INTRODUCTION

Reactive oxygen species (ROS) are oxidizing, highly reactive and unstable molecules containing oxygen. They are produced during normal cellular metabolism as by-products of respiration in the mitochondria. They include hydroxyl radical (OH•), superoxide anion (O₂•−), hydrogen peroxide (H₂O₂), 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 their 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\textsuperscript{-}), an oxidant obtained from the breakdown of H\textsubscript{2}O\textsubscript{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\textsubscript{2}- and H\textsubscript{2}O\textsubscript{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].

**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 powder 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 3\textsuperscript{rd} 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 \(\mu\text{g/mL}\)) 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\textsubscript{18} column (4.6 mm \(\times\) 250 mm) packed with 5 \(\mu\text{m}\) 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 \(\mu\text{L}\) 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 \(\mu\text{m}\) 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].

**Tapinanthus globiferus** and *Zanthoxylum zanthoxyloides* are plants commonly used as folkloric 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.
LOD and LOQ were calculated as 3.3 and 10 \( \sigma/S \), respectively, where \( \sigma \) 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 \( \pm \) 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 (0089.0.245.000-12). 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 \( \mu \)g/mL). Hydrogen peroxide (100 \( \mu \)M) 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 Shingi [22] with slight modifications. Briefly, 2.5 \( \mu \)L of different concentrations of the extracts (10-150 \( \mu \)g/mL) was added to leukocytes suspension (497.5 \( \mu \)L) and incubated in the presence or absence of hydrogen peroxide (2 mM) + azide (1 mM), for 3 h at 37\(^\circ\)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 \( \mu \)L of leukocytes suspension was mixed with 50 \( \mu \)L of 0.4% trypan blue solution and left for 5 min. The cell viability was determined microscopically (\( \times 400 \) 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 \( \mu \)g/mL) (200 \( \mu \)L) were mixed with 625 \( \mu \)L of potassium phosphate buffer solution (0.2 M, pH 6.6) and 625 \( \mu \)L of potassium ferricyanide (1%, w/v), followed by incubation at 50\(^\circ\)C for 20 min. The reaction was stopped by adding 625 \( \mu \)L of trichloroacetic acid solution (10%, w/v) and then centrifuged at 5000 \( \times \)g for 10 min. A known volume (625 \( \mu \)L) of the upper layer solution (obtained after centrifugation) was taken in another test tube and mixed with 625 \( \mu \)L of distilled water, then, 250 \( \mu \)L 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 \( \mu \)g/mL) was used as a positive control.

**Statistical Analysis**

Values were expressed as mean \( \pm \) 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](image-url)
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*. *H₂O₂* (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].

**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 *H₂O₂* + azide were used to inhibit catalase activity in leukocytes and consequently detect the toxicity induced by *H₂O₂*. *H₂O₂* + 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 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. globiferus | Z. zanthoxyloides | LOD (μg/mL) | LOQ (μg/mL) |
|-----------------|---------------|-------------------|-------------|-------------|
| Rutin           | 40.25         | 9.14±0.1          | 0.03        | 48.09±0.03  |
| Quercetin       | 50.11         | 7.08±0.02         | 0.03        | 4.87±0.06   |
| Kaempferol      | 60.18         | -                 | 0.03        | 4.23±0.01   |
| Gallic acid     | 11.92         | 2.35±0.1          | 0.03        | 4.87±0.06   |
| Chlorogenic acid| 23.86         | 6.83±0.1          | 0.03        | 4.23±0.01   |
| Caffeic acid    | 25.09         | 1.98±0.03         | 0.03        | 4.87±0.06   |

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

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

| Treatment (H₂O₂, NC) | Extract concentration (μg/mL) | 0     | 1     | 2     | 3     | 4     | DI    |
|----------------------|-------------------------------|-------|-------|-------|-------|-------|-------|
| Control (H₂O₂, 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 |
| *H₂O₂* (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                  | 94.03±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 | 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±0.22 |
| 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 (H₂O₂), †P<0.001 versus control. *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
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""/ferricyanide complex to the Fe" 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 antioxidant activity. In the agreement to this, Amarowicz and Troszynska [33] demonstrated a direct relationship between antioxidant activity and 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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