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(RESEARCH ARTICLE)

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Evaluation of antioxidant potential and toxicological safety of ethanol extract of *Morinda citrifolia* leaf

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Abstract

This study explores the potential of *Morinda citrifolia* leaf through a modified ethanol extraction method which employed variation in the percentage of ethanol used for the leaf's extraction that differs from previous approaches. With established reports from the fruits for its antioxidant capacity, we looked into its leaf to investigate its effectiveness and safety. Based on the pathogenesis of oxidative stress induced ailments, the studies evaluate the in-vivo antioxidant and toxicological effects of the *M. citrifolia* leaf by determining DPPH, ABTS, superoxide, nitric oxide radical scavenging abilities, reducing power, lipid peroxidation, β-carotene bleaching inhibition and metal chelating potentials of *M. citrifolia*. We carried out LD_{50} and sub-chronic (28-day) toxicity assay and histopathology examination of the essential organs. The results of the study showed that ethanolic extracts of *M. citrifolia* (EMC) has strong potential as an effective antioxidant and it has little or no toxicological effect. The results align with previous studies carried out on the fruits of *M. citrifolia*. This study provided preliminary evidence that supports the potential of the ethanolic extracts of *M. citrifolia* leaf for further studies as a viable antioxidant agent that can be incorporated as supplements or developed from its bioactive components as a modern drug for oxidative stress induced ailments.

Keywords: *Morinda citrifolia;* Antioxidant potential; Toxicological safety; Toxicity; Oxidative stress; Immunostimulatory

1. Introduction

Morinda citrifolia (popularly known as Noni) is a member of the Rubiaceae family and is considered one of the most important medicinal plants due to its various applications. Studies have reported that the different parts of Noni contain about 160 phytochemicals with proven nutraceutical properties (Ali et al., 2020), which allows it a wide range of therapeutic which includes anticancer, antidiabetic, anti-fungal, anti-epileptic, anti-inflammatory, antioxidant and immunostimulatory amongst many others (Ali et al., 2020). All these benefits offer a good market with its fruits and leaves primarily sold as juices, tea, and capsules (Singh, 2012).

Plants have always played a significant role in the advent of modern medicine; likewise, they are available in nature and inexpensive making them more accessible for use within the low-income class and developing countries. Hence, proper studies can provide us with cheaper alternatives to standard therapies with lesser side effects (Salmerón-Manzano et al., 2020). This problem, amongst other factors, necessitates effective methods by which these adults can control the epidemic.

Most of the antioxidant and toxicological studies that have been carried out using *M.citrifolia* have focused more on the potential of the fruit; however, recent experiences with local traditional medicine experts have pointed more towards

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the viability of the leaves. Different studies have shown that the leaves, roots, and fruits are the most sought-after parts for therapeutic remedies, with the leaves being the most preferred (González et al., 2010; Yabesh et al., 2014). Despite the popularity and increase in traditional alternatives, there is a need to ascertain the safety of herbs consumed to ensure a net-positive effect. It is reported that patients often do not disclose to their physicians that they use herbal alternatives to supplement drugs (Mehta et al., 2008). Hence, we ought to ensure these herbs are safe for the public to use as they become increasingly popular. It is also known that most patients combine conventional drugs with herbal supplements (Dasgupta, 2019). A careful study of the reported herbs would help to manage effectively, the correct herbal dosage with its use based on the clinical conditions (Choi et al., 2016). We aim to study the ethanolic extracts of *M.citrifolia* leaves using a modified ethanolic extraction method and use some in-vivo assays to determine the extended viability of the leaves for antioxidant purposes and possible toxicity effects that could affect its potential for incorporation into modern medicine or jeopardize the safety of its popular consumers.

2. Materials and Methods

2.1. Plant Materials

The Noni leaves were obtained from a farm on the outskirts of Owo, Southwestern Nigeria. They were identified and authenticated by a taxonomist in the Department of Plant Science and Biotechnology, Achievers University, Nigeria. The leaves were washed thoroughly with distilled water, air-dried at room temperature for 72 hours, and pulverized into a fine-powdered form using an electric blender for further use.

2.2. Extraction, concentration, and lyophilization

An 80% acidified ethanol solution was prepared, the powdered samples were immersed for extraction for 24 hours at 40°C to extract the bioactive components in the leaves effectively. After 24 hours, the mixture was filtered using a muslin cloth, subsequently filtered and subjected to evaporation using a rotary evaporator to remove the ethanol and concentrate the *M. citrifolia* extract samples. This extraction method was modified with variation in the type of solvent, extraction time, temperature, and the use of additional steps such as fractionation/purification steps, depending on the specific requirement of the study or the compound(s) of interest. The concentrated samples were frozen, then lyophilized and stored in air-tight bags in a freezer until use. Ethanol extraction method was used due to its ability to extract a wide range of polar and semi-polar compounds including phenolics, flavonoids, and alkaloids. The uniqueness of ethanol extraction lies in balance of safety, efficiency and broad –spectrum extraction capacity.

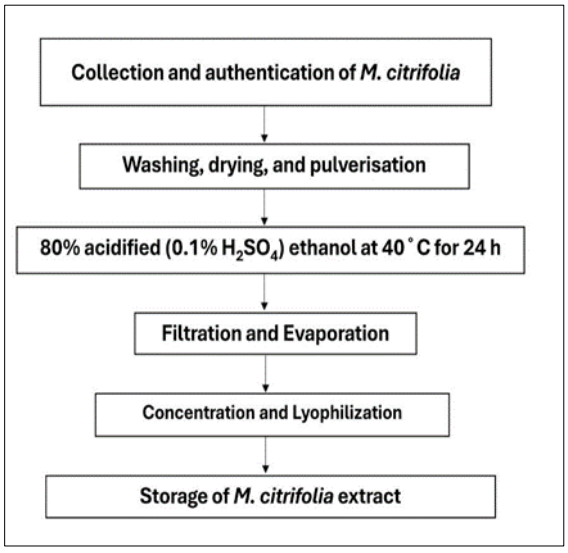


Figure 1 Flow chart showing the extraction process for *M. citrifolia* leaves

2.3. Characterization of *M. citrifolia* extract

The samples were then characterized by HPLC and subjected to phytochemical screening. HPLC is used for the separation, identification, and quantification of individual components such as anthraquiones, scopoletin, and damnacanthal. Phytochemical screening is a preliminary tests to identify classes of compounds such as alkaloids, flavonoids, phenolics, saponins, tannins and glycosides.

2.4. LD_{50} and Sub-chronic (28 days) Toxicity Assay

The LD_{50} and Sub-chronic (28 days) were carried out using the modified method of (Lorke, 1983). The 35 rat models with body weights of 150±20g were divided into seven experimental groups and were administered increasing doses of the *M. citrifolia* extract per body weight. The different dosages used in this study are: Group 1: Control; Group 2: 10 mg/kg BWT EMC; Group 3: 100 mg/kg BWT EMC; Group 4: 1000 mg/kg BWT EMC; Group 5: 1600 mg/kg BWT EMC; Group 6: 2900 mg/kg BWT EMC; and Group 7: 5000 mg/kg BWT EMC. After 28 days, the rat models were sacrificed, and the kidney and liver were collected for function assays and histopathology examinations.

2.5. Hepatic Function Assays, Kidney Function Assays, and Histopathology Examination

After the study, serum samples were collected from the rat subjects. The serum levels of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), and Gamma-Glutamyl Transferase (GGT) were determined for hepatic functions. At the same time, blood urea, nitrogen, and creatinine concentrations were used to assess kidney health status. They were determined using the Randox assay kits, respectively. Subsections of the liver and kidney from each group were administered with different dosages, collected, and fixed overnight with 10% formalin for histopathological examinations.

2.6. In-vitro Antioxidant potential studies

The potentials of the extracts as antioxidants substitutes were determined using modified methods from the various assays designed to evaluate the capacity of the plants extracts to neutralize free radicals, scavenge reactive oxygen species (ROS), and reduce oxidants. Here are common methods used to assess the antioxidant activity of *Morinda citrifolia* extract; The DPPH radical scavenging activity assay was done by the method of (Leong & Shui, 2002); ABTS radical scavenging activity assay (Re et al., 1999); superoxide radical scavenging activity was done using the NBT reduction method; nitric oxide radical scavenging activity assay by (Marcocci et al., 1994) method; ferric reducing power assay was determined by (Oyaizu, 1986) method; inhibition of Fe^{2+}-induced lipid peroxidation assay was determined by (Ohkawa et al., 1979); inhibition of β-carotene bleaching (ß-CB) (β-carotene-linolenic Acid assay) and metal chelating activity was determined by ferrous ion chelating activity assay.

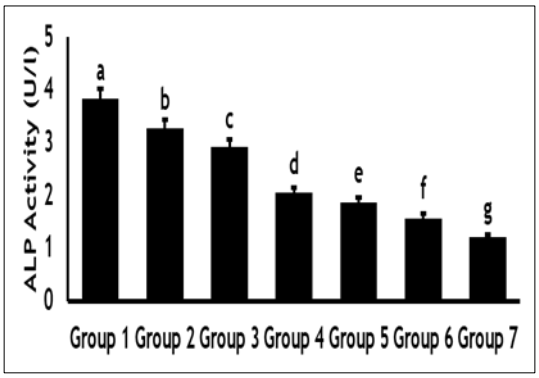
2.7. Statistical Analysis

All analyses were carried out in triplicate, representing the results as mean ± SE. The statistical analysis was conducted using one-way ANOVA. The ANOVA test indicate whether there are significant differences in antioxidant activity among the different concentrations.

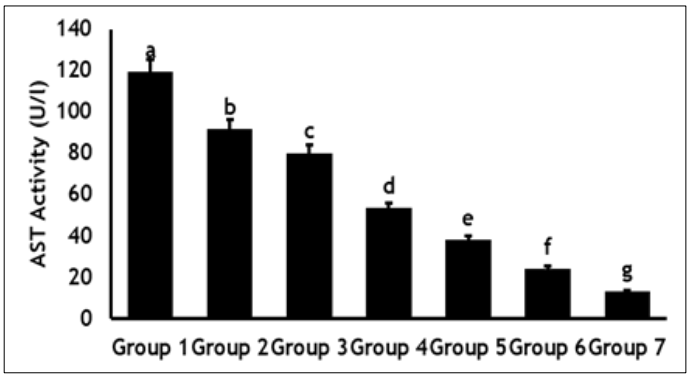
3. Results and Discussion

3.1. LD_{50} Sub-Chronic (28 Days) Toxicity Assay

3.1.1. Liver Function Assay



(A)



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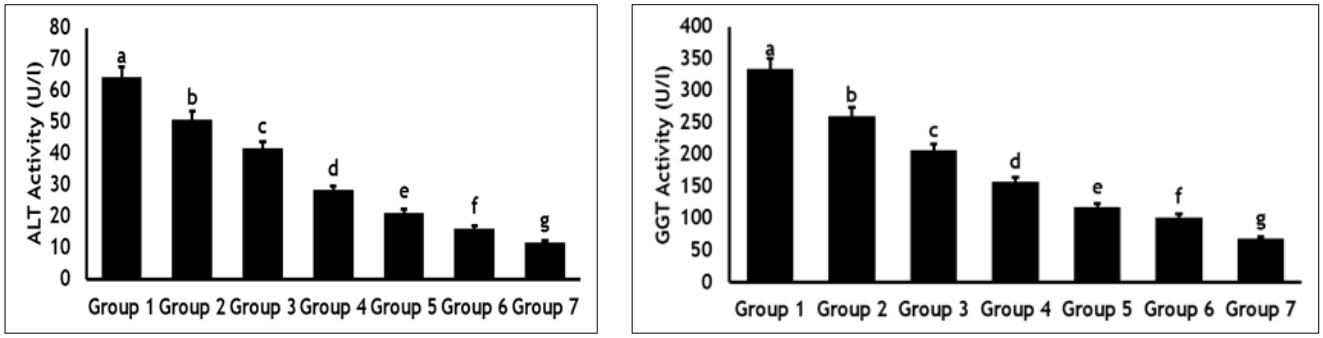


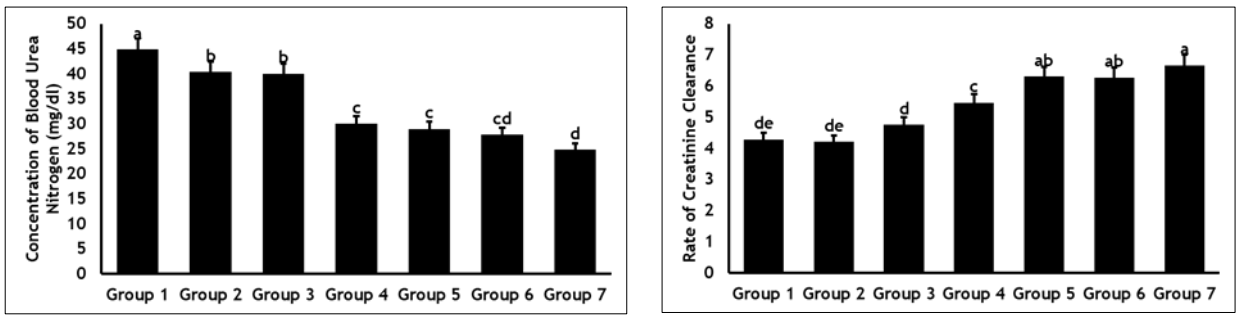
(C) (D)

Figure 2 The effects of EMC on (A) ALP activity, (B) AST activity, (C) ALT activity and (D) GGT activity

The liver is responsible for essential physiological functions, including the metabolism of toxic materials. Liver function tests estimate any potential damage that toxins can induce on the liver (Dasgupta, 2019). Some of the commonly tested enzymes serve as markers since they are released when hepatocytes are damaged; these enzymes include aspartate aminotransferase (AST), alanine aminotransferase (ALT), g-glutamyl transferase (GGT), and alkaline phosphatase (ALP) A pattern of decreasing enzyme levels was noticed across all dosages in a dose-dependent fashion. These results imply that the M*. citrifolia* extract has a hepato-protective effect on the liver, which could be due to its already-established antioxidant effects (Figure 2A-D)

From the results, we could suggest that the group fed 1000mg/kg BWT would be the optimal dose; however, severe toxicity was not established at 5000mg/kg BWT.

3.1.2. Kidney Function Assay



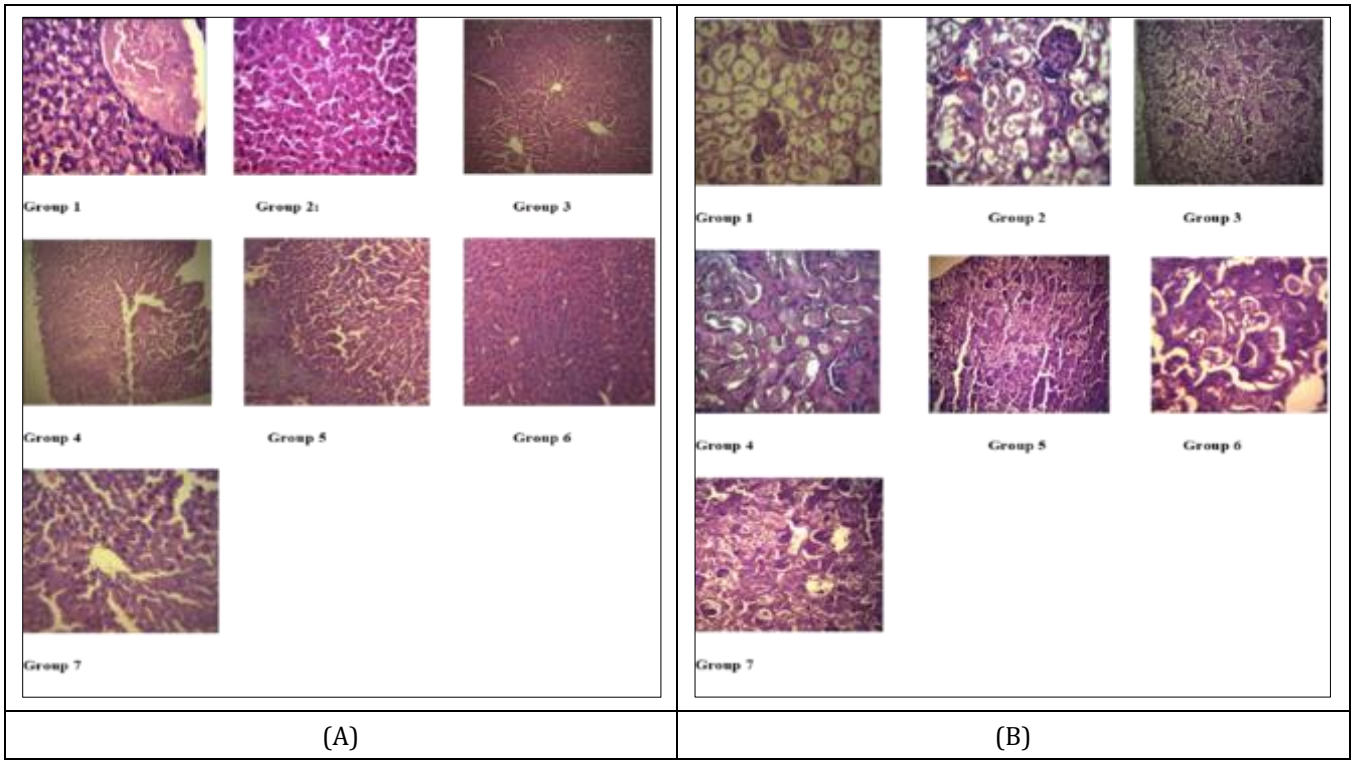
(A) (B)

Figure 3 The effects of EMC on (A) the concentration of Blood Urea Nitrogen and (B) the rate of creatinine clearance

Like the liver, the kidney is also affected by toxic compounds based on its activities surrounding blood flow, urine concentration, and excretion processes (Bartoli, 2016). Blood Urea Nitrogen (BUN) and creatinine have been used for an extended period as indicators of the whole kidney function, although with low sensitivity in clinical settings (Xie et al., 2013).

Decreasing levels of BUN in a dose-dependent manner suggests improved kidney health and function (Figure 3A). There were also increased creatinine clearance levels across the groups (Figure 3B). To complement the liver function tests, the obtained results from the kidney function tests strengthen the overall health of the essential organs. While further studies might be required to establish these effects confidently, we could propose that *M. citrifolia* leaves extracts are not toxic as hepatotoxicity had been reported in the fruit extracts (Achkar et al., 2019; Stadlbauer et al., 2005). With proper dosage administration and monitoring, the leaves extract could serve as a better substitute available freely. This also agrees with the study by Nayak et al. (2009), in which healing activities were reported with no toxicity.

3.2. Histopathological Examinations



(A) (B)

Figure 4 (A) Histological section of the liver and (B) Histological section of the kidney showing the effects of EMC

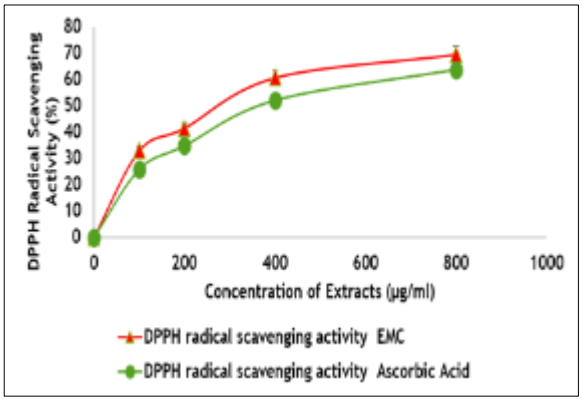
In the control group, the images show moderate portal, central venous, and sinusoidal congestion. The image suggests congestions depict an impairment to regular liver function and an increased resistance to blood flow. This should generally serve as a baseline but cannot accurately represent what other groups show regarding toxicity since we do not expect changes in the control group. In groups fed with 10mg/kg BWT to 5000mg/kg BWT, we do not see visible lesions, which infers that there was no significant sign of damage caused by the toxicity of the extracts. Hence, we could consider them safe (Figure 4A).

The control group 1 shows diffuse renal tubular degeneration from the images obtained. This depicts some baseline degeneration not induced by the *M. citrifolia* leaves extract. In groups that were administered 10 and 100mg/kg BWT, there was severe diffuse tubular degeneration and necrosis of the renal tubules. There are protein casts in their lumen. There is also moderate to severe interstitial congestion. The image suggests increased damage and obstruction of the tubules, while the interstitial congestion depicts compromised blood flow and possible inflammation. For 1000mg/kg BWT dosage, severe congestion of the renal interstitium is present. The increased amount of proteinaceous material in the lumen of the renal tubules suggests impairment of renal function due to the multiple proteinaceous material and possible toxicity. However, at 1600mg/kg BWT, there is mild congestion of the renal cortex, which results in better congestion and improvement in the impairment of renal function compared to previous dosages. At dosages 2900 and 5000mg/kg BWT, no visible lesions or damage were observed (Figure 4B)

While the results from groups fed dosages 10-1000mg/kg BWT do not fully agree with results obtained from the kidney function assays earlier, dosages 1600-5000mg/kg BWT represent improved health when compared to the 10-1000mg/kg BWT representing the potentials of the *M. citrifolia* extract. However, the reasons for the discrepancies are not established, and further studies could be conducted to resolve the differences in kidney function assays and the histopathological results.

Aside from the lesions and visible changes, we do not see notable differences in the weights of the organs. However, the high dosages beyond 1000mg/kg BWT could have long-term lethal effects on these essential organs.

3.3. Antioxidant Activities



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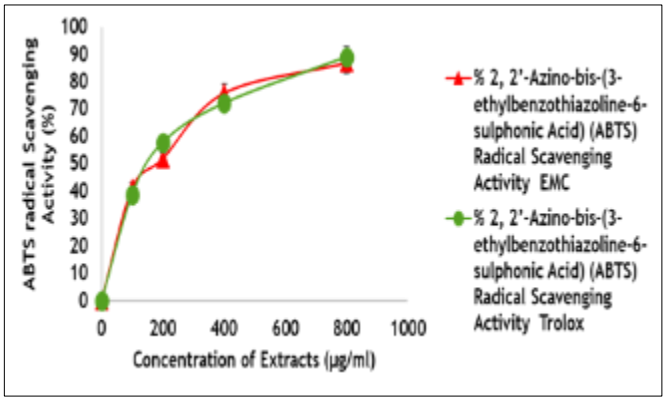

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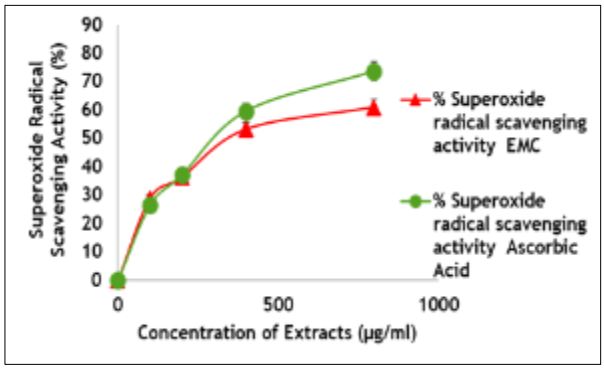

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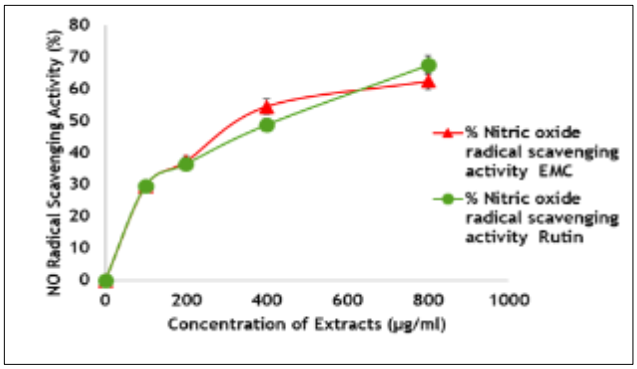
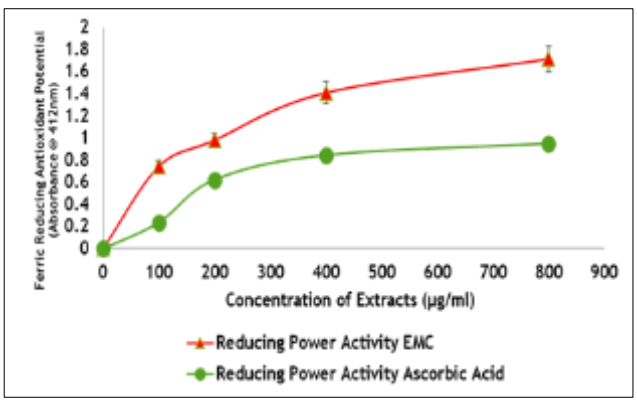


(D)

Figure 5 The effects of EMC on (A) DPPH radical scavenging activity, (B) ABTS radical scavenging activity (C) Superoxide Radical Scavenging Activity and (D) Nitric oxide radical scavenging activity

DPPH and ABTS are synthetic radicals used to assess herbal extracts' free radical scavenging activity (Li et al., 2017). Our results show an overall increase in DPPH radical scavenging activity with an increase in concentration (Figure 5A), demonstrating the potential of M. *citrifolia* extracts as a better antioxidant compared to the standard ascorbic acid. For ABTS, our results indicate that EMC and Trolox have almost equal potency in neutralizing ABTS radical action. In a dose-response relationship, we see the same potency at the low concentration and a slight increase in trolox's ABTS activity at high concentrations (Figure 5B). At medium concentration, we see a slight increase in the ABTS radical activity of *M. citrifolia*, suggesting its optimum concentration.

In SOD, we see an increase in the radical scavenging activity of ascorbic acid at higher concentrations (Figure 5C). The NO radical scavenging activity results show a similar pattern to the ABTS radical scavenging activity assay (Figure 5D).



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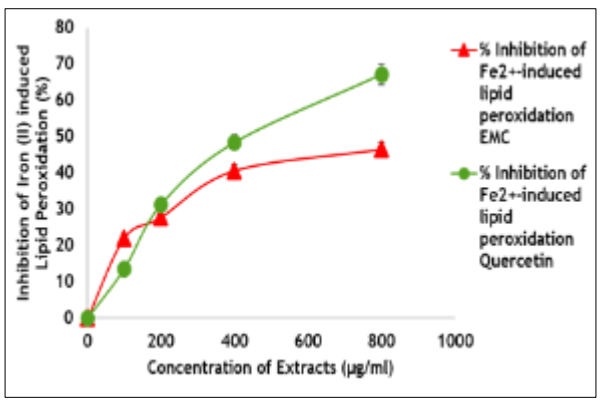

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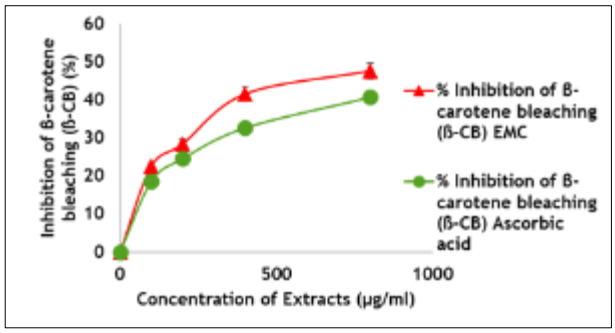

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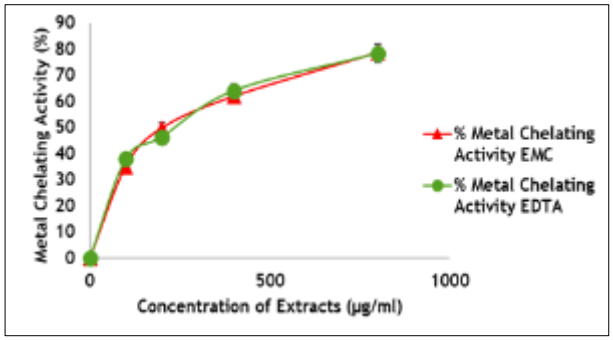

(D)

Figure 6 The effect of EMC on (A) Ferric reducing antioxidant potential, (B) Inhibition of Iron (II) induced lipid peroxidation, (C) Inhibition of β-carotene bleaching and (D) metal chelating activity

Reducing power activity measures the total antioxidant capacity by electron-donating capacity (Li et al., 2017); the intensity of the color change observed typically shows this. This suggests that *M. citrifolia* extract has more ability to donate electrons than ascorbic acid, which is the established standard based on its antioxidant properties. At the highest concentration of the extract and ascorbic acid, *M. citrifolia* seems to have double the reducing power activity of ascorbic acid and is, hence, highly potent (Figure 6A).

Quercetin is one of the commonly used flavanols, and it has been reported to lead to a decrease in lipid peroxidation (Vinayagam & Xu, 2015). Our assay results show that EMC has lower antioxidant activity potential than Quercetin, which means it is less effective at neutralizing free radicals and is less potent at inhibiting lipid peroxidation (Figure 6B). Although at low concentrations, we see an increased activity of EMC compared to Quercetin, that activity does not seem to hold.

The β-carotene bleaching assay depends on the loss of color in β-carotene due to oxidation. The inhibition of this activity by neutralizing the reactive oxygen species demonstrates its antioxidant and toxicological capacity (Dawidowicz & Olszowy, 2010). EMC showed lower antioxidant capacity to prevent the oxidative degradation of B-carotene compared to the ascorbic acid standard (Figure 6C). Overall, the reduced antioxidant capacity could be due to the extraction method used, which could influence the total phenolic content of the extract (Sarikurkcu et al., 2020).

Metal chelating activity is essential for controlling heavy metals in biological systems, minimizing their harmful effects and excessive ROS formation. This helps improve antioxidant capabilities an minimizing oxidative damage (Gulcin & Alwasel, 2022). EDTA is a potent metal chelator and the almost equal activity of EMC from varying concentrations indicates that it is just as powerful as EDTA, limiting the generation of free radicals (Figure 6D). This further affirms the antioxidant properties of EMC, which has already been established from previous results. Overall, we see a pattern where *M. citrifolia* leaf extract is effective as an antioxidant agent.

4. Conclusion

In conclusion, the ethanolic extracts of *M. citrifolia* showed strong potential as an effective antioxidant and it has little or no toxicological effect which can be explored in further studies. This study showed comprehensive results of the required markers that are considered in evaluating the potentials of the *M. citrifolia* extracts and would be recommended for further in-vitro studies to establish the efficacy of *M. citrifolia* extracts.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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