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Effect of Herbal Combination and Natron Addition on the Antioxidant Properties of *Guiera senegalensis* J. F. Gmel (Combretaceae) Aqueous Leaf Extract

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ABSTRACT

*Guiera senegalensis*is among the medicinal herbs traditionally used for the management of maternal conditions in Katsina State, Nigeria. In the course of its preparation, *G. senegalensis* is, in most cases, mixed with *Ipomoea asarifolia* and *Euphorbia balsamifera*. Small amount of natron is often added in order to enhance the palatability of the herbal preparation. In this study, we investigated the impact of these two phenomena on the antioxidant properties of *G.senegalensis.*Phenolic antioxidant contents were assessed using Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) calorimetric assays. Antioxidant activity on the other hand was evaluated using 2,2'-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity, Ferric Reducing Antioxidant Power (FRAP) and Total Antioxidant Capacity (TAC) assays. Herbal combination and natron addition were found to significantly ($P < 0.05$) affect the antioxidant properties of *G. senegalensis.*High antioxidant properties were observed when *G. senegalensis* was extracted alone compared to when extracted in combination with either *I. asarifolia or E. balsamifera* or both. Although it increased the TPC and TFC values, natron addition was also found to be detrimental to the antioxidant activities of *G. senegalensis.*

Keywords: Antioxidant, *Guiera senegalensis,* herbal combination, natron, maternal healthcare

INTRODUCTION

Oxidative stress has been linked with pathogenesis of adverse pregnancy outcomes including preeclampsia and fetal growth restriction (Mistry and Williams, 2011). Preeclampsia, characterized by hypertension, edema and/or proteinuria is one of the leading causes of maternal mortality and preterm deliveries in the world (Al-jameil *et al*., 2014). About 790 maternal death per 100,000 live births have been attributed to preeclampsia (Wagner, 2004). Reactive oxygen species (ROS) have been identified as major key players in preeclampsia because of their involvement in lipid peroxidation, oxidative damage of biomolecules and cellular dysfunction. They are also believed to initiate maternal vascular endothelial dysfunction (Padmini and Lavanya, 2012). Studies have revealed that reduced antioxidants and increased oxidative stress are associated with adverse birth outcomes in maternal complications such as preeclampsia and preterm deliveries (Kilari *et al*., 2014). Free radicals are generated in the body as a result of normal metabolic processes. However, oxidative stress occurs when there is imbalance between the free radical and built-in antioxidants such as glutathione peroxidase, catalase, superoxide dismutase, uric acid, bilirubin, vitamins A and E, etcetera (Sharma *et al*., 2014).

To overcome oxidative stress, body cells are equipped with special machineries such as Heat Shock Proteins (HSPs) which coordinate mechanisms aimed at protecting cells from stressful environmental, pathological or physiological stimuli. During preeclampsia however, such proteins are compromised, resulting in excessive oxidative stress (Padmini and Lavanya, 2012). Calcium, vitamins C and E are the common supplements employed to increase the level of endogenous antioxidants. However, studies revealed that the use of those supplements do not reduce the incidence of preeclampsia. As such, natural antioxidants capable of protecting cells from several diseases attributed to free radicals are much preferred(Sharma *et al*., 2014). For this reason, antioxidants particularly from herbal origin may be of special benefit for women in developing countries like Nigeria, where there is great burden of morbidity and mortality associated with preeclampsia.

Phenolic compounds including flavonoids possess antioxidant property because of their

ability to scavenge free radicals thereby maintaining balance between oxidants and antioxidants (Sreeramulu *et al*., 2013). Natural antioxidants from herbs continue to receive attention in the scientific community because of safety issues associated with synthetic antioxidants some of which are believed to be carcinogenic (Ajiboye *et al*., 2013).

An ethnobotanical study revealed that women in Katsina State, Nigeria employ the services of medicinal plants for their traditional maternal health care because of their affordability, accessibility and fear of excessive side effects associated with orthodox drugs(Kankara *et al*., 2015). Majority of these herbs are used for general wellbeing during pregnancy which means managing and preventing many ailments associated with oxidative stress. *Guiera senegalensis* appears to be the most widely used herb used for general wellbeing during pregnancy in the study area. However, *Guiera senegalensis*is in most cases prepared in combination with *Ipomoea asarifolia* and *Euphorbia balsamifera*. Although the concept of multiple herbal therapy is gaining popularity because of the belief that poly-herbal combination is more effective due to assumed synergetic effect of various herbs present in the multi-component herbal preparations (Padmanabhan and Jangle, 2012; Wang *et al*., 2014). This belief is however, being contemplated because some individual herbs may have an adverse effect on the overall efficiency of the poly-herbal mixture due to chemical and physical interactions which may result in antagonism (Johnson and Ayoola, 2015).

During the herbal preparation of *Guiera senegalensis*, a small amount of reddish-white natron (trona), locally known as "*jar-kanwa*" is added in the preparation mixture in order to decrease the bitterness and enhance the palatability of the medicinal plants. In spite of the availability of literature supporting the medicinal value of natron (Sodipo, 1993; Ajiboye *et al*., 2013), its efficacy and safety is of great concern because of its high sodium content which is believed to have prooxidant properties (Ford *et al*., 1998; Payne *et al*., 2010). Despite the repo rted cases on the implication of natron consumption on human health (Davidson *et al*., 1974), no report was found on its impact on the antioxidant properties of medicinal plants.

Taking these into consideration, this study was conducted to evaluate the impact of herbal combination and natron addition on the antioxidant properties of the medicinally valuable herb, *G. senegalensis.*

MATERIALS AND METHODS

Materials

All chemical reagents used in this study are were of analytical grades. Further dilutions were conducted where necessary.

Samples Collection, Identification and Preparation

Gueira senegalensis leaves, *Euphorbia balsaminifera* twigs and *Ipomoea asarifolia* leaves were collected from Umaru Musa Yar'adua University, Katsina campus. All the species were identified and authenticated by Professor Munier Abdel-Ghani of Biology Department, Umaru Musa Yar'adua University, Katsina. Voucher specimens were prepared and deposited at the herbarium of the Biology Department.The identified samples were air dried in the laboratory and the dried samples were ground to powder using a mill (Retsch, SM100 comfort Hann, Germany). The powder obtained from the different samples were individually packaged and stored in the dark, at an ambient temperature.

Experimental Design

Single factor experiments were used in this study. The effect of herbal combination on the antioxidant properties was investigated using Single Herbal formulation (SHf), Double Herbal formulation (DHf) and Triple Herbal formulation (THf) as follows:

Table 1: Herbal Combination

S/No	Formulation	Combination
1	SHf-Gs	*Guiera senegalensis* alone
2	SHf-Ia	*Ipomoea asarifolia* alone
3	SHf-Eb	*Euphorbia balsamifera* alone
4	DHf-Gs+Ia	*G. senegalensis* + *I. asarifolia* (1:1 w/w)
5	DHf-Gs+Eb	*G. senegalensis* + *E. balsamifera* (1:1 w/w)
6	DHf-Ia+Eb	*I. asarifolia* + *E. balsamifera* (1:1 w/w)
7	THf-Gs+Ia+Eb	*G. senegalensis* + *I. asarifolia* + *E. balsamifera* (1:1:1 w/w)

For the evaluation of the effect of natron addition, the extraction material was serially supplemented with different concentrations (0, 2.5, 5, 10 and 20mg/ml) of aqueous natron solution.

Dried powder (60mg) of each sample and of different combinations at equal proportions were put in 1000 cm^3 conical flask, in each case, covered with paraffin. These were then extracted with

deionized water (600 cm^3) in a temperature-controlled water bath shaker (WNB 7- 45, Memmert, Germany) at a constant speed (60 shake/min) and temperature (40°C) for 1 hour. The crude extracts were then filtered through Whatmann No. 1 filter paper (Whatmann International Ltd, England). Filtrates were collected and lyophilized using a freeze dryer. The lyophilized extracts were used for the estimation of phenolic antioxidants and the evaluation of antioxidant activities of each sample using various biochemical assays.

Determination of Total Phenolic Content (TPC)

Total Phenolic Content (TPC) was determined using Folin-Ciocalteu's (FC) method as reported by Thoo *et al*. (2010), with slight modifications. 500µL of dilute crude extract was mixed with 500µL of 10-fold diluted Folin-Ciocalteu reagent. After 3 minutes, 400µL of sodium carbonate anhydrous was added and vortexed. After 2 hours of incubation in dark, at room temperature. Absorbance was measured at 765nm against a blank (prepared by replacing plant extract with deionized water) using a UV/VIS spectrophotometer (Lambda 25, ParkinElmer, Singapore). Measurements were calibrated to a standard curve of prepared gallic acid solution (10 – 100µg/ml) with equation y = 0.01x – 0.009 (R^2 = 0.999). The TPC was then expressed as milligram of gallic acid equivalent (GAE) per 100g of dry weight (DW).

Determination of Total Flavonoid Content (TFC)

Total Flavonoid Content (TFC) was determined using the aluminium chloride calorimetric assay method, as reported by Kaur and Mondal (2014). 125µL of each crude extract was mixed with 625µL deionized water and 37.5µL of 5% sodium nitrite. Each mixture was allowed to stand for 6 minutes before 75µL of 10% aluminium chloride-6-hydrate was added thereafter. After 5 minutes, 250µL sodium hydroxide solution was added. 137.5µL deionized water was then added and mixed. Absorbance was measured on each sample immediately at 510nm against a blank (prepared by replacing plant extract with deionized water). Measurements were calibrated to a standard curve of prepared quercetin solution (0 – 800µg/ml) with equation y = 0.0000x + 0.003 (R^2 = 0.981). The TFC was then expressed as milligram quercetin equivalent (QE) per 1g dry weight (DW).

Determination of 2,2'- diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant capacity through DPPH scavenging activity was determined according to the protocol reported by Tan *et al*. (2013). Methanolic DPPH stock solution was prepared by dissolving 4mg of 2,2'- diphenyl-1- picrylhydrazyl (DPPH) powder into 100ml absolute methanol. The working solution was obtained by mixing 50ml stock solution with 20ml methanol in order to obtain an absorbance of 1.00±0.02 unit at the 517nm wavelength. 100µL of the various concentrations of the plant extract was mixed with 900µL methanolic DPPH solution before being allowed to stand in the dark at room temperature for 30 minutes. After 30 minutes incubation, absorbance was measured at 517nm using a UV/VIS spectrophotometer. Percentage DPPH scavenging activity was determined using Equation 1.

$$\text{DPPH scavenging activity (\%)} = [(A_0 - A_{30})/A_0] * 100 \quad (1)$$

Where A_0 = Absorbance at time 0; A_{30} = Absorbance after 30 minutes.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was carried out using the protocols reported by Benzie and Strain (1996). The stock solution included 300mM acetate buffer (3.1g $C_2H_3NaO_2$.$3H_2O$ and 16mL $C_2H_4O_2$), pH 3.6, 10mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40mM HCl and 20mM $FeCl_3$. $6H_2O$ solution. Fresh FRAP working solution was prepared by mixing acetate buffer, TPTZ solution and $FeCl_3$. $6H_2O$ solutions in the ratio of 10:1:1, respectively. The FRAP solution was warmed at 37°C for 30 minutes in water bath (GFL 1004 Burgwedel, Germany) before it was used. 100µL of aqueous plant extract was allowed to react with 1000µL of the FRAP solution in the dark for 30 minutes. Absorbance of the colored product (ferrous tripyridyltriazine complex) was measured at 593nm wavelength using the UV/VIS spectrophotometer against a blank (prepared by replacing plant extract with deionized water). Measurements were calibrated using a linear standard curve of prepared ascorbic acid solution (5 – 35mg/ml) with equation y = 0,045x + 0.395 (R^2 = 0.996). The results were expressed as milligram ascorbic acid equivalent (AAE) per 100g dry weight (DW). Additional dilution was needed when the FRAP value measured was over the linear range of the standard curve.

Total Antioxidant Capacity (TAC)

Total antioxidant capacity was determined using phosphomolybdate assay procedure, asreported by Mohammed *et al*. (2014). The working solutions consist of 600mM sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. 1000µL of the working

solution was mixed with 100µL aqueous plant extract (1mg/ml) and incubated in a water bath (GFL 1004 Burgwedel, Germany) at 95°C for 90 minutes. The mixture was allowed to cool to room temperature before absorbance was measured at 695nm using the UV/VIS spectrophotometer against blank (prepared by replacing the plant extract with deionized water). Measurements were calibrated to a linear standard curve of prepared ascorbic acid solution (100 – 700µg/ml) with equation y = 0.0016 + 0.0222 (R^2 = 0.999) and results were expressed as milligram ascorbic acid equivalent (AAE) per 1g dry weight (DW). Additional dilution was needed when the TAC value measured was over the linear range of the standard curve.

Statistical Analyses

Results were analyzed using SPSS software (version 20) and expressed as mean ± standard deviation of 3 replicate. One- way analysis of variance (ANOVA) with Duncan's test was carried out to test statistical significance of the various levels of treatments. Significant levels were defined where P < 0.05. Pearson correlation between variables was also established using the SPSS software (version 20).

Results and Discussion

The Effect of herbal combination on the phenolic antioxidants (TPC and TFC) and the antioxidant properties (DPPH, FRAP and TAC) is presented in Figure 1. The results demonstrate that both herbs showed considerable antioxidant properties individually. Their combination however, significantly (P<0.05) affects all the parameters checked. In all the assays, highest effects were observed when *G. senegalensis* was extracted singly. It is also important to note that interaction of *G. senegalensis* with either *I. asarifolia* or *E. balsamifera* had adversely affected the performance of the former in all the assays. More so, combination of all the three species did not change the scenario, as the values obtained are significantly lower that recorded when *G. senegalensis* was treated singly. These findings suggest that both *I. asarifolia* and *E. balsamifera* have antagonistic effect on *G. senegalensis.* Phenolic and flavonoid compounds have been linked with the antioxidant properties of fruits, vegetables and herbs (Yang *et al*., 2009). Change in the antioxidant properties observed in this study may therefore be attributed to the alteration of the structure and consequently the release of phenolic and flavonoid compounds present in *G. senegalensis.* This is further supported by the significant correlation observed between phenolic compounds TPC and TFC) and antioxidant properties (Table 2). Adverse interaction effects of medicinal plants are also attributed to the presence of tannins in herbal preparations, which can impede the presence of alkaloids and proteins and some enzymes such as cytochrome P450 (Williamson, 2001). It was also reported that antioxidant activity of phenolic compounds is affected by the number and positions of phenolic hydroxyls (Chaillou and Nazareno, 2006). Results obtained in this study are contrary to the popular belief that phytochemicals interact synergistically to neutralize the effect of free radicals (Williamson, 2001;Jacobo-Velázquez and Cisneros-Zevallos, 2009). Some previous studies also reported the antagonistic behavior of some medicinal plants. Johnson and Ayoola (2015) for example reported that a combination of *Kigelia africana* and *Alafia bateri* resulted in antagonism in their antioxidant property. In another study involving *Acanthus montanus*, *Emilia coccinera*, *Hibiscus rosasinensis* and *Asystasiagangetica*, it was found that single herbal formulation yielded radical scavenging ability better than some combined herbal formulations (Ojiako *et al*., 2015). In the contrary, combination of *Aloe vera*, *Bacopamonniera*, *Moringa oleifera* and *Zingiberofficinale* yielded more antioxidant activities than the individual species which is attributed to the additive and synergetic actions of the phytochemicals present in the extract (Padmanabhan and Jangle, 2012). In another study, it was reported that a combination of herbs showed higher antioxidant capacity than the individual eight Traditional Chinese herbs (Yang *et al*., 2009). Relating this to the previous studies, it is clear that herbal combination does not always enhance antioxidant property.

The addition of natron into the herbal combination significantly affects the antioxidant properties of *G. senegalensis* (Figure 2). While extraction of phenolic antioxidants (TPC and TFC) was enhanced following the addition of the natron, especially at 5.0mg/ml loading, both antioxidant activities (DPPH, FRAP and TAC) were however, adversely affected. Being alkaline in nature, sodium containing compounds increase the pH of the solutions they are found in. Alteration of antioxidant properties observed in this study, could therefore, be as a result of change in pH brought by the sodium containing natron. It was earlier reported that polyphenol compound are degraded following alkaline treatment (Cheng *et al*., 2009). Elevated TPC and TFC values observed following natron treatment could therefore, be explained by the fact that trace amounts of metals that might be present in the natron could form complexes with the substances in the mixture, thereby increasing the absorbance values obtained for TPC and TFC(Psotová *et al*., 2003).Transition metals are also believed to play a potential role as catalysts of oxidative processes by enhancing the formation of hydroxyl radicals and hydroperoxide decomposition during the Fenton reaction (Halliwell *et al*., 1997). Miller *et al*. (2008) also report that an alkaline treatment can compromise the content phenolic compounds and antioxidant properties of cocoa (*Theobroma cacao*). Alkaline

pH was also found to have adverse effect on the antioxidant properties of both *Rosemanirus officinalis*, *Thymus vulgaris* and *Origanum majorana*(Gawlik-dziki and Świeca, 2007). In an *in vivo* study, natron was found to suppress the built-in antioxidant systems in male rats (Ajiboye *et al*., 2013). In another study, it was also found to impair the lipid profile and heart tissues in female albino rats (Muhammad *et al*., 2014). Beside promoting oxidative stress, natron was also found to have some medical and nutritional implications (Ford *et al*., 1998; Latunde-dada, 2015).

Results on the Pearson correlation analysis are presented in Table 1. In the table, it can be seen that significant linear relation exists between the phenolic antioxidants (TPC and TFC) and their antioxidant activities (DPPH, FRAP and TAC) under the influence of the herbal combination(s). Significant linear relations also exist between TPC and FRAP (R^2 = 0.701), TFC and FRAP (R^2 = 0.774) as well as between TFC and FRAP (R^2 = 0.563). It is also interesting to note that significant negative correlations were observed between TFC and DPPH (R^2 = -0.577), between TFC and FRAP (R^2 = -0.652) and also between TFC and TAC ((R^2 = -0.782) under the influence of the natron addition. This has partly substantiated the assumption that the elevated values of TPC and TFC observed may not necessary be the actual TPC and TFC values but rather the absorbance of complexes formed as a result of the natron addition. The correlations in the results indicate that the phenolic antioxidant play a significant role in the antioxidant properties of *G. senegalensis*. Previous studies also reported significant relations between phenolic compound and antioxidant activities of many medicinal plants. Ibrahim *et al*. (2013), for example, reported strong correlation (R^2 = 0.929) between phenolic compounds and their antioxidant activity in *Streblusasper*. TPC also showed significant positive relations with DPPH and FRAP (R^2 = 0.996 and 0.985, respectively) in another study involving *Boerhaviadiffusa*(Avasthi *et al*., 2014). Significant positive relations were also observed between some phenolic compounds and the antioxidant activities of some medicinal rhizomes (Yan and Asmah, 2010). Thoo *et al*. (2010) however, reported weak correlations between selected phenolic compounds and their antioxidant properties in*Morinda citrifolia*.

CONCLUSIONS

This study revealed that both *Ipomoea asarifolia* and *Euphorbia balsamifera*canhave detrimental effects on theantioxidant properties of *Guiera senegalensis.* Taking into consideration, stated reports on the toxicity of *I. asarifolia* and *E. balsamifera* and the findings of this study, it brings into light the health concern that can be with preparing herbal remedies that can contain both *G. senegalensis*and natron.

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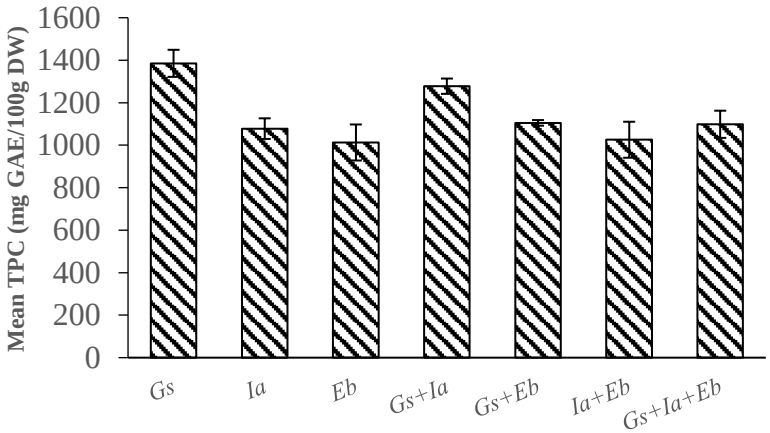
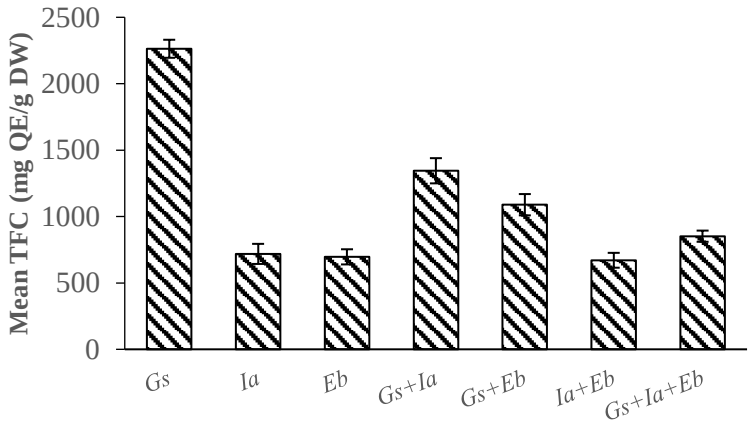
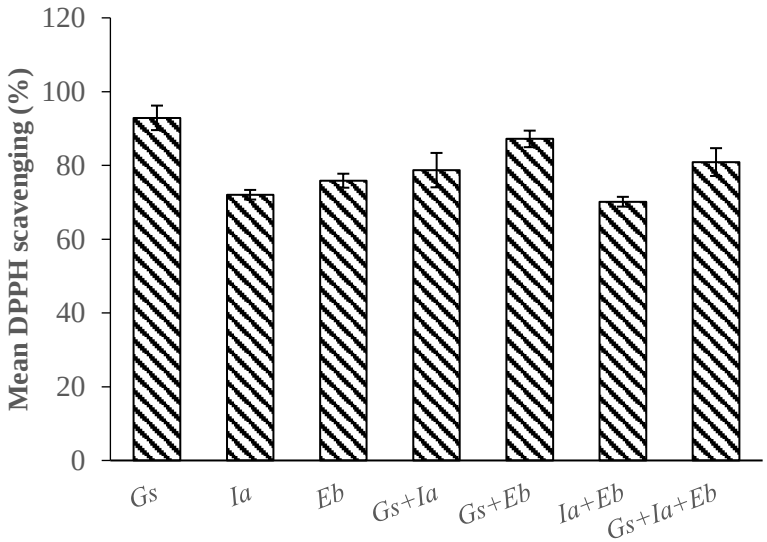
Table 2: Correlation Analysis Between Assays as Affected by Different Extraction Conditions

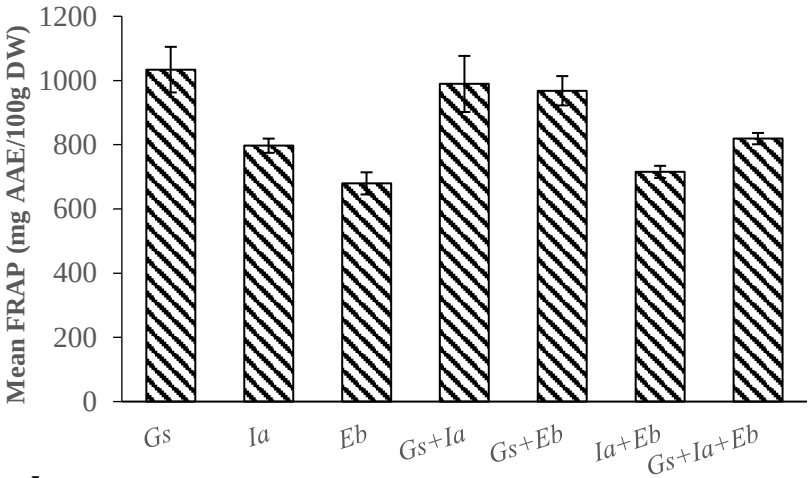
	Herbal combination				Natron additon			
	TFC	DPPH	FRAP	TAC	TFC	DPPH	FRAP	TAC
TPC	0.862**	0.360	0.701*	0.412	0.296	0.584*	0.448	-0.021
TFC		0.426	0.774**	0.563**		-0.577*	-0.652**	-0.782**
DPPH			0.398	0.013			0.951**	0.643**
FRAP				0.610**				0.736**


Mean TPC (mg GAE/100g DW)
1600
1400
1200
1000
800
600
400
200
0
Gs
Ia
Eb
Gs+Ia
Gs+Eb
Ia+Eb
Gs+Ia+Eb

a


Mean TFC (mg QE/g DW)
2500
2000
1500
1000
500
0
Gs
Ia
Eb
Gs+Ia
Gs+Eb
Ia+Eb
Gs+Ia+Eb


Mean DPPH scavenging (%)
120
100
80
60
40
20
0
Gs
Ia
Eb
Gs+Ia
Gs+Eb
Ia+Eb
Gs+Ia+Eb



d

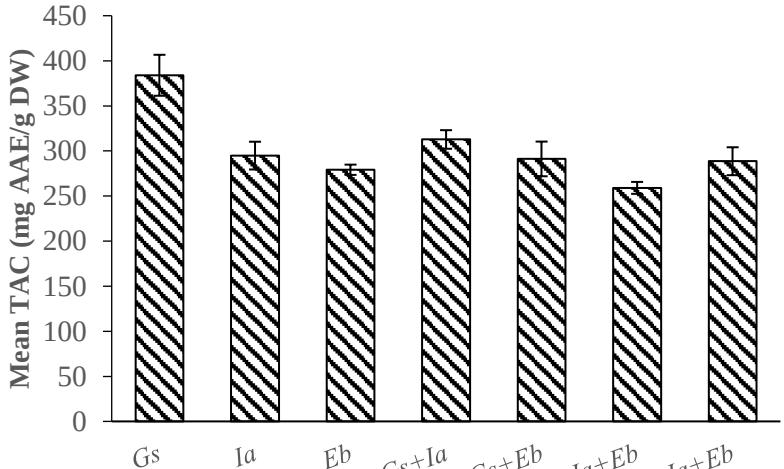


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Figure 1: Effect of herbal combination on (a) Total Phenolic Content, (b) Total Flavonoid Content, (c) DPPH radical scavenging, (d) Ferric reducing Antioxidant Power and (e) Total Antioxidant Capacity of *Guiera senegalnsis*leaves water extract.

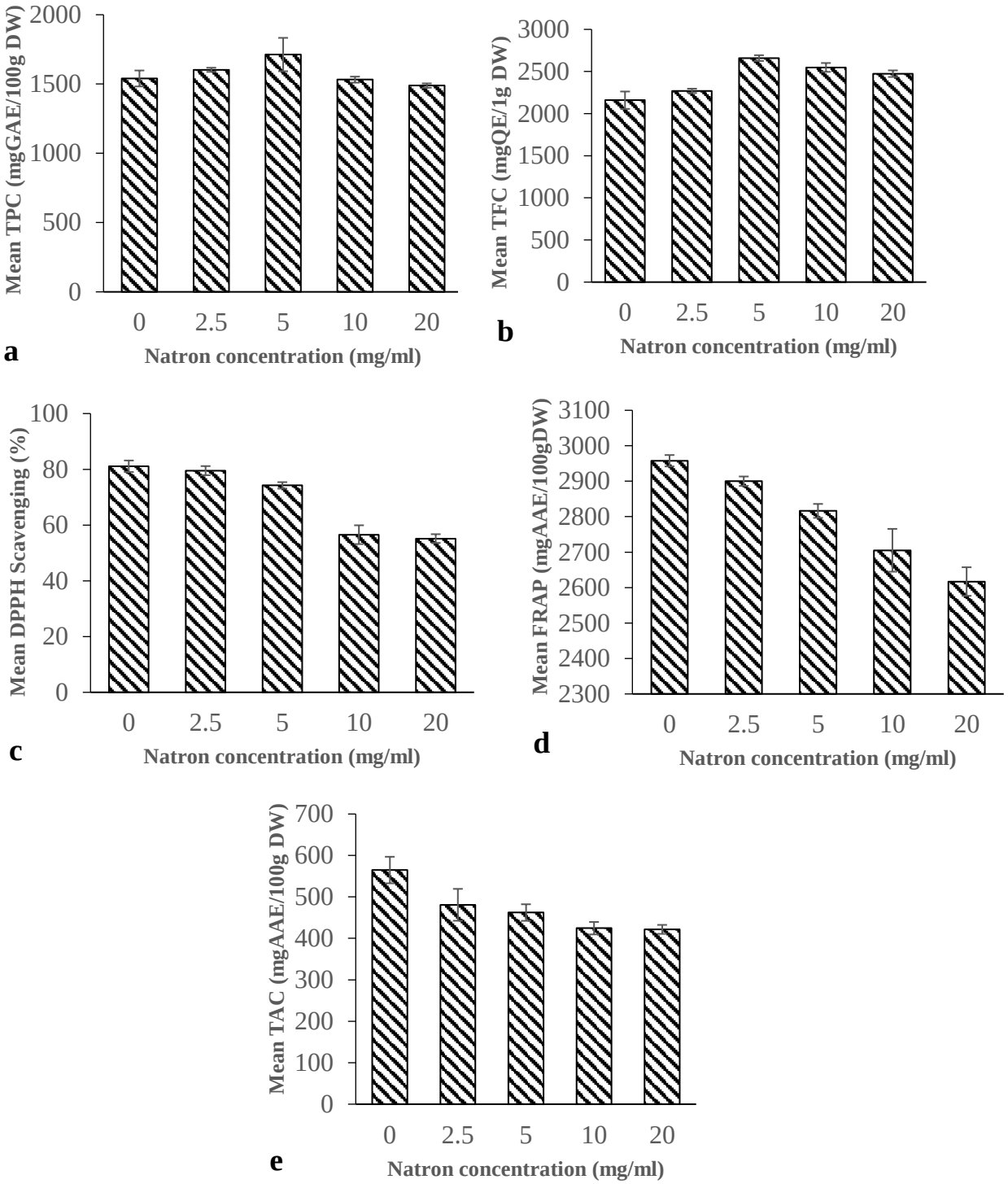

Figure 2: Effect ofnatron addition on (a) Total Phenolic Content, (b) Total Flavonoid Content, (c) DPPH radical scavenging, (d) Ferric reducing Antioxidant Power and (e) Total Antioxidant Capacity of *Guiera senegalnsis*leaves water extract.

TPC: total phenolic content; TFC: total flavonoid content; DPPH: DPPH-radical scavenging activity; FRAP: ferric reducing antioxidant power; TAC: total antioxidant capacity.

*Significant level at P< 0.05

** Highly significant level at P< 0.01

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