Antioxidant and *in vitro* membrane stabilization activities of leaf extract of *Gossypium barbadense* L. (Malvaceae)

Chibuamam E. Ilechukwu[1], Mubo A. Sonibare[1,], Oluwafemi G. Oluwole[2], Abayomi M. Ajayi[2], Solomon Umukoro[2]
[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria
[2]Department of Pharmacology and Therapeutics, Faculty of Basic Medical Sciences, University of Ibadan, Ibadan, Nigeria

Corresponding author: Mubo A. Sonibare,
E-mail: sonibaredeola@yahoo.com; Phone: +2348134901273

ABSTRACT

Background: *Gossypium barbadense* L. (GB) is used in the treatment of various diseases such as convulsions, jaundice, gastrointestinal disorders, and rheumatism in African traditional medicine.

Objectives: This study aimed at evaluating the antioxidant and *in vitro* membrane stabilization activities of leaf extracts of GB.

Methods: *Gossypium barbadense n*-hexane (GBHE), dichloromethane (GBDC), ethyl acetate (GBET) and methanol (GBME) extracts were assayed for phytochemical constituents, DPPH free radical scavenging activity, cytotoxicity using brine shrimp lethality assay as well as *In vitro* red blood cell membrane stabilization assay (MSA)

Results: Phytochemical screening of GB revealed the presence of cardiac glycosides, tannins, flavonoids, and steroids. The GBME had the highest phenolic (196.8 ± 3.72 mgGAE/g sample), flavonoid content (75.1 ± 1.97 mgRE/g sample) and DPPH scavenging activity (IC_{50} of 3.56±0.82 µg/mL). Also, GBHE and GBME showed the highest and lowest cytotoxicity, respectively. The GBET demonstrated significant MSA of 83.71% at 1mg/mL, while Indomethacin gave 68.30% protection.

Conclusion: The high contents of flavonoids and phenolics in *Gossypium barbadense* could be responsible for its antioxidant property and red blood cell membrane stabilization activity.

Keywords: Antioxidant, anti-inflammatory, *Gossypium barbadense*, membrane stabilization, phenolic content

Activités antioxydantes et *in vitro* de stabilisation membranaire de l'extrait des feuilles de *Gossypium barbadense L.* (*Malvaceae*)

Chibuamam E. Ilechukwu[1], Mubo A. Sonibare[1,], Oluwafemi G. Oluwole[2], Abayomi M. Ajayi[2], Solomon Umukoro[2]
[1]Département de Pharmacognosie, Faculté de Pharmacie, Université d'Ibadan, Ibadan, Nigéria
[2]Département de pharmacologie et de thérapeutique, Faculté des sciences médicales de base, Université d'Ibadan, Ibadan, Nigéria

Auteur de correspondance : Mubo A. Sonibare,
Email: sonibaredeola@yahoo.com; Téléphone: +2348134901273

RESUME

Contexte : Le *Gossypium barbadense* L. (GB) est utilisé dans le traitement de diverses maladies telles que les convulsions, la jaunisse, les troubles gastro-intestinaux et les rhumatismes dans la médecine traditionnelle africaine.

Objectifs : Cette étude visait à évaluer les activités antioxydantes et *in vitro* de stabilisation membranaire des extraits de feuilles de GB.

Méthodes : Les extraits de *Gossypium barbadense n*-hexane (GBHE), de dichlorométhane (GBDC), d'acétate d'éthyle (GBET) et de méthanol (GBME) ont été testés pour leurs composants phytochimiques, leur activité de piégeage des radicaux libres, leur cytotoxicité, à l'aide d'un essai de crevette de saumure de même qu'un test *In vitro* d'un essai de stabilisation de la membrane des globules rouges (MSA)

Résultats : Le test phyto-chimique de GB a révélé la présence de glycosides cardiaques, de tanins, de flavonoïdes et de stéroïdes. Le GBME avait le plus haut indice phénolique (échantillon 196,8 ± 3,72 mgGAE/g), la teneur en flavonoïdes (échantillon 75,1 ± 1,97 mgRE/g) et l'activité piégeant la DPPH (IC_{50} de 3,56±0,82 µg/mL). En outre, GBHE et GBME ont montré la cytotoxicité la plus élevée et la plus faible, respectivement. Le GBET a démontré une MSA significative de 83,71% à 1 mg/mL, tandis que l'indométacine a donné une protection de 68,30%.

Conclusion : Les teneurs élevées en flavonoïdes et en composés phénoliques dans le *Gossypium barbadense* pourraient être responsables de sa propriété anti-oxydante et de l'activité de stabilisation de la membrane des globules rouges.

Mots-clés : Antioxydant, anti-inflammatoire, *Gossypium barbadense*, stabilisation membranaire, contenu phénolique

INTRODUCTION

Gossypium barbadense L. (Malvaceae) is a perennial shrub that grows to about 1 - 3 m tall.[1] The plant is mainly grown for its cotton lint, which is used in textile and clothing, and it represents approximately 5% of the world fiber production.[2] The plant is native to South America and is now distributed from Senegal to Nigeria and widely cultivated in the tropics.[1] It is employed in traditional African folk medicine for the treatment and management of various forms of ailments including conjunctivitis, convulsions, jaundice, gastrointestinal 00disorders, rheumatism, wounds and sexually transmitted infections such as gonorrhea.[3] Studies on the biological activities of *G. barbadense* revealed that the plant possessed antimalarial [4], antibacterial [5] and blood-pressure-lowering effects.[6]

It has antifungal properties, probably due to its gossypol content, which apparently makes it less susceptible to insect damage.[7] Also, its use as a male anti-fertility drug has been reported.[8] The pulped young shoots are used against palpitations, and the fiber in dressings on wounds.[9]

In view of the several medicinal application of the plant, the present study evaluated the antioxidant and *in vitro* anti-inflammatory activity of *G. barbadense* leaf extract. The brine shrimp lethality assay and red blood cell membrane stabilizing activity were employed for *in vitro* cytotoxicity and anti-inflammatory activities.

MATERIALS AND METHODS

Reagents and chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, indomethacin and Folin-Ciocalteau Reagent (Sigma Aldrich, Germany). Other chemicals used were of analytical grade.

Plant materials

The leaves of *G barbadense* were harvested from cultivated plants in Alomaja town in Ibadan East Local Government Area, Ibadan, Nigeria in May, 2015. The plant was identified and authenticated by Mr. Patrick Agwu in the Department of Pharmacognosy Herbarium, University of Ibadan (DPHUI), with voucher specimen deposited under the reference number: DPHUI 1627.

Preparation of extracts

The leaves were cleaned and shade-dried for three weeks and ground to coarse powder using a mechanical mill. The powdered *G. barbadense* (0.8 kg) was successively extracted at room temperature (28-32°C) by cold maceration, using solvents with increasing polarity (*n*-hexane, dichloromethane, ethyl acetate and methanol. The extracts were filtered using a Buchner funnel and concentrated *in vacuo* (Buchi 23112R000 Rotary evaporator, India). The dried extracts were weighed and used for the calculation of the extraction yield and then stored under refrigeration (4°C) until used for analysis.

Phytochemical screening

Powder and extracts (GBHE, GBDC, GBET and GBME) of *G. barbadense* were screened for the presence of secondary metabolites using standard methods reported in literature[10,11]. Alkaloids were screened for using Mayer's and Dragendorff reagents, saponins with froth and emulsification tests, flavonoids with the Schinoda Test, cardiac glycosides with Keller-Kiliani and Kedde tests, tannins with ferric chloride reagent and anthraquinones with ammonia test.

Determination of total phenolic content

Folin Ciocalteu reagent was used for analysis of total phenolics content (TPC).[12] The phenolic concentration of the extracts was evaluated from a gallic acid calibration curve within range of 15.63-500 µg/mL (R^2 = 0.9934). The extract (0.2 mL) in methanol (1.0 mg/mL) was mixed with 0.4 mL of Folin-Ciocalteu reagent. The solution was allowed to stand at 25°C for 5-8 min before adding 4.0 mL of 7.0% Sodium carbonate solution and made to 10 .0 mL with distilled water. The mixture was allowed to stand for 120 min before its absorbance was measured at 725 nm (INESA 752N). Total phenolic content was expressed as mg gallic acid equivalents per gram (mgGAE/g) of sample.

Determination of total flavonoid content

Aluminum chloride colorimetric method was used for total flavonoid determination.[13] The extracts of each plant material were prepared in the concentration of 1mg/mL in 50% methanol. Initially, 10% $NaNO_2$ solution (0.3 mL) was added to each test tube; at 5 min, 10% $AlCl_3$ (0.3 mL) was added; and at 6 min, 1M NaOH (2 mL) was added. After allowing the reaction mixture to stand for 30 min at room temperature, the absorbance of the reaction mixture was read at 510 nm (INESA 752N). The calibration curve was constructed by preparing Rutin solutions at concentrations (31.3 to 1000 µg/ mL) in methanol. All determinations were performed in triplicates and total flavonoid content (TFC) expressed as milligrams of Rutin equivalent (RE) per gram of extract (mg RE/ g sample).

DPPH free radical scavenging activity

The DPPH free radical scavenging activity was assessed according to previously described methods.[14,15] Ascorbic acid was used as control. The extracts and control (2 mL) at various concentrations (3.125, 6.25, 12.5, 25, 50, 100, µg/mL) were separately added to 2 mL of freshly prepared DPPH solution (0.1 mM) in methanol. The mixture was incubated in the dark for 30 min at room temperature and the scavenging effect on the DPPH radical was read using a spectrophotometer (INESA 752N) at 517 nm. All determinations were performed in triplicates.

The percentage inhibition of DPPH free radical scavenging activity was calculated using the following equation:

$$\% \text{ inhibition} = \left[\frac{(\text{Absorbance of blank} - \text{Absorbance of test sample})}{\text{Absorbance of blank}}\right] \times 100$$

The antioxidant activity of each sample was expressed in terms of IC_{50} (micromolar concentration required to inhibit DPPH radical formation by 50%), which was calculated from the linear regression curve. Ascorbic acid was used as positive control measured in terms of hydrogen donating or radical scavenging ability using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH)

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Brine shrimp lethality

The extracts were routinely evaluated in a test for lethality to brine shrimp larvae. Brine shrimp eggs (*Artemia salina*) were hatched in natural seawater obtained from the Bar Beach, Ikoyi, Lagos, Nigeria, and incubated for 48 h in seawater. After hatching, 10 active shrimps (the *nauplii*) were collected and treated with selected concentrations (five dilutions, 1 - 1000 µg/mL) of the plant extracts. The 50% lethal concentration (LC_{50} value) at 95% confidence interval was calculated for each of the plant extracts, using a non-linear regression curve. This was based on the modified method of Ramachandran et al.[16]

Preparation of red blood cell

Blood was collected following the cardiac puncture of a healthy male Wistar rat (150 g), that had been anaesthetized using pentobarbital (35 mg/kg bw). The blood was collected into equal volume of Alsevier's solution (Dextrose 2%, Sodium citrate 0.8%, citric acid 0.05%, Sodium chloride 0.42% and distilled water 100 mL) and centrifuged with isotonic solution at 3000 rpm for 5 min. The clear plasma (supernatant) and the white buffy layer (consisting of the white blood corpuscles) were removed by means of a micropipette, and the packed cells re-washed with isotonic solution. A 10% red blood cell (RBC) suspension was then prepared from it, and was refrigerated till it was ready for use.

Membrane stabilizing activity

The membrane stabilizing activity assay was carried out according to the modified method of Sadique et al.[17] using 10% (v/v) erythrocyte suspension, while Indomethacin (1 and 0.25 mg/mL) was used as standard drug. Various concentrations (1, 0.5, 0.25 and 0.125 mg/mL) of the extracts (0.5mL) was prepared. Tween 80 was used to solubilize the water insoluble extracts. To each concentration, 2 mL hypotonic solution, 2.2 mL isotonic solution and 0.3 mL of 10% RBC suspension were added. Drugs were omitted in the blood control, while the drug control did not contain the erythrocyte suspension. The 5 mL reaction mixture was then incubated at ± 56°C on a water bath for 30 min and subsequently cooled with cold water before centrifuging the tubes at 4000 rpm for 5 min at room temperature. The hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm.

The percentage of RBC membrane stabilization or protection was calculated by using the following formula:

$$\% \text{ Membrane stabilization} = \left[\frac{(\text{Mean control} - \text{Mean treatment})}{\text{Mean control}}\right] \times 100$$

All determinations were performed in triplicates, and the mean values determined.

Data analysis

The tests were performed in triplicate and results are representatives of the mean ± standard errors of the mean. All of these results have been submitted to the statistical analysis of variance (ANOVA) at 0.05% probability level.

RESULTS

Phytochemical screening result

Phytochemical screening results indicate varying distribution of alkaloids, tannins, flavonoids, flavones, cardiac glycosides, steroids and saponins in the leaves and the extracts, whereas anthraquinones were absent (Table 1).

Total phenolic content, total flavonoid content and DPPH radical scavenging activity

The TFC ranged from 9.4 ± 3.10 to 75.1 ± 1.97 mg RE/g in

G. barbadense extracts, while the TPC ranged from 32.9 ± 1.27 to 196.8 ± 3.72 mgGAE/g (Table 2). The maximum flavonoid / phenolic ratio was found in GBME (0.38). The DPPH (2,2-diphenyl-1-picrylhydrazyl radicals) scavenging activity of *Gossypium barbadense* extracts is shown in Figures 1 and 2. All the extracts showed the capacity to scavenge the DPPH radicals. *Gossypium barbadense* methanol (GBME) and *n*-hexane (GBHE) extracts gave the highest and least free radical scavenging activity (FRSA) of 95.29% and 62.81%, respectively at the highest concentration of 50 µg/mL, when compared with the standards (Ascorbic acid: 99.6% and Gallic acid: 94.5% at the same concentration). These results were corroborated with their IC_{50} values (the concentration of the extracts required for 50% scavenging of the DPPH radicals). Hence, GBME showed the highest antioxidant activity with the least IC_{50} of 3.56 ± 0.82 µg/mL, whereas GBHE with the least activity had the IC_{50} of 27.35 ± 0.09 µg/mL[24]. Interestingly, both GBET and GBME showed even greater antioxidant activity than the standards (Ascorbic acid, IC_{50} of 6.90 ± 0.18 µg/mL ; Gallic acid, IC_{50} of 8.60 ± 0.65 µg/mL), with their lower IC_{50} values (GBET, IC_{50} of 3.85 ± 0.53 µg/mL; GBME, IC_{50} of 3.56 ± 0.82 µg/mL).

Brine shrimp lethality activity

Table 3 shows the *in vitro* cytotoxicity of *G. barbadense* leaf extracts in the BSL assay, expressed as LC_{50} values, alongside the confidence limits at 95% interval. The cytotoxicity values ranged from 681.12 to 1863.57 µg/mL.

Membrane stability activity

The percentage membrane stabilizing profiles of the various extracts *G. barbadense* on red blood cell exposed to both heat and hypotonic induced lyses are shown in Table 4. The GBHE exhibited a minimum membrane stability of 15.68% whereas GBET exhibited maximum activity of 83.71%, at the lowest and highest concentrations of 0.25 and 1 mg/mL, respectively. The standard anti-inflammatory drug, Indomethacin exerted maximum membrane stability of 68.30% at 1mg/mL.

Table 1: Phytochemical screening of *Gossypium barbadense* extracts

Tests	Observation	Powdered drug	GBHE	GBDC	GBET	GBME
Ikaloids						
Mayer's	Milky precipitate	+	-	-	+	-
Dragendorff's	Reddish brown precipitate	-	+	+	++	+
Anthraquinones:						
free	No colour change	-	-	-	-	-
combined		-	-	-	-	-
Tannins	Greenish black colour	+++	+	-	+	+
Flavonoids	Reddish colour	+++	+	+	+	++
Flavones	Orange colour	+	-	-	+	++
Cardiac glycosides: Kedde(unsaturated lactones)	Brown purple colour	++	-	-	+	++
Keller-kiliani (2-deoxy sugar)	Reddish brown ring with a green lower acetic layer	+	-	++	+	+++
Steroids (Liebermann Burchard's Reaction)	A reddish brown ring	-	-	-	-	++
Saponins:						
Frothing	Stable froth formation	+	-	-	-	++
Emulsion	Emulsion formation with olive oil	++	-	-	-	++

GBHE: G. barbadense n-hexane extract, GBDC: *G. barbadense* dichloromethane extract, GBET: *G. barbadense* ethyl acetate extract, GBME: *G. barbadense* methanol extract

Table 2: Total phenolic contents and total flavonoid contents of G. barbadense extracts

Sample	TFC (mg RE/g sample)	TPC (mgGAE/g sample)	Flavonoids/Phenolic
GBHE	9.4 ± 3.10	32.9 ± 1.27	0.28
GBDC	18.4 ± 1.37	96.4 ± 3.49	0.19
GBET	40.7 ± 0.95	172.9 ± 29.33	0.24
GBME	75.1 ± 1.97	196.8 ± 3.72	0.38

Values are expressed as mean ± standard error of the mean (SEM) (n=3), analyzed individually in triplicates. GBHE: G. barbadense n-hexane extract, GBDC: G. barbadense dichloromethane extract, GBET: G. barbadense ethyl acetate extract, GBME: G. barbadense methanol extract

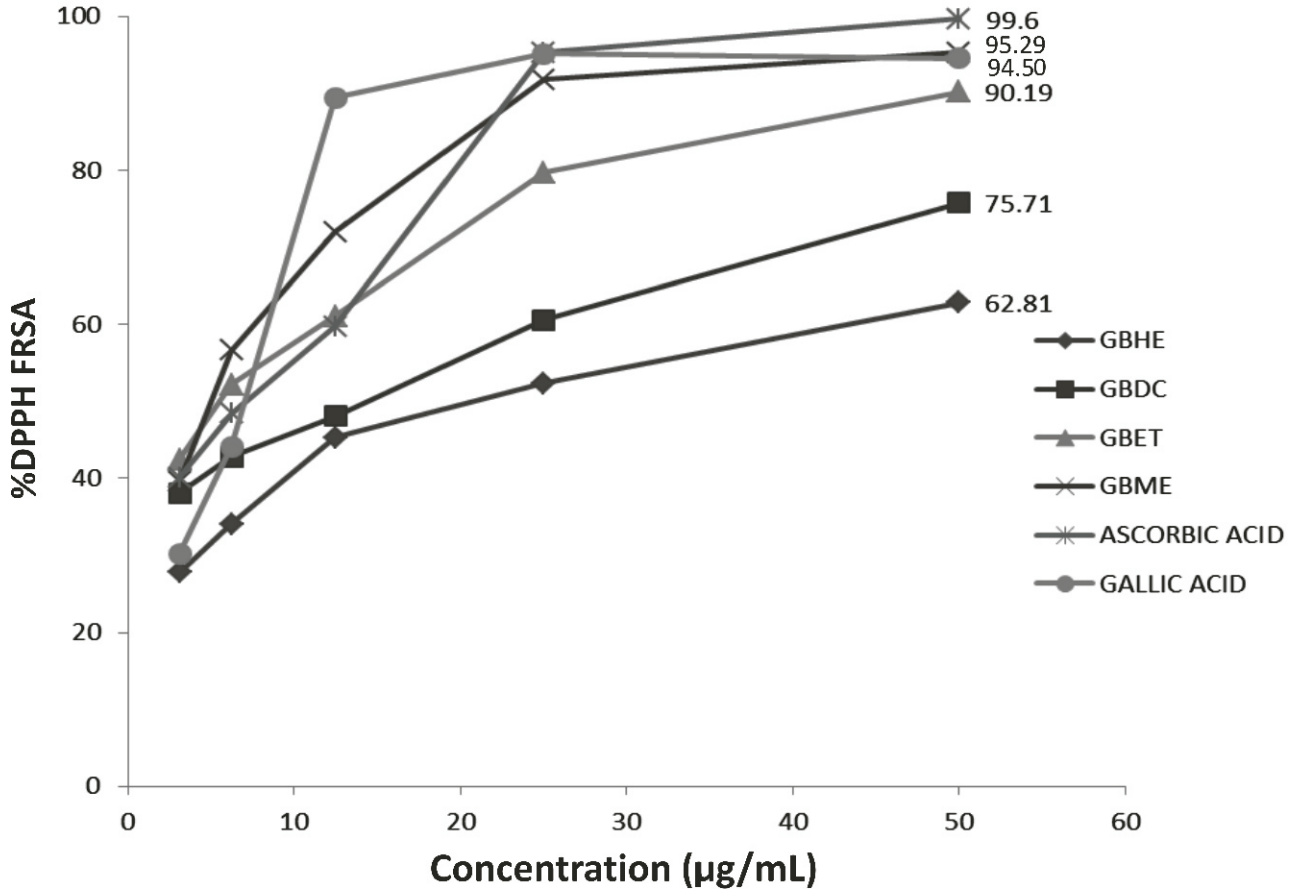

Figure 1: Percentage DPPH free radical scavenging activity (FRSA) of *Gossypium barbadense*

GBHE: G. barbadense n-hexane extract, GBDC: G. barbadense dichloromethane extract, GBET: G. barbadense ethyl acetate extract, GBME: G. barbadense methanol extract

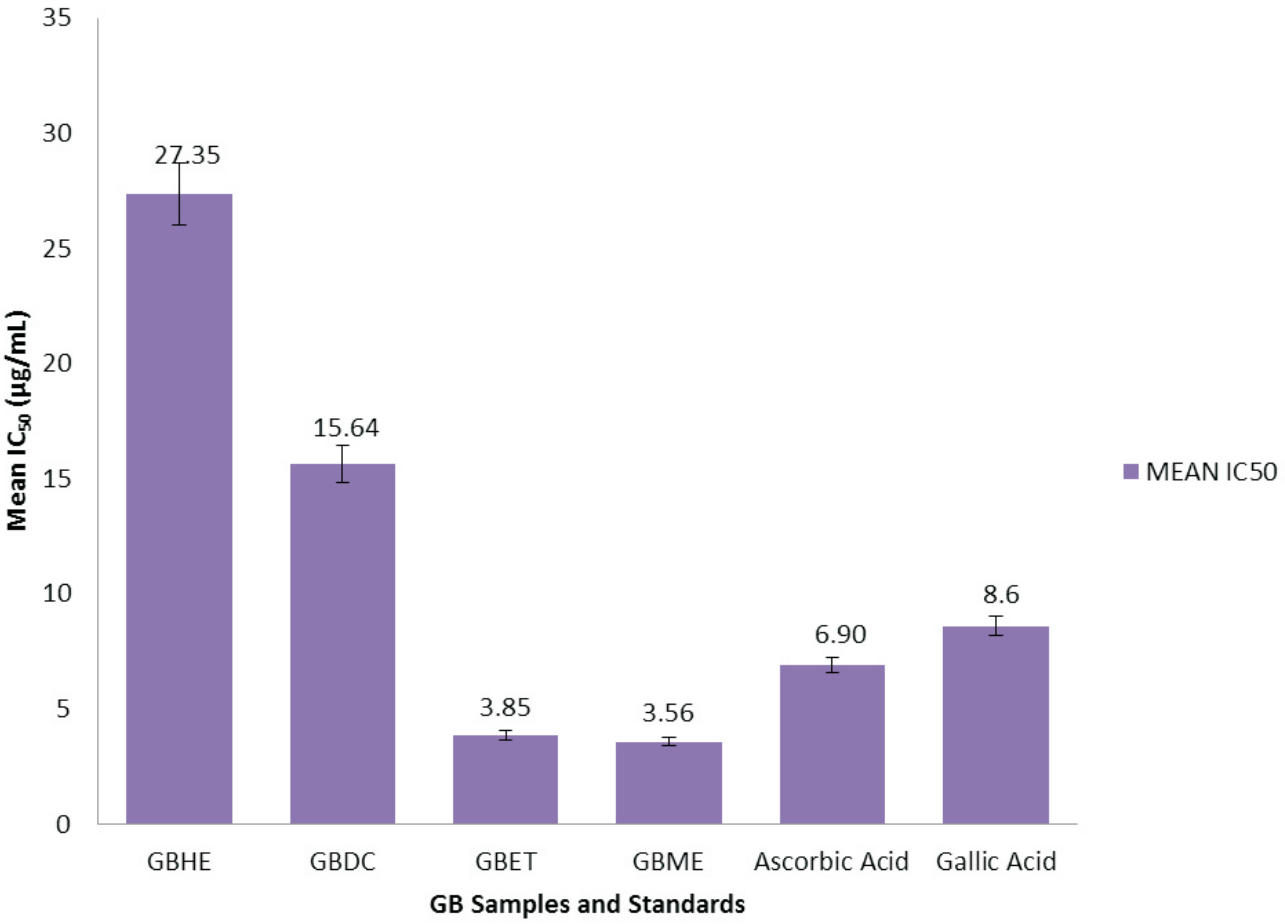

Figure 2: Median inhibitory concentration (IC_{50}) values of the DPPH free radical rcavenging activity of *Gossypium barbadense*

*GBHE: G. barbadense n-hexane extract, GBDC: G. barbadense dichloromethane extract, GBET: G. barbadense ethyl acetate extract, GBME: G. barbadense methanol extract*Table 3: Brine shrimp lethality assay of *Gossypium barbadense* extracts

Extracts	LC_{50}(µg/mL)	95 % Confidence Interval
GBHE	681.12 ± 30.21	621.91 - 740.33
GBDC	1252.09 ± 39.31	1175.04 - 1329.14
GBE T	753.26 ± 10.30	733.07 - 773.45
GBME	1863.57 ± 519.86	844.64 - 2882.50
Cyclophosphamide	529.30 ± 37.24	456.31 - 602.29

GBHE: G. barbadense n-hexane extract, GBDC: G. barbadense dichloromethane extract, GBET: G. barbadense ethyl acetate extract, GBME: G. barbadense methanol extract

Table 4: Percentage membrane stability activity of G. barbadense extracts

Treatment	Concentration (mg/mL)	Mean Absorbance ± SEM	% Membrane Stability
GBHE	1	0.302 ± 0.01	38.31
	0.5	0.389 ± 0.02	20.45
	0.25	0.412 ± 0.02	15.68
GBDC	1	0.160 ± 0.02	67.35
	0.5	0.129 ± 0.05	73.62
	0.25	0.222 ± 0.02	54.67
GBET	1	0.080 ± 0.02	83.71
	0.5	0.102 ± 0.04	79.21
	0.25	0.340 ± 0.03	30.40
GBME	1	0.222 ± 0.04	54.67
	0.5	0.267 ± 0.01	45.47
	0.25	0.332 ± 0.02	32.04
INDO	1	0.155 ± 0 .01	68.30
	0.25	0.213 ± 0.01	56.44

GBHE: G. barbadense n-hexane extract, GBDC: G. barbadense dichloromethane extract, GBET: G. barbadense ethyl acetate extract, GBME: G. barbadense methanol extract, INDO- Indomethacin

DISCUSSION

The presence of higher total phenolics and flavonoids in the methanol extract of *G. barbedense* could be laying credence to the fact that the amount of the antioxidant components that can be extracted from a plant material is not only affected by the sample type, but by the extraction procedure, as well as the efficiency of the extracting solvent.[13] It was observed that in the determination of both TPC and TFC, an increase in polarity of the extracting solvent led to increase in the total flavonoid and phenolic contents, such that the *n*-hexane extracts had the least amount, whereas the methanol extracts had the highest of both total flavonoid and total phenolic contents. This is in line with the report of Sultana et al.[13], that phenolics are often extracted in higher amounts in more polar solvents.

Phenolics or polyphenols, ubiquitously present in plants are known for their wide spectrum of physiological functions such as antioxidant, antimutagenic and antitumor activities as well as ability of modifying gene expression.[18,19] Flavonoids are the most common and widely distributed group of plant phenolic compounds, characterized by a benzo-?-pyrone structure. Phenolic compounds contribute to the overall antioxidant activities of plants mainly due to their redox properties, which enable them act as reducing agents, hydrogen donors, singlet and triplet oxygen quenchers or metal chelators, which may be linked to their chemical structures.[20] The general mechanism by which phenolic compounds exert their antioxidant activity is by neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.[21,22]

The DPPH is a stable nitrogen-centered free radical, which is violet in methanol solution, and turns yellow (diphenylpicrylhydrazine) upon reduction by either the donation of a hydrogen atom or an electron.[23] The scavenging activity is expressed as a percentage of the ratio of the decrease in absorbance of the test solution to that of DPPH solution void of the extracts. It has been established that free radical scavenging activity of many plant extracts is mainly due to the presence of phenolic and flavonoid compounds.[24], although the presence of other antioxidant secondary metabolites in the extracts such as volatile oils, carotenoids, and vitamins contribute to their antioxidant capacity.[21] Hence, the high phenolics and flavonoid contents of the ethyl

acetate and methanol extracts of *G. barbadense* (GBET and GBME) may explain the high levels of antioxidant activity observed.

Brine shrimp lethality (BSL) assay is a reliable, low-cost, rapid general bioassay, requiring no special equipment and a relatively small amount of sample to perform. It can detect a broad spectrum of pharmacological activities in higher plants, and is also used to screen for substances that are toxic to zoologic systems, since bioactive compounds are almost always toxic at high doses.[25] The GBME had been previously evaluated using the BSL assay, which gave LC_{50} of 3585.0 µg/mL.[26], as against the LC_{50} of 1863.57 µg/mL obtained in this study. It is noteworthy that the successive extraction method used in our study may account for the difference in LC_{50} values obtained. This LC_{50} suggests relatively non-toxicity of the extracts. Gossypol, a major constituent of *Gossypium* sp. (a lipid soluble polyphenolic compound isolated from cotton seed oil) has been shown to possess cytotoxic properties[8]. However, its effect was not apparent in this study, suggesting its absence in the plant part (leaves) tested. Different levels of cytotoxicity has been reported for other *Gossypium* species; *Gossypium arboreum* L. with LC_{50} of 94.1 and *G. hirsutum* L. with LC_{50} of 257.2 µg/mL, respectively.[26]

The membrane stabilizing activity of the extracts revealed that the plant contains principles that protect erythrocyte membranes effectively, with the highest constituents in the ethyl acetate extract. Also, it was noted that all the extracts showed a dose-dependent membrane stabilizing activity over all the concentration ranges, with the exception of GBDC. The red blood cell membrane stabilization assay was used in this study to screen for anti-inflammatory activity *in vitro*, because the erythrocyte membrane is analogous to the lysosomal membrane. Therefore, it can be extrapolated that any extract or compound that stabilizes the erythrocyte membrane may as well stabilize the lysosomal membrane.[27 30] The excellent membrane stabilization activity exhibited by the ethyl acetate extract (GBET) could be due to its phytoconstituents. Certain saponins and flavonoids have been reported to exert profound stabilizing effect *in vitro* and *in vivo* on lysosomal enzymes. Tannins and saponins have also been reported to possess the ability to bind cations, thus stabilizing the erythrocytes and other biological macromolecules membranes.[17,31]. This study corroborates the findings of Sabiu *et al*[32], who reported marked dose-dependent protection of bovine erythrocytes against hypotonic and heat-induced damages, by the aqueous extract of the plant (which is polar). From our study, using gradient extraction methods, it becomes obvious that the bulk of the phytoconstituents responsible for membrane stabilization lies in the ethyl acetate region (moderately polar). The GC-MS of the aqueous extract of the plant revealed α-bergamotene, ocimene, stearate, n-palmitate, erucate, margarate, aqua cera, phytol and cis-myrtanol as the major compounds in the plant.

Further studies would reveal the exact compounds within the GBET, which are responsible for this activity.

CONCLUSION

The present study helps to clearly demonstrate that *Gossypium barbadense* contains flavonoids and phenolics, responsible for antioxidant property of the plant. The extracts are rather non-cytotoxic to brine shrimp. The ethyl acetate extract of the plant showed good red blood cell membrane stabilization, so the plant could be regarded as a natural source of anti-inflammatory agents. However, further research is needed to isolate and identify the compounds responsible for these biological activities.

ACKNOWLEDGEMENTS:

This study was partially supported through University of Ibadan Senate Research Grant- SRG/FP/2010/4[A].

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