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Original Research

Effects of *Tapinanthus globiferus* and *Zanthoxylum zanthoxyloides* extracts on human leukocytes *in vitro*

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ABSTRACT

Objective: This study aimed at investigating the genotoxicity and cytotoxicity effect of *Tapinanthus globiferus* and *Zanthoxylum zanthoxyloides* to human leukocytes. In addition, the reductive potential and the chemical composition of the two plant extracts were also determined. **Materials and Methods:** Human leukocytes were obtained from healthy volunteer donors. The genotoxicity and cytotoxicity of *T. globiferus* and *Z. zanthoxyloides* were assessed using the comet assay and trypan blue exclusion, respectively. The antioxidant activity of the plant extracts was evaluated by the reducing power assay. Furthermore, high-performance liquid chromatography-diode array detector was used to characterize and quantify the constituents of these plants. **Results:** *T. globiferus* (10-150 μg/mL) was neither genotoxic nor cytotoxic at the concentrations tested, suggesting that it can be consumed safely at relatively high concentrations. However, *Z. zanthoxyloides* showed cytoxicity and genotoxicity to human leukocytes at the highest concentration tested (150 μg/mL). In addition, the total reducing power of *T. globiferus* was found higher than *Z. zanthoxyloides* in potassium ferricyanide reduction. Both plants extract contained flavonoids (rutin and quercetin) and phenolic acids (chlorogenic and caffeic). **Conclusion:** The results obtained support the fact that some caution should be paid regarding the dosage and the frequency of use of *Z. zanthoxyloides* extract.

KEY WORDS: Cytoxicity, genotoxicity, high performance liquid chromatography-diode array detector, *Tapinanthus globiferus, Zanthoxylum zanthoxyloides*

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INTRODUCTION

Reactive oxygen species (ROS) are oxidizing, highly reactive and unstable molecules are containing oxygen. They produced during normal cellular metabolism as by-products of respiration in the mitochondria. They include hydroxyl radical (OH•), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and singlet oxygen [1]. Cumulative evidence suggests that ROS play important roles in signal transduction, sensing of oxygen tension and regulation of functions controlled by oxygen concentration [2]. They are also involved in boosting the immune system [3]. However, ROS can be harmful when its cellular levels exceed the level of cellular antioxidants, which results in oxidative stress. Oxidative stress would eventually cause injury to cellular macromolecules such as membrane lipids, proteins and nucleic acids, thereby affecting the normal functioning of cells.

DNA is one of the major targets of ROS in living cells and tissues. ROS induces DNA mutations that can cause or lead to

cancer and age-related disorders [4]. Hydroxyl radicals ($OH^{\bullet}$), an oxidant obtained from the breakdown of H_2O_2 is majorly responsible for DNA damage. It reacts with DNA molecule causing DNA protein cross-links, DNA strand breaks and alkali-labile sites [4,5], which may lead to permanent damages that can cause severe biological consequences [6]. Furthermore, Shi *et al.* [7] revealed that $O_2^{\bullet -}$ and H_2O_2 are capable of inducing strand-breaks and oxidation of DNA bases.

Further, studies have shown that DNA damage can be minimized or prevented by the use of natural antioxidants such as vitamin C, vitamin E, carotenoids, flavonoids, and other polyphenolic compounds, by scavenging or inactivating ROS. Particularly, natural compounds exhibit protective effects when used in oxidative stress-induced DNA damage [8]. Furthermore, plants rich in antioxidants have been shown to protect ROS-induced oxidative DNA damage [9].

Tapinanthus globiferus and *Zanthoxylum zanthoxyloides* are plants commonly used as folkoric medicine and highly consumed in the Nigeria and Cameroon. *T. globiferus* known as mistletoe (in English) belongs to the family Loranthaceae. It is a woody, spreading shrub with blackish, smooth stems made rough by the presence of lenticels. It is popularly called "afomo" in South Western Nigeria whereas, Z. *zanthoxyloides* (family, Rutaceae) is commonly known as candle wood. The root of Z. *zanthoxyloides* is used as antibacterial toothbrush in South Western Nigeria, and the decoction of its leaves and roots is used to wash wounds for healing. In addition, the bark of the plant is used in the treatment of intestinal worms and edema. Likewise, *T. globiferus* is commonly consumed for the treatment of hypertension, ulcers, diabetics, weakness of vision, and for promoting muscular relaxation before delivery. Recent studies revealed that the plants exhibit a variety of pharmacological activities including antitrypanosomal [10,11], antimicrobial [12], anti-inflammatory [13] activities, and are rich in antioxidants [14].

Human leukocytes are used to evaluate DNA damage, repair studies and genotoxicity using comet assay because leukocytes are obtained in a relatively non-invasive way and do not require tissue disaggregation [15]. Comet assay is highly sensitive for *in vitro* genotoxicity test methods on leukocytes [16] and is of particular importance for safety evaluation. For instance, genotoxicity can be a consequence of long-term exposure to very low levels of chemicals and have a hereditary and delayed-onset nature that may lead to major consequences at the population level [17].

Considering the growing interest in the use of medicinal plants to treat and/or prevent various diseases associated with free radicals, there is an urgent need to provide information on toxicity risk-assessment of plants extracts. Therefore, the present study aimed at investigating the possible genotoxic and cytotoxic potential of *T. globiferus* and Z. *zanthoxyloides* in human leukocytes. A further attempt was made to determine the reducing potential (conversion of Fe (III) to Fe (II)) of these plants as well as their chemical characterization.

MATERIALS AND METHODS

Chemicals

All chemicals used including solvents were of analytical grade.

Plants Collection and Extraction Procedure

The leaves of *T. globiferus* and stem bark of Z. *zanthoxyloides* were obtained from Ogbomoso, Nigeria in 2013 and were identified by Dr. Ogunkunle of the Botany Unit, Department of Pure and Applied Biology (Ladoke Akintola University of Technology, where the specimen was deposited). The dried leaves and stem bark were pulverized into a powdery form, after which 100 g of *T. globiferus* and 100 g of Z. *zanthoxyloides* were macerated at room temperature with ethanol (70%) and extracted for 3 days. The combined ethanolic extract of each sample was filtered on the 3rd day and the solvent was fully evaporated under reduced pressure to give a green solid for *T. globiferus* and yellow solid for Z. *zanthoxyloides*. The ethanolic extract of *T. globiferus* was then suspended in water, while, that of Z. *zanthoxyloides* was suspended in ethanol in order to prepare different concentrations (10-150 μg/mL) used in the experiments.

Quantification of Some Flavonoids and Phenolic Compounds by High Performance Liquid Chromatography-Diode Array Detector (HPLC)

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% of B until 2 min and changed to obtain 25%, 40%, 50%, 60%, 70% and 100% B at 10, 20, 30, 40, 50 and 65 min, respectively, following the method described by Laghari *et al.* [18] with slight modifications. The extracts of *T. globiferus and* Z. *zanthoxyloides* were analyzed, at a concentration of 5 mg/mL. The presence of six phenolics compounds was investigated, namely, gallic, chlorogenic and caffeic acids and the flavonoids quercetin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and ultraviolet (UV) absorption spectrum with those of the commercial standards. The flow rate was 0.6 mL/min, injection volume 40 μL and the wavelength were 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.031-0.250 mg/mL for kaempferol, quercetin and rutin; and 0.006-0.250 mg/mL for gallic, caffeic and chlorogenic acids. All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves as defined by ICH [19].

LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Obtension of Human Leukocytes

Heparinized venous blood was obtained from healthy volunteer donors from the Hospital of the Federal University of Santa Maria (UFSM), Santa Maria, RS, Brazil (age 25 ± 10). This work was carried out in accordance with the Guidelines of the Ethical Committee of UFSM and approved by the Institutional Review Board of UFSM (0089.0.248.000-12). Differential erythrocyte sedimentation with dextran was used to separate leukocytes of the blood as previously described [20].

Genotoxicity evaluation of T. globiferus and Z. zanthoxyloides using comet assay

The comet assay was performed under alkaline conditions according to the method of Santos *et al.* [21]. Briefly, peripheral leukocytes were incubated for 3 h in the absence or presence of plant extract, at different concentrations (10-150 μg/mL). Hydrogen peroxide (100 μM) was used as a positive control, while water was used as negative control (NC). After incubation and electrophoresis, one hundred cells per sample were randomly selected and visually scored according to tail length into five classes: (1) Class 0: Undamaged, without a tail; (2) Class 1: With a tail shorter than the diameter of the head (nucleus); (3) Class 2: With a tail length 1-2 times the diameter of the head; (4) Class 3: With a tail longer than 2 times the diameter of the head and (5) Class 4: Comets with no heads. Comets with no heads and images with nearly all DNA in the tail or with a very wide tail were excluded from the evaluation because they probably represent dead cells. DNA damage was presented as DNA damage index (DI) and it is based on the length of migration. The DI was calculated from cells in different damage classes as follows: DI = n1 + 2n2 + 3n3 + 4n4. Where, n1-n4 represents the number of cells with level 1-4 of damage. The slides were analyzed under blind conditions by at least two individuals.

Cytotoxicity evaluation of T. globiferus and Z. zanthoxyloides by trypan blue

The toxic effects of *T. globiferus and Z. zanthoxyloides* toward leukocytes were determined as described by Mischell and Shiingi [22] with slight modifications. Briefly, 2.5 μL of different concentrations of the extracts (10-150 μg/mL) was added to leukocytes suspension (497.5 μL) and incubated in the presence or absence of hydrogen peroxide (2 mM) + azide (1 mM), for 3 h at 37°C in a water bath. Hydrogen peroxide (2 mM) + azide (1 mM) was used as a positive control whereas distilled water was used as NC. After the incubation, a volume of 50 μL of leukocytes suspension was mixed with 50 μL of 0.4% trypan blue solution and left for 5 min. The cell viability was determined microscopically (×400 magnification) using a hemocytometer and was calculated as the number of living cells (i.e., those not stained with trypan blue) divided by the total number of cells multiplied by 100.

Reducing Power Assay

The Fe^{3+} reducing power of the extracts was determined according to a modified method of Mathew and Abraham [23]. Various concentrations of *T. globiferus and Z. zanthoxyloides* (10-150 μg/mL) (200 μL) were mixed with 625 μL of potassium phosphate buffer solution (0.2 M, pH 6.6) and 625 μL of potassium ferricyanide (1%, w/v), followed by incubation at 50°C for 20 min. The reaction was stopped by adding 625 μL of trichloroacetic acid solution (10%, w/v) and then centrifuged at 5000 ×*g* for 10 min. A known volume (625 μL) of the upper layer solution (obtained after centrifugation) was taken in another test tube and mixed with 625 μL of distilled water, then, 250 μL of ferric chloride solution (0.1%, w/v) was added and mixed well. The absorbance was measured at 700 nm in a spectrophotometer. The blank was prepared by the same procedure without plant extracts. Ascorbic acid (10-150 μg/mL) was used as a positive control.

Statistical Analysis

Values were expressed as mean ± standard error of the mean. One-way ANOVA, followed by Benferroni post-test was used to evaluate the differences among the groups. The results were considered as statistically significant for $P < 0.05$.

RESULTS

Phytochemical Constituents

The HPLC analysis was used to identify and quantify the presence or absence of phenolic acids and flavonoids from the leaf extract and stem bark of *T. globiferus*, and *Z. zanthoxyloides* respectively. The results of HPLC profile indicate that both plant extracts contain chlorogenic and caffeic acids, rutin and quercetin [Figure 1]. However, gallic acid, present in the leaf extract of *T. globiferus*, was absent in the stem bark of *Z. zanthoxyloides*. Similarly, kaempferol, absent in the

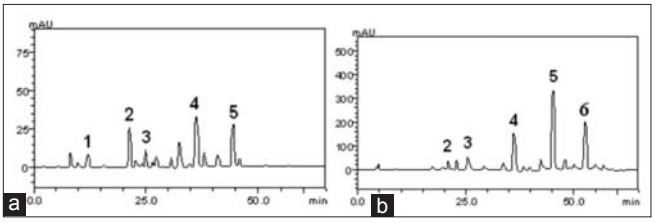

Figure 1: High performance liquid chromatography (HPLC) profile of the leaf extract of *Tapinanthus globiferus* (a) and *Zanthoxylum zanthoxyloides* stem bark extracts (b). Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6). The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by diode array detector spectra (200-500 nm). Calibration curve for gallic acid: Y = 11611x + 1468.8 (r = 0.9999); chlorogenic acid: Y = 14762x + 1257.5 (r = 0.9997); caffeic acid: Y = 11526x + 1293.1 (r = 0.9995); rutin: Y = 13035x – 1045.9 (r = 0.9998); quercetin: Y = 15105x – 1192.3 (r =0.9998) and kaempferol: Y = 15223x – 1303.9 (r = 0.9999). All chromatography operations were carried out at ambient temperature and in triplicate

leaf extract of *T. globiferus*, was present in the stem bark of *Z. zanthoxyloides* [Figure 1 and Table 1]. These compounds were identified by comparing their retention times and UV spectra to that of authentic standards analyzed under identical analytical conditions. Quantitative HPLC analysis showed that the rutin was the major component in *T. globiferus* (9.14 ± 0.1 mg/g) while caffeic acid was the minor (1.98 ± 0.03 mg/g). However, the major component found in *Z. zanthoxyloides* was quercetin (48.09 ± 0.03 mg/g), while chlorogenic acid (4.23 ± 0.01 mg/g) was the minor [Table 1].

Effects of *T. globiferus* and *Z. zanthoxyloides* on DNA Damage

Table 2 shows the comet assay results obtained after exposure of human leukocytes to various concentrations (10-150 μg/mL) of *T. globiferus and Z. zanthoxyloides*. H_2O_2 (positive control) induced a significant increase in DNA migration when compared to NC ($P < 0.001$), as evidenced by the DI [Table 2]. Ethanol used as a vehicle for *Z. zanthoxyloides* did not have any effect on DNA migration in comparison with the NC ($P > 0.05$). There was no significant difference in the DI when the cells were treated with *T. globiferus* (10-150 μg/mL) when compared to NC ($P > 0.05$). However, a statistically significant increase in DNA DI was observed at 150 μg/mL of *Z. zanthoxyloides*. Generally, when the human leukocytes were exposed to both plant extracts (10-150 μg/mL), the majority of leukocytes examined on slides were undamaged (Class 0). Few leukocytes showed minor DNA damage (Class 1) and very few showed a large amount of DNA damage (Class 2-4) [Table 2].

Table 1: Qualitative and quantitative analyses of some flavonoids and phenolic compounds from the leaf extract of *T. globiferus* and *Z. zanthoxyloides* stem bark extract by HPLC-DAD

Compounds	$t_{R\ (min)}$	*T. globiferus*		*Z. zanthoxyloides*		LOD	LOQ
		mg/g	%	mg/g	%	(μg/mL)	(μg/mL)
Rutin	40.25	9.14±0.1	0.83	18.25±0.06	2.01	0.022	0.074
Quercetin	50.11	7.08±0.02	0.7	48.09±0.03	4.82	0.028	0.092
Kaempferol	60.18	-	-	26.03±0.07	2.61	0.031	0.103
Gallic acid	11.92	2.35±0.13	0.23	-	-	0.017	0.056
Chlorogenic acid	23.86	6.83±0.1	0.61	4.23±0.01	0.42	0.036	0.119
Caffeic acid	25.09	1.98±0.03	0.19	9.02±0.08	0.92	0.009	0.028

Results are expressed as mean±standard deviations of three determinations. LOD: Limit of detection, LOQ: Limit of quantification, t_R: Retention time, *T. globiferus: Tapinanthus globiferus, Z. zanthoxyloides: Zanthoxylum zanthoxyloides*, HPLC-DAD: High performance liquid chromatography-diode array detector

Effects of *T. globiferus* and *Z. zanthoxyloides* on Leukocytes Viability

In order to assess the toxicity of *T. globiferus and Z. zanthoxyloides* on human leukocytes, cellular viability was evaluated following exposure, by using the trypan blue assay dye exclusion method. The H_2O_2 + azide were used to inhibit catalase activity in leukocytes and consequently detect the toxicity induced by H_2O_2. H_2O_2 + azide used as positive control, caused a significant decrease in cell viability (approximately 48% decrease) when compared to control [Figure 2a and b; $P < 0.05$]. *T. globiferus* at all the concentrations tested did not have any effect on cell viability [Figure 2a], whereas, *Z. zanthoxyloides* at the highest concentration (150 μg/mL) exhibited a significantly decrease [Figure 2b] when compared to control ($P < 0.05$). It should be noted that 150 μg/mL of *Z. zanthoxyloides* concentration was genotoxic and cytotoxic to human leukocytes.

Reducing Power Potential of *T. globiferus* and *Z. zanthoxyloides*

As depicted in Figure 3, *T. globiferus and Z. zanthoxyloides* showed increased absorbance with increased concentrations, which indicates increased ferric reducing power. However, the reducing potential of both extracts was lower than that of ascorbic acid used as standard antioxidant. The reducing power of the extracts and ascorbic acid decreased in the order ascorbic acid > *T. globiferus* > *Z. zanthoxyloides*.

Table 2: Effect of *T. globiferus* and *Z. zanthoxyloides* on human leukocytes

Treatment	Extract concentration (μg/mL)	Levels of DNA damage					DI
		0	1	2	3	4	
Control (H_2O, NC)	0	96.25±0.14	3.125±0.23	0.5±0	0.125±0.12	0±0	4.500±0.20
Etanol (vehicle)	-	95.91±0.19	3.52±0.23	0.48±0	0.141±0.12	0±0	4.480±0.32
H_2O_2 (PC)	100 μM	71.2±0.26	22.57±0.01	3.87±0.36	1.25±0.28	1.38±0.26	40.01±1.22*
T. globiferus	10	96.25±0.25	3.125±0.47	0.375±0.12	0.25±0.14	0±0	4.625±0.23
	25	95.75±0.14	3.875±0.31	0.375±0.23	0±0	0±0	4.625±0.23
	50	94.75±0.25	4.5±0.35	0.75±0.14	0±0	0±0	6.000±0.20
	100	94.5±0	5.25±0.25	0.375±0.23	0±0	0±0	6.000±0.28
	150	94±0.35	5.625±0.37	0.375±0.23	0±0	0±0	6.375±0.47
Z. zanthoxyloides	10	96.25±0.25	2.87±0.55	0.37±0.24	0.37±0.12	0.12±0.12	5.25±0.32
	25	95.87±0.24	2.62±0.43	1.25±0.24	0.37±0.12	0±0	6.00±0.2
	50	95.12±0.37	4.75±0.32	0.12±0.12	0±0	0±0	5.00±0.45
	100	94.87±0.12	4.37±0.4	0.75±0.32	0±0	0±0	5.87±0.42
	150	94.12±0.24	4.87±0.59	0.87±0.43	0±0	0±0	7.00±0.45#

The results are mean±SEM of $n=4$ independent experiments. Water was used as NC while hydrogen peroxide was used as PC. *$P<0.001$ versus control (H_2O), #$P<0.001$ versus PC. *T. globiferus* did not have any effect on DNA damage at the concentrations tested. DI: Damage index, *T. globiferus: Tapinanthus globiferus, Z. zanthoxyloides: Zanthoxylum zanthoxyloides*, SEM: Standard error of the mean, NC: Negative control, PC: Positive control

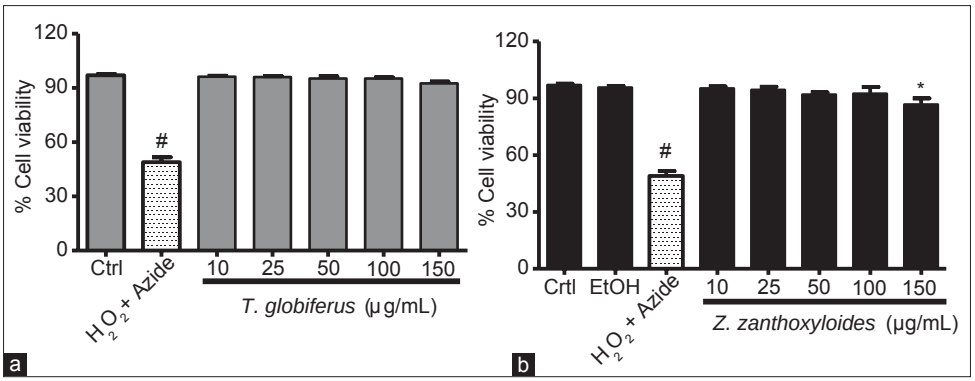

Figure 2: Survival of leukocytes treated with *Tapinanthus globiferus* (a) and *Zanthoxylum zanthoxyloides* (b) for 3 h. Results are expressed as mean ± standard error of the mean, $n = 4$. H_2O_2 (2 mM) + azide (1 mM) was used as positive control. #$P < 0.001$ versus control (Ctrl), *$P < 0.05$ versus Ctrl. *T. globiferus* was not cytotoxic to leukocytes at the concentrations tested, while, *Z. zanthoxyloides* does at the highest concentration

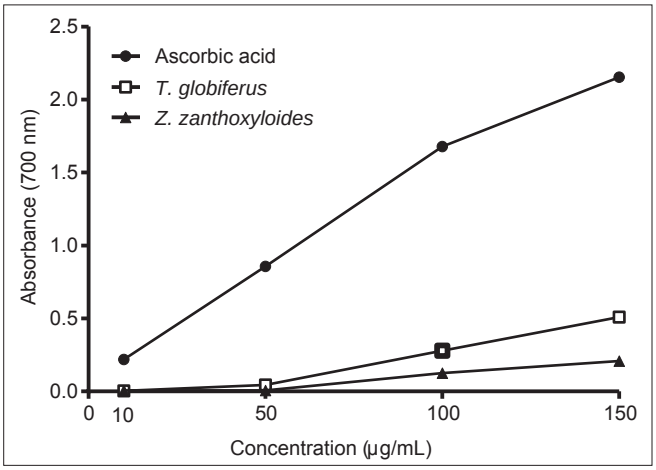

Figure 3: Reductive ability of the leaves extract of *Tapinanthus globiferus* and *Zanthoxylum zanthoxyloides* stem bark extract versus ascorbic acid. Values expressed in absorbance are the mean ± standard error of the mean of $n = 4$ performed in duplicates

DISCUSSION

Although medicinal plants are regarded as safe, there is increasing evidence that plant extracts and/or their chemical constituents can have toxic effects [24]. Therefore, the toxicity evaluation of plant extracts used in folk medicine is highly recommended. In the present study, the genotoxicity and cytotoxicity effects of *T. globiferus* and *Z. zanthoxyloides* were investigated in human leukocytes, as well as their reducing potential. The results demonstrated that *T. globiferus* was neither genotoxic nor cytotoxic to human leukocytes at all the concentrations tested. However, *Z. zanthoxyloides* was genotoxic and cytotoxic at the highest concentration tested (150 µg/mL). These results indicate that the use of *T. globiferus* at relatively high concentrations could be regarded as safe. The genotoxicity and cytotoxicity effects of *Z. zanthoxyloides* at the highest concentration tested leads to DNA damage, an indication of the presence of chemical constituents which interacted with DNA, leading to damage. Another explanation could be a synergistic interaction of compounds within the plant extracts resulting in the observed damage to DNA [25]. Although the comet assay has been criticized for the agarose concentration [15,26], it has become the most popular method for measuring DNA damage of various sorts, including oxidative damage inflicted by ROS [16,26].

Natural antioxidants found in plants and vegetables are extensively studied for their ability to protect the organism and cells from the deleterious effects induced by oxidative stress [27-29]. In previous studies, *T. globiferus* and *Z. zanthoxyloides* have shown antioxidant activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and iron chelating activities [30,31]. In this study, the reductive potential of *T. globiferus* and *Z. zanthoxyloides* was determined on the basis that this assay has a different mechanism of action in relation to DPPH and iron chelating assays. In addition, reducing power of a compound is associated with antioxidant activity and may serve as a significant reflection of its potential antioxidant capacity [20,32]. This assay is based on the reduction of Fe^{3+}/ferricyanide complex to the Fe^{2+} form in the presence of antioxidant. The reduction is observed by the change of the yellow test solution to green or blue color depending on the reducing power of antioxidant samples. In addition, a higher absorbance indicates a higher ferric reducing power. Here, *T. globiferus* and *Z. zanthoxyloides* showed increased ferric reducing power with an increased concentration as ascorbic acid, indicating that both plant extracts have antioxidant activity. In the agreement to this, Amarowicz and Troszynska [33] demonstrated a direct relationship between reducing power and antioxidant activity. Consequently, the reducing power of these plant extracts may be associated with the antioxidant activity of phenolic acids and flavonoids found in these extracts.

CONCLUSION

The safety evaluation of *T. globiferus* and *Z. zanthoxyloides* revealed that *T. globiferus* (10-150 µg/mL) was neither genotoxic nor cytotoxic to human leukocytes following 3 h exposure. This indicates that its popular use in infusion might be considered safe for consumption. In contrast, *Z. zanthoxyloides* at the highest concentration tested (150 µg/mL) showed genotoxicity and cytotoxicity effects, therefore not safe for consumption. Both plants showed antioxidant activity as evidenced by their

reducing power potential, which can be attributed at least, in part, to their flavonoid and phenolic contents.

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