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Antifungal Activities Of Essential Oils Of Cymbopogon Citratus, Ocimum Gratissimum And Eucalyptus Globulus Against Botrytis Cinerea, Penicillium Expansum And Potential Application As Biocontrol Agents During Fruit Cultivation

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Abstract – The present work aims to study the effectiveness of selected medicinal and food plant extracts (EtOH, DCM) including essential oils (*Eucalyptus globulus, Ocimum gratissimum, Cymbopogon citractus)* on the mycellian growth of toxinogenic moulds responsible for fruit alteration. Antifungal tests were carried out with the different extracts to assess their potential antimicrobial activities against *Botrytis cinerea* and *Penicillium expansum* responsible for fruits deterioration in several regions of the world.

In vitro antifungal tests have shown that essential oils have pronounced antifungal activities at low concentrations of 0.3µl/ml, 1.3µl/ml and 5µl/ml respectively for *Cymbopogon citractus*, *Eucalyptus glogulus* and *Occimum gratissimum*, while non-volatile extracts exert growth inhibition of 50% on average on *Botrytis cinerea*. This study suggests use as biocontrol agents for plant pests during fruit cultivation that plants present themselves as a promising alternative for the fight against toxinogenic fungi.

Keywords – Antifungal Activities,Essential Oils, Cymbopogon Citratus, Ocimum Gratissimum, Eucalyptus Globulus, Botrytis Cinerea, Penicillium Expansum, Biocontrol Agents, Fruit Cultivation

I. Introduction

Most fruits as tomatoes, and oranges are highly perishable products, especially during the postharvest period, and a major loss is caused by fungal pathogens (Spadaro and Gullino, 2010). *B. cinerea* is the most common postharvest pathogen of grapes and attacks more than 230 plant species and although fungicides exist for its control, many classes of fungicides have failed due to its genetic plasticity and *P. expansum* is often found on rotting apples, pears, and cherries, but it is also common on walnuts, pecans, hazelnuts, and acorns in most regions of the world, resulting in severe postharvest losses (Nally et al. 2012; Filtenborg, O. et al, 1996; Sakhr ajouz, 2009, Brian williamson, 2007) and cause important economic problem to agricultur and horticultur crops (Keller et al., 2003, Sakhr Ajouz, 2009). These fungi two are the major cause of fruits diseases and produce mycotoxin which account for up to 70 % crop loss without adequate protection (Popp et al. 2013). *P. expansum* is of concern especially in fruit products because of its production of patulin. Specific regulation for patulin at a level of 50 µg/kg has been set by most countries in Europe, and several quantitative methods have been developed (Majerus, P et al, 2002; Ritieni, A. et al., 2003). In Africa (Côte d'Ivoire), some toxicological analyses carried out on three (3) beverages revealed that the level of contamination is three (3) to five (5) times higher than the European recommendations on the maximum tolerable level of patulin in fruit juices and derived beverages (Ehouman Ano Guy Serge et al, 2020). However, these regulations and methods deal only with patulin, and *P.*

expansum produces many other toxic metabolites. These mycotoxins are responsible for acute and sometimes fatal poisoning, especially in livestock, or chronic poisoning.

In the fight against these toxic moulds, chemical plant protection products have many advantages. However, their use can cause environmental and public health problems, especially since the risks inherent in some of them are poorly assessed (Jovana deravel et al, 2014). Plants produce a variety of medicinal components that can inhibit the growth of pathogens, and a considerable number of studies have been conducted to evaluate the antimicrobial activity of extracts and essential oils of medicinal plants (Hernández díaz, L., 2001). Natural products have diverse chemical characteristics that can influence the evaluation of antifungal activity,

The purpose of this work is to assess the antifungal potential of certain plants of the Beninese flora as an alternative to the use of synthetic pesticides in order to fight against post fruit harvesting as well as evaluating the combination of plant extracts with other environmentally friendly methods. Thus, an ethnobotanical survey was conducted among traditional practitioners and farmers and cited plants species were analyzed for the antifungal potential. To assess the toxicological potential, *in vitro* cytotoxicity assays were performed using human keratinocytes (HaCaT Alpha cell line).

II. Material and method

Ethnobotanical survey

In the Republic of Benin, as in most African countries, traditional medicine is the first line of defense and is integrated into the health system. According to the World Health Organization, more than eighty percent of the population depends on traditional medicine, particularly because of the high cost of allopathic pharmaceuticals (World Health Organization, 2001). In order to obtain and document information regarding the traditional therapeutic uses of the plants that are the focus of our study, an ethnomedical survey was conducted among traditional practitioners in February 2019 in northern Benin. The survey was conducted at in several regions and in total Seventy-five (75) indigenous traditional medicine practitioners were identified based on the recommendation of older community residents (leaders, community agents, and representatives of rural associations), and interviewed using a previously prepared questionnaire. All participants were informed of the survey and written consent was obtained before personal visits were made. The questionnaires were designed in French, and addressed to traditional healers in their local dialect for those who did not speak French. The main questions focused on general knowledge of plants with high antibiotic power used for the treatment of mycoses and also plants used in the management of plagues and fungi that attack crops including vernacular names as well as the mode of application.

Identification of the plant material was done in the field using the Analytical Flora of Benin (Akoègninou et al., 2006) and verified at the Herbarium of the University of Abomey-Calavi (UAC) Benin.

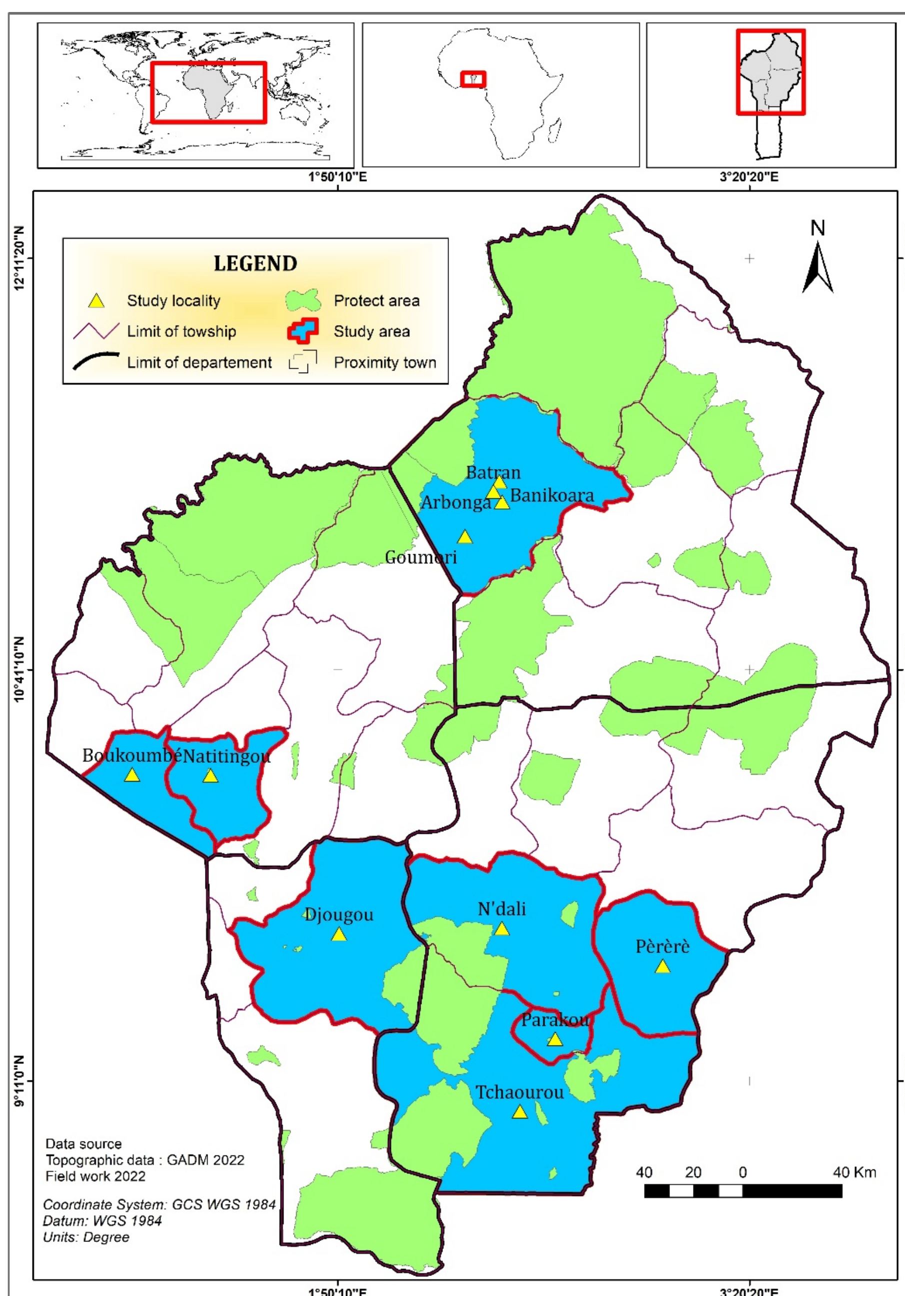

Figure 1. Geographical description of the study area. Map of septentrional region showing the location of Boukoumbé, djougou, N'dali, Batran, Arbonga, Goumori, Banikoara, Kérou, Péréré, Parakou, and Natitingou districts.

During May 2019. The samples were authenticated at Abomey Calavi University (UAC), Benin. Voucher specimen (ID-number AB-05-2019) have been deposited at the Inter-Regional University of Industrial Engineering Biotechnologies and Applied Sciences (IRGIB Africa University), Cotonou, Benin. A certificate for exportation was obtained from the Ministry of Agriculture and Husbandry.

Plants extracts preparation

The plants used were harvested in the Republic of Benin in the indicated area (Figure **1**). The bark of the trunk, root bark and leaves of the plants were collected and dried at laboratory temperature 25°C, protected from the sun. The dry specimens were then powdered using an electronic grinder and packaged in jars.

Preparation of ethanolic extracts and Dichloromethane: Ten grams (10 g) of powdered substance of each plant part was macerated in 100 ml ethanol (96 % V/V) or dichloromethane for 24 hours. The macerate was filtered with Whatman filter paper. The filtrate was concentrated in vacuum at 30°C and stored at 4°C until further use.

Preparation of essential oils: The extraction of essential oils (E.O) was done by hydro-distillation using the apparatus described by Sedef nehir el et al, 2014. Distillation was performed using dry leaves to water ratio of 1:10 for 4 hours minimum. The essential oil was collected in amber colored vials and stored at 4°C for future use. The yield depend to the plants and the haverest period.

Fungal Material

Culture of fungi on Potatose Dextrose Agar (PDA) petri dish

Preparation of the PDA medium

24 g of potato dextrose (Sigma-Aldrich) was mixed with 15 g of Aga agar (ROTH®) in 1 liter of distilled water. the medium was Autoclave for 30 minutes at 121°C and pour into Petri dishes (Greiner bio-one®)

Inoculation of Petri dishes

Place an agar disk of an actively growing fungus (4 to 6 days old) with a diameter of about 5 mm on a PDA plate. The fungal culture is done in the dark at 23-25°C and the agar plates prepared without fungal culture were stored at 4 ° C.

Preparation of conidia of Botrytis cinerea and Penicillium expansum

For the preparation of conidia of Botrytis cinearea and Penicillium expansum, conidia are collected directly on the surface of mature PDA petri dishes (10 to 12 days) by leaching with 1ml of a solution of Tween 80 to 0,3% and counted in the Neubauer® numeration chamber with quadruple determination. The number of conidia was calculated according to the following formula.

Number of conidia per ml = counted conidia* 10^4 * dilution

Antifungal activity

Microbial Strains

The antimicrobial activity was evaluated using the following strains: *Botrytis cinerea* and *Penicillium expansum.* The strains were provided by the Fraunhofer Institute for Cell Therapy and Immunology, Antimicrobial Agents Unit (Leipzig, Germany).

Chemicals and reagents

Potatose dextrose Broth (PDB, Sigma-Aldrich), Potatose Dextrose Agar (PDA, Sigma-Aldrich) and Agar (ROTH®) were purchased and prepared and used according to the manufacturers' instructions. (DMSO; ≥ 99.7%) and dimethylformamide (DMF) were obtained from Sigma-Aldrich, Steinheim, Germany.

Determination of Minimum Inhibitory Concentration (MIC)

The determination of the plants extracts MIC on the strains used in the biological assays was determined by the broth microdilution method (R. Cleeland and E. Squires, 1991). One hundred milliliters (100 μL) of liquid medium PDB was transferred into the wells of a 96-well microdilution plate. For the control medium, 200 µl PDB were pipetted into the wells and for the positive control 100 µl PDB and 100 µl conidial suspension containing 10^3 conidia per ml of PDB. The rest of the wells were filled each up to 100 µl of conidia suspension and 100 µl of prediluted extract in PDB at different concentrations according to the double concentration method. Some wells are filled with 100 µl of prediluted extract were mixed with 100 µl of PDB. These correspond to the background of the absorbance measurement and were included in the evaluation as a correction factor. The microtiter plates were sealed and incubated in the dark at room temperature. According to the growth curve, the absorbance was then measured at 72 and 96 hours and at 620 nm using the microplate reader; Epoch2 BioTek.

Cytotoxicity assay

Chemicals and reagents

DMEM high glucose medium, fetal bovine serum (FBS) and non-essential amino acids were purchased from Biowest, Nuaille, France. Penicillin/streptomycin (P/S) was procured from Biochrom AG, Berlin, Germany. Phorphol-12-myristate-13- acetate, lipopolysaccharide (LPS) from Escherichia coli (serotype 0111:B4; impurities < 1% protein, Lowry), budesonide, triton-X, thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), dimethyl sulfoxide (DMSO; ≥ 99.7%) and dimethylformamide (DMF) were obtained from Sigma-Aldrich, Steinheim, Germany.

Cell culture

Immortalized human keratinocyte cells (HaCaT) (Boukamp et al., 1988) which is epidermal keratinocyte line that has been used for investigation of multistep carcinogenesis in human cells (M. kulesz-martin et al., 2010) were purchased from CLS Cell Lines Service GmbH, Eppelheim, Germany and cultured in Dulbecco's Modified Eagles Medium (DMEM, Biowest, Nuaillé, France) supplemented with 1% penicillin/streptomycin (P/S, Biowest), 10% fetal calf serum (FCS, Biowest) in cell culture flasks (Sarstedt Nümbrecht, Germany) at 37° C in a humidified atmosphere at 5% CO2. Experiments were conducted at a cell confluency of 80–90%. Cells were seeded at 10^5/200 µL after detachment using trypsin/EDTA (0.05%) solution (Biowest), in 96 well plates and incubated for 24 h , the supernatants were removed, cells were treated with plant extracts at the indicated concentrations and incubated in the same conditions. After 24 hours, the cell-free supernatant was collected for LDH and the cells was use for MTT.

Cell Viability Assay

The effect of plants extracts on the viability of HaCaT cells was assessed using the MTT assay.

after collecting the supernatant, cells were treated with an MTT solution (0.3 mg/mL in DPBS) and incubated for 2 h. The resulting formazan salt was dissolved with 500 µL SDS lysis buffer (20 g SDS, 40 mL DMF, ad 100 mL aqua dest. pH = 4,7). The control well is treated with the culture medium, and in another well, cells are lysed with triton. After an incubation overnight, the amount of formazan was quantified spectrophotometrically at 570 nm using an Infinite® M 200 plate reader (TecanGroup Ltd. Mannedorf, Switzerland).

The percentage cell growth in the presence of the five plant extracts was determined as follows:

$$\text{Cell growth \%} = 100 \times (\text{mean absorbance in treatment wells})/(\text{mean absorbance in control wells})$$

Data analysis

Data analysis was performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). Data is expressed as mean ± SEM; n represents the number of independent experiments/different animals used. Statistical analysis was performed by one-way analysis of variance (ANOVA). Concentration-response-curves were obtained by non-linear least-square fit analysis, whereby ICx describes the concentration of extract that induced x% inhibition.

III. RESULT AND DISCUSSION

Essential oils

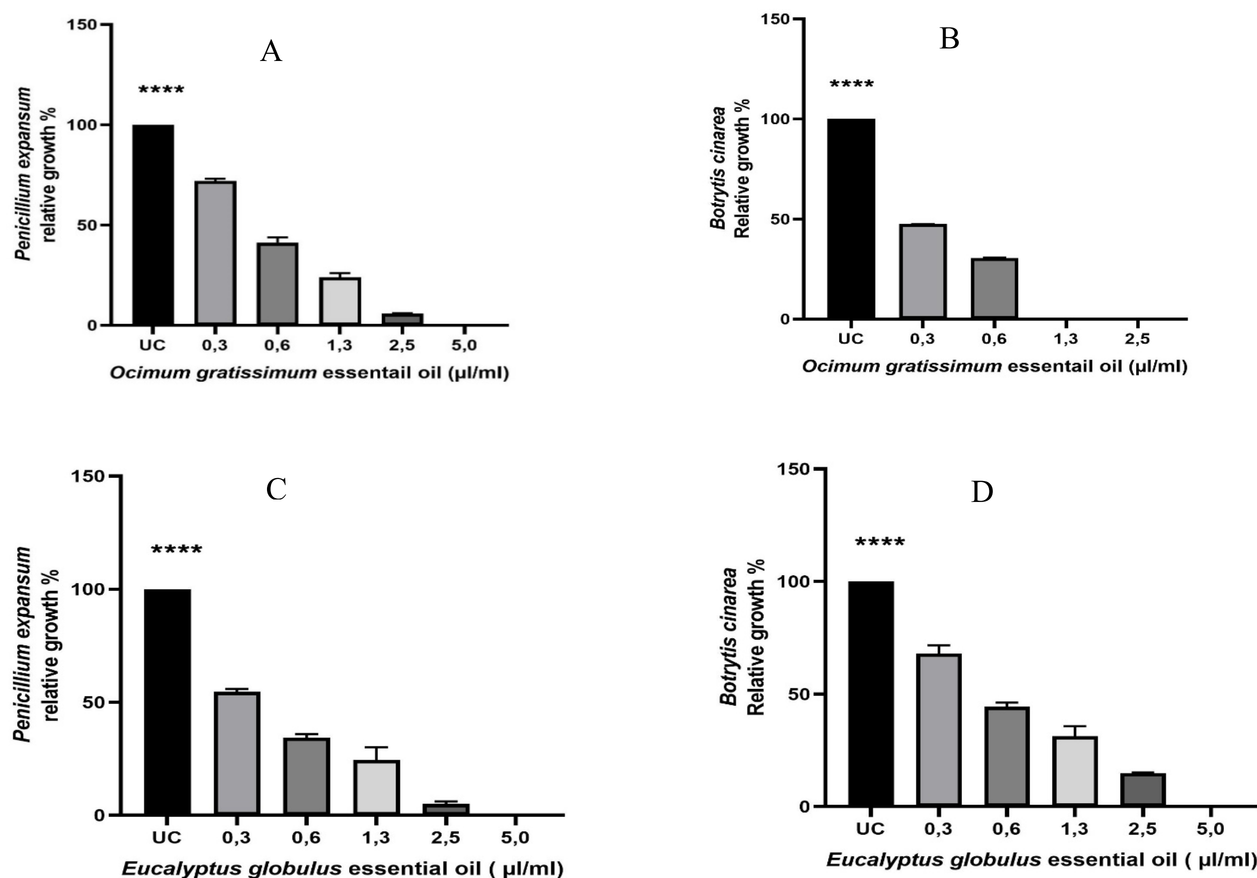

Figure 1: Influence of *Ocimum gratissimum and Eucalyptus globulus* essential oil (0.3 µl/ml – 5 µl/ml; 96 hours incubation time) on *Penicillium expansum* and *Botrytis cinerea* mycelium growth. Data are presented as ±SEM, ****P<0.0001vs control, n=4.

The minimal inhibition concentration for *Ocimum gratissimum* was MIC = 5 µl/ml and MIC = 1.3µl/ml respectively for *Penicillium expansum* and *Botrytis cinerea* confirming the bioactivity of EO of fresh leaves of *O. gratissimum* from Benin as an aflatoxin inhibitor and fungal growth suppressor (Euloge S. Adjou et al. 2013). Eugenol was the compound responsible for antifungal activity of the essential oil of *O. gratissimum* (Terezinha de Jesus Faria et al. 2006).

The minimal inhibition concentration for *Eucalyptus globulus* was MIC= 5 µl/ml for *Penicillium expansum* and *Botrytis cinerea.*

The evaluation of the antifungal activity of the essential oils showed that they have a very strong activity against germs tested with cymbopogon *citractus* essential oil which was 100% effective against all fungal strains tested at the concentration of 0.3 µl/ml followed *by Eucalyptus globulus* and finally *Ocimum gratissimum*. These three oils are real candidates for the fight against toxinogenic fungi.

DCM and EthOH extracts

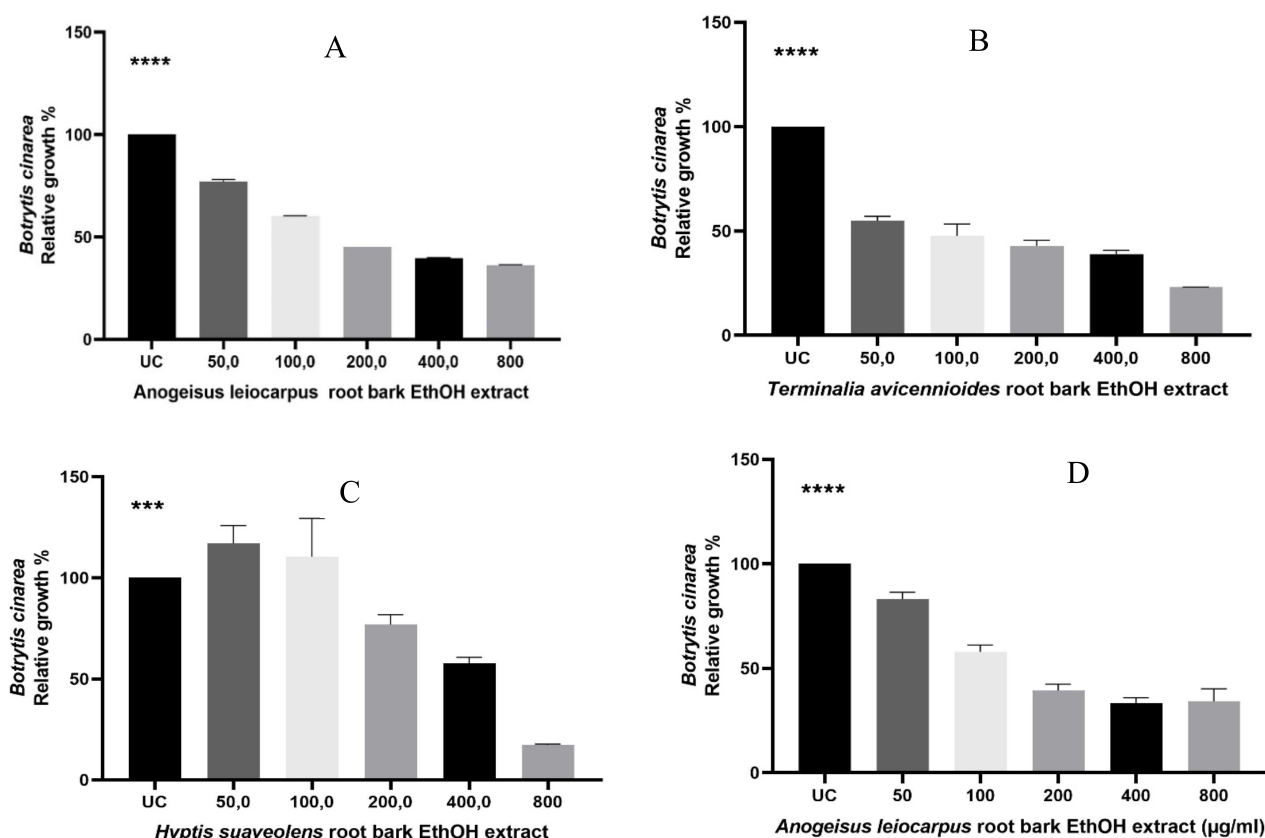

Figure 3: Influence of *Anogeisus leiocarpus* root bark EthOH extract (A); *Terminalia avicennioides* root bark EthOH extract (B) and *Hyptis suaveolens* leaves EthOH extract on *Penicillium expansum* and *Botrytis cinerea* mycelium growth.

The relative growth were determine using microdilution in 96 wells plates the extracts were applied from 50 µg/ml to 800 µg/ml. Data are presented as ±SEM, P<0.0001vs control, n= 4. The reading was taking 96 H after extracts application.

The ethanolic and dichloromethane extracts from the different plants tested demonstrated antifungal activities on *Botrytis cinerea* but we did not obtain MIC on all concentrations tested. Nevertheless, we obtained inhibitions of more than 50% on concentrations ranging from 100 µg/ml to 800 µg/ml, which means that all these plants have low antifungal activity on all test species. These can be used despite this as is the case with extracts of *Reynoutria sachalinensis* which between veraison and harvest, reduced the incidence of B. cinerea in grape clusters by 50% (Schilder et al., 2002).

Cytotoxicity assay

The MTT assay has become the most common cytotoxic and anti-proliferative assay for evaluating test substances (Mosmann, T.R, 1983). Cytotoxicity assays such as MTT able to detect alterations in cellular structure and/or functions, including lethal cytotoxicity indicating a potential to cause cell and tissue injury including eye injury (*Bryan ballantyne, 2006)*

Cell Viability Assay

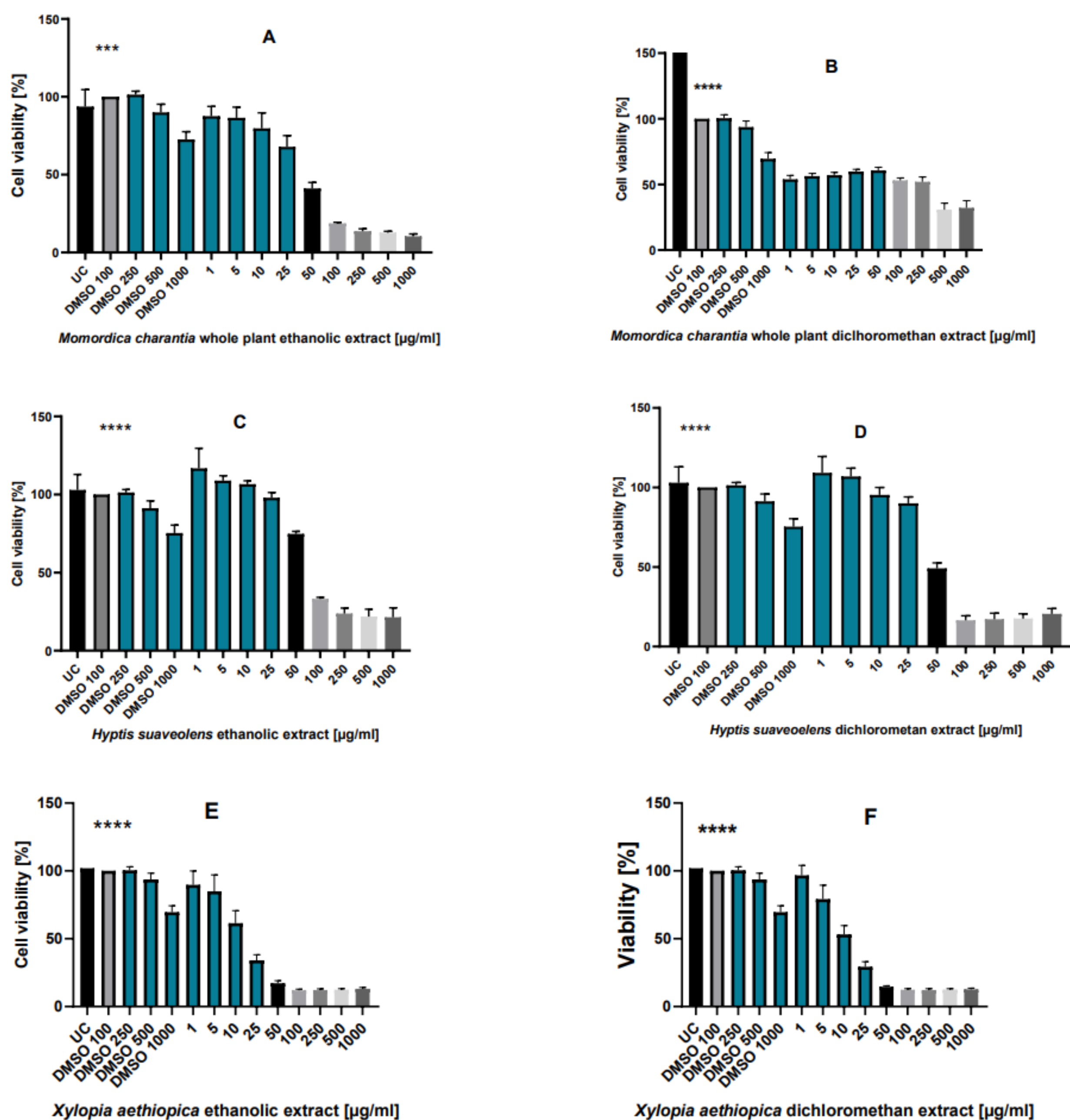

Figure 4: Cell viability assay using MTT test with *M. charantia* ethanolic extract (A), *M. charantia* dichloromethan extract (B), *H. suaveolens* ethanolic extract (C), *H. suaveolens* dichloromethan extract (D), *X. aethiopica* ethanolic extract (E), *X. aethiopica* dichloromethane extract (F).

Cell viability were determine using MTT test in 96 wells plates. The plants extracts were applied from 1 µg/ml to 1000 µg/ml. Data are presented as ±SEM, P<0.0001vs control, n= 6.

The ethanolic extract of *Momordica charantia* is more toxic than the dichloromethane which from 50 µg/ml showed a reduction of more than 50% in cell death, these results are related to other publications on *Momordica charantia* or it is proven

that the plant has a cytotoxic effect, which significantly inhibited DNA and protein synthesis in human peripheral blood lymphocytes (Licastro, F. ;1980),

The *H. suaveolens* dichloromethane extract was found to be more toxic than the ethanolic extract, which showed a reduction in cells of 66.65% and 50.87% respectively at 100 µg/ml for EthOH extract and 50 µg/ml for DCM extract.

The dichloromethane and ethanolic extracts of *X. aethiopica* showed toxicity from 10 µg/ml and 25 µg/ml, respectively.

IV. Conclusion

Essential oils extracted from fresh leaves of *Ocimum gratissimum*, *Cymbopogon citractus*, *Eucalyptus globulus* had high effect on the growth inhibition of *Penicillium expansum* and *Botrytis cinerea* which are fruits pathogens at concentrations ranged from 0.3 µg/ml to 5 µg/ml. This essential oil could be used as natural antimicrobial agent in the fight against moulds species responsible for biodeterioration. With regard to the dichloromethane and ethanolic extracts of the different plants *Anogeissus leiocarpus*, *Hyptis suaveolens*, *Terminalia avicennioides*, we have more than 50% innihbition especially for *B. cinerea*. In addition the study of cytotoxicitis has shown that these different plants in concentrations test its not harmful to the human skin. For the practical use of plants extract as novel fungal-control agent, further research is needed on safety issues for human health and this product acceptability.

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Declaration of competing interest

The authors declare that there is no conflict of interest

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