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Efficacy of methanol extracts of *Lawsonia inermis* L. and *Cymbopogon citratus* Stapf. against fungal isolates from poultry farms in Anambra State, Nigeria

Chito Clare EKWEALOR[1*], Anthonia Nkiruka MBA[2], Vivian Nonyelum ANAKWENZE[1], Chikaodili Gladys ANAUKWU[1], Ikechukwu Amechi EKWEALOR[1]

[1]Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, P. M. B. 5025, Awka, Anambra State, Nigeria. [2]Department of Microbiology/Parasitology, David Umahi Federal University of Health Sciences, Uburu, Ebonyi State, Nigeria.

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Abstract
Fungal diseases in poultry constitute a challenge to poultry productivity and public health risk to poultry workers. The aim of this research was to evaluate the efficacy of *Lawsonia inermis* L. and *Cymbopogon citratus* Stapf. leaves against fungal isolates from poultry farms. Susceptibility of fifteen species of fungi to methanol extracts of the plants leaves were assessed using disc diffusion method. Discs impregnated with different concentrations of these extracts were placed on Sabouraud Dextrose Agar plates inoculated with the test organisms. Discs with 2% DMSO and 1.25 mg/ml Nystatin served as negative and positive controls. The plates were incubated at 25°C for 48 hours and diameter of zone of inhibition measured in millimeters. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) were determined using broth dilution method. Ten moulds (66.7%) were susceptible to methanol extract of *L. inermis* while eight (53.3%) were inhibited by *C. citratus*. MIC and MFC of the extracts were recorded at ≥12.5 mg/ml and ≥50 mg/ml respectively. The leaves exhibited fungicidal activities against most tested moulds and could be explored as alternative and safer sources of new antifungal drugs.

Keywords: *Lawsonia inermis*; *Cymbopogon citratus;* Poultry farms; Antifungal activity

INTRODUCTION

Fungal infections have emerged as a world-wide health challenge in the poultry industry [1], causing direct and indirect harm to the workers as well as high morbidity, mortality and production losses of the birds [2,3]. Within the poultry house, fungi may be present in settled dust, bioaerosols derived from soil, dust, droppings, mouldy feed, and to a lesser extent, from the birds themselves [4]. Birds become infected during hatching, by inhalation of the fungal spores from contaminated hatchery machines or contaminated litter [5,6]. Infection can spread through direct contact either with other birds or workers which can be influenced by characteristics of the building, type of task, type of litter and poultry production, type of breeding, method of feed distribution, type of system operated and airflow velocity [7]. Most

*Correspondence. *E-mail*: c.ekwealor@unizik.edu.ng *Tel*: +234-8035080790. ISSN 0189-8442
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of the fungal diseases of poultry occur sporadically but occasionally in the form of outbreak [8].

The genera of fungi associated with poultry farm diseases include; *Acremonium, Fusarium, Lichtheimia, Aspergillus, Trichoderma, Penicillium, Scopulariopsis, Curvularia, Alternaria,* yeast, such as *Histoplasma, Cryptococcus,* and most commonly *Candida* species, *Microsporium,* and *Trichophyton* [9,10,11]. Many of these fungi produce mycotoxins which can substantially affect animal performance causing symptoms which are most times not recognized as mycotoxin related, resulting in reduced productivity of the birds and mortality in extreme cases [12,13,14].

It has been reported that a large proportion of the antimicrobials in current use for treatment of microbial infections are derived from natural products [15]. Treatment of fungal infections is still insignificant compared to outstanding advances already established in the treatment of bacterial and viral diseases of poultry [8]. There is great difficulty in production of antifungal drugs for use in birds and humans and the few available ones do have many side effects. Recent research has also shown increase in resistance by fungi to currently used antifungal drugs [16]. These antifungal compounds not only target fungal pathogens but also act on targets found in mammalian cells which may result in toxicity or adverse drug interactions [17], hence, the need for alternative source.

Medicinal plants have been used in conventional medicine for treatment of both human and animal fungal infections. These plants are rich in many biologically active, secondary metabolites like tannins, saponins, phenolics compounds and terpenoids, which may be prospective natural antimicrobial agents that may serve as an alternative, effective, cheap and safe antimicrobial agents, for treatment of common microbial infections [16,18,19]. Only estimated fraction of 250,000 – 500,000 plants have been screened for their phytochemical constituents with infinitesimal percentage submitted for biological investigations [19].

Natural products derived from medicinal plants have been well known for their contributions in developing modern drugs as well as herbal formulations in traditional medicine. These medicinal plants remain the primary source of treatment of diseases in developing countries and about 60% of world's populations depend on them [18]. In some Asian, Latin American and African countries, 80% of the populations depend on traditional medicine for primary health care [20]. In Nigeria, 50.481% of the populations live in rural areas [21] and most do not have access to quality healthcare [22] but resort to medicinal plants for their health needs.

The acceptability of natural plants as alternative source of health care has prompted researchers into investigating the antimicrobial activities of these medicinal plants [23]. It has been recorded that the side effects seen with other drugs are absent with medicinal plants, they show synergistic effect when they contain multifunctional molecules and have safety potentials [24]. With the increased prevalence of fungi resistance to antifungal drugs, it is essential to evaluate antifungal effect of leaves of *Lawsonia inermis* L. and *Cymbopogon citratus* Stapf. against pathogenic moulds isolated from birds, feeds, poultry workers and environment.

Lawsonia inermis L. (Lythraceae) commonly known as Henna or Mehndi, is native to tropical and subtropical regions of Africa, Asia, Australia, South-East Africa, Middle East, Arab [25]. It is cultivated for its leaves although stem, bark, roots, flowers and seeds have also been used in traditional medicine [26,27]. The plant is gray-white, glabrous and has many branches with spine. Leaves are opposite, entire, subsessile, elliptical and broadly lanceolate, having depressed veins on the rim of calyx tube. The

flowers have fragrance and are small in white or pinkish colour. The plant is reported to contain carbohydrates, proteins, flavonoids, tannins and phenolic compounds as well as alkaloids, terpenoids, quinones, coumarins, xanthones and fatty acids [25,26,28,29]. It has a colour agent known as Lawsom, with the chemical formular $C_{10}H_6O_3$, which is its most effective component [30].

Indian women use it as a fashion in every occasion to dye their hair, decorate the palms, nail and feet. Men also use it to dye their hair and beard [31]. In the northern part of Nigeria, the plant is called 'lali' and is also primarily used to dye different parts of the body [32]. In addition to its use as cosmetic, it has many health benefits [25]. The fungicidal effect of Henna has long been known [25,27,33]. It is also known for being famous in anticancer, antibacterial, antimalarial, antipyretic, antioxidant and anti-inflammatory activities, hepatoprotective ability and wound and burn healing [27,34] The astringent nature of the leaves, make it useful in cure of sunburn and other rashes on the body while the bark can help in treatment of skin diseases such as athlete's foot, eczema and ringworm [31].

Cymbopogon citratus Stapf. **(**Gramineae**) is** popularly known as citronella grass or lemon grass [35]. It is indigenous to tropical and semi-tropical areas of Asia but cultivated in South and Central America, Africa, India and other tropical countries [36]. Lemon grass is a tufted perennial grass growing to a height of 1 meter with numerous stiff leafy stems arising from short rhizomatous roots. It can be dried and powdered or used fresh. The compounds identified in *Cymbopogon citratus* are mainly terpenes, alcohols, ketones, aldehyde and esters. Some of the reported phyto-constituents are essential oils that contain Citral α, Citral β, Nerol Geraniol, Citronellal, Terpinolene, geranyl acetate, geraniol, Myrecene (an antibacteria and a pain reliever) and Terpinol Methylheptenone [36,37]. Other phytoconstituents such as flavonoids and phenolic compounds, which consist of luteolin, isoorientin 2'-O-rhamnoside, quercetin, kaempferol and apiginin have also been identified in it. The lemon-like scent of *C. citratus* have been linked to the existence of cyclic Monoterpene (citral) [36,37]. Studies also indicate that *Cymbopogon citratus* possesses various pharmacological activities such as anti-amoebic, antibacterial, antidiarrheal, antifilarial, antifungal, antihypertensive, antioxidant and anti-inflammatory properties [38]. *It* is also commonly used in teas, soups and curries and also suitable for poultry, fish and seafood [39].

The study evaluated the antifungal efficacy of *Lawsonia inermis* L. and *Cymbopogon citratus* Stapf. against fungal isolates from six poultry farms in Anambra State, Nigeria.

EXPERIMENTAL METHODS

Fungal isolates. Fifteen (15) fungal species (*Aspergillus chevalieri Mangiin, Aspergillus conicus, Aspergillus fumigatus, Aspergillus flavus, Aspergillus tubingenses, Aureobasidium pullulans, Cunninghamella bertholletiae, Curvullaria verruculosa, Fusarium oxysporum, Lichtheimia corymbifera, Penicillium citrinum, Paecillomyces varioti, Syncephalis aggregate, Scopulariopsis brevicaulis* and *Trichoderma erinaceum)* isolated from birds, feeds and poultry workers in six poultry farms in Anambra State, Nigeria which were identified based on detailed studies of their macroscopic, microscopic and genetic features were used for the study.

Collection and identification of plants. Leaves of *Lawsonia inermis* and *Cymbopogon citratus* were obtained from matured plants in the morning hours from Keffi town, in Nasarawa State and Umunya in Anambra State, Nigeria respectively. They were identified in the Department of Botany, Nnamdi Azikiwe University, Awka and

voucher specimens NAUH-252A for *Lawsonia inermis* L. and NAUH-187A for *Cymbopogon citratus* Stapf. lodged in the herbarium.

Preparation / extraction of crude extracts. Soxhlet extraction of the plant extract was carried out according to methods reported in published sources [32, 40], using methanol as a solvent. Healthy leaves of the medicinal plants were washed with distilled water, dried in an oven at 40°C and ground into fine powder. Fifty grams (50 g) of the powder was used for the extraction with 500 mL methanol using a Soxhlet extractor. Thereafter, the solvent was removed with a rotary evaporator apparatus. The crude extracts were stored in a freezer, at -4°C, for fungal susceptibility studies.

Inoculum preparation and antifungal activity of plants extracts. The inoculum was prepared by growing a four-day old fungal isolate on SDA. This was aseptically scrapped and transferred into a tube containing 10 mL sterile water, vigorously shaken and diluted Ten-fold [41].

In vitro antifungal activity of the extracts was evaluated by the disc diffusion method. The concentrated methanol extract (20mg) was dissolved in 1 mL of 2% dimethyl sulphoxide (DMSO) and serially diluted two-fold in sterile water. The different concentrations (20 mg, 10 mg, 5 mg and 2.5 mg) were impregnated on 6 mm diameter paper discs. A 0.1 mL of 10^{-6} dilution of the inoculum suspension (equivalent to 1.5×10^{-6} sfu/mL) was inoculated on Sabouraud Dextrose Agar plates using the spread plate method and allowed to dry. Discs which had been impregnated with methanol extract were thereafter placed gently on the surface of the SDA using a pair of sterile forceps. The sensitivity tests were done in duplicate plates. Disc impregnated with 2% dimethyl sulphoxide (DMSO) and another impregnated with 1.25 mg/mL Nystatin [42] served as negative and positive controls respectively. The petri dishes were incubated at 25°C for 48 hours and diameter of the zone of inhibition measured in millimeters using a calibrated meter rule.

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of methanol extracts. Minimum inhibitory concentrations (MIC) of the different plant extracts were determined by broth dilution method described by McGinnis [43]. Methanol extract (200 mg) were dissolved in 1 mL of 2% dimethylsulfoxide (DMSO) and serially diluted two fold to concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL. Standardized suspension, 0.1 mL (1.5×10^{-6} sfu/mL dilution) of the test organism was aseptically inoculated into each of the dilution tubes and incubated at 25°C for seven days. Tubes with 2% dimethyl sulphoxide (DMSO) and another with 1.25 mg Nystatin [42] served as negative and positive controls respectively. The tubes were observed for growth and MIC recorded as the lowest concentration of the plant extract in the tube that failed to show any visible fungal growth.

Minimum fungicidal concentration (MFC) of the plant extract was determined by plating-out a loopful from tubes of MIC without visible fungal growth onto sterile plates of Sabouraud Dextrose Agar. The plates were incubated for seven days at 25°C and observed for growth. The MFC was taken as the lowest dilution of the extract that showed no visible growth on SDA plate [32,44].

RESULTS

The susceptibility of the fungal isolates to methanol extracts of the medicinal plants is represented in Table 1. As observed, methanol extract of *Lawsonia inermis* was active against all the isolates except *Aspergillus chevalieri Mangin, Aspergillus tubingense, Aureobasidium pullulans, Cunninghamella bertholletiae* and *Curvullaria verruculosa* at

20 mg/mL with zones of inhibition ≥9.4 mm (Table 1).

Similarly, methanol extract of *Cymbopogon citratus* was active against test isolates except *Aspergillus chevalieri Mangin, Aspergillus tubingense, Aureobasidium pullulans, Cunninghamella bertholletiae, Lichtheimia corymbifera, Scopulariopsis brevicaulis* and *Trichoderma erinaceum.* However, it was observed that, the extract completely inhibited the growth of *Penicillium citrinum* at concentration of 5 mg/mL. It is important to observe that *Aureobasidium pullulans* that is resistant to methanol extracts of *L. inermis* and *C. citraus* leaves is also resistant to the antifungal drug, Nystatin (Tables 1). The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of methanol extracts of *Lawsonia inermis* and *Cymbopogon citratus* against the fungal isolates are represented in Table 2. It was observed that both plants extracts had MIC of 50 mg/mL (Table 2) against all the fungal isolates tested except for *L. inermis* whose MIC was 25 mg/mL for *Trichoderma erinaceum* and 12.5 mg/mL for the activity of *C. citratus* on *Penicillium citrinum.* While the two extracts have an MFC of 100 mg/mL against all the fungal isolates, *L. inermis* had 50 mg/mL for *Trichoderma erinaceum* and *Cymbopogon citratus,* 25 mg/mL for *Penicillium citrinum* (Table 2)

DISCUSSION

Fungal infections have been reported to cause significant economic losses to the poultry industry either due to their direct effects in causing diseases and mortality or production of mycotoxins [2]. Unfortunately, poultry workers are subject to a number of potential health hazards including fungal respiratory diseases due to high level of contamination by fungal spores found in poultry houses [45]. Chronic exposure to fungal spores can also produce allergic responses in sensitized birds resulting in illness and decreased productivity [2]. These fungal diseases are usually treated with antifungal agents though long-term use can lead to fungal resistance. Drug resistant fungi have become a major global health challenge [46], hence, the need for natural compounds capable of exerting antifungal activity without resistance.

Table 1: Susceptibility of fungi isolates to methanol extracts of *Lawsonia inermis* and *Cymbopogon citratus*

Species of fungi	Diameter zone of inhibition (mm)									
	L. inermis (mg/mL)				*C. citratus* (mg/mL)				Nystatin (mg/mL)	DMSO (2%)
	20.0	10.0	5.0	2.5	20.0	10.0	5.0	2.5	1.25	
Aspergillus. Chevalieri mangiin	+	+	+	+	+	+	+	+	20.0	+
Aspergillus conicus	9.4	+	+	+	22.3	18.1	15.5	13.0	20.0	+
Aspergillus fumigatus	18.2	16.2	14.5	+	20.3	17.1	15.0	1.2	CC	+
Aspergillus flavus	13.1	11.0	9.2	+	14.6	12.0	10.5	8.0	20.0	+
Aspergillus tubingenses	+	+	+	+	+	+	+	+	24.0	+
Aureobasidium pullulans	+	+	+	+	+	+	+	+	+	+
Cunninghamella bertholletiae	7	+	+	+	+	+	+	+	33.0	+
Curvullaria verruculosa	+	+	+	+	18.4	15.6	13.1	10.2	+	+
Fusarium oxysporum	17.0	15.2	13.5	+	13.5	11.2	9.2	+	20.5	+
Lichtheimia corymbifera	12.4	10.2	8.5	+	+	+	+	+	17.0	+
Penicillium citrinum	13.2	11.4	9.1	+	CC	CC	CC	+	+	+
Paecillomyces varioti	22.1	20.3	17.2	+	19.4	13.1	11.0	9.2	25.0	+
Syncephalis aggregate	20.3	16.4	14.2	+	20.1	17.3	15.1	12.2	22.0	+
Scopulariopsis brevicaulis	15.2	12.4	10.1	+	+	+	+	+	+	+
Trichoderma erinaceum	31.0	25.2	21.1	+	+	+	+	+	28.0	+

+ = Not inhibited, CC = Cleared completely, Diameter of disc used = 6 mm, DMSO = Dimethyl Sulphoxide

Table 2: Minimum inhibitory concentration and minimum fungicidal concentration of methanol-extracts of *Lawsonia inermis* and *Cymbopogon citratus*

Species of fungi	Minimum inhibitory concentration (mg/mL)				Minimum fungicidal concentration (mg/mL)	
	L inermis	*C citratus*	Nystatin (1.25)	DMSO (2%)	*L inermis*	*C citratus*
Aspergillus chevalieri mangiin	ND	ND	-	+	ND	ND
Aspergillus conicus	50	50	-	+	100	100
Aspergillus fumigatus	50	50	-	+	100	100
Aspergillus flavus	50	50	-	+	100	100
Aspergillus tubingenses	ND	ND	-	+	ND	ND
Aureobasidium pullulans	ND	ND	+	+	ND	ND
Cunninghamella bertholletiae	ND	ND	-	+	ND	ND
Curvullaria verruculosa	ND	50	+	+	ND	ND
Fusarium oxysporum	50	50	-	+	100	100
Lichtheimia corymbifera	50	ND	-	+	100	ND
Penicillium citrinum	50	12.5	+	+	100	25
Paecillomyces varioti	50	50	-	+	100	100
Syncephalis aggregate	50	50	-	+	100	100
Scopulariopsis brevicaulis	50	ND	+	+	100	ND
Trichoderma erinaceum	25	ND	-	+	50	ND

ND: Not determined, + = Not inhibited, - = inhibited, DMSO = Dimethyl Sulphoxide

Medicinal plants rich in bioactive and secondary metabolites have been used in traditional systems for treating both human and animal fungal infections [16,18,19,47]. From available literature, many researches have been carried out on the antifungal activities of *L. inermis* and *C. citratus* against *Candida* spp. and dermatophytes [32,38,40,48,49], but not much on non-dermatophyte moulds isolated from human samples [23,50].

Methanol extract of *L. inermis* inhibited ten out of the fifteen moulds tested with diameter zone of inhibition ≥9.4 mm. The inhibitory activity observed is supported by a published work [51], in which antifungal effects of methanol extract of five medicinal plants were tested against 10 pathogenic isolates and *C. albicans* (B017). In this source, it was reported that methanol extract of *L. inermis* showed the highest percentage (76 – 88%) inhibition of mycelial growth when compared with the other four plants. The result is also corroborated by an earlier publication [23] in which a strong activity of methanol extracts of *L. inermis* against non-dermatophyte moulds isolated from farmers with onychomycosis was observed. Similarly, others in their research findings [40], reported high susceptibility of three different yeasts and moulds to methanol extracts of the *L. inermis*, and attributed the antifungal activity to naphthoquinone and tannin found in the plant. It has been reported that quinones have the ability to receive one or two electrons from the microorganisms, which create radial anion intermediates that are exceptionally reactive and cause oxidative stress in the microbial cells [52]. High, activity of methanol extract of *L. inermis* against *A. flavus* has also been reported [53]. A similar study using ethanol as solvent [54] reported high sensitivity of *Fusarium oxysporum, Penicillium* sp. and *Aspergillus flavus* to extracts of *L. inermis*.

Methanol extract of *C. citratus* inhibited eight out of the fifteen moulds tested. The inhibitory effect of the extract agrees with the work of many researchers who reported the antifungal effect of *C. citratus* on different fungi [38,55,56,57]. Parveen *et al*. [57] and Aourach *et al*. [56] reported inhibition of *Fusarium oxysporun* isolated from plant roots

by *C. citratus* extract which corroborates the result obtained in this study.

In this study, *A. flavus* was inhibited by methanol extract. This is in line with the result of a study [56], which also reported that methanol extract of *C. citratus* was more effective against *A. flavus*. In contrast to our findings, one publication [58] reported no inhibitory activity of the methanol extract of *C. citratus* leaves against *A. flavus* from herbal drugs. Minimum Inhibitory Concentration of ≤50 mg/mL and Minimum Fungicidal Concentration of ≥50 mg/mL were recorded against all the tested moulds that showed susceptibility to methanol extract of *L. inermis* while MIC of ≤50 mg/mL and MFC of ≥25 mg/mL were reported for *C. citratus.*

Conclusion. The methanol extracts of *L. inermis* and *C. citratus* showed antifungal activities against fungal isolates from six poultry farms in Anambra State, Nigeria. The bioactive ingredients in these medicinal plants can be extracted and used for production of plant based antifungal agents that may be cheaper, safer and devoid of antifungal resistance.

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