

Original Research Article

Isolation and Antimicrobial Activity of Bioactive Methylated Flavanone from Methanol Stem-Bark extract of *Nauclea diderichii* (Rubiaceae)

ABSTRSCT

The chromatographic investigation of the ethyl-acetate soluble fraction of methanol stem bark extract of *Nauclea diderichii* using a combination of silica and sephadex LH-20 led to the isolation of a methylated flavanone. The structure of this new compound was determined using both 1 and 2D nuclear magnetic resonance (NMR). The antimicrobial activities of the crude fractions were also investigated using agar diffusion and broth dilution techniques. Clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria were used for the studies. The crude fraction showed activity against *Staphylococcus aureus*, *Streptococcus pyrogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Samonella typhi*, but were tested doses below detectable limits (BDL) in methicillin resistant *S. aureus*, *Bacillus subtilis*, *Corynobacterium ulcerans*, *Escherichia coli* and the fungus *Candida albicans*. The low concentration for minimum inhibitory concentration (MIC, 1.25 µg/ml) and minimum bactericidal concentration (MBC, 2.25 µg/mL), while minimum fungicidal concentration (MFC, 5.00 µg/mL) suggesting that the compound has a good antimicrobial activity against the susceptible organisms and validates the ethno medicinal use of the *N. diderichii* in the treatment of various bacterial infections, including the opportunistic HIV.

Key words: *Nauclea diderichii*, antimicrobial, nuclear magnetic resonance (NMR). Microbes, secondary metabolites.

INTRODUCTION

Bioactive plant secondary metabolites have been found to be active against a wide variety of micro-organisms and have shown great promise in the treatment of many intractable diseases like the opportunistic infectious diseases and even the recent Ebola virus. Most of the plant secondary metabolites have been investigated for their chemical components and some of the isolated compounds have been shown to posse diverse and interesting biological activities (Gupta, 1994; Eja *et al.*, 2007).

Malaria, diarrhoea and dysentery are health challenges (Nwankwo *et al.*, 2005; Emokpae *et al.*, 2006) and is believed that *Nauclea diderichii* possesses medicinal properties effective in their management since decoctions of this plant are used for their cure in Mubi North and South LGAs of Adamawa State North-Eastern Nigeria.

Nauclea diderichii (De Wild & Dur) Meril (Rubiaceae). Its commercial name is *bilinga* and common name is African peach, is reputed for its numerous medicinal uses as a tonic and fever medicine, chewing stick, toothaches; dental caries, septic mouth, malaria, diarrhoea and dysentery cure (Lamidi 1995).

Most European countries similarly witness a seemingly unimpeded increase of antimicrobial resistance in the major Gram-negative pathogens which could unavoidably lead to loss of therapeutic treatment options (European Centre for Disease Prevention and Control (ECDC), 2012).

Natural products have been the most significant source of drugs and drug leads in history (Cragg and Newmann, 2005). The emergence of multidrug resistance in human and animal pathogenic bacteria as well as undesirable side-effects of certain antibiotics has triggered immense interest in the search for new antimicrobial / antifungal / antiviral drugs of plant origin (Ahmed and Beg, 2001). In theory, bacteria will continue to develop resistance once exposed to any antimicrobial agents, thereby imposing the need for a permanent search, develop and advancement of new drugs (Silver and Bostian, 1993) and higher plants represent a potential source of novel antibiotic prototypes (Meurer–Grimes *et al.*, 1996).

In Mubi LGAs, North Eastern Nigeria, the stem bark of *bilinga* are used (oral decoction), for the treatment of malaria and dysentery /diarrhoea both in children and adults (per community).

In our search for bio-active compounds from plants, we hereby report the isolation and antimicrobial effect of a new methylated flavanone from the ethyl acetate soluble fraction of the methanol extract of the stem-bark of *Nauclea diderichii*, thus viewing the plant as a potential source for phytomedicine development.

MATERIALS AND METHODS

Identification, collection and preparation of plant: Fresh samples of the stem bark of the *African peach* were obtained from Michika and Bali in Adamawa and Taraba States, respectively, in September, 2011 and were identified in the Biological Sciences Department of Adamawa State University Mubi. The FHI number is 0194 and a specimen of the plant was deposited in the herbarium. The sample (1.4kg) was air dried in the laboratory before pounding to a fine powder using pestle and mortar to about 70 mesh sizes and then stored in a dry sterile bottles.

Extraction: 450g of the powdered stem bark was accurately weighed and percolated with 10L of distilled methanol for 72hrs. After which there was decantation, filtration, and concentration using rotary evaporator (R110) at 35°C to obtain methanol soluble fractions, (F_m01), labelled, F_m0R. [30.5g] and this was kept in the refrigerator for further separation. It was later macerated with different solvents of increasing polarities starting with petroleum ether, chloroform, ethyl-acetate and methanol to have their corresponding fractions.

Thin layer chromatography (TLC): The ethyl-acetate soluble portion of the methanol extract of *N. diderichii*, was subjected to TLC using pre-coated aluminum plates. The solvent systems used were ethyl-acetate: methanol (9:1). The spots on TLC were visualized under ultraviolet (UV), spraying with 5% sulphuric acid in methanol followed by heating at a temperature of 110°C for 5 minutes.

Chromatographic separation

Column chromatography, silica gel (60 -80): Ethyl acetate soluble fraction (2.5 g) was mounted on a glass column (75 × 3.5 cm)). The column was eluted continuously using petroleum ether, ethyl acetate and methanol mixture by gradient elution technique; 20 ml each was collected as eluates and the progress of elution was monitored using TLC. A total of 15 fractions (ND-01- ND-15) from 200 collections were made; similar fractions were combined and further purified by Sephadex LH-20 gel filtration column chromatography. In both cases, the progress of separation was monitored using TLC.

Gel filtration: Further purification of ND-06 was undertaken using sephadex LH-20 eluted with ethyl acetate. The progress of separation was monitored using TLC. Repeated gel filtration led to the isolation of a compound coded ND-06.

Structure determination: Spectral structure determination was conducted at the School of Chemistry, University of Kwazulu-Natal, South Africa using UV light at 356 nm, infra-red (IR) was recorded at 3239.9 cm⁻¹, NMR was run with 400 MG-Htz Bruker NMR machine and data confirmed using MestReNova^(R).

Antimicrobial assay: The microorganisms tested included *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Corynebacterium ulcerans*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi* and *Candida albicans*. All the organisms were clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

Reference drug: Sperfloxacin 5 mcg/disc made by Himedia Laboratories Pvt. Ltd. Mumbai, India was used as the reference drug.

Susceptibility studies: Preliminary antimicrobial activity of the compound was carried out using stock concentration of 20 µg/ml. The microorganisms were maintained on agar slant. The inocula were prepared by inoculating the test organisms in nutrient broth and incubating them for 24 h at 37°C for the

bacteria, while for *C. albicans*, Sabouraud Dextrose broth was used and was incubated for 48 h. After incubation, the broth cultures were diluted to 1:1000 for the Gram-positive bacteria and 1:5000 for the Gram-negative bacteria. One milliliter of the diluted cultures was inoculated into a sterile molten nutrient agar at 45°C and poured into sterile petri-dish. Similarly, 1 mL of the diluted fungal suspension was poured into sterile Sabouraud dextrose agar plates and the excess sucked up with Pasteur pipette. These were swirled gently and allowed to solidify. Wells were bored into the solidified inoculated nutrient agar plates using cork borer of 6 mm diameter. The wells were filled with 0.1 mL of the compound. Sparfloxacin standard disc was also placed on the agar plate. 1 h was allowed for the compound to diffuse into the agar after which the plates were incubated overnight at 37 and 25°C for bacteria and fungi, respectively. At the end of incubation period, diameter of inhibition zone was measured using transparent ruler and recorded. The compound and standard antibiotic were tested in duplicate and mean zones of inhibition were computed.

Minimum inhibitory concentration (MIC)

MIC was determined using broth dilution method (Volleková *et al.*, 2001). Two fold serial dilutions of the compound were made to obtain concentrations of 5.00, 2.50, 1.25, 0.625 and 0.312 µg/ml. 0.2 mL suspension of standard inoculum of each organism was inoculated to the different concentrations of the compound. The test tubes were then incubated at 37°C for 24 h after which they were observed for growth. Inhibition of growth was indicated by a clear solution. The MIC was defined as the lowest concentration of the compound inhibiting the visible growth of each microorganism.

Minimum Bactericidal Concentration (MBC) / Minimum Fungicidal Concentration

The contents of the MIC tubes and the preceding tubes in the serial dilution were sub-cultured into appropriately labeled nutrient agar plates by dipping a sterile wire loop into each test tube and streaking the surface of the labeled nutrient agar plates. The plates were then incubated at 37°C for 24 h after which they were observed for colony growth. The lowest concentration of the subculture with no growth was

118 considered as minimum bactericidal concentration / minimum fungicidal concentration (Volleková *et al.*,
119 2001).

120 RESULTS AND DISCUSSION

121 **EXTRACTION:** The crude methanol extract (850 g) of *N. diderichii* stem bark after extraction, was
122 macerated with petroleum ether, chloroform and ethyl acetate to give the following residues and
123 weights.

124 **Table 1:** Extractive values of macerated methanol extract of *N. diderichii* stem bark

Extract	Colour	Weight (g)	Recovery yield (%)
Pet. Ether	Light brown	3.00	10.52
Chloroform	Pale yellow	6.63	17.80
Ethyl-acetate	Green	4.8	15,65
Methanol	Brown	24.4	28.70

125

126 SPECTRAL DATA

127 **Ultraviolet (UV):** In ND-06, the UV absorption band using a 356 nm UV spectral lamp, giving a white
128 spot is indicative of weak auxochromes that are pH dependent like OH. The C=C stretching frequency of
129 the cyclic molecule could be seen at 1604cm⁻¹.

130 **Infrared (IR):** The IR spectra of the isolated compound indicated prominent absorption frequencies
131 characteristic of certain functional groups: The band of medium intensity at 3239.9 cm⁻¹ suggests the
132 presence of hydroxyl group(s). The broad band of very strong intensities at 2950 and 2852.76 cm⁻¹ are

characteristic of C-H stretching of aliphatic groups e.g. CH₃, CH₂, and CH etc Overtones and combination tones of lower and weak frequency bands are displayed at 1530.8 cm⁻¹. Their appearance and position in a spectrum can be gainfully used for identification of some structural features. The C-H bending vibrations of the CH₃ and CH₂ groups are observed at 1429.6 and 1359.0 cm⁻¹.

Nuclear Magnetic Resonance (NMR)

Proton (¹H): The chemical shift of any proton is determined by its chemical environment: The cluster of methyl and methylene protons as observed in the spectra suggests the flavanonal nature of the compounds. DEPT, 90, 45 and 135, provides a clue to the various methyls, methylenes, methines and quaternary carbons. The hydroxyl proton is again confirmed at 4.0969 ppm in the proton NMR spectral.

Carbon (¹³C): *Compound NDK12:* Spectra data of ¹H, ¹³C, HSQC, HMBC, COSY, DEPT, NOSEY and LC-MS spectroscopy confirm the structure to be a methylated flavanone (Figure 1), and this was confirmed and further justified by ¹³C NMR data simulated by MestReNova^(R) ¹³C were within ± 03 ppm (Reynolds et al., 1986; Tinto et al., 1992; Jain and Bari, 2010).

The NMR spectrum (Figure1) indicated thirteen quaternary, three methine, seven methylene, and five methyl carbons and there are other side groups attached on the flavanone skeleton. The hydroxyl moiety is further confirmed by the proton nuclear magnetic resonance ¹H-NMR by the band at 4.7350. The appearances and positions of other combination peaks /bands in the spectrum have been gainfully used for identification of some structural features.

Mass Spectra of ND-06: Mass spectrum of ND-06: The mass spectrum of peak 0.11minutes from the Gas Chromatogram showed the parent ion $[M^+]$ peak at M/Z 419.1 which corresponds to the molecular formula $C_{28}H_{32}O_3$ showing characteristics of mass spectrum for a methylated flavanone and on the bases of the (GC-MS and the inbuilt main library (C:/Database/NIST05.LIB search and confirmation of the compound(s) (Figure1) was confirmed as being present in the plant.

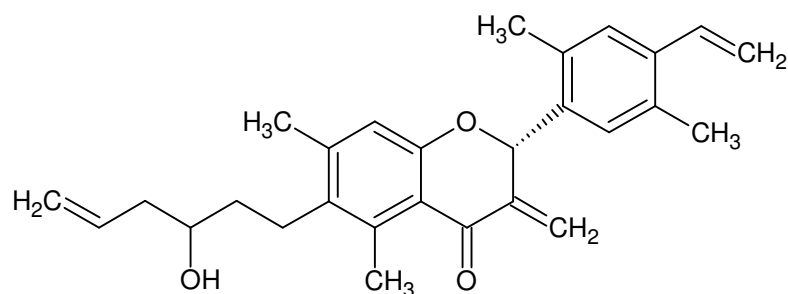


Figure 1: Suspected structure (A Methylated flavanone)

Table 2: Zone of inhibitions for Petroleum ether, Chloroform, ethyl acetate and methanol extracts of the stem bark of *N. diderichii* on fourteen micro-organisms.

Micro-organisms	Zones of inhibition (mm)						
	Pet. Ether	Chloro.	Ethyl Act.	Methanol	Sparflo	.Flocon	DMSO
Methicillin R.S.A	-	25± 0.48	-	21± 0.68	33± 0.68	-	-
<i>Staphylococcus aureus</i>	15± 0.11	27± 0.76	21± 0.63	22± 0.46	35± 0.38	-	-
<i>Streptococcus pyogenes</i>	-	-	-	-	32± 0.67	-	-
<i>Bacillus subtilis</i>	14± 0.32	26± 0.72	20± 0.82	23± 0.85	40± 0.64	-	-
<i>Corynebacterium ulceras</i>	-	24± 0.43	20± 0.84	21± 0.73	28± 0.74	-	-
<i>Escherichia coli</i>	17± 0.41	27± 0.62	21± 0.50	22± 0.62	30± 0.46	-	-

<i>Proteus mirabilis</i>	-	22± 0.91	-	-	28± 0.54	-	-
<i>Proteus vulgaris</i>	-	-	-	-	28± 0.47	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	28± 0.56	-	-
<i>Salmonella typhi</i>	17± 0.51	27± 0.76	20± 0.85	21± 0.85	32± 0.55	-	-
<i>Klebsiella pneumoniae</i>	19± 0.81	25± 0.82	21± 0.54	25± 0.63	40± 0.45	-	-
<i>Candida albicans</i>	-	2± 0.32	-	-	-	24± 0.65	-
<i>Candida stllatoidea</i>	-	-	-	-	-	27± 0.78	-
<i>Candida tropicalis</i>	-	24± 0.71	20± 0.56	21± 0.65	-	21± 0.36	-

162 Results: Mean of three trials ± Standard error, (-) = Resistant Strain, Sparflo = Sparfloxacin (Bacteria

163 Control): 10µg/ml, Flucona = Fluconazole (Fungal control): 20µg/ml

164 **Table 3:** Minimum Inhibitory Concentration (MIC) of Petroleum ether, Chloroform, ethyl acetate and methanol extracts of the stem bark of *N.*
 165 *diderichii* on fourteen micro-organisms

Micro-organisms	Concentration (mg/mL)																			
	Petroleum Ether					Chloroform					Methanol					Ethyl acetate				
	5.00	2.50	1.25	0.625	0.312	5.00	2.50	1.25	0.625	0.312	5.00	2.50	1.25	0.625	0.312	5.00	2.50	1.25	0.625	0.312
Methicillin R.S.A	-	-	-	-	-	-	-	1.25	+	++	-	-	1.25	+	++	-	-	-	-	-
<i>Staphylococcus aureus</i>	5.00	+	+	++	+++	-	-	1.25	++	+++	-	-	1.25	+	++	-	2.50	+	++	++
<i>Streptococcus pyogenes</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bacillus subtilis</i>	5.00	+	+	++	+++	-	-	1.25	+	++	-	2.25	-	+	++	-	-	1.25	+	++
<i>Corynebacterium ulceras</i>	-	-	-	-	-	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++
<i>Escherichia coli</i>	-	2.25	+	++	+++	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++
<i>Proteus mirabilis</i>	-	-	-	-	-	-	-	1.25	+	++	-	-	-	-	-	-	-	-	-	-
<i>Proteus vulgaris</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Salmonella typhi</i>	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++
<i>Klebsella pneumoniae</i>	-	2.25	+	++	+++	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++
<i>Candida albicans</i>	-	-	-	-	-	-	-	1.25	-	-	-	-	-	-	-	-	-	-	-	-
<i>Candida stllatoidae</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Candida tropicalis</i>	-	-	-	-	-	-	-	1.25	+	++	-	-	1.25	+	++	-	-	1.25	+	++

166 Key: - = no turbidity, (No growth), + = turbidity (Light growth), ++ = (Moderate growth), +++ = (High growth) MIC (Values Given) = Minimum
 167 inhibitory concentration, Sparflo = Sparfloxacin (Bacteria Control): 10µg/ml, Flucona = Fluconazole (Fungal control): 20µg/ml

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169 **Table 4:** Minimum Bactericidal Concentration (MBC)/ Minimum Fungicidal Concentration (MFC) of Petroleum ether, Chloroform, ethyl acetate
170 and methanol extracts of the stem bark of *N. diderichii* on some fourteen micro-organisms

Micro-organisms	Concentration (mg/mL)																			
	Pet. Ether					Chloroform					Methanol					Ethyl acetate				
	5.00	2.50	1.25	0.625	0.312	5.00	2.50	1.25	0.625	0.312	5.00	2.50	1.25	0.625	0.312	5.00	2.50	1.25	0.625	0.312
Methicillin R.S.A	-	-	-	-	-	-	-	MIC	+	++	MIC	+	++	+++	+++	-	-	-	-	-
<i>Staphylococcus aureus</i>	MIC	+	++	+++	++++	-	MIC	+	++	+++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
<i>Streptococcus pyrogenes</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Bacillus subtilis</i>	MIC	+	++	+++	++++	-	MIC	+	++	+++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
<i>corynebacterium ulceras</i>	-	-	-	-	-	MIC	+	++	+++	++++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
<i>Escherichia coli</i>	MIC	+	++	+++	++++	-	MIC	+	++	+++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
<i>Proteus mirabilis</i>	-	-	-	-	-	MIC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Proteus vulgaris</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Salmonella typhi</i>	MIC	+	++	+++	++++	-	MIC	+	++	+++	MIC	+	++	+++	++++	MIC	+	++	+++	++++
<i>Klebsella pneumoniae</i>	MIC	+	++	+++	++++	-	MIC	+	++	+++	-	MIC	+	++	+++	MIC	+	++	+++	++++
<i>Candida albicans</i>	-	-	-	-	-	MIC	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Candida stllatoidae</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Candida tropicalis</i>	-	-	-	-	-	MIC	+	++	+++	++++	MIC	+	++	+++	++++	MIC	+	++	+++	++++

171 Key: - = no turbidity, (No growth), + = turbidity (Scanty colonies growth), ++ = (Moderate growth), +++ = (High growth), ++++ = (v. heavy
172 colonies growth) MBC / MFC = Minimum Bactericidal Concentration / Minimum Fungicidal Concentration.

In Table 2 most of the microbes showed sensitivity to the extracts though at different levels of inhibition but for *Streptococcus pyrogenes*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and the fungus *Candida stellatoidea*, where the extracts do not show any inhibition zones. Petroleum ether extract was seen not to be active on most of the pathogens e.g. Methicillin Resistant *Staphylococcus aureus*, or (Methicillin R.S.A), *Streptococcus pyrogenes*, *Corynebacterium ulceras*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and the fungi, *Candida albicans*, *Candida stellatoidea* and *Candida tropical*. Thus one can also infer from the table that the chloroform extract has the highest activity, followed by the ethyl acetate, then the methanol and lastly the petroleum ether.

It was observed from Table 3 that the minimum inhibitory concentration (MIC) was 1.25 mg/mL generally. This result, also showed that the chloroform fraction had the highest anti-microbial reactivity (at this concentration, it has the widest spectrum of affected microbes) followed by the ethyl acetate fraction, the methanol and lastly the petroleum ether fraction of the stem bark of the plant. This shows that the secondary metabolites from the plant are highly active on the tested microorganisms. The present investigation clearly reveals the antibacterial / anti-fungal nature of this plant and portrays it as a potential source of useful drugs thereby suggesting that it could be exploited in the management of diseases caused by these bacteria /fungi in human and plant systems (Raghavendra et al., 2006).

MBC /MFC, (Table 4), were carried out to check whether the bacteria and the fungi were killed or only their growth was inhibited. The average value was 2.25mg/mL. MFC was found to be 5.00mg/mL and was effective for chloroform, ethyl-acetate and methanol

Currently, no single drug is effective for the treatment of multidrug resistant malaria and combination therapy includes artemisinin derivatives such as artesunate (David *et al.*, 2004) or mixtures with older drugs such as atovaquone (Deprez-Poulain and Melnyk, 2005), proguaml (Jones and Good, 2006) combination malarone / maldox (Winter *et al.*, 2006; Taylor and white, 2004).

Unfortunately, first report on drug resistance to arteminin-derivatives (Jambou *et al.* 2005) and to drug combination therapies (Wichmann *et al.*, 2004) have already appeared. So, in the absence of a functional, safe and widely available malaria vaccine, efforts to develop new anti-malaria drugs continue.

CONCLUSION

Present study showed that the stem bark of *N. diderichii* contains an antimicrobial flavanone as one of its constituents. The antimicrobial activity by *N. diderichii* is significant and may serve as a lead towards the development of more potent, safe and cost effective antimicrobial agents. It also validates the ethno medicinal use of the plant in the treatment of various bacterial infections, including typhoid, diarrhoea and may be enhanced to cure even the current Ebola. To our knowledge, this is the first reported methylated flavone from *N. diderichii*.

The present work should be scaled- up and further work carried out with it on the malaria parasite to confirm whether the active metabolites would be effective singly or in synergy.

Clinical and nutritional studies are recommended for the above indigenous plants so as to be able to advice locals in the various locations of the ill effects of the plants. Equally, the phytochemistry of these plants from different locations could be equally compared and research should be carried out on other parts of the plants to ascertain whether the entire plant possess medicinal properties.

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