells as the core membrane (BARREIROS et al., 2006).

Below is a simplified equation for the Fenton reaction of hydrogen peroxide with transition metals (M) (Cu^{+} or Fe^{+2}):

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO\bullet + HO^{-}$$

In the case of $SnCl_2$ (5 mg/ mL), the inhibition halo was reduced by 50 % when combined with the extract (50 mg/ mL). The extract somewhat protected the bacteria from the tin salt effect by exhibiting antioxidant activity. It should be emphasized that in this stage the wine coloring extract, soon after being applied to the disc along with the stannous chloride, changed color to purple and in reading all the disks acquired coloration near the transparent, indicating most probably a change of pH of the medium, because the anthocyanin staining is sensitive to pH variation and the extract has a large amount of anthocyanins derived mainly from delphinidines and cyanidines. The results show that the H_2O_2 (3%) associated with the extract (50 mg/ mL), contrary to the previously reported results for $SnCl_2$, increased the effect of hydrogen peroxide, increasing the inhibition halo diameter. Indicating that the association between them generates pro-oxidants or that there are substances present in the extract that do not interact directly with hydrogen peroxide, but present a similar mechanism capable of triggering oxidative stress.

In general, the extract in the presence of small amount of the oxidizing agent presented antioxidant activity and in a large amount of pro-oxidant agents presented synergism.

As the methodology was developed and adapted by the laboratory, no external work was found for comparative purposes of methodologies for the same species or other species of the same genus. Despite this, the data found in the present study are in agreement with what has already been reported in the literature for the species, presenting antibacterial potential.

Significant results were found when the extract was tested together as some antibiotics used in the clinic. It did not present positive or negative interference when tested for inhibitors of protein synthesis (chloramphenicol) and DNA (norfloxacin).

Chloramphenicol is a broad spectrum bacteriostatic agent, potent inhibitor of bacterial protein synthesis. As a mechanism of action reversibly binds the 50s subunit of the bacterial ribosome and inhibits peptide bond formation (DECK & WINSTON, 2012a).

The quinolones, class to which norfloxacin belongs, have as a mechanism of action to block the synthesis of bacterial DNA by inhibiting topoisomerase II (DNA gyrase) and IV. Inhibition of DNA gyrase prevents relaxation of DNA that is positively coiled and required for normal transcription and replication. On the other hand, inhibition of topoisomerase IV interferes with the separation of replicated chromosomal DNA in the respective daughter cells during cell division. They are effective in treating urinary tract infection and bacterial diarrhea caused, for example, by E. coli (DECK & WINSTON, 2012b).

As the extract did not show synergistic activity for any of the antibiotics mentioned above, not justifying a possible association for resistance cases.

In contrast, the extract showed significant synergistic activity with inhibitors of bacterial wall synthesis, the penicillins amoxicillin and ampicillin.

Penicillins have the chemical structure of the presence of a β-lactam ring (carrying a secondary amino substituent) fused to a thiazolidine ring. Substituents may vary in the amino group by determining the antibacterial activity and pharmacological properties of the molecule. The aminopenicillins present a broad spectrum of performance, as tested in this work, and have improved activity against Gram-negative organisms (DECK & WINSTON, 2012c).

Amoxicillin is used to treat urinary tract infections, sinusitis, otitis, and lower respiratory tract infections. Ampicillin is useful for treating severe infections caused by susceptible organisms, including anaerobes, enterococci, *L. monocytogenes*, and Gram-negative cocci negative β-lactamase strains and bacilli such as *E. coli* and *Salmonella sp* (DECK & WINSTON, 2012a).

Amoxicillin and ampicillin are the most active antibiotics, the oral β-lactams, against pneumococci. They are also available in combination with β-lactamase inhibitors such as clavulanic acid and sulbactan. The addition of these β-lactamase inhibitors extends the activity of these penicillins including β-lactamase-producing S. aureus strains as well as β-lactamase-producing Gram-negative bacteria (DECK & WINSTON, 2012a).

Studying medicinal plants and their antibacterial properties are important for the pharmaceutical industry, as new substances can be discovered that become prototypes, for the production of synthetic or semi-synthetic analogues, or substances that do not have a significant bactericidal potential, but reduce the frameworks of resistance, mentioned in the review of this work, and that has become the great problem in the treatment of infectious diseases in the present days.

In the present study, the effect of resveratrol on the mechanism of action of resveratrol (polyphenol) and its methylated derivative pterostilbene (Yang, Shih-Chun et al. both phenolic compounds, as well as protocatecuic acid (phenolic acid) can be proposed by many researchers as a chemical that confers the antimicrobial properties of the hibiscus extract (DA-COSTA-ROCHA et al., 2014). a probable mechanism of action.

Yang et al. (2017) performed a very complex and illuminating study with in vivo, in vitro, and proteomic analysis models to try to explain the likely mechanism of action and therapeutic efficacy of resveratrol and its methylated derivative against strains MRSA and *P.*

aeruginosa. Both presented mechanisms of action are slightly different, with pterostilbeno produced more significant results than resveratrol during the study and possibly its mechanism of action occurs by inducing rupture of the cell membrane. YANG et al. (2017) showed that the methylated derivative of resveratrol triggered bacterial membrane disturbance resulting in increased cell permeability. It was also able to change protein expression, promoting a negative regulation (result different from that found in resveratrol) of chaperones and GAPDH (YANG et al., 2017). Such proteins are useful in cell wall regulation, antibiotic resistance and oxidative stress. The interaction of these compounds with these structures and bacterial membrane can inhibit the growth of the pathogen and lead to cell death (YANG et al., 2017). This was the most complete study of a natural phenolic compound (isolated) and its methylated derivative, attempting to exclude its possible mechanism of action (YANG et al., 2017).

The extract tested in the present study showed synergistic activity with cell wall inhibitors and no interaction with inhibitors of protein and DNA synthesis, indicating that its mechanism of action may be close to that of pterostilbene that acts at the level of bacterial membrane destabilizing it and leading the cell to rupture and oxidant activity triggering a process of oxidative stress intracellularly in the bacterium.

The assertions made are assumptions, since further studies would be necessary not only to isolate and test protocatecuic acid, but more complex analyzes of the gene expression of bacteria would be needed to suggest a more precise mechanism of action. Despite this, the study can confirm the bactericidal activity attributed to the hibiscus, already described in the literature, and its possible use as complementary agent in the treatment of infections caused by Escherichia coli.

5. CONCLUSION

The plant material presented a mean value of total solids of 92.21g / 100g and average humidity of 7.79 %. The aqueous extract of the calves of *Hibiscus sabdariffa L.* showed flavonoid phytochemicals, saponins and coumarins, low antioxidant potential (2560 µmol trolox / 100 g dry basis) when compared to the literature and the presence of the anthocyanins delphinidin (unidentified) and cyanidin -3-glycoside in high concentrations. On the antibacterial, cytotoxic and genotoxic activity, it presented activity as monotherapy only in the volume of 24 µL (50 mg/ mL) and synergistic activity with the bacterial cell wall inhibitory antibiotics (amoxicillin and ampicillin/ sulbactan) and hydrogen peroxide indicating its possible mechanism of action of interaction with the cell membrane of *E.coli* and to be a possible auxiliary agent in cases of resistance to these antibiotics presented by these bacteria.

ACKNOWLEDGMENTS

We thank the Foundation for Research Support of the State of Rio de Janeiro (FAPERJ), for the support to the research carried out in the laboratory of Chemical and Biological Analysis (LAQB) of the Foundation Center University State of the West Zone (UEZO).

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