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Investigation of phytochemicals and antioxidant activities in the leaves methanolic extract from *Moringa oleifera* plants grown in Bangladesh

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Abstract

Moringa oleifera is a plant with various medicinal benefits. Medicinal properties attribute to the presence of various antioxidants. However, antioxidant activities of *Moringa oleifera* leaves from different regions of the world have different ranges. This study was designed to evaluate the antioxidant activity of *Moringa oleifera* grown in Bangladesh. Dried leaves of *Moringa oleifera* were extracted in methanol. Qualitative and quantitative analyses were done for the presence and content of phytochemicals of *Moringa oleifera* leaves methanolic extract (MOLME). Total phenolics and flavonoids contents were determined as 97.33 ± 1.12 mg/gm of dry extract as GAE and 32.03 ± 2.53 mg/gm of dry extract as CAE, respectively. Antioxidant activities were determined in terms of DPPH, ABTS and lipid peroxidation inhibition assay. IC₅₀ values of DPPH, ABTS and lipid peroxidation inhibition were found 554.47 ± 5.21 µg/ml, 41.33 ± 0.07 µg/ml and 743.14 ± 6.67 µg/ml respectively. Phytochemical contents and antioxidant properties from different regions were compared with the findings of our study. It was observed that, phytoconstituents content and antioxidant potentials of *Moringa oleifera* vary with different geographical locations. Quality of soil, which is controlled by various factors such as nutrients, water content *etc.*, leads to the variations in phytochemical content and antioxidant properties of MOLME.

Keywords: MOLME, phytochemicals, antioxidants, geographical positions

1. Introduction

The notion of plant foods always reduce the risk of developing chronic diseases is partly underpinned by the fact that plants act as reservoir of diverse phytochemicals with enormous antioxidant potentials^[1]. Chronic diseases like atherosclerosis, cancer, diabetes, cardiovascular disease, ageing and inflammatory diseases can be caused by imbalance between formation and neutralization of reactive oxygen species (ROS) which damage nucleic acids, proteins and lipids^[2, 3]. The harmful effects of these reactive oxygen species or free radicals causing potential biological damage is termed as oxidative stress. Antioxidants help to inhibit free radicals production to prevent oxidative stress, thus prevents those chronic diseases^[3, 4]. Biological systems have antioxidant defence mechanisms such as catalase, superoxide dismutase, peroxidase glutathione system *etc.* which protect oxidative damages and repair enzymes to remove damaged molecules^[5]. However, these defence mechanisms often hit the skids; hence regular intake of antioxidants through dietary means is required^[5]. Synthetic antioxidants, which are currently used in the food industry, are responsible for liver damage and carcinogenesis^[5-8]. On the contrary, phytoantioxidants have lesser side effects as compared to their synthetic counterparts. Thus, persistent search for novel natural antioxidants becomes inevitable^[9]. Of note, plants are the reservoir of natural antioxidants.

Moringa oleifera, also known as drumstick tree or horseradish tree. The plant is indigenous to Africa, Arabia, South America, Caribbean Islands and Indian subcontinent including Bangladesh^[10]. It is widely used in ethnobotany and is thought to cure a variety of diseases^[10]. *Moringa* has long been a part of Ayurvedic medicine in India and is understandably referred to as the "Miracle Tree". Even if all parts of the *Moringa* are edible, leaves and pods are used most frequently. The leaves of the plant are utilized as a nutritional supplement, are considered to boost the immune system^[11]. The leaves can be eaten fresh, cooked, or stored as a dried powder for later use as a food additive. Many important bioactive compounds like quercetin, kaempferol, zeatin, campesterol, sitosterol *etc.* which attribute to the various medicinal properties of *Moringa*^[12]. *Moringa* is a universally recognized medicinal plant, however, in the context of that geographical position affects the production of phytochemicals with regard to the amount and type, even with its bioactivities,

examining the plant for its bioactive components from different regions of the world in search of variations in active compounds, certainly makes sense [13]. There are few reports on the identification of phytochemicals and antioxidants of *Moringa oleifera* leaves from Nigeria, India, Jamaica, Pakistan, Malaysia, Egypt, and Indonesia [14-20]. Though a previous study was reported from this region long ago [21], only DPPH radical scavenging assay and some qualitative phytochemical tests were not sufficient to draw a comparison with others. Therefore, focusing in detail analysis, the present study was undertaken to investigate and compare the phytochemical content and antioxidant potentials of leaves of *Moringa oleifera* of Bangladesh.

2. Materials and Methods

2.1 Chemicals and reagents

Folin-ciocalteu reagent (FCR), Sodium carbonate, Gallic acid, Catechin, Sodium hydroxide, Ascorbic acid, DPPH (2, 2-diphenyl-1-picryl-hydrazyl), ABTS• (2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid, Trichloro acetic acid (TCA), Thiobarbituric acid (TBA) were purchased from Sigma, MO, USA. Aluminum chloride and Sodium nitrite were purchased from Carl Roth, Germany. All other chemicals used in this study were of analytical grade.

2.2 *Moringa oleifera* leaves methanolic extract (MOLME) preparation

Moringa oleifera leaves extract was prepared according to the method as described by Olayaki *et al.*, 2015 with slight modifications [22]. Mature leaves were collected from the local area and authenticated by Department of Botany, University of Rajshahi, Bangladesh. The leaves were then rinsed with clean water, and air-dried at room temperature. After complete drying, leaves were grinded into coarse powder. 100gm of the powder was soaked in 400ml of Methanol. After 24 hours of stirring, supernatant was separated kept in a beaker. 100ml methanol was further added and previous step was repeated. The resulting extract was filtered through Whitman No.1 filter paper. Afterwards, the solvent was evaporated and the residue was kept in 4°C until further analysis.

2.3 Qualitative phytochemical analysis

Qualitative analysis of phytochemicals such as alkaloids, carbohydrates, flavonoids, glycosides, triterpenoids, resins, saponins, steroids, tannins and Coumarins was carried out by dissolving samples in specific reagents using the method as described by Sivaraman *et al.*, 2010 [23].

2.4 Quantitative phytochemical analysis

2.4.1 Determination of total phenolics

Total phenolics content was measured using the method as described by Kubola *et al.*, 2011 [24]. Briefly, 2.25ml of Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) was mixed with 300µl of extract and kept for 5 minutes at room temperature. Then, 2.25ml of sodium carbonate (60g/l) solution was added to the mixture and the absorbance was taken at 725nm after 90 minutes of incubation at room temperature. Gallic acid was used as standard and total phenolics content was measured in terms of gallic acid equivalent per gram of dry weight (mg GAE/g dry extract) based on the standard curve. Total phenolics content of MOLME was calculated using the following formula $(C \times V) \div m$, where 'C' is the concentration of gallic acid, established from calibration curve in mg/ml; 'V' is the volume of plant extract in ml and 'm' is the weight of dry plant extract in

gram. Total phenolics content was reported as mg GAE/g dry extract of extract by reference to a standard curve ($y = 0.007x + 0.029$ $R^2 = 0.999$) (Fig 1A).

2.4.2. Determination of total flavonoids

Total flavonoids content was determined using the method described by Bakar *et al.*, 2009 with slight modifications [25]. Firstly, 0.50ml of crude extract (1mg/ml DW) were mixed with 2.25ml of distilled water and then 0.15ml of 5% NaNO₂ solution; 0.3ml of 10% AlCl₃ solution was added after 6 min of incubation, and the mixture was allowed to stand for 5 minutes. Then, 1ml of 1M NaOH solution was added. Then the mixture was vortexed and absorbance was measured immediately at 510nm. Total flavonoids content was calculated from calibration curve and the results were expressed as catechin equivalent per gram of dried sample (mg CAE/gm). Total flavonoids content of MOLME was calculated using the following formula $(C \times V) \div m$, where 'C' is the concentration of catechin, established from calibration curve in mg/ml; 'V' is the volume of plant extract in ml and 'm' is the weight of dry plant extract in gram. Total flavonoids content was reported as mg CAE/gm of dry extract by reference to a standard curve ($y = 0.003x + 0.044$ $R^2 = 0.988$) (Fig 1B).

2.5 *In vitro* antioxidant assays

2.5.1 DPPH free radical inhibition assay

Antioxidant activity was determined by the DPPH method as described previously by Bakar *et al.*, 2009 with some modifications [25]. In short, a solution of DPPH (3ml, 60µM) prepared in methanol was mixed with 1ml sample. Then the reaction mixture was vortexed and left at 25°C in the dark for 30 minutes. Ascorbic acid was used as reference compound. Absorbance at 517nm was taken with a spectrophotometer using methanol as a blank. Radical scavenging activity was measured using following formula:

$$\% \text{ of Inhibition} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$$

Where, A control is the absorbance of the control (DPPH radical solution without test sample) and A sample is the absorbance of the test sample.

2.5.2 ABTS free radical inhibition assay

The method of Re *et al.*, 1999 was used to determine ABTS radical scavenging activity [26]. ABTS radical was obtained by reacting 7mM ABTS stock solution with 2.45mM potassium persulfate solution and the mixture was left in the dark at room temperature for 13 hours before use. Solution of ABTS radical (stable for 2 days) was diluted with water to obtain an absorbance of 0.70±0.02 at 734nm. Then, 1ml of various concentrations of sample was mixed with 3.0ml of ABTS++ solution. After 6 minutes of incubation absorbance was measured at 734nm. Ascorbic acid was used as positive control. The formula for calculating ABTS Radical scavenging activity:

$$\% \text{ of Inhibition} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$$

Where, A control is the absorbance of the control (ABTS radical solution without test sample) and A sample is the absorbance of the test sample.

2.5.3 Lipid peroxidation inhibition assay

The ability of MOLME extract to inhibit lipid peroxidation was determined according to the method as described by

Mosbah *et al.*, 2018 with slight modification [27]. Homogenate was prepared from freshly excised mice liver mixing with 10X cold PBS then centrifuged at 3000 rpm for 10 minutes. Extract and each fraction were added to 100 μ l of 10mM ferrous sulphate followed by the addition of 100 μ l previously prepared homogenate. Then the mixture was incubated at 37°C for 1 hour. The reaction was stopped by the addition of 500 μ l 28% TCA, followed by 380 μ l of TBA and the mixture was then heated at boiling temperature for 20 min. After centrifugation at 3000 rpm for 10 minutes, the intensity of the malondialdehyde-TBA complex in the supernatant was measured at 535nm. Lipid peroxidation inhibition was assessed by the following formula:

$$\% \text{ of Inhibition} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$$

Where, A control is the absorbance of the control (Lipid radical solution without test sample) and A sample is the absorbance of the test sample.

2.6 Statistical analysis

All values were expressed as mean \pm SD (Standard deviation).

3. Results

3.1 Qualitative analysis of phytochemicals

The results of phytochemical screening of MOLME are shown in Table 1. Alkaloids, carbohydrates, flavonoids, glycosides, resins and tannins were detected in the plant extract.

3.2 Total phenolics and flavonoids contents

Total phenolics and flavonoids contents were determined 97.33 \pm 1.12mg/gm of dry extract as GAE and 32.03 \pm

2.53mg/gm of dry extract as CAE, respectively shown in Table 2.

3.3 *In vitro* antioxidant activity of MOLME

3.3.1 DPPH scavenging activity

At the concentration of 800 μ g/ml the percentage inhibition for MOLME and reference compound ascorbic acid (AA) were 68.77 \pm 0.89 and 94.43 \pm 0.42 respectively. MOLME exhibited concentration dependent DPPH inhibition potential. The IC₅₀ values of MOLME and AA were 554.47 \pm 5.21 μ g/ml and 184.831 \pm 9.53 μ g/ml respectively. The slope of the DPPH reduction percentage plot was used as an indicator of antioxidant capacity (Fig 2).

3.3.2 ABTS radical scavenging activity

At the concentration of 50.0 μ g/ml, inhibition percentages of MOLME and AA were 58.27 \pm 0.37 and 98.92 \pm 0.10 respectively. The inhibitory activity of MOLME and AA were consistent with the increase of concentration (Fig 3). IC₅₀ values of both MOLME and AA n compound against ABTS were 41.33 \pm 0.07 μ g/ml and 14.08 \pm 1.20 μ g/ml respectively.

3.3.3 Lipid peroxidation inhibition activity

MOLME displayed reducing capacity against lipid peroxidation in a concentration dependent manner (Fig 4) though it was low relative to AA. The inhibition percentage of MOLME was 1.26 \pm 0.722 to 70.78 \pm 0.551 at concentrations ranged from 50 μ g/ml to 1000 μ g/ml while AA showed maximum lipid peroxidation inhibition of 88.08 \pm 0.826% at the concentration of 1000 μ g/ml.

IC₅₀ values of MOLME and AA for DPPH, ABTS and lipid peroxidation inhibition are given in Table 3.

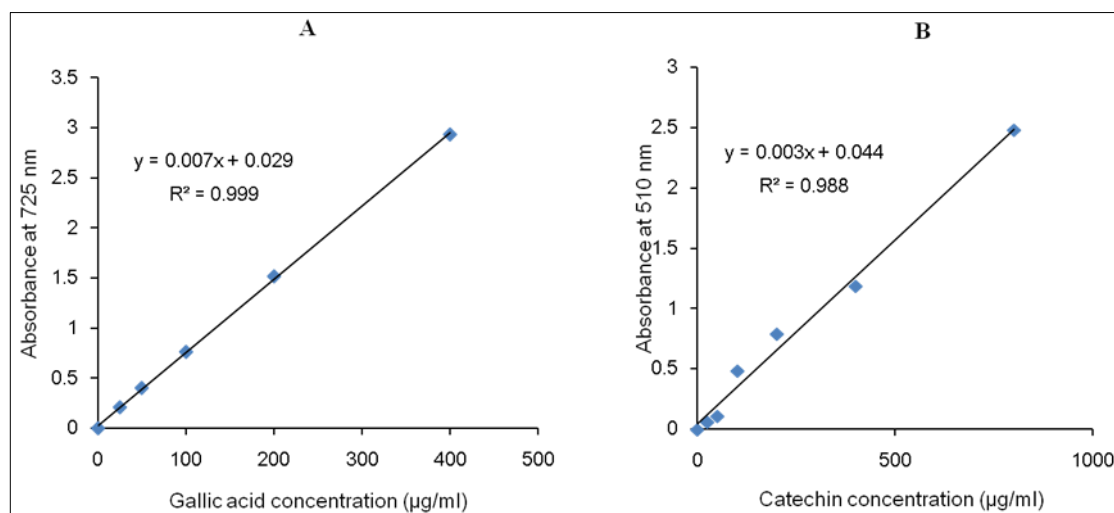


Fig 1: Standard curve of gallic acid (A) and catechin (B) for the determination of total phenolics and total flavonoids respectively. The plot represents the mean \pm SD from three experiments.

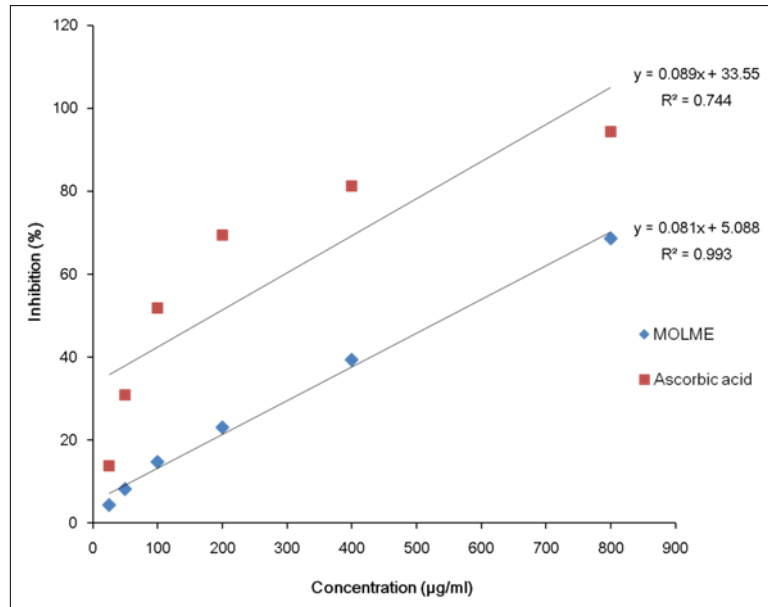


Fig 2: DPPH free radical inhibition activity of MOLME and Ascorbic acid. The results were expressed as the percentage of DPPH inhibition. The plot represents the mean \pm SD from three experiments.

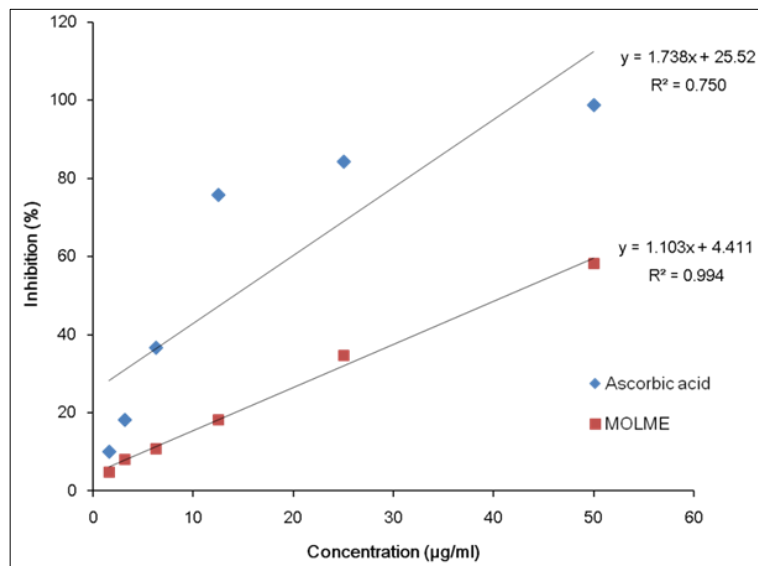


Fig 3: ABTS free radical inhibition activity of MOLME and Ascorbic acid. The results were expressed as the percentage of ABTS inhibition. The plot represents the mean \pm SD from three experiments.

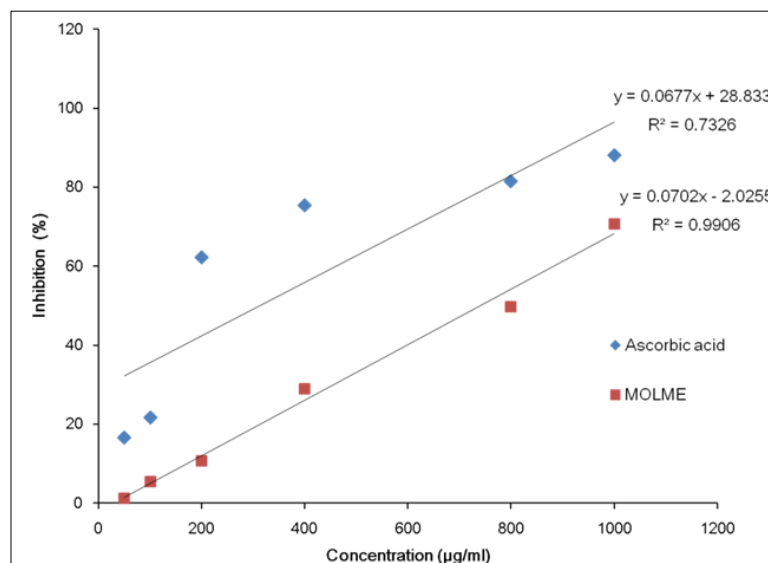


Fig 4: Lipid peroxidation inhibition activity of MOLME and Ascorbic acid. The results were expressed as the percentage of lipid peroxidation inhibition. The plot represents the mean \pm SD from three experiments.

Table 1: Qualitative analysis of phytochemicals of MOLME.

Tests for compounds	Name of the tests	Presence of the compound
Alkaloids	Dragendorff's Test	+
	Hager's test	+
	Wagner's test	-
	Mayer's test	-
Carbohydrates	Anthrone's test	+
	Benedict's test	+
	Fehling's test	+
	Molisch test	+
Flavonoids	Flavonoid test	+
Glycosides	Glycosides test	+
Triterpenoids	Test for triterpenoids	-
Resin	Test for Resins	+
Saponins	Test for Saponins	-
Steroids	Liebermann-Burchard's test	-
	Salkowski reaction	-
Tannins	Test for tannin	+
Coumarins	Test for coumarins	-

'+' indicates presence and '-' indicates absence of phytoconstituents.

Table 2: Phytochemical contents of MOLME

Total phenolics	97.33 ± 1.12 mg/g GAE ^a
Total flavonoids	32.03 ± 2.53 mg/g CAE

GAE: gallic acid equivalent; CAE: catechin equivalent.

^a Data were expressed as mean ± standard deviation (SD). (n=3)

Table 3: Antioxidant activity of MOLME and Ascorbic acid in different assay

	IC ₅₀ values ^a (µg/ml)		
	DPPH	ABTS	Lipid peroxidation
MOLME	554.47 ± 5.21 ^b	41.33 ± 0.07	743.14 ± 6.67
Ascorbic acid	184.83 ± 9.53	14.08 ± 1.20	315.97 ± 4.93

^a IC₅₀ value: Effective concentration required for 50 % of a maximum inhibition.

^b Data were expressed as mean ± standard deviation (SD). (n=3)

4. Discussion

Phytochemical contents and antioxidant properties of *Moringa oleifera* leaves extract were assessed previously in India, Nigeria, Jamaica, Malaysia, Indonesia, Egypt, and Pakistan. However, this seems to be first study regarding detailed analysis of phytochemical content and antioxidant activity of *Moringa* leaves sourced from Bangladesh except for a study measuring just DPPH radical scavenging activity and qualitative analysis of phytochemicals. Different agroclimatic conditions from different geographical positions can result in diverse phytochemical content and as it is well established that phytochemical content attributes to the antioxidant activities therefore, resulting difference in phytoconstituents might cause ultimate variations in antioxidant potentialities [28]. In this study, phenolics content of MOLME was found to be 97.33mg of GAE which is significantly less than the phenolics content of *M. oleifera* leaves extract from Egypt and Nigeria [19, 29]. Total phenolics contents were 113.3mg and 129.44mg of GAE in Nigeria and Egypt respectively. However, Sharad *et al.*, (2017) from Rajasthan, India reported least phenolic content of 9.58mg among the reported regions [15]. Consistently, results of the same study suggested lowest flavonoid content of 2.3mg. On the other hand, result of our study showed 32.03mg of total flavonoids which was closer (20.43mg) to the findings of Erian *et al.*, (2016) from Egypt [19]. Moreover, flavonoids content from Nigeria reported to be 91.2mg of CAE [29]. Considering the variations in phytochemical contents of *Moringa* leaves from different region around the world, it is overt that antioxidant properties must also vary with different geographical positions. Antioxidant property of MOLME was

determined in terms of DPPH, ABTS and Lipid peroxidation inhibition assays. IC₅₀ values from different tests were taken into consideration to identify and compare antioxidant potentials of MOLME. The IC₅₀ value is the concentration required to reduce 50% of the free radicals. Low IC₅₀ values correspond to high antioxidant activity [16]. IC₅₀ of 554.47 ± 5.21µg/ml in DPPH inhibition assay was found in our study which was quite comparable to the finding (610µg/ml) of Sharad *et al.*, 2017 from Rajasthan, India [15] and to the finding (720µg/ml) of Abdul aziz *et al.*, 2015 from Malaysia [18]. However, another group of investigators from Indonesia reported significantly less IC₅₀ value in DPPH scavenging assay, which was 49.30µg/ml [20]. Results of these investigations postulated greater antioxidant potentials in the leaf of *Moringa oleifera* grown in Indonesia than the plant grown in Bangladesh, India, and Malaysia. In ABTS scavenging assay, we found an IC₅₀ of 41.33 ± 0.07µg/ml which was slightly comparable to the IC₅₀ value (11.73µg/ml) of *Moringa* leaves grown in Indonesia [20]. IC₅₀ values differ from test to tests and region to region. We further found, in case of lipid peroxidation assay 50 % inhibition of free radicals occurred at the concentration of 743.14 ± 6.67µg/ml of MOLME. Lack of literature regarding lipid peroxidation inhibition assay suggests more works with lipid peroxidation from different regions in the world.

Siddharaj and colleagues from India reported that different agroclimatic conditions can result in different antioxidant capacities [11]. However, that study was not focused on the quality of soil which is also an important factor affecting the quality and quantity of bioactive principles in plants. There seems to be no direct study yet done on the effect of other soil

factors such as pH, soil nutrient levels, and soil type on the antioxidant capacity in *Moringa* leaves [11]. Therefore analyses of soil factors could be beneficial in providing a broader picture of the impact of environmental conditions on antioxidant capacity in *Moringa oleifera*. Researchers in Pakistan investigated the impact of seasonal and geographical changes on antioxidant activity of *Moringa* leaves. Not surprisingly, it was found that antioxidant activity varies depending on season and location of the plant from where it was collected [17]. Therefore, aforementioned factors might contribute to the variation of phytochemical contents and antioxidant potentials of *Moringa oleifera* leaves.

5. Conclusion

Numbers of studies are going on to identify potent antioxidants for health and food industry. Antioxidants may be derived from animals and synthetic chemical preparations, but antioxidants from plants are with low adverse effects and universally available. *Moringa oleifera* is a medicinally important, commonly consumed plant and has known to be a true source of natural antioxidants. In this study we intended to evaluate phytochemical and antioxidant contents of *Moringa oleifera* leaves and also compared these outcomes with other findings from different agroclimatic conditions in the world. We have drawn a comparison of phytochemical and antioxidant contents among India, Nigeria, Egypt, Indonesia, Malaysia and Bangladesh. In the light of these findings, it can be concluded that phytoconstituents of *Moringa oleifera* differ from parish to parish, perhaps depending on the nature of soil. Further studies are required to identify specific factors causing this variation which could be beneficial to use this plant more effectively as a true source of natural antioxidants.

Conflict of Interest: The authors declare no conflict of interest.

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