OPEN ACCESS
Saudi Journal of Medical and Pharmaceutical Sciences
Abbreviated Key Title: Saudi J Med Pharm Sci
ISSN 2413-4929 (Print) |ISSN 2413-4910 (Online)
Scholars Middle East Publishers, Dubai, United Arab Emirates
Journal homepage: https://saudijournals.com/journal/sjmps/home

Original Research Article

Evaluation of *In Vitro* Antifungal Activity of Different Stem Extracts and Fractions of *Lophira Lanceolata*

Abubakar Muhammad Amali[1*] and Junaidu Mustapha[2]

[1]Department of Pharmacology and Toxicology Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto, Nigeria
[2]Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto, Nigeria

DOI: 10.36348/sjmps.2019.v05i08.002 | **Received:** 28.06.2019 | **Accepted:** 24.07.2019 | **Published:** 25.08.2019

***Corresponding author:** Abubakar Muhammad Amali

Abstract

Lophira lanceolata is a plant is commonly used in traditional medicine for the treatment of dermatosis, toothache, muscular tiredness and menstruel pain. This research was aimed at evaluating the *in-vitro* antifungal potential of different extracts and fractions of *Lophira lanceolata* stem-bark. The stem bark of the plant was collected, air-dried, powdered and extracted with methanol, ethanol and aqueous solvents using maceration method. The crude methanol extract (CME) was fractionated into n-hexane (HF), ethyl acetate (EF), n-Butanol (BF) and aqueous (AF) portions and were tested against pathogenic fungus strains namely; *Aspergillus Niger, Candida albican, Trichophyton mentagrophyte* and *Trichophyton rubrum.* The agar well – diffusion methods were used to determine the diameter of the zones of inhibition using 96 wells micro titer plates. The minimum inhibitory concentration and minimum fungicidal concentration were also determined. The methanol extract was found to be more effective against fungi tested, ethanol and aqueous extract which showed moderate effect against the test organisms. The methanol extract was found to be inhibitorier compared to ethanol and aqueous extracts. The results obtained revealed that, stem bark of *Lophira lanceolta* may contain phytochemicals that possesses antifungal activity.

Keywords: Stem bark, *Lophira lanceolta*, methanol extract, antifungal, inhibitory concentration.

INTRODUCTION

Plants are the richest sources of drugs of traditional, pharmaceutical intermediates, nutraceuticals, food supplements, modern medicines and chemical entities for synthetic drugs [1]. According to World Health Organization (WHO), plant extracts or their active constituents are used as folk medicine in traditional therapies of approximately 80% of the world`s population. Medicinal plants are used as antibiotic agents [2]. Plants are the important potent biochemists and have been components of phytomedicine.

A large number of medicinal plants are used in modern medicine where they occupy a very significance place as raw material for important drugs. Plants used in traditional system of medicine in pharmaceutical houses are collected from wild sources. Plants are known to play a significant role in maintaining human health and improving the quality of human life for thousands of years and have served humans well as valuable components of medicines, seasonings, beverages, cosmetics and dyes [3]. The plant is well distributed north western Nigeria including Sokoto. *Lophira lanceolata* a multipurpose tree. Its seeds are eaten, but more commonly in the past than at present, now they are mainly used to extract edible oils. The oil also has cosmetic and medicinal uses and is suitable for making soap. The wood is hard and heavy and is locally used e.g. for mortars, railway sleepers and in bridge construction [4]. The plant was reported have Anti-plasmodial and antioxidant [5].

Fungi are increasingly recognized as major pathogens in critically ill patients. Candida spp. and Cryptococcus spp. are the yeasts most frequently isolated in clinical practice. The most frequent filamentous fungi isolated are *Aspergillus spp.,* but *Fusarium spp., Scedosporium spp. Penicillium spp.* and Zygomycetes are increasingly seen. Several reasons have been proposed for the increase in invasive fungal infections, including the use of antineoplastic and immunosuppressive agents, broad-spectrum antibiotics, and prosthetic devices and grafts, and more aggressive surgery. Patients with burns, neutropenia, HIV infection and pancreatitis are also predisposed to fungal infection [6]. Antifungal also known as an antimycotic,

pharmaceutical is fungicide or fungistatic used to treat and prevent mycoses such as athlete's foot, ringworm, candidiasis, serious systemic infections such as cryptococcal meningitis, and others. Such drugs are usually obtained by a doctor's prescription, but a few are available over-the-counter and they are associated with number of side effects. The increase in infectious diseases and resistance to antifungal drugs called for the development of newer, safe and effective medicines. The search for biologically active compounds extracted from traditionally used plants is relevant. This research is aimed to investigate the *in vitro* antifungal activity of different extracts and fractions of *Lophira lanceolata* stem bark using Agar-well diffusion and 96-well micro dilution techniques.

MATERIALS AND METHOD

Materials/equipments

Spectrophotometer, drying oven, Incubator, Autoclave, sabouroud dextrose agar, sabouroud dextrose broth, 96 Well microtiter plates, micro pipette, Multichannel micro pipette and sterile teats.

Collection, identification and drying of plant materials

Fresh stem bark of *Lophira lanceolata* was purchased from Bukkuyum local government of Zamfara state, Northern part of Nigeria in May 2018. The plant was identified and authenticated by Dr. H. E. Mshelia at the Department of Pharmacognosy and Ethnomedicine, Usman Danfodiyo University, Sokoto, Nigeria. A voucher with number PCG/UDUS/OCHA/0001 was preserved at the herbarium for future reference. The stem bark was dried for about one week away from sunlight to avoid possible damage of their Phyto-constituents. The stem bark was grinded in to fine powder and store appropriately until needed for extraction.

Preparation of extract

The fresh stem bark of *Lophira lanceolata* was air dried and grinded using motar and pestle. Three hundred grams (300) of *Lophira lanceolata* were divided into 3 portions. First portion 100g of the powdered dried plant was extracted with 500 mL of methanol by maceration at room temperature for 48 hours. Second portion 100g of the powered dried plant were extracted with 500 mL of ethanol by maceration at room temperature for 48 hours. Third portion 100g of the powered dried plant was extracted with 500 mL of Distilled water by maceration at room temperature for 48 hours. The mixture was then filtered using Whatman no. 1 filter paper. The filtrates were dried using incubator at 40°C.

Percentage yield was calculated using the formular

$$\% \text{ Yield} = \frac{\text{Mass of extract}}{\text{Initial mass}} \times 100$$

Liquid-liquid fractionation

The crude methanol extract was partitioned using different solvents based their polarity. The solvents used are n-hexane, ethylacetate, n-butanol and water. The fractionation was done using separating funnel, 5 g of the crude methanol extract was first suspended separately in 50mL distilled water and was successively extracted with the organic solvent of increasing polarity (250mL twice each) from the least polar, n-hexane, ethylacetate, n-butanol, yielding n-hexane, ethylacetate, n-butanol and the aqueous soluble fractions respectively. The obtained fractions were dried using rotary evaporator and the dried fractions were kept for subsequent tests. The water insoluble fraction was treated with n-hexane and chloroform to exhaust the marc.

Preparation of test organisms

The test fungal pathogens (*Aspergillus niger*, *Candida albicans*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*) were obtained at Department of pharmaceutics and pharmaceutical microbiology, faculty of pharmaceutical science, UsmanDanfodiyo University, Sokoto, Sokoto state. All the test fungi were maintained on fresh plates of sabouraud dextrose agar (SDA) media with regular sub-culturing. Standard antifungal agent was purchased at HealthPlus pharmacy, Abuja, Nigeria.

Preparation of serial dilution

10% DMSO was used to dissolve all the extracts. Test tube 18 were provided for the extract of *Lophira lanceolate* stem bark, the first 6 test tube were for methanol extract, second 6 test were for ethanol extract and third 6 test tube were for aqueous extract. The first 6 test tube were labelled as 1000 mg/mL, 500 mg/mL, 250 mg/mL, 125 mg/mL, 62.5 mg/mL, 31.25 mg/mL. The first test 1000 mg of *Lophira lanceolate* stem bark extract was weighed and transferred into the first test tube, DMSO 1 ml was measured and transferred into the test tube containing 1000 mg of the extract. 0.5 ml of the DMSO were measured and transferred into each of the 5 test tube. 0.5 ml were measured from the 1 test tube and transferred to the 2 test tube, 0.5 ml were measured from the 2 test tube and transferred to the 3 test tube, 0.5 ml were measured from the 3 test tube and transferred to the 4 test tube, 0.5ml were measured from the 4 test tube and transferred to the 5 test tube, 0.5 ml were measured from the 5 test tube and transferred to the 6 test tube, 0.5 ml were measured from the 6 test tube and discarded. This procedure was repeated for ethanol, aqueous, n-hexane, n-butanol and ethylacetate

Determination of diameter zones of inhibition

Preparation of the media

For one plate, Sabouroud dextrose agar 1 gram were weighed and dissolved in 15 ml distilled water, sterilized in an autoclave for 15 minutes, it was allowed

to cooled and 0.2 mg of chloramphenicol were added to inhibit bacterial growth. It was poured in to the sterile petri dishe on the bench and allowed to solidify for 30 minutes

Evaluation of anti-fungal activity

The antifungal activity was evaluated following the method described by Zhang *et al* [7]. The media were prepared into 24 petri dishes, first 6 petri dishes *Candida albican* were spread on the media by sterile cotton wool swab. Six wells were prepared on each petri dish with 8mm cork borer. Briefly 0.5 ml of various concentrations (1000, 500, 250, 125, 62.5, 31.125 mg/mL) of methanol extract were transferred in separate wells of the 1 petri plates, the procedure was repeated for ethanol and aqueous extract, n-butano fraction, n-hexane fraction and ethylacetate fraction for 2 -6 petri dishes. The procedure was repeated for *Aspergillus niger*, *Trichophyton mentagrophyte* and *Trichophyton rubrum*. Fluconazole (µg/mL) were used as positives control and DMSO were used as negative control. The plates were allowed to stand for 1 hour and incubated at 25 °C for 48 – 72 hours. After the incubation the plates were observed for formation of clear incubation zone around the well indicates the presence of antifungal activity. The zone of inhibition was calculated.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

Preparation of the media

For one bottle, Sabouroud dextrose broth 0.33 gram were weighed and dissolved in 10 ml of distilled water sterilized in an autoclave for 15 minutes, it was allowed to cool.

Fungal inoculum preparation

Two colonies of the fungi (*Aspergillus Niger*, *Candida albican*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*) were isolated from the slant prepared and inoculated in to 4 different bottles of broth prepared, one bottle serve as control and inoculated for 48 hours.

The culture was scraped with sterile scalpel and with 10ml of sterile water. The suspension was adjusted spectrophotometrically to an absorbance 0.1 at 530nm. Each tube was inoculated with 1ml of fungal suspension and diluted with 19 mL of normal saline.

Antifungal activity was measured by a quantitative micro spectrophotometric assay [8]. Growth inhibition was measured in 96-well microtiter plates at 530 nm. For the first fungi inoculum Aspergilus niger. Two 96-well microtiter plate were used, 1st plate Sabouroud dextrose broth 100µL was dispensed in the 96 wells, the 1st and 2nd wells (A & B) 100µL various concentration of ethanol extract (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.4mg/mL) and 100µL of standard fungal inoculum were dispensed in the wells. 3rd Wells (C) SDB 100µL and standard fungicidal agent fluconazole at 0.2 mg/mL (Satish *et al.*, 2007) 100µL were used as a positive control. 4th and 5th wells (D & E) 100µL of various concentration of methanol extract (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.4mg/mL) were dispensed in the wells. 100µL of standard fungal inoculum were dispensed in the wells. 6th wells (F) SDB 100µL only was used as negative control. 7th& 8th wells (G & H) 100µL of various concentration of water extract (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.4mg/mL) and 100µL were dispensed in the wells. 2nd plate Sabouroud dextrose broth 100µL of was dispensed in the 96 wells, the 1st and 2nd wells (A & B) 100µL various concentration of n-butanol fraction (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.4mg/mL) and 100µL of standard fungal inoculum were dispensed in the wells. 3rd wells (C) SDB 100µL and standard fungicide fluconazole at 0.2 mg/mL (Satish *et al.*, 2007) 100µL were used as a positive control. 4th and 5th wells (D & E) 100µL of various concentration of n-hexane fraction (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.4mg/mL) were dispensed in the wells. 100µL of standard fungal inoculum were dispensed in the wells. 6th wells (F) SDB 100µL only was used as negative control. 7th& 8th wells (G & H) 100µL of various concentration of ethylacetate fraction (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.4mg/mL) and 100µL were dispensed in the wells. The same procedure was repeated for *Candida albican*, *Trichophyton mentagrophytes* and Trichophyton *rubrum*. The plates were left standing for 30 minutes and allowed the spores to sediment, after which absorbance was measured at 530 nm in a spectrophotometric plate reader. After 48 hours of incubation at 27 °C, growth was recorded by measuring absorbance. All assays for antifungal activity were carried out at least in duplicate. Growth inhibition [7] was determined based on the equation.

$$\text{Percentage growth inhibition} = \frac{\Delta C - \Delta T}{\Delta C} \times 100$$

Where ΔC is the corrected absorbance of the control micro-culture at 530 nm

ΔT is the corrected absorbance of the test microculture.

The corrected absorbance values equal the absorbance at 530 nm of the culture measured after 48 hours minus the absorbance at 530 nm measured after 30 minutes.

The MIC was regarded as the lowest concentration of the extract that did not show any viable growth after 48 hours incubation (compared with control)

The MFC was determined using the method of [9]. The wells which showed no visible growth after 48 hours incubation was sub-cultured on free SDA plates and incubated at 25 ℃ for 48 hours. The MFC was regarded as the lowest concentration of the extract that prevented the growth of any fungal colony on the solid media.

The wells were carried out in duplicate for each extract (methanol, ethanol, water, n-hexane fraction, n-butanol fraction and ethyl acetate fraction) and inoculated at 25 ℃ for 48 hours. Thses procedure was repeated for all standard fungal inoculum.

RESULTS

Table-1.0: The result of percentage yield of extract obtained from 100g of dried powder of stem bark of L. lanceolate

S.No.	Plant extracts	Yield (g)	Yield (%)
1	**Methanol**	**15.23**	**15.23**
2	**Ethanol**	**12.98**	12.98
3	**Aqueous**	**6.25**	6.25

Liquid- liquid fractionation

The result of percentage yield of most active methanol fractions of *Lophira lanceolata* obtained from 10g of methodic crude extract in table 1.1

Table-1.1: Percentage yield of fractions of methanol extract of stem bark *Lophira lanceolata*

S. No.	Fraction	Quantity (g)	Percentage yield (%)
1	n-hexane	0.6	12
2	n-butanol	1.3	26
3	Ethylacetate	0.8	16
4	Aqueous	1.5	30

Diameters of zones of inhibition

The result of zones of inhibition of extracts and fractions of *Lophira lanceolata*is presented in Table 1.2-1.5 respectively.

Table-1.2: Diameter zones of inhibition of Methanol extract *Lophira lanceolata*

S.No	Organisms	Zones of inhibition (mm)/concentration mg/mL						Fluconazole	DMSO
		1000	500	250	125	62.5	31.25	(µg/mL)	0
1	*C. albican*	16±0.58	13±0.24	12±0.32	11±0.57	8±0.18	-	17.67±4.03	0
2	*A. niger*	27±0.41	25±0.23	21±0.581	8±0.10	15±0.00	12±0.32	24.00±0.82	0
3	*T. mentagrophte*	21±0.58	17±0.57	15±0.18	14±0.49	11±0.43	9±0.25	23.67±1.64	0
4	*T. rubrum*	28±0.23	24±0.13	22±0.45	20±0.17	16±0.00	13±0.33	26.33±1.73	0

Key - = No zone of inhibition; Fluco = Fluconazole DMSO = Dimethyl sulfoxide

Table-1.3: Diameter of zones of inhibition of Ethanol extracts *Lophira lanceolata*

S.No.	Organisms	Zones of inhibition (mm)/concentration mg/mL							
		1000	500	250	125	62.5	31.25	Fluconazole	DMSO
1	*C. albican*	16±0.58	12±0.24	11±0.57	9±0.50	8±0.45	-	17.67±4.03	0
2	*A. niger*	21±0.23	17±0.10	15±0.46	14±0.0	11±0.33	9±0.25	24.00±0.82	0
3	*T. mentagrophyte*	19±0.00	15±0.32	13±0.15	11±0.57	10±0.25	8±0.45	23.67±1.64	0
4	*T. rubrum*	24±0.25	20±0.41	16±0.17	12±0.58	9±0.42	-	26.33±1.73	0

Key - = No zones of inhibition; Fluco = Fluconazole, DMSO = Dimethyl sulfoxide

Table-1.4: Zones of inhibition of Aqueous extract *Lophira lanceolata*

S.No.	Organism	Zones of inhibition (mm)/concentration mg/mL						
		1000	500	250	125	62.5	Fluconazole	DMSO
1	*C. albican*	16±0.00	14±0.27	10±0.34	-	-	17.67±4.03	0
2	*A. niger*	17±0.56	15±0.37	13±0.42	11±0.15	-	24.00±0.82	0
3	*T. mentagrophyte*	16±0.29	13±0.30	10±0.58	-	-	23.67±1.64	0
4	*T. rubrum*	20±0.41	17±0.45	15±0.33	12±0.25	10±0.27	26.33±1.73	0

Key - = No zones of inhibition; Fluco = Fluconazole, DMSO = Dimethyl sulfoxide

Table-1.5: Diameter zones of inhibition (mm) of methanol fraction of *Lophira lanceolata*

S.No.	Organism	n-hexane	n-butanol	Ethylacetate	Fluco.	DMS0
1	*Candida albican*	13±0.57	11±0.24	10±0.00	17.67±4.03	0
2	*Aspergillus niger*	10±0.45	12±0.23	10±0.37	24.00±0.82	0
3	*Trichophyton mentagrophyte*	10±0.58	9±0.00	13±0.53	23.67±1.64	0
4	*Trichophyton rubrum*	11±0.49	10±0.25	11±0.08	26.33±1.73	0

Key: Fluco = Fluconazole DMSO = Dimethyl sulfoxide

MIC AND MFC OF PLANT EXTRACTS AND FRACTIONS

The MIC and MFC of extracts and fractions of *Lophira lanceolata* are presented in Table 1.6 and 1.7

Table-1.6: MIC and MFC of Plant extract (mg/mL) of *Lophira lanceolata*

S.No.	Organism	Methanol		Ethanol		Aqueous		Fluco.	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
1	*Candida albican*	125	500	125	250	250	500	31.25	31.25
2	*Aspergillus niger*	125	125	125	125	125	250	31.25	62.50
3	*Trichophyton mentagrophyte*	*125*	125	500	500	125	250	62.50	62.50
4	*Trichophyton rubrum*	62.5	125	125	125	125	250	31.25	62.50

Key: Fluco = Fluconazole

Table-1.7: MIC and MFC of Methanol fractions (mg/mL) of *Lophira lanceolata*

S.No.	Organism	Methanol		Ethanol		Aqueous		Fluco.	
		MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
1	*Candida albican*	1000	1000	1000	-	1000	-	31.25	31.25
2	*Aspergillus niger*	500	500	1000	1000	1000	1000	31.25	62.50
3	*Trichophyton mentagrophyte*	500	500	1000	-	1000	-	62.50	62.50
4	*Trichophyton rubrum*	250	500	1000	1000	1000	1000	31.25	62.50

DISCUSSION

The present study revealed that the methanol, ethanol and aqueous stem bark extracts of *Lophira lanceolate* possess antifungal activity against four different fungi tested, *Canidida albican*, *Aspergillus niger*, *Trichophyton mentagrophyte* and *Trichophyton rubrum*. The methanol extracts of the plant demonstrated higher anti-fungal activity compared to the ethanol and aqueous extracts. Different solvents have been demonstrated to have the ability to extract different phytochemical constituents depending on their polarity and solubility in the solvent. More of the phytochemical constituents could have been extracted by methanol than other solvents used in the study. The exhibited antifungal properties of *Lophira lanceolata*can be attributed to the presence of phytochemical constituents Nabila [10]. Similar finding on *in vitro* antifungal activity on *Psidium guajava* was reported by Doughari [11]. The findings also justify the used of the plant in the treatment of vaginal irritation and discharges reported by Egwaikhide [12]. Similar finding was also reported by Demirel [13]. Only few organisms show resistant against the ethanol extract. The methanol fractions, n-hexane, n-butanol and Ethylacetate, n-hexane fraction of methanol, *Candida albican* showed the highest activity, followed by *T. rubrum*, *T. mentagrophyte* and *Aspergillus niger*as showed in Table 1.6. The n-butanol fraction of methanol, Aspergillus *Niger* showed more sensitivity, followed by *C. albican*, *T. rubrum* and *T. mentagrophyte* showed in Table 1.6. The Ethylacetate fraction of methanol extract, *T. mentagrophyte* showed the highest sensitivity, followed by T. *rubrum*, *Candida albican* and Aspergillus *Niger* as showed in Table 1.6. Similar finding was reported by Kandhasamy [14]. *In vitro* antifungal activity of *Naravelia zeylenica*, Ethylacetate fraction activity demonstrated highest activity compared with the other fungi tested.

Minimum inhibitory concentration (MICs) of the stem bark extract is the lowest concentration which inhibit the growth of the organism and were determined using the broth dilution. Both the extract and the fraction showed various ranges of MIC from 62.5mg/mL to 1000mg/mL. Minimum fungicidal concentration (MFCs) of the stem bark extract is the lowest concentration that killed at least 99.9% of the organism. The extract showed various ranges of MFC from 125mg/mL to 500mg/mL and the fraction showed ranges of concentration from 500mg/mL to 1000mg/mL and some of them showed resistant. Similar result was reported by Doughari [15].

CONCLUSION

From the above studies, the stem bark of *Lophira lanceolate* has antifungal activity against four different fungal species *C. albican*, *A. niger*, *T. mentagrophytes*, and *T. rubrum* indicating its broad spectrum of activity. However, this finding provides and insight into the usage of this plant in the traditional

treatment of foot infection, intestinal parasitism and fungal infection. Thus the stem bark of this plant can be exploited further to isolate the active phytochemical(s) responsible for the observed activity.

REFERENCES

1. Hammer, B. H. L. B., Hansen, L. B., & Nørskov, J. K. (1999). Improved adsorption energetics within density-functional theory using revised Perdew-Burke-Ernzerhof functionals. *Physical Review B*, *59*(11), 7413.
2. Padmini, E., Valarmathi, A., & Rani, M. U. (2010). Comparative analysis of chemical composition and antibacterial activities of Mentha spicata and Camellia sinensis. *Asian J. Exp. Biol. Sci*, *1*(4), 772-781.
3. Agaie, B. (2004). The role of phytochemicals in optional health. *Journal of the National Academy for Child Development*, *11*(1), 27-29.
4. Pegnyemb, D. E., Messanga, B. B., Ghogomu, R., Sondengam, B. L., Martin, M. T., & Bodo, B. (1998). A new benzoylglucoside and a new prenylated isoflavone from Lophira lanceolata. *Journal of natural products*, *61*(6), 801-803.
5. Onyeto, C. A. (2014). Anti-plasmodial and antioxidant activities of methanol extract of the fresh leaf of Lophira lanceolata (Ochnaceae). *African Journal of Biotechnology*, *13*(16).
6. Eggimann, P., Garbino, J., & Pittet, D. (2003). Epidemiology of Candida species infections in critically ill non-immunosuppressed patients. *The Lancet infectious diseases*, *3*(11), 685-702.
7. Zhang C. Q., Liu Y. H., Wu H. M., Xu B. C., Sun P. L., Xu Z. H. (2012). Baseline sensitivity of Pestalotiopsis microspora, which causes black spot disease on Chinese hickory (Carya cathayensis), to pyraclostrobin. Crop Prot. 42 256–259.
8. Broekaert, W. F., Terras, F. R., Cammue, B. P., & Vanderleyden, J. (1990). An automated quantitative assay for fungal growth inhibition. *FEMS Microbiology Letters*, *69*(1-2), 55-59.
9. Rotimi, V. O., Al-Sweih, N. A., & Feteih, J. (1998). The prevalence and antibiotic susceptibility pattern of gram-negative bacterial isolates in two ICUs in Saudi Arabia and Kuwait. *Diagnostic microbiology and infectious disease*, *30*(1), 53-59.
10. Benhammou, N., Bekkara, F. A., & Panovska, T. K. (2008). Antioxidant and antimicrobial activities of the Pistacia lentiscus and Pistacia atlantica extracts. *African Journal of Pharmacy and Pharmacology*, *2*(2), 022-028.
11. JH, D., & Obidah, J. S. (2008). *In vitro* antifungal activity of stem bark extracts of Leptadenia lancifolia. *International Journal of Integrative Biology*, *3*(2), 111.
12. Egwaikhide, P. A., Okeniyi, S. O., & Gimba, C. E. (2009). Screening for anti-microbial activity and phytochemical constituents of some Nigerian medicinal plants. *Journal of Medicinal Plants Research*, *3*(12), 1088-1091.
13. Demirel, Z., Yilmaz-Koz, F. F., Karabay-Yavasoglu, U. N., Ozdemir, G., & Sukatar, A. (2009). Antimicrobial and antioxidant activity of brown algae from the Aegean Sea. *Journal of the Serbian Chemical Society*, *74*(6).
14. Kandhasamy, M., & Arunachalam, K. D. (2008). Evaluation of *in vitro* antibacterial property of seaweeds of southeast coast of India. *African journal of Biotechnology*, *7*(12).
15. Doughari, J. H., & Nuya, S. P. (2008). Vitro antifungal activity of deterium microcarpum. *Pakistan Journal of Medical Sciences*, *24*(1), 91.