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In vitro antimicrobial appraisal of the potentials of *Morinda lucida* against some selected bacteria

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Abstract

The bioactive compounds from plant extract represent a potent alternative to combating the increasing antimicrobial resistance (AMR) to synthetic drugs. This study aimed to investigate the phytochemical, and antibacterial activities of *Morinda lucida*. Leaves of *M. lucida* was extracted successively using n-hexane, acetone, methanol and distilled water. The crude plant extracts were evaluated for their antibacterial activity against pathogens found in food and other sources such as *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhi, *Escherichia coli*, and *Klebsiella* spp. using the agar well diffusion technique. Minimum inhibitory concentration (MIC) was carried out using the microbroth dilution technique. The phytochemical analysis showed the presence of alkaloids and sterols in all extracts. Flavonoid was only present in the n-Hexane extract. The result showed that n-Hexane extract had the widest zone of inhibition against *P. aeruginosa* - 13.67 mm (12.5 mg/mL MIC), *B. subtilis* - 15 mm (12.5 mg/mL MIC), while acetone extract was most active against *S. enterica* ser. Typhi - 24.67 mm (6.25 mg/mL MIC), *S. aureus* - 24.83 mm (3.13 mg/mL MIC), *E. coli* - 13.33 mm (1.56 mg/mL MIC) and *Klebsiella* spp. - 18.33 mm (12.5 mg/mL MIC). These findings indicated that n-Hexane and acetone extract of *M. lucida* exhibited significant antimicrobial activity. Therefore, further research should be carried out to isolate the bioactive compounds with potentials in developing new drug leads against drug-resistant microbial strains.

1. Introduction

Antimicrobial resistance (AMR) is a situation where microorganisms evolve to a state of non-susceptibility upon exposure to established antimicrobial agents resulting in persistent infection by resistant microbes (Hay *et al*., 2018). The impact of AMR on economies and health care systems of a nation is significant and limits the productivity of patients and handlers as a result of the extended hospital admission which may require a sophisticated treatment approach that may be more expensive (Dadgostar, 2019). The continued occurrence of AMR and multidrug resistance (MDR) in pathogenic microbes are of great concern as there are no effective antimicrobials to combat such pathogens (Tanwar *et al.*, 2014; Aslam *et al*., 2018; Hay *et al*., 2018; Talebi Bezmin Abadi *et al*., 2019). Some of these pathogens of concern are transmitted through contaminated food. Thus, the need to find new antimicrobial agents that are effective against pathogenic microbes. Before the 16th century, plants were used for prophylactic and therapeutic purposes and around the 16th century the advent of iatrochemistry changed the approach to the treatment of patients. Recent trends have seen a reduction in the efficacy of synthetic drugs and their increasing detrimental side effect. This has resulted in sourcing for treatment with the use of natural products (Petrovska, 2012). Medicinal plants have been engaged in treating ailments in Africa for centuries. In line with World Health Organization's (WHO) report, nearly 80% of people living in developing countries adopt the administration of herbal medicine in primary healthcare practice (World Health Organization, 2013).

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South-west Nigeria is host to *Morinda lucida*. The plant is rich in nutrients and is available all year round. It has been reported to be rich in Vitamin A, E and K. It has been reported to have antibiotic, anti-parasitic and antiviral components. Infusions or plasters and decoctions of parts of the plant are used to treat different fevers, stomach aches, diabetes, cerebral congestion, gonorrhoea, dysentery, ulcers, hypertension, and leprosy (Agyare *et al.,* 2013; Adeleye *et al.,* 2018). The plant is locally known in the southwest region of Nigeria as "Oruwo" (Osuntokun, 2015). This current study explored continuous successive extraction for stratification of extract based on polarity, to establish the antimicrobial properties of the different phytochemical components of *M. lucida*.

2. Materials and methods

The leaves of *M. lucida* were collected from the Omu-Aran forest in Kwara State, Nigeria. Identification of the plants was done at the Department of Plant Biology, the University of Ilorin and a voucher specimen was archived with the reference number UILH/001/913/2021 (*Morinda lucida*).

2.1 Extraction

A total of 600 g of *M. lucida* leaves were washed and dried at 16-18°C. The leaves were pulverized to a fine powder and stored in an air-tight container. The pulverized material was extracted using the modified method by Rubab *et al*. (2021). The dried pulverized plant leaves were extracted successively in n-hexane, acetone, methanol and water, by soaking in 6 L of each solvent. Maceration was done in each solvent for three days while shaking at 100 rpm to increase the extraction rate at ambient temperature (27±2.5°C). Extraction solvent was removed from each extract with the aid of a rotary evaporator under reduced pressure and the concentrated extracts were weighed and stored in a glass container (Rubab *et al*., 2021).

2.2 Phytochemical screening

2.2.1 Qualitative phytochemical screening

Crude solvent-free extracts of *M. lucida* leaves were analyzed for the presence of phytochemicals - alkaloids, saponins, cardiac glycoside, sterols, phenols, terpenoids, quinones, flavonoids and tannins using the established method described by (Ali *et al*., 2018).

2.2.2 Quantitative phytochemicals screening

2.2.2.1 Determination of total alkaloid

The gravimetric technique previously described in the literature was modified for use in this work (Onochie *et al*., 2020). A total of 5 g of each extract was dissolved in 50 mL of 10% ethanolic acetic acid solution. The solution was stirred for about 10 mins and allowed to stand for a period of 4 hrs before filtering. The filtrate collected from the solution was allowed to evaporate to 25% of its initial volume after which the alkaloid present in the solution was precipitated with concentrated NH_4OH (drop-wise addition). The precipitate was removed from the solution with a filter paper and washed with 1% NH_4OH solution. The resulting precipitate was dried at 60°C in a hot air oven for 30 mins to evaporate all liquid from the precipitate and reweighed to get the alkaloid content of each extract.

2.2.2.2 Determination of total phenolic content

The total phenolic content of the leaf extracts was quantified using the Folin Ciocalteu technique as reported by Singleton *et al*. (1999). The standard was gallic acid. A gallic acid calibration curve was made using varying concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. Then, 50 µL of crude extract was measured into 250 µL Folin-Ciocalteu reagent and mixed with 3 mL of double distilled water and allowed to stand for 5 mins on the bench. About 750 µL of 20% Na_2CO_3 was added to the standing mixture and mixed for 2-4 mins after which it was incubated at room temperature for 30 mins. A UV-visible spectrophotometer was used to measure the absorbance at 760 nm in triplicate. The total phenolic content was evaluated from the calibration curve.

2.2.2.3 Determination of total tannin content

The total tannin content of the leaf extracts was derived using a slightly modified Folin-Ciocalteu assay technique. A Gallic acid calibration curve was made using varying concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. Then, 5 mL of already prepared leaf extract, was added to 5 mL of distilled water, Na_2CO_3 (35%) and Folin-Ciocalteu reagent (500 µL) was mixed for 15 mins and allowed to stand for 30 mins at ambient temperature. A UV-visible spectrophotometer was used to measure the absorbance for standard solutions against blank at 725 nm and the total tannin content was extrapolated from the calibration curve and expressed as mg/g (Singleton *et al.,* 1999).

2.2.2.4 Determination of total saponin content

A total of 5 g of the leaf extract was dissolved in 100 mL of 20% acetic acid (in ethanol) and left to stand in a water bath at 50°C for 24 hrs. The resulting solution was filtered and concentrated in a water bath to a quarter of the initial volume. Concentrated NH_4OH was added drop-wise to the solution until it was completely precipitated. The solution containing the precipitate was allowed to stand for the precipitate to settle before the precipitate was filtered and weighed. The saponin content was

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weighed and calculated as mg/g of the sample (Ali *et al*., 2018).

2.2.2.5 Determination of total terpenoid content

A total of 2 g of the leaf extract were weighed into a beaker containing 50 mL of 95% ethanol for 24 hrs. The leaf extract was filtered and the collected filtrate was re-extracted using petroleum ether (60-80℃) and concentrated to dryness. The resulting mass from drying was taken as total terpenoids and estimated in mg/g (Bashua and Oladunmoye, 2017).

2.3 Organisms used in this study

The bacteria used for this study were *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* serovar Typhi (ATCC 20971), *Escherichia coli* (ATCC 25922) *Klebsiella* spp.

2.4 Antimicrobial screening

2.4.1 Antimicrobial activity of extracts

The antimicrobial potential of all crude extracts (200 mg/mL) was tested using the agar well diffusion technique. A modified method by Zofuo *et al*. (2019) was used. A 24-hr old culture on nutrient broth was used as inoculum and the closed mesh was streaked on solidified Mueller-Hinton agar plates for every organism in replicates. A 9 mm cork borer was used to make four wells in the Mueller-Hinton agar. Each well was filled with 100 µL of each leaf extract. Ciprofloxacin was used as the positive control and DMSO as the negative control. The prepared plates were allowed to stand for the diffusion of the extract into the medium before incubating at 37°C for 18 - 24 hr. The plates were observed for zones of inhibition and the antimicrobial activity was recorded as the mean of the diameters of the zones of inhibition (Zofou *et al.,* 2019).

2.4.2 INT colourimetric assay for MIC determinations

The MIC of leaf extracts of *M. lucida* on *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 6633), *P. aeruginosa* (ATCC 9027), *S. enterica* serovar Typhi (ATCC 20971), *E. coli* (ATCC 25922) *Klebsiella* spp. were done using INT colourimetric micro broth dilution as described by Ohikhena *et al*. (2017) with some modification. The leaf extracts were dissolved in DMSO to a concentration of 200 mg/mL. Mueller Hinton Broth (100 µL) was dispensed into all 96 wells of the microtitre plates. A two-fold dilution of plant extract (100 µL) was made across the well rows, till the tenth dilution was achieved. A total of 20 µL broth containing the test organism was introduced into each well. The plates were covered and incubated at 37°C for 16-18 hrs. The assay was done in replicates. DMSO served as the negative control. 10 µL of 0.2 mg/mL p-iodonitrotetrazolium chloride (INT) was added to all wells after 18 h and further incubated for another 30 mins. The MIC was recorded as the least concentration in wells that could not reduce the yellow INT solution to pink (Ohikhena, Wintola, and Afolayan, 2017).

3. Results

3.1 Qualitative phytochemical screening

The extraction procedure yielded four different extracts one for each extraction liquid used (n-hexane, acetone, methanol and water). The agents responsible for antimicrobial activities in plants are the phytochemicals. The qualitative phytochemical analysis of the leaf extract from the four solvents was carried out. The analysis revealed the presence of Alkaloids and sterols in all extracts and water extract has the highest number of phytochemicals including alkaloids, saponins, cardiac glycoside, sterol, phenols terpenoids, quinones and tannins lacking only flavonoids. N-hexane extract tested positive for only a few phytochemicals, namely, alkaloids, sterols and flavonoids which could be a result of the polarity of the solvent (Table 1).

Table 1. Qualitative analysis of different extracts of *Morinda lucida*

Extracts	Alkaloids	Saponins	Cardiac glycoside	Sterols	Phenols	Terpenoids	Quinones	Flavonoids	Tanins
n-Hexane	+	-	-	+	-	-	-	+	-
Acetone	+	+	+	+	+	+	-	-	+
Methanol	+	+	+	+	+	+	-	-	+
Water	+	+	+	+	+	+	+	-	+

+: present, -: absent

3.2 Quantitative phytochemical screening

The availability and quantity of the phytochemical constituent of plants play a major role in their activity when used in local concoctions. Some selected phytochemicals present in the extracts were quantified (Table 2) and it was observed that the acetone extract had the highest quantity of phenols (0.06±0.34 mg/g) and tannins (0.06±0.32 mg/g) per gram of extract. The methanol extract had the highest quantity of Alkaloids (0.06±0.004 mg/g) and terpenoids (0.05±0.01 mg/g), while the water extract had the highest quantity of saponins (0.12±0.1 mg/g) (Table 2).

3.3 Antimicrobial activity of extracts

Compared to other extracts the acetone extract had the highest activity against *S. enterica* ser. Typhi (24.67

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Table 2. Quantitative analysis of different extracts of *Morinda lucida*

Extracts	Alkaloids (mg GAE/g)	Saponins (mg GAE/g)	Phenols (mg GAE/g)	Terpenoids (mg GAE/g)	Tannins (mg GAE/g)
n-Hexane extract	0.02±0.005	-	-	-	-
Acetone extract	0.04±0.007	0.09±0.02	0.06±0.34	0.04±0.01	0.06±0.32
Methanol extract	0.06±0.004	0.06±0.02	0.04±0.28	0.05±0.01	0.04±0.28
Water extract	0.02±0.004	0.12±0.1	0.03±0.39	0.03±0.01	0.01±0.03

mm), *S. aureus* (24.83 mm), *E. coli* (13.33 mm) and *Klebsiella* sp (18.33 mm). While the n-hexane extract displayed the widest zone of inhibition against *P. aeruginosa* (13.67 mm) and *B. subtilis* (15 mm) (Table 3).

3.4 INT colourimetric assay for MIC determinations

The MIC of the leaf extract was examined against six different isolates and the MIC varied against the different isolates. The n-hexane leaf extract had the lowest MIC (6.25 mg/mL) against *S. enterica* ser. Typhi (Table 4) while the acetone leaf extract had the lowest MIC (12.5 mg/mL) against *P. aeruginosa* (Table 6). In the case of *S. aureus* (Table 5), Acetone extract had the lowest MIC (3.125 mg/mL) next to which was n-hexane extract (6.25 mg/mL). The MIC of all four extracts in broth against *Klebsiella* spp. was 12.5 mg/mL showing similar potentials across all extracts (Table 7). Acetone extract had a MIC of 1.56 mg/mL against *E. coli* while other extracts had MICs of 12.5 mg/mL (Table 8). Three of the leaf extracts (in n-hexane, acetone and water) shared the lowest MIC (12.5 mg/mL) against *B. subtilis* (Table 9).

4. Discussion

Time and evolution have allowed bacterial pathogens to attain a state of resistance to previously potent antibacterial agents. Some of these organisms access the human host through food, water and other sources. These trends call for the discovery of new, effective and safe antibacterial agents. Plants extracts have been reported to be a suitable source for new antibacterial agents due to their use in traditional medicine for centuries. Plants are rich in phytochemicals that could have a prophylactic or therapeutic effect on health. They have been studied widely to ascertain potency and to decipher how they confer these health benefits.

Table 3. Antimicrobial susceptibility testing using agar well diffusion

	Zone of inhibition (mm)					
Test Organisms	*P. aeruginosa*	*S. enterica* serovar Typhi	*S. aureus*	*E. coli*	*B. subtilis*	*Klebsiella* spp.
N-hexane extract	13.67	0	16.67	8	15	17
Acetone extract	11.17	24.67	24.83	13.33	9.67	18.33
Methanol extract	7.33	15.5	18.67	4.67	0	0
Water extract	7.5	22	14.27	0	13.17	14
CPR	30.17	43	26.33	36.33	33.33	25.33
DMSO	0	0	0	0	0	0

CPR: ciprofloxacin, DMSO: dimethyl sulfoxide

Table 4. Minimum inhibition concentration (MIC) for *S. enterica* ser. Typhi in different concentrations of *Morinda lucida* extracts.

	50 mg/mL	25 mg/mL	12.5 mg/mL	6.25 mg/mL	3.13 mg/mL	1.56 mg/mL	0.78 mg/mL	0.39 mg/mL	0.2 mg/mL
n-Hexane extract	-	-	-	-	+	+	+	+	+
Acetone extract	-	-	-	-	+	+	+	+	+
Methanol extract	-	-	-	+	+	+	+	+	+
Water extract	-	-	+	+	+	+	+	+	+

-: No growth, +: Growth present

Table 5. Minimum inhibition concentration (MIC) for *S. aureus* in different concentrations of *Morinda lucida* extracts.

	50 mg/mL	25 mg/mL	12.5 mg/mL	6.25 mg/mL	3.13 mg/mL	1.56 mg/mL	0.78 mg/mL	0.39 mg/mL	0.2 mg/mL
n-Hexane extract	-	-	-	-	+	+	+	+	+
Acetone extract	-	-	-	-	-	+	+	+	+
Methanol extract	-	-	-	+	+	+	+	+	+
Water extract	-	-	+	+	+	+	+	+	+

-: No growth, +: Growth present

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Table 6. Minimum inhibition concentration (MIC) for *P. aeruginosa* in different concentrations of *Morinda lucida* extracts.

	50 mg/mL	25 mg/mL	12.5 mg/mL	6.25 mg/mL	3.13 mg/mL	1.56 mg/mL	0.78 mg/mL	0.39 mg/mL	0.2 mg/mL
n-Hexane extract	-	-	-	+	+	+	+	+	+
Acetone extract	-	-	-	+	+	+	+	+	+
Methanol extract	-	-	+	+	+	+	+	+	+
Water extract	-	-	+	+	+	+	+	+	+

-: No growth, +: Growth present

Table 7. Minimum inhibition concentration (MIC) for *Klebsiella* spp. in different concentrations of *Morinda lucida* extracts.

	50 mg/mL	25 mg/mL	12.5 mg/mL	6.25 mg/mL	3.13 mg/mL	1.56 mg/mL	0.78 mg/mL	0.39 mg/mL	0.2 mg/mL
n-Hexane extract	-	-	-	+	+	+	+	+	+
Acetone extract	-	-	-	+	+	+	+	+	+
Methanol extract	-	-	-	+	+	+	+	+	+
Water extract	-	-	-	+	+	+	+	+	+

-: No growth, +: Growth present

Table 8. Minimum inhibition concentration (MIC) for *E. coli* in different concentrations of *Morinda lucida* extracts.

	50 mg/mL	25 mg/mL	12.5 mg/mL	6.25 mg/mL	3.13 mg/mL	1.56 mg/mL	0.78 mg/mL	0.39 mg/mL	0.2 mg/mL
n-Hexane extract	-	-	-	+	+	+	+	+	+
Acetone extract	-	-	-	-	-	-	+	+	+
Methanol extract	-	-	-	+	+	+	+	+	+
Water extract	-	-	-	+	+	+	+	+	+

-: No growth, +: Growth present

Table 9. Minimum inhibition concentration (MIC) for *B. subtilis* in different concentrations of *Morinda lucida* extracts.

	50 mg/mL	25 mg/mL	12.5 mg/mL	6.25 mg/mL	3.13 mg/mL	1.56 mg/mL	0.78 mg/mL	0.39 mg/mL	0.2 mg/mL
n-Hexane extract	-	-	-	+	+	+	+	+	+
Acetone extract	-	-	-	+	+	+	+	+	+
Methanol extract	-	-	+	+	+	+	+	+	+
Water extract	-	-	-	+	+	+	+	+	+

-: No growth, +: Growth present

This current study revealed good antibacterial activity of the different plant extracts against selected gram-positive and gram-negative bacteria. The presence of phytochemicals such as alkaloids, saponin, cardiac glycoside, sterols, phenols, terpenoids, quinones, flavonoids and tannins in these plant extracts could be responsible for the antimicrobial activities observed. Reports have shown that alkaloids exhibit antibacterial, antiproliferation, insecticidal, antiviral and antimetastatic effects on diverse cancer cells both in vitro and in vivo (Qiu *et al*., 2014; Hussain *et al*., 2018; Chikowe *et al.,* 2020). The glycoside and saponin constituent of the plant leaf extracts indicates the plant's potential to confer protection against pathogens and exhibit antimicrobial, antiulcer, anti-inflammatory, and antidiarrheal activities (Prakash *et al.,* 2020). Terpenoids have been implicated to possess antimicrobial, anticarcinogenic, hepaticidal, antimalarial, anticancer activities and anti-ulcer activities. Flavonoids (e.g. rutin and quercetin) have antiviral, anti-inflammatory and antihistaminic effects. The presence of this phytochemical may be responsible for the health benefits derived from *M. lucida* when used in traditional medicine to treat diverse ailments as claimed by the African Traditional Medicine practitioners.

Quantitative analysis of the phytochemicals present in the leaf extract of this plant revealed the acetone extract had the highest phenolic content (total phenols and tannins) per mg of GAE/g of leaf extract, which could be responsible for the superior antimicrobial effect when compared to extract from other solvents. Plant-derived phenolics including tannins have been implicated for their diverse biological activities which include their inhibitory and bactericidal activities against selected bacteria of medical importance (Mandal *et al.,* 2017; Bouarab-Chibane *et al*., 2019; Maisetta *et al*., 2019).

The extraction solvent plays an important role in identifying the active fractions or compounds in plant material. The extraction of the plant leaf was done in solvent successions, starting from the least polar solvent to the most polar. Though the plant leaf is traditionally extracted using water, this study reported a better activity from acetone extracts *in-vitro*. This is in tandem with reports that most antimicrobial compounds found in plant materials are non-polar or have intermediate polarity, thus making acetone extract most potent against

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bacteria. This may be a result of the polar and nonpolar characteristics of acetone as an extraction solvent (Eloff, 2019).

The agar well antimicrobial susceptibility testing revealed that the acetone extract had a higher zone of inhibition against *S. enterica* ser. Typhi, *S. aureus, E. coli* and *Klebsiella* spp. (24.67 mm, 24.83 mm, 13.33 mm and 18.33 mm respectively). The activity against *S. aureus* was almost similar to ciprofloxacin (26.33). The result is in line with the report from several works on the antimicrobial potentials of *M. lucida* (Fakoya *et al.,* 2014; Temitope *et al.,* 2016).

The acetone extracts had the lowest MIC of all extracts against *Escherichia coli* and *Staphylococcus aureus* (1.56 mg/mL and 3.1 mg/mL respectively) and were only equalled by n-hexane extract against *S. enterica* ser. Typhi and *P. aeruginosa*, the activities against *E. coli* are in tandem with earlier reports (Osuntokun, 2015; Olawuwo *et al.*, 2019). This is a confirmation of Eloff's report on the antimicrobial activities of plant compounds characterized by intermediate polarity or non-polar (Eloff, 2019).

4. Conclusion

In conclusion, the findings in this work have been able to support the ethnobotanical claims on the use of *M. lucida* by rural dwellers in Nigeria. The activity of the extracts against all selected isolates which cuts across Gram-positive and negative bacteria of medical importance with its best activity against *E. coli* (acetone extracts) showed the plant can be considered as a source of a new antibiotic in the search for alternative antimicrobial agents in the continued rise of drug-resistant bacteria. The method of extraction engaged in this study can be compared with others for the best approach to the extraction of active ingredients in the plant leaf. It is recommended that further studies be done to elucidate the active compound in the plant and isolate it for further work. This is the first time the extraction technique used in this work has been used for this plant and the result has shown that it can be considered a viable technique to study the activity of plant extracts against microbes. This work provides a phytochemical explanation for the herbal use of *M. lucida* in African Traditional Medicine. The effective inhibition of pathogenic bacteria by acetone extract at low MIC suggests that further detailed study could lead to the discovery of some potent antibiotic agents.

Conflicts of interest

The authors declare no conflict of interest.

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