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Antibacterial Activities of Metal-Natural Extract Complexes

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Metal-based compounds consist of an organic substituent as the ligand and an inorganic substituent as the metal center. Natural products, particularly natural extract, are well known to exhibit excellent biological activities, while the inorganic substituents are usually metal ions. Metal ions, especially transition metals, exhibit different oxidation states and can interact with any ligands. Advances in inorganic chemistry provide better opportunities to use metal-based compounds as therapeutic agents. The mode of action of these metal-based compounds on living organisms is different from non-metals. The unique properties of metal ions should be exploited to design new compounds. *Pluchea indica (L.) Less* (locally known as Beluntas), *Clinacanthus nutans* (locally known as Belalai Gajah), and *Phyllanthus niruri* (locally known as Dukung Anak) are medicinal plants that possess various biological properties. Incorporation of transition metal ions (Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}) into the crude extracts from these medicinal plants would give metal-natural extract complexes of the plants. Leaves of *Pluchea indica (L.) Less*, *Clinacanthus nutans*, and *Phyllanthus niruri* were collected and extracted with either methanol or ethanol and reacted with various transition metal salts to yield metal-natural extract complexes. The metal-natural extract complexes were assessed for their antibacterial activities using quantitative and qualitative antibacterial assays against four pathogenic bacteria, which were *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 117788), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922). The antibacterial assays showed that the biological activities of the metal-natural extract complexes were enhanced and selective towards selected target bacteria. It was found that the identity of transition metal ions plays an important role in enhancing the bioactivity exhibited by the metal-natural extract complexes. The results showed that these metal-natural extract complexes of *Pluchea indica (L.) Less*, *Clinacanthus nutans*, and *Phyllanthus niruri* are potential alternative antibacterial agents.

Key words: *Pluchea indica (L.) Less*; *Clinacanthus nutans*; *Phyllanthus niruri*; Beluntas; Belalai Gajah; Dukung Anak; metal-natural extract complexes; antibacterial agent

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Pluchea indica (L.) Less (locally known as Beluntas), *Clinacanthus nutans* (locally known as Belalai Gajah), and *Phyllanthus niruri* (locally known as Dukung Anak) are well known medicinal plants in Malaysia that have been used in traditional remedies for their wide range of pharmacological activities. The leaf extract of *Pluchea indica (L.) Less* was found to possess pronounced anti-inflammatory activities [1], antiproliferative activities [2], and antioxidant activities [3-4]. The extracts of *Clinacanthus nutans* are well known to display antiviral activities [5-6], anti-inflammatory activities [7], antioxidant activities [8], and cytotoxic activities [9]. *Phyllanthus niruri* has been reported to show antioxidant and hepato-protective activities [10-11], as well as antimalarial activities [12]. Despite all the distinctive biological properties exhibited by these three medicinal plants, they possess one common biological property-antibacterial activity. In fact, they are commonly used as an antidote for bites, various infections, burns and scalds, infectious diseases, and dysentery [13-17].

A thorough search from the literature showed that various solvents had been used for extraction. However, the quality of extracts varies as the concentration and composition of the phytochemicals vary. This is exhibited by either the antioxidant and biological properties (antibacterial, antifungal or cytotoxic studies) or qualitative and quantitative phytochemical screening tests. It was found that methanol was the ideal solvent used to extract *Pluchea indica (L.) Less* [18-19] and *Phyllanthus niruri* [20], while 95% ethanol was selected as the ideal solvent to extract *Clinacanthus nutans* [21]. Polar solvents such as methanol and ethanol yielded the strongest DPPH radical scavenging and FRAP. The extraction procedure used was Soxhlet extraction for at least 36 hours.

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Metal-based compounds have made their way into the pharmaceutical and medicinal industries since these compounds exhibit remarkable biological properties [22]. Structurally, they are a combination of an organic substituent (ligand) and an inorganic substituent (metal ion). Organic substituents, e.g., natural products, are known to exhibit excellent biological activities, either the crude extract or the isolated compounds. Inorganic substituents, e.g., metal complexes, are documented for their remarkable biological activities as potent antibacterial properties compared to the parent ligands [23]. To date, no work has been done to synthesize metal-natural extract complexes.

Transition metal ions, in particular, have excellent ligation as they possess vacancies in *d* and *f* orbitals that facilitate coordination with ligands. Complexation of the natural extracts from these traditional herbs with transition metal ions will yield metal-natural extract complexes derived from *Pluchea indica (L.) Less*, *Clinacanthus nutans*, and *Phyllanthus niruri*, respectively. These complexes were expected to be more thermodynamically stable and capable of undergoing any purification, isolation, and crystallization processes compared to the parent organic constituents (e.g., phytochemicals, flavonoids, lignans, alkaloids etc.). These metal-natural extract complexes were expected to display more potent antibacterial activities than the existing bioactivities exhibited by the crude extracts (parent compounds).

MATERIALS AND METHODS

Plant Collection and Authentication

Fresh leaves of *Pluchea indica (L.) Less*, *Clinacanthus nutans*, and *Phyllanthus niruri* were collected from several areas in Kuantan City, Pahang, Malaysia. The leaves were authenticated by Dr. Shamsul Khamis, the Coordinator of Biodiversity Unit, Institute of Bio-science, Universiti Putra Malaysia. All chemicals and solvents were of analytical grade and were used as received.

Extraction of *Pluchea indica (L.) Less*

Leaves were separated from the branches, washed with water and oven-dried at 40°C, powdered, and sieved through 100 meshes. Fibers and unwanted materials were discarded after sieving. Approximately 10 g of pulverized powder was extracted with methanol. The extraction was performed at a ratio of 1:15 (w/v) using a Soxhlet extractor for 36 hours. Then, the solvent was evaporated under reduced pressure using a rotary evaporator to obtain a semi-solid residue. Obtained residues were stored in a fridge at 4°C and fractionated within two days.

Extraction of *Clinacanthus nutans*

Leaves were separated from the branches, washed with water and oven-dried at 40°C, powdered, and sieved through 100 meshes. Approximately 10 g of pulverized powder was extracted with 95% ethanol. The extraction was performed at a ratio of 1:10 (w/v) using a Soxhlet extractor for 48 hours. The ethanolic extract of *C. nutans* was filtered using Whatman No. 1 filter paper and was concentrated in vacuo using a rotary evaporator to obtain the residue. Obtained residues were stored in a fridge at 4°C and fractionated within two days.

Extraction of *Phyllanthus niruri* sp.

Leaves were separated from the branches, washed with water and oven-dried at 40°C, powdered, and sieved through 100 meshes. Approximately 10 g of pulverized powder was extracted with methanol. The extraction was performed at a ratio of 1:8 (w/v) using a Soxhlet extractor for 36 hours. The methanolic extract was evaporated using a rotary evaporator and dried by a vacuum pump. The solid residue obtained was kept in a fridge at 4°C and fractionated within two days.

Fractionation of Extracts

Fractionation was done from 10 g of plant extract in 100 mL of distilled water with dichloromethane in a ratio of 1:2. Fractionation was done repeatedly until the green bottom layer removed was pale in color or colorless for the effective removal of chlorophyll. The fractionated extract was kept at 4°C and used within a week.

Synthesis of Metal-natural Extract Complexes

Approximately 5.0 mL of fractionated extract was added with either ethanolic or methanolic solution (depending on the solvent used to extract the leaves) of 0.01 g of metal salt and refluxed for 8 hours. The precipitate formed was filtered, washed with cold ethanol/methanol, and kept dry under silica gel until further analyses. Transition metal ions used were Cu^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+}, respectively. The natural extracts (solvent used) were *Pluchea indica (L.) Less* (methanol), *Clinacanthus nutans* (95% ethanol), and *Phyllanthus niruri* (methanol), respectively.

Metal Content Analysis using Atomic Absorption Spectroscopy

The percentage of metal in the complexes was determined using Perkin Elmer AAnalyst 700 Atomic Absorption Spectrophotometer. All complexes (except for the cobalt and zinc complexes) were accurately weighed and digested using concentrated

nitric acid. A sample was heated until a clear solution was obtained. This solution was quantitatively transferred and made up to 10 mL with 5% nitric acid. Calibration curves were obtained from the prepared standard metal solutions using an appropriate range of concentration. All the zinc complexes were sticky and unable to be retrieved for digestion. The cobalt complexes were not digested and not analyzed due to the absence of the cobalt hollow cathode lamp.

Antibacterial Activity

Antibacterial activity was evaluated using qualitative and quantitative antibacterial assays at Kulliyyah of Science, IIUM.

Target Microorganisms

Four pathogenic bacterial strains were used to test the biological potential of the metal-natural extract complexes. They were *Staphylococcus aureus* (ATCC 29213), *Bacillus cereus* (ATCC 117788), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922). The sources of the microbes and culture maintenance were as previously described in [24].

Qualitative Antibacterial Assay

Antibacterial activities of the extracts were qualitatively determined by a modified disc diffusion method [24]. A lawn of microorganisms was prepared by pipetting and evenly spreading the inoculum (10^{-4} cm^3, adjusted turbidometrically to $10^5 – 10^6$ cfu cm^3 (cfu: colony forming units)) onto nutrient agar (NA) set in petri dishes. 6 mm in diameter Whatman No. 1 filter paper discs were impregnated with stock solutions of the respective compounds (500 µg cm^{-3}) and dried under sterile conditions. The dried discs were then placed on the inoculated agar plates. The plates were inverted and incubated for 24 hrs at 37°C. All assays were performed in triplicate. Antibacterial activity is indicated by clear inhibition zones around the discs. Commercially available streptomycin sulphate (10 µg/ disc) was used as a positive antibacterial control, while ethanol/methanol was used as a negative control.

Quantitative Antibacterial Assay

Positive antibacterial compounds with inhibition zone ≥ 15 mm of the disk diffusion assay were subjected to the quantitative measurement of microbiostatic (inhibitory) activity as described by Hufford [25]. The lowest concentration which completely inhibited visible bacterial growth was recorded as the minimum inhibitory concentration (MIC, µg cm^{-3}).

RESULTS AND DISCUSSION

Extraction and Synthesis of Metal-natural Extract Complexes

All natural extracts were fractionated to remove chlorophylls, which are abundant in fresh green leaves. Removal of chlorophylls is essential because chlorophylls are bioactive compounds that can stimulate tissue growth, speed up the progression of wound healing, and prevent the invasion of bacteria [26]. Chlorophylls would also act as an organic substituent and potential ligand to complex with the metal ions if not removed effectively.

Table 1 summarizes the physical properties of the metal-natural extract complexes. All the complexes obtained were dark in color compared to the metal salts and the natural extracts because transition metal ions have incomplete *d*-orbitals that allow electron promotion from lower energy orbitals to higher energy orbitals when provided with sufficient energy. These energy differences transmit visible lights, except the ones absorbed by the substance. The metal content, as shown in Table 1, assured the presence of metal ions in all the complexes. The percentage of copper obtained was in the range of 0.12 – 0.18%, while nickel was about 0.99 – 1.80%. The ligands attached to the metal ions were expected to be very large with heterodonor atoms of oxygen and nitrogen because most of the secondary metabolites of the extracts were composed of hydroxyl and amine groups that would facilitate the formation of coordination bonds upon binding with the metal ions. Therefore, the presence of metal ions confirmed that the metal-natural extract complexes were successfully synthesized.

Table 1. Physical properties of synthesized metal-natural extract complexes

Metal-natural extract complexes	*Type of solvent used in extraction*	*Color of product*	*Metal content (%)*
Cu(II)- *Clinacanthus nutans* complex	95% ethanol	Dark blue-green	0.12 %
Co(II)- *Clinacanthus nutans* complex	95% ethanol	Dark brown red	na
Ni(II)- *Clinacanthus nutans* complex	95% ethanol	Dark bronze	1.06%
Zn(II)- *Clinacanthus nutans* complex	95% ethanol	Dark yellow	na
Cu(II)-*Pluchea indica (L.) Less* complex	methanol	Dark blue-green	0.18%
Co(II)-*Pluchea indica (L.) Less* complex	methanol	Dark red	na
Ni(II)-*Pluchea indica (L.) less* complex	methanol	Dark brown	1.80%
Zn(II)-*Pluchea indica (L.) less* complex	methanol	Dark yellow	na
Cu(II)- *Phyllanthus niruri* complex	methanol	Dark blue-green	0.15%
Co(II)- *Phyllanthus niruri* complex	methanol	Dark brown	na
Ni(II)- *Phyllanthus niruri* complex	methanol	Dark bronze	0.99%
Zn(II)- *Phyllanthus niruri* complex	methanol	Dark yellow	na

na – not analyzed

Biological Activities of Natural Extracts and their Metal-natural Extract Complexes

Table 2 shows the qualitative antimicrobial assay results. All the natural extracts were found to be biologically active against all the bacteria tested. It is noteworthy that most of the compounds, specifically the metal-natural extract complexes were highly active compared to the commercial antibiotic, streptomycin sulphate. From the results, the methanolic natural extract of *Pluchea indica (L.) Less* was most active with the largest zone of inhibition for all the tested bacteria compared to the ethanolic natural extract of *Clinacanthus nutans* and the methanolic natural extract of *Phyllanthus niruri*. *Pluchea indica (L.) Less* was mentioned to be biologically active due to the presence of saponin that acts as an antibacterial agent in the extract and thus assists the inhibition effect against bacteria [27]. Overall, all the metal-natural extract complexes showed enhanced antibacterial activities in comparison to their parent compounds (natural extracts), except for Co(II)- and Ni(II)- *Clinacanthus nutans* complexes that showed a decrease in activity against *E. coli* when compared to the ethanolic natural extract of *C. nutans*. The decreased activity could be correlated to the relationship between the individual metals that could exhibit antagonistic effects [28]. The other reason could be due to the metal-based compound–membrane components interaction [28]. The zone of inhibition for Co(II)-*Pluchea indica (L.) Less* complex against *P. aeruginosa* and Ni(II)-*Pluchea indica (L.) Less* complex and Ni(II)-*Phyllanthus niruri* complex against *S. aureus* was the same as exhibited by the natural extracts, which showed that the antibacterial activities of these metal-natural extract complexes remained unaltered.

Table 2. Qualitative antimicrobial assay results (mean ± SD, n = 3)

Compounds	Zone of inhibition (mm)			
	***E. coli* (ATCC 25922)**	***P. aeruginosa* (ATCC 27853)**	***S. aureus* (ATCC 29213)**	***B. cereus* (ATCC 117788)**
Clinacanthus nutans extract	15.0 ± 0.5	17.0 ± 0.0	12.0 ± 0.4	10.0 ± 0.0
Pluchea indica (L.) Less extract	19.0 ± 0.2	21.0 ± 0.0	16.0 ± 0.2	15.0 ± 0.5
Phyllanthus niruri extract	16.0 ± 0.5	18.0 ± 0.2	13.0 ± 0.0	12.0 ± 0.2
Cu(II)- *Clinacanthus nutans* complex	18.0 ± 0.5	23.0 ± 0.5	19.0 ± 0.0	16.0 ± 0.4
Co(II)- *Clinacanthus nutans* complex	14.0 ± 0.0	20.0 ± 0.5	13.0 ± 0.0	11.0 ± 0.5
Ni(II)- *Clinacanthus nutans* complex	11.0 ± 0.0	18.0 ± 0.0	14.0 ± 0.3	11.0 ± 0.5
Zn(II)- *Clinacanthus nutans* complex	17.0 ± 0.2	21.0 ± 0.4	17.0 ± 0.5	13.0 ± 0.5
Cu(II)-*Pluchea indica (L.) Less* complex	26.0 ± 0.5	28.0 ± 0.2	24.0 ± 0.5	23.0 ± 0.2
Co(II)-*Pluchea indica (L.) Less* complex	20.0 ± 0.0	21.0 ± 0.0	18.0 ± 0.2	19.0 ± 0.0
Ni(II)-*Pluchea indica (L.) Less* complex	22.0 ± 0.0	23.0 ± 0.0	16.0 ± 0.0	18.0 ± 0.0
Zn(II)-*Pluchea indica (L.) Less* complex	24.0 ± 0.2	26.0 ± 0.5	21.0 ± 0.0	21.0 ± 0.5
Cu(II)- *Phyllanthus niruri* complex	23.0 ± 0.4	25.0 ± 0.5	22.0 ± 0.5	19.0 ± 0.4
Co(II)- *Phyllanthus niruri* complex	19.0 ± 0.0	21.0 ± 0.3	15.0 ± 0.5	13.0 ± 0.0
Ni(II)- *Phyllanthus niruri* complex	17.0 ± 0.0	21.0 ± 0.0	13.0 ± 0.5	15.0 ± 0.3
Zn(II)- *Phyllanthus niruri* complex	21.0 ± 0.2	24.0 ± 0.0	21.0 ± 0.5	17.0 ± 0.5
Streptomycin sulphate	19.0 ± 0.0	17.0 ± 0.0	21.0 ± 0.0	21.0 ± 0.0

From Table 2, the metal-natural extract complexes were most active as Cu(II) complexes, followed by Zn(II) complexes. Co(II) and Ni(II) complexes had interchangeable patterns selectively either against the different strains of bacteria or the identity of the natural extracts. Chelation with Cu(II) significantly enhanced the antibacterial activities selectively towards both the gram-positive bacteria (*S. aureus* and *B. cereus*) and the gram-negative bacteria (*E. coli* and *P. aeruginosa*). Previous literatures are also in agreement that copper complexes have a good therapeutic property as an anti-inflammatory, as well as an antibacterial [29]. It is postulated that the enhancement in activity upon chelation is the consequence of the non-ionic nature of all complexes, which might facilitate their diffusion through the biological membrane [30]. In other theories, complexation enhances the lipophilic character of the central metal atom, which favors its permeation through the lipid layers of the cell membranes and enhances bioactivity [31]. Singh et al. [32] also portrayed similar trends of bioactivity results upon complexation of ligands with metal ions. Cu(II) ion has the smallest in-plane radius among divalent, first-row transition metals, which results in a stronger ligand binding that plays the primary role in ligation

and transport upon chelation [33]. This is further supported by the findings that showed biological activity very strongly correlates with electronic and transport factors and that the bioactivity of the transition metal complexes is mirrored by the charge density of the metal ions [34]. Complexation with metals is an important factor that tetracyclines could traverse the outer membrane of gram-negative enteric bacteria through the OmpF and OmpC porin channels, as positively-charged cation coordination complexes, leading to accumulation in the periplasm, where the metal ion-tetracycline complexes presumably dissociate and diffuse through the lipid bilayer regions of the inner cytoplasmic membrane [35-36]. This mechanism could be similar to the metal-natural extracts here as well.

The MIC values (as shown in Table 3) showed that all the natural extracts and the metal-natural extract complexes were able to inhibit the gram-negative bacteria (*E. coli* and *P. aeruginosa*) at lower concentrations compared to the gram-positive bacteria (*S. aureus* and *B. cereus*). The selectivity against these two gram-negative bacteria would probably be due to the thin layer of peptidoglycan possessed by the gram-negative bacteria compared to the thicker layer of peptidoglycan in the gram-positive bacteria.

Table 3. Minimum Inhibitory Concentration (MIC) of natural extract and their metal-natural extract complexes (mean, n = 3)

Compounds	**MIC value (μg/mL)**			
	***E. coli* (ATCC 25922)**	***P. aeruginosa* (ATCC 27853)**	***S. aureus* (ATCC 29213)**	***B. cereus* (ATCC 117788)**
Clinacanthus nutans extract	0.333	0.333	1.000	1.000
Pluchea indica (L.) Less extract	0.111	0.111	0.333	0.333
Phyllanthus niruri extract	0.333	0.333	1.000	1.000
Cu(II)- *Clinacanthus nutans* complex	0.037	0.037	0.111	0.333
Co(II)- *Clinacanthus nutans* complex	0.333	0.111	0.333	0.333
Ni(II)- *Clinacanthus nutans* complex	0.333	0.111	0.333	0.333
Zn(II)- *Clinacanthus nutans* complex	0.111	0.037	0.333	0.333
Cu(II)-*Pluchea indica (L.) Less* complex	0.012	0.004	0.037	0.111
Co(II)-*Pluchea indica (L.) Less* complex	0.111	0.037	0.111	0.333
Ni(II)-*Pluchea indica (L.) Less* complex	0.111	0.111	0.111	0.333
Zn(II)-*Pluchea indica (L.) Less* complex	0.111	0.012	0.111	0.333
Cu(II)- *Phyllanthus niruri* complex	0.111	0.037	0.011	0.111
Co(II)- *Phyllanthus niruri* complex	0.333	0.111	0.333	0.333
Ni(II)- *Phyllanthus niruri* complex	0.333	0.111	0.333	0.333
Zn(II)- *Phyllanthus niruri* complex	0.333	0.111	0.333	0.333
Streptomycin sulphate	0.001	0.001	0.001	0.001

CONCLUSION

The present study showed that the metal ions were successfully incorporated into the metal-natural extracts complexes of *Pluchea indica (L.) Less*, *Clinacanthus nutans*, and *Phyllanthus niruri*. These complexes have displayed their potential as potent antibacterial agents against human bacterial infections caused by the organisms mentioned above compared to the natural extracts (the parent compounds). Thus, the incorporation of the metal ions has indeed increased the antibacterial properties of the natural extracts.

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