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ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF ETHANOLIC EXTRACTS OF *TECOMA STANS, GLYPHAEA BREVIS, GARCINIA KOLA, ZANTHOXYLUM MACROPHYLLA* AND *GONGRONEMA LATIFOLIUM* ROOT BARK

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ABSTRACT

Background: The *in vitro* antioxidant and free radical scavenging activities of *Tecoma stans* (*TS*), *Glyphaea brevis* (*GB*), *Garcinia kola* (*GK*), *Zanthoxylum macrophylla* (*ZM*) and *Gongronema latifolium* (*GL*) root bark were assayed by measuring the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical, superoxide anion ($O_2^{\cdot-}$) radical and nitric oxide ($NO^{\cdot}$) radical scavenging capabilities of the extracts. **Materials and Methods:** The plants obtained from Nsukka, Nigeria, were identified and analysed using standard reagent and equipments. Data analysis was done with SPSS version 20.0 and significant level was set at $P<0.05$. **Results:** *TS* was most efficient at concentration dependent DPPH radical inhibitory potential, maximally inhibiting 66.99±2.13% compared to ascorbic acid (AA) (90.27±1.81%) at 500µg/ml. *GS* was most efficient at maximal inhibition of 68.92±3.68% compared to AA (91.33±6.22%) at 1000µg/ml. *GK*, inhibits 63.33±3.12% of DPPH compared to AA's 90.27±1.81% at 500µg/ml. *ZM* was most efficient, maximally inhibiting 65.93±2.63% compared to AA (91.33±6.22) at 1000µg/ml, while *GL* was most efficient, maximally inhibiting 59.47±0.55 compared to AA (91.33±6.22%) at 1000µg/ml. The IC_{50} was 69.02, 210.37, 206.53, 110.41 and 415.91µg/ml respectively compared to AA (37.06µg/ml). The extracts respectively inhibited the formation of reduced nitro blue tetrazolium (NBT). *TS*, *ZM*, and *GL* showed a

maximum $O_2^{\cdot-}$ inhibitory activity of 49.15±1.21, 40.40±2.33 and 41.70±3.35% respectively, compared to quercetin (91.33±5.38%) at 1000µg/ml. *GB* and *GK* showed maximum inhibitory activity of 29.41±0.19 and 47.40±0.09%, compared to quercetin (90.27±12.70%). The IC_{50} for $O_2^{\cdot-}$ inhibition of *TS*, *GB*, *GK*, *ZM* and *GL* were 387.25, 4226.86, 562.34, 2582.26 and 408.10µg/ml respectively compared to quercetin 42.65µg/ml. $NO^{\cdot}$ scavenging capacity of *TS* and *ZM* were concentration dependent, scavenging most efficiently (117.2±2.41 and 115.09±1.11%) at 250µg/ml compared to α-tocopherol (138.5±2.90%), while *GL* scavenged 91.02±2.69% compared to α-tocopherol (109.21±1.17%) at 200µg/ml. **Conclusion:** The results suggest that the extracts possess significant ($p<0.05$) antioxidant and free radical scavenging capabilities, hence beneficial to health.

KEYWORDS: Ascorbic acid, α-tocopherol, antioxidant, free radicals, scavenging.

INTRODUCTION

Medicines for prophylactic or therapeutic purposes largely come from a group of plants and plant parts. The therapeutic constituents of medicinal plants are determined by the active substances found to be present in their organs and these include; alkaloids, flavonoids, glycosides with physiologic effect on humans and animals. Antioxidants are class of compounds perceived to play vital role in preventing certain types of damages chemically induced by excess free radicals. They are molecules whose reactions are very crucial for life such that they inhibit the oxidation of other molecule of which in their absence the actions of free radicals resulting from oxidative stress can be life threatening. Plants and animals posses an intricate system of antioxidants such as glutathione, vitamin C, vitamin E and antioxidant enzymes such as catalase, superoxide dismutase as well as various peroxidases. Imbalance in antioxidant levels against free radicals results to oxidative stress which damages cells and cell contents. Reactive oxygen species has long been implicated as a component of the killing response apparatus of immune cells to microbial invasion and these reactive molecules are formed by numerous mechanisms that result in oxidative stress. The scavenging of these free radicals generated from oxidative stress to an extent helps in fighting against cancer, heart disease, stroke and other immune compromising diseases as reported by Yi-fang *et al*., 2012 and Aruoma, 2014.

MATERIALS AND METHODS

Chemicals

Chemicals used in the course of this study were of analytical grade. Solvents such as hexane, ethanol and ethyl acetate were purchased from EMD Biosciences (Gibbstown, NJ, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, sodium nitroprusside (SNP), naphthylethylenediamine dihydrochloride, phosphoric acid, L-ascorbic acid, phosphate buffered saline (PBS), sulphanilamide, acetic acid, gallic acid, quercetin, methionine, riboflavin and ethylenediaminetetraacetic acid (EDTA) were all purchased from Sigma Chemical Co. (St. Louis, MO).

Plants root barks were obtained from Nsukka, Enugu State and Isuofia Anambra State, both located in Eastern Nigeria.

Equipments

UV-Spectrophotometer (Jenway 6305), weighing balance (Ohaus), water bath (Genlab Ltd).

Methods

Crude extract preparation of plant root barks

Root barks of the respective plants were air-dried at room temperature and milled to fine powder. The resultant fine powder was subjected to extraction with absolute ethanol. The ethanol extract was concentrated using a rotary evaporator and stored for use at 4℃ (Gülçin, 2005; Elmastas *et al*., 2016).

In vitro Quantitative DPPH radical-scavenging assay

DPPH free radical scavenging activity of the plant extracts were ascertained according to the method reported by Gyamfi and his co-researchers with a few modifications (Awah *et al*., 2012). About 2.0 ml of the extract solution at different concentrations diluted two-fold (2–250 µg/ml) in ethanol was mixed with 1.0 ml of 0.3 mM DPPH in ethanol. The mixture was shaken vigorously and allowed to stand in the dark for 25 min at room temperature. Each test sample solution (2.0 ml) and 1.0 ml of ethanol was used to prepare the blank solutions while 1.0 ml of 0.3 mM DPPH solution in addition with 2.0 ml of ethanol was used to prepare the negative control. L-ascorbic acid served as the positive control. After this, the absorbance of the assay mixture was measured at 518 nm against each blank with an Agilent 8453E UV-visible spectrophotometer. Lower absorbance reading of the reaction mixture indicated higher

radical scavenging activity. DPPH radical scavenging activity was calculated using the equation:

where A_0 is the absorbance of the control, and A_s is the absorbance of the tested sample. The IC_{50} value represented the concentration of the extract that caused 50% inhibition of DPPH radical and was calculated by linear regression of plots, where the abscissa represented the concentration of tested sample and the ordinate the average percent of inhibitory activity from three replicates.

***In vitro* Quantitative Superoxide radical ($O_2^{\cdot-}$)-scavenging assay**

The superoxide radical ($O_2^{\cdot-}$)-scavenging assay was ascertained based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) (Beauchamp and Fridovich, 2014) and the method used by Martinez *et al.* (2001) to determine superoxide dismutase with a few modifications (Awah *et al.*, 2012). About 3.0 ml each of the reaction mixture contained 0.05 M phosphate buffered saline (PBS) (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, NBT (75 µM) and 1.0 ml of test sample solutions (10–250 µg/ml). Thereafter, the tubes were kept in front of a fluorescent light (725 lumens, 34 watts) for 20 min after which absorbance was read at 560 nm. The entire reaction assembly was enclosed in an aluminium foil coated box. Identical tubes containing reaction mixtures which served as blanks were kept in the dark. The estimated percentage inhibition of superoxide generation was ascertained by comparing the absorbance of the control against those of the reaction mixture containing test sample as obtained by the equation:

where A_0 is the absorbance of the control, and A_s is the absorbance of the tested sample.

***In vitro* Quantitative Nitric oxide radical ($NO^{\cdot}$) scavenging assay**

Nitric oxide ($NO^{\cdot}$) generated from sodium nitroprusside (SNP) was ascertained according to the method of Marcocci *et al.*, (1994). Briefly, the reaction mixture (5.0 ml) containing SNP (5 mM) in phosphate buffered saline (pH 7.3), with or without the plant extract at different concentrations, was incubated at 25^{o}C for 180 min in front of a visible polychromatic light source (25watts tungsten lamp). The $NO^{\cdot}$ radical thus generated interacted with oxygen to produce the nitrite ion (NO_2^{-}) which was assayed at 30 min intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with

sulphanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was measured at 546 nm. The nitrite generated in the presence or absence of the plant extract was estimated using a standard curve based on sodium nitrite solutions of known concentrations. Each experiment was carried out at least three times and the data presented as an average of three independent determinations.

RESULTS

Free radical Scavenging Activity (*in vitro*)

Effect of extracts on 2,2-diphenyl-1-picryl hydrazyl (DPPH) radicals

All extracts presented significant dose-dependent DPPH radical scavenging capacity as shown in figure 1. *Tecoma stans* is efficient, inhibiting 66.99±2.13% of DPPH at a concentration of 500 µg/ml compared to ascorbic acid which inhibited 90.27±2.75% at same concentration. *Glyphaea brevis*, *Garcinia kola*, *Zanthoxylum macrophylla*, and *Gongronema latifolium* are all efficient, inhibiting respectively 68.92±3.68%, 63.16±0.60%, 65.93±2.63%, and 59.47±0.55% DPPH at a concentration of 1000 µg/ml compared to ascorbic acid which inhibited 91.33±6.22% at the same concentration. The IC_{50} values for DPPH radical inhibition of the extracts were 69.02µg/ml, 210.37 µg/ml, 206.53 µg/ml, 110.41 µg/ml and 415.91 µg/ml respectively, when compared to that of ascorbate 37.06 µg/ml.

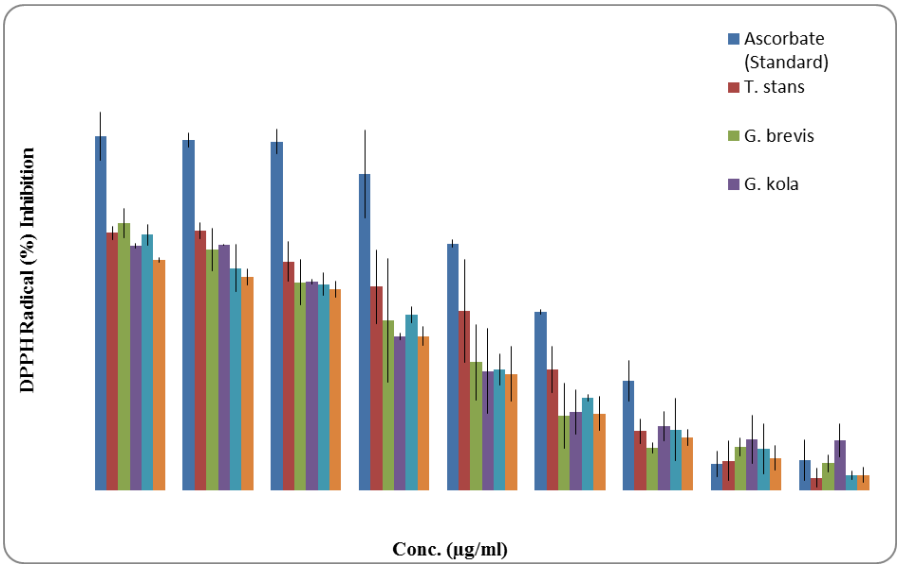


Fig. 1: Effect of extracts on 2,2-diphenyl-1-picryl hydrazyl (DPPH) radicals

Data presented as Mean ± SEM of triplicate evaluation; (n=3)

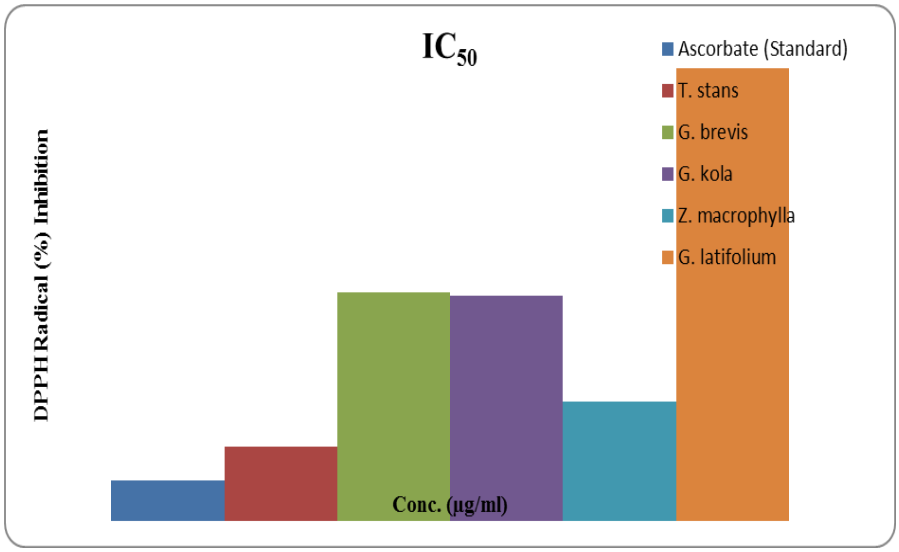


Fig. 2: IC_{50} DPPH Radical (%) inhibition

Effect of extracts on superoxide anion ($O_2^{\cdot-}$) radical

The plant extracts inhibited the formation of reduced Nitro blue tetrazolium (NBT) in a dose-related manner. As presented in figure 3, *Tecoma stans* showed the maximum $O_2^{\cdot-}$ anion inhibitory activity of 49.15±1.21% compared to quercetin (91.33±5.38%) at the concentration of 1000 µg/ml. The $O_2^{\cdot-}$ scavenging effect of the extracts could culminate in the prevention of $^{\cdot}OH$ radical formation since $O_2^{\cdot-}$ and H_2O_2 are required for $^{\cdot}OH$ radical generation. The IC_{50} for $O_2^{\cdot-}$ anion inhibition of *Tecoma stans*, *Glyphaea brevis*, *Garcinia kola*, *Zanthoxylum macrophylla* and *Gongronema latifolium* were 387.25 µg/ml,4226.86 µg/ml, 562.34 µg/ml, 2582.26 µg/ml and 408.10 µg/ml respectively compared to quercetin 42.65 µg/ml.

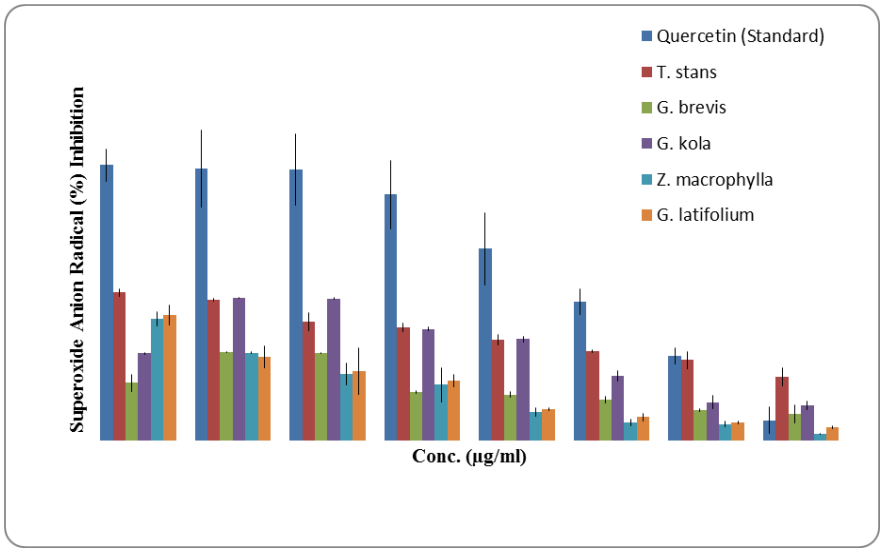


Fig. 3: Effect of extracts on superoxide anion ($O_2^{\cdot-}$) radical

Data presented as Mean ± SEM of triplicate evaluation; (n=3)

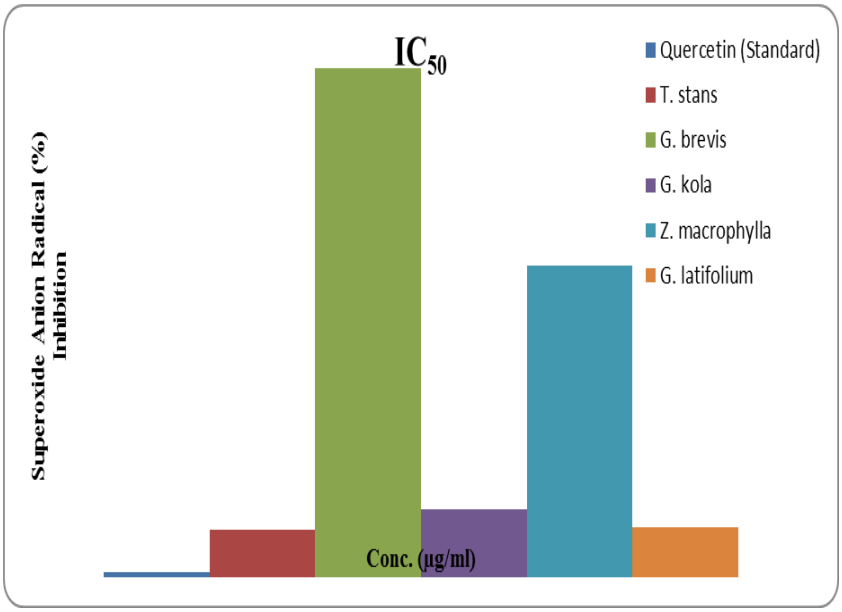


Fig. 4: IC_{50} of superoxide anion ($O_2^{\cdot-}$) radical (%) inhibition

Effect of extracts on nitric oxide ($NO^{\cdot}$) radical production

Nitric oxide ($NO^{\cdot}$) released from sodium nitro-prusside (SNP) has a strong $NO^{\cdot}$ character which can alter the structure and function of many cellular components. From the study, it reveals that the phenol-rich extracts in SNP solution decreased levels of nitrite, a stable oxidant product of $NO^{\cdot}$ liberated from SNP as presented in figure 5. The extracts exhibited strong $NO^{\cdot}$ radical scavenging activity leading to the reduction of the nitrite concentration in the assay medium, a possible protective effect against oxidative damage. The $NO^{\cdot}$ scavenging capacity was concentration dependent with 250 µg/ml of the extracts scavenging most efficiently compared to α-tocopherol.

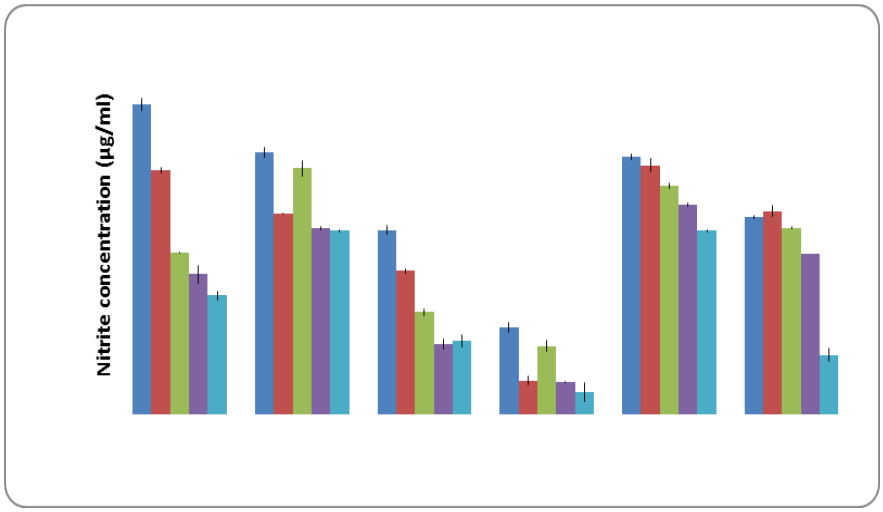


Fig. 5: Effect of extracts on nitric oxide ($NO^{\cdot}$) radical production

Data presented as Mean ± SEM of triplicate evaluation; (n=3)

APPENDIX

Table 1: Effect of extracts on DPPH anion radical

Conc. (µg/ml)	DPPH Radical (%) Inhibition					
	Ascorbate (Standard)	*T. stans*	*G. brevis*	*G. kola*	*Z. macrophylla*	*G. latifolium*
1000	91.33±6.22	66.37±1.75	68.92±3.68	63.16±0.60	65.93±2.63	59.47±0.55
500	90.27±1.81	66.99±2.13	62.15±5.40	63.33±0.12	57.35±6.01	55.13±2.06
250	89.91±3.17	59.03±5.13	53.65±5.89	53.76±0.60	53.19±2.88	51.88±2.00
125	81.52±11.24	52.57±9.51	43.84±15.84	39.74±0.85	45.31±2.00	39.78±2.48
62.5	63.68±0.92	46.19±13.27	33.07±9.70	30.85±11.00	31.19±3.94	30.11±7.03
31.25	46.09±0.64	31.24±5.88	19.27±8.35	20.26±5.68	23.98±0.88	19.86±4.33
15.63	28.23±5.19	15.31±3.13	11.02±1.35	16.67±3.75	15.75±8.01	13.74±2.11
7.82	6.90±3.37	7.70±5.13	11.22±2.31	13.25±6.16	10.83±6.46	8.42±3.19
3.90	7.90±5.23	3.31±2.45	7.03±2.09	12.91±4.23	3.99±1.04	4.05±1.87
IC_{50}	37.06	69.02	210.37	206.53	110.41	415.91

Data presented as Mean ± SEM (n=3).

Table 2: Superoxide anion radical ($O_2^{\cdot-}$) inhibition by extracts

Conc. (µg/ml)	Superoxide Anion Radical (%) Inhibition					
	Quercetin (Standard)	*T. stans*	*G. brevis*	*G. kola*	*Z. macrophylla*	*G. latifolium*
1000	91.33±5.38	49.15±1.21	19.22±2.81	29.06±0.36	40.40±2.33	41.70±3.35
500	90.27±12.70	46.70±0.48	29.41±0.19	47.40±0.09	29.26±0.24	27.77±3.65
250	89.91±11.78	39.47±2.94	29.02±0.16	47.14±0.27	22.33±3.62	23.11±7.80
125	81.52±11.23	37.60±1.27	16.14±0.39	36.99±0.63	18.64±5.62	20.01±2.03
62.5	63.68±12.01	33.52±1.61	15.32±0.97	33.69±0.99	9.60±1.37	10.56±0.43
31.25	46.09±4.19	29.77±0.48	13.75±1.06	21.57±1.62	6.25±1.12	7.95±1.22
15.63	28.23±2.69	26.76±2.81	10.19±0.48	12.82±2.15	5.63±1.04	6.22±0.46
7.82	6.90±4.32	21.19±6.19	8.82±3.00	11.80±1.44	2.33±0.08	4.56±0.41
IC_{50}	42.65	387.25	4226.86	562.34	2582.26	408.10

Data presented as Mean ± SEM (n=3).

Table 3: Effect of extract on the accumulation of nitrite upon decomposition of sodium nitroprusside (SNP); 5 mM at 25°C

Conc. (µg/ml)	Nitrite concentration					
	α-tocopherol (Standard)	*T. stans*	*G. brevis*	*G. kola*	*Z. macrophylla*	*G. latifolium*
250	138.5±2.90	117.2±2.41	82.46±2.04	39.00±2.43	115.09±1.11	88.1±0.74
200	109.21±1.17	89.77±0.18	64.17±0.99	15.22±1.87	111.4±3.00	91.02±2.69
150	72.31±0.44	110.0±3.42	45.79±1.66	30.71±2.41	102.3±1.25	83.42±0.44
100	62.75±4.02	83.11±0.71	31.48±2.43	14.57±0.29	93.79±0.81	71.65±0.07
50	53.23±2.09	82.1±0.48	33.24±2.73	10.11±4.16	82.23±0.42	26.74±3.01

Data presented as Mean ± SEM (n=3).

DISCUSSION

Phenolics are compounds that are well known for their anti-oxidative potentials, hence reported to be useful in managing diseases relating to oxidative stress e.g. diabetes (Awah *et al*., 2012).

The antioxidant activity of anti-diabetic and anti-inflammatory plants was investigated using superoxide anion radical (Robak and Gryglewski, 2013). The extracts inhibited the formation of reduced NBT in a dose-related manner. From figure 3, *Garcinia kola* showed the maximal $O_2^{\cdot-}$ anion inhibitory activity of 47.40±0.09% compared to quercetin (90.27±12.70%) at the concentration of 500µg/ml. The concentration of extract that inhibited 50% of the DPPH and superoxide anion radicals (IC_{50}) was used to determine the potency of the extracts. The lower the IC_{50} value, the better the extracts potency. From the results, the ethanolic plant extracts were seen to be efficient inhibitors of different free radicals compared to standard antioxidant. The IC_{50} values were significantly lower for *T. stans* root bark extract compared to other extracts for both DPPH radical and $O_2^{\cdot-}$ anion inhibition. Superoxide is highly implicated in the pathogenesis of diabetes and is thought to be responsible for the oxidative damage of the pancreatic cells resulting to reduction in insulin production in type 1 diabetes. Excess glucose also leads to generation of free radicals, as such the ability of these plant extracts to scavenge free radicals to have a highly beneficial effect in the management of diabetes.

Nitric oxide (NO^-) released from sodium nitroprusside (SNP) has a strong oxidizing character which can alter the structure and function of many cellular components as reported and reviewed by Beckman and Koppenol, (2016). The extracts exhibited strong NO^- radical scavenging activity leading to the reduction of the nitrite concentration in the assay medium, a possible protective effect against oxidative damage. The NO^- scavenging capability was concentration dependent with 1 mg/mL. The *T. stans* and *Z. marcophylla* scavenged most efficiently compared to other plant extracts. This suggests that the plant has a potent nitric oxide scavenging activities. The ability of the extracts to quench NO^-, could be highly useful in preventing the formation of the very harmful peroxynitrite between superoxide anion and nitric oxide (Pacher *et al*., 2014) and these could be very helpful in protecting pancreatic cell from oxidative damage.

CONCLUSION

These results therefore suggest that the plant root bark extracts posses good antioxidant potentials and therefore could be used to manage disease conditions like pile and diabetes whose progression involves oxidative damage by possibly protecting the β-cells of the Islet of Langerhans from oxidative damage.

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