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LAURUS NOBILIS L. A NATURAL ALTERNATIVE AGAINST *ASPERGILLUS FLAVUS* AND AFLATOXINS

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ABSTRACT

Essential oils are natural compounds that have been used for thousands of years for their smell, medicinal properties and food preservation. Several are used today in the food industry. This study focuses on the anti-fungal properties of the essential oil of noble laurel leaves (*Laurus nobilis*) especially against *Aspergillus flavus*, the main producer of aflatoxins, which contaminate agricultural products, especially cereals, during their storage. The action of noble laurel essential oil, extracted by hydrodistillation of leaves, on the development and production of aflatoxins was evaluated *ex vivo* by microatmosphere and aromatogram tests and in vivo on rice and maize, locally produced, directly grown and under storage conditions. The essential oil of bay leaf totally inhibited the development of *Aspergillus flavus* with the microatmosphere test at a concentration of 0.1µl/ml and an inhibition diameter of 5 cm for the same concentration. *In vivo* test showed a reduction in the development, sporulation and pigmentation of the fungus on rice with no aflatoxin production. In response to this activity, the composition of *Laurus nobilis* essential oil was evaluated by gas chromatography. It revealed the presence of several bioactive molecules mainly eugenol (42.498%), myrcene (29.09%, chavicol (9.21%), limonene (5%) and 1.8-cineole (1.87%). The results of this study reveal the possible use of noble laurel essential oil as an alternative control agent against fungal deterioration and aflatoxin contamination of foodstuffs during storage.

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INTRODUCTION

The essentials oils are natural compound used since millenary for their odors as contact between human and gods, as cure and conservation solution (Stewart, 2007) Lot of them are employed in food industry. Differents studies (Retnouwati *et al.,* 2003), (Gnonlonfin *et al.,* 2013) (Algabri *et al.,* 2018) althought show that some of spices and aromatics plants are not subject to mycotoxins contamination. Regarding food safety problem like crops contamination by mycotoxins, secondaries metabolites produced by fungi, some of essential oils as the ones extracted from *Ocimum sanctum*, *Ocimum tenuiflorum*, *Ocimum basilicum (Shipra Rastogi et al., 2007; Abou El-Soud et al.,* 2012; Pragyanshree et Mehta, 2013; Wajiha Iram *et al*., 2016) which have antimicrobial properties are used to protect crops. (Arora and Pandey, 1977; Di Leo Lira *et al.,* 2009; Nehir El *et al.,* 2014; Simi *et al.,* 2004). *Laurus nobilis* essential oil has also antimicrobial properties (Algabri *et al.,* 2018; Caputo *et al.,* 2017; Merghni *et al.,* 2016; Nehir El *et al.,* 2014). Some studies showed that the essential oil of *L. nobilis* had strong anti-bacterial effects against Gram positive bacteria : *S. aureus*, *Staphylococcus epidermidis* and *Streptococcus faecalis* and Gram negative bacteria: *Pseudomonas aeroginosa, Shigella flexneri, Klebsiella pneuomoniae, Salmonella typhi, Serratia marcescens* and *E. coli* were (Moghtader and Farahmand 2013 ; Ouibrahim *et al.* (2013) and antifungal effects on *Glomus deserticola, Glomus intraradices and, Eurotium*, *Aspergillus sp. (Aspergillus niger)*, *Penicillium* genus (Geeta and Reddy 1990; Guynot *et al.* 2003) ; *Fusarium moniliforme* (*Gibberella fujikuroi*), *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Phytophthora*

capsici (Muller Riebau *et al.,*1995 ; Gokalp, 2016; *Chahal et al., 2017).* In the same way, Gnonlonfin *et al.* (2010) shown that *Laurus nobilis* dry leaf sold in Benin and Togo markets are not contaminated by aflatoxins, a carcinogenics mycotoxins, mainly produced by species of *Aspergillus* section *Flavi*. In tropical countries, aflatoxin is the first mycotoxin that contaminate crops and storage food. This study shows the antifungal property of essential oil of this plant against *Aspergillus flavus* and aflatoxins production and the different possible applications in food and crop preservation.

MATERIALS AND METHODS

Laurus nobilis: Laurus nobilis *leaves have been taken from a plant cultivated without chemical compound as protection, in a private garden at one kilometer to the beach in Cotonou. The harvest have been done in june 2018. The plant specie have been identified by National herbier of the University of Abomey-Calavi (N°AAC1513/HNB).*

Fungus strain: Fungal strains used for the different tests are the refence strains of *Aspergillus flavus* var. *flavus* (AFc5) isolated and partially sequenced by Adjovi *et al.* (2013) in cassava chips. A fresh culture on malt extract agar is realized.

Chemical reageant and media: All the media used were from Oxoid Ltd. and Hampshire, UK and the reagents and solvents used were from Merck (Darmstadt, Germany). Aflatoxins standards (AFB1, AFG1, AFB2, AFG2) were purchased from Sigma Aldrich Chemicals (St. Louis, MO).

Essential oil extraction: Fresh leaves (500 g) of *Laurus nobilis* plant were thoroughly washed with distilled water, then ground in a Waring blender and subjected to hydrodistillation in Clevenger's apparatus for 3 hours according to the standard procedure described in the European Pharmacopoeia. The volatile fraction (EO) was separated and stored in clean brown glass vial. (Di Leo Lira *et al.,* 2009).

***Laurus nobilis* L. of Benin essential oil composition:** The composition has been determinated by gaz chromatography (GC) as described by Kpoviessi *et al.,* 2011. Analysis of the oils was performed by GC-FID. The gas phase chromatography analysis was firstly carried out on a DELS/IGC 121C equipped with a CP WAX 52 CB (25 m x 0.3 mm, df: 0.25mm) column, an injector (split/splitless) and a flame ionization detector (FID). The temperature of the injector and the detector were 240°C and 250°C, respectively. The temperature programming was as follows : isothermal at 50°C for 5 min followed by 2°C/min gradient until 220°C. Carrier gas was N with a flow rate of 1 mL/min under 0.8 bar pressure. The volume of oil injected was 0.5 mL. The GC was a Hewlett Packard 5890 equipped with an ionization detector (FID) and a split/splitless injector was secondly used for compound separation with a glass capillary column (30 m x 0.25 mm) coated with DB-5 phase thickness 0.25 mm or glass capillary column (30 m x 0.25 mm) coated with Supelcowax (0.20 mm film thickness). For semi-quantification purposes, the normalised peak area abundances without correction factors were used.

Antifungical activity test: The antifungal activity of laurel EO was determined using three different tests. For all tests, microbial suspensions were adjusted to 10^7 cfu.mL^{-1} and spread in Malt extract agar using a sterile handle. Negative controls were prepared using only the dimethylsulphoxide DMSO solution used to dilute EO, whereas positive control was prepared with pure EO. The antifungal activity was evaluated by strictly measuring of the radius of inhibition zones to the nearest millimetre. All experiments were conducted in triplicate. The moulds subcultured were incubated at 25 °C ± 1 °C. The mycelial growth was appreciated every day by measuring the average of two perpendicular diameters passing by the middle of the EO deposit point, from the first day till the fifth (5 days minimum). The antifungal activity was evaluated by the following formula :

Antifungal activity (AA) = (Dc-Dt)/Dc X 100

Dc is the diameter of growth in control plate (witness) (Petri dish without essential oil) and Dt is the diameter of growth in the plate containing tested antifungal agent (Balouiri *et al.*, 2016).

Test of determination of the fungistatic or fungicidal activity: Fungistatic or fungicidal activity was evaluated using the experimental concentrations where neither no growth, or germination was observed. This test consists in taking the mycelial disc not germinated at the end of the incubation of the Petri dish and reintroducing it in a new culture medium (former one) nine without natural extract. If the mycelial growth is always inhibited, the fungicidal activity of the natural extract and in the contrary case, it's spoken about the fongiostatic activity (Alitonou *et al*, 2012).

Disk-diffusion method / Aromatogram: The test is the paper disk-diffusion method (NCCLS, 2002). (Ramos *et al.*, 2011; Balouiri *et al.*, 2016). Inoculum was spread on a MEA plate. Subsequently, filter paper disks (6mm; Whatman no. 1) were placed in the surface of Petri dishes and impregnated with 50 µL of EO at different concentrations. EO was diluted into 0.1% dimethylsulphoxide DMSO.

Agar-well diffusion method: According to the procedure used in disk-diffusion method, the agar plate surface is inoculated by spreading the fungical inoculum over the entire agar surface. Then, a hole with a diameter of 6 mm is punched with a sterile tip, and 50 µL of the Laurel EO at differents concentrations is introduced into the well. Then, agar plates are incubated (Balouiri *et al.*, 2016).

Micro-area: According to Zaika (1988), micro-area test is a technique similar to aromatograms. The method consists in placing the disc impregnated with essential oil in the centre of the lid of the Petri dish, spilled during the experiment. This means that the latter is no longer in contact with the agar medium (Pibiri, 2006). A disc of Whatman paper (6 mm in diameter) impregnated with 50 µl of essential oil is placed in the centre of the lid of a Petri dish containing the seeded MEA agar. After 1 hour of diffusion, the Petri dish is incubated for 3 to 5 days at 25°C. (Pibiri, 2006).

Test *in vivo:* Two different tests have been done : a direct contact test and a volatils compounds test. These tests have been done on a sample of local rice and a sample of imported rice. The two types of rice have been contaminated by 100µl of an inocculum of 10^7CFU.mL^{-1} of *Aspergillus flavus*. For direct contact test, 50µl of EO have been spread on contaminated rice and for volatil compound test, storage

Table 1. Antifungal activity

	Concentration (EOµl/µL)	Inhibition diameter (cm)	AA	Macroscopic	Microscopic
Wells	0,25	5.8	69%	Dark green, fine powder with a little white aerien mycelium	Small spherical head, biserial ¾ cover, scarce spherical and smooth conidia ; undeveloped conidiophore
	0.2	5.7	68%		
	0,125	2.8	33%		
	0.1	2.3	27%		
Discs	0,25	5.3	63%	Dead leaf green, light down, aerial mycelium	Uniserial, small head 1/3 cover, vesicle hemispherical ; many spherical single conidia ; undeveloped conidiophore
	1/5	5.3	63%		
	0.125	3.1	37%		
	0.1	2.9	35%		
Micro-area	0,25	6.2	74%	Dead leaf green, little coloured, light down in places and fine powder, highly developed aerial mycelium	Biserial elongate head 1/3 cover ; Scarce small spherical conidial ; undeveloped conidiphore
	0,1	3.9	46%	Leaf green, fine powder, scace aerial mycelium	Biserial, full and big spherical head total cover ; many spherical and smooth enchain conidia ; developed conidiophore
Control inoculum To		0	0%	Dark green, powdered with big grain ; without aeral mycelia	Biserial ; big spherical head entierely cover ; many long enchain spherical and smooth conidia ; presence of pseudopodal cell ; developed conidiophore
Control DMSO		0	0%	Dark green, powdery colony with large grains, absence of aerial mycelium	Biserial ; big spherical head entierely cover ; many long enchain spherical and smooth conidia ; presence of pseudopodal cell ; developed conidiophore

Figure 2. Test in rice after 3 days, a) negative control ; b) test in imported rice ; c) test in local rice ; d)positive contrôle *Laurus nobilis* EO property to inhibit aflatoxins production has been evaluated by direct contact test and volatils compounds test in rice sample

Table 2. Aflatoxin in Tests in vivo

	AFB1 (EO Direct test)	AFB1 (Volatils compounds test)
Negative contrôle (Rice+EO)	-	-
Positive contrôle (Rice+AF)	+	+
Local rice	-	-
Imported rice	-	-

conditions have been feing with a water solution of EO put into storage box at room temperature.

Aflatoxins Detection: Aflatoxin were extracted from contaminated samples according to the method of Seitz and Mohr (1977) with some modifications. Briefly, a ground of each sample (10 g) was mixed with 50 mL of chloroform and macerated for 30 min. The mixture was filtered through Whatman filter paper N° 1. The eluate was dried down at 60°C and then reconstituted in 50 µL of methanol. The extracts (10 µl) were analyzed by using thin layer chromatography (TLC) on silica gel plate type (1.05553. DC-Alufolien Kieselgel 60 MERCK) to show the presence of different aflatoxins (B1, B2, G1, G2). The plates were developed with ether-methanol-water (96:3:1, v/v/v). Aflatoxin was identified on the basis of co-migration with aflatoxins standards and by their fluorescent color characteristic under UV 360 nm illumination.

Statistical analysis: Statistical analysis was carried out by using SPSS 19 software. Data were analyzed by analysis of variance (ANOVA) and differences among the means were determined for significance at $P \leq 0.05$ using Tukey's multiple range test.

RESULTS

Essential oil extraction

- The rent of extraction is very low (6%).
- The extraction of 500 g of fresh leaf permit to have 3 mL of essential oil. The extraction with the dry leaf is lower than fresh.

Activity against *Aspergillus flavus:* All the three tests have shown the activity of EO of Laurel against *A. flavus* development and pigmentation.

Test *in vivo:* Effect of *Laurus nobilis* EO on *Aspergillus flavus* have been tested on local rice and imported rice. The results show that EO have not inhibited completly the fungi development but have affected pigmentation.

Table 3. *Laurus nobilis* L. from Benin EO composition

Molecules	Concentration	Lis-Balchin (2006)	(Ramos *et al.*, 2012)	Sedef Nehir El *et al.*, 2014
Monoterpene hydrocarbons				
alpha thujène	0.134%	-	0.5	3.45
alpha pinène	0.638%	7	2.3	38.98
béta pinène	0.079%	4	1.8	32.35
oct-1-èn-3-ol*	1.748%	-	-	-
Myrcène	29.090%	-	0.6	-
alpha phellandrène	0.170%	-	0.2	1.58
delta-3-carène	0.230%	-	0.8	-
para cymène	1.261%	-	-	12.79
Limonène	5.001%	Trace	-	-
béta phellandrène	0.342%	-	-	-
1,8-cinéole*	1.870%	40	-	630.24
(E)-béta ocimène	0.139%		0.1	-
cis oxyde de linalol*	0.125%		-	-
<6,7>epoxymyrcène*	0.147%		-	-
Hydrogenated monoterpenes				
Linalool*	1.952%	10-12	8.4	2.40
terpinene 4-ol*	0.660%	-	2.6	-
alpha terpinéol*	0.644%	9	2.7	-
Décanal*	0.203%		-	-
Chavicol*	9.211%	1-5	-	12.8
Eugénol*	42.498%	1-5	1.2	12.15
Sesquiterpenes				
beta caryophyllene	0.099%	-	0.7	-
(E,E)-alpha farnesene	0.071%	-	-	-
delta cadinene	0.117%	-	0.9	-

Table 4. Molecules activities

Molecules	Familly	Activity	Concentration (%)
Linalool	Monoterpen	Antiseptic, antifungal (Aspergillus species) and antiviral ; activity against Candida fluconazole résistant	1.952
Carvacrol	Phenols	Activities against fungi, *Candida albicans*, Saccharomyces cerevisiae, Aspergillus niger	
Myrcene	Monoterpen	Antibacterial (against *S. aureus*) Inoue *et al.*, 2004	29.090
Limonene	Terpene	Anticarcinogen, antitumorale	5.001
Chavicol (déméthylation of eugénol)	phenol-methyl-éther (Allyl phenol)	Anti-histaminic ; antibacterial	9.211
Eugenol (4-allyl-2-méthoxyphénol.)		antiseptic and anesthetic (tuberculosis and pulmonary gangrene) (Lebeau P., Courtois G. ; Traité de pharmacie chimique – Tome II, Masson et C^{ie}, 1929)	42.491
1,8-cineole or eucalyptol	Monoterpene	Activities against fungi and fungal metabolites production	1.870
oct-1-en-3-ol	alkenyl alcohol	octenol or mushroom alcohol is a molecule that attracts certain biting insects. A cytotoxic to human embryonic stem cells; a neurotoxic to Drosophila; an endocrine disruptor. It plays a role of a fungal metabolite	1.748
Para-cymene	Monoterpene hydrocarbon	Antioxydant ; antiinflammatory ; carvacrol precursor ; inactivate Escherichia coli O157:H7	1.261

Figure 2 shown the test after three days. The AFB1 is not detected in all tested culture except the positive control (rice with *A. flavus*) (Table 2).

EO composition: The determination of mains compounds of the essential oil of Laurel is presented on the table 3 with composition from another studies. Table 4 shows the activities of mains molecules found in Laurel essential oil from Benin.

DISCUSSION

Aflatoxins are carcinogenics mycotoxins produced by *Aspergillus* section *Flavi*. It is produced during crops storage. Cereals are the most subjects of this contamination. Some studies are interested in figthing aflatoxins in food and crops, but other figth alternatives are most encouraged. This study has focused on the aflatoxin figth with the use of EO. Gnonlonfin *et al.* (2010) have shown the absence of aflatoxin contamination in some spices and aromatics herbal such as bay leaf (Tassou, 2006).

Laurel is firstly known for its effects on the nervous system and is midly narcotic (Lis-Balchin *et al.*, 1998). Their uses include infections of respiratory system, flu, dental infection, angina, diarrhoea. Laurel activities is due to its bactericide and fungicide properties particularly chronic pathologies (Mailhebiau, 1995). Some studies shown bay leaf as the most active against bacteria like *Campylobacter*, *Salmonella*, *E. coli*, *Staphylococcus aureus* and *Listeria* and have activities against fungi *Aspergillus niger* (95%), *Aspergillus ochraceus* (80%), *Fusarium culmorum* (69%).(Lis-Balchin, 2006). In this study the antifungal and antiaflatoxin activity of Laurel essential oil is shown. The different tests made show the fungicide activity against *A. flavus* by aromatogram (disc test), well-test and micro-area test. The best activity was made and revealed by the micro-area test (74% inhibition at 12.5 µL EO) followed by the well-test (69%) and disc (63%). No significant difference between well-test and disc test. This first step supposed that the activity of Laurel E.O. is due to volatile compound. The results of the gas chromatography analysis showed the presence of several antimicrobial molecules, some of which are in small quantities (see Table 3) such as

eucalyptol (1.8%), myrcen (5%), linalol (1.8%) ; and others in large proportions such as eugenol (42%) and chavicol, a molecule resulting from the demethylation of eugenol (9%) which are proven antimicrobials. The compositon of *Laurus nobilis* used in this study is different from this decribed by Lis-Balchin *et al.*, 2006 ; Ramos *et al.*, 2012 ; Neith *et al.*, 2014. The different bay Laurel EO composition found in previous studies is likely related to several factors known to affect the chemical composition of EOs, such as genetic, climatic, seasonal and geographic (Papageorgiou *et al.*, 2008). According to some research, which has focused on the application of eugenol, a study has shown that the addition of a small amount of eugenol to the controlled atmosphere applied for packaging grapes improves the preservation of taste and nutritional properties while slowing microbial development. This antimicrobial activity by aerosolization could explain the results obtained with laurel essential oil in the micro-area test performed in this study. The high content of eugenol in bay leaf essential oil is believed to be responsible for this activity. Other work on this essential oil had shown a content of less than 2% or between 1-5% (Lis-Balchin, 2006). Some oils such as cloves (72-88 eugenol) are very corrosive with very high antimicrobial activity.

In another studies, the extracts of *L. nobilis* showed highest antifungal activity against *A. niger* and *C. albicans* with inhibition zone diameters of 20-32 mm/15ml (Erturk 2006). Essential oil of bay leaf was tested *in vitro* against two foodborne fungi belonging to the dominant mycobiota of stored rice, *Fusarium culmorum* and *F. verticillioides*, The result showed that bay essential oil possessed great potential to control both fungal pathogens (Rosello *et al.* 2015). (Geeta and Reddy 1990; Guynot *et al.* 2003) demonstrated by biological assays the potential of bay leaf essential oils against species belonging to *Eurotium*, *Aspergillus* and *Penicillium* genus that fungitoxicity against *Fusarium moniliforme* (*Gibberella fujikuroi*), *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Phytophthora capsici* was due to different concentrations of the phenolic fraction in the essential oils (Muller Riebau *et al.*, 1995 ; Chahal *et al*, 2017). Abou El-Soud *et al.*, suggested that *O. basilicum* L. at concentration of 1000 ppt could supress *A. flavus* fungal growth and inhibit markedly aflatoxin B1 production which could be attributed to its rich content of linalool, 1,8-cineol and eugenol (*Abou El-Soud et al.,2012)* This work can supposed that antiaflatoxin effects of *Laurus nobilis* essential oil could mainly due to linalool, 1,8-cineol and eugenol and charvicol. In conclusion, these results reveal that *Laurus nobilis* essential oil collected in Benin is rich in oxygenated monoterpene and monoterpene hyrocarbons compounds showed higher antifungal properties. Its also confirm the antifungal activities of *Laurus nobilis* essential oil and its potential use to prevent *Aspergillus flavus* development and aflatoxins production during cereals storage. In some of all, this work show that bay laurel EO have potential applicability as a natural alternative to synthetic food preservatives to enhance food safety and increase its shelf life by usage like (par son utilisation en aérosole grâce à ces composés volatiles qui lui confère ces propriétés).

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