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In vitro antioxidant and antiglycation studies on african mistletoe (*T. bangwensis* (*Engler* and K. Krause), Danser) and ring worm plant (*S. alata* (Linn.) Roxb) from Nigeria

Kingsley C. Patrick-Iwuanyanwu[1&2], Sajjad Ali[2&3*], Muhammad Iqbal Choudhary[2], Muhammad Ismail[3], Saima Rasheed[2]

[1]*Department of Biochemistry (Toxicology Unit), Faculty of Chemical Sciences, College of Natural and Applied Sciences, University of Port Harcourt, Port Harcourt, Rivers State, Nigeria*

[2]*HEJ Research Institute of Chemistry, ICCBS, University of Karachi Pakistan*

[3]*Department of Chemistry, Karakoram International University, Gilgit, Gilgit-Baltistan, Pakistan*

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Abstract

The antioxidant activity of crude methanolic extracts and fractions of African mistletoe (*Tapinanthus bangwensis*) and Ring worm plant (*Senna alata*) leaves from Nigeria was investigated. In an *in vitro* free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide scavenging (SOS) activity and chelation of iron (II) ion [Fe^{2+}] in the presence of ethylenediaminetetraacetic acid (EDTA), ethyl acetate (EtOAc) fractions of both plants showed antioxidant activity in DPPH (83.95±0.04 and 66.35±0.07%), SOS activity (86.10±0.03 and 83.14±0.07%) for *T.bangwensis* and *S.alata* respectively compared to control (*n*-propyl gallate) which showed 90.31±0.01% inhibition). Inhibition of Fe^{2+} chelation showed 84.29±0.06 and 81.59±0.04% for *T.bangwensis* and *S.alata* respectively when compared to control, EDTA (97.60±0.07%). Furthermore, in an advanced glycation end products (AGEs) study, the *in vitro* inhibition (≥50%) was highest in EtOAc fraction of *S.alata* (57.06±3.20 and 65.53±1.76%) at 0.25 and 0.5 mg/mL, respectively when compared to the control, rutin (86.00±1.50%). In conclusion, results of the study showed possible antioxidant as well as antiglycation potential of leaves of *Tapinanthus bangwensis* and *Senna alata*.

***Corresponding Author:** Sajjad Ali ✉ sajjad.ali@kiu.edu.pk

Introduction

There has been an increased attention towards natural antioxidants (Naimiki, 1990). These antioxidants have been described as vital substances which possess the ability to protect the body from damage caused by free radical-induced oxidative stress (Ozsoy *et al.,* 2008). These reactive oxygen species (ROS) include a number of chemically reactive molecules derived from oxygen (Halliwell, 1996; Halliwell, 1999; Betteridge, 2000) produced in the cells by cellular metabolism and other exogenous environmental agents. At low levels, ROS are known to act as important signalling molecules and play positive roles such as in energy production, phagocytosis, regulation of cell growth and synthesis of biologically important compounds. However, ROS may also be very damaging as they can induce oxidation of lipids, causing membrane damage, decreasing membrane fluidity and leading to cancer *via* DNA mutation (Cerutti, 1994; Ames *et al.,* 1995; Apel and Hirt, 2004). Therefore, it is pertinent that a potent scavenger of these ROS may serve as possible prevention against free radical mediated diseases (Pietta, 2000).

Advanced glycation end-products (AGEs) are formed as a result of a non-enzymatic reaction in which glucose forms adducts with proteins, lipids and nucleic acids (Semba *et al.,* 2010). AGEs accumulate naturally as a result of chronological aging, but this process is greatly accelerated under conditions of hyperglycaemia and oxidative stress (Dimitrova *et al.,* 2010). Protein glycation and advanced glycation end-products (AGEs) are accompanied by increased free radical activity that contributes towards the biomolecular damage in diabetes. Studies suggest that interaction of AGEs with AGE-specific receptors (RAGEs) alter intracellular signalling, gene expression, release of pro-inflammatory molecules and free radicals that contribute toward the pathology of diabetic complications (Ahmed, 2005). There is considerable interest in anti-glycation-rich compounds derived from medicinal plants because of their therapeutic potential and strong inhibitory activity (Materska and Perucka, 2005). They are well-known contributors to the pathophysiology of aging and diabetic chronic complications (Lunceford and Gugliucci, 2005). Increased glycation during hyperglycaemia can cause intra- or inter- molecular cross linking of proteins as they accumulate AGEs. It has been shown that build up of cross-linked AGEs on long-lived proteins may underlie the development of complications affecting diabetes and ageing (Ahmed, 2005). Furthermore, the levels of serum advanced glycation end-products reflect the severity of these complications whereas therapeutic interventions aimed at reducing AGEs can inhibit or delay their progression (Monnier, 2003). Several plants have been reported to show antiglycation activities in BSA-glucose model (Gutierrez *et al.,* 2010).

As nature and natural sources, for instance, plants, animals, microbes, and minerals have remained a veritable source of bioactive constituents with therapeutic values. Reports on the free radical scavenging activity and antiglycation properties on both *T.bangwensis* and *S.alata* are either not available or scanty (Yakubu and Musa, 2012; Bassey *et al.,* 2012). Since the present study was conducted to identify the fraction with medicinal potency which could lead to the discovery of some lead constituents from *T.bangwensis* and *S. alata* to address the challenges of death leading diseases like diabetes and cancer. Therefore, in this study antioxidant and antiglycation properties of *T.bangwensis* and *S. alata* were evaluated by estimating their free radical scavenging activity against 1,1- diphenyl-2-picrylhydrazyl (DPPH), superoxide scavenging (SOS) activity and chelation of Fe^{2+} ion in the presence of EDTA and by BSA- glucose model, respectively.

Materials and methods

Materials

Bovine Serum Albumin (BSA) was purchased from Merck Marker Pvt. Ltd. Rutin, Methyl glyoxal (MGO) (40% aqueous solution), sodium dihydrogen phosphate (NaH_2PO_4), and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Sigma

Aldrich. The *β*-nicotinamide adenine dinucleotide (reduced form) (NADH) was obtained from Research Organics (USA); phenazine methosulphate (PMS), nitro blue tetrazolium (NBT), and 1, 1-diphenyl picryl hydrazyl (DPPH) are products of Sigma (Germany); sodium monohydrogen phosphate, sodium dihydrogen phosphate and ethanol were purchased from Merck (Germany), and dimethyl sulfoxide (DMSO) from AVONCHEM (UK).

Collection and Authentication of Plant Materials

The authentic plants materials of *Tapinanthus bangwensis* and *Senna alata* were collected in June 2009 from Delta Park, University of Port Harcourt, Port Harcourt, Rivers State. The plant materials were identified by Dr. N. L. Edwin-Wosu of the Department of Plant Science and Biotechnology, University of Port Harcourt. Voucher specimens were deposited with the Herbarium of the Department of Plant Science and Biotechnology, University of Port Harcourt, Port Harcourt, Rivers State.

Extraction of the Plant Leaves

The sun-dried leaves of *T.bangwensis* (2 Kg) and *S.alata* (1.5 Kg) were separately pulverized into a fluffy mass. One kilogramme each of the powdered leaves of *T.bangwensis* and *S.alata* was respectively extracted with 8 litres of 80 % methanol (MeOH) using Soxhlet extractor for 24 hrs. The extract was evaporated to dryness under reduced pressure (below 40 °C) by using rota evaporator to yield crude methanolic extracts of *T.bangwensis* (700 g) and *S.alata* (550 g).

Fractionation of the Extracts

Forty grams each of the dried crude methanolic extract of both *T.bangwensis* (695 g) and *S.alata* 545 g) was dissolved separately in distilled water (200 mL) in conical flasks (250 mL). It was further fractionated by successive solvent extraction with *n*-hexane, ethyl acetate (EtOAc) (2 x 100 mL) and *n*-butanol (BuOH) saturated with H_2O (3 x 100 ml). Each extract was evaporated to dryness under reduced pressure to yield *n*-hexane (210, 121 g), ethyl acetate (139, 127 g), *n*-butanol (114, 109 g) and aqueous fractions (140, 182 g) of *T.bangwensis* and *S.alata,* respectively. The extraction scheme for both the plant species was same and shown in Fig.1.

DPPH Radical Scavenging Assay

The scavenging effect of crude MeOH extracts and fractions (*n*-hexane, ethyl acetate, *n*-butanol, and aqueous) of leaves of *T.bangwensis* and *S.alata* on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured according to the method of Shimada *et al.* (1992) with some modifications. This method was performed by taking 95 µL of DPPH solution and 5 µL of sample in a micro titer plate. DPPH solution was prepared in ethanol and sample (500µg) was dissolved in DMSO. The final well concentration for DPPH was maintained at 300 µM and for sample at 500 µg/mL. Reaction mixture was incubated at 37 °C for 30 minutes, and the final absorbance was recorded on microplate reader at 515 nm. Radical quenching efficacy of compounds was determined by comparison with DMSO treated blank (control). A lower absorbance represented a higher DPPH scavenging activity. The results were calculated as the percentage of free radical-scavenging effect according to the following formula:

Radical Scavenging activity (%) RSA= {1-(AS/AC)} x 100

Where, AS, and AC are the absorbance of the sample and control, respectively.

Superoxide Anion Radical Scavenging Assay

The scavenging activity of the fractions of crude methanolic extracts (*n*-hexane, ethyl acetate, *n*-butanol, and aqueous) of leaves of *T.bangwensis* and *S.alata* was determined in a 96-well micro titer plate. In each reaction well was added 10 µL of test compound (500 mg/mL), 40 µL of NADH (100 µM), 40 µL of NBT, 20 µL of phenazinemethosulphate (PMS), nitro blue tetrazolium (NBT) and 90 µL of phosphate buffer (100 µM, pH 7.4).

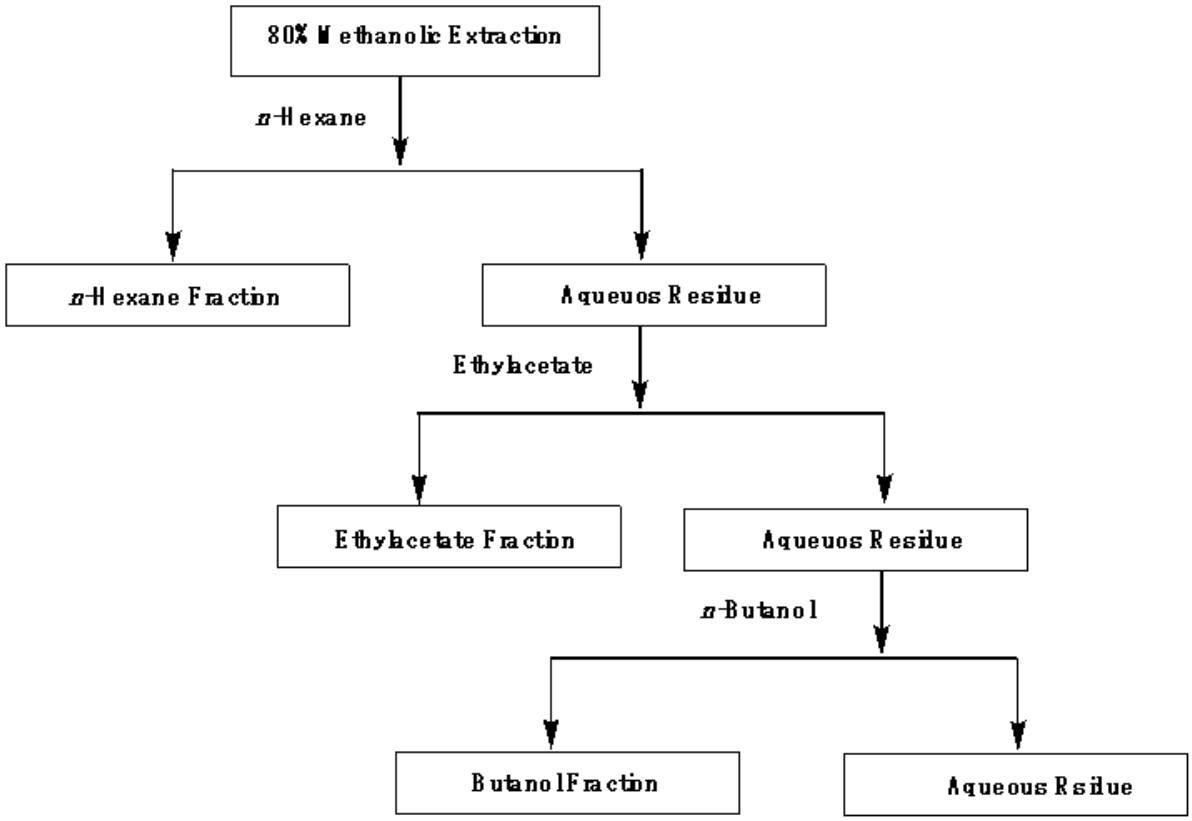


Fig. 1. Scheme for solvent extraction of *T.bangwensis* and *S.alata*.

Buffer was used for the preparation of reagents, whereas DMSO was used for the test compound. The experiment was carried out at room temperature 25 ^{o}C and generation of superoxide was monitored spectrophotometrically at 560 nm. A control sample was set up in parallel for the comparison with the test sample, since the decrease in absorbance of test sample was an indication of its antioxidant activity. The positive was *n*-propyl gallate. Triplicate tests were conducted for each sample. The results were calculated as the percentage of radical-scavenging activity according to the following formula:

Radical Scavenging activity (%) RSA= {1-(AS/AC)} x 100

Where, AS, and AC are the absorbance of the sample and control, respectively.

Iron Chelation Assay

The chelating activity of the extracts for iron (II) ions (Fe^{2+}) was determined according to the method of Dinis *et al.,* (1994). The sample, 5µL was added to 35 µL of 0.025 µM $FeCl_2$. The reaction was initiated by the addition of 60 µL of 0.04 µM ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the solution was then measured spectrophotometrically at 562 nm. EDTA was used as a positive control. All tests were carried out in triplicate. The percentage inhibition of ferrozine–Fe^{2+} complex formation was obtained using the formula:

Iron (II) ion chelating activity = $[(A_o - A_1) / A_o] \times 100$

where A_o was the absorbance of control and A_1 the absorbance of samples.

Antiglycation Assay

The assay was carried out by using the method described by Rahbar and Figarola (2003) with slight modifications. Triplicate samples of BSA 10 mg/ml, 14 mM MGO, 0.1 M phosphate buffer (pH 7.4) containing NaN_3 (30 mM) was incubated under aseptic conditions, (in such a way that each well of 96-well plate contained 50 µL BSA solution, 50 µL

MGO, and 20 µL test sample) at 37°C for 9 days in the presence or absence of various concentrations of the test samples. After 9 days of incubation, each sample was examined for the development of specific fluorescence (excitation, 330 nm; emission, 440 nm), on a microtitre plate spectrophotometer (Spectra Max, Molecular Devices, USA). Rutin was used as a positive control (IC_{50} = 294±1.50µM).

The percent inhibition of AGE formation in the test sample versus control was calculated for each inhibitor by using the following formula:

% inhibition= (1- fluorescence of test sample/ Fluorescence of the control group) x 100

Statistical Analysis

The experimental data was expressed as ± standard deviation (±SD). Statistical analysis was performed by using one-way analysis of variance (ANOVA).

Results

Antioxidant studies

The result of the DPPH free radical scavenging activity of methanolic extracts and fractions (*n*-hexane, ethylacetate, *n*-butanol and aqueous) are shown in Fig. 2. It was observed that the EtOAc fractions of *T.bangwensis* and *S.alata* showed the highest free radical scavenging activity among the experimental groups (83.95 and 66.35 % respectively). This was followed by the aqueous fraction of *T.bangwensis* (70.60 %) and methanolic extract of *S.alata* (62.99 %). The hexane fractions of the leaves of both *T. bangwensis and S. alata* showed the lowest DPPH free radical scavenging activity (49.25 and 37.79% respectively). In general, the EtOAc fractions of the leaves of both *T.bangwensis* and *S.alata* exhibited the highest free radical scavenging potential among the test groups when compared with the control *n*-propyl gallate which showed 90.31 % free radical scavenging activity (Fig. 2). The result of superoxide anion scavenging assay in this study showed that the EtOAc fractions of both plants exhibited the highest free radical scavenging activity. Of the two plants, EtOAc fraction of *T.bangwensis* had the highest percentage inhibition (86.10 %) while the EtOAc fraction of *S.alata* recorded 83.14 %.

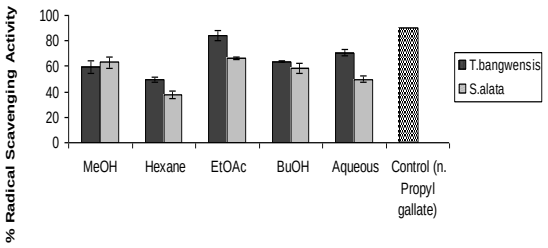


Fig. 2. DPPH free radical scavenging activity of extracts of *T.bangwensis* and *S.alata*.

This was followed by the BuOH fraction (83.88%) among the extracts of leaves of *T.bangwensis* and the aqueous fraction (72.08 %) among the extracts of the leaves of *S.alata*. All these activities are comparable to standard antioxidant compound, *n*-propyl gallate (90.31 %) as shown in Fig. 3.

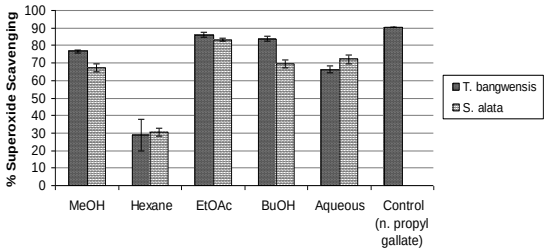


Fig. 3. Superoxide scavenging activity of the extracts of *T.bangwensis* and *S.alata*.

The hexane fractions of the leaves of both plants showed a moderate activity, (28.67 and 30.30 %) for *T.bangwensis* and *S.alata* respectively. The iron (III) ion (Fe^{3+}) chelating activities of samples showed that the EtOAc fractions of leaves of *T.bangwensis* and *S.alata* exhibited highest inhibition (82.49 and 81.59 % respectively) which is comparable to standard chelating agent EDTA (97.60 %) (Fig. 4); this was followed by the BuOH fractions which recorded 81.13

and 71.29 % for *T.bangwensis* and *S.alata,* respectively. The hexane fractions of the leaves of both plants showed a moderate inhibition. Inhibition of hexane fractions of *T.bangwensis* and *S.alata* were found to be 41.91 and 41.78 % respectively. In general, the trend in percentage inhibition was EtOAc > BuOH > Aqueous > MeOH > Hexane for both *T.bangwensis* and *S.alata*.

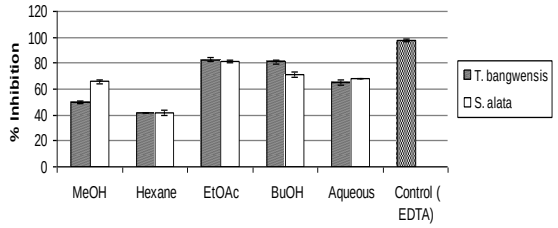
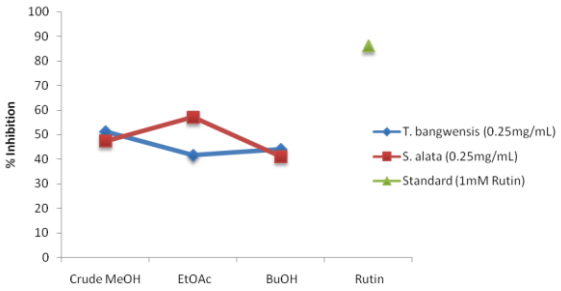


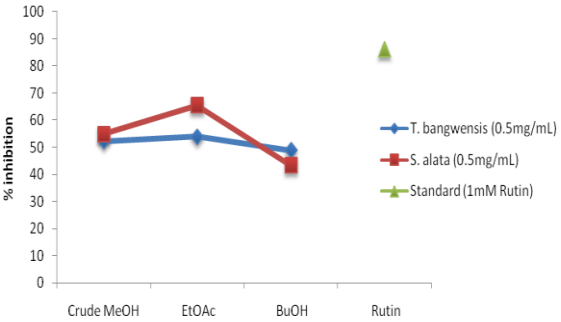
Fig. 4. Iron (III) ion chelating activity of extracts of *T.bangwensis* and *S.alata*.

The results of the advanced glycation end products (AGEs) study are shown in Figs. 5 and 6. There was a significant inhibition of greater than 50 % of AGEs formation at a concentration of 0.25 mg/mL by ethyl acetate (EtOAc) fraction of *S.alata* (57.06±3.20 %) followed by methanolic extract of *T.bangwensis* (51.27 ±2.48 %). However, the methanolic extracts and fractions of both plants at 10 mg/mL showed significant (p≤0.05) inhibition of greater than 50 %. The ethyl acetate (EtOAc) fraction showed the highest inhibition of AGEs (65.53 ±1.76 %) followed by methanolic extract for *S.alata* whereas the ethyl acetate fraction showed the highest inhibition (54.94 ±1.12 %) followed by methanolic fraction (52.21 ±1.10 %) for *T.bangwensis* when compared to the control, rutin (86.15 %). In general, the EtOAc fractions of leaves of both *T.bangwensis* and *S.alata* showed the most significant percent inhibition of AGEs at both concentrations (0.25 and 0.5 mg/ml) while the least percent inhibition of AGEs was exhibited by the BuOH fractions of both *T.bangwensis* and *S.alata*.



Values are expressed as average percentage inhibition of AGE formation ± SD of three independent experiments performed in quintuplicate. $p \leq 0.05$ compared to control.

Fig. 5. Inhibition of protein glycation (AGEs formation) by methanolic extracts and fractions of *T.bangwensis* and *S.alata* at 0.25mg/kg.



Values are expressed as average percentage inhibition of AGE formation ± SD of three independent experiments performed in quintuplicate.

Fig. 6. Inhibition of protein glycation (AGEs formation) by methanolic extracts and fractions of *T.bangwensis* and *S.alata* at 0.5mg/kg.

Discussion

The present study shows that methanol extracts and its fractions of *T.bangwensis* and *S.alata* exhibited antioxidant property by the reduction of DPPH radicals to the corresponding hydrazine in a stoichiometric manner in which the unpaired electrons of DPPH are converted to paired ones which in effect is the action of the antioxidants (Sanchez-Moreno *et al.,* 1999; Sanchez-Moreno, 2002). DPPH radical is a stable free radical, which has been widely used to evaluate the free radical scavenging effects of natural antioxidants (Blois, 1958; Lee, 2003). The ability of the EtOAc fractions of *T.bangwensis* and *S.alata* (83.95 % and

66.35 % respectively) and BuOH fractions (63.53 % and 58.55 % respectively) to scavenge DPPH radicals suggest that they are electron donors and can react with free radicals to convert them to more stable products and terminate radical chain reaction (Hamad *et al.,* 2010). However, *n*-hexane fraction showed the weakest radical scavenging activity (49.25 and 37.79 % respectively). Again, this may be attributed to the non-polar nature of the solvent, hexane. The results of this study showed that the EtOAc fractions of both plants exhibited strong radical scavenging activity with *T.bangwensis* showing the strongest (86.1 %) amongst the other fractions. Superoxides are produced from molecular oxygen due to oxidative enzymes (Sainami *et al.,* 1997) of the body as well as *via* non-enzymatic reactions such as autoxidation by catecholamine (Hemmani and Parihar, 1998). The probable mechanism of scavenging the superoxide anions may be by the scavenging effect of polyphenolic compounds present in the plants after generation of superoxide anion radicals in the *in-vitro* reaction mixture. The results obtained in iron (III) ion reduction capacity in metal chelating assay corroborate those obtained in the DPPH and superoxide anion scavenging assays. Both EtOAc and BuOH fractions showed higher iron (III) ion reduction. These *in-vitro* results clearly show EtOAc, BuOH and MeOH fractions as potent free radical scavengers and antioxidants among other fractions. This observation may be due to the presence of polyphenols and other antioxidant constituents present in the leaf extracts of *T. bangwensis* and *S. alata* respectively. Krishna *et al.* (2010) had earlier reported that fractions showing the highest phenolic /flavonoid content had highest iron (III) ion reduction whereas fractions showing lowest phenolic content had the lowest iron (III) ion reduction activity.

The methanolic extracts and fractions (EtOAc and BuOH) of *T. bangwensis* and *S. alata* showed an inhibition of non-enzymatic glycation activities greater than 50 % at concentration as low as 0.25 mg/mL. Studies have shown that a good correlation exists between free radical scavenging capacity and AGE inhibitory activity *in vitro* (Matsuda *et al.,* 2003; Yokozawa and Nakagawa, 2004). This suggests that they exert their inhibitory activity by interrupting the aut-oxidative pathways. There is also growing evidence that production of ROS is increased in diabetic patients and that oxidative stress is associated with diabetic complications (Gutiérrez *et al.,* 2010). It therefore implies that the antiglycation properties exhibited by methanolic extracts and fractions (EtOAc and BuOH) of *T.bangwensis* and *S.alata* at the concentrations (0.25 and 0.5 mg/mL) investigated may be attributed to their inhibitory activity by interrupting the oxidative pathways (Gutierrez *et al.,* 2010). They also observed a high content of phenolic compounds in plants with high antiglycation properties. Similarly, Materska and Perucka (2005) have reported that purified phenolic compounds (including flavones, flavonones, proanthocyanidins and other phenolics) and phenolic-rich plant extracts have been found to have strong inhibitory activity. Thus, the inhibition of AGEs exhibited by the ethyl acetate fractions of both *T.bangwensis* and *S.alata* may be attributable to the high level of phenolic compounds present in *T.bangwensis* and *S.alata* respectively.

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