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Formulation and Antioxidant Activity Evaluation of *Spondias mombin* - *Abelmoschus esculentus* Mucilage Emulsion

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Abstract

Plant gums and mucilage have wide applications in pharmaceutical formulations as emulsifying agents. But these are quite costly, therefore there is always the need for cheaper alternatives. In this study, Okra mucilage from *Abelmoschus esculentus* (L.) Moench pods was used to formulate *Spondias mombin* L. leaf extract emulsion. The emulsion was assessed for the effect of the mucilage on antioxidant activity of the extract using four standard *in vitro* assays. Phytochemical screenings of the mucilage as well as physico-chemical and microscopic characterization of the emulsion were also carried out. The mucilage contained carbohydrates, reducing sugars, terpenoids and unsaturated lactones. The pH of the resulting emulsion was 6.88. The size of the oil globules were small with the average size of 4. 8µm. The extract had superior 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, hydrogen peroxide radical scavenging and ferric ion reducing capacity compared to the emulsion ($p \leq 0.05$). In addition, the emulsion had a better metal chelating activity compared to the extract ($p \leq 0.05$). Okra mucilage may have reduced the antioxidant activity of the extract suggesting its incongruity in this emulsion formulation.

Key words: Antioxidant, emulsion, formulation, mucilage.

Introduction

Pharmaceutical formulations are stable preparations comprising of the active pharmaceutical ingredient (API) and the excipients that ensure that the API is released at the right time and to the proper site of action. Excipients are inert ingredients that impact desirability on these preparations either as bulking agents, fillers, viscosity imparting agents, lubricants, anti-adherents, sweeteners, colorants, binders, disintegrants, emulsifying agents, suspending agents and so on (Aulton and Taylor, 2017). Excipients not only serve to increase the palatability of pharmaceutical dosage forms but also fulfil multiple roles such as to increase serum drug bioavailability, delay drug release to reduce adverse effects and ensure exact API delivery to specific site of drug action (Jaimini and Kothari, 2012). In these preparations, the excipients serve to increase the weight, patient acceptability and consistency of the formulation (Avachat *et al*., 2011). Excipients range widely in nature. They may be but not limited to inert carbohydrates, naturally occurring sugars, synthetic mineral salts, water, naturally occurring oils, mineral oils, naturally occurring mineral matter and preservatives. Majority of excipients are derived from synthetic or semi-synthetic sources that may be expensive to source. Hence, it is needed to use cheaper naturally sourced excipients. Natural

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excipients are mostly derived from plants and minerals. The most widely used natural excipients are gums and mucilages because of their multiple roles in formulations. A natural gum or mucilage may act as a binder and disintegrant in the same formulation. Examples include Acacia gum which may serve as a binder because of its adhesive properties but at the same time, absorbs water from the surrounding medium to facilitate break up of a tablets. In the same formulation, it may also serve as a wetting agent because of its hydrophilicity thus aiding the drug dissolution, absorption and bioavailability in the circulation. However, because of the multipurpose use of this Acacia gum, it is in high demand in the pharmaceutical industry, thus this has increased its price in the market. Due to the exorbitant prices of these gums, there is a need for use of more affordable and suitable alternatives to function in pharmaceutical formulations. Okra is a natural thickener obtained from pods of *Abelmoschus esculentus* (L.) Moench (Fam. Malvaceae) are used to prepare soups for consumption with staple meals. It is a vegetable grown in tropical and sub-tropical regions of the world but is indigenous to North East Africa especially Ethiopia and Sudan (Gemede *et al*., 2013). Okra mucilage has been used as a binder and retardant in sustained-release diclofenac tablets (Naveen *et al*., 2013). It also caused sustained-release of propranolol hydrochloride in tablet dosage forms (Zaharuddin *et al*., 2014). Okra mucilage was also used to retard the release of naproxen sodium tablets at 1-5% concentration (Hussain *et al*., 2017). It was also used to formulate a stable oil-in-water emulsion of n-hexadecane at acidic pH (Ghori *et al*., 2014). Okra mucilage is made up of a polysaccharide that consists of galactose, rhamnose and galacturonic acid subunits with some acetylation (Hussain *et al*., 2017). Herbal preparations are in increasing use by many people in the world (WHO, 2013) and while practitioners make their formulations, they prefer the use of multi-component natural herbs and vegetables (Oiseoghaede *et al*., 2016). *Spondias mombin* L. (Fam. Anacardiaceae) is a small deciduous tree that grows in humid tropical climates (Castner *et al.*, 1998). The leaves are rich in vitamins such as ascorbic acid, niacin, riboflavin and thiamine as well as tannins, saponins and flavonoids which make it a very strong antioxidant (Igwe *et al*., 2010). The leaves have been shown to contain 19.35mg/100g ascorbic acid (Njoku and Akumefula, 2007) which makes it a good candidate for this study. This study aims to evaluate effect of *A. esculentus* mucilage on antioxidant property of *S. mombin* extract in a formulated emulsion of the extract with the mucilage used as the emulsifying agent using various *in vitro* antioxidant assays. The study was also designed to physically and microscopically characterize formulated emulsion, perform phytochemical screening on the mucilage and assess if emulsion to be formulated can be fit for therapy against human oxidative conditions.

Materials and Methods

Chemicals and reagents: Potassium ferricyanide, trichloroacetic acid, ferric chloride (Sigma Aldrich Germany) 1,1-diphenyl-2-picrylhydrazyl (DPPH) crystals and orthophenanthroline (Merck KGaA, Darmstadt, Germany) were purchased from source as indicated. Phosphate buffer (0.1M, pH 7.4), phosphate buffer (pH 6.6) and ethanol were prepared at and obtained from Department of Pharmacognosy laboratories, University of Lagos. Absolute ethanol and methanol (EMD Milliform Corporation, Germany) and Rhodamine B dye (Macklin Industries, Shanghai, China) were purchased while hydrogen peroxide (20 volume) used was a standard proprietary product bought off the shelf.

Plant collection, identification and preparation: Plants were purchased from Ijegun market, Lagos state, Nigeria in August and September 2019. Okra pods and *S. mombin* leaves were authenticated at the Department of Botany, University of Lagos where the plants were assigned voucher specimen number LUH 6203 and 8999, respectively. Voucher specimens were then deposited at the herbarium. The plant materials were garbled, cleaned and dried in the oven at 72h, powdered and stored in air-tight containers until needed for the study (Ng *et al*., 2012).

Extraction of plant materials: 770.22 g of powdered Okra pods were transferred into a container with 500 ml distilled water, heated at 60°C and continuously stirred for approximately 4h. The concentrated solution was filtered through a muslin cloth for several times and cooled to room temperature. Extracted gum was isolated by precipitating in absolute ethanol. The isolated gum was placed in several steel containers and dried to attain a constant weight at 35-45°C in hot air oven. The mucilage was then powdered and stored in dry containers (Kulkarni *et al*., 2002). *S. mombin* leaves were cleaned then dried in the oven (Ng *et al*., 2012). 1246.55g of powdered leaf was macerated in 10l of 70% ethanol in an amber colored Winchester bottle for 72h after which the clear filtered ethanol extract was concentrated in the rotary evaporator set at 45°C to dryness. The yield of extract was calculated in relation to the dried plant materials.

Phytochemical screenings: The Okra mucilage extract was subjected to qualitative chemical investigation to test for the presence of various phytochemicals using standard procedures and reagents (Sofowora, 1993).

Emulsion formulation: The emulsion of the test material was formulated by using the following formula.

S. mombin leaf extract	37.5% w/v
Okra mucilage	0.5% w/v
Methyl cellulose	0.5% w/v
Liquid paraffin	20% v/v
Distilled water to	100 ml

This was done using a modified Forbes method (Suthar and Barbhaiya, 2015). 1g of the mucilage and methylcellulose were weighed and transferred into the mortar and triturated by a pestle till a fine powder was formed. 75mg *S. mombin* was weighed and transferred into a beaker and gradually dissolved with a minute quantity of distilled water and warmed till an appreciable quantity dissolved. The resulting mixture was then filtered and filtrate transferred into the mortar in aliquots and triturated till a homogenous mixture was formed. 40ml~~s~~ liquid paraffin was added in little quantities till a homogenous paste was observed. This was then transferred into a calibrated bottle and distilled water added to the mark. The bottle was also shaken to ensure proper emulsification. This is equivalent to 0.375mg/ml (37.5%) solution. Emulsion was refrigerated and reconstituted for use. Serial dilutions with methanol were made from this emulsion for use in the assays.

Preparation of S. mombin aqueous stock solution: 200 mg of the *S. mombin* extract was weighed using an analytical balance and transferred into a beaker and gradually dissolved with a minute quantity of distilled water and warmed till an appreciable quantity dissolved. The resulting mixture was filtered, and the filtrate was made up to 200 ml with distilled water. This is equivalent to 1 mg/ml (100%) solution and was used as representative of the aqueous fraction of *S. mombin.* This was then refrigerated until used. This concentration (1 mg/ml) was used because it had comparable antioxidant activity with ascorbic acid at the same concentration as evidenced in a previous study (Ogunye, 2018). Serial dilutions were made with methanol from this preparation for use in the assays.

Characterization of emulsion

Microscopy: 0.35g water-soluble Rhodamine B dye was weighed and dissolved in 100 ml distilled water. A 1:1 solution of the emulsion and the diluted dye was prepared and a drop of the mixture was placed on a slide and covered with a cover slip. It was mounted on a microscope and viewed at x 10 and x 40 objective.

Micrometry: The size of oil globules of the already mounted emulsion-dye mixture above was measured using an already calibrated eyepiece micrometer (1 division=2.4µm). 10 globules were measured and grouped into small, medium and large sized granules. Mean size each globule group was calculated and expressed as mean size ± standard deviation (SD).

Measurement of pH: 30 ml of *S. mombin – A. esculentus* emulsion was measured in measuring cylinder and transferred into a beaker to test for its

pH using a pH meter. The same procedure was repeated for *S. mombin* stock solution.

Antioxidant assays

DPPH free radical scavenging activity: The antioxidant activity of the extract was evaluated based on the radical scavenging effect of the stable 1, -diphenyl-2-picrylhydrazyl (DPPH)-free radical activity by a method employed by Blois (1958). The extracts (0.0125-0.2%) were prepared from the stock solution using suitable dilution. 0.1mM of DPPH was prepared in methanol and 1 ml of this solution was mixed with 3 ml of sample solution in test tubes in triplicates. Absorbance was measured at 517 nm using UV-VIS Spectrophotometer. Methanol (3 ml) with DPPH solution (0.1mM, 1 ml) was used as control. This was also repeated for the emulsion. Methanol was used as blank. % inhibition was calculated using the following equation.

Inhibition of DPPH = $(A_C - A_A)/A_C \times 100\%$

Where A_C is the absorption of the control sample and A_A is the absorption of tested extract (Eruygur *et al*., 2017).

Hydrogen peroxide scavenging assay: This was carried out by a method employed by Gayathri *et al.* (2014). The extracts (3 ml) were prepared in methanol at various concentrations (0.0125-0.2%) and mixed with 1.8 ml of 40 mM hydrogen peroxide prepared in phosphate buffer (0.1M, pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230nm against blank solution containing phosphate buffer without hydrogen peroxide. The experiments were carried out in triplicates. This was also repeated for the emulsion.

% Inhibition = $(A_C - A_A)/A_C \times 100\%$ Eq. 2

Where A_C is the absorption of the control sample and A_A is the absorption of tested extract.

Ferric ion reducing antioxidant capacity: Using a modified method as employed by Adesegun *et al.* (2009), ferric ion reducing capacity of the extract solution and emulsion was assessed. The extracts (0.0125-0.2%) in 0.5 ml of methanol was mixed with phosphate buffer (0.25 ml, pH 6.6) and potassium ferricyanide (0.25 ml 1%). The mixture was incubated at 50°C for 20 min. A portion (0.5ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 1600 g for 10 min. The upper layer of the solution was mixed with distilled water (0.5 ml) and $FeCl_3$ (0.1mls, 1%) and the absorbance was measured at 700 nm. The experiments were carried out in triplicates and also done for the extracts. Increased absorbance of the reaction mixture indicated increased reducing power.

Metal chelating activity: The reaction mixture contain 1 ml 0.05% orthophenanthroline in methanol, 2 ml ferric chloride 200 μM and 2 ml of various concentrations (0.0125-0.2%) of the solution and emulsion. The mixture was incubated at ambient temperature for 10 min, and then the absorbance of the same was measured at 510 nm. The chelated iron formed a red chromophore in solution and an increase in absorbance indicated increased activity. The experiment was performed in triplicate (Qureshi *et al*., 2010).

Statistical analysis: All tests were done in triplicates and analyzed using Microsoft Excel expressed as mean ± standard deviation (SD) and compared using Repeated Measures (RM). Two-way Analysis of Variance (ANOVA) by GraphPad prism 8 Software as well as Bonferroni multiple comparison post-test were used to compare against the *S. mombin* solution. Differences between assay results were assessed at 95% confidence interval ($p \leq 0.05$).

Results and Discussion

Extract yield: The yields of *A. esculentus* and the *S. mombin* extracts were 25.75% and 2.31%, respectively as shown on Table 1. The high yield of the *S. mombin* leaves was expected because the extracting solvent was highly polar and would extract the abundant polar constituents of the *S. mombin* leaves. The yield of the *S. mombin* was similar to that reported by hydroethanolic extraction of the leaves in a similar study which recorded 23.5±4.4 % yield (Cristofoli *et al*., 2018). However, the mucilage yield was quite low compared to a recent study that used

The percentage inhibition w

similar method but different precipitating solvent, acetone (Farooq *et al.*, 2013). The yield was 11.44% w/w which was over 4-fold the yield obtained in this study. The large difference may be attributable to the different solvents used.

Phytochemical screening: The mucilage was observed to contain terpenoids, reducing sugars, carbohydrates and unsaturated lactones and lacked saponins, alkaloids, flavonoids, tannins, deoxysugars and anthraquinones as shown on Table 2. In a related study, it was seen to contain only carbohydrates similar to this study (Kumar *et al.*, 2009). It also had similar results in that it also lacked saponins, alkaloids, flavonoids, tannins and deoxysugars. In contrast, this study showed presence of terpenoids, reducing sugars and unsaturated lactones. The presence of carbohydrates and reducing sugars corresponds to work done by some researchers (Farooq *et al.*, 2013). Deoxysugars and unsaturated lactones are hallmark of cardiotonic glycosides. However, unsaturated lactones were present and deoxysugars were absent in this study.

Table 1. Calculation of yield of extract.

Plants	Dry plant material (g)	Extract (g)	% Yield
Abelmoschus esculentus	770.22	17.78	2.31
Spondias mombin	1246.55	320.94	25.75

Table 2. Results of phytochemical screening of mucilage.

Sample	Saponins	Alkaloids	Terpenoids	Tannins	Shinoda	Fehling's	Keller-Killiani	Borntrager’s			Kedde's	Molisch
								Free	O	C		
AEM	-	-	+	-	-	+	-	-	-	-	+	+

+ = present, - = absent; AEM= *A. esculentus* mucilage.

Emulsion stability: Stability of formulations is critical to prevent coalescence and phase separation. Utilization of an oil phase that will stabilize the *S. mombin* extract is crucial in formulation development. Liquid paraffin emulsion BP was utilized as the base formulation with the dual suspending agent capacity of *A. esculentus* mucilage and methyl cellulose. A concentration of 0.5% w/v *A. esculentus* extract and 0.5% methylcellulose led to a 1%w/v total suspending agent concentration. Herb-excipient interaction may have led to the perceived decrease in the antioxidant effect of the *S. mombin* extract. This occurred due to formation of globules with increased integrity in the emulsion that did not optimally release the *S. mombin* active responsible for anti-oxidant activity. The formulation however exhibited high stability.

Microscopy, micrometry and pH measurement: Microscopy revealed the emulsion as an oil-in-water emulsion with the background taking the color of the water-soluble Rhodamine dye and the yellow to brown colored oil globules on the foreground as observed in Figure 1. Micrometry showed that the oil globules were majorly of the small-sized type with average size of 4.8 µm. The size range of globules was 4.8-21.6 µm as seen on Table 3. This is identical with what was reported in literature with size ranges from 6-23 µm (Ghori *et al.*, 2014). The pH measurement gave a value of slightly acidic pH of 6.88 as seen on Table 4 which was very close to neutral and may be considered safe as it may not cause gastric ulceration associated with acidic pH. Emulsification was at slightly acidic pH comparable to what was seen in literature (pH 3) as reported by Ghori *et al*. (2014).

Antioxidant assays

DPPH radical percentage inhibition: A concentration-dependent increase in DPPH radical

scavenging activity was observed for both extract and emulsion as seen in Table 5. The extract and emulsion had comparable DPPH activity at 0.0125%, $p \leq 0.05$ ($p > 0.05$). However at 0.025%, the extract was a better DPPH radical scavenger than the emulsion, $p \leq 0.05$ ($p \leq 0.01$). This trend was maintained between 0.05-0.2% with the extract having better DPPH activity than the emulsion, $p \leq 0.05$ ($p \leq 0.0001$). This means that the extract was a better DPPH radical scavenger than the emulsion (formulated with the mucilage), due to entrapment of the extract globules in the emulsion matrix (Ilomuanya *et al.*, 2018). This indicated that the introduction of the mucilage in the formulation reduced the DPPH activity of the extract. The DPPH activity exhibited however is still adequate for its use as an antioxidant preparation.

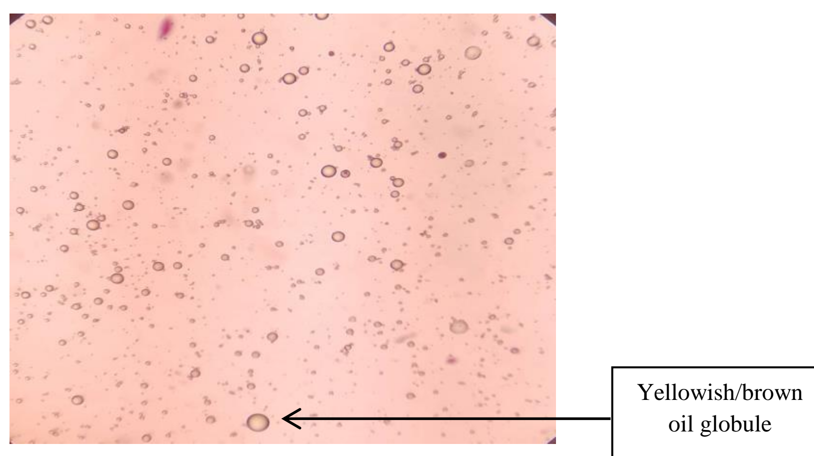

Figure 1. Microscopic study of *S. mombin – A. esculentus* mucilage emulsion oil globules (x 100).

Table 3. Micrometric studies.

Size	Range size (No. of divisions)	No. of globules	Mean size ± SD (µm)
Small	1-3	6	4.80±2.16
Medium	4-6	3	11.20±1.39
Large	7-9	1	21.60±0.00

Table 4. Measurement of pH.

Sample	pH
S. mombin solution	3.87
S. mombin – A. esculentus emulsion	6.88

Table 5. Percentage inhibition in DPPH assay at 517 nm.

Concentration	0.0125	0.025	0.05	0.1	0.2
S. mombin solution	27.17±2.78	46.09±4.05	61.30±5.42	85.39±1.45	88.76±0.29
S. mombin - A. esculentus emulsion	28.50±5.60[a]	25.26±1.60[c]	18.91±6.36[e]	39.29±2.52[e]	40.12±7.79[e]

Data expressed as mean ± SD at 5 concentrations, $n = 3$; [e] = Significantly different from *S. mombin* solution at $p \leq 0.05$ ($p \leq 0.0001$); [c] = Significantly different from *S. mombin* solution at $p \leq 0.05$ ($p \leq 0.01$); [a] = Not significantly different from *S. mombin* solution at $p \leq 0.05$ ($p > 0.05$).

Hydrogen peroxide radical scavenging inhibition: There was a concentration-dependent decrease in hydrogen peroxide radical scavenging activity for both extract and emulsion as seen in Table 6. This is an abnormal trend as there should be an increase in activity as concentration increases but this was the reverse. The extract had a better hydrogen peroxide radical scavenging at 0.0125% and 0.1%, $p \leq 0.05$ ($p \leq 0.001$) and at 0.025% and 0.05%, $p \leq 0.05$ ($p \leq 0.0001$). However, they had statistically similar activity at 0.2%, $p \leq 0.05$ ($p > 0.05$). The extract still had better activity than the emulsion at 0.2%. This implies that the extract was a better hydrogen peroxide radical scavenger than the emulsion (formulated with the mucilage). This indicated that the introduction of the mucilage in the formulation also diminished the hydrogen peroxide radical scavenging activity of the extract. This negative activity observed in both extract and emulsion showed they may not be suitable for use in scavenging hydrogen peroxide radicals.

Ferric ion reducing capacity: A concentration-dependent increase in reducing capacity was observed as represented on Table 7. The extract and emulsion exhibited similar activity at 0.0125-0.1%, $p \leq 0.05$ ($p > 0.05$) but extract had better activity than emulsion at 0.2%, $p \leq 0.05$ ($p \leq 0.05$). Thus, it can be deduced that at lower concentration the activities of extract and emulsion are similar but extract had better activity at high concentration. This also suggests that the use of the mucilage as emulsifier in the formulation caused a decline in the ferric ion reducing capacity of the extract because the extract still had a better activity than the emulsion. However, the reducing capacity of the emulsion will still suffice its use as an antioxidant preparation.

Table 6. Hydrogen peroxide radical scavenging inhibition at 230 nm.

Concentration	0.0125	0.025	0.05	0.1	0.2
S. mombin solution	51.09±3.05	39.43±12.12	1.60±2.79	-63.35±9.65	-105.21±5.39
S. mombin - A. esculentus emulsion	-41.96±10.43[d]	-83.70±8.45[e]	-132.7±23.51[e]	-152.3±6.46[d]	-118.9±34.96[a]

Data expressed as mean ± SD at 5 concentrations, $n = 3$; [e] = Significantly different from *S. mombin* solution at $p \leq 0.05$ ($p \leq 0.0001$); [d] = Significantly different from *S. mombin* solution at $p \leq 0.05$ ($p \leq 0.001$); [a] = Not significantly different from *S. mombin* solution at $p \leq 0.05$ ($p > 0.05$).

Table 7. Ferric ion reducing capacity at 700 nm.

Concentration	0.0125	0.025	0.05	0.1	0.2
S. mombin solution	0.206±0.102	0.287±0.103	0.355±0.044	0.559±0.004	1.194±0.391
S. mombin - A. esculentus emulsion	0.248±0.001[a]	0.466±0.051[a]	0.625±0.096[a]	0.504±0.062[a]	0.667±0.219[b]

Data expressed as mean ± SD at 5 concentrations, $n = 3$; [b] = Significantly different from *S. mombin* solution at $p \leq 0.05$ ($p \leq 0.05$); [a] = Not significantly different from *S. mombin* solution at $p \leq 0.05$ ($p > 0.05$).

Table 8. Analysis for metal chelating activity at 510 nm.

Concentration	0.0125	0.025	0.05	0.1	0.2
S. mombin solution	0.034±0.002[b]	0.038±0.006[a]	0.041±0.002[c]	0.056±0.005[c]	0.058±0.003[e]
S. mombin - A. esculentus emulsion	0.056±0.011	0.054±0.012	0.075±0.004	0.092±0.002	0.115±0.017

Data expressed as mean ± SD at 5 concentrations, $n = 3$; [e] = Significantly different from *S. mombin* solution at $p \leq 0.05$ ($p \leq 0.0001$); [c] = Significantly different from *S. mombin* solution at $p \leq 0.05$ ($p \leq 0.01$); [b] = Significantly different from *S. mombin* solution at $p \leq 0.05$ ($p \leq 0.05$); [a] = Not significantly different from *S. mombin* solution at $p \leq 0.05$ ($p > 0.05$).

Metal chelating activity: There was a concentration-dependent increase in metal chelating activity as shown on Table 8. The extract and emulsion exhibited similar activity at 0.025%, $p \leq 0.05$ ($p > 0.05$). However, this effect was isolated because the emulsion was better than the extract at other concentrations – at 0.0125%, $p \leq 0.05$ ($p \leq 0.05$), at 0.05 and 0.1%, $p \leq 0.05$ ($p \leq 0.01$) and at 0.2%, $p \leq 0.05$ ($p \leq 0.0001$). This indicates that inclusion of the mucilage in the formulation increased the metal chelating activity of the extract.

Conclusions

Naturally sourced plant gums and mucilage have been used in various capacities as excipients in pharmaceutical formulations, including the emulsions. The widely used plant gums and mucilage are however becoming expensive due to their broad acceptance and multipurpose uses in the industry. Hence, a need arises for safe, affordable and viable alternatives. This study has shown that Okra mucilage is a good emulsifier but may have reduced the antioxidant activity of *S. mombin* extract when formulated as emulsion. The emulsion however exhibited good stability and thus can benefit from future studies that would reduce the extract entrapment to increase antioxidant activity. The evidence in this study will direct future research effort at sourcing for other natural plant-based gums and mucilage that can be used in formulating herbal preparations with good drug release and stability profiles.

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Conflict of Interest

The authors declare no conflict of interest

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