African Journal of Biotechnology Vol. 10(41), pp. 8117-8121, 3 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.330
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

In vitro antilisterial properties of crude aqueous and n-hexane extracts of the husk of *Cocos nucifera*

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Accepted 25 April, 2011

The *in vitro* antilisterial activities and time kill regimes of crude aqueous and n-hexane extracts of the husk fiber of *Cocos nucifera* were assessed. The aqueous extracts were active against 29 of the 37 test *Listeria* isolates while the n-hexane extracts were active against 30. The minimum inhibitory concentrations (MICs) of all the susceptible bacteria ranged between 0.6 and 2.5 mg/ml for the aqueous fraction and between 0.6 and 5.0 mg/ml for the n-hexane extract. The average log reduction in viable cell count in the time kill assay ranged between 0.32 Log_{10} and 3.2 Log_{10} cfu/ml after 4 h of interaction, and between 2.6 Log_{10} and 4.8 Log_{10} cfu/ml after 8 h interaction in 1 × MIC and 2 × MIC (aqueous extract); and between 2.8 Log_{10} and 4.8 Log_{10} cfu/ml after 4 h of interaction, and 3.5 Log_{10} to 6.2 Log_{10} cfu/ml after 8 h interaction in 1 × MIC and 2 × MIC for the n-hexane extract. The extract was bactericidal against one of the test bacteria at 1 × MIC and against three of the test bacteria at 2 × MIC for the 8 h interaction period for the aqueous extract, while for the n-Hexane fraction; the extract was bactericidal against all the five test bacteria at both MICs after the 8 h interaction period. We suggested that the crude aqueous and n-hexane extracts of the husk of *C. nucifera* could be bacteriostatic or bactericidal depending on the time of exposure and concentration.

Key words: *Cocos nucifera,* n-hexane extract, aqueous extract, minimum inhibitory concentration, time-kill.

INTRODUCTION

Listeriosis is a serious disease of humans. The overt form of the disease has mortality rate which is greater than 25% (Kenneth, 2008) and it is a serious infection caused by eating food contaminated with *Listeria* species. Listeriosis has been recognized as an important public health problem in the United States (Kenneth, 2008). The disease affects primarily pregnant women, newborns, and adults with weakened immune systems (Kenneth, 2008). The causative agents in most instances appear to be members of the indigenous microbiota and, thus, the infections might be seen as endogenous (Socransky and Haffajee, 2002). The two main clinical manifestations are sepsis and meningitis. Meningitis is often complicated by encephalitis, a pathology that is unusual of bacterial infections (Kenneth, 2008). *Listeria* species have many opportunities to enter food production and processing environments. Consequently, outbreaks and sporadic cases of Listeriosis have been traced to different food-stuffs, such as dairy products (Allerberger and Wagner, 2010; Denny et al., 2008).

The use of medicinal plants in the treatment of infections caused by *Listeria* pathogen remains an important approach to the development of new antimicrobial drug. Esquenazi et al. (2002) reported the antibacterial, antifungal and antiviral properties of the husk fibre extract of the northeastern Brazil *Cocos nucifera* plant and an extensive range of several medicinal uses of this plant has been reported (Duke, 1992). In recent years, resistance to multiple drug in both human and plant pathogenic microorganisms have deve-loped due to indiscriminate use of conventional antibiotics commonly applied in the treatment of infectious diseases (Loper et al., 1999). This situation has encouraged further exploration for new antimicrobials from various sources, including medicinal plants (Cordell, 2000) that could be used in the treatment of infections by drug resistant

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pathogens.

C. nucifera (Aracaceae, English name: coconut palm) is an important food crop and medicinal plant mostly found in the tropical and subtropical countries. It belongs to the family *Palmae*. The coconut palm is found throughout the tropics, where it is very important to the local people. It is particularly important in the low islands of the Pacific where, in the absence of land-based natural resources, it provides almost all the necessities of life: food, drink, oil, medicine, fibre, timber, thatch, mats, fuel and domestic utensils (Edward and Craig, 2006). For good reason, it has been called the “tree of heaven” and “tree of life” and till now, it still remains an important economic and subsistence crop in many small Pacific island states (Edward and Craig, 2006).

The husk fiber of *C. nucifera* has been reported to be rich in catechin and epicatechin together with condensed tannins, which confers to its aqueous extract a potent antioxidant characteristics (Alviano et al., 2004). It has also been reported to have antibacterial, antiviral, antidysenteric, antifungal, antileishmanial, antilympho-proliferative and antineoplasic activities (Esquenazi et al., 2002; Mendonca-Filho et al., 2004; Kirszberg et al., 2003; Koschek et al., 2007). Moumita and Adinpunya (2007) observed that the husks of the coconut palm are discarded as waste and it is considered as one of the major agro wastes of the tropical countries, hence this study will definitely open up a scope for the future utilization of these agro waste for therapeutic purposes. In Brazil, the husk fibre decoction is used in traditional medicine for treatment of diarrhea and arthritis (Esquenazi, 2002) and in India, heating the coconut shells gives oil that is used against ringworm infections (Chakraborty, 2008).

To the best of our knowledge, this is the first report on the antilisterial activities of the crude n–hexane extract of the husk of *C. nucifera*. In this study, we explored the potentials of the crude aqueous and n-hexane extracts of the husk of *C. nucifera* for the treatment of listerial infection.

MATERIALS AND METHODS

The plant specimens were collected from the vicinity of the Research Farm of the Obafemi Awolowo University, Ile Ife, Nigeria and identified by the curator of the Herbarium at the Department of Botany, Obafemi Awolowo University, and a voucher specimen was deposited in the herbarium.

Preparation of extracts

The husk of the coconut was sun-dried, milled and sieved manually to obtain the fine powdered particles. About 50 g dried powdered coconut husk was added to 200 ml of 95% n-hexane using Soxhlet extraction method at room temperature and for 48 h. The mixture was then filtered using Whatman 1 filter paper. The filtrates of each extraction were pooled together and concentrated to dryness *in vacuo* using a rotary evaporator, STRIKE 202 model manufactured by Steroglass S.R.L Company Italy to remove the n-hexane. The concentrated extract was then allowed to dry at room temperature to a constant weight. For the aqueous extract, about 50 g of the powdered extract was dissolved in 500 ml of sterile distilled water for 24 h with shaking. The resultant extracts were centrifuged at 3000 rpm for 5 min at 4°C. The supernatant was filtered through a Whatman No. 1 filter paper and the filtrate was lyophilized.

Test bacterial strains

The bacterial isolates used in this study included 37 *Listeria* isolates which were isolated from waste-water effluents in the Eastern Cape Province, South Africa as part of the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The isolates comprise of *Listeria ivanovii* (33), *Listeria grayi* (3), and *Listeria monocytogenes* (1) (Odjadjare et al., 2010).

Antibacterial susceptibility test

The susceptibility screening of the test bacteria to both crude extracts and standard antibiotics were done in accordance with the method of Irobi et al. (1994) and Akinpelu et al. (2008). The inoculum size of each test strain was standardized at 5×10^5 cfu/ml using McFarland Nephelometer standard. Sterile Mueller-Hinton agar plates were seeded with test bacterial strains and allowed to stand at 37°C for 3 h. Wells were then bored into the agar media using a sterile 6 mm cork borer and the wells were filled with the solution of the extracts and antibiotics, taking care so as not to allow spillage of the solution onto the surface of the agar. The plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of the extract and antibiotics into the media and thereafter incubated at 37°C for 24 h, after which they were observed for zones of inhibition. The effects of the extracts on the test bacterial isolates were compared with those of tetracycline and ampicillin standard antibiotics at a concentration of 1.0 mg/ml and 10.0 µg/ml respectively.

Determination of minimum inhibitory concentration (MIC)

The MIC of the crude aqueous and n-hexane extract was carried out using the method of Akinpelu and Kolawole (2004). Two-fold dilutions of the extracts were prepared and 2.0 ml aliquot of different concentrations of the solution were added to 18 ml of pre-sterilized molten Mueller-Hinton agar at 40°C to give final concentration regimes of 5.0 to 0.156 mg/ml. The media were then poured into sterile Petri dishes and allowed to set. The surfaces of the media were allowed to dry under a laminar flow before streaking with 18 h old bacterial cultures. The plates were later incubated at 37°C for up to 72 h after which they were examined for the presence or absence of growth. The MIC was taken as the least concentration of extracts that prevented the visible growth of the test bacteria.

Time-kill assay

Determination of the kill rate of the crude extracts was done following the procedure described by Okoli and Iroegbu (2005). Inocula were prepared following the described guidelines of EUCAST (2003). The resultant suspension were diluted 1:100 with fresh sterile broth and used to inoculate 50 ml volumes of Mueller Hinton broth incorporated with extracts at MIC and 2 × MIC to a

Table 1. Anti-listerial activities of crude aqueous and n-hexane husk extracts of *C. nucifera*.

Isolate identity	Inhibition zone (mm) / MIC			
	Aqueous extract	n-Hexane extract	AMP	TET
L. ivanovii LEL_1	15 / 2.5	18 / 0.625	21	31
L. ivanovii LEL_2	15 / 0.625	16 / 1.25	26	30
L. ivanovii LEL_3	15 / 0.625	19 / 0.625	25	34
L. ivanovii LEL_4	20 / 2.5	24 / 2.5	22	40
L. ivanovii LEL_5	- / ND	12 / 2.5	25	34
L. ivanovii LEL_6	12 / 0.625	- / ND	30	32
L. ivanovii LEL_7	14 / 1.25	18 / 1.25	21	29
L. ivanovii LEL_8	12 / 1.25	- / ND	27	29
L. ivanovii LEL_9	16 / 2.5	21 / 0.625	24	38
L. ivanovii LEL_{10}	- / ND	14 / 0.625	22	30
L. ivanovii LEL_{14}	15 / 1.25	21 / 2.5	34	40
L. ivanovii LEL_{15}	15 / 1.25	12 / 1.25	30	36
L. ivanovii LEL_{17}	15 / 2.5	18 / 0.625	25	32
L. ivanovii LEL_{18}	- / ND	12 / 2.5	20	22
L. ivanovii LEL_{30}	- / ND	15 / 1.25	21	29
L. ivanovii LAL_1	13 / 0.625	- / ND	20	28
L. ivanovii LAL_2	- / ND	- / ND	22	35
L. grayi LAL_3	12 / 2.5	14 / 0.625	28	33
L. ivanovii LAL_4	14 / 0.625	20 / 2.5	26	41
L. ivanovii LAL_5	18 / 2.5	20 / 5.0	28	44
L. ivanovii LAL_6	16 / 2.5	15 / 2.5	21	30
L. ivanovii LAL_7	- / ND	- / ND	20	31
L. monocytogenes LAL_8	13 / 1.25	16 / 1.25	20	34
L. ivanovii LAL_9	13 / 0.625	15 / 0.625	42	42
L. ivanovii LAL_{10}	17 / 0.625	16 / 1.25	32	46
L. ivanovii LAL_{11}	12 / 1.25	12 / 1.25	24	40
L. grayi LAL_{12}	13 / 1.25	12 / 2.5	24	26
L. ivanovii LAL_{14}	16 / 2.5	14 / 2.5	22	34
L. grayi LAL_{15}	12 / 0.625	14 / 2.5	24	26
L. ivanovii LDB_3	17 / 0.625	18 / 5.0	50	46
L. ivanovii LDB_6	14 / 1.25	14 / 2.5	21	23
L. ivanovii LDB_7	12 / 0.625	- / ND	25	22
L. ivanovii LDB_8	12 / 0.625	14 / 1.25	26	38
L. ivanovii LDB_9	12 / 2.5	- / ND	28	32
L. ivanovii LDB_{10}	- / ND	14 / 2.5	38	34
L. ivanovii LDB_{11}	15 / 2.5	15 / 5.0	40	42
L. ivanovii LDB_{12}	- / ND	13 / 2.5	29	34

-: No bacterial activity; MIC, minimum inhibitory concentration; ND, not determined; AMP, ampicillin; TET, tetracycline.

final cell density of approximately 5×10^5 cfu/ml. The flasks were incubated at 37°C on an orbital shaker LM- 5730R manufactured by Cococno Lab. Equipment, Taiwan at 120 rpm. A 500 µl sample was removed from the cultures at the appropriate time interval of 0, 4 and 8 h, respectively, and transferred to 4.5 ml of nutrient broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compounds carry-overs from the test suspensions. The suspensions were diluted serially and 100 µl of the diluted samples were plated on Mueller Hinton agar plates and incubated at 37°C for 24 h. Controls included extract-free Mueller Hinton broth seeded with the test inoculum. The numbers of colony were counted using the electronic colony counter.

RESULTS AND DISCUSSION

The results of the antilisterial activities of the crude aqueous and n–hexane extract of the husk of *C. nucifera* against the test organisms are shown in Table 1. Twenty-nine (29) of the test organisms were susceptible to the aqueous extract, while thirty (30) were susceptible to the n-hexane extract both at the screening concentration of 25 mg/ml. The diameters of the zones of inhibition ranged between 12 and 17 mm for the aqueous extract; and

Table 2. Nature of inhibition of crude aqueous and n-hexane extracts of *C. nucifera* husk against *Listeria* pathogens.

Susceptible isolate	Aqueous extract					n-Hexane extract				
	MIC (mg/ml)	Log_{10} Kill (MIC)		Log_{10} Kill (2*MIC)		MIC (mg/ml)	Log_{10} Kill (MIC)		Log_{10} Kill (2*MIC)	
		4 h	8 h	4 h	8 h		4 h	8 h	4 h	8 h
L. ivanovii LEL_3	0.625	0.32	2.6	2.8	3.0*	0.625	3.2*	4.8*	4.0*	5.2*
L. ivanovii LEL_9	2.5	1.4	2.8	3.2*	4.8*	0.625	2.6	2.8	2.4	4.8*
L. ivanovii LEL_{15}	NA	NA	NA	NA	NA	2.5	2.8	3.0*	3.8*	5.6*
L. ivanovii LEL_{17}	2.5	0.48	2.8	2.2	3.4*	0.625	4.0*	4.0*	4.8*	5.0*
L. ivanovii LEL_{30}	NA	NA	NA	NA	NA	1.25	3.2*	3.5*	4.6*	6.2*

MIC, Minimum inhibitory concentration; *, bactericidal effect; NA, no activity.

between 12 and 24 mm for the n-hexane extract; while those of the two control antibiotics (ampicillin and tetracycline) ranged between 20 and 50 mm; 22 and 46 mm, respectively (Table 1). The minimum inhibitory concentrations (MICs) of the extract against the susceptible bacteria generally ranged between 0.6 and 5.0 mg/ml. Specifically, MICs of the aqueous extract ranged between 0.6 and 2.5 mg/ml, while that of the n-hexane was between 0.6 and 5.0 mg/ml (Table 1).

Emergence of food borne bacterial illnesses caused by strains of *Listeria* pathogens continues to be a significant threat to public health, and thus, there is need for a reliable preservative and control measure that limits the proliferation of this psychotropic pathogen in refrigerated food and drinks. The development of suitable control strategies for this pathogen would be achieved from the availability of functional and effective antilisterial control measures. Many natural compounds found in dietary and medicinal plants, such as extracts of herbs and fruits, and essential oils of various spices, possess antimicrobial activities against *Listeria* pathogens (Kim et al., 1995 and Hao et al., 1998). Hence, our findings on the antilisterial efficacy of *C. nucifera* further corroborate the potentials of the plant remedies in antilisterial infections.

C. nucifera is a well known antimicrobial solution, but a review of literature on the extract found antimicrobial activity against a range of bacteria, for example Esquenazi et al. (2002) reported the antibacterial, antifungal and antiviral properties of the husk fibre extract of the north eastern Brazil *C. nucifera* plant, these studies have not been elaborate enough and have emphasized on few bacteria strains such as *Staphylococcus aureus* which though pathogenic is not a known food borne pathogen, hence, there is need for the assay on the antilisterial efficacy of this plant. The results from this study show the antilisterial activity of the n-hexane extract to be stronger than that of the aqueous extract, virtually against all the test bacteria. This is in accordance with the findings of Al-Reza et al. (2009), who revealed a strong antilisterial effect of the n-hexane extract of *Zizyphus jujuba* against all strains of *L. monocytogenes* tested with zones of inhibition ranging between 11 and 18 mm.

The aqueous extract had lower antilisterial activity in comparison with the n-hexane extract in support of other reports (Esquenazi et al., 2002; Zakaria et al., 2006). Results from this study also suggest n-hexane to be a better solvent for the extraction of bioactive compounds of *C. nucifera* than water. Al-Reza et al. (2009) in their superoxide radicals scavenging activity assay observed the extract of n-hexane to be relatively efficient in extracting bioactive compounds in terms of potency and diversity of compounds extracted.

The stronger antilisterial activities of the n-hexane extract as compared to the aqueous extract observed in this study could be attributed to the documented chemical composition of the plant as well as the concentration of the extracted solvent isolated from the plant. For example, Alviano et al. (2004) has reported the husk fiber of *C nucifera* to be rich in catechin and epicatechin together with condensed tannins which confers on it potent antioxidant characteristics. Also Paschuka et al. (1998) and Peng et al. (2001) in their studies indicated that catechin; one of the compounds present in the extract of *C. nucifera* plant is capable of inhibiting tumor cell lines.

The results of time-kill studies are presented in Table 2. Data are presented in terms of the log_{10} cfu/ml reduction in viable count. For the aqueous extract, average log reduction in viable cell count ranged between 0.32 Log_{10} and 4.8 Log_{10} cfu/ml after 8 h interaction in 1 × MIC and 2 × MIC. For the n-hexane extract, the log reduction ranged between 2.4 Log_{10} and 6.2 Log_{10} cfu/ml after 8 h interaction in 1 × MIC and 2 × MIC. The greatest reductions in cell counts achieved with the aqueous extract was on *L. ivanovii* LEL_9 with the average log reduction of 4.8 Log_{10} cfu/ml, while the greatest reduction achieved by the n-hexane extract was on *L. ivanovii* LEL_{30} with the average log reduction of 6.2 Log_{10} cfu/ml. Log reduction in viable cell counts of *L. ivanovii* LEL_9 was 4.8 Log_{10} cfu/ml after 8 h of interaction at 1 × MIC, the log reduction was constant until after 8 h even at 2 × MIC, thus suggesting that the total population of the bacteria had been wiped out by the forth hour at both 1 × MIC and 2 × MIC.

The time-kill characteristics of the two extracts were assessed against some selected listerial isolates. The

effectiveness of an antibacterial agent is measured by its ability to inhibit and kill bacteria (Nostro et al., 2001). At higher concentration (2 × MIC) and longer duration of interaction (8 h), more bacteria were killed, thus corroborating the observation of Rhodes (2004) in his comparison study on the antilisterial properties of red grape juice and red wine.

The n-hexane extract showed good bactericidal activity at 2 × MIC against the 4 test *L. ivanovii* isolates after 4 h of exposure, and after 8 h, all the bacteria were eliminated. For the aqueous extract, bactericidal activity was observed against 3 of the tested *Listeria* strains at a concentration of 2 × MIC after 8 h exposure period. Alviano et al. (2004) had reported a similar finding on the bactericidal efficiency of the husk fiber aqueous extract of *C. nucifera* plant which revealed a 64.1% reduction in the bacterial count. The results of the time kill studies suggest that the effect of the extract of *C. nucifera* could either be static or cidal depending on concentration and duration of exposure, also the plant represent a promising source of chemotherapeutic agents against *Listeria* pathogens.

In vitro time-kill assays are expressed as the rate of killing by a fixed concentration of an antimicrobial agent and are one of the most reliable methods for determining tolerance (Nostro et al., 2001). The *in vitro* data corroborates the reported efficacies of the different crude extracts of *C. nucifera* on a wide range of micro-organisms. For example, Wager et al. (2008) reported on the efficacy of this plants in the prevention and treatment of oral and periodontal diseases caused by some planktonic organism, thus supporting the folkloric uses of this plant in the treatment of different topical ailments (Nostro et al., 2001). The use of plant extracts with medicinal properties represents a concrete alternative for the treatment of different pathological conditions.

Conclusion

The levels of antilisterial activities observed suggest that the plant is a potential source of bioactive compounds that could be relevant in antilisterial drugs formulation which is a subject of on-going research in our group. Consequently, our study will also open up a scope for future utilization of the agro wastes from the husk of coconut plant for therapeutic purposes.

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