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Faatimah Hanna Jahally
Faculty of Agriculture, University of Mauritius, Réduit, Mauritius

Daneshwar Puchooa
Faculty of Agriculture, University of Mauritius, Réduit, Mauritius

Correspondence
Faatimah Hanna Jahally
Faculty of Agriculture, University of Mauritius, Réduit, Mauritius

Assessing the antimicrobial and antioxidant properties of *Moringa oleifera*: A comparative study between deionised water and Zamzam water as solvent

Faatimah Hanna Jahally and Daneshwar Puchooa

Abstract
The aim of this study was to assess the antimicrobial and antioxidant properties of different *M. oleifera* leaf extracts while exploiting the potential of using Zamzam water as a solvent for the phytochemical extraction. Six solvents were used in the extraction protocol: deionised water (DW), Zamzam water (ZW), deionised water/ethanol (DWE), Zamzam water/ethanol (ZWE), deionised water/acetone (DWA) and Zamzam water/acetone (ZWA). The antimicrobial activities of the extracts were tested against the following microorganisms: *S. aureus*, *B. cereus*, *B. subtilis*, *P. aeruginosa*, *E. coli*, *P. mirabilis* and *C. albicans*. The least MIC value reported was 6.25 mg/ml with *B. subtilis* with ZWA while having an MBC value of 50 mg/ml. The highest phenolic content (122.18± 0.45 mg GAE/g extract) as well as the flavonoid content (316.0 ± 6.5 mg QE/g extract) was observed with ZWA. With the DPPH assay, the IC_{50} values varied from 5.362 ± 0.146 mg/ml to 0.617 ± 0.005 mg/ml with ZWA having the lowest value. Overall, the acetone extracts showed higher biological activity in almost all the assays performed. Interesting findings were also obtained when the DPPH assay was done; revealing the possibility of the two types of water used affecting the results obtained.

Keywords: *M. oleifera*, Zamzam water, deionised water, DPPH assay, acetone extracts

1. Introduction
Moringaceae is a monogeneric family having *Moringa* as the only genus [1]. *Moringa oleifera* is one such plant that have medicinal properties and stimulates the cardiac and circulatory systems, possess cholesterol lowering properties, oncolytic, anti-inflammatory, antihypertensive, antipyretic, diuretic, hepatoprotective, antiepileptic, antiulcer, antidiabetic, antispasmodic, antioxidant, antibacterial and antifungal activities [2] and can treat sexually-transmitted diseases, malnutrition and diarrhoea [3].
Although the sources of freshwater on the earth is limited with only 2.8% of the total water found on the planet being freshwater, Zamzam water is found to be continuously flowing since 2000BC in the valley of Makkah; a city located in the western part of Saudi Arabia [4]. Zamzam water is found to have higher concentrations of both cations and anions than normal water [4]. Studies revealed that Zamzam water has anticancer properties [5, 6], antioxidant properties [4, 7], anti-inflammatory characteristics [4], and stimulates aquaporins [8], endometrial growth [9], and formation of immunoglobulin [4].
Most of the previous works that have been done in Mauritius using *Moringa oleifera* were on the coagulant property of its seeds and the literature found on the antimicrobial and antioxidant properties of the latter was scarce. Hence, this work focuses on exploiting the bioactive properties of locally grown *Moringa oleifera* and also on evaluating the potential of using Zamzam water as a solvent for the extraction of phytochemicals from the leaves of *Moringa*.

Materials and Methods
Collection of plant material
The leaves of *Moringa oleifera* were collected from the southern region of Mauritius; most precisely in the village of Camp-Diable during the month of September. The latter was subjected to shade drying for two days and then was grounded into powder and stored at room temperature until further use.

Plant material extraction
The solvents used were: deionised water, Zamzam water, ethanol/deionised water (80/20 v/v), ethanol/Zamzam water (80/20 v/v), acetone/deionised water (80/20 v/v) and acetone/Zamzam water (80/20 v/v).

The extraction protocol [10] used was as follows: 10 g of the plant powder was soaked in 200 ml of each solvent for 3 days at room temperature on an orbital shaker at 200 rpm. The extracts were then filtered using Whatman no.1 filter paper under vacuum. The aqueous ethanolic and aqueous acetone extracts were dried by the rotary evaporator followed by freeze drying. The water extracts were frozen at -20 ºC and then freeze-dried.

All of the crude plant extracts used were dissolved in deionised water or Zamzam water respectively. The extracts used were: ethanolic extracts dissolved in deionised water (DWE) and Zamzam water (ZWE), acetone extracts dissolved in deionised water (DWA) and Zamzam water (ZWA) and deionised water extract dissolved in deionised water (DW) and Zamzam water extract dissolved in Zamzam water (ZW).

Phytochemical analysis

The phytochemicals were qualitatively detected through standard protocols: coumarins, anthraquinones, saponins, tannins [11], terpenes, cardiac glycosides [12], phenols [13], alkaloids [14], flavonoids [15], carotenoids [16], quinone [17] and anthocyanin [18].

The Folin-Ciocalteu assay [19] was performed to calculate the total phenolic content (TPC) of the different plant extracts, and for the determination of the flavonoid content (TFC), the Aluminium chloride method [20] was followed.

Antimicrobial screening

The microorganisms that were used in this study were obtained from the Microbiology Lab at the Faculty of Agriculture, University of Mauritius and were as follows: *S. aureus* (ATCC 29213), *B. subtilis*, *B. cereus* (ATCC 10876), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *P. mirabilis* (ATCC 12453) and *C. albicans* (ATCC 10231).

Prior to any antimicrobial assay performed, each bacterium/fungal strain was incubated in 10 mL of MHB/PDB overnight and the culture was incubated at 37 °C for the bacterial species and at 24 °C for the fungal specie. The PDB was supplemented with chloramphenicol to inhibit bacterial growth [21]. Also, all the extracts were filter sterilised using the membrane filters of micro pore size 40 µm.

Agar disc diffusion method

The disc diffusion assay [22] was done as a preliminary screening test to detect for the presence of antimicrobial activity with the extracts at a given concentration.

All of the microbial cultures were standardised by dilution with sterile MHB/PDB spectrophotometrically using the 0.5 McFarland as reference.

In this test, 0.1 ml of each of the standard microorganism was spread onto the MHA/PDA plate. Then, 10 µl of each of the extracts (50 mg/ml) were added on sterile paper discs of about 6mm

in diameter and were left to air dry before seeding them onto the agar. The 24 petri plates were then incubated at 37 °C (for the bacteria) and 24 ºC (for the fungus) for one day. The positive control in this test was Ampicillin (25µg/disc) for the bacterial sample and nystatin (50 µg/mL) for the fungal species. The negative controls were deionised water and Zamzam water.

Minimum inhibitory concentration test

The broth micro-dilution method was performed using a 96-well microtitration plate for the MIC assay [23]. With the bacterial species, the extracts were made to a final concentration of 200 mg/ml; while with the fungal species, the concentration was made to 400 mg/ml.

The microbial cultures were standardised spectrophotometrically using the 0.5 McFarland as reference. The bacterial suspensions were further diluted (1:100) to obtain a concentration of 3×106 cells while the fungal culture was diluted to yield a concentration of 5.0 x 102 to 2.5 x 103 cells per ml [24].

50 µl of the tested plant extracts for each microorganism was diluted two-fold serially with 50 µl of sterile MHB/PDB in a 96-well microplate. A two-fold dilution of chloramphenicol (10 µg/mL) was used as positive control against each bacterium and with the fungus, nystatin was used (50 µg/mL). Deionised water and Zamzam water were used as negative control. Finally, 50 µl of the respective broth was added in each well such that the final concentration of the plant extract obtained in the first well was 50 mg/ml for the bacterial species and 100 mg/ml for the fungal species.

The MIC plates were incubated at 37 °C for the bacterium and at room temperature for the fungus. The minimum inhibiting concentration of the extracts (MIC) inhibiting total bacterial/fungal growth was noted after 24 hr for the different microorganisms. The results were compared with the negative controls and the positive control. Bacterial growth was assayed colorimetrically by adding 40 µl of 0.2 mg/ml iodonitrotetrazolium violet (INT) to each well, and was then incubated again at 37 °C for 30 min [23] while with the fungal species, the observations were made visually [25].

Minimum bactericidal/fungicidal concentration

To determine the minimum bactericidal or fungicidal concentration [26], the samples which showed no growth in the MIC plates were taken and sub-cultured on MHA or PDA respectively. The plates were incubated at 25 ºC for the fungi and 37 ºC for the bacteria. Observations were made after 24 hours. The least concentration that did not produce any bacterial colony was taken as MBC/MFC. For the negative controls, the MBC/MFC was not determined as the solvents did not inhibit bacterial/fungal growth.

Antioxidant activity

The 2,2-dipheny-1-dipycryl hydrazyl (DPPH) radical scavenging activity was performed to evaluate the antioxidant properties of the plant extracts [27].

DPPH radical scavenging activity

The ratio of extract: DPPH (v/v) used was 1:5 instead of 1:10 in this study. In 0.1mM solution of DPPH prepared in methanol, 500µl of the extracts (0.05, 0.2, 0.8, 3.2 and 12.8 mg/mL) was added to 2.5 ml of the DPPH solution. The mixtures were vortexed and allowed to stand in the dark for 20 minutes at room temperature. Then the absorbance was read at 517 nm and a standard curve using ascorbic acid as reference was plotted. The DPPH scavenging activity was calculated as follows:

$$\text{DPPH scavenging activity (\%inhibition)} = \frac{A0 - A1}{A0} \times 100$$

Where, the absorbance of the control reaction is A0, and the absorbance in presence of all of the extract samples and reference is A1. Triplicates were done with all the test samples. The concentration of the extract needed for inhibiting 50% of the DPPH radical was determined and the value was expressed as IC50. A low IC50 value indicates a high antioxidant capacity and vice versa.

Statistical Analysis

The ANOVA was used to test for differences among the extracts. The Tukey test was carried out in parallel (p=0.05) to make pairwise comparisons of means of the treatments. The regression analysis together with Pearson correlation method were done with the extracts in response to the performed assays. Analyses were done using the Microsoft Excel 2013 and the statistical software MINITAB Express version 1.5.0.0.

Results and Discussions

Phytochemical analysis

In all of the extracts, terpenes, phenols and flavonoids with traces of alkaloids and cardiac glycosides were present; while coumarins, anthocyanin and saponins were absent (Table 1). Anthraquinone was observed only in the acetone extracts while quinone and carotenoids were found in both the ethanolic and acetone extracts except in the water extracts (Table 1).

Table 1: Phytochemical screening of the different leaf extracts

Phytochemicals	DW	ZW	DWE	ZWE	DWA	ZWA
Coumarins	-	-	-	-	-	-
Terpenes/steroids	++	++	++	++	++	++
Phenol	++	++	++	++	++	++
Anthocyanin	-	-	-	-	-	-
Alkaloids	+	+	+	+	+	+
Saponins	-	-	-	-	-	-
Anthraquinone	-	-	-	-	+	+
Flavonoids	++	++	++	++	++	++
Carotenoids	-	-	+	+	+	+
Quinone	-	-	+	+	+	+
Tannins	+(H)	+(H)	++	++	++	++
Cardiac glycosides	+	+	+	+	+	+

Key: '-': absent, '+': traces, '++': present, 'H': only hydrolysable tannins present.

In this study, the ZWA had both the highest TPC (*Fig 1*) and TFC (*Fig 2*) while the DW extract had the lowest polyphenolic content. From a paper [28], the TPC of aqueous *Moringa* leaf extract was found to range from 36.02 ± 0.01 mg GAE/g plant material (tender leaves) to 45.81 ± 0.02 mg GAE/g plant material (mature leaves) whereas in the current work, the aqueous extracts exhibited a higher phenolic content (*Fig 1*). This difference could have been observed because the maturity of the leaves were not considered in this study. The ratio of mature to young leaves in the sample could have been greater, hence explaining the high polyphenolic content. In another paper [29], the TPC of both the aqueous and ethanolic extracts were lower than that of the extracts in the current work. A probable reason for this occurrence could be with regard to the solvent used; 95% ethanol was used instead of 80%. Since polyphenolic extraction is facilitated when more polar solvent is used [30], this explained the higher polyphenolic content of the extracts in this study.

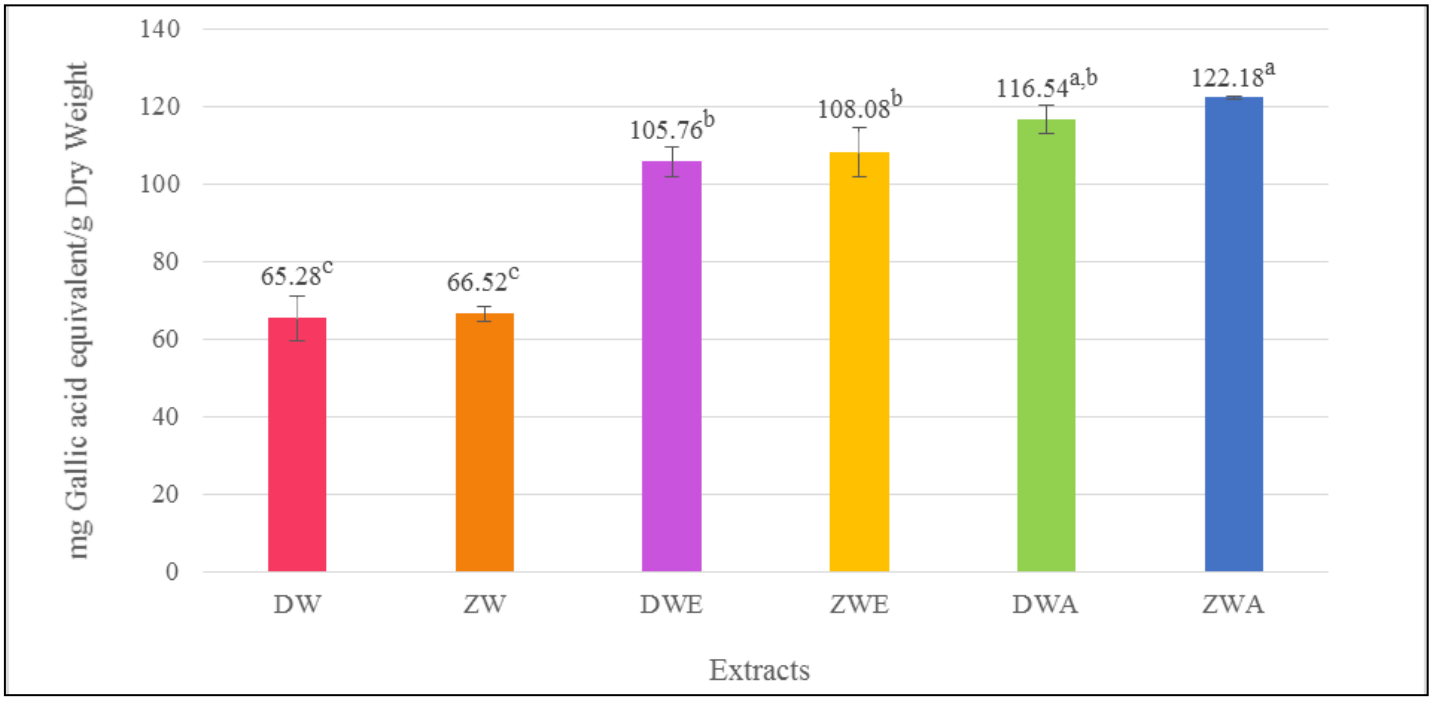

Fig 1: Total phenolic content of all the extracts.

The results were expressed as mean ± standard deviation (n=3). Significant differences were observed in TPC of the extracts ($p<0.05$), except for values with the same superscript letter.

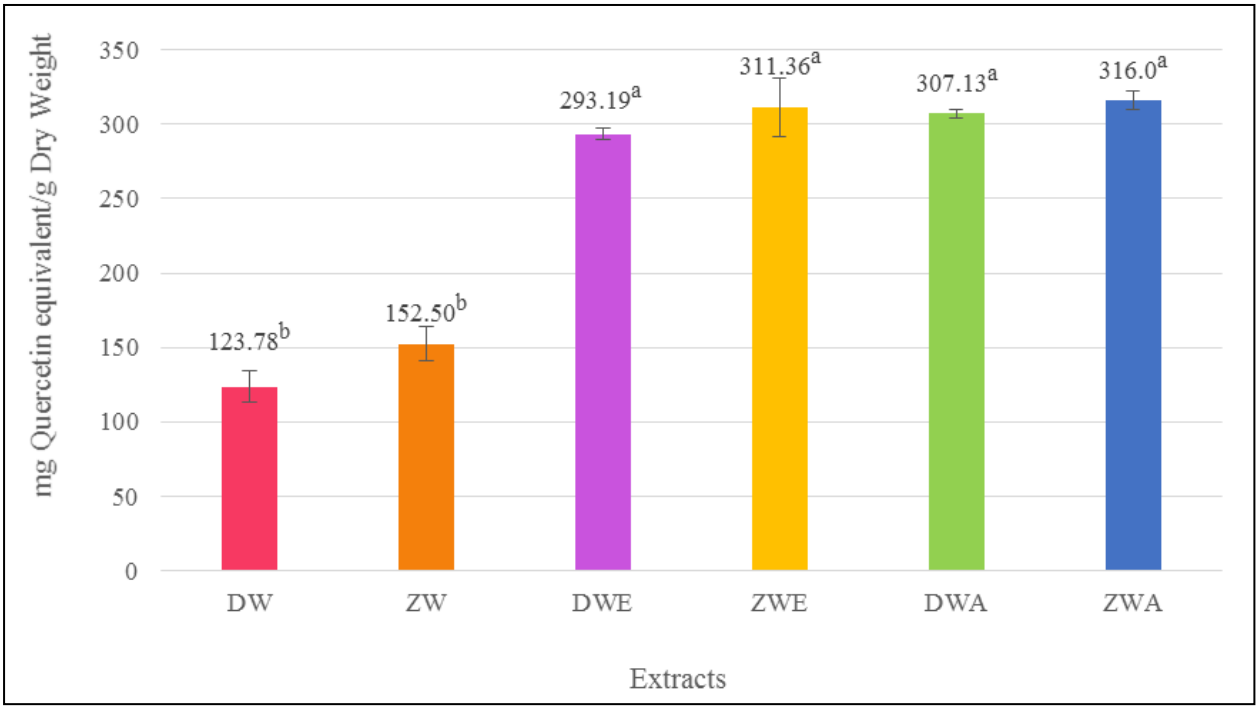

Fig 2: Total flavonoid content of all the extract.

The results were expressed as mean ± standard deviation (n=3). Significant differences were observed in TPC of the extracts ($p<0.05$), except for values with the same superscript letter.

Antimicrobial activity

From *Table 2*, the lowest zone of inhibition recorded was 0.3 ± 0.3 mm which was observed with the DW and ZW extracts for the bacteria: *S. aureus*, *B. cereus* and *E. coli*. However, those values obtained are not reliable as only one plate among the triplicates showed this negligible clear region.

Table 2: The susceptibility of the bacterial species to the crude plant extracts at a final concentration of 50 mg/ml.

Extracts	Diameter of zone of inhibition (mm)[a]			
	S. aureus	***B. cereus***	***E.coli***	***P. aeruginosa***
DW	0.3 ± 0.3	-	0.3 ± 0.3	-
ZW	0.3 ± 0.3	0.3 ± 0.3	-	-
DWE	-	1.0 ± 0.0	-	1.0 ± 0.0
ZWE	-	2.0 ± 0.0	1.0 ± 0.0	2.0 ± 0.0
DWA	-	2.0 ± 0.0	1.0 ± 0.0	-
ZWA	-	1.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
Ampicillin	16.0 ± 0.0	2.0 ± 0.0	14.0 ± 0.0	12.0 ± 0.0

Key: '-'= no inhibition. The values were expressed as mean ± SE of the triplicates. [a] Diameter of inhibition zones excluding diameter of disc of 6 mm.

Data on the bacterial species *B. subtilis* and *P. mirabilis* and the fungus *C. albicans* are missing in the above table since no zones of inhibitions were noticed with the plant extracts except with their positive controls. It should be noted that the inoculum size used was 1–2 × 10^8 bacterial cells and 1–2 × 10^6 fungal cells using the 0.5 McFarland standard and that the incubation time was 24 hours. According to M02-A11 protocol, the incubation time for the bacterial sample is usually 16-18 hours [31]. The incubation time used in this study could have caused over growth of the bacteria making it difficult to observe any probable inhibition zone which could have been formed.

From *Table 3*, it can be seen that only four extracts responded against all the microorganisms in the MIC test which were the acetone and ethanol extracts. The presence of phytochemicals like quinone [32, 33] or the higher polyphenolic content of those extracts [34] could have supported their antimicrobial activity.

Table 3: The MIC and MBC values of the bacterial species and fungal species.

Microorganisms	MIC/MBC values (mg/ml)			
	ZWE	**DWE**	**ZWA**	**DWA**
S. aureus	50.00 ± 0.00	50.00 ± 0.00	50.00 ± 0.00	50.00 ± 0.00
B. cereus	50.00 ± 0.00	50.00 ± 0.00	50.00 ± 0.00	50.00 ± 0.00
B. subtilis	50.00 ± 0.00	50.00 ± 0.00	6.25 ± 0.00 (50)	12.5 ± 0.0
P. aeruginosa	6.25 ± 0.00	25.00 ± 0.00	50.00 ± 0.00	-
E. coli	50.00 ± 0.00	-	50.00 ± 0.00	50.00 ± 0.00
P. mirabilis	-	-	50.00 ± 0.00	50.00 ± 0.00
C. albicans	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00

Key: '-': no activity. MBC values are within brackets (). All the values were expressed as means ± standard deviation (n=3).

It was also observed that the extracts that did not show any antimicrobial activity with *S. aureus*, *B. subtilis* and *P. mirabilis* in the agar disc diffusion method responded when the MIC test was done (*Table 3*). This could have occurred due to the inoculum size used in microdilution test (3×10^6 cells); which is lower than that used in the agar disc diffusion assay. It should also be noted that ZW and DW extracts were missing from the table, indicating the possibility of false positive results in the agar disc diffusion assay which was previously done. The lack of antimicrobial activity of the DW and ZW extracts could be explained due to their phytochemical contents (Table 1) whereby anthraquinones, quinones and carotenoids were present in the other extracts that worked. Also the TPC and TFC values of these two extracts were considerably low when compared with the other extracts (*Fig 1* and *Fig 2*) which could be another reason for the absence of the antimicrobial activity. From *Table 3*, it was however observed that only ZWA exhibited bactericidal properties and that too, with only one microorganism: *B. subtilis*.

From the ANOVA test conducted, no significant differences were found between the mean MIC values of the four extracts: DWE, ZWE, DWA and ZWA ($p < 0.05$) *(Fig* **3**). The high standard deviation observed was due to the uneven sampling number of each extract and also because of the high MIC value noted with *C. albicans* (*Table 3*) with all the extracts which acted as an outlier (*Fig* **3**).

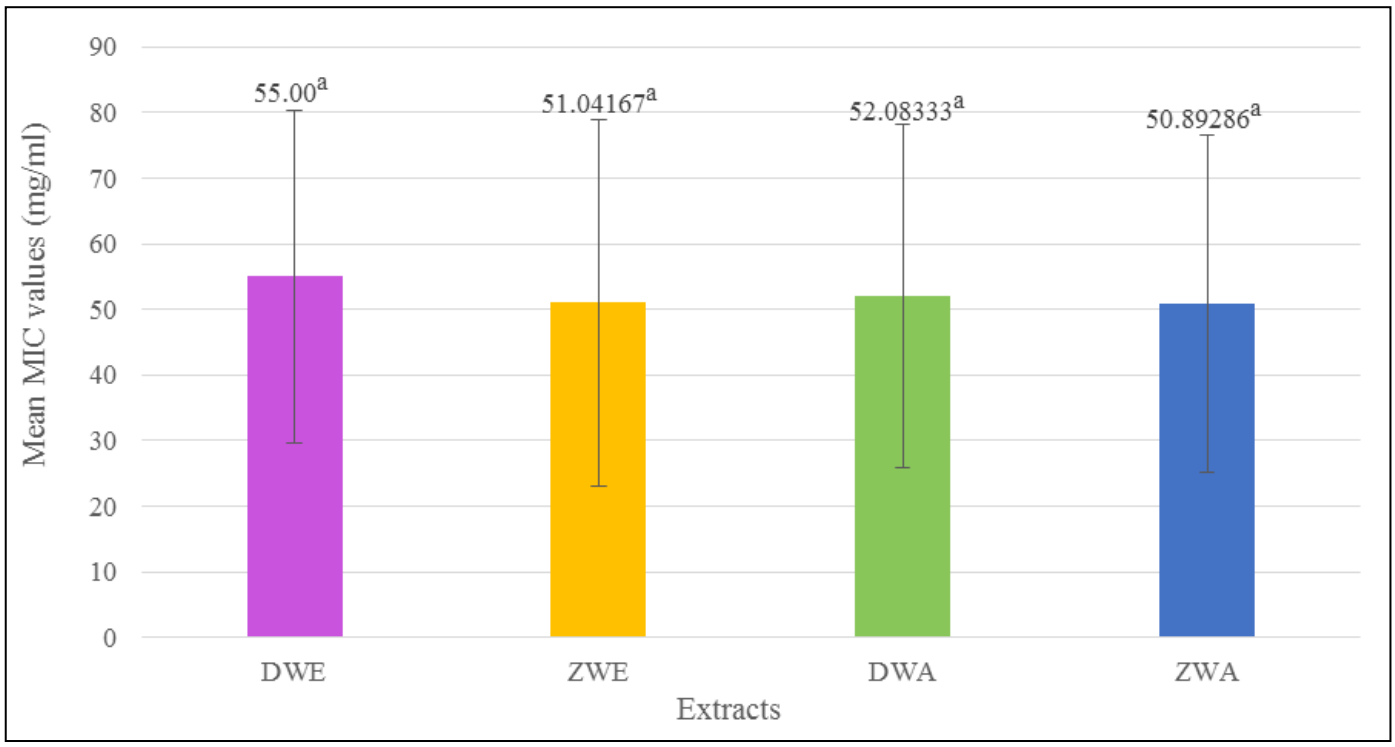

Fig 3: The mean MIC of all the extracts.

Values are expressed as mean ± standard deviation (n= 15 for DWE, n= 18 for ZWE and DWA and n= 21 for ZWA).

Relationship between polyphenolic content and antimicrobial activity

To determine whether the polyphenols affected antimicrobial activity, as mentioned in literatures [34], the Pearson correlation method was done in parallel with the regression analysis. A positive linear correlation (r^2= 0.2982) was found between the total phenolic content and the inhibitory effect of the extracts (Fig 4). Similarly, the regression analysis performed between the total flavonoid content and inhibitory effect of extracts showed a positive linear correlation (r^2 = 0.4963) between the two factors (Fig 5). The fact that the flavonoid content of the extracts (Fig 2) were higher than their phenolic content (Fig 1) explained the higher r^2 value obtained in the correlation of TFC against inhibitory activity. It could thus be concluded that the antimicrobial activity of the extracts depended more on the TFC rather than on the TPC.

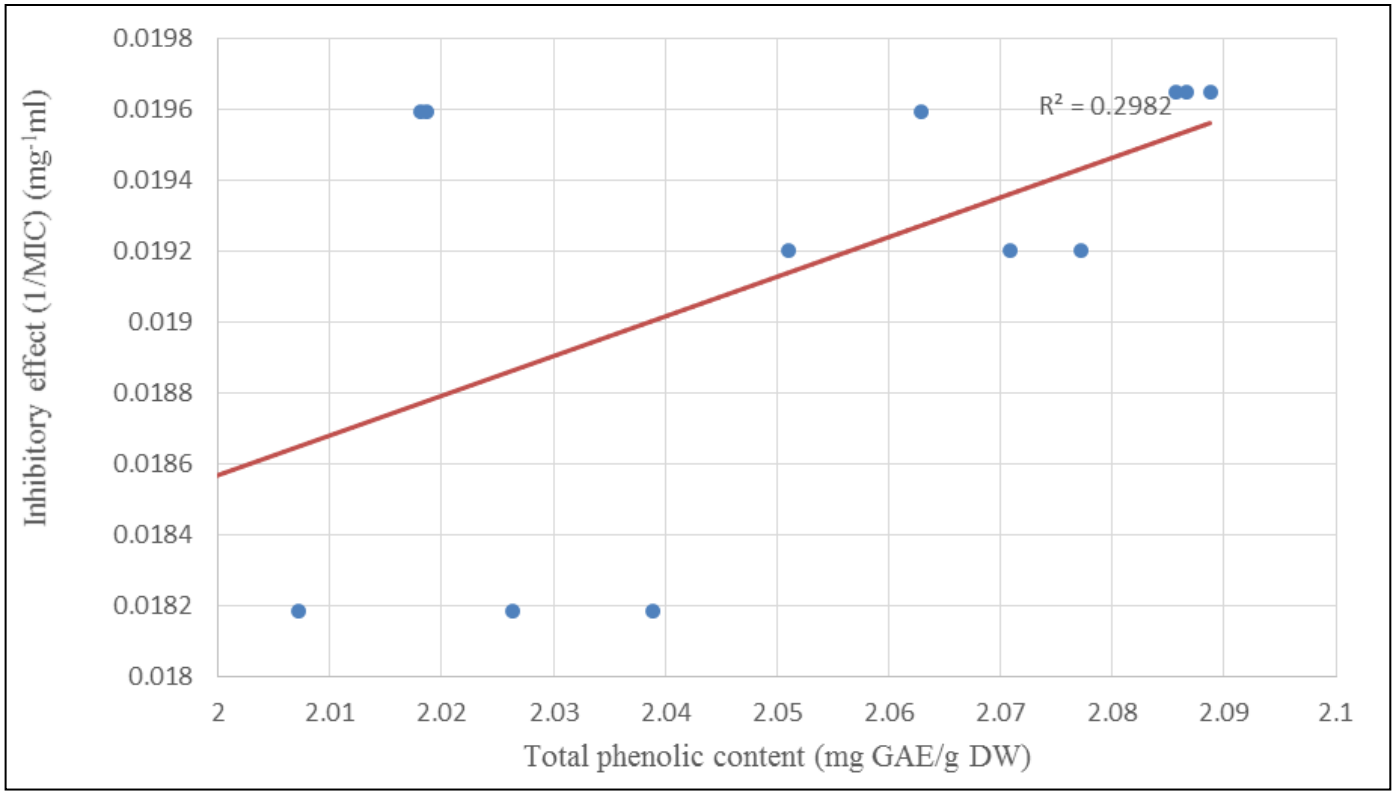

Fig 4: The fitted line plot linear regression model of inhibitory effect against TPC.

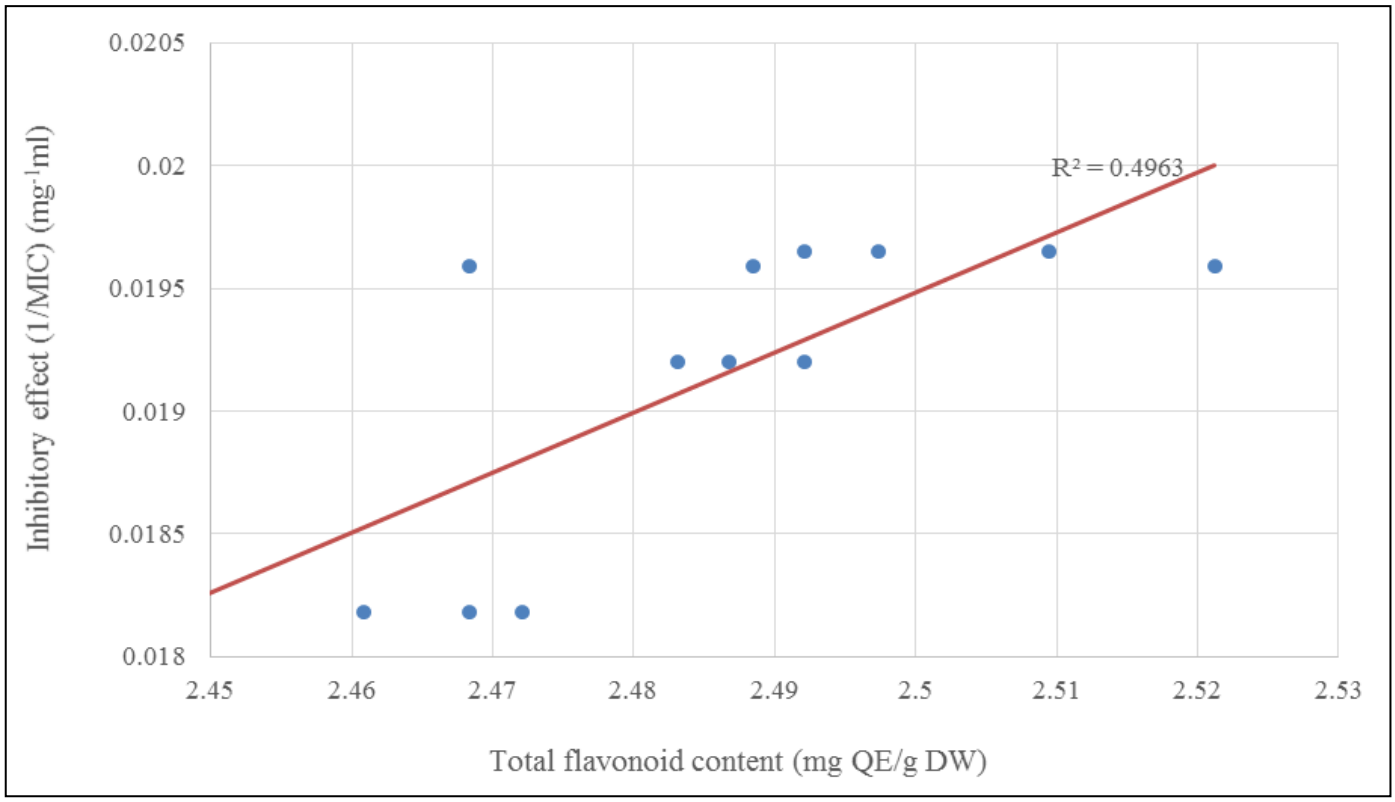

Fig 5: The fitted line plot linear regression model of inhibitory effect against TFC.

Antioxidant activity

A growing interest is being shown in the phytochemicals that have the ability to prevent or delay the oxidation of certain molecules to be used as active ingredients in complementary medicines [35]. In this study, the DPPH radical scavenging assay was used to determine the antioxidant potential of *Moringa*. The mechanism of reaction in this assay is based on the single electron transfer (SET).

From the results obtained, the IC_{50} values varied from 5.362 ± 0.146 mg/ml to 0.617 ± 0.005 mg/ml with the extract DW having the highest value; indicating lowest scavenging capacity while ZWA having the lowest value; indicated highest scavenging activity (*Fig* 6). Ascorbic acid had an IC_{50} of 0.0056 mg/ml and was found to be 110-fold more potent than ZWA.

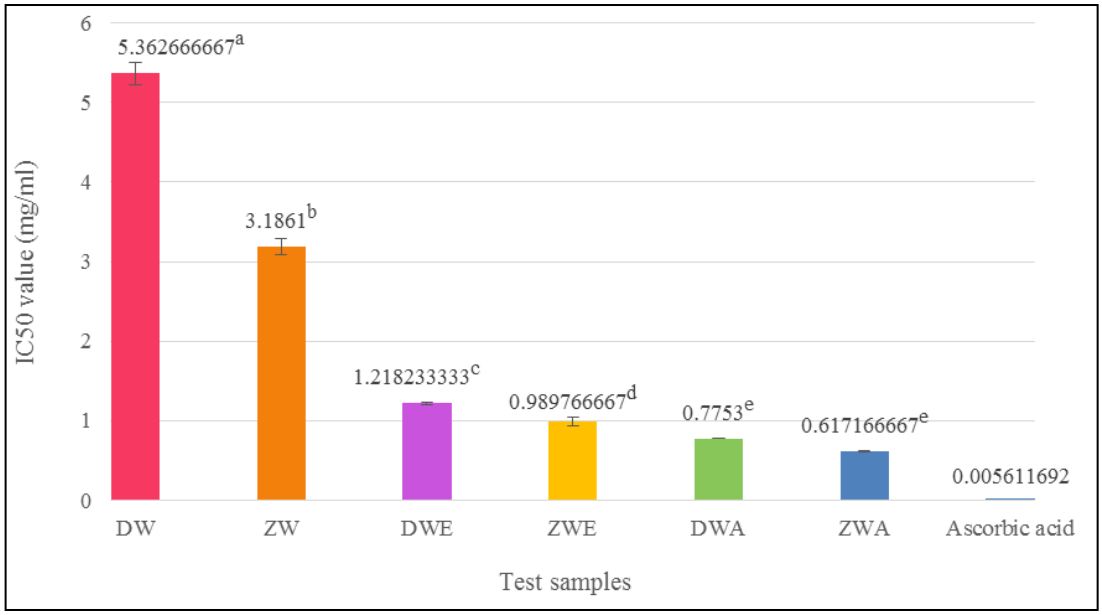

Fig 6: The DPPH IC_{50} values of the extracts and the standard ascorbic acid.

The IC_{50} values are expressed as mean ± standard deviation (n=3). Significant differences were observed in the IC_{50} values of the extracts ($p<0.05$), except for values with the same superscript letter.

The variation in the scavenging activity between the extracts (*Fig* 6) could be associated to the solvent in which they were extracted [34] or dissolved [36]. It could be observed that the extracts that were dissolved in Zamzam water showed higher scavenging capacity than those that were dissolved in deionised water. As mentioned earlier [36], the activity of the antioxidants may be interfered by hydrogen bonds which are formed between the solvent and the latter. Based on this statement, it could be possible that the Zamzam water that contained a higher number of electrolytes [4] than the deionised water could have influenced the antioxidant activity.

According to a paper [37], flavonoids and phenols which are extracted by water do not show antimicrobial activity and they are significant only in antioxidant studies; thus explaining the response of the water extracts to the antioxidant assay (*Fig* **6**). The fact that the acetone extracts contained more phytochemicals (*Table* 1) could explain the reason for the higher scavenging activity when compared to the other extracts [38].

Relationship between polyphenolic content and antioxidant activity

Previous literatures mentioned that phytochemicals like phenolic compounds [38] and flavonoids [39] are known to possess antioxidant properties. Indeed, a positive linear relationship was found to be present between polyphenolic content and scavenging activity of the extracts (*Fig 7* and Fig 8). The scavenging activity was computed as inverse of the IC_{50} value because a low IC_{50} value indicated a high scavenging capacity. In this study, a positive linear correlation (r^2= 0.8662) was observed between the total phenolic content and DPPH scavenging capacity (*Fig 7*). This shows that extracts having higher phenolic content have the ability to scavenge DPPH to a greater extent than those that have poor TPC. Similarly, a positive linear interaction (r^2=0.7835) was found between the total flavonoid content and the DPPH scavenging activity (Fig 8). Hence, it could be concluded that the antioxidant activity of the extracts is significantly dependent on their phenolic and flavonoid content.

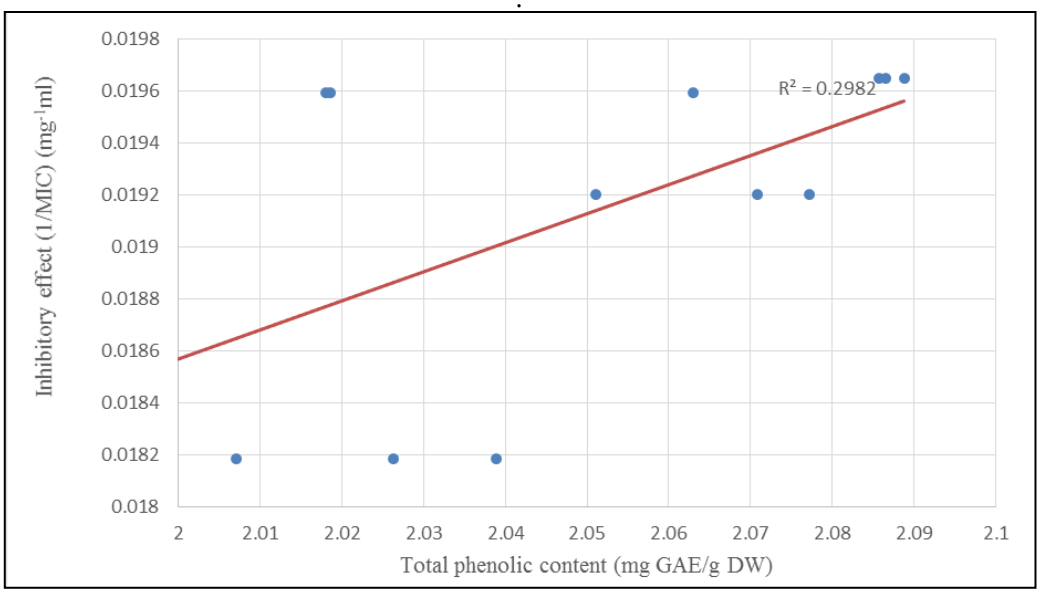

Fig 7: The fitted line plot linear regression model of DPPH scavenging capacity against TPC.

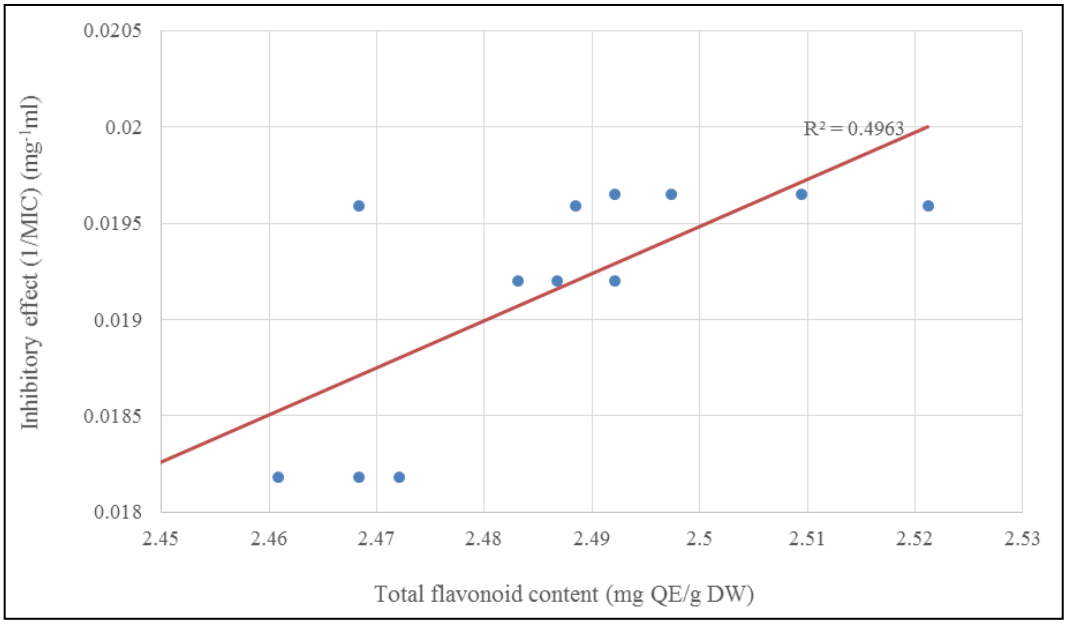

Fig 8: The fitted line plot linear regression model of DPPH scavenging activity against TFC.

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