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Phytochemical Analysis and Antibacterial Effect of *Medicago sativa* and *Moringa oleifera* on Selected Bacteria Isolates

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Authors' contributions

This work was carried out in collaboration among all authors. Author GEI designed the study, wrote the protocol, managed the literature searches and wrote the first draft of the manuscript. Author CGE performed the statistical analysis and author NCO managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

In vitro antibacterial activities of the crude leaf extract of *Moringa oleifera* and *Medicago sativa* were investigated against some bacterial isolates (*Escherichia coli, Staphylococcus aureus, Bacillus cereus* and *Pseudomonas aeroginosa*) using the agar well diffusion and broth dilution techniques. The ethanolic crude extract of both plants exhibited significant inhibitory action against all the isolates tested at an initial concentration of 200 mg/ml except for *Bacillus cereus.* The minimum inhibitory concentrations exerted by the ehthanolic extract of *Moringa oleifera* against the bacterial isolate ranged from 6.25 mg/ml and 50 mg/ml with *Escherichia coli* recording the least while *Medicago sativa* varied from 12.5 mg/ml and 100 mg/ml. the plant leaf extract compared favourably

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with the control antibiotics used in the study. The phytochemical compounds observed in the leaf extracts are flavonoids, tannin, terpenoids, saponins and alkaloids. Tannin was the phytochemical that had the least concentration in the leaves of the plants. The quantitative yield of the bioactive compounds of the leaf extracts showed that *Medicago sativa* has highest yield of flavonoid at 3.81% yield and 4.51% for *Moringa oleifera.* The significant antibacterial activities exhibited by the ethanolic extract of the plants confirmed the therapeutic potentials of these plants in the treatment of various infections in herbal medicine.

Keywords: Ethanolic extract; antibacterial activity; minimum inhibitory concentration; leaf extract; phytochemicals.

1. INTRODUCTION

Plants are independent of other species in terms of food since they perform the producers' function in the ecosystem. On the earth, there are a lot of various types of plants. For a number of reasons, including oxygen production, soil conservation, food production, and lumber production, plants are vital to life. A variety of diseases have traditionally been treated with plants. Herbs have less adverse effects than other types of medications, which makes them more popular and increases public confidence in plant-based medicines. Various illnesses can be treated with plant extracts, leaves, bark, roots, and other parts [1].

In the past, pathogenic microorganisms were thought to be a major factor in human disease and mortality. “Due to the indiscriminate use of commercial antimicrobial medications frequently utilized in the treatment of such disorders, multiple drug resistance among pathogenic bacteria has recently been on the rise. Other causes include the use of antibiotics in animal husbandry and environmental superbugs that spread resistance genes to susceptible bacteria” [2]. “With increasing drug resistance among bacteria, efforts are being made to seek out new therapies. Phytotherapy is one of the most promising therapies for many diseases. Indeed, the collection and screening of medicinal plants can be helpful in areas with high potential for growth of medicinal plants” [3]. “The best-known approach to combating bacterial diseases involves the use of antibiotics. During the last decades, the overuse of antibiotics resulted in selective pressures that led to the widespread appearance of antibiotic-resistant microorganisms. Each of the antibiotics in use has generally inadequate efficacy and a number of serious adverse effects. It is imperative to investigate new antimicrobial agents that are more effective and less toxic than these antibiotics. From this perspective, the application of herbal compounds may potentially hold great promise. Plant-based antimicrobial drugs are difficult to isolate and identify because bioactive molecules frequently co-occur in complicated combinations with other secondary metabolites. Additionally, because these compounds are so scarce, it is crucial to improve their antibacterial capabilities” [4].

“Microbes are the most common cause of infectious diseases which participate in about half percent of the death cases in animals. As well as morbidity and mortality due to diarrhea in many developing countries which act as a major problem. The infections due to variety of bacterial etiologic agents such as pathogenic *Escherichia coli (E. coli), Salmonella spp.* and *Staphylococcus aureus (S. aureus)* are most common. Also systemic fungal infections due to *Candida albican (C. albican)* have emerged as important causes of morbidity and mortality” [5]. “Many antibacterial substances discovered and extracted from medicinal plants have been shown to be highly effective against both Gram-positive and Gram-negative bacteria” [4].

“Due to its potent medicinal ingredients and pharmacological activity, the *Moringa* species is widely employed in medicine. The most common species of *Moringa* genus is *Moringa oleifera* which has rich sources of various phytochemical compounds including glucosinolates and has antibacterial activity” [6]. One of the species in the family Moringaceae, It is indigenous to the continents of Africa, Arabia, South Asia, South America, the Himalayan area, India, Pakistan, the Pacific, and the Caribbean Islands. The plant known as *M. oleifera*, also known as the miracle tree, ben oil tree, horseradish tree, drumstick tree, and "Mother's best friend," has become a naturalized species in numerous tropical and subtropical places across the world. "Drumstick" is the popular name for the plant. It is a small to medium-sized tree that grows to a height of around 10 meters in the sub-Himalayan region.

"The tree has an open crown of drooping, frail branches, feathery foliage with tripinnate leaves, and thick corky, whitish bark. It is a small, quickly growing evergreen or deciduous tree that typically reaches heights of 10 to 12 meters..The *Moringa oleifera* plant provides a rich and rare combination of zeatin, quercetin, kaempferom and many other phytochemicals" [7]. "*Moringa oleifera* is one of the most commonly and widely used plants in its crude form in Nigeria, and many parts of the world. It has been established that every part of the plant (Leaves, Flowers, Roots, pods, and seeds) are used for the treatment of various ailments such as toothache, common cold, diarrhoea, and oedema [2].

Medicago sativa, also known as alfalfa and lucerne, comes from the Fabaceae family. *M. sativa* is used as a food additive in the United States, Russia, North Africa and China because of their high vitamin content. It produces secondary metabolites, such as coumarins, isoflavones, naphthoquinones, alkaloids and saponins that have nematocidal, cytotoxic and antimicrobial effects" [3].

The objectives includes ; to investigate the antibacterial activity of ethanolic and aqueous extracts of *M. oleifera* and *Medicago sativa* leaves on clinical isolates, to determine the effect of the combined extracts of the leaves on the isolates, and to determine the minimum inhibitory concentration of the leaves on the test organisms.

2. METHODOLOGY

2.1 Extraction of Plant Material

The leaves of the plants (*Medicago sativa* and *Moringa oleifera*) were plucked, rinsed with water and were shade dried at room temperature (32 – 35°C) to constant weight over a period of 5 days. The dried leaves were ground into powder using a mortar and pestle. 25 g of the powdered leaves were separately extracted in 500 ml conical flasks with 90% ethanol (ethanolic extraction) and water (aqueous extraction) .50g of each leaf powder was added to 200ml of 90% ethanol and 200ml of water. "The conical flasks were plugged with rubber corks, then shaken at 120 rpm for 30 minutes and allowed to stand at room temperature for 5 days with occasional manual agitation of the flask using a sterile glass rod at every 24 hour. The extracts were separately filtered using sterile Whatman no. 1 filter paper. These extracts (ethanolic and aqueous) were used in further process" [7].

2.2 Source of Microorganisms

The organisms used were *Escherichia coli, Pseudomonas aeroginosa, Staphylococcus aureus,* and *Bacillus cereus*. The organisms were obtained from the Microbiology Laboratory of Department of Science Laboratory Technology, Federal Polytechnic Ohodo, Enugu State Nigeria.

2.3 Screening of the Extract for Antibacterial Activity

The preliminary study of antimicrobial activity of different extracts of *Moringa oleifera* and *M. sativa* was performed by using agar well diffusion. 20 ml of sterile Mueller Hinton Agar (Hi-Media) was prepared and poured into sterile petri plates. After solidification, it was placed into the incubator at 37°C for 24 hrs to test for media sterility. 0.2 ml of the standardized inoculum was dropped onto the media using a sterile syringe and emulsified using sterilized bent glass. With the aid of a sterile standard 6 mm cork borer, wells were bored at equidistant positions. The different concentrations of the extracts were introduced into the different holes and the last hole contained the diluent, dimethyl sulfide (DMSO) as well as levoflaxin (antibiotic) which was used as the positive and negative control respectively. This procedure was repeated for all the test organisms and allowed for 30 minutes on the bench and then incubated for 24 hours at 37°C [2,8].

2.4 Determination of MIC and MBC

By dissolving 50 mg in 2.5 ml of ethanol, cold water and hot water, sterilizing through a Millipore filter, and loading their necessary amount over sterilized filter paper discs (8 mm in diameter), various concentrations of the effective plant extract (50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 mg/ml) were prepared individually. The pathogenic strains of bacteria were suspended on Mueller-Hilton agar and planted into sterile Petri dishes. The Mueller-Hilton agar plates were placed on top of the loaded filter paper discs that contained various amounts of the useful plant extract. The plates were incubated at 35°C for 24 hrs after being maintained at 5°C in the refrigerator for 2 hrs. By using a Vernier caliper, the inhibition zones were measured and recorded in relation to the quantities of the potent plant extracts [8].

The minimum bactericidal concentration (MBC) was determined by subculturing the test dilution onto fresh drug-free solid medium and incubating

further for 18 to 24 hrs. The highest dilution that yielded no single cell colony on the solid medium was taken as the minimum bactericidal concentration [8].

2.5 Qualitative Phytochemical Analysis

1. **Test for Alkaloids:** A known quantity of the extract, 0.1 mg was added to 6 ml of dilute hydrochloric acid and boiled, after boiling, it was cooled and filtered. The filtrate was divided into three portions and subjected to the following tests.

To the first portion, 2 drops of Dragendorff's reagent were added. The formation of a red precipitate indicated the presence of alkaloids.

To the second portion, 2 drops of Meyer's reagent were added. A creamy white precipitate indicated the presence of alkaloids.

To the third portion, 2 drops of Wagner's reagent were added. A reddish-brown precipitate indicated the presence of alkaloid [9].

2. **Test for Saponin:** To detect the presence of saponin, 5 mL of distilled water was added to 1 mL of extract and vortexed for 10 min. The formation of a foam column that did not disappear with the addition of HCl was evaluated as positive for saponin [10].
3. **Test for Tannin:** The extract, 1 ml was added to 10 ml of deionised water and then treated with 3 drops of ferric chloride. A greenish-brown precipitate indicated the presence of tannins [9].
4. **Test for Flavonoids:** A quantity of the extract was boiled in ethylacetate (10 ml) for 3 minutes, filtered and cooled. Then the filtrate (4 ml) was shaken with 1ml of dilute ammonia solution. An intense yellow colouration indicated the presence of flavonoids [9].
5. **Test for Steroids (Salkowki's test):** 5 drops of concentrated H_2SO_4 were added to 1 ml of each extracts in a separate test tube. The formation of a reddish brown colour was taken as a positive reaction [11].

2.6 Qualitative Phytochemical Analysis

1. **Alkaloids:** 5 g of the plants sample was grabbed in a beaker and then solution of C_2H_5OH and 10% of CH_3CO_2H of 200 ml was to plant sample. The mixture was encrusted and allowed to stand for 4 hrs then filtered in a water bath until it reaches 1/4 of the native volume, extract was enabled to become concentrated then conc. NH_4OH was added until the precipitation was completed. The precipitate collected and wiped with dilute NH_4OH and finally filtered. Then dried and weighed the alkaloid which is sublimate.
2. **Flavonoids:** 10 g of the leaves samples was separated with 100 ml of 80% aqueous methanol at room temperature. Through filter paper the whole solution was filtered then the filtrate relocated into a water bath and solution was evaporated into dryness. Weighed the sample until a constant weigh.
3. **Tannins:** 0.5 g of the leaves samples was weighed into a 50 ml plastic bottle. 50 ml of distilled was included and agitated for 1 hr. The sample was then filtered into a 50 ml volumetric flask and made up to mark. 5 ml filtered sample was then pipette out into test tube and assorted with 2 ml of 0.1 M $FeCl_3$ in 0.1 M HCl and 0.008 M $K_4Fe(CN)_6.3H_2O$. With a spectrophotometer at 395 nm wavelength within 10 min. Measure the absorbance of the sample [12].
4. **Terpenoides:** The extract (1 g) was marcarated with 50 ml of ethanol and filtered. To the filtrate (2.5 ml), 2.5 ml of 5% aqueous phosphomolybdic acid solution was added and 2.5 ml of concentrated H_2SO_4 was gradually added and mixed. The mixture was left to stand for 30 min and then made up to 12.5 ml with ethanol. The absorbance was taken at 700 nm.
5. **Saponin:** The extract (1 g) was macerated with 10 ml of petroleum ether and decanted into a beaker. Another 10 ml of the petroleum ether was added into the beaker and the filtrate evaporated into dryness. The residue was dissolved in 6 ml of ethanol. The solution (2 ml) was put in a test tube and 2 ml of chromogenic solution added into it. It was left to stand for 30 min and the absorbance was read at 550 nm.
6. **Test for Reducing Sugar:** The extract (1 g) was macerated with 20 ml of distilled water and filtered. To 1 ml of the filtrate, 1 ml of alkaline copper reagent was added. The mixture was boiled for 5 min and allowed to cool. Then 1 ml of phosphomolybdic acid reagent and 2 ml of distilled water was added and the absorbance read at 420 nm [9].

3. RESULTS AND DISCUSSION

3.1 Results

Table 1. Qualitative phytochemical analysis of extract of *Moringa oleifera* and *Medicago sativa* plants

Phytochemicals	*Moringa oleifera*		*Medicago indica*	
	Ethanolic extract	Aqueous extract	Ethanolic extract	Aqueous extract
Tannins	+	++	-	-
Steroids	+	++	-	+
Flavonoids	+	++	+	-
Alkaloids	-	+	-	-
Saponins	+	++	-	-

Keys: + minute concentrations; ++ moderate concentrations

Table 2. Quantitative phytochemical analysis of extract of *Moringa oleifera* and *Medicago sativa* plants

Phytochemicals	*Moringa oleifera*		*Medicago indica*	
	Ethanolic extract	Aqueous extract	Ethanolic extract	Aqueous extract
Tannins	0.26 ± 0.00	0.10 ± 0.10	1.40 ± 0.05	0.00 ± 0.00
Terpenoids	2.38 ± 0.05	4.12 ± 1.2	1.87 ± 0.06	0.00 ± 0.00
Reducing sugars	4.25 ± 0.10	2.12 ± 0.34	2.00 ± 0.17	1.08 ± 0.10
Flavonoids	4.51 ± 0.13	5.22 ± 0.30	3.81 ± 0.01	0.00 ± 0.00
Alkaloids	0.00 ± 0.00	6.31 ± 0.60	3.52 ± 0.10	0.00 ± 0.00
Saponins	1.75 ± 0.00	2.36 ± 0.05	1.26 ± 0.01	0.00 ± 0.00

Table 3. MIC and MBC values (mg/ml) of ethanolic extract of *Moringa oleifera* and *Medicago sativa* plants against isolates

Plants	organisms	100	50	25	12.5	6.25	3.12	1.56	0.78	MIC	MBC
Moringa oleifera	*E. coli*	-	-	-	-	+	+	+	+	6.25	12.5
	S. aureus	-	-	-	+	+	+	+	+	12.5	25
	B. cereus	-	-	+	+	+	+	+	+	25	50
	P. aeroginosa	-	-	+	+	+	+	+	+	25	50
Medicago sativa	*E. coli*	-	-	-	+	+	+	+	+	12.5	25
	S. auereus	-	+	+	+	+	+	+	+	50	100
	P. aeroginosa	-	+	+	+	+	+	+	+	50	100

Keys:
+ Growth of the organism indicated by turbidity in the broth medium,
- Absence of the test organism shown by no form of turbidity in the medium

Table 4. MIC and MBC values (mg/ml) of hot water extract of *Moringa oleifera* and *Medicago sativa* plants against isolates

Plants	organisms	100	50	25	12.5	6.25	3.12	1.56	0.78	MIC	MBC
Moringa oleifera	*E. coli*	-	+	+	+	+	+	+	+	50	100
	S. aureus	-	+	+	+	+	+	+	+	50	100
	B. cereus	+	+	+	+	+	+	+	+	100	100
	P. aeroginosa	+	+	+	+	+	+	+	+	100	100
Medicago sativa	*E. coli*	-	-	+	+	+	+	+	+	25	50
	S. auereus	-	+	+	+	+	+	+	+	50	100
	P. aeroginosa	-	+	+	+	+	+	+	+	50	100

Keys:
+ Growth of the organism indicated by turbidity in the broth medium,
- Absence of the test organism shown by no form of turbidity in the medium

Table 5. MIC and MBC values (mg/ml) of cold water extract of *Moringa oleifera* and *Medicago sativa* plants against isolates

Plants	organisms	100	50	25	12.5	6.25	3.12	1.56	0.78	MIC	MBC
Moringa	*E. coli*	-	+	+	+	+	+	+	+	50	100
oleifera	*S. aureus*	+	+	+	+	+	+	+	+	100	100
Medicago	*E. coli*	+	+	+	+	+	+	+	+	100	100
sativa	*S. auereus*	+	+	+	+	+	+	+	+	100	100

Keys:
+ Growth of the organism indicated by turbidity in the broth medium,
- Absence of the test organism shown by no form of turbidity in the medium

3.2 Discussion

In this study, the leaves of these plants have appreciable amount of the phytochemical, hence their medicinal values. *Moringa oleifera* had the highest amount of alkaloids when compared with those of *Medicago sativa.* This trend was observed for saponin and tannin which justifies the pharmaceutical and therapeutic potentials of the plants and their products [13]. Tannin is also known to possess immuno stimulating activity [9].

[14] reported that "a number of plants used in traditional medicines for rejuvenation therapy and chronic ailments have been shown to stimulate immune responses". "Saponin are either triterpenoid or steroidal glycosides proven as important phyto-constituent with different pharmacological activities such as antiallergic, cytotoxic etc effects" [15].

According to [16], both alkaloids and flavonoids have antimicrobial activities. Phytoconstituents such as saponin and phenolic compounds have also been reported to inhibit bacterial growth. "The secondary metabolites exert antimicrobial activity through different mechanisms. Tannins form irreversible complexes with proline rich protein, resulting in the inhibition of cell protein synthesis and the flavonoids complex with extracellular soluble proteins and with bacterial cell wall proteins while lipophilic exert antimicrobial activity by disrupting microbial cell membrane" [17].

The growth of all the pathogenic microorganisms used for the test was inhibited by the ethanolic extracts of the *Moringa oleifera.* The inhibition zone ranged from 8.5 mm to 22.0 mm. *E. coli* was susceptible to the ethanol extract of *Moringa oleifera* at 200 mg/ml concentration while at a similar concentration *Medicago sativa* for the same organism a diameter zone of inhibition of 19.5 mm was obtained.

The study shows that the plants studied possess antimicrobial properties, with greater antimicrobial efficacy when used synergistically. "*Moringa* leaves contain a variety of bioactive substances, and the antibacterial, antifungal, antiviral, and antiparasitic properties of its many preparations have been thoroughly established. However, some studies claim that chemical compounds found in *Moringa* leaves, such as pterygospermin, moringine, and benzyl isothiocyanate, were what caused the plant's antimicrobial effects" [13]. "Due to the enhanced activity of some apigenin derivatives, which have been found to be most effective against both Gram-positive and Gram-negative bacteria like *Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa*, apigenin is thought to be a future green chemical to combat the problem of antibiotic resistance" [18]. The study also validates the local use of *Moringa oleifera* for medicinal purposes in treating infectious diseases caused by Gram negative bacteria such as gastrointestinal infections, diarrhoea etc. [19] reported that "the reason for a greater activity of ethanol over the aqueous extract could be attributed to the polarity of the solvent which was responsible for the extraction of a wide range of phytochemicals that potentiates the pharmacological activity of plant extracts. They stated also that the polarity of ethanol gives it the ability to penetrate cell membrane to extract intracellular ingredients from plants and also, since most phytochemicals are mostly aromatic or saturated compounds which are uncharged, they can easily be extracted by charge or polar solvents".

4. CONCLUSION

The findings for this research showed that the leaves from the plants studied had pharmacological effect of the phytochemical constituents such as alkaloids and flavonoids as well as the antimicrobial activity of the plant which explains the rationale for the use of these plant seeds in the treatment of infections in traditional medicine. The results of *in-vitro* antimicrobial analysis of *Moringa oleifera* and

Medicago sativa leaf extract showed that, both plants possess potential antimicrobial strength evidenced by inhibition of growth of the test bacteria used in the study. Hence these plants are efficacious and contain natural compounds that could be used in the treatment of bacterial infections. It's important to keep in mind that although laboratory studies on these natural antibacterial agents have yielded encouraging results, more research and clinical trials are still required to confirm their effectiveness and safety for use as therapeutic agents. Furthermore, due to the fact that extraction techniques and plant sources can greatly affect the quantity and bioavailability of active chemicals, employing plant extracts as antibiotics should be done with caution. Before utilizing moringa or alfalfa leaf extracts for antibacterial reasons, like with any natural medicine, it is essential to speak with a healthcare provider.

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To my colleague, who was part of the planning of the leaf collection may your lovely soul rest with the Lord.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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