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Intitulé

Activités biologiques de quelques ressources végétales de la Numidie Orientale et leur valorisation dans la conservation du blé *Triticum durum* Defs.

Presentée par : CHIBI Asma

Directrice de thèse : HASSAINE Amina (MCA, Université Badji Mokhtar - Annaba)

Devant un jury composé de :

Dr.SLIMANI Abderachid	Président	Université Badji Mokhtar - Annaba
Pr.HACINI Nesrine	Examinatrice	Université Chadli Bendjedid- El Taref
Dr. NECIB Asma	Examinatrice	Université Med Cherif Messaadia-Souk Ahras
Dr. BRINIS Amir	Examinateur	Université Badji Mokhtar - Annaba

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You have always been by my side to support and encourage me. May this work express my gratitude and affection.

The most beautiful sister, Queen Aya

my brothers Ayhem and Anis

To all my family and friends

CHIBI Asma

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PHOTOS

Abstract

This work firstly focuses on the study of the fungal process associated with stored wheat grains (3 varieties treated and 5 untreated with a fungicide) and plants. Mycological analysis revealed a diverse fungal flora, including mycotoxic species. 80 strains were detected; including 53 identified belonging to 18 genera *Fusarium, Alternaria, Aspergillus, Penicillium, Cladosporium, Rhizopus, Absidia, Rhizoctonia, Trichoderma,* Microdochium, *Purpureocillium, Acremonium, Rhizomucor, Helminthosprium, Geotrichum, Sclerotina, Ulocladium and Chaetomium*. In this context, this document presents the results on the evaluation of the biological activities of certain plant resources from Eastern Numidia and their exploitation in the conservation of wheat *Triticum durum* Defs. 4 EOs and 8 botanical extracts from 8 cultivated plants from Algeria (*Cupressus sempervirens, Salvia rosmarinus, Eucalyptus polybractea, Lantana camara, Morus alba, Rubus ulmifolius, Dittrichia viscosa* et *Thuja orientalis*) in comparison with sodium bicarbonate and two fungicides. The chemical analysis of EOs by GC/MS/MS made it possible to identify 65 compounds for *C. sempervirens* and *L. camara*, as well as 45 compounds for *S.rosmarinus* and *E. polybractea*. The results of the antibacterial activity of these EOs against six pathogenic bacteria as *Pseudomonas aeruginosa* ATCC27853, *Staphylococus aureus* ATCC25923, *Echerichia coli* ATCC25922, *Salmonella sp., S. aureus, P. aeruginosa* and one yeast *Candida albicans* presented great antimicrobial activities against most of them at minimum inhibitory concentration (MIC) values ranging from 0.91 to 8.7 mg/mL. Determination of antifungal activity of EOs against ten wheat endophyte strains from genera *Penicillium, Cladosporium, Aspergillus, Alternaria, Trichoderma* and *Fusarium* was carried out on solid PDA medium. The results approve the antifungal activity of these EOs at a concentration of 300µl. However, the study also looked at the antifungal effectiveness of the extracts. While methanolic extract of *M. alba* leaves and aqueous extract of *D. viscosa* leaves showed very promising antifungal effect, followed by methanolic extract of *R. ulmifolius*, ethanolic extract of *T. orientalis* and *L. camara*, that of the methanolic combination between Morus and Rubus is the weakest. Notably, sodium bicarbonate gave positive results compared to the two chemical fungicides (Agriconazole and Vidan) on the same fungi tested. Furthermore, the investigation of insecticidal activity against a model of stored wheat pest, *Tribolium castaneum*, by fumigation and repellent tests demonstrated the dependence of the effectiveness of these activities on the concentration and the exposure time. It should be noted that unlike the activity of plant extracts, fumigation of EOs gave better results since the highest concentration tested gave 100% insecticidal activity. The botanical extracts used appear to be a promising, effective, economical and ecological alternative for the biological treatment of fungi and insects, with remarkable antibacterial power.

Key words: Plant-extracts, Essential oils, Antibacterial activity, Insecticidal activities, Antifungal activity, Stored wheat, Fungi, Biological treatment

Résumé

Ce travail porte en permier sur l'étude du cortège fongique associé aux grains de blé stockés (3 variétées traitées et 5 non traités par un fongicide) et aux plantes. L'analyse mycologique a permis de mettre en évidence une flore fongique diversifiée, y compris des espèces mycotoxiques. 80 souches ont été détectées; dont 53 identifiées appartenant à 18 genres *Fusarium, Alternaria, Aspergillus, Penicillium, Cladosporium, Rhizopus, Absidia, Rhizoctonia, Trichoderma,* Microdochium, *Purpureocillium, Acremonium, Rhizomucor, Helminthosprium, Geotrichum, Sclerotina, Ulocladium et Chaetomium.* Dans cette optique, ce document présente les résultats sur l'évaluation des activités biologiques de quelques ressources végétales de la Numidie Orientale et leur exploitation dans la conservation du blé *Triticum durum* Defs. 4 HEs et 8 extraits botaniques retirés de 8 plantes cultivées d'Algérie (*Cupressus sempervirens, Salvia rosmarinus, Eucalyptus polybractea, Lantana camara, Morus alba, Rubus ulmifolius, Dittrichia viscosa* et *Thuja orientalis*) en comparaison avec le bicarbonate de sodium et deux fongicides. L'analyse chimique des HEs par GC/MS MS a permis d'identifier 65 composés pour *C. sempervirens* et *L. camara*, ainsi que 45 composés pour *S.rosmarinus* et *E. polybractea*. Les résultats de l'activité antibactérienne de ces HEs contre les six bactéries pathogènes *Pseudomonas aeruginosa* ATCC27853, *Staphylococus aureus* ATCC25923, *Echerichia coli* ATCC25922, *Salmonella sp, S. aureus, P. aeruginosa* et une levure *Candida albicans* ont présenté de fortes activités antimicrobiennes contre la plupart d'entre elles à des valeurs de concentration minimale inhibitrice (CMI) allant de 0,91 à 8,7 mg/mL. La détermination de l'activité antifongique des HEs de dix souches fongiques du blé des genres *Penicillium, Cladosporium, Aspergillus, Alternaria, Trichoderma* et *Fusarium* a été effectuée sur un milieu PDA. Les résultats confirment l'activité antifongique de ces HEs contre les champignons des grains de blé stockés à une concentration de 300µl. Cependant, l'étude a également porté sur l'efficacité antifongique des extraits. Alors que l'extrait méthanolique de feuilles de *M. alba* et l'extrait aqueux de feuilles de *D. viscosa* ont montré un effet antifongique très prometteur, suivi par l'extrait méthanolique de *R. ulmifolius*, l'extrait éthanolique du *T. orientalis* et *L. camara,* celui de la combinaison méthanolique entre Morus et Rubus est la plus faible. Le bicarbonate de sodium a notamment donné des résultats positifs par rapport aux deux fongicides chimiques (Agriconazole et Vidan) sur les mêmes champignons testés. En outre, l'étude de l'activité insecticide contre un model de ravageurs du blé entreposé, *Tribolium castaneum*, par fumigation et par repellent, a démontré que l'efficacité de ces activités dépendait de la concentration et de la durée d'exposition. Il convient de noter que la fumigation a donné de meilleurs résultats que les tests répulsifs du faite que la concentration la plus élevée testée a donné 100% de mortalité, à l'inverse de l'activité des extraits. Les extraits botaniques utilisés apparaissent comme une alternative prometteuse, efficace, économique et écologique pour le traitement biologique des champignons et les insectes, avec un pouvoir antibactérien remarquable.

Mots clés : Extraits de plantes, Huiles essentielles, Activités antimicrobiennes, Activités insecticides, Activité antifongique, Blé stocké, Champignons, Traitement biologique

ملخص

يركز هذا العمل في المقام الأول على دراسة الموكب الفطري المرتبط بحبوب القمح المخزنة (3 أصناف معالجة و5 غير معالجة بمبيد فطري) ونباتاتها، أين كشف تحليل الفطريات عن وجود مجموعة متنوعة من الفطريات، بما في ذلك المنتجة للسموم. لقد تم الكشف عن 80 سلالة و تحديد 53 منها الى الاجناس ال18 التالية *Fusarium*, *Alternaria*, *Absidia*, *Aspergillus, Penicillium, Cladosporium, Rhizopus, Rhizoctonia, Trichoderma, Microdochium, Purpureocillium, Acremonium, Rhizomucor, Helminthosprium, Geotrichum, Sclerotina, Chaetomium.* و *Ulocladium* ومن هذا المنطلق، تعرض هذه الوثيقة نتائج تقييم النشاط البيولوجي لبعض الموارد النباتية من نوميديا الشرقية واستغلالها في المحافظة على محصول القمح *Triticum durum* Defs ، 4 زيوت اساسية و 8 مستخلصات نباتية مأخوذة من 8 نباتات مزروعة في الجزائر *(Cupressus sempervirens, : Salvia rosmarinus, Eucalyptus polybractea, Lantana camara, Rubus ulmifolius, Dittrichia* (*Thuja orientalis* و *viscosa* بالمقارنة مع بيكربونات الصوديوم واثنين من مبيدات الفطريات. أتاح التحليل الكيميائي للزيوت الاساسية بواسطة GC/MS/MS تحديد 65 مركبًا لـ *L. camara* و*C. sempervirens* بالإضافة إلى 45 مركبًا لـ *E. polybractea* و*S.rosmarinus*. تائج النشاط المضاد للبكتيريا للزيوت الاساسية ضد ستة أنواع من البكتيريا *الممرضة Pseudomonas aeruginosa* ATCC27853, *Staphylococus aureus* ATCC25923, *Echerichia coli* ATCC25922, *Salmonella sp, S. aureus, P. aeruginosa* وخميرة واحدة *Candida albicans*, أظهرت أنشطة قوية مضادة لمعظم للميكروبات عند قيم التركيز المثبط الأدنى التي تتراوح من 0.91 إلى 8.7 ملغم / مل لقد تم إجراء تحديد النشاط المضاد للفطريات للزيوت الأساسية لعشرة سلالات فطرية من القمح لأجناس *Penicillium, Cladosporium, Aspergillus, Alternaria, Trichoderma* et *Fusarium.* على وسط PDA .

تؤكد النتائج فعالية هذه الزيوت ضد فطريات حبوب القمح المخزنة بتركيز 300 ميكرولتر. نظرت الدراسة أيضًا الى الفعالية المضادة للفطريات للمستخلصات، حيث أظهر المستخلص الميثانولي لأوراق *M. alba* والمستخلص المائي لأوراق *D. viscosa* تأثيراً واعداً جداً، يليه المستخلص الميثانولي لـ *R. ulmifolius* ، *والمستخلص الإيثانولي لـ l. camara* و*T.orientalis* أما المزيج الميثانولي بين *Rubus* و*Morus* فهو الأضعف , كما أعطى بيكربونات الصوديوم نتائج إيجابية بشكل ملحوظ مقارنة بالمبيدين الكيميائيين (أجريكونازول وفيدان) على نفس الفطريات التي تم اختبارها. بالإضافة إلى ذلك، أظهرت دراسة نشاط المبيدات الحشرية ضد نموذج من آفة القمح المخزونة *Tribolium castaneum* ، عن طريق التبخير والطرد، أن فعالية هذه الأنشطة تعتمد على التركيز ومدة التعرض. تجدر الإشارة إلى أن التبخير أعطى نتائج أفضل من اختبارات المواد الطاردة حيث أن أعلى تركيز تم اختباره أعطى نسبة موت 100%، على عكس نشاط المستخلصات. كما يبدو أن المستخلصات النباتية المستخدمة هي بديل واعد وفعال واقتصادي وبيئي للمعالجة البيولوجية للفطريات والحشرات، مع قوة ملحوظة مضادة للبكتيريا.

الكلمات المفتاحية: المستخلصات النباتية، الزيوت الاساسية، أنشطة مضادات الميكروبات، أنشطة مبيدات الحشرات، نشاط مضاد للفطريات، القمح المخزن، الفطريات، المعالجة البيولوجية

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Wheat is the oldest and most important grain in the world's food supply. It is the staple crop of North Africa, where its consistent availability and affordability are central aspects of regional food security. Cereal crops continually suffer from multiple diseases that spread throughout the world. The main wheat diseases are especially caused by fungal pathogens (Figueroa *et al.,* 2018), such as *Septoria tritici* blotch, stripe rust, leaf rust, Fusarium head blight and powdery mildew. Habitually, fungi present in crops are either "field fungi" (or plant pathogens), which invade and produce their toxins before harvest; or "storage" (or saprophytic) fungi and become a problem after harvest. However, the original source of both classes of fungi is in the field and typical examples of the field fungi that occur on the grain until harvest are the species belonging to genus Alternaria, Cladosporium, Microdochium, Trichoderma, Helminthosporium and Fusarium (Felšöciová, *et al*., 2021).

In each of the pre-harvest and post-harvest operations, wheat faces various hurdles that affect the final quality of the grains. Stored grain products are subject to microbial contamination, associated toxins and insect attacks, leading to changes in their essential nutrients and major economic losses. Microbial invasion is one of the main causes of grain quality deterioration (Fleurat-Lessard, 2017). Grains are easily contaminated by various microorganisms during production, harvesting, transportation and storage, among which fungi are the main microbial species affecting the quality and safety of the grains. *Fusarium*, *Aspergillus*, *Penicillium*, and *Alternaria* are filamentous fungal species found in grains (Balendres *et al*., 2019). However, fungi belonging to the genera *Alternaria*, *Cladosporium*, *Fusarium*, and *Helmintosporium* have been reported as contaminants of grains in the field, while *Aspergillus*, *Penicillium*, *Eurotium*, and *Mucor* are mainly reported to contaminate grains in storage conditions (Doyle *et al*., 2020). These fungal genera include many species that can produce toxigenic secondary metabolites (mycotoxins), which can cause severe carcinogenicity, mutagenicity, genetic toxicity, growth and reproduction toxicity, immunotoxicity, and neurotoxicity in humans and animals (Luo *et al*., 2021). The most frequently detected mycotoxins in wheat grain are deoxynivalenol (DON), fumonisins (Fs), and zearalenone (ZEN), produced by *Fusarium* species, and aflatoxins (AFs) and ochratoxin A (OTA) produced by *Aspergillus* and *Penicillium* species, respectively (Pandey *et al.,* 2022).

It is also important to highlight that stored product pests inflict various damages and cause economic losses by direct consumption, spoilage and loss of product quality. The granary weevil, *Sitophilus granarius* (L.) (*Coleoptera: Curculionidae*), the lesser grain borer, *Rhyzopertha dominica* (F.) (*Coleoptera: Bostrichidae*), the confused flour beetle, *Tribolium confusum* (Jacquelin du Val) (*Coleoptera: Tenebrionidae*) and the red flour beetle, *Tribolium castaneum* (Herbst) (*Coleoptera: Tenebrionidae*) are some of the major pests of stored grains in the world which share some common traits in terms of surviving on small amounts of food and living in cryptic places such as cracks and crevices in storage environment. After all, *Tribolium castaneum* is the most widespread and polyphagous stored-product insect pests (Aboelhadid and Youssef, 2021) affecting mainly stored food products, such as grains, flour, and cereals, among others, with reports on contaminating 246 food products (Skourti *et al*., 2019). The adult and immature forms are categorized as secondary pests which feed on grains or seeds previously damaged in storage conditions. The contamination of infested stored products by body fragments and toxins (e.g., methyl-1,4-benzoquinone, ethyl-1,4-benzoquinone, methoxybenzoquinone) may have a negative impact on consumers. *T. castaneum* is an important international pest of stored products and has been extensively researched to improve pest management programs.

The significance of biodiversity for human kind is immense, ranging from the direct consumption of plants and animals for sustenance, healthcare, construction, and other purposes to aesthetic, cultural, recreational, and research values (Baranitharan *et al*., 2018). Plants, in particular, are a prolific source of bioactive organic compounds and manufacture a range of secondary phytochemicals (Teklić *et al*., 2021; Jamwal *et al*., 2018). Worldwide, the green earth concept determines the use of natural products in daily life. The actual synthetic chemicals, commonly used to control pathogen strain, raise serious preoccupations related to human health (Falleh *et al*., 2019). Currently, plant extracts essential oils (EOs) and their components are attracting more attention in the commercial food sector due to their unique aroma, flavors and biological properties (Burt, 2004). These botanical extracts are obtained by different extraction methods (ethanol, water, methanol…etc) and each of them may contain valuable bioactive compounds which have a multitude of biological activities. EOs are considered under the 'generally recognized as safe' (GRAS) label by the US-FDA (Ju *et al.*, 2018), including basil, cinnamon, clove, coriander, ginger, rosemary, lavender, menthol, oregano, rose, sage, and thyme EOs. Chemically, EOs and extracts are a rich mixture of numerous bioactive chemical components such as alkaloids, terpenes, terpenoids, and

phenolic compounds (Jamwal *et al*., 2018; Samadi *et al*., 2021). They are worldwide known for their proven biological activities including antimicrobial, antifungal, antioxidant, antiviral, antiparasitic and insecticidal properties (Shaaban, 2020; Masyita *et al*., 2022; Pop *et al*., 2019). The major sources relevant for industrial use are from *Alliaceae*, *Apiaceae*, *Poaceae*, *Lamiaceae, Myrtaceae, Cupressaceae*, *Asteraceae*, *Lauraceae*, Zingiberaceae, *Pinaceae* and *Rutaceae* plant families and the extraction can be made from different part of plants: flowers, leaves, fruits, seeds, grasses, roots, rhizomes, wood, bark, gum, tree blossoms, bulbs, and dried flower buds (Moghaddam and Mehdizadeh, 2017).

There is no longer any doubt that biopesticides have great potential to combat bacterial and fungal pathogens as well as storage grain insects. This study aimed firstly, to explore fungal diversity in stored wheat grains and infected wheat plants in order to develop an inventory of the fungal flora. The second objective was to highlight the biological activities of certain plant resources of Eastern Numidia: eight natural plants (*Eucalyptus polybractea, Cupressus sempervirens, Lantana camara, Salvia rosmarinus, Morus alba, Rubus ulmifolius, Thuja orientalis* and *Dittrichia viscosa*) growing in different environments and frequently used in ethnomedicine were used to prepare 8 extracts and 4 EOs for their valorization in the conservation of wheat *Triticum durum* Defs. EOs and plant-extracts constitute a promising source for novel environmentally safe bactericides, fungicides and insecticides. However, the antibacterial activity of the selected EOs is tested, with particular attention to the fungicidal activity of both EOs and extracts in comparison with sodium bicarbonate and two chemical fungicides. In the context of managing stored grain insect pests, another stated objective concerns the insecticidal activity (fumigant and repellent) of these selected extracts and EOs against the red flour beetle *T. castaneum*. In parallel, we tested the biological effect (antifungal and insecticide) of these extracts alone or even combined.

The study is divided into three chapters where each contains a literature review and methodology: sample, context, data analysis methods and discussion. After a general introduction which presents the objective of the study, the first chapter consists of revealing the fungal procession associated with wheat (plants and cereals). The second chapter presents the antibacterial activity of four selected EOs, the antifungal activity of selected EOs and eight plant-extracts and the last highlights the insecticidal activity of these EOs and extracts against *Tribolium castaneum*. Finally, the study is closed with a general conclusion, perspectives and bibliographical references.

CHAPTER 1:
FUNGAL PROCESSION ASSOCIATED WITH WHEAT

CHAPTER 1: Fungal procession associated with wheat

Introduction

Cereals, the historical basis of the Mediterranean diet, still occupy today an important place both in the agricultural and food production in Algeria and in the food consumption of households. Wheat is one of the most cultivated and consumed basic cereal in the world (Tilman *et al*., 2011). Crop preservation is "the art of preserving the original quality of grains and preventing their deterioration for a specified period of time, whether they are kept for on-site use or transported for final processing together" (Kiaya, 2014). Wheat is typically stored for one or more years after harvest to supply the domestic grain industry and meet export and import needs. One of the major management challenges associated with grain storage is preventing fungal growth on/in canned wheat (Fleurat-Lessard, 2017). These fungi can cause significant yield losses in wheat grain in the field and storage facilities, particularly due to their ability to produce mycotoxins, rendering the crop unfit for consumption (Chen *et al*., 2019; Lee and Ryu, 2017). Under favorable microbiological conditions, wheat grains are infected by fungi both in the field prior to harvest and during storage, as they provide a favorable environment for their growth. In particular, genera such as Alternaria, Cladosporium, Fusarium, Aspergillus, and Penicillium are known to infect food grains during storage, leading to loss of quantity and quality worldwide (Rasooli *et al.,* 2006) and produce toxic or carcinogenic metabolites (Mannaa and Kim, 2017). The main objective of this study was to isolate and identify fungi from durum wheat plants and stored grains.

1. Presentation and main characteristics

Fungi are a kingdom with 99,000 species divided into about 10 phyla, and their classification is reviewed on a regular basis (McLaughlin *et al*., 2009; Blackwell, 2011). They are aerobic organisms that can be found in all ecosystems and have beneficial biological activities (Musavi and Balakrishnan, 2014). Fungi are thought to be the biosphere's second most species-rich kingdom, after insects (Cordier, 2012). Their development is influenced by their surroundings. They are achlorophyllous eukaryotic microorganisms with a mycelial structure and thallus composed of numerous branched filaments (Hassouni, 2007). Fungi are heterotrophs (Botton *et al*., 1990) forming a very heterogeneous group whose common essential characteristic is heterotrophic nutrition by absorption, which can take the form

saprophytism, parasitism or symbiosis (Nasraoui, 2006). Microscopic fungi (molds) use their mycelial structure with branched filaments called hyphae often septate or aseptate branches (Stajich *et al.*, 2009), which spread by polar extension (Steinberg *et al.*, 2017). Fungi include macroscopic (Macromycetes) and microscopic (Micromycetes) species (Tabuc, 2007), which includes the yeasts, rusts, smuts, mildews, molds, and mushrooms. From a structural point of view, there is a wide variety of fungi. They are classified into two broad categories: the most common is the vegetative form in the form of unicellular yeast and the multicellular mycelial form consisting of hyphae (Redecker, 2002). Some species have the ability to adopt the two forms, yeast and mycelial (dimorphism) (Ruiz-herrera et Campos-Góngora, 2012), while others are restricted to one forms (Jennings and Lysek, 1996). However, while fungi are beneficial for many processes, they can also cause certain diseases (Pointing and Hyde, 2001).

2. Morphology and Structure

Filamentous fungi consist of a vegetative structure known as thallus. It can exist in either a unicellular state (such as yeast) or a filamentous form (such as mold). Under specific conditions, some yeast can develop filamentous structures, also known as pseudomycelium. The thallus is composed of tangled filaments or hyphae, which creates a system called the mycelium. Filamentous fungi grow long 2–10 μm thin filaments (hyphae) into intricate network structures (mycelium) that are observable to the naked eye and can grow to the centimeter to meter scale (Hüttner *et al*., 2020). In certain molds, like Mucor, the cells lack a transverse partition resulting in a coenocytic or "siphoned" thallus, while in others, like Aspergillus, the thallus is partitioned or "septate" (Fig1). The partitions, commonly known as septa, are perforated for intercellular communication. The morphological features of these microorganisms are related to their nutrient substrate. Hyphal extension and branching enable substrate colonization (Lecellier, 2013).

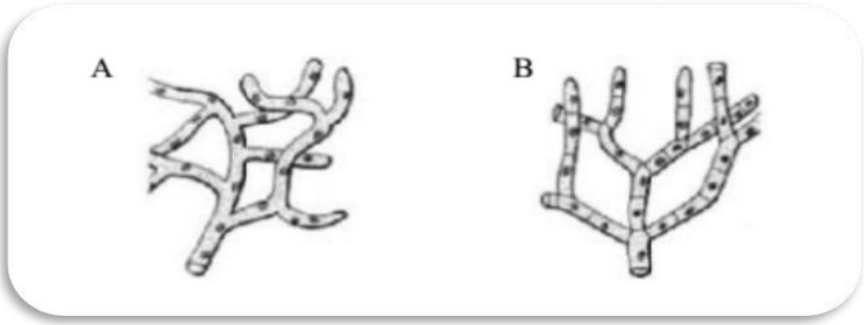


Figure 1. Hyphae structure: (A) coenocytic hyphae, (B) septate hyphae (Leclerc *et al.*, 1983)

3. Ecology of fungi

Fungal proliferation in nature is facilitated by the filamentous mode of growth and the secretion capacity of proteins and primary and secondary metabolites (Wösten, 2019). They typically develop as saprophytes in nature. They contribute to the degradation and recycling of both organic and mineral matter (Fernandez *et al*., 2020). Filamentous fungi are heterotrophic and cosmopolitan, and as such, inhabit diverse environments, whereby they engage in unique interactions with their surroundings and develop particular lifestyles. The success of fungi to adapt and proliferate in diverse ecological niches is dependent on their ability to respond to changes in the environment. They secrete a wide variety and large amount of enzymes and can be involved in degradation of cellulose (Wösten, 2019). These include pectinases, transferases, cellulases, peroxidases, and laccases (Verma *et al*., 2019). Fungi also have essential roles as components of the microbiota, where they act as symbionts, endophytes, parasites, or saprotrophs (Wösten, 2019; Bonfante *et al*., 2019).

Saprophytic fungi comprise the largest group of fungi that nourish themselves on decaying organic material, such as animal and plant debris. They are generally considered a primary engine of the decomposition process (Maltz *et al*., 2017). Parasites (e.g. pathogens of human, animals, plants, insects and fungi themselves), on their side, derive their energy from living organic matter found in plants, trees, and animals. Most are harmless recyclers but some can cause significant damage to plants (Sandhu *et al*., 2021) and affect animal (Nenoff *et al.*, 2019) including human health (Dolma *et al*., 2018). Parasites infecting plants employ three typical feeding strategies (O'Keeffe *et al*., 2021). Biotrophic parasite keeps host cells alive to extract resources from them. Necrotrophic parasites kill host cells to access their resources, creating necrotic tissue while they grow within their host. Finally, hemibiotrophic parasites infect as biotrophs, and then switch to become necrotrophic. In nature, fungi exist in symbiotic relationships with such diverse species as plant and algae, to form mycorrhizae and lichens, respectively. Mycorrhizas are among the most important biological interkingdom interactions (Genre *et al*., 2020). Mycorrhizal fungi form a beneficial relationship between plants and microorganisms (Chen *et al.*, 2018): a fungus takes nutrients from the host plant to complete its growth and development. At the same time, it helps the plant absorb water and nutrients and impart stress resistance. In fine, fungal endophytes are asymptomatic inhabitants of plant tissue and are reported from all parts of plants (Sarsaiya *et al*., 2019). Endophytic microorganisms promote plant growth and provide protection against pests and pathogens through different mechanisms (Busby *et al*., 2016).

4. Reproduction

Fungal reproduction is complex, reflecting the differences in life styles and genetic makeup within this diverse kingdom of organisms. Although certain fungi reproduce asexually by fragmentation, fission, and budding, the majority reproduce sexually through the generation of spores (Fig2). Sexual reproduction often occurs in response to adverse environmental conditions.

- **Asexual reproduction:** this method involves only a single parent in the reproduction. The whole process can be broadly categorized into three key mechanisms:

  1.1. **Fragmentation:** Some forms belonging to Ascomycotina and Basidiomycotina multiply by breakage of the mycelium, and mycelial fragments can form new colonies

  1.2. **Budding:** A bud arises as a papilla on the parent cell and then after its enlargement separates into a completely independent entity.

  1.3. **Asexual spores:** Are another common way for fungi to propagate asexually. These spores contain genetic material from the parent fungi and can survive harsh conditions, allowing them to colonise new areas.

- **Sexual reproduction**, in which two mycelia of opposite sexes meet. A haploid mycelium meets another mycelium of complementary polarity, resulting in fusion of the cytoplasm and the formation of a new diploid mycelium. Sexual reproduction in the fungi consists of three sequential stages: plasmogamy, karyogamy, and meiosis. With the exception of Deuteromycotina (Fungi imperfecti), we find sexual reproduction in all groups of fungi.

During asexual and sexual reproduction processes spores are the essential structures. The spores formed after meiosis are called meiospores (ascospores, basidiospores and sporangiospores) and those resulting from mitosis, called mitospores (mitospores, zoospores, aplanospores, conidia, uredospores).

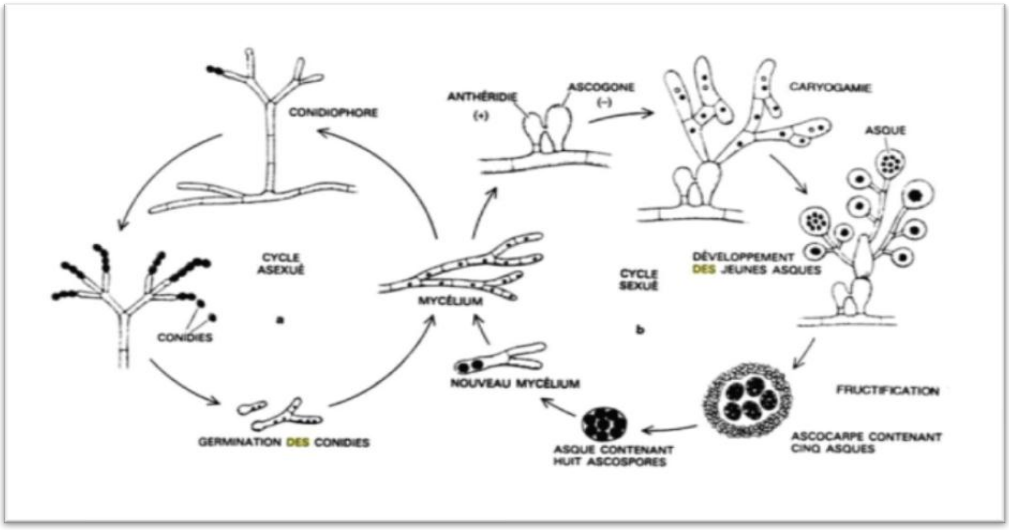


Figure 2. Schematic diagram of asexual and sexual reproduction of a mold (Lecellier, 2013)

5. Fungal Classification

Fungal taxonomy has undergone major changes. Early classifications included several groups of heterotrophic eukaryotes including a clade considered "true fungi", or "Eumycota" (Whittaker, 1969). Early on, four major phyla were defined within the true fungi, based on their morphological and reproductive traits: Chytridiomycota, Zygomycota, Ascomycota and Basidiomycota (Whittaker, 1969). More recently, molecular phylogenies and the advent of environmental-sequencing-based technologies allowed the recognition and describtion of new fungal species. The most up-to-date taxonomy comprises the described diversity of Eumycota, dividing it into nine major lineages (monophyletic clade): Opisthosporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Zoopagomycota, Mucoromycota, Glomeromycota, Ascomycota and Basidiomycota (Tedersoo *et al*., 2018) (Fig3). The eternal topic of fungal taxonomy is to reconstruct the Fungal Tree of Life (FTOL), where ideally all fungal species are well-delimited, formally described and correctly assigned to their particular positions. Generally, the Ascomycota is the largest group of microscopic fungi with 15 classes, 68 orders, 327 families and approximately 64,163 species (Kirk *et al.*, 2008). It includes lichen fungi (Carlile *et al.*, 2001), plant pathogens, and yeasts that are beneficial to humans such as *Saccharomyces cerevisiae*. The Basidiomycota group includes most of the mycorrhizal fungi with carpophores, in addition to plant and yeast parasites. This group develops non-flagellate spores at the tips of specialized cells called basidia, which get dispersed by wind. The Chytridiomycota group comprises species that produce uniflagellate spores or zoospores that reflect a predominantly aquatic lifestyle. This group constitutes the

oldest evolutionary lineage of fungi (James *et al*., 2006). The Glomeromycetes group includes strict plant symbiotic and biotrophic fungi that form arbuscular mycorrhizae. In fine, the Zygomycota group is relatively recent and includes many saprotrophs and insect parasites. This phylum includes approximately 900 species (Botton *et al.*, 1990).

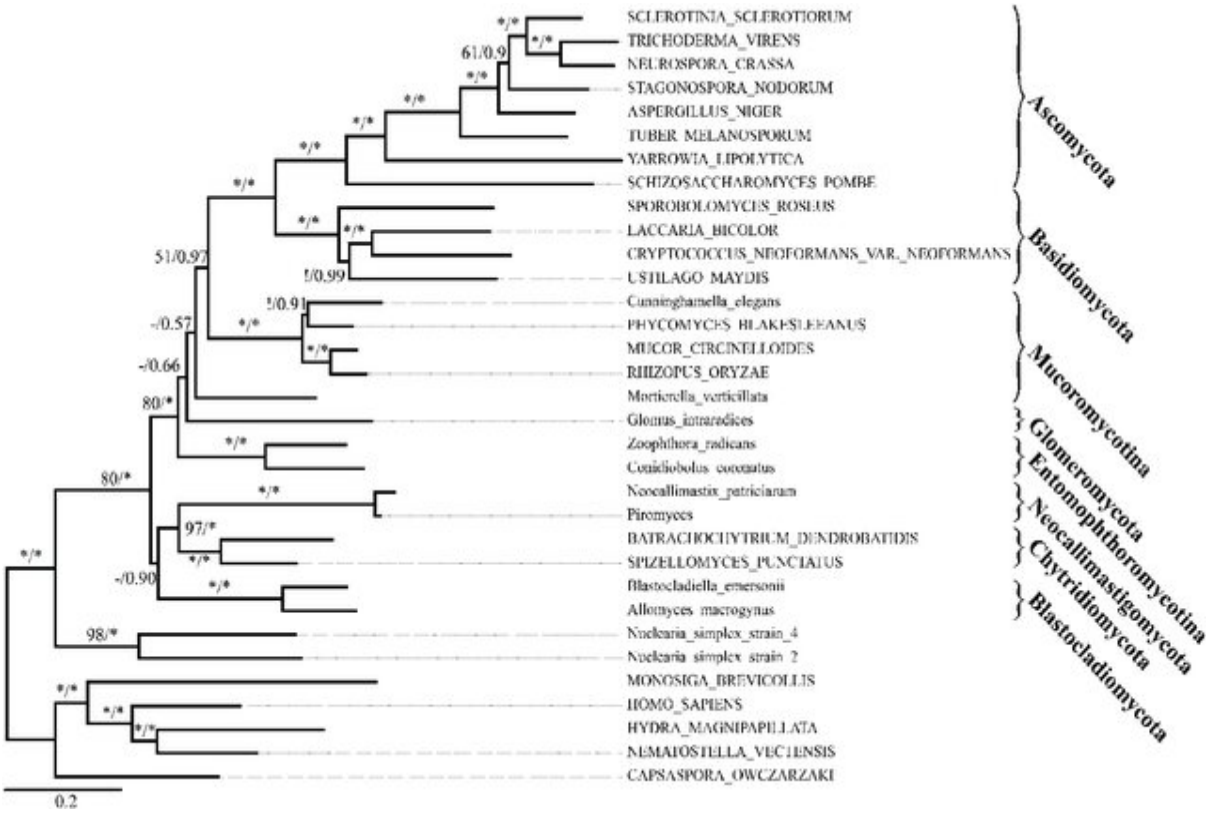


Figure 3. Phylogenic classification of fungi (*In* Ebersberger *et al.*, 2011)

6. Economic Importance of Fungi

Filamentous fungi are thus getting increasing attention as workhorses in industrial production. They are excellent organisms as cell factories for production of a variety of products (Fig4). They naturally produce efficient enzymes for the decomposition and conversion of biological material. They also produce different compounds, many of which can have interesting commercial applications. In agriculture, fungi play a significant role, including plant growth and protection. For example, the use of fungi as biofertilizers is one emerging area which is getting more attention as it is proving its importance by enhancing plant growth and productivity by diverse plant growth-promoting traits including production of phytohormones, siderophores, and hydrolytic enzymes; making available different nutrients; and protecting plants against pathogens (Kour *et al.* (2019). Another essential agricultural fungal type is the endophytic fungi that colonize plant tissue. The complex

involves modulating the plant's defense mechanism in terms of inhibiting phytopathogens and stimulating the growth of the plants even under biotic and abiotic stress conditions (Galindo-Solís et Fernández, 2022). Several fungi are used as insect pests biocontrol agents, known as biopesticides, against different agricultural pest insects (Sindhu *et al.*, 2017), because they show an excellent potential to defend against disease crops and attenuate the unfavorable conditions that can affect plant growth and stimulate plant growth (Lahlali *et al.*, 2022).

In the food industry, they have been used since ancient times for various purposes, such as fermentation, production of enzymes, and as a source of food (Amara et El-Baky, 2023). They have long been used to produce traditional fermented foods such as cheese, bread, soy sauce, tempeh and mold-cheeses. Filamentous fungi play an increasing role in industrial production of organic acids, enzymes, flavors, vitamins, colorants and proteins, especially enzymes at large scales. Their metabolism assures obtaining large quantities of amylases, proteases, pectic enzymes, galactosidases, lipases, chitinases, or lignocellulolytic enzymes (Copetti, 2019). Currently, fungi exhibit great potential for developing strategies that enhance environmental protection. They are involved in practices such as the bioremediation of pharmaceutical compounds, agricultural wastes, or degradation of various pollutants. Mycoremediation as a form of bioremediation may be an eco-friendly technique for decontamination of polluted environmental matrices because of its simplicity and highly efficient implementation process (Dey *et al.*, 2022). Fungi indubitably dominate the biotechnology sphere for being an optimal candidate to produce several products such as textiles, biofuels, building materials, wastewater treatment, and sustainable meat substitutes (Hüttner *et al.*, 2020).

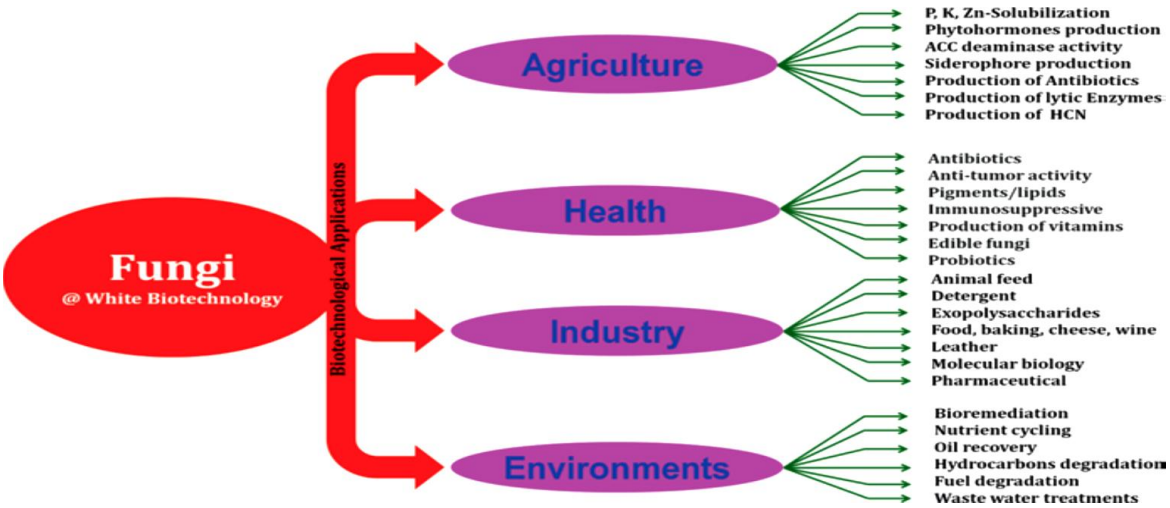


Figure 4. Biotechnological applications of fungi and their value-added products in agriculture, health, industry, and environments (Kour *et al.*, 2019)

7. Phytopathogenic fungi: the case of fungal diseases of wheat

Phytopathogenic fungi have been devastating threats throughout the history of agriculture. Their infections cause an enormous spectrum of disease symptoms. However, wheat plants can be infected by a variety of fungal pathogens, which cause losses totaling about 20% of global production (Kuzdraliński *et al*., 2017). Among the most common wheat fungal diseases, we include rusts (caused by *Puccinia* spp.), the Septoria leaf blotch complex (*Zymoseptoria tritici* and *Parastagonospora nodorum*), powdery mildew (*Blumeria graminis*), wheat blast (*Pyricularia oryzae* Triticum lineage), and several afflictions incited by species of Fusarium (Thierry *et al.,* 2020). Fusarium head blight (FHB) is one of the most relevant fungal diseases of wheat caused by different *Fusarium spp* (Summerell, 2019). Several studies showed that FHB epidemics on wheat take place sporadically. *Fusarium graminearum* is the main pathogen of FHB worldwide (Summerell, 2019), with a significantly contribution of *F. graminearum, F. culmorum, F. avenaceum, F. poae, F. tricinctum, F. equiseti and F. sporotrichioides* (Xue *et al*., 2019). In addition *F. pseudograminearum, F. praegraminearum, F. dactylidis, F. langsethiae, F. cerealis, F. subtropicale,* and non-toxigenic species *Microdochium nivale* have also been described as FHB pathogens (Pereira *et al*., 2018). The various fungal diseases affect all parts of the plant, including roots, stems, leaves, nodes, glumes and head. Wheat crop is also affected by number of seed-borne pathogens, which can reduce its global production to significant extent. Rusts are among the most devastating diseases of wheat. The three species of black, yellow and brown rusts attack both soft and hard wheat. Wheat is affected by three different types of rust diseases; leaf rust (caused by *Pucina triticina* Eriks), stripe rust or yellow rust (caused by *P. striiformis* Westend. f. sp. *tritici Eriks*), and stem rust (caused by *P. graminis* Pers:Pers. f. sp. *tritici Eriks*). Stem rust is the most dangerous form of rust to wheat crops, and when attacking a variety that has no genetic resistance, losses to stem rust can be total (Soko *et al*., 2018). The stored seeds are more prone to be attacked by fungi; approximately 15 to 18 species of Penicillium, Aspergillus, Alternaria, Bipolaris, Curvularia and Fusarium have been reported as important contaminants of wheat grains (Sadhasivam *et al.,* 2017). These fungi invade the seeds after their harvest the so-called “storage fungi” or under field conditions and remain alive for years (Ulziijargal *et al*., 2019). Generally, seed-borne fungi that reduce seed and grain quality are commonly divided into three ecological general groups—field fungi, intermediate and storage fungi—depending on whether the infection occurs mostly before or after harvest (Tab1).

Table 1. Main micromycetes groups of cereals (Botton *et al*., 1990)

Ecological group	Genus
Field fungi	*Alternaria, Fusarium, Epicoccum, Septoria*
Intermediate fungi	*Cladosporium, Aureobasidium, Mucor, Rhizopus, Absidia, yeasts*
Storage fungi	*Aspergillus, Eurotium, Penicillium, Wallemia, Scopulariopsis, Byssochlamus*

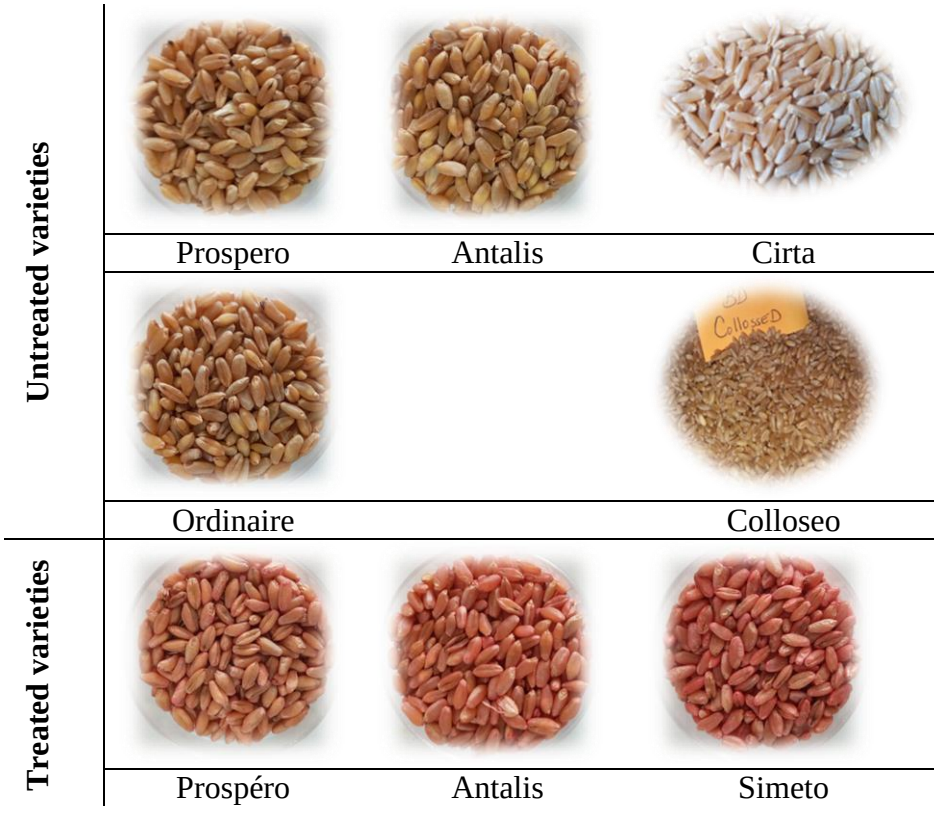
8. Material and methods

This chapter of the study aimed to explore the fungal diversity in wheat stocked grain and infected wheat plant to develop a fungus load inventory and to serve as a database for future research. To achieve this, an analysis of numerous parameters regulating fungal growth in stored wheat grains is carried out, followed by a microbiological analysis.

8.1. Plant material

The plant material consists of various varieties of durum wheat. We used, on the one hand, wheat grains stored for a year, sampled randomly: three varieties of them (Prospéro, Antalis and Simeto) are treated with a fungicide and the other five varieties (Prospéro, Antalis, Ordinaire, Colloseo and Cirta) are untreated (Photo1). All these varieties were supplied by the CCLS (Cereals and Dried Vegetables Cooperative) of Annaba.

Photo 1. Wheat grains varieties used



In the other hand, the infected wheat plants were harvested from wheat fields from Annaba in April 2021 (Photo 2).

Photo 2. Samples of infected leaves, spikes, and roots (Chibi, 2021)

8.2. Physicochemical quality of seeds

8.2.1. Seed physical condition

This requires counting the number of broken seeds in a test sample of 100 seeds taken randomly, which is representative of each sub-sample to be analyzed.

8.2.2. pH measure

To estimate the acidity or alkalinity of the wheat seed samples, create a solution by adding 45 ml of distilled water to 5 g of wheat seed. The pH of solution is then measured using the pH meter after placing it for an hour with continuous stirring**.**

8.3. Physiological parameters

8.3.1. Estimation of Germination Capacity

The germination capacity or germination power of seeds is the percentage of grains that have germinated over 3 and 10 days under controlled germination conditions. The purpose of the germination test is to obtain an estimate of the biological state of the grain, which might reflect internal contamination. This percentage can provide information regarding the grain storage conditions (Boulal *et al*., 2011). The experiment involves randomly selecting 25 seeds

from each variety, disinfecting the surface using 2% bleach for two minutes, and then rinsing twice using distilled water. Afterward, the seeds are placed in Petri dishes containing distilled water-soaked blotting paper, where they are left to germinate. The percentage of germination (FG) can be calculated using the following formula: $\mathbf{FG} = \frac{\mathbf{NG}}{\mathbf{NTG}} \times \mathbf{100}$

Where, NG denotes the number of germinated seeds, and NTG is the total number of seeds used in the experiment.

8.4. Microbiological parameters

8.4.1. Culture conditions

Leaves, spikes, stems, roots and grains with disease symptoms were washed to remove surface debris and disinfected with 2% bleach for 5 minutes. After two washes with sterile distilled water and drying with sterile absorbent paper, the specimens were cut into pieces of about 1 cm and 10 diseased wheat grains were aseptically transferred into 90 mm sterilized Petri dishes containing PDA medium and incubated at temperature 28°C. The culture media used are frequently used for research and enumeration of molds, as well as maintenance of collection strains and transplanting (Botton *et al.*, 1990) (Tab 2).

Table 2. The common fungal culture media used

PDA (Potato Dextrose Agar) culture medium
- Potatoes……...……..200g. - Dextrose…….20g. - Agar .....20g - Distilled water ……....……1000ml. pH 6,8
Malt Extract Agar (MEA, d'après Blakeslee, 1915)
- Malt Extract ..……..20g - Peptone……1g - Glucose…….20g - Agar……..15g - Distilled water……..1000ml pH : 5,6
Sabouraud
- Glucose…….....…….20 g - Peptone…….....……10 g - Agar….....20 g - Distilled water…….....1000 ml PH = 6,5

8.4.2. Purification and conservation

After incubation of Petri dishes for 5 days at 28°C, several molds have developed. The isolation and purification of isolates is a very delicate step that plays an important role in the identification stage. The purification of the strains is done by a series of sub culturing which consists in aseptically transferring a microorganism into a new and sterile medium to maintain it in pure culture (Botton *et al*., 1990). The fungi, once purified, are kept in agar inclined tubes on common media such as PDA, or MEA (Botton *et al*., 1990). The strains must be transplanted every 8 to 10 months (Botton *et al*., 1990).

8.4.3. Identification of fungal strains

The identification remains the most difficult operation in Mycology; it aims to classify fungal strains by genera and species according to the identification criteria. It is based on two aspects: microscopic and macroscopic. The isolated strains were identified based on complete determination keys of Botton *et al*. (1990), Domch *et al*. (1980).

The fungus cultures were observed by visual observation and with a magnifying glass writing down their morphological characteristics. The consistency of the colony (fluffy, woolly, cottony, flaky, powdery...etc); the color of the front and back of the Petri dish; the size (diameter) of the colony; the pigmentation; the shape of the outline (regular, irregular, lobed, jagged, filamentous,...etc) as well as the surface (flat, wrinkled, cerebrospinal) and the presence or absence of droplets (exudate).

Under hygienic and aseptic conditions, the preparation of fungal material for microscopic observation is done by diluting a mycelial fragment in a drop of physiological water on a sterile slide, then covering the preparation with a slide. Microscopic observation is based on morphological characters, we note: fruiting bodies, types of spores, aspect of the thallus, size, color and arrangement of pores. The observation is carried out with a light microscope «LEIKA ICC50 HD» at magnifications: (×10), (×20), (×40). The differentiated organs will be observed with immersion at magnification (×100).

8.4.4. Centesimal frequency

The centesimal frequency represents the proportion of individuals of a specific species in relation to the total individuals of all species present. The formula used to calculate centesimal frequency is as follows:

$$\text{The centesimal frequency} = \frac{\text{Number of repetitions of each species}}{\text{Total number of species recorded}}$$

9. Results and Discussion

9.1. Results

9.1.1. Physicochemical quality of seeds

9.1.1.1. Seed physical condition

The physical condition of 100 grains of eight wheat varieties taken at random was assessed (Fig 5). The result of broken grains in the treated varieties ranged from 22% for both Simeto and Prospero, and 15% for Antalis. While the broken grains in the untreated varieties ranged from 58% for Prospero, followed by Colloseo (33%) and Antalis (32%), those of Ordinaire, have the lowest number (11%).

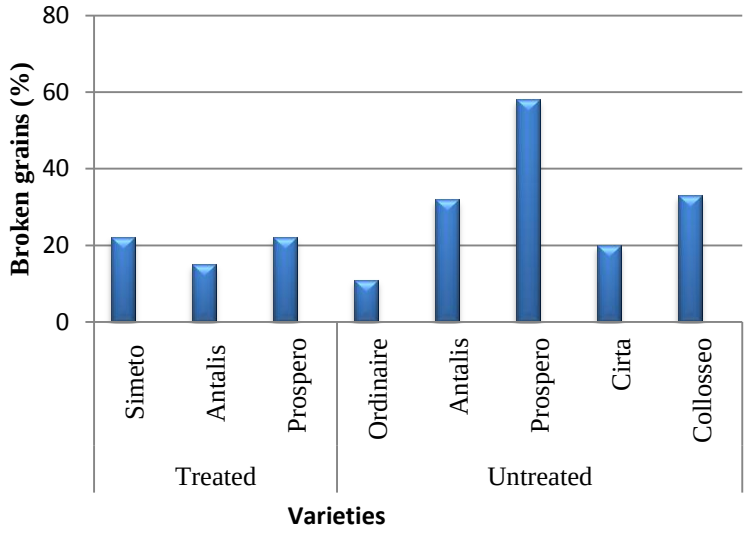


Figure 5. Wheat broken grain

9.1.1.2. pH measure

The pH values of the different grain samples illustrated in Figure 6, demonstrate that Simeto and Colloseo have a slightly basic pH of respectively 7.35 and 7.62. The results the rest of treated varieties were slightly acids ranging respectively from, 6.3 and 6.34 for Antalis and Prospero. Likewise, the pH of the grains of untreated varieties is close to 6 for Prospero Ordinaire and Antalis, while Cirta have recorded the values of 7.2.

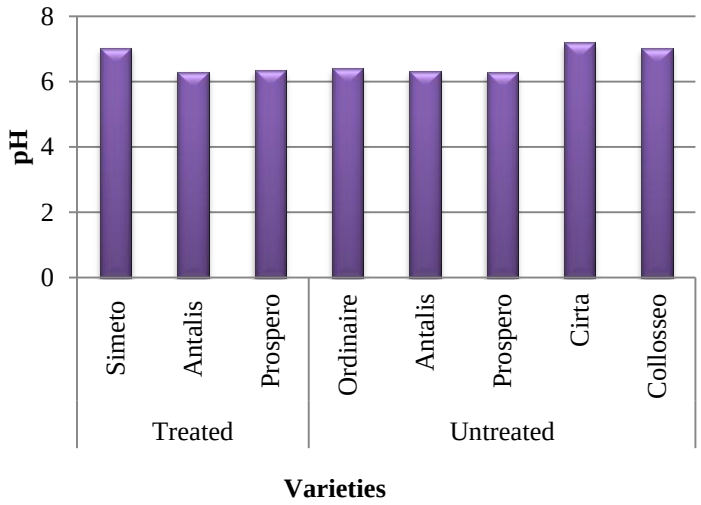


Figure 6. pH of wheat grain

9.1.2. Physiological parameters

9.1.2.1. Estimation of germination capacity

After 3 days of sowing, we notice a breakthrough followed by a growth of the different seeds. However, the germination rate of the untreated varieties Prospero, Cirta, Ordinaire and Colloseo recorded the highest rates up to 80% (Fig 7). Regarding the treated varieties, we see that Simeto and Antalis gave a similar value of 44% and Prospéro scored 20%.

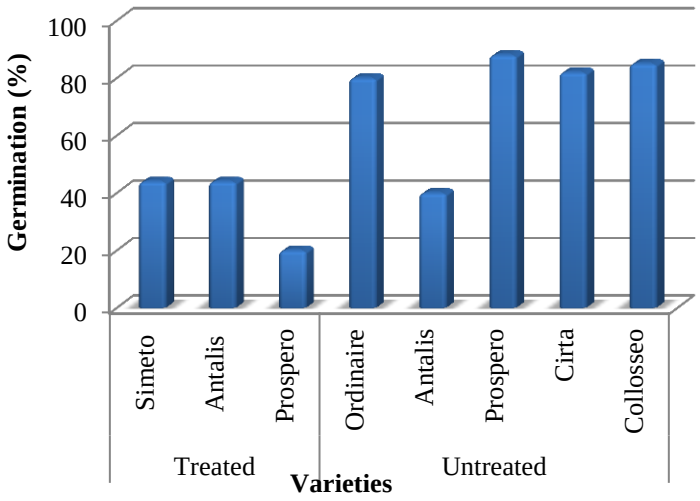


Figure 7. Germinating power of seeds after 3 days

After 10 days of germination of the eight varieties of wheat, we noticed an advanced growth of the seedlings (Fig 8). We notice a high germination rate of untreated varieties (up to 88%); while Ordinaire recorded 100% germination rate. The treated varieties, Simeto

noted a value of 68%, and Antalis the lowest values of 44%. We note that some wheat grains are contaminated.

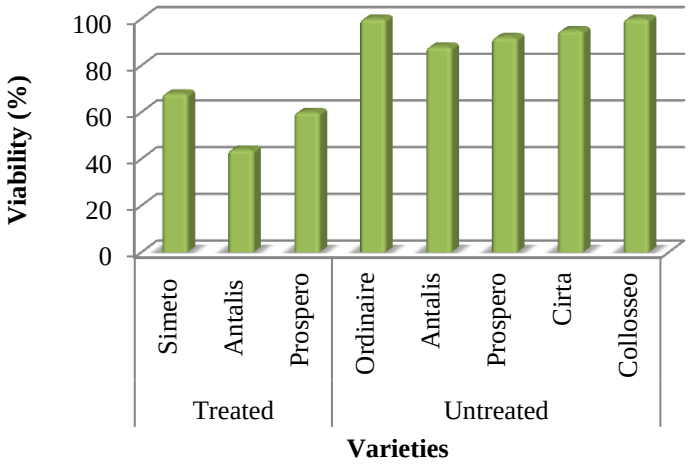


Figure 8. Viability of wheat seeds after 10 days

The plants are gradually exposed to the ambient atmosphere for 3 days of germination (Photo 3). We note advanced growth of untreated grain plants (shown at the bottom of Photo3), as well as the appearance of fungal contamination.

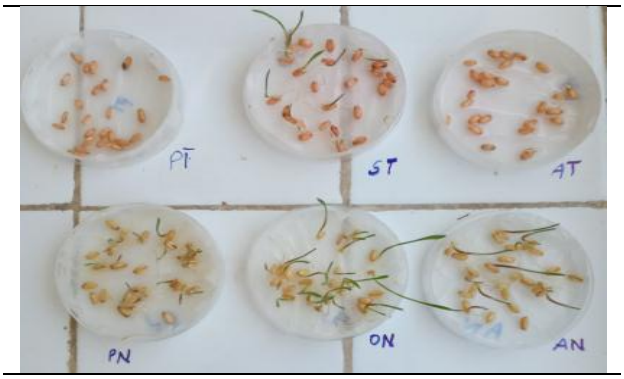


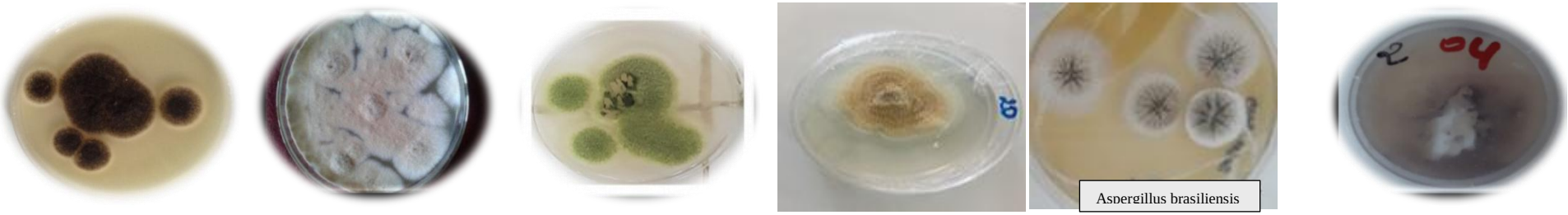
Photo3. Petri dish germination test

9.1.3. Microbiological parameters

9.1.3.1. Identification of fungal strains

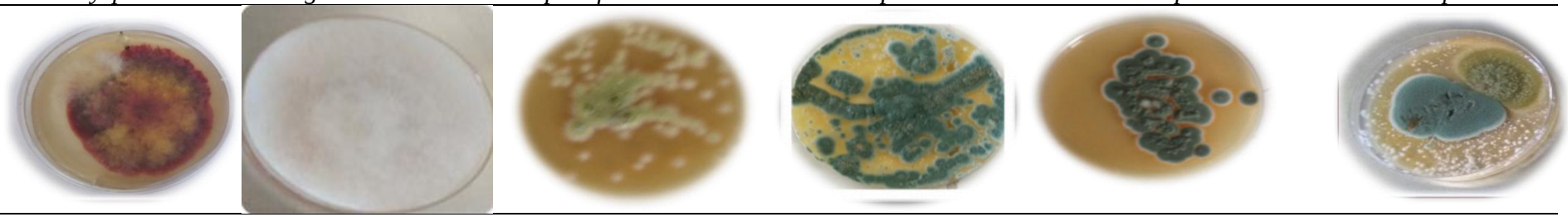
The study of the fungal procession associated with wheat allowed us to highlight a diversified fungal flora (Photo 4). The mycoflora of plant parts and seeds were determined morphologically. The macroscopic characteristics of some distinctive fungal strains are recorded in all of the following photos:

Photo 4. Macroscopic characteristics of isolated strains



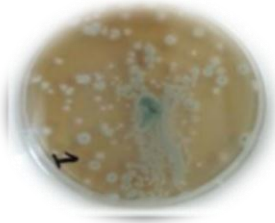
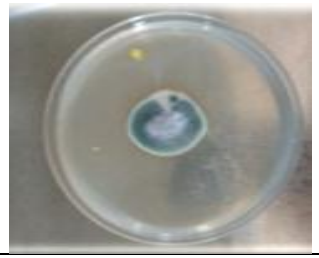
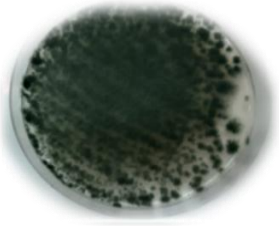
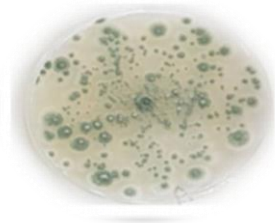
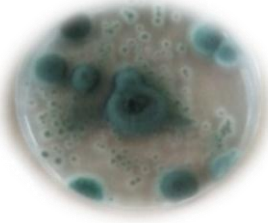
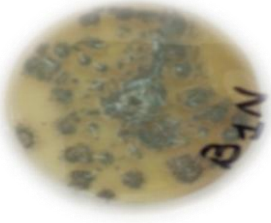
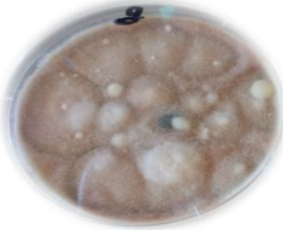
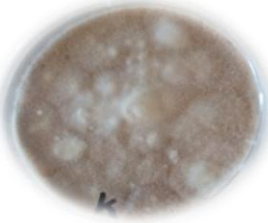
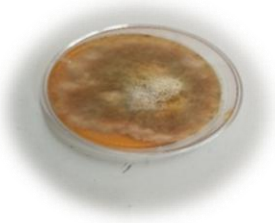
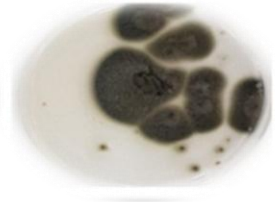
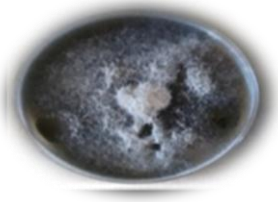
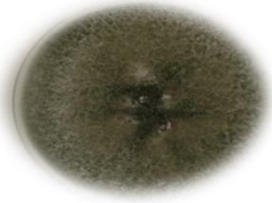
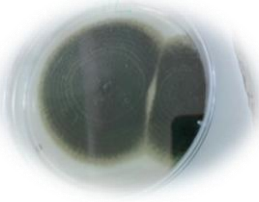
Aspergillus niger	*A. terreus*	*A.flavus*	*A.ochraceous*	*A. brasiliensis*	*Fusarium. solani*

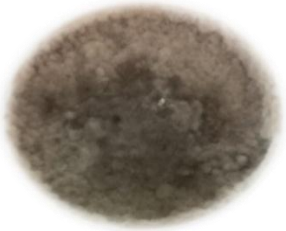
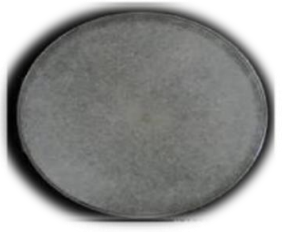
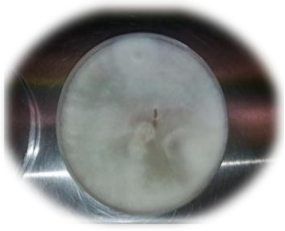
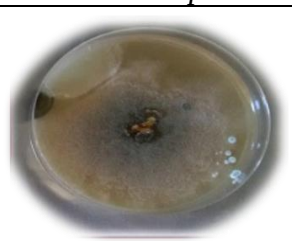
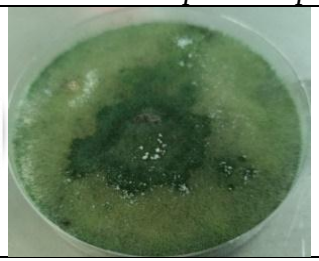
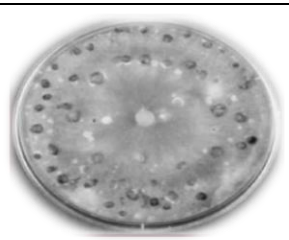
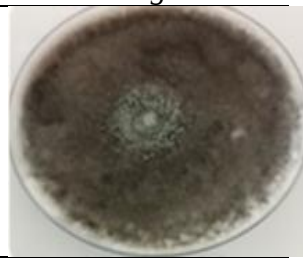
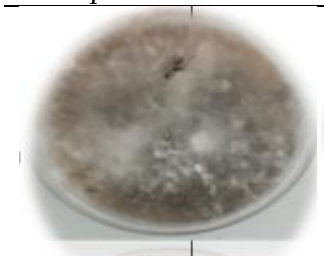
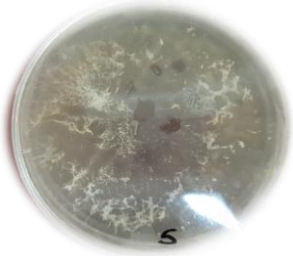
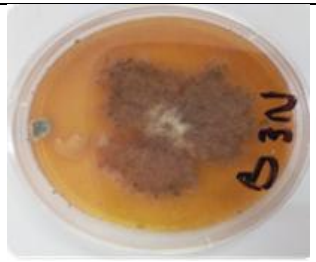
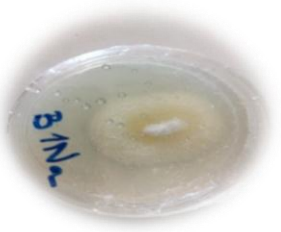
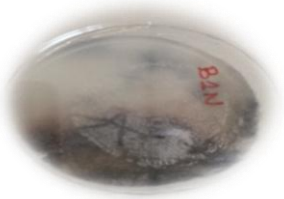
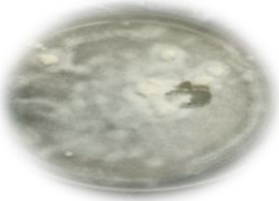
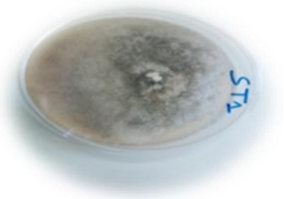
F. oxysporum	*F. graminearum*	*F. proliferatum*	*F. poae*	*F. sp1*	*F. sp 2*



F. culmorum	*Microdochium nivale*	*Penicillium notatum*	*P. chrysogenum*	*P. aurantiogriseum*	*P.expansum*

Not identified

					
P. digitatum	*P. sp 1*	*P. sp 2*	*P. sp 3*	*P. sp 4*	*P. sp5*
					
P. sp 6	*P. sp7*	*P. sp8*	*P. sp9*	*Purpureocillium lilacinum*	*Purpureocillium sp1*
					
Purpureocillium sp2	*Purpureocillium sp3*	*Rhizoctonia sp1*	*R. sp2*	*R. sp 3*	*R. sp4*
					
Cladosporium herbarum	*Cladosporium cladosporioides*	*Acremonium sp*	*Acremonium murorum*	*Alternaria alternata*	*Alternaria triticina*

					
Alternaria sp1	*Rhizomucor sp*	*Helminthosporium sp*	*Geotrichum candidum*	*Rhizopus stolonifer*	*Absidia glauca*
					
Chaetomium tetraspermum	*Rhizopus oryzae*	*Trichoderma viride*	*Sclerotina sclerotorum*	*Ulocladium sp*	*Not Identified*
					
Not Identified	*Not Identified*	*Not Identified*	*Not Identified*	*Not Identified*	*Not Identified*
					
Not Identified	Not Identified	Not Identified	Not Identified	Not Identified	Not Identified

The mycological analysis revealed a wide range of fungi. Indeed, 80 strains were discovered, of which 53 identified strains (Tab 3) belonging to 18 different fungal genera *Fusarium, Alternaria, Aspergillus, Penicillium, Cladosporium, Rhizopus, Absidia, Rhizoctonia, Trichoderma,* Microdochium, *Purpureocillium, Acremonium, Rhizomucor, Helminthosprium, Geotrichum, Sclerotina, Ulocladium and Chaetomium*. Identification for some fungal strains reached the "genus" stage only and some strains could not be identified.

Table 3. Identified fungal isolates

N°	Species	N°	Species
1	*Aspergillus niger*	28	*P.sp9*
2	*A.flavus*	29	*Purpureocillium lilacinum*
3	*A.ochraceous*	30	*Purpureocillium sp1*
4	*A.terreus*	31	*Purpureocillium sp2*
5	*A. brasiliensis*	32	*Purpureocillium sp3*
6	*Fusarium* oxysporum	33	*Rhizoctonia sp1*
7	*F. poae*	34	*R. sp2*
8	*F. graminearum*	35	*R. sp3*
9	*F. solani*	36	*R. sp4*
10	*Microdochium nivale*	37	*Cladosporium cladosporioides*
11	F. proliferatum	38	*Cladosporium herbarum*
12	*F. culmorum*	39	*Acremonium murorum*
13	F. sp1	40	*Acremonium sp*
14	F. sp2	41	*Alternaria alternata*
15	*Penicillium chrysogenum*	42	*A. triticina*
16	*P. notatum*	43	*Alternaria sp*
17	*P. aurantiogriseum*	44	*Rhizomucor sp*
18	*P. expansum*	45	*Sclerotina sclerotorum*
19	*P. digitatum*	46	*Ulocladium sp*
20	*P.sp1*	47	*Chaetomium tetraspermum*
21	*P.sp2*	48	*Rhizopus stolonifer*
22	*P.sp3*	49	*Rhizopus oryzae*
23	*P.sp4*	50	*Trichoderma viride*
24	*P.sp5*	51	*Absidia glauca*
25	*P.sp6*	52	*Geotrichum candidum*
26	*P.sp7*	53	*Helminthosporium sp*
27	*P.sp8*		

9.1.3.2. Centesimal frequency

The identified fungal strains (Fig 9) reveal a dominance of the genus Penicillium with the highest frequency of appearance of 29.85%. This one contains 13 species of which 8 species are only identified at the rank of genus. Those identified are *P. chrysogenum, P. notatum, P. aurantiogriseum, P. expansum* and *P. digitatum.* The Fusarium genus, in turn,

comes in second position with an appearance rate of 15.49%. This genus contains 6 identified fungal species which are *Fusarium oxysporum*, *F. poae*, *F. graminearum*, *F. solani*, *F. proliferatum* and *F. culmorum* and only 2 unidentified species. However, both Aspergillus and Rizoctonia have a similar frequency of appearance of 7.69%. The first genus contains 5 identified species, while the second is composed of 4 identified species to the rank of the genus. They are respectively *Aspergillus niger*, *A. flavus*, *A. ochraceous*, *A. terreus* and *A. brasiliensis*, as well as 4 species of Rizoctonia.

The centesimal frequency of the genus Purpureocillium is 5.77%. It contains 4 fungal species, one of which is identified, namely *Purpureocillium lilacinum*. The Alternaria genus, for its part, recorded a rate of 4.68%. It contains 3 fungal species, two of which are identified; these are *Alternaria alternata* and *A. triticina*. The first group of fungal genera Cladosporium, Acremonium and Rhizopus recorded a similar centesimal frequency of 3.85%. They contain 2 fungal species for each genus. The second group is composed of 9 fungal genera, with a similar frequency of 1.29%. They are only represented by single species namely *Absidia glauca*, *Geotrichum candidum*, *Helminthosporium sp*, *Trichoderma viride*, *Sclerotina sclerotorum*, *Ulocladium sp*, *Chaetomium tetraspermum*, *Rhizomucor sp* and *Microdochium nivale.*

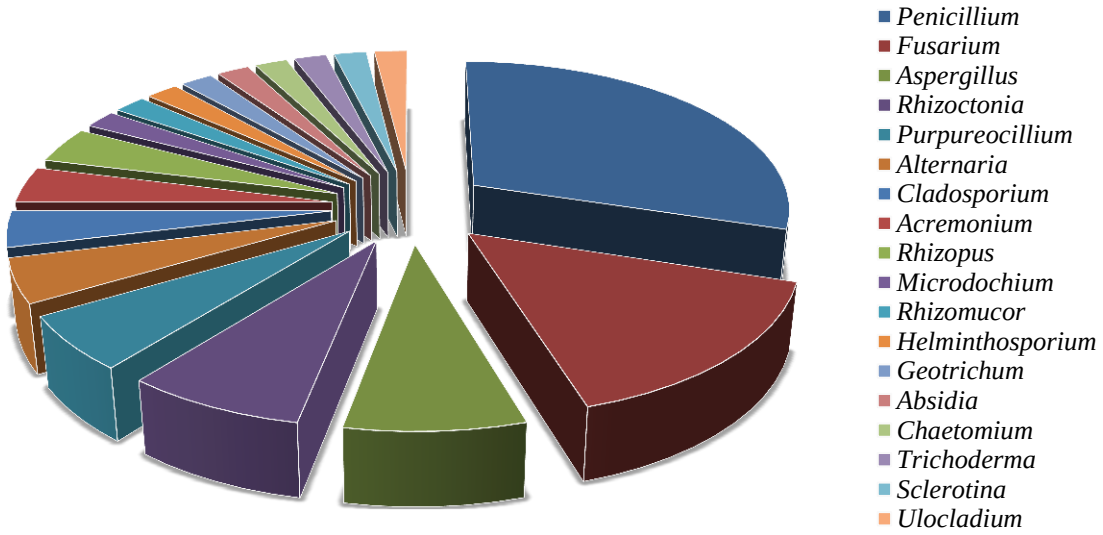


Figure 9. Frequency rates of different fungal genera isolated

The fungal taxa listed in this study are represented by 3 phyla: Ascomycota, Basidiomycota, and Zygomycota (Tad 4). However, the Sordariomycetes class dominates with 5 fungal species associated with durum wheat, followed by the Dothideomycetes class

with 4 species. The phylum Zycomycota is represented by 3 fungal species, on the other hand the Basidiomycota are represented by only 2 fungal species.

Table 4. Taxonomy of identified fungal strains

Phylum	**Class**	**Family**	**Genus**
Ascomycota	Eurotiomycetes	Trichocomaceae	Aspergillus
			Penicillium
	Sordariomycetes	Nectriaceae	Fusarium
		Amphisphaeriaceae	Microdochium
		Ophiocordycipitaceae	Purpureocillium
		Hypocreaceae	Trichoderma
		Chaetomiaceae	Chaetomium
	Dothideomycetes	Davidiellaceae	Cladosporium
		Pleosporaceae	Alternaria
		Massarinaceae	Helminthosporium
		Pleosporaceae	Ulocladium
	Leotiomycetes	Sclerotiniaceae	Sclerotina
	Saccharomycetes	Dipodascaceae	Geotrichum
Basidiomycota	Agaricomycetes	Ceratobasidiaceae	Rhizoctonia
		Hypocreaceae	Acremonium
Zygomycota	Zygomycetes	Mucoraceae	Rhizomucor
			Rhizopus
		Cunninghamellaceae	Absidia

9.2. Discussion

This part of our study deals on one hand with the state of wheat grains depending on the variety and the conditions regulating fungal growth in these stored grains. On the other hand, it concerns isolation and identification of fungal species associated with wheat. Grain quality depends largely on the grain type and its end use. It includes a range of properties that can be defined in terms of physical (moisture content, total damaged kernels, broken kernels, breakage susceptibility), sanitary (fungi and mycotoxin count, insects, toxic seeds, pesticide residue, odor, dust), and intrinsic (oil content, protein content, hardness, feed value, viability, storability) quality characteristics.

The results given on the physicochemical quality of stored wheat grains indicate that the samples analyzed contain a percentage of broken grains higher than the percentage set by the International Food Standards for Durum Wheat (CODEX ALIMENTARIUS) (2019), which require that the quality of wheat does not exceed a percentage of 6% broken grains. This increase in the number of broken grains could be explained by several factors that could influence the state physics of the wheat grains particularly that of the local area, such as poor

harvest conditions, the characteristics of each variety, the mechanical failures of the devices and especially the shocks inflicted on grains during mechanical transport to silos. We have to notice that the wheat seeds of Antalis and Prospero have large seed size than the seeds of other varieties. The presence of broken grains can only encourage the development of contaminant sources and therefore, affect the quality of stored grains. pH is one of the most important predictors of fungal richness. Specific fungal strains typically have a wider pH optimum, often covering a range of 5–9 pH units without significant inhibition of growth (Bahram *et al*., 2018). Our results confirm that the samples in the present study constitute a favorable environment to the development of fungi.

In this context, we must recall that in our study we used 3 varieties of wheat treated with a fungicide. It has been proven that fungicides deleted some species and did not affect the development of others (Rozhkova *et al.,* 2021). However, we want, first, to see the effect of fungicides on the growth and development of sprouts. Our results show that treated seeds have slow growth compared to untreated seeds. These results suggest that applying fungicides on wheat seeds, causes inhibition and/or toxicity leading to a reduction in germination and growth parameters of our varieties. The treatment of seeds with a fungicide could be a kind of stress (Mamenko et Kots, 2022). Our results are similar to those obtained by Hoose *et al.* (2022) using fungicide seed coatings and also Chibis *et al.* (2019), on wheat treated with 4 fungicides Komfort, AltSil, Terrasil and Alcasar. In general, each wheat varieties have precocity of germination specific to its nature, because each one has its own germination requirements (Penfield, 2017). This is how among the varieties of seeds wheat experienced, only Ordinaire has the best germination

The following approach can be considered the most important part of the study. It consists of isolating and identifying the mycoflora associated with different varieties of seeds wheat (treated and untreated), as well as from infected plant parts. First of all, it should be noted that storage period and poor sanitary conditions can strongly contribute to the dispersal of fungal spores, thus causing grain contamination (Smiri *et al.,* 2021). Mycological analysis revealed the presence of 80 pathogenic fungal species of which only 53 were identified, belonging to 18 different fungal genera *Fusarium, Alternaria, Aspergillus, Penicillium, Cladosporium, Rhizopus, Absidia, Rhizoctonia, Trichoderma, Microdochium, Purpureocillium, Acremonium, Rhizomucor, Helminthosporium, Geotrichum, Sclerotina, Ulocladium and Chaetomium*. In general, the fungal community present in stored wheat seeds can contain numerous saprophytic, endophytic and pathogenic species, which has been

confirmed by previous studies of Al-Bedak *et al*. (2020) and Kesho *et al*. (2020). The data on fungal contamination obtained in the current study revealed the presence of various storage and field fungi. Some of the mold species come also from the soil (Emin *et al.*, 2022). Typical examples of the field fungi (that occur on the grain until harvest) are the species belonging to genus Alternaria, Cladosporium, Microdochium, Trichoderma, Helminthosporium and Fusarium (Felšöciová, *et al*., 2021). Molds from genera Aspergillus and Penicillium predominate in the group of storage fungi (occurring after harvesting) (Emin *et al.*, 2022). Research shows that the major mycotoxigenic fungi in a variety of wheat seeds are *Aspergillus sp., Fusarium sp*., and *Penicillium* sp., which are capable of producing a wide range of mycotoxins (Taniwaki *et al*., 2018). It is, therefore, possible that *Aspergillus niger* and *Penicillium spp*. were carried in contaminated grain from the field to storage through contact with soil at harvest (Aklaku *et al*., 2020). They have been reported to reduce the seed germination and seed loss during storage (Jamadar et Chandrashekhar, 2015). Recent literature demonstrate that fungal genera are usually found in grain stored in the form of spores, in accurate quantities during transportation and storage and could be survive for several years (Ulziljargal *et al*., 2019). Among the fungal diversity isolated from wheat seeds and plant parts such as the dominant genera Fusarium, Penicillium, and Aspergillus: we found that 9 genera have low frequencies as Helminthosporium, Chaetomium, and Geotrichum. This finding are supporting by Minati et Mohammed-Ameen (2020). Overall, fungi belonging to Ascomycota were in a dominant position. However, many previous studies conducted by Minati et Mohammed-Ameen (2020); Sui *et al.* (2022) and Sun *et al*. (2020) indicated the same conclusion. After analyzing wheat samples, Penicillium was determined to be the most common species of molds. Our results are in agreement with the finding of Al-Nash (2023), Emin *et al.* (2022) and Felšöciová *et al*. (2021). However, the most occurring airborne spores belonging to *Penicillium*, *Aspergillus*, and *Alternaria* have been demonstrated as the most widespread fungi detected (Jean Phellipe *et al*., 2019).

Aspergillus genera are common in warm areas and have a high ability to adapt to high temperatures that rise during the summer season, as well as its capacity to produce masses of conidia (In Minati et Mohammed-Ameen, 2020). In addition, the presence of *A. flavus* can result in the reduction of grain quality and quantity and the production of toxic secondary metabolites (Mohapatra *et al.*, 2017). Aflatoxin produced by Aspergillus species in cereal grains both in the field and in storage (Lei *et al.*, 2023). The high frequency and abundance of Penicillium and Aspergillus in our study could be due to inadequate farming practices,

poor quality feed, and poor storage conditions. This situation becomes even more complicated when climatic conditions are favorable for the development of mold.

In the present study, the grains and plant parts of the analyzed wheat varieties were colonized mainly by *Fusarium oxysporum, F. poae, F. graminearum, F. solani, F. proliferatum and F. culmorum*. According to the research, *F. graminearum* and *F. culmorum* are considered to be the most common (Leslie *et al*., 2021). *Fusarium spp* are saprophytes, plant pathogens of a variety of hosts including wheat, and the most mycotoxigenic species. Alternaria and Cladosporium are broadly distributed fungal genera including saprophytic, endophytic and pathogenic species. They are well adapted to be spread easily over long distances, they are cosmopolitan and widely present in all various types of plants, mostly isolated from air, soil, seeds, grains, food, paint, textiles and other organic matter (Yehia *et al*., 2020).

Conclusion

The data obtained in the present study indicate that wheat grains during storage or in the field can be attacked by molds. In fact, broken grains are more easily invaded by fungi. The present study contributes to the knowledge of the mycobiota of wheat, which is involved in the presence of numerous saprophytic, endophytic, pathogenic and toxigenic fungi. Our fungal inventory consists of 53 species belonging to 18 genera, with a dominance of *Penicillium, Aspergillus* and *Fusarium.*

CHAPTER 2:
MICROBIAL ACTIVITIES OF PLANT-BASED PRODUCTS

CHAPTER 2: Microbial activities of plant-based products

Introduction

The use of plants for their beneficial effects dates back to the first steps in human history. Plants are the most important source of bioactive molecules. According to Van Wyk (2015), more than 5400 plant species are used in traditional medicine in Africa, but only 10% have been commercially developed to some extent. Today, all cultures around the world have extensive knowledge of plant medicine because of accumulated experience from previous generations. Plants are a rich source of secondary metabolites, which are used in pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides (Al-Snafi, 2022). Those metabolites, also known as phytochemicals, can be found in various parts of plant, including stems, leaves, roots, seeds, fruits, and flowers.

Plant extracts have raised a big interest for their implication in traditional medicine due to their biological properties such as anti-inflammatory, antimicrobial, antiallergic, antiviral, anticancer, antimutagenic and anti-oxidant activities (Leite *et al*., 2019; Sadh *et al.*, 2018). Essential oil, an important category of plant extracts, has a multidirectional action mode and a variety of biological activities (Wojtunik-Kulesza *et al*., 2019). Essential oils (EOs), mostly aromatic and naturally occurring volatile organic compounds throughout all regions of the plant such as seeds, flowers, peel, stem, bark and the whole plants (Bhavaniramya *et al*., 2019). Nowadays, plant extracts are considered to be the most pressing sources of biomolecules, which can be screened from plant parts. They refer to products formed through an extraction and separation process where plants are used as raw materials. Recently, the interest in plant extracts and EOs produced by medicinal and aromatic plants has focused on their biocontrol potential against plant pests and diseases. The growing number of studies related to the herbicidal, insecticidal, acaricidal, nematicidal and antimicrobial effects of plant-based products demonstrate their effectiveness and suitability as sustainable and environment-friendly biopesticides.

1. Definition and concept

Plant extracts (PEs) and Essential oils (EOs) have been continuously used as a source of bioactive molecules that have potential applications in traditional medicine, food, perfumery and cosmetics due to their unique properties (Jugreet *et al*., 2020). The main difference

between essential oils and extracs is the process. While both are extracted from different parts of the plant, the process is very different. EOs needs to be extracted through distillation, while extracts are soaked in a liquid to isolate the flavour. Both of them are secondary metabolites and most of oil formed PE are commonly called essential oils, which are mixed oil compounds with variable chemical compositions and concentrations.

- Medicinal plant extracts, 'plant extract', 'botanical extract', 'herb extract' or 'medicinal plant extract' are secondary plant metabolites and refers to an active natural chemical compound that is formed through an extraction and separation process where plants are used as raw materials (dry or fresh plant) using an appropriate solvent and standard extraction procedure. Generally the active compounds of the plant are soluble in water or oil and the original components of the plants are not changed. Extracts may be derived from whole plants or from specific parts of plants such as leaves, stems, barks, roots, flowers, and/or fruits and may be either "total extracts" or "selective extracts" with final choices based on available evidence of efficacy. Each extract may contain hundreds of different chemicals, in liquid, semi-solid or dry powder form and within the complex mixture, individual chemicals such as vincristine, vinblastine, hyoscyamine, hyoscine, pilocarpine, forskolin and codeine, may have performance characteristics which differ from their properties when in isolation even at comparable concentrations (Atanasov *et al*., 2021). Plant extracts usually contain phytochemical, compounds, and macromolecules to serve as an essential bioactive compound source for numerous programs related to drug discovery.
- Essential oils are also plant extracts. Generally, plants can synthesize two kinds of oils: fixed and essential oils. Fixed oils are triacylglycerols or triglycerides. Essential oils, also known as essences, volatile oils, etheric oils, ethereal oil (Aramesh et Ajoudanifar, 2017) or aetheroleum, are complex natural mixtures of volatile, lipophilic, and odoriferous substances commonly found in aromatic plants. Essential oils are major complex compounds of aromatic terpenes, isoallyl and allyl phenols, and related esters produced in specialized secretory tissues in plants (Sharifi-Rad *et al.*, 2021). The oil secreting glands are located in fruits, flowers, seeds, wood, leaves, roots, barks and sometimes present throughout the plant (Naeem *et al*., 2018). The majority of essential oils are colorless or pale yellow, liquid at room temperature, and less dense than water, with very few exceptions (cinnamon, sassafras, and vetiver). The oil bears the name of the plant from which it is derived; for example, citrus oil or lavender oil. Such oils were called essential because they were thought to represent the very essence of odour and flavor. Due to their volatility, they can easily be extracted by

different method from different natural sources. Choice of extraction method depends upon the characteristics and components needed for the purposes. Various organs of aromatic plants are utilized to distill EOs, for example, seeds (Caraway, Cumin, and Coriander), leaves (Mint, Thyme, Sage, Rosemary, Oregano, Basil, Celery, and Parsley), fruits (Anise, Fennel, and Lemon), flowers (Rose and Rosemary), bark (Cinnamon), cloves or buds (Clove and Garlic), and rhizomes (Ginger).

2. Chemical constituents of plant extracts

Plant extracts and EOs contain a varied range of chemicals (secondary metabolites) such as terpenoids, phenolic compounds, nitrogen-containing compounds, and various organic acids (Hussein et El-Anssary, 2018). These chemicals are responsible for their unique nature and perceived biological activity of plant extracts. The phytochemical composition of EOs is generally less complex than that of extracts generated by solvent extraction. While extracts typically contain several thousand different natural compounds, EOs are limited to a few dozen. Phytochemical screenings of plant extracts are preliminary tests conducted to detect the presence of bioactive compounds or secondary metabolites (tannins, saponins, flavonoids, alkaloids, phenols, glycosides, steroids and terpenoids) in an extract according to standard protocols (Lawal *et al.*, 2019) (Fig 10). The plant extracts basically consist of two classes of compounds, the terpenes and phenylpropenes (Bakkali *et al*., 2008).

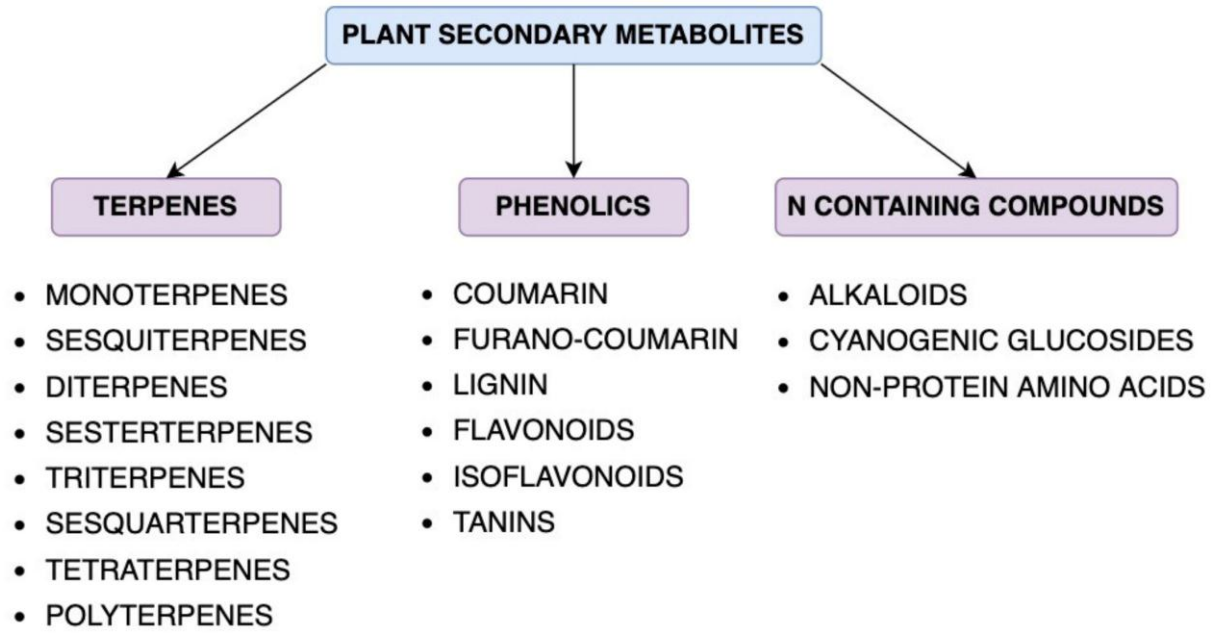


Figure 10. The major secondary metabolites in plant extract (Twaij et Hasan, 2022)

Among the secondary metabolites, EOs are the most studied and are of great commercial importance. EOs are complex mixtures of volatile organic compounds, which are usually composed of more than 500 chemicals (Liang *et al*., 2023). Their composition can vary within the same botanical genus, and such variations can also be observed within the same species. Thus, some chemicals are found in a variety of plant EOs, such as camphene and linalool. Therefore, the characteristic odor of an essential oil directly depends on its chemical composition. Some substances are unique to some EOs, such as menthol and camphor. In general, essential oils are composed of numerous components at different concentrations, but some of them may contain more different substances. However, two or three components are usually present in large proportions (20–70%) compared to other constituents present in small concentrations (Chouhan *et al*., 2017). For example, 1,8-cineole or eucalyptol is the major component(70–90%) of *Eucalyptus globulus* Labill. EO (Boukhatem *et al*., 2020). Typically, the major components of essential oils are the main components responsible for their biological properties and provide the typicality of its smell (Dhifi *et al.*, 2016). Nevertheless, minor compounds may also play an important role in bioactivity, either by potentiating the action of major components or through antagonistic or additive effects (Perricone, 2015).

Generally, pure essential oils can be subdivided into two distinct chemical classes: terpenes and phenylpropanoids. Furthermore, terpene compounds can be divided into two main categories: (1) hydrocarbons, mainly the mono-, sesqui-, and diterpenes; and (2) their oxygenated compounds, for instance, alcohols, oxides, aldehydes, ketones, phenols, acids, esters, and lactones (Angane *et al.*, 2022); (Wani *et al.*, 2021). Terpenes (pinene, myrcene, limonene, terpinene, *p*-cymene) are a class of natural products found in EOs, constituting of numerous isoprene units (C_5H_8) merging to form a hydrocarbon molecule, while terpenoids (oxygen-containing hydrocarbons) are defined as modified class of terpenes with different functional groups and oxidized methyl groups moved or removed at various positions (Perveen, 2018). The chemical structures of the major constituents of EOs are depicted in Figure 11.

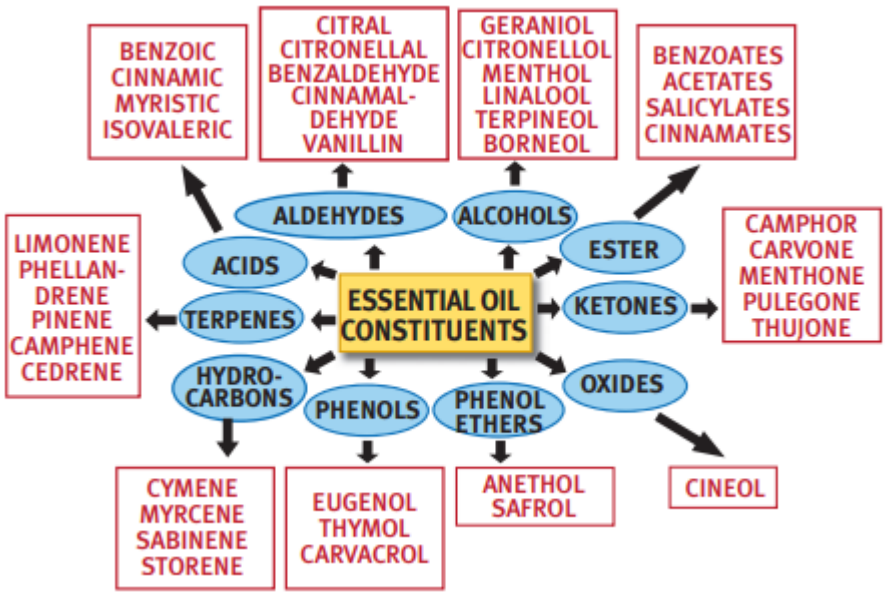


Figure 11. Heterogeneous chemical groups present in EO (Handa *et al*., 2008)

3. Methods used in extraction of medicinal plants

Extraction is the most important first crucial step in any natural product research. This involves the separation of medically active parts of plant tissues using selective solvents following standard procedures. Such extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular marc. The products thus obtained are relatively complex mixtures of metabolites. These include classes of preparations called decoctions, infusions, fluid extracts, tinctures, pill extracts, or powdered extracts. Such preparations are commonly called galenics. The resulting extract contains a complex mixture of numerous medicinal plant metabolites, such as alkaloids, glycosides, terpenoids, flavonoids and lignans. In order to be used as a modern medicine, an extract can be further processed by various fractionation techniques to isolate individual chemical entities such as vincristine, vinblastine, pilocarpine, forskolin and codeine.

Solvents used for the extraction of bioactive molecules from plants are chosen based on the polarity of the solute of interest. Multiple solvents can be used sequentially in order to limit the amount of analogous compounds in the desired yield. The polarity, from least polar to most polar, of a few common solvents is as follows: Hexane < Chloroform < Ethylacetate < Acetone < Methanol < Water (Altemimi *et al*., 2017).

Several methods were used in the extraction of medicinal plants (Fig 12) such as maceration, infusion, decoction, percolation, digestion and Soxhlet extraction, superficial extraction, ultrasound-assisted, and microwave-assisted extraction. In addition, thin-layer

chromatography (TLC), high-performance liquid chromatography (HPLC), paper chromatography (PC), and gas chromatography (GC) were used in separation and purification of the secondary metabolites (Ingle *et al.*, 2017). Generally, there are two main steps that are applied to analyze plant-extracts: (1) extraction/distillation, which takes several hours, and (2) chemical analysis, which takes several minutes. The choice of an appropriate extraction method depends on the nature of the plant material, the solvent used, the pH of the solvent, the temperature and the solvent-to-sample ratio (Ingle *et al.*, 2017).

3.1. Conventional extraction methods

- In the **maceration** process, the whole or coarsely pulverized crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble material has dissolved. The mixture is then clarified by filtration or decantation after standing. This technique is used for the extraction of thermolabile compounds, which require maceration in warm water to release their EOs, such as chokeberry, and strawberry (Zhang *et al*., 2018).
- This extraction process is called **digestion,** if moderate heat is used to decrease the viscosity of extraction solvent and enhance the removal of secondary metabolites. This method is suitable for plant materials that are readily soluble (Ingle *et al.*, 2017).
- Fresh **infusions** are prepared by macerating the crude drug for a short time with cold or boiling water. This method is suitable for extraction of easily soluble bioactive constituents. Additionally, it is a suitable method for preparing fresh extract before use. The solvent to sample ratio is typically 4:1 or 16:1 depending on the intended use (Ingle *et al.*, 2017).
- In **decoction**, the crude drug is boiled in a specified volume of water for a defined time (usually about 15 min); it is then cooled and filtered. This process is suitable for the extraction of water-soluble and heat-stable constituents. The ratio of solvent to crude drug is usually 4:1 or 16:1 (Ingle *et al.*, 2017).
- **Percolation** is the most frequently used procedure to extract active ingredients in the preparation of tinctures and fluid extracts.
- **Distillation methods** are represented by three types: hydrodistillation, direct steam distillation (Khan et Dwivedi, 2018), and water and steam distillation methods. Hydrodistillation is the simplest and oldest method of oil extraction, which includes a heating source, vessel, condenser, and a decanter to collect the condensate. The process involves complete immersion of the material directly into boiling water. The oil released from the oil

glands/ducts/cells in the plant tissue due to the influence of boiling water and steam, and indirect cooling with water condenses the vapor mixture of water and oil.

➢ **Soxhlet extraction** is an exhaustive extraction technique widely applied to bioactive compounds from many natural sources that are sufficiently thermally stable. The extraction is carried out in a special device called a Soxhlet apparatus. The extraction solvent flows continuously through the matrix, boiling and condensing, with the sample collected in the hot solvent. To obtain a satisfactory yield from this technique as well as to avoid the loss of volatile compounds, the right choice of solvent is imperative. The extraction period is generally long, thus leading to the destruction of certain heat-labile compounds (Wong *et al*., 2014).

3.2. Alternative extraction methods

➢ **Supercritical fluid extraction** is the most modern and sophisticated. It uses carbon dioxide (CO_2) at their supercritical stage. Many solvents can be used as the method brings the solvent at its temperature and pressure above its thermodynamic critical point. This method is based on the fact that gas at the supercritical state can enter throughout the plant material like a gas and dissolve component like a liquid. After the extraction procedure, the EOs compounds are mixed with the supercritical fluid (in liquid form). The separation is performed by reducing temperature and increasing the pressure up to room conditions (Chenni *et al.,* 2016).

➢ **Microwave-assisted extraction** is a variant of the distillation method where the heating source has been changed from the normal electric heating cap by the microwave. In this method, materials are immersed in a solvent and exposed to microwave energy. As the materials are heated up, high pressure is generated within the cell walls of the materials. The high-pressure build-up and severe thermal stress within the glands cause swelling, stretching, and rupture of the cell walls, which facilitates the release of constituents. However, this method requires a larger quantity of organic solvent and is not considered environment friendly compared to the Supercritical fluid method.

➢ **Ultrasound-assisted extraction (UAE)** is a simple, efficient, and inexpensive method that uses ultrasonic wave energy for extraction. The extraction mechanism involves cavitation by the solvent, heat transfer through the cell walls, and breakdown of microscopic bubbles. The cavitation effect is due to the passage of ultrasonic waves, which can lead to cell destruction and improve the extraction rate of EOs in a shorter time. UAE is considered one of the more suitable methods for the extraction of thermolabile, unstable, and other natural products (Chemat *et al.,* 2017).

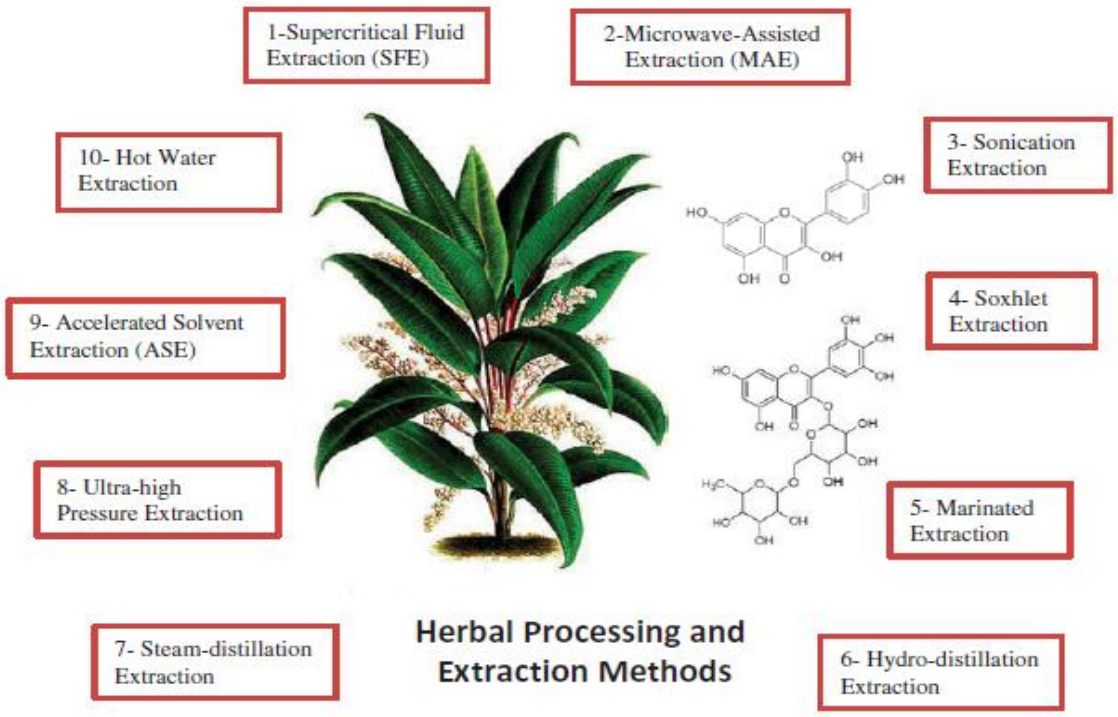


Figure 12. Different techniques for extracting plant metabolites (Mohammad Azmin *et al.*, 2016)

4. Identification and characterization

Phytochemical screenings are preliminary chemical tests conducted to detect the presence of both primary and secondary metabolites in an extract. Several qualitative analyses have been used to detect the presence of alkaloids, flavonoids, tannins, saponins, flavones, sterols, terpenes, cardiac glycosides, protein, carbohydrates, and fats using Standard methods (Tab 5).

Since plant extracts usually occur as a combination of different types of phytochemicals with different polarities, their separation still remains a major challenge for the identification and characterization process. It is common to isolate these bioactive compounds to use a number of different separation techniques such as Thin Layer Chromatography (TLC), Column Chromatography (CC), Flash Chromatography, Sephadex chromatography and High Performance Liquid Chromatography (HPLC). The pure compounds isolated are then used for the determination of structure and biological activity. Beside that, non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (MAbs), Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds.

Table 5. Some phytochemical screening tests of a plant extract (from literature)

Group	Test
Alkaloids	Dragendoff's reagent, Wagner's reaction, Mayer's reaction, Hager's reaction, Marme's reaction, Kraut's reaction, Scheibler's reaction, Tannic acid reaction, Reineckate salt reaction, Sonnenschein's reaction.
Flavonoids	Lead acetate, Alkaline reagent test (NaOH), Shinoda test, H_2SO_4 test
Terpenoids	Salkowski's test
Phenols	Ferric chloride test, Lead acetate test
Saponnins	Frothing test
Tannins	Ferric chloride, Lead subacetate
Balsam	Balsam
Resins	Copper acetate, Acetate/H_2SO_4
Anthraquinone	Borntrager's test

5. Biological activities of plant-extracts

Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. Natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have served as the basis for the development of new lead chemicals for pharmaceuticals. EOs and extracts are of great importance in various industries due to the presence of large amounts of volatile, aromatic, and bioactive compounds (Samadi *et al*., 2021). Furthermore, their biological activities have been studied, such as antitumor, antioxidant, antimicrobial, antiviral, antimutagenic, antidiabetic, anticarcinogenic, and anti-inflammatory activities (Guo *et al*., 2019; Karpinski, 2020) and play an essential role in the pharmaceutical, food, agricultural, cosmetic, and health industries (Aćimović *et al*., 2022).

5.1. Antibacterial activity

The antibacterial activity of plant-extracts mainly depends on their chemical structures, principal constituents and effective dose (Elshafie *et al*., 2023). Their powerful antimicrobial capabilities are commonly affected by the presence of some ingredients including terpenoids (aliphatic alcohols, aldehydes, ketones, acids) and some phenolic compounds (isoflavonoids). The antibacterial activity varies greatly among plant species, and also, in the case of each plant type, it may fluctuate depending on the type of solvent used for extraction and the genus of bacteria tested (Manilal *et al*., 2020). The possible mechanisms wherein EOs interfere with bacterial proliferation may involve the following Figure (13): (1) the disintegration of the bacterial outer membrane or phospholipid bilayer, (2) alteration of the fatty acid composition, (3) increase in membrane fluidity resulting in leakage of potassium ions and protons; (4)

interference with glucose uptake, and (5) inhibition of enzyme activity or cell lysis (Cho *et al.*, 2020). Antibacterial action kinetics may achieve values that i) only inhibit the bacterial growth (bacteriostatic) or ii) may be used at either high concentrations or are inherently more aggressive and their action results in a decline in the number of bacterial cells (bactericide). Based on the results of some previous studies, plant-extracts, especially EOs are more active against Gram-positive bacteria than Gram-negative bacteria (Moghaddam *et al*., 2018).

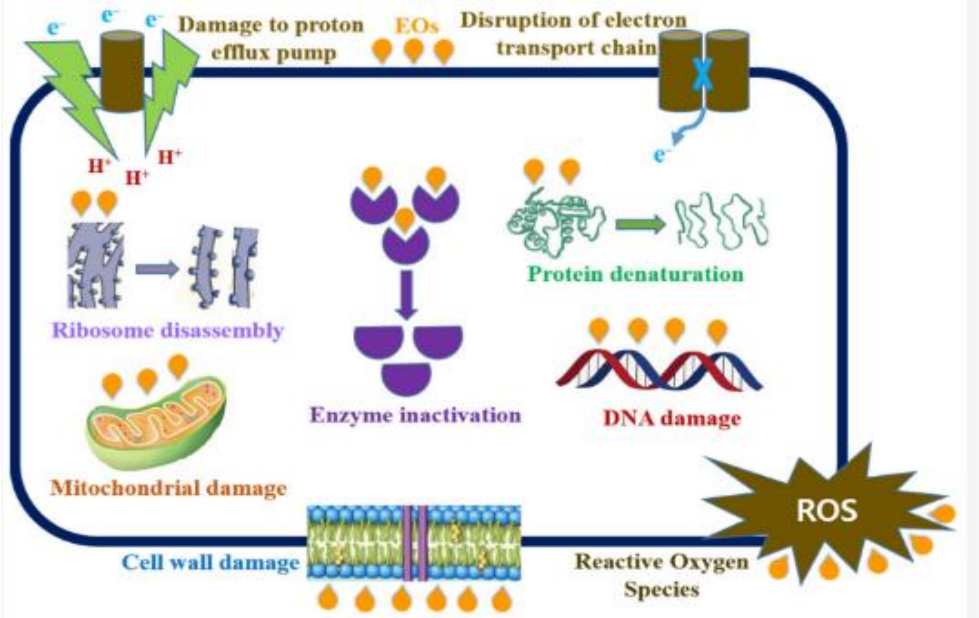


Figure 13. Proposed mechanism of antibacterial action of EOs (Basavegowda et Baek, 2021)

5.2. Antifungal activity

Different aromatic plant-extracts and essential oils showed potent antifungal effects against different pathogenic fungi, including yeasts. The antifungal activity of plant extracts depends mainly on the content of phenols, terpenes and alkaloids (Acheuk *et al*., 2022). Plant-extracts and EOs act on fungal cell structures in a manner similar to that described for their antibacterial activity: they cause changes in functions that are essential for microbial survival. The cell wall of fungi may be considered as the prime target for selectively toxic antifungal agents because of its chitin structure, which is absent in human cells. The antifungal activity of EO might be caused by the properties of terpenes/terpenoids that due to their highly lipophilic nature and low molecular weight are capable of disrupting the cell membrane, causing cell death or inhibiting the sporulation and germination of fungi. Additionally, many plant bioactive metabolites had been confirmed to show antifungal activity or greatly increase the antifungal action of existing antifungal drugs by synergistic action (Loi *et al.*, 2020).

According to Freiesleben and Jager (2014), the antifungal agents can deactivate the fungus by disrupting the structure and function of membranes or organelles of fungal cell and/or inhibiting the nuclear material or protein synthesis (Fig14). The effect of thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum zeylanicum*), rosemary (*Rosmarinus officinalis*) and marjoram (*Origanum majorana*) essential oils a synergism of their possible double and triple combinations, on *Penicillium expansum* and *Botrytis cinerea* (Nikkhah *et al.*, 2017). The strength of the biocidal effect of plant extracts varies and depends on the type of extract and the microorganism on which it acts. Literature data indicate that the chemical composition of plant extracts varies depending on the plant species and the sampling site (Nazzaro *et al.*, 2017).

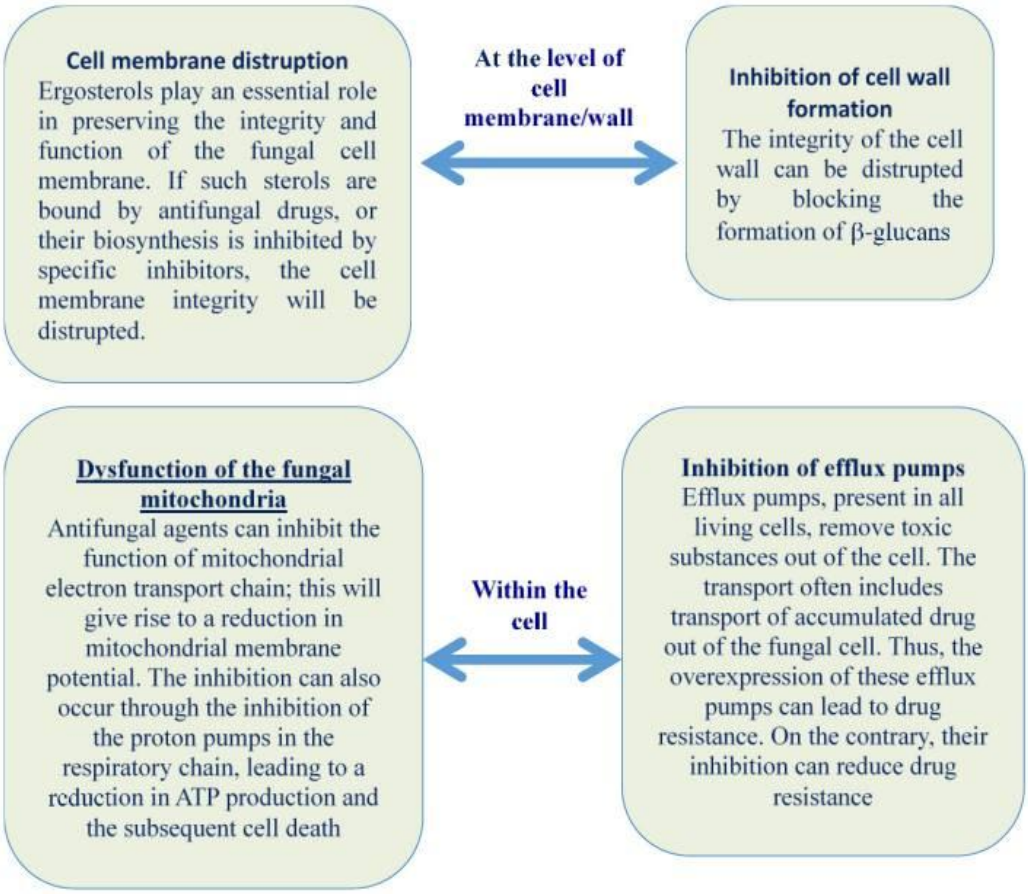


Figure 14. EOs action against fungi (Freisesleben et Jager, 2014)

5.3. Antioxidants activity

From a chemical point of view, antioxidant activity is defined as the ability of a given compound, present in small amounts, to protect an easily oxidizable material from oxidation (Amorati et Valgimigli, 2018). Some EOs have an important role in reducing oxidative stress and often used to prevent several chronic diseases. DPPH (2,2-diphenyl-1-

picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (ferric ion reducing antioxidant potential) and FC (Folin-Ciocalteu), are radical compound which can assist in the measurement of antioxidant activity through color change. The extra reactive oxygen species and excess free radicals produce a case of oxidative stress, in which different macromolecules are damaged in the cell, causing numerous health ailments such as aging, cancer, diabetes, arteriosclerosis, and Alzheimer's disease (Hao *et al*., 2021). Oxidative stress is a phenomenon caused by the interaction, production, and accumulation of oxidative free radicals and associated ROS, such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. Among the main constituents of EOs, terpenoids, sulfur-containing components, and phenolic compounds such as carvacrol, thymol, eugenol, and linalool, possess the highest scavenging activity of EOs (Golmakani *et al*., 2018). The majority of natural antioxidants are phenolic and terpenolic compounds with the most important groups being flavonoids, tocopherols, and phenolic acids (Ju et *al*., 2022). Aqueous tea extract is one of the most common natural antioxidants used in the food industry due to its rich content of catechins, tannins and flavonoids without affecting the food flavor, as reported by Novilla *et al.* (2022).

5.4. Anti-Inflammatory activity

Recent decades have shown that terpenes and terpenoids are physiologically important to alleviate various symptoms caused by inflammation, most likely by inhibiting multiple pathological steps in the inflammatory process (Kim *et al.*, 2020). Inflammation is a protective response of the host to non-self objects, usually generated by microbial infection and/or tissue damage. Dysregulation of inflammatory responses can lead to acute and chronic inflammatory diseases that cause excessive or long-lasting tissue damage (Chen *et al*., 2017). The application of terpenes and terpenoids to alleviate inflammation has been shown successful in the mitigation of respiratory inflammation, atopic dermatitis, arthritis and neuroinflammation (Kim *et al.*, 2020). Several studies have revealed biological applications of EOs in controlling the inflammatory response, based on their use in traditional medicine. A good anti-inflammatory propreties have assigned to *Pinus densiflora, Pinus koraiensis, Cinnamomum subavenium* and *Chamaecyparis obtusa* (Yang *et al*., 2021), *Curcuma longa, Zingiber officinale, Rosmarinus officinalis* and *Borago officinalis*, which have promising applications in some clinical aspects (Ghasemian *et al.*, 2016).

5.5. Anticancer activity

Cancer is a complex disease that can take many forms. Approximately 200 types of tumors can affect all tissues of the body (Kushi *et al*., 2012), each resulting from the

acquisition of abnormal characteristics by cells. Essential oils (EOs) induce programmed cell death of cancer cells via apoptosis, necrosis, arrest of cell cycle, and dysfunctioning of main cell organelles. Several molecules present in EOs; in particular, phenols (such as carvacrol, thymol and eugenol), alcohols (such as linalool), and aldehydes (such as cinnamaldehyde) possess antitumor properties (Swain *et al*., 2023). Natural products, such as irinotecan, vincristine, vinblastine, etoposide and paclitaxel from plants are widely used in various cancer therapies (Huang *et al*., 2021). The ability of EOs to inhibit cancer cell growth without affecting healthy cells is linked to their aptitude to activate specific molecular targets that induce cell death.

6. Material and methods

Thanks to their chemical composition, plant-extracts including EOs have numerous biological activities. This study aimed to better document the diversity of Algerian plants and highlight their extracts or essential oils. In particular, we explores (1) the methods of extraction of the plant biomass (plant-extracts or EOs); (2) chemical composition of EO; (3) to evaluate the antifungal activity of EOs and plant-extracts, in comparaison of (4) sodium bicarbonate and (5) two synthetic fungicides (Vidan 25EC and Agriconazole 25% EC) on stored wheat fungi, also (6) to emphasis the antibacterial activity. It is also necessary to propose the use of a combination of two plant extracts in the hope of obtaining a reinforced overall effect.

After a meticulous investigation of the literature, no data has been reported on the biological activity of EO of *E. polybractea* and the use of sodium bicarbonate on wheat storage fungi. Wherease, the specific microbial activity of *Morus alba* and *Rubus ulmifolius* have not been mentioned in the search results.

6.1. Products

- Mueller-Hinton (MH) agar was purchased from CONDA Bacteriology
- Absolute methanol and ethanol used for the extraction were from Sigma-Aldrich and were of analytical reagent grade.
- Sodium bicarbonate was obtained from Spectrum™ Chemical
- The fungicide were purchased from authorized stores and stored according to manufacturer's instructions.

6.2. Plant material

Eight species of Algerian medicinal plants collected for this study, are mentioned in the table below. The samples were dried in the shade at room temperature in a dry place protected from humidity in order to preserve the integrity of its molecules as much as possible. After drying, the sampled plant parts were finely ground in an electric mill and then stored in shaded, hermetically sealed glass bottles until extraction.

Table 6. Information about the plants sampled

Scientific name	Common name	Plant parts	Sample location	Year of sampling
Salvia rosmarinus	Rosemary	Aerial	collected in the precincts of the University Badji Mokhtar Annaba (Northeast Algeria)	February 2021
Lantana camara	Lantana	Leafy branches		
Cupressus sempervirens	Cypress			
Rubus ulmifolius	Elmleaf blackberry or Blackberry	Mature leaves		
Morus alba L.	White mulberry			
Dittrichia viscosa L.	False yellowhead			
Platycladus orientalis	Thuja orientalis	Fruits		
Eucalyptus polybractea	Eucalyptus	Leaves	Algiers forest (Northern Algeria)	January 2019

6.3. Summary biography of plants

6.3.1. Rosemary (*Salvia rosmarinus*)

Rosemary is a fragrant, evergreen aromatic shrub with needle-like leaves and two-lipped, purplish-blue and white flowers belonging to the *Lamiaceae* family (Tab7) (Photo 5) is endemic to the Mediterranean region. Rosemary has therapeutic properties and has been used in the folk medicine, pharmaceutical, cosmetics industries, mainly for its antioxidant, anti-inflammatory, antimicrobial, antispasmodic, analgesic properties (Waller *et al.*, 2020) and also as an important spice, flavouring agent, and food preservative (Tan et McClements, 2021). Rosemary EOs extracted from rosemary leaves is rich in active compounds such as rosmarinic acid carnosol, and carnosic acid, which can inhibit the growth of microorganisms, and contains polyphenols that can enhance the antioxidant capacity (Veenstra & Johnson, 2021).

Table 7. Scientific classification of Rosemary (Begum *et al.*, 2013)

Kingdom	*Plantae*
Sub kingdom	*Tracheobionta*
Super division	*Spermatophyta*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Sub class	*Asteridae*
Order	*Lamiales*
Family	*Lamiaceae*
Genus	*Salvia*
Species	*Salvia rosmarinus Spenn., 1835*

Photo5. Natural rosemary (Chibi, 2021)

6.3.2. Lantana (*Lantana camara* Linn)

Lantana is an attractive ornamental, aromatics evergreen belonging to the family *Verbenaceae* (Tab 8). The plant is a low erect or subscandent vigorous shrub with tetrangular stem, stout recurved pickles (Photo 6) and a strong odour of black currents. The leaves are oval, acute and subacute, crenate-toothed, rough above, scabrous on both sides. However, the leaves and stem are covered with rough hairs. Small flower held in clusters (called umbels). Colour usually orange, sometime varying from white to red in various shades and the flower usually change colours as they ages. Inflorescences are produced in pairs in the axils of opposite leaves. Inflorescences contain 20-40 sessile flowers. Root system is very strong and it gives out new fresh shoots even after repeated cuttings. This plant has been used in traditional medicine for several thousand years and is reported for various medicinal propertie viz, anticancer, anti-inflammatory, antidiabetic, anthelmintic, antibacterial, and antifungal (Wu *et al*., 2020). The medicinal potential of *Lantana camara* may be attributed to some of the chemicals in it, including Lantanoside, linaroside and camarinic acid, caryophyllene-like bicyclic terpene, lantanine, and verbascoside (Battase et Attarde, 2021). It contains various toxic pentacyclic triterpenes, the most abundant of which are lantadene A, lantadene C, and icterogenin, although metabolites may also be toxic (Cullen et Stalker, 2016).

Table 8. Taxonomic classification of Lantana (Cronquist, 1981)

Kingdom	*Plantae*
Subkingdom	*Tracheobionta*
Division	*Magnoliophyta*
Class	*Asteridae*
Subclasse	*Lamiales*
Family	*Verbenaceae*
Genus	*Lantana*
Species	*L. camara* L., 1753

Photo 6. Natural Lantana (Chibi, 2021)

6.3.3. Cypress (*Cupressus sempervirens*)

Known as Mediterranean or common cypress, it's an ornamental tree and a member of the Cupressaceae family (Tab 9), with a distinctive aroma and rich in essential oils. It's a medium-sized coniferous evergreen tree to 35 m tall, with a very variable crown shape, from columnar to spread with level branches and variably loosely hanging branchlets, dark green foliage and small ovoid brown cones (Photo7). Medical examination found that *Cupressus sempervirens* contained antioxidant, anticancer, antifungal, antibacterial, antiparasitic, antiviral, insecticidal, anticoagulant, estrogenic, healing and numerous properties (Batiha *et al.*, 2023); (Fadel *et al.*, 2021). With respect to these advantages, *C. sempervirens* is widely used as a cosmetic ingredient in perfumery and soap-making, including its essential oil distilled from shoots. Previous studies on *C. sempervirens* EO chemical composition reported 10–67 compounds identified depending on the plant organ used for EO extraction (Fadel *et al.*, 2021). EOs obtained from the leaves of this species are characterized by α-pinene and δ-3-carene as major components (Almadiy et Nenaah, 2022); (Fadel *et al.*, 2021).

Table 9. Taxonomic classification of Cypress (In Batiha *et al*., 2023)

Kingdom	*Plantae*
Subkingdom	*Viridiplantae*
Infrakingdom	*Streptophyta*
Superdivision	*Embryophyta*
Division	*Tracheophyte*
Subdivision	*Spermatophytin*
Class	*Pinopsida*
Subclass	*Pinidae*
Order	*Pinales*
Family	*Cupressaceae*
Genus	*Cupressus*
Species	*Cupressus sempervirens L. 1753*

Photo7. Natural Cypress (Chibi, 2021)

6.3.4. Eucalyptus blue mallee (*Eucalyptus polybractea*)

It is a mallee eucalypt from the large genus *Eucalyptus* (*Myrtaceae*) (Tab 10) that typically grows to a height of 8–10 m and forms a lignotuber. It has rough, fibrous or flrom aky, greyish to brownish bark on the lower part of the trunk, smooth greyish to brownish bark above that is shed in ribbons. Young plants have bluish to glaucous, linear to lance leaves. Adult leaves are the same shade of bluish green on both sides, lance-shaped (Photo 8). The flowers are white and the fruit is a woody, cup-shaped. Various species of *Eucalyptus* are recognized for their high biomass production, rapid growth rate, good adaptation to various environmental conditions, and excellent wood quality to produce paper and derived products (Ballesta *et al*., 2020). In turn, some species of the genus (e.g., *E. polybractea*) have received particular attention as sources of essential oils for use in pharmaceutical and cosmetic products (Chandorkar *et al.,* 2021). Eucalyptus blue mallee is multi-stemmed tree, which is widely cultivated in Australia for the production of Eucalyptus oil. Eucalypt plants have been used in traditional medicine in Australia for thousands of years. The leaf extracts, including the essential oil (mainly 1,8-cineole and eucalyptol) (Aldoghaim *et al*., 2018), are currently widely used in perfumery and cosmetic products and to a lesser extent as a therapeutic agent. The current medicinal use is based on the range of biological effects exhibited by the oils in vitro, including antioxidant, anti-inflammatory, analgesic, and antimicrobial activities

Table 10. Taxonomic classification of Blue mallee (Website1)

Kingdom	*Plantae*
Division	*Magnoliophyta*
Class	*Magnoliopsida*
Order	*Myrtales*
Family	*Myrtaceae*
Genus	*Eucalyptus*
Species	*E. polybractea R.T.Baker*

Photo 8. Natural Blue mallee (website2)

6.3.5. Blackberry (*Rubus ulmifolius*)

Rubus ulmifolius, commonly known as Elm Leaf Blackberry or Blackberry, is a deciduous shrub belonging to the *Rosaceae* family (Tab 11), well-known for its edible fruits (blackberries). The plant is a fast-growing shrub (Photo 9) that can reach a height of 3 to 4 meters, often scrambling with bristly or prickly stems bearing simple, lobed, palmate or pinnate leaves and 5 white or pink petalled flowers followed by juicy, sometimes edible fruits. The plant can be cultivated in gardens, borders, and hedges. Rubus fruits are very appreciated by consumers for its color, flavor, and taste and also for the rich composition in bioactive compounds (Cassidy, 2018; Braga *et al.*, 2018). Blackberries and their derivatives have been used since ancient times in traditional medicine, but recently knowledge about their health-beneficial components has received much attention, especially due to their richness in different bioactive compounds, with the presence of vitamins, minerals, fiber, phenolic compounds, flavonoids, and anthocyanins (Weli *et al*., 2020). It has been reported that Rubus genus has anti-inflammatory, analgesic, antipyretic, antidiabetic, anti-tumor, wound-healing, anti-cancer, and antibacterial effects (Bhuyan et Dutta, 2021; Boscaro *et al*., 2022). The plant has been used for many generations in the treatment of various ailments such as gastrointestinal illness, diabetes, bacterial and fungal infections; wound healing, and ulcers (Bhuyan et Dutta, 2021).

Table 11. Taxonomic classification of Blackberry (Evans *et al.*, 2007)

Kingdom	*Plantae*
Infra-kingdom	*Viridaeplantae*
Phylum	*Tracheophyta*
Sub-phylum	*Spermatophytina*
Infra-phylum	*Angiospermea*
Class	*Magnolipsida*
Ordrer	*Rosales*
Family	*Rosaceae*
Genre	*Rubus*
Species	*R. ulmifolius* schott, 1818

Photo 9. Natural *Rubus* (Chibi, 2021)

6.3.6. White mulberry (*Morus alba L.*)

Morus alba, known as white mulberry, common mulberry and silkworm mulberry and belonging to the *Moraceae* family (Tab12), is a fast-growing, small to medium-sized mulberry tree that grows between 10–20 m tall, with a picturesque habit, a wide crown and very varied foliage (Photo 10). The leaves are generally shiny and thin with alternating lengths and shapes depending on the age of the tree. Young shoots tend to produce leaves with deep, detailed lobes. However, older shoots are not lobed and are serrated or irregular at the edges. The tree produces petalless, unisexual flowers grouped in clusters called catkins and pollinated primarily by wind dispersal. Both sexes of catkins are generally present on each tree. The fruits produced are abundant and resemble those of blackberries. Their color is typically white but can sometimes be pinkish-violent. The berries are poisonous when unripe due to chemical called latex, which is toxic to humans, but are a rather pleasant fruit to enjoy when ripe. The white mulberry fruits have shown to possess vast amounts of highly beneficial biologically active ingredients. They have therefore been widely used in traditional medicine. Research has revealed the presence of several bioactive compounds in the mulberry fruits, such as alkaloids and flavonoids, which are considered effective antioxidants and anthocyanins have been associated with major prospective pharmacological health benefits, such as anti-cholesterol, anti-obesity and hepatoprotective effects (Zhang *et al*., 2018). The White Mulberry (*Morus alba*) has been cultivated for centuries in many places for its fruit and properties of its leaves, including its utility as a fodder crop for the silkworm, *Bombyx*

mori (Samami *et al*., 2019). The plant is a very good source of ascorbic acid, more than 90% of which is present in reduced form, and also contains carotene, vitamin B1, folic acid, folinic acid, tannins, flavonoids and saponins, which act as natural antioxidants (Rodrigues *et al*., 2019).

Table 12. Taxonomic classification of White Mulberry (Sadiq *et al*., 2008)

Kingdom	*Plantae*
Sub-kingdom	*Tracheobionta*
Phylum	*Magnoliophyta*
Class	*Magnoliopsida*
Sub-class	*Hamamelidae*
Ordrer	*Urticales*
Family	*Moraceae*
Genre	*Morus*
Species	*Morus alba L.*, 1753

Photo 10. Natural Morus (Chibi, 2021)

6.3.7. False yellowhead (*Dittrichia viscosa L.*)

Dittrichia viscosa L. (W. Greuther) known as False Yellowhead belongs to the *Asteraceae* family (Tab 13); is a common ruderal plant of the Mediterranean region (Parolin *et al.*, 2014). It's an erect, perennial, soft-wooded shrub, measuring 1 to 1.5 m high. Its leaves are greyish-green and elliptical and serrated on the edge. The leaves are half-wrapped around the stem. The golden yellow flower heads resemble daisies, with radiating petal-shaped florets. The flowers are surrounded by narrow, triangular, sticky bracts (Photo 11). Several hundred of these flower-heads can be produced in branched clusters towards the tips of the stems. This shrub's bioactive compounds include flavonoids, triterpenoids guaianolides, sesquiterpenes, sesquiterpene acids, lactones, and essential oils (Özkan *et al*., 2019). According to ethnobotanical studies, *D. viscosa* is known for its traditional use in the treatment of cancer in the Mediterranean region and is used is also as medicine (wound healing, herniated disc, stomachache, kidney pain, kidney stones, skin, hair, and eye ailments), food, and dye (Özkan *et al*., 2019). Young stems and leaves are fully covered with glandular hairs that exude sticky foul-smelling oil.

Table 13. Taxonomy of Dittrichia (Dupont et Guignard, 2007)

Kingdom	Plantae
Phylum	Spermatophyta
Class	Magnoliopsida
Ordrer	Asterales
Family	Asterasceae (Compositae)
Genre	Dittrichia
Species	*Dittrichia viscosa*

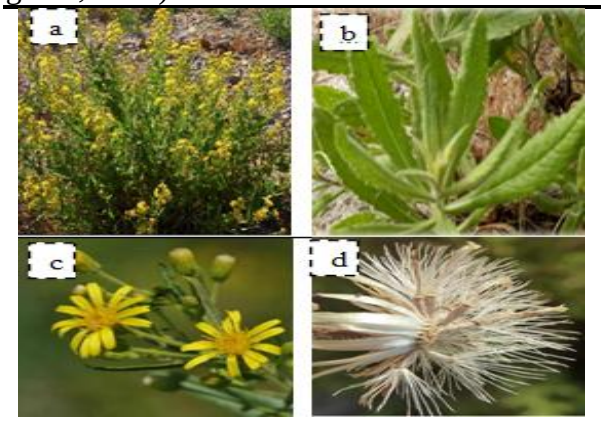


Photo 11. Plant (a), leaves (b), flowers (c) and fruits (d) of Dittrichia (Chibi, 2021)

6.3.8. Oriental Thuja (*Platycladus orientalis L.)*

Commonly known as *Biota orientalis*, *Thuja orientalis* or oriental arborvitae, is a small monoecious evergreen tree up to 20 m high (Photo 12) and belongs to the *Cupressaceae* family (Tab14). The crown is ovoid-pyramidal in young individuals, broadly rounded or irregular in older individuals. Plants tend to open with age. The leaves of the plant are mainly present at the terminal branches and are very small in size, scaly, imbricate, bright green in color and the shape of the leaves is obovate-rhomboid, with a linear gland on the underside. The fruit or strobile female part of the plant is ovoid, erect, with mucronate and glaucous scales; in the male, the strobilus are globular in shape, yellow in color and erect; the seed part of the plant is thick, wingless and ovoid in shape. The leaf part of the plant has a bitter taste and has many uses as gastric, diuretic, astringent, tonic and anti-fever actions. The interest in plant extract is still increasing since their bioactive compounds have shown beneficial effects on health and the human body (Yu *et al*., 2021). Essential oils, phenolics, anthocyanins, carotenoids and vitamins of the medicinal plants are well known to have potent biological activities (Yener *et al*., 2020).

Table 14. Taxonomic classification of *Thuja orientalis (In* Rajpurohit *et al.*, 2022)

Kingdom	Plantae
Phylum	Spermatophyta
Clade	Tracheophytes
(Unranked)	Gymnosperms
Division	Pinophyta
Class	Pinopsida
Ordrer	Pinales
Family	Cupressaceae
Sub-family	Cupressoideae
Genre	Platycladus
Species	*Platycladus orientalis* (L.)

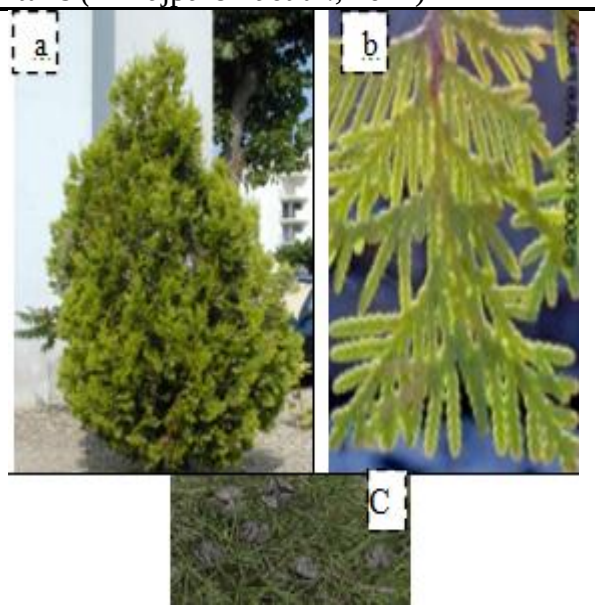


Photo 12. Plant (a), leaves (b) and fruits (c) of Thuja (Chibi, 2021)

6.4. Plant based essential oils

6.4.1. Plant material

The four aromatic plants selected for extraction of EOs were *Eucalyptus polybractea*, *Lantana camara*, *Cupressus sempervirens* and *Salvia rosmarinus*. The selection was made on the basis of their grouping in the families of aromatic plants, namely, Myrtaceae, Verbenaceae, Cupressaceae and Lamiaceae traditionally use in the treatment of various forms of diseases.

6.4.2. Extraction, yield and organoleptic properties of EOs

Extraction methods used in this study is hydrodistillation using the Clevenger apparatus, where the hydrated sample (100g of dried plant material) is heated to vaporize volatile constituents. The vapors of water laden with essential oils condense in a refrigerant and are collected in a separatory funnel. The oil less dense than water are collected by simple decantation and dried on anhydrous sodium sulphate (Na_2SO_4) before analysis. The essential oil was preserved and stored in well-sealed opaque bottles in a refrigerator at 4 °C.

The yields of EO of each plant were expressed in g relative to 100 g of dry vegetable matter; it was calculated according to Equation:

$$Yield\ (\%) = \frac{\text{Amount of extracted oil (g)}}{\text{Amount of dry vegetal matter mass (g)}} \times 100$$

For organoleptic properties of different EOs: the colour and aroma were carried out.

6.4.3. Chemical composition of EOs

GC/mass spectrometry (GC/MS) was carried out with a Hewlett Packard 5890 II /MSD 5973 system equipped with a DB-5MS column (30 m × 0.25 mm, film thickness 0.5 µm; J&W) following the above operative column and conditions; helium flow was fixed at 1 mL/minute. It was operating in electron impact (EI) mode at 70 eV, 300 µA, with an ion-source temperature of 220°C and a quadrupole temperature of 250°C. Samples were injected in splitless mode. Mass spectra were scanned in the range 33–500 m/z.

6.5. Plant-extracts

6.5.1. Plant material and extract preparation

Five natural plants were used to prepare 8 extracts (Tab 15). Twenty grams of powdered plant material was kept in 250 mL conical flask and added individually 200 mL of solvent such as water, methanol or ethanol. The mouth of the conical flask was covered with aluminum foil and kept in a reciprocating shaker for 72 hours for continuous agitation at 150 rounds per minute (rpm) for thorough mixing and also complete elucidation of active materials to dissolve in the respective solvent. Then, the extract was filtered by using muslin cloth followed by Whatman n° 1 filter paper. The solvent from the extract was removed using a rotary evaporator with a water bath temperature of 40°C. Finally, the concentrated extract was stored at −4°C until further analysis.

6.5.2. Percent yield of plant-extracts

Percent yield of all extracts were calculated using following formula:

$$Yield\ (\%) = \frac{\text{Weights of solvent free extract (g)}}{\text{Dried extract weight. (g)}} \times 100$$

For every sample triplicates flasks were used for extraction and results were expressed as mean ± standard deviation (SD) (n = 3).

Table 15. List of plant extracts used in this study

N°	Botanical name	Family	Extracted part	Plant-extract
1	***Morus alba***	Rosaceae	Leaves	Aqueous
2				Methanolic
3	***Rubus ulmifolius***	Rosaceae	Leaves	Methanolic
4				Aqueous
5	***M. alba + R. ulmifolius***			Methanolic combinaison (50/50, v/v)
6	***Lantana camara***	Verbenaceae	Leaves	Ethanolic
7	***Dittrichia viscosa***	Asteraceae	Leaves	Aqueous
8	**Thuya**		Cones	Ethanolic

6.6. Evaluation of antimicrobial activity of EOs

The effectiveness of the essential oils was evaluated against six bacterial strains (3 standard reference strain and 3 clinical isolates), involved in food poisoning and infectious diseases: two Gram-positive bacterial strain *Staphylococus aureus* ATCC25923, *S. aureus*, and four Gram-negative bacterial strain *Pseudomonas aeruginosa* ATCC27853, *Echerichia coli* ATCC25922, *Salmonella sp.*, *P. aeruginosa*. In the present study, as we tested the antibacterial activity of multiple clinical strains, we also integrated clinical *Candida albicans* (opportunistic pathogenic yeast) and experienced the effect of different EOs on its growth. All strains were provided by Ibn Rochd, Annaba Hospital. Cell cultures maintenance of these strains was carried out in Mueller-Hinton (MH) agar and incubated at 37°C for 24 h to obtain fresh cultures.

The antimicrobial activity is evaluated by the aromatogram method (Dayal and Purohit, 1971) which makes it possible to determine the sensitivity of the various bacterial species with respect to the given essential oil. Briefly, the MH agar plate was inoculated with adjusted bacterial suspension (10^8 CFU/mL) (McFarland turbidity standard of 0.5), then blotting paper discs 6 mm in diameter, previously impregnated with 10 µl of the following concentration 1, 1/2, and 1/3 essential oils diluted in dimethyl sulfoxide (DMSO). However, DMSO was used as a negative control and an antibiotic disc: Gentamicin (GN) as positive control. After that, plates were refrigerated at 4 °C for two hours to allow the essential oils to diffuse into agar, followed by incubation at 28°C for 24 h; the antibacterial activity of each sample was evaluated by measuring the zone of inhibition diameter (ID) expressed in millimeters (mm). Inhibition zones with diameters of <12, 12 to 16 and > 16 mm were classified as having low,

moderately active, and highly active antibacterial activity, respectively (Indu *et al.*, 2006). A dilution agar method was used to determine MIC of each oils. According to Suliman (2011), MIC <0.1 mg/mL, 0.1 - 0.5 mg/mL, 0.6 – 1.5 mg/mL and ≥1.6 mg/mL, EOs were classified as very strongly inhibitory, highly inhibitory, moderately inhibitory and low inhibitory, respectively. The tests were performed in triplicate.

6.7. Antifungal activity

6.7.1. Antifungal activity of plant-extracts and EOs

Among the fungal strains mentioned in the previous chapter, 10 fungal strains (from stocked wheat grains) are subject to the effectiveness of both plant-extracts and EOs. These are the following strains (Tab 16). Note that we had to replace two fungal species (Alternaria and Trichoderma) by *Rhizopus oryzae* and *Aspergillus terreus* after losing them.

Table 16. Tested fungi

Aspergilus niger	*Fusarium solani*
Aspergilus flavus	*Penicillium expansum*
Alternaria alternata	*Penicillium sp1.*
Cladosporium herbarum	*Penicillium sp2.*
Fusarium graminearum	*Trichoderma viride*
Rhizopus oryzae	*Aspergillus terreus*

6.7.1.1. Antifungal activity of EOs

The bioassays was performed by the direct contact of the fungus with the potato dextrose agar (PDA) culture medium containing the EO in Petri dishes (90 mm), dissolving essential oils at concentrations of 100, 150, 200, 250, and 300 µl, in previously sterilized PDA growth medium flasks and distributed into Petri dishes. Mycelial prickle of each tested fungi from young cultures is placed in the center of the Petri dishes. Plates were incubated in the dark at 28°C for 3 and 7 days. Petri dishes controls (without EO) are included in the tests. The mycelial diameter of the fungus was evaluated by measuring the diameter of the colony with a calipet, until the control reached the edge of the plate. Inhibition tests were performed in triplicate and inhibition activity was determined as follows: 20 mm, very strong activity; 21–40 mm strong activity; 41–60 mm moderate activity and > 61 mm weak activity.

6.7.1.2. Antifungal activity of plant-extracts

All the extracts were screened for their antifungal activities against the isolated fungi. The sets of five dilutions (100, 150, 200, 250 and 300 µl) were prepared. Each concentration was added to Petri dishes (90 mm) with PDA culture medium previously sterilized. Mycelial prickle of the corresponding strain from young cultures is placed in the center of the Petri dishes, which is than incubated in the dark at 28°C for 3 and 7 days. In addition, is included an absolute control (PDA + fungus). The radical colony diameters of the fungus were evaluated by measuring with a caliper, until the control reached the edge of the plate. All bioassays were performed in triplicate.

6.7.2. Effect of sodium bicarbonate

A. Presentation of sodium bicarbonate

Name: Sodium bicarbonate

Synonyms:

* Soda bicarbonate
* Sodium hydrogen carbonate
* Monosodium carbonate
* Sodium hydrogen carbonate
* Vichy salt
* Bicarbonate of soda

Sodium bicarbonate (SBC) is an inorganic chemical compound with the formula $NaHCO_3$. It is a salt composed of a sodium cation (Na^+) and a bicarbonate anion (HCO_3^-). It's a white crystalline powder or lumps and has a slightly salty, alkaline taste (bitter). Sodium bicarbonate is a versatile product commonly used in cooking and baking, to absorb odors, cleaning, gardening, it's an ecological product. It has weak disinfectant properties (Malik et Goyal, 2006) and it may be an effective fungicide against some organisms (Zamani *et al*., 2009) because it alkalinizes the medium. Sodium bicarbonate (SBC) is a general food additive, has less risk of phytotoxicity at the low concentrations at which it is used (1–4%) (Usall *et al.*, 2008) and is Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration (FDA, 2017).

B. Antifungal activity of sodium bicarbonate

The in vitro antifungal activity of SBC was tested by direct contact. In brief, SBC at concentrations of 100, 200, 300 mg/ml was added to sterilized PDA to generate media. After solidification of the medium, mycelial prickle of each tested fungi from young cultures is

placed in the center of the Petri dishes. Plates were incubated in the dark at 28°C for 3 and 5 days. Petri dishes controls (without SBC) are included in the tests. The mycelial diameter of the fungus was evaluated by measuring the diameter of the colony, until the control reached the edge of the plate.

6.7.3. Effect of chemical fungicides

A. Fungicides used

Fungicides are chemical or biological substances that kill or neutralize pathogenic fungi. They are also called mycocides or antifungal products, which they can be abiotic (chemicals) or biotic (bacteria, fungi).

- **VIDAN 25**

VIDAN 25 (Photo13) is a fungicide of the TRIAZOLE family, whose active ingredient is Triadimenol which acts by contact and systemic action. This fungicide has a triple preventive, curative and eradicating action, and also; it has a mode of action on the biosynthesis of sterols.VIDAN 25 has beneficial effects on the plant by stimulating its vegetation. This fungicide is used on several crops such as fruit trees and cereals, artichokes, tomato, grapevine, chili and bell pepper (Tab17).

Photo 13. VIDAN Fungicide

Table 17. Chemical properties and use of VIDAN

Active ingredient	Triadimenol
Formulation	Soluble concentrate
Concentration	25%
Crops	Artichoke, tomato, grapevine, pepper, melon ,Fruit trees, Cereals
Diseases	Oidium, Moniliosis, Rusts
Doses	25-50 ml/hl, 40 ml/hl, 500 ml/ha

➢ **Agriconazole 25% EC**

Agriconazole (Photo14) is a systemic fungicide belonging to the TRIAZOLE chemical family used for the control of different fungal diseases (Tab18). Applied at the first signs of the disease. The second application must be made at the latest before the grain is milky ripe.

Photo 14. Agriconazole

Table 18. Chemical properties and use of Agriconazole

Active ingredient	Difenoconazole
Formulation	Soluble concentrate
Concentration	25%
Crops	Cereals , tomato
Diseases	Brown rust on wheat , Powdery mildew on peppers
Doses	0.5 L/ha

B. Antifungal activity of fungicides

This activity is the same reported with SBC except that the two fungicides are liquid, so the final concentrations of 100, 200, 300 µl were added to sterilized PDA to generate media.

6.8. Statistical analysis

All tests were carried out in triplicate, and analysis of variance was performed by ANOVA procedures, Tukey test, a P value of 0.05 was regarded as significant.

7. Results and discussion

7.1. Percentage yields of plant-extracts

The extraction yield is a measure of the solvent efficiency to extract specific components from the original material. It will give an idea about the extractability of the plant studied under different conditions (Lahmar *et al*., 2018). The percentage crude extract yield shown in Table 19 is based on the weight of dried and ground plant materials. The data obtained revealed that Lantana ethanolic extract has the highest extract yield (35%), followed by Dittrichia aqueous extract (32%). The combined methanolic extract of (*M.alba* + *R. ulmifolius*) scored 25.70% while the ethanolic extract of thuja and aqueous extract of Morus recorded 22%. Rubus methanolic extract gave the lowest yield (11.46%). The solvents most commonly used for phytochemical extraction from plant tissue are water, ethanol, methanol,

acetone, and ether or a mixture of these (Amakura, 2017). Water extraction is the safest, least expensive, and most environmentally friendly method. Polysaccharides, proteins, polyphenols, and glycosides, which are soluble in water, are separated during water extraction. Although ethanol or methanol may be selected as a suitable solvent for the separation of active ingredients, such as phenol, plant tissues contain numerous biologically active compounds that require alternate extraction solvents depending on the plant species. Additionally, extraction yield is the most important factor in selecting a solvent and is affected by extraction time, temperature and sample composition, among other factors such as species, the time of harvest, the age of the plant, the part subjected to distillation and the extraction technique (Gil-Martín *et al.*, 2022).

Table 19. The percentage yields of crude extracts

Plants	**Extracts**	**Yields (%)**
M.alba	Aqueous	22
	Methanolic	18,2
R.ulmifolius	Aqueous	15,6
	Methanolic	11,46
M.alba+ R.ulmifolius	Methanolic combination (50/50, v/v)	25,70
Lantana camara	Ethanolic	35
Dittrichia viscosa	Aqueous	32
Thuya	Ethanolic	22

7.2. Extraction, yield and organoleptic properties of EOs

Essential oils (EOs) are aromatic and their fragrances play a particularly important role in increasing the attractiveness of the EOs. The following table (Tab 20) shows some of the organoleptic properties of the essential oil obtained (color, odor) from different plant.

Table 20. Aroma profile of EOs

	S. rosmarinus	***C. sempervirens***	***L. camara***	***E. polybractea***
Part expressed	Aerial parts	leafy branches	leafy branches	leaves
Appearance	Liquid	Liquid	Liquid	liquid, fluid and clear
Color	light yellow	light yellow	varied from yellow to orange	transparent to pale yellow
Odor	aromatic camphorated	fresh and woody odor with a lemony note	similar to that of davanone	spicy, woody

The yields of essential oils *Cupressus sempervensis*, *L. camara*, *S. rosmarinus* and *E. polybractea* gave variable results expressed in (%) on the following table:

Table 21. Yields of EOs

EOs	Yield (%)
Cupressus sempervensis	0.66
L. camara	0.76
S. rosmarinus	0.68
E. polybractea	0.80

7.3. Chemical composition of EOs

7.3.1. *Cupressus sempervirens* EO

The EO yield from leafy branches of *C. sempervirens* prepared by hydrodistillation method was 0.66%. Selim *et al*. (2014), obtained an oil yield of 2.6%, v/w from the aerial parts of *C. sempervirens* L. collected in the random gardens in Sakaka, Aljouf (Saudi Arabia), using the Clevenger hydrodistillation method. Qaralleh *et al.* (2021) reported that the oil yield is at least 0.26% (w/w) using steam-distillation method on leaves of *C. sempervirens* L. from Dhana Natural Reserve, Al-Tafilah (Jordan). Briefly, the oil yield of this plant has been researched before and it seems that is no prior research discussing the differences among different plant organs at different times (example young or mature leaves, cones and different seasons).

GC/MS analysis revealed that the oil contain 65 components (Tab 22) with 2-Pinene (20.44%), 3-Carene (11.98%), Cedrol (5.75%), alpha.-Terpinyl acetate (5.34%), 4-Isopropylidene-1-cyclohexe (3.51%), Bicyclo[3.1.1]heptane, 6,6-dimethyl (3.46%) were the major compound of the EO. Previous studies on *C. sempervirens* leaves EO chemical composition reported that α-pinene and δ-3-carene as major components (Fadel *et al*., 2021); (Almadiy et Nenaah, 2022); (Akermi *et al*., 2022). These results are in agreement with the ones obtained in this study. The analysis by GC-MS and GC-FID techniques showed the presence of 65 compounds where the major constituents were α-pinene (68.0%), *epi*-cedrol (6.1%), α-terpenyl acetate (3.5%) and germacrene D (2.5%) in *C. sempervirens* oil growing in the Aures region of Algeria (Fadel *et al.,* 2021). Akermi *et al*. (2022) showed that the gas chromatography analysis exhibited 27 different components in Tunisian *C. sempervirens*, as α-pinene (38.47%) and δ-3-carene (25.14%) are the major components of the *C. sempervirens* EO.

Table 22. Chemical composition (%) of Cypress EO from Algeria

Peak	R.Time	Area%	Name
1	7.883	20.44	2-Pinene
2	8.039	1.60	Bicyclo[2.2.1]heptane, 7,7-dimethyl
3	8.917	0.30	1,3,5-Cycloheptatriene, 3,7,7
4	9.179	2.33	alpha.-phellandrene (
5	9.347	1.81	2(10)-Pinene
6	10.178	3.46	Bicyclo[3.1.1]heptane, 6,6-dimethyl
7	11.672	11.98	3-Carene
8	12.077	1.95	Benzene, 1-methyl-4-(1-methylethyl
9	12.404	3.34	D-Limonene
10	13.885	1.05	.gamma.-Terpinene
11	15.656	3.51	4-Isopropylidene-1-cyclohexe
12	16.245	0.50	Linalool
13	16.428	0.29	trans-Verbenol
14	18.623	0.40	3-Cyclohexene-1-carboxaldehyde
15	19.134	0.39	Bicyclo[3.3.2]decan-9-one
16	19.406	0.17	p-Mentha-1,5-dien-8-ol
17	20.399	1.47	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl
18	20.845	0.64	Benzenemethanol, .alpha.,.alpha.,4-trimethyl
19	21.123	0.53	alpha.-Terpineol
20	21.855	0.44	cis-Verbenol
21	23.443	0.30	Dill ether
22	23.878	0.58	Benzene, 1-methoxy-4-methyl-2-(1-methylethyl
23	24.520	0.21	Linalyl acetate
24	25.931	0.54	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate,
25	26.605	1.69	Cyclohexanol, 1-methyl-4-(1-methylethylidene)-
26	27.095	0.35	4,7,7-Trimethylbicyclo[4.1.0]hept-3-en-2-one
27	27.595	0.38	4-Decenal, (E)-
28	28.653	1.62	(1R,3R,4R,5S)-1-Isopropyl-4-methylbicyclo[3.1.0]hexan
29	29.304	5.34	.alpha.-Terpinyl acetate
30	30.261	0.27	Copaene
31	30.904	0.21	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetr
32	31.534	0.40	Longifolene
33	31.900	0.96	(3R,3aR,7R,8aS)-3,8,8-Trimethyl-6-methyleneoctahydro
34	32.223	0.99	Caryophyllene
35	32.628	0.28	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyc
36	33.726	0.85	alpha.-Humulene
37	34.140	0.78	(+)-epi-Bicyclosesquiphellandrene
38	35.029	3.24	(-)-Germacrene D
39	35.528	0.63	gamma.-Muurolene
40	35.801	0.88	alpha.-Muurolene
41	36.379	1.05	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-
42	36.848	2.14	delta.-Cadinene
43	37.112	0.26	Copaene

44	38.024	0.62	2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydro-naphthalen
45	39.172	0.25	Caryophyllene oxide
46	39.414	0.27	(3R,3aR,5S,6R,7aR)-3,6,7,7-Tetramethyloctahydro-3a
47	40.270	5.75	Cedrol
48	40.423	1.22	(1R,2R,4S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetri
49	41.066	0.57	4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl
50	41.534	1.38	Tau-Cadinol acetate
51	41.772	0.38	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-
52	42.128	1.53	.alpha.-Cadinol
53	42.620	0.19	Caryophyllene oxide
54	43.045	0.33	((4aS,8S,8aR)-8-Isopropyl-5-methyl-3,4,4a,7,8,8a-hexahyd
55	43.297	0.52	(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol
56	49.475	0.19	2-Isopropyl-4a-methyl-8-methylenedecahydro-1,5-naphthalen
57	52.840	0.20	m-Camphorene
58	53.151	0.38	Phenanthrene, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a
59	54.258	2.58	Phenanthrene, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a-
60	56.255	1.12	Phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-
61	57.021	0.17	(4aS,4bR,10aS)-7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a
62	57.804	0.57	A'-Neogammacer-22(29)-ene
63	59.141	0.33	(12Z)-abienol
64	61.192	0.21	1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(
65	63.898	0.71	2-Phenanthrenol, 4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-

7.3.2. *L. camara* EO

The extraction yield of the EO from leafy branches of Algerian *L. camara* Linn was of 0.76%. Some previous works have studied EOs of different parts of this plant (leaves, flowers, fruits, and stems). The hydrodistillation of these organs produced EOs, whose yields fluctuated during harvest period. For example, leaf EO yields obtained from Ivorian *L. camara* by Nea *et al.* (2020) was 0.04–0.12% (v/w), Sajid *et al*. (2021), obtained by steam hydro-distillation process a yield of 0.23 g/100 g of fresh leaves, collected from the Botanical Garden of the University of Agriculture Faisalabad (UAF), Faisalabad, Pakistan. The variability of EO yield might arise from climatic and seasonal parameters, the hydrodistillation and plant storage conditions as well as from environmental and edaphic constraints (Nea *et al.*, 2020). According to Liambila *et al*. (2021), environmental conditions in the drier season favour the production of more dominant compounds than in the rainy season. About the composition of *L. camara* EO, 65 constituents were identified. The gas chromatography/mass-spectrometric (GC-MS) analysis results for the essential oil are summarized in Table 23. The major components of EO were Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl) (10.22%), Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-(7.89%), 5-Hepten-3-one, 2-(5-ethenyltetrahydro-5-methyl-2-fur (7.83%), 1,4,7,-

Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z- (6.93%), (-)-Germacrene D(5.49%), (1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0] undec (5.02%), Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate (5.00%). Previous studies reported caryophyllene as the main constituent of the EO of *L. camara* leaves in various regions with such as caryophyllene oxide (7.5%) in Saudi Arabia (Khan *et al*., 2016a), (E)-β-caryophyllene (39.9%) in Cote d'Ivoire (Nea *et al*., 2020), β-caryophyllene (35.70%) in Algeria (Zoubiri et Baaliouamer, 2012) and also caryophyllene (24.33%) in Tunisia (Gaid *et al*., 2023). Nevertheless, previous studies highlighted cis-3-hexen-1-ol and 1-hexanol as key constituents of *L.camara* EO from Saudi Arabia (Khan *et al*., 2016a), (E)-nerolidol and davanone from respectively Cuba and Nepal (Satyal *et al*., 2016).

Table 23. Chemical composition of Algerian Lantana EO

Peak	R.Time	Area%	Name
1	6.405	0.03	Tricyclo[2.2.1.02,6]heptane, 1,7,7-trimethyl-
2	6.627	0.96	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl
3	7.040	4.13	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
4	7.671	2.41	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-
5	7.885	0.03	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl
6	9.246	10.22	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)
7	10.013	1.27	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
8	10.652	0.76	.alpha.-phellandrene (only name in Wiley6)
9	11.071	3.22	3-Carene
10	11.365	1.02	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-
11	11.822	1.46	Benzene, 1-methyl-4-(1-methylethyl)-
12	12.191	5.00	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate
13	13.265	0.98	1,3,6-Octatriene, 3,7-dimethyl-, (E)-
14	13.807	1.71	.gamma.-Terpinene
15	14.415	0.03	trans-Arbusculone
16	15.428	0.80	4-Isopropylidene-1-cyclohexene
17	15.940	0.01	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl
18	16.269	2.64	Linalool
19	17.240	0.02	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis
20	18.383	0.50	1,7,7-trimethylbicyclo[2.2.1]heptan-2-one
21	19.363	0.01	p-Mentha-1,5-dien-8-ol
22	19.623	0.27	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, (1S-endo)-
23	20.285	0.68	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-
24	21.002	0.06	(1S)-1,3,3-trimethylnorbornan-2-ol
25	21.233	0.07	Ethanol, 2-(3,3-dimethylcyclohexylidene)-,
26	21.440	0.03	Estragole
27	21.915	0.02	(1R,6R)-3-methyl-6-propan-2-yl-1-cyclohex-2-enol
28	23.557	0.03	Butanoic acid, 2-methyl-, hexyl ester
29	23.815	0.01	Butanoic acid, 3-methyl-, hexyl ester

30	28.414	0.31	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-
31	29.004	0.40	.alpha.-Cubebene
32	29.780	0.11	1,2,4-Metheno-1H-indene, octahydro-1,7a-dimethyl-5-(
33	30.225	0.90	Copaene
34	30.629	0.26	Cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl
35	31.027	3.93	1-Methyl-1-ethenyl-2,4-bis(1'-methylethenyl)cyclohexane
36	31.777	0.42	1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8
37	32.496	7.89	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-
38	32.768	2.96	(+)-epi-Bicyclosesquiphellandrene
39	33.129	0.85	Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-
40	33.896	6.93	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
41	35.002	5.49	(-)-Germacrene D
42	35.693	5.02	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undec
43	36.217	3.85	beta.-Bisabolene
44	36.834	1.76	delta.-Cadinene
45	37.146	0.66	4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl
46	37.615	0.18	Cyclohexene, 4-[(1E)-1,5-dimethyl-1,4-hexadien-1-yl]-
47	37.940	0.25	Caryophyllene oxide
48	38.155	0.57	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-,
49	38.481	1.67	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, [S-(Z)]-
50	39.015	0.49	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimet
51	39.228	1.16	Caryophyllene oxide
52	39.628	7.83	5-Hepten-3-one, 2-(5-ethenyltetrahydro-5-methyl-2-fur
53	40.320	1.84	Humulenol-II
54	40.840	0.31	Butanoic acid, 3-methyl-, 1,7,7-trimethylbicyclo[2.2.1]
55	41.142	1.71	Humulene epoxide I
56	41.490	1.45	Kauran-18-al, 17-(acetyloxy)-, (4.beta.)-
57	41.948	0.77	Isospathulenol
58	42.338	0.34	Santrolinatriene
59	42.840	0.67	8.alpha.,11-Elemadiol
60	43.590	0.14	1H-Naphtho[2,1-b]pyran-8(4aH)-one, 3-ethenyldeca
61	44.012	0.14	Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-
62	44.491	0.17	(S,E)-6-Hydroxy-6-methyl-2-((2S,5R)-5-methyl-5-vinyltetrah
63	54.124	0.04	Phenanthrene, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a
64	56.199	0.01	Phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl
65	57.961	0.16	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(

7.3.3. Rosemary EO

The yield of rosemary aerial parts EO obtained during hydrodistillation was 0.68%, while Sakar *et al*. (2023) in Taounate province (Morocco) have obtained from wild and cultivated rosemary EO using the Clevenger hydrodistillation method respectively 01.93% and 01.10%, also Elyemni *et al*. (2019), in Fez (Morocco) have obtained 0.37%. The yield acquired from Tunisian rosemary EO was 0.91%. However, although rosemary is native to temperate Mediterranean regions, variation in EO yield is governed by many factors including

internal chemical properties (bonding) and external physical properties (temperature and pressure) (Ali *et al.,* 2019). According to Sakar *et al*. (2023), the extraction technique and rosemary's origin (wild or cultivated) impacted rosemary essential oil yield. Results of chemical profiling were shown in Table 24. The EOs chemical composition was strongly dominated by Phenol, 2-methyl-5-(1-methylethyl)- (35.47%), Eucalyptol (17.04%), Thymol (14.36%), 1,7,7-trimethylbicyclo[2.2.1]heptan-2-one (5.04%), (1R)-2,6,6-Trimethylbicyclo [3.1.1]hept-2-ene (4.74%). Our findings are comparable with previously published literature on rosemary EO profiling. in recent work of Sakar *et al.* (2023) in Morocco, founded that rosemary EO was dominated by 1,8-cineole (148 mg/mL), camphor (40 mg/mL), *α*-pinene (28 mg/mL), *α*-terpineol (10.6 mg/mL), borneol (8.40 mg/mL), camphene (8.14 mg/mL) and limonene (6.64 mg/mL). Also, according to Jafari-Sales et Pashazadeh (2020), the dominant compound of rosmary oil were 1.8 Cineole (21.8%), α-pinen (18.7%), Camphor (14.6%), Linalool (13.4%) and Camphene (7.2%), while Rahmouni *et al*. (2019), founded that eucalyptol 26.45%, camphor 15.51%, α- and β-pinene 21.31% were the major compounds. Results of Saraiva *et al.* (2021) of volatile composition of *S. rosmarinus* represented the dominant of α-pinene (21.90%), myrtenyl acetate (19.82%), prenol (12.46%), β-linalool (11.44%). In Ainane *et al.* (2019) results, the chemical composition of the essential oil performed by gas chromatography coupled with mass spectrometry (GC-MS), revealed that the majority compounds are: (-)-camphor31.16%, β-Caryophyllene 18.55% and 2,4-hexadiene, 3,4- dimethyl-, (Z, Z) 9.08%.

Table 24. Chemical composition (%) of Algerian rosemary EO

Peak	R.Time	Area%	Name
1	6.559	0.21	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-
2	6.857	4.74	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
3	7.533	2.19	Camphene
4	9.005	3.84	2(10)-Pinene
5	9.636	0.75	Linalool
6	9.882	0.72	beta.-Myrcene
7	10.447	0.15	2-Propanol, 1-(2-methoxy-1-methylethoxy)-
8	10.688	0.15	2-Propanol, 1-(2-methoxy-1-methylethoxy)-
9	10.886	0.21	3-Carene
10	11.253	0.52	alpha.-Terpineol[.alpha,alpha.,4-trimethyl-3-cyclohexene-1-methanol]
11	11.713	1.07	Benzene, 1-methyl-4-(1-methylethyl)-
12	11.962	1.08	D-Limonene
13	12.107	17.04	Eucalyptol
14	13.709	0.41	.gamma.-Terpinene
15	15.381	0.24	4-Isopropylidene-1-cyclohexene

16	15.939	0.14	Borneol
17	16.121	0.51	Linalool
18	17.006	0.52	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-
19	18.385	5.04	1,7,7-trimethylbicyclo[2.2.1]heptan-2-one
20	19.615	1.54	Borneol
21	19.724	0.24	Cyclohexanemethanol, .alpha.,.alpha.-dimethyl-4-methylene-
22	20.268	0.51	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-
23	21.007	1.21	alpha.-Terpineol
24	22.142	0.15	Anisole, 2-isopropyl-4-methyl-
25	25.060	0.38	Phenol, 2-methyl-5-(1-methylethyl)-
26	25.449	14.36	Thymol
27	25.550	0.16	3-Methyl-4-isopropylphenol
28	26.055	35.47	Phenol, 2-methyl-5-(1-methylethyl)-
29	30.201	0.17	Copaene
30	31.195	0.48	1H-Cyclopropa[a]naphthalene, 1a,2,3,3a,4,5,6,7b-octahydro-1,1,3a,7-tetramethyl-,
31	31.632	0.17	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-
32	32.174	2.40	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-
33	32.577	0.50	Neoalloocimene
34	32.913	0.38	4-ethyl-2-methoxy-6-methyl-phenol
35	33.683	0.48	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
36	34.731	0.15	.gamma.-Muurolene
37	35.236	0.27	1H-Cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR
38	35.760	0.13	.alpha.-Muurolene
39	36.090	0.20	.gamma.-Cadinene
40	36.522	0.19	delta.-Cadinene
41	36.744	0.21	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-
42	38.787	0.25	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha
43	38.970	0.14	sospathulenol
44	39.162	0.18	Caryophyllene oxide
45	52.026	0.16	(E)-1-[(1R)-2,6,6-trimethyl-1-cyclohex-2-enyl]-1-penten-3-one

7.3.4. *E.polybractea* EO

The yield acquired from the EO leaves of Algerian *E. polybractea* was of 0.80%, which is lower than the result of Bush *et al.* (2022) of 7.5% from Australia. It is obvious that there are differences in the EO yields, which is acceptable since it depends on several biotics (the harvest period, the type of organs harvested, soil and rainfall), intrinsic (genetics, subspecies, and plant age) or extrinsic factors, such as climate and cultivation conditions (geographical origin) or isolation methods (Fleming *et al.*, 2002).

The chemical composition of the EO of *E. polybractea* by GC/MS revealed 45 compounds (Tab25). The most prominent compounds were Eucalyptol (34.87%), alpha.-phellandrene (13.10%), alpha.-Terpineol (12.17%), (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (5.49%) and Terpinen-4-ol (5.18%). However, the chemical composition analysis showed that *E. polybractea* dominated by monoterpenoids compounds. Previous studies revealed the existence of different principal constituents. Our results are different from those recorded by Poli *et al.* (2018) that shown the domination of p-cymene (25.5%), cryptone (11.42%). In some works, other chemotypes have been found with predominant major compounds was 1,8-Cineole recorded by Aldoghaim *et al.* (2018) and Bush *et al*. (2022).

Table 25. Chemical composition (%) of Algerian *E. polybractea* EO

Peak	R.Time	Area%	Name
1	6.664	2.33	alpha.-phellandrene (only name in Wiley6)
2	7.033	5.49	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
3	7.619	0.61	Camphene
4	9.268	13.10	alpha.-phellandrene (only name in Wiley6)
5	10.052	1.83	2(10)-Pinene
6	11.784	3.11	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
7	12.943	0.29	Benzene, 1-methyl-4-(1-methylethyl)-
8	13.440	34.87	Eucalyptol
9	13.952	0.42	1,3,6-Octatriene, 3,7-dimethyl-, (E)-
10	14.350	1.69	gamma.-Terpinene
11	15.510	1.00	5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol
12	16.016	0.80	4-Isopropylidene-1-cyclohexene
13	16.240	0.78	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-
14	17.291	0.23	Linalool
15	18.305	0.49	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-
16	19.888	0.45	p-Menth-2-en-1-ol
17	20.550	2.75	alpha.-Terpineol
18	20.808	5.18	Terpinen-4-ol
19	21.568	0.21	2-Cyclohexen-1-one, 4-(1-methylethyl)-
20	22.105	12.17	.alpha.-Terpineol
21	23.154	0.27	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans-
22	24.278	0.33	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
23	25.105	0.20	p-Menthane-1,2,3-triol
24	25.799	0.32	trans-Ascaridol glycol
25	26.012	0.18	Bicyclo[2.2.1]hept-2-ene, 2,3-dimethyl-
26	26.782	0.22	trans-Ascaridol glycol
27	27.635	0.29	2H-Pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl-
28	30.032	0.29	Cyclohexene, 1-(1,1-dimethylethoxy)-2-methyl-
29	30.265	0.20	Ylangene
30	31.020	0.27	Copaene

31	32.246	0.63	1-Methyl-1-ethenyl-2,4-bis(1'-methylethenyl)cyclohexane
32	33.082	1.45	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-
33	33.776	0.24	Neoalloocimene
34	34.727	1.96	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
35	34.954	0.28	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR
36	35.178	0.73	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]
37	35.583	0.94	beta.-Selinene
38	36.768	1.42	1.beta.,4.beta.H,10.beta.H-Guaia-5,11-diene
39	38.132	0.27	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-
40	38.991	0.24	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-
41	39.203	0.39	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.
42	39.835	0.45	5-Oxatricyclo[8.2.0.04,6]dodecane, 4,12,12-trimethyl-9-methylene-, (1R,4R,6R,
43	40.266	0.17	Guaiol
44	41.432	0.31	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene
45		0.17	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.

7.4. Antimicrobial activity

The results confirm the great antibacterial activity of EOs against different strains tested including *Candida albicans* (Fig 15; Fig 16). As in all essential oils in general, the factors that influence the different proportions of constituents include the location/region of cultivation, the part of the plant collected, the period of plant development in the EO extraction, and varieties of the species (Ibrahim *et al*., 2017; Powers *et al*., 2018). It appears that the negative control (DMSO) showed no antibacterial activity. However, antibacterial activity of *E. polybractea, L. camara,* EOs can be classified as highly active against all tested strains. The Minimal Inhibitory Concentration (MIC) of EOs derived from *E. polybractea*, *Lantana camara*, *S. rosmarinus*, and *C. sempervensis* for bacteria have a range of 0.91 to 9 µg/ml (Tab26).

According to the results (Fig 15), ***C. sempervensis*** essential oil (CSEO) demonstrated antibacterial activity against various bacterial strains. The lowest MIC value of 2.17 µg/ml was observed against both *P. aeruginosa* ATCC27853 and *Staphylococus aureus ATCC 25923*. *Salmonella sp.* followed with a slightly higher MIC value of 2.9 µg/ml (Fig 16). Whereas, *E. coli ATCC 25922, S. aureus* and *P. aeruginosa* have the highest MIC value

of 8.7 µg/ml, suggesting it required a higher concentration of the EO for inhibition. A previous investigation of Qaralleh *et al.* (2021) reported that CSEO from Jordan exhibited a lower MIC when tested against Gram-positive bacteria, with a value of 370 µg/mL and demonstrated a higher MIC range of 2000–3000 µg/mL when tested against Gram-negative bacteria. Our results showed variable value MIC on the tested bacterial strains. CSEO was highly active against two Gram-negative bacteria *Salmonella sp* with an ID of 34±3.35 mm and *P. aeruginosa* ATCC 27853 with an ID of 17±2.82 mm and towards one gram-positive bacterium *S. aureus* ATCC 25923 (ID of 21±2.82 mm), whereas it's moderately active against *P. aeruginosa* (16±2.12 mm) and low active against both *S. aureus* (ID of 11±0.00 mm) and *E. coli* ATCC 25922 with ID of 10±0.70 mm. The effectiveness of CSEO (10 µl/disc) was assessed and compared to gentamicin used as conventional drugs. Results show a great sensibility of bacterial strains against gentamacin than CSEO. Notably, *Salmonella sp* displayed lower susceptibility to the antibiotics compared to the EO, as indicated by a wider zone of inhibition observed for the EO treatment. In general, the tested EO is effective and competes with the examined antibiotics. In partial agreement with our findings, CSEO has demonstrated remarkable antibacterial activity against all bacterial species, whilst it was observed that *C. sempervirens* exhibited a slightly higher inhibitory activity against Gram-positive bacteria (Al Mouhajer *et al.*, 2017). Previous investigations of Layal *et al*. (2020) determined the ID for *P. aeruginosa* (7 mm) and *S. aureus* (12 mm) after the application of CSEO. The differences between our findings and the results of other authors may be due to the different origins of the microorganisms as well as the different origins of CSEO and its acquisition method. Ibrahim *et al.* (2017) analyzed the activity of CSEO on seven selected species (*S. aureus, S. epidermidis, S. pyogenes, E. coli, Klebsiella pneumoniae, Proteus vulgaris, P. aeruginosa* and *Shigella boydii*), and reported strong antibacterial effects were observed. Argui *et al*. (2021) also detected remarkable antibacterial activity against *S. aureus.* Al-Mijalli *et al*. (2023) determined the antibacterial activity of CSEO against *P. aeruginosa* (18.15 ± 0.68 mm), *S.aureus* (17.11 ± 0.28 mm) and *E. coli* (8.05 ± 0.56 mm). In our work, we detected almost similar zones of inhibition which nevertheless support the assertion that the CSEO has an important potential for the control of clinical bacterial species. Previous investigations have highlighted the bioactive chemical constituents of CSEO in the effectiveness of antibacterial activity (Al Mouhajer *et al.*, 2017. In case of *C.albicans*, CSEO has very strong antifungal activity (ID of 41±4.94 mm) and MIC of 2.9 µg/ml. Results obtained of Galovičová *et al.* (2023) confirmed with an ID of 11.33 ± 1.15 mm (very low compared to our result).

In the present study, great MIC values of **rosemary** essential oil (REO) (9 µg/ml) were detected for *S. aureus* ATCC 25923, MIC of 4.5 µg/ml for *E. coli* ATCC 25922, *Salmonella sp* and *Pseudomonas aeruginosa* and for *P. aeruginosa* ATCC 27853 and *S. aureus* an MIC value of 3 µg/ml (Fig16). Other studies reported the effectiveness of REO depending on whether the MIC of cultivated REOs are in the range of 0.315–2.5 mg/L and those of wild rosemary are between 0.625 and 5 mg/L of *S. aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *E. coli* ATCC 8739 and *P. aerugenosa* ATCC 9027 (Elyemni *et al.*, 2022). The antibacterial activity of rosemary essential oil (REO) has been tested on Gram-negative and Gram-positive strains (Fig 15). Indeed, EO provoked an inhibition of the growth of both Gram-negative bacteria *S.aureus* ATCC 25923 and *S. aureus* with inhibition diameters (ID) of the order of 11±1.41 and 26±3.35 mm and Gram-positive strains: *P. aeruginosa* ATCC 27853, *P. aeruginosa, E. coli* ATCC 25922 and *Salmonella sp.* with ID of the order of 15±1.41, 15±2.12, 26± 4.49 and 9±1.41mm, respectively. Results showed that REO had varying degrees of growth inhibition against bacterial strains (Table 20). Thus, it was highly active against *E. coli* ATCC 27853 and *S. aureus*, moderately active against the two species of *P.aeruginosa* and low active against *S.aureus* ATCC 25923. All inhibitory diameters are inferior to those of the positive control gentamicin, except, *E. coli* ATCC 25922 and *S. aureus.* Litterature data showed that rosemary EO had a more powerful effect on Gram-negative bacteria than Gram-positive bacteria (Yeddes *et al*., 2022; Valková *et al.*, 2021; Ben Arfa *et al*., 2022). In fact, this effect could be due to differences in cell membrane compositions which play the role of a barrier against macromolecules. In comparison with the results of Ben Arfa *et al*. (2022), the inhibitory action of Tunisian EOs against the growth of *E. coli* was 28.05 mm (superior to our findings), while ID of *Salmonella sp, S. aureus and P. aeruginosa* (5.3 ± 0.6 , 10.3 ± 0.6 and 7.0 ± 1.0 mm, respectively) were considerably inferior than our results. Generally, rosemary EOs exhibit strongest inhibitory efficiency against the growth of bacterial strains. On the other hand, EO exhibited the least antifungal activity against *C.albicans* with an ID of 9±2.12 mm, slightly superior to Valková *et al.* (2021) (8.0 ± 1.0 mm). Previous study by Jafari-Sales et Pashazadeh (2020) showed that rosemary EOs contains various compounds that can have antibacterial activity. It has been described that, in general, aromatic nuclei with polar functional groups provide the antimicrobial activity for essential oil (Guimarães *et al.*, 2019). The anticandidal activity of REO exhibits an MIC of 4.5 µg/ml and an ID of 9 mm. El-Baz *et al.* (2021) reported a MICs ranging from 16–2000 µg/Ml.

E. polybractea showed the highest effect with zone of inhibition between 20 and 40 mm for all selected species of bacteria (Fig 15). Study by Tian *et al*. (2020) on *Eucalyptus cloeziana, E. umbellata and E. benthamii* indicated that they are highly antibacterial agent. The results of antibacterial activity of Algerian *E. polybracteae* EOs (EPEO) revealed that all bacterial strains were ranked extremely sensitive according to the ID values expressed in mm, and their ID are greater than those of the positive control gentamicin. EPEO exhibited an inhibition of the growth of Gram-positive bacteria *S.aureus* ATCC 25923 and *S. aureus* with ID of 20 ± 0.50 and 40 ± 2.0 mm, respectively. Aldoghaim *et al.* (2018), reported that the largest zone of inhibition was observed for 50 µL of Australian EPEO against *S. aureus* ATCC 29213 (29.5 ± 0.7 mm) and Jeddi *et al.* (2023) from Morocco an ID of 18.4 ± 0.655 mm, which is lower than antibacterial activity of Algerian EPEO. In contrast, the IDs of Gram-negative bacteria *P. aeruginosa* ATCC 27853 (20±3.00 mm), *P. aeruginosa* (27±2.50 mm), *E. coli* ATCC 25922 (21±2.50 mm) and *Salmonella sp* (23±1.50 mm) were also lower than those of *Escherichia coli* ID from Aldoghaim *et al.* (2018), with (16.7 ± 0.6 mm) and Jeddi *et al*. (2023) with (17.13 ± 2.85 mm). EPEO exhibited great anticandidal activity with large ID which reached 29 mm and MIC of 2.3 µg/ml (Fig 16). Aldoghaïm *et al.* (2018) showed that the sensitivity of *Candida albicans* ATCC 90028 to EPEO (MIC% v/v) determined by the broth microdilution test was 8% (v/v).

Lantana camara essential oil (LCEO) showed promising antibacterial activities and comparable with standard antimicrobial agents. Although LCEO is highly active against *P.aeruginosa* and *P.aeruginosa* ATCC 27853 (27 ± 2.00 mm), followed by *E. coli* ATCC 25922 (26 ± 2.51 mm), *S. aureus* ATCC 25923 (25 ± 3.05 mm), *S. aureus* (21 ± 1.52 mm), it has moderate inhibitory action on *Salmonella sp.* (14 ± 2.08 mm) (Fig 15). Compared to gentamicin, all bacterial strains are sensitive to LCEO, except *Salmonella sp*. The MICs were 4.55 µg/ml for three bacterial strains and 2.77 µg/ml for the other tested bacterial strains (Fig 16). The findings contradict previous results of similar research that suggest that LCEO have strong antibacterial activity against *E. coli* ATCC 25922 with an ID of 22±1.41mm, *S. aureus* ATCC 2523 (14.5±0.71), *Salmonella infantis* SKN 557 (8.5±0.71) and *Pseudomonas aeruginosa* ATCC 9027 (6±00 mm) (Semdé *et al*., 2018). El Baroty *et al*. (2014) found that the essential oil of *L. camara* fresh leaves was not active on *S.aureus* ATCC 25923, *E. coli, Salmonella typhimurium, P. aeruginosa* ATCC 9027 and *Candida albicans*. In another study LCEO showed weak inhibitory activity against Gram-negative bacteria: *E. coli* (ATCC25922), *Klebsiella pneumoniae (ATCC13883)* and *P. aeruginosa* (ATCC27853) and

Gram-positive bacteria: *Staphylococcus aureus* (ATCC25923) and *Streptococcus pneumoniae* (ATCC49619) (Mbayo-Marsi *et al*., 2021). Findings from this study corroborate those reported by Deena and Thoppil (2000) who observed that LCEO had antibacterial activity towards both Gram-positive as well as negative bacteria. However, the anti-bacterial effect of essential that essential oil of dried leaves of *L. camara* was more active on gram-positive bacteria than gram-negative bacteria (Semdé *et al*., 2018). The ability of essential oil to exhibit anti-bacterial activity is contributed by its different components whose modes of action involve several targets on the bacterial cell. LCEO inhibited *Candida albicans* with MICs value of 2.77 µg/ml and a large ID of 23±1.52 mm. It seem's that the yeast is more sensitive to LCEO than gentamicin. Mbayo-Marsi *et al.* (2021) reported that LCEO was inactive on this yeast, with MIC of 3.28 mg/ml. The variation of the antimicrobial activitiy between the all investigated EOs samples can be attributed to their chemical composition, in particular to their abundant compounds.

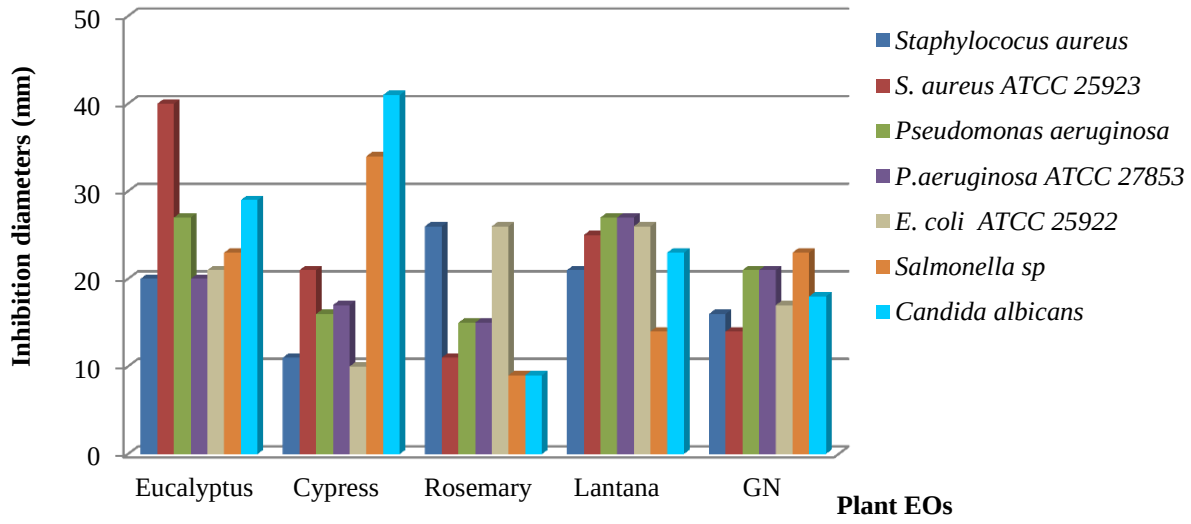


Figure 15. Antimicrobial activity of different plant EOs

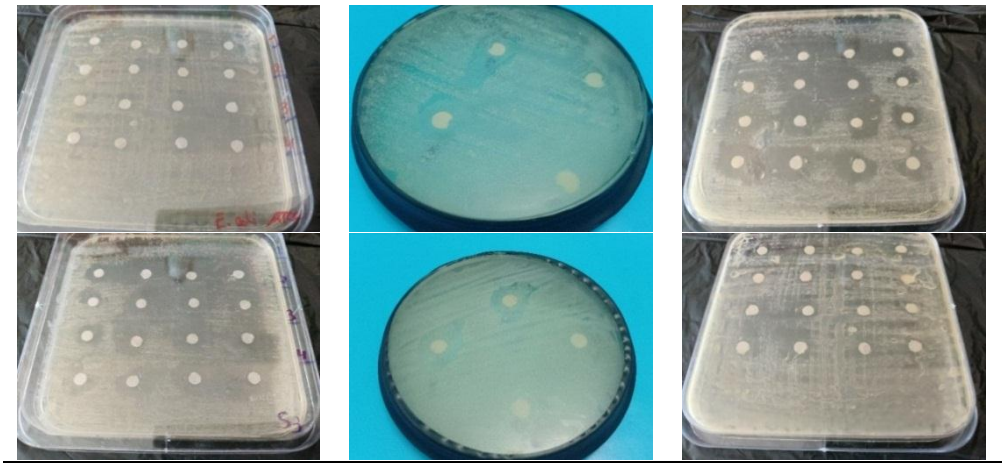


Figure 16. Antibacterial activity of EOs

Table 26. Minimal Inhibitory Concentration of essential oils

Strains	Eucalyptus	Cypress	Rosemary	Lantana
Staphylococus aureus	4.6	8.7	3	4.55
***S. aureus* ATCC 25923**	3.06	2.17	9	4.55
***Pseudomonas* aeruginosa**	4.6	8.7	4.5	4.55
***P.aeruginosa* ATCC 27853**	2.3	2.17	3	2.77
***E. coli* ATCC 25922**	3.06	8.7	4.5	2.77
Salmonella sp	3.06	2.9	4.5	2.77
Candida albicans	2.3	2.9	4.5	2.77

7.5. Antifungal activity

7.5.1. Antifungal activity of EOs

The antifungal activity of plant EOs extracted by Clevenger apparatus from four different species (Cypress, Rosemary, Blue mallee and Lantana) was investigated against ten common fungi isolated from stored wheat grains and compared to a control (Fig 21). In general, diameters of fungal growth (DFG) were inversely proportional to the concentrations of different EOs and control, which was 90 mm. It is considered that plant EOs possess an antifungal property that could be a result of a single compound or of a combination of compounds acting synergistically or antagonistically (Tariq *et al*., 2019). According to Santos *et al*. (2020), fungi of the genera *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. can damage stored seeds, reducing their quality, as these fungi develop in tissues of embryos, causing discoloration and seed rot. Jeldi *et al.* (2022) report that the mechanism of action of EOs appears to involve disturbing the cell wall and plasma membrane, leading to cell lysis and leakage of intracellular compounds, and ultimately cell death. This study indicated that the antifungal activity is variable depending on the dose, fungal strain and tested oils. It

should be noted that the Penicillium controls reach the edge of the Petri dish after 3 days of incubation (DFG of 90 mm) while the rest of the fungal strain controls require 4 days. The solid medium EO dilution method is superior to the diffusion method for assessing antifungal effect (Klančnik *et al*., 2010) due to its ability to visualize fungal growth on the petri dish, resulting in clearer results.

The results of the antifungal activity of ***Cupressus sempervirens*** EO (CSEO) (concentration 100, 150, 200, 250 and 300 µl) against ten stored wheat grains fungi on agar plates (Fig 17) revealed that CSEO had a variant inhibitive effects on the growth of fungal species and the increase of oil concentration leads to increase of growth inhibition, hence; decrease of the colony diameter, and concentration of 300 µl proved the optimum results. Based on our findings, the DFG of seven fungi ranged between 79 to 10 mm, while the three Penicillium species were resistant. Regarding *A. flavus, Alternaria alternata*, *Cladosporium herbarum* and *Trichoderma viride*; CSEO has very strong activity, with a DFG of 16, 14, 17 and 10 mm, respectively. In their work, Azzaz *et al*. (2019) reported the effect of cypress volatile oil against the growth of *Aspergillus niger* and *Fusarium oxysporum* for 2 weeks. While Aspergillus at 100 mg/ml, shows a DFG of 13 mm, Fusarium at 75 mg/ml presents an ID of 7 mm in the first week and then disappears. Previous work of Alfazairy (2004), CSEO displayed insignificant antifungal activity against *Aspergillus sp*., *Penicillium* sp., *Fusarium* sp., and *Mucor* sp., as tested by the agar disk diffusion method, which support our results. In contrary, CSEO experienced against seven fungi (*A. niger, A. flavous, A. fumigatus, F. solani, F. oxysporium, P. digitatum*, and *C. terreus*) shows after 96 hour a DFG of 5.7 mm against *F. solani* to 29 mm against *P. digitatum* (Mahmood *et al*., 2013). Regarding Amri *et al.* (2013) results, CSEO show a significant antifungal activity against 8 cultivated crop fungi (*Fusarium culmorum, Fusarium oxysporum, Fusarium equisiti, Fusarium verticillioides, Fusarium nygamai, Botrytis cinerea, Microdochium nivale var. nivale* and *Alternaria sp*). Essential oils isolated from *Cupressus sempervirens* var. *dupreziana* leaves were tested for antifungal activity against 10 agricultural fungal species (*Gibberella avenacea, Fusarium culmorum, Fusarium oxysporum, Fusarium subglutinans, Fusarium verticillioides, Fusarium nygamai, Rhizoctonia solani, Microdochium nivale, Alternaria alternaten* and *Fusarium culmorum*). Results of in vitro antifungal test assays showed that oils significantly inhibited the growth of 10 plant pathogenic fungi (Amri *et al*., 2013; Al-Snafi, 2016).

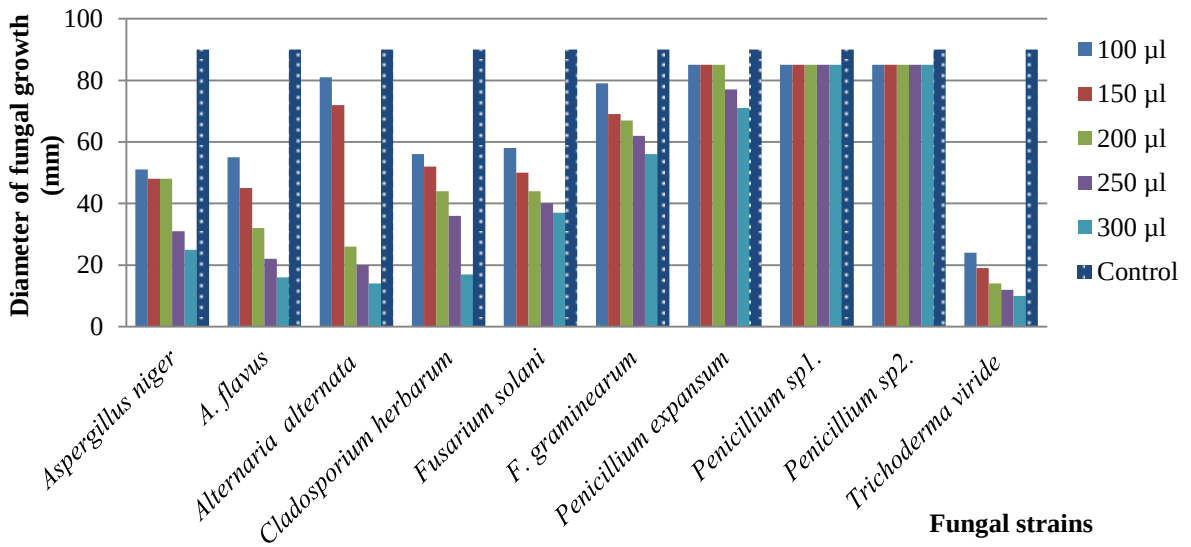


Figure 17. Antifungal activity of CSEO

Therefore, the *in vitro* antifungal activity of **rosemary** EO (REO) on inhibiting mycelial growth of the ten fungal pathogens of stored wheat grains was investigated. Antifungal activity varied from weak to very strong at 300 µl, with particular attention to two species of the genus Penicillium and Cladosporium which were found to be very resistant (DFG of 85 mm) (Fig18). Overall, as the concentration of EO increased, the inhibitory effect on the growth of seven tested fungi species gradually increases. Contrary results of the present study were found in the research of Nakada-Freitas *et al*. (2022) studying REO; they related no efficiency in the control of *A. flavus* at 25 µL mL^{-1} concentration. Wherease, Lee *et al.* (2020) concluded efficiency in the control of *A. flavus* using rosemary essential oil at 25 µl/ml concentration. Baghloul *et al*. (2017), reported that REO with 1,8-cineole major compound (63.6%), has a fungistatic activity on foods contaminated by *A. niger* at a minimum inhibitory concentration of 0.5%. Valková *et al*. (2021), support our result obout the resistance of Penicillium to REO. In their work, REO (125, 250, and 500 µL/L) displayed no inhibitory impact on the growth of *P. crustosum*, and *P. citrinum* and *P. expansum* were significantly ($p < 0.05$) inhibited by the EOs in the highest concentrations.

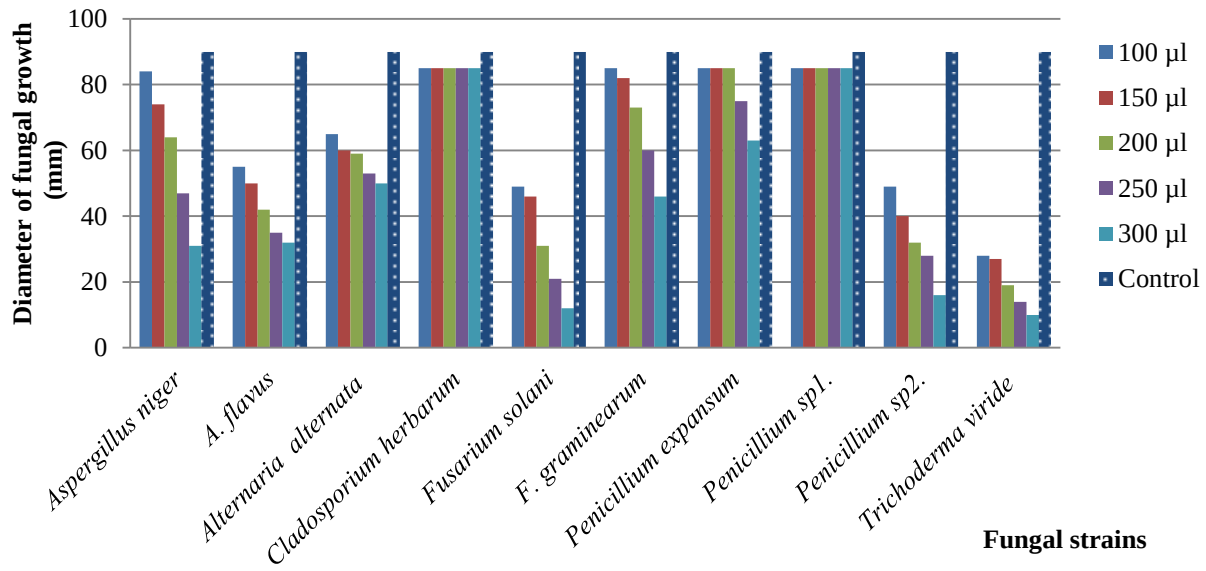


Figure 18. Antifungal activity of Rosemary EO

The majority of the detected fungi in stored wheat grain *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* are common mycotoxigenic fungi. Our results (Fig19) revealed that the growth inhibition of fungi strains depends on the type and concentration of the EO used. Remarkable antifungal activity was observed for ***Eucalyptus polybracteae*** essential oil (EPEO), which decreased the colony diameter of *P. sp*2 and *T. viride* from 71 mm (100 µl) to 9 mm (300 µl) and from 87 mm (100 µl) to 17 mm (300 µl), respectively. EPEO displayed insignificant antifungal activity against *A.niger, Cladosporium herbarum, P.expansum* and *P. sp1*. The efficacy of EPEO against microorganisms could in part be attributed to their volatile bioactive oil components (Sharma *et al*., 2022). Previous research of Schrodera *et al*. (2017) revealed the resistant of *Penicillium sp* and *Aspergillus sp* against eucalyptus oil. In contrast to our study, the results of Pedrotti *et al*. (2022) showed that *Eucalyptus staigeriana* oil was effective in reducing the incidence and severity of black rot caused by *Alternaria alternata* in preventive and curative treatments at different concentrations, indicating resistance of *Alternaria alternata*. In general, the composition of the fungal microbiota of wheat grains found in the current study agrees with previous studies of Kumari *et al*. (2019); Al Bedak *et al*. (2020). Eucalyptol is a bioactive terpenoid plant constituent and its antifungal properties have been explored previously by Ivanov *et al.* (2021).

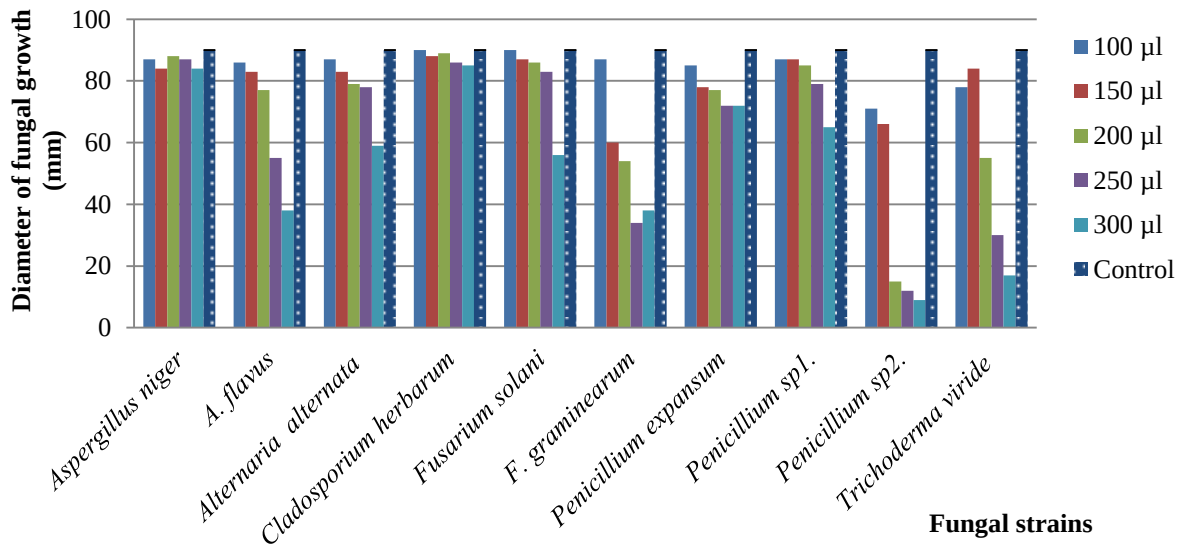


Figure 19. Antifungal activity of *E. polybractea* EO

In the present work, ***Lantana camara*** essential oil (LCEO) shows very strong activity against most of the fungal strains (Fig20). This finding has been supported by many previous studies (Nyiro *et al*., 2020 and Bhattarai and Jha, 2016). The growth diameters of six fungi (DFG) at 300 µl were ranging between 6 and 22 mm. Both *Penicillim expansum* and *P. sp2* were the most resistant and their growths were not affected (DFG = 85 mm), whereas, P. sp1 moderately affected and its growth diameter was 47 mm. According to the search results, there is an effectiveness of LCEO against Aspergillus. Essential oil of *Lantana camara* showed also effective anti-fungal activity against *Alternaria alternata* and *Fusarium oxysporum* at different concentration (10, 15 and 20 mg/ml) which is supported by Bhattarai and Jha (2016), who showed that LCEO remarkably inhibited the growth of Colletotrichum, Fusarium and *Alternaria spp*. Nyiro *et al*. (2020) reorted that this EO strongly inhibited the growth of *F. oxysporium* (DI=44 mm). The fungi counteracted the oil by developing more pigmentation, sclerotia, concentric rings and some aerial mycelia which indicate the presence of stress factors in its growth. *Aspergillus spp* and *Fusarium spp* growth on food items results in the production of mycotoxins. Nawaz *et al.* (2016) supported our results that LCEO has strong antifungal activity against *Aspergillus niger, Penicillium digitatum, Aspergillus nidulans, Cladosporium herbarium* and *Rhizopus nigricans* by disc diffusion method, except three species of Penicillium in our work were resistant. According to Nawaz *et al.* (2016), organic volatile compounds like cyclic hexadepsipeptide compound might be responsible for their anti-fungal activity. Moreover, this activity is due to the presence of different bioactive constituents such as Eicosane, squalene, β-ionone, caryophyllene oxide, β-

caryophyllene, hexanoic acid and tiglic acid, a mixture of lantanilic, camaric acids, and lantadene (DelgadoAltamirano *et al.*, 2019). A research article suggests that lantadene A and boswellic acid isolated from the leaves of *Lantana camara* have the potential to control phytopathogenic Fusarium species (Seepe *et al.*, 2022). Another study mentioned in the search results that isothiocyanate, a component of LCEO, was effective against Penicillium species (Swamy *et al.*, 2016). A search result mentioned a study indicating that isothiocyanate, which is a constituent of essential oil from *Lantana camara*, was effective against Trichoderma species (Nurby *et al.*, 2011). In the scientific literature, few data exists on the antifungal effect of Lantana essential oil compared to that of extracts from different parts of the plant.

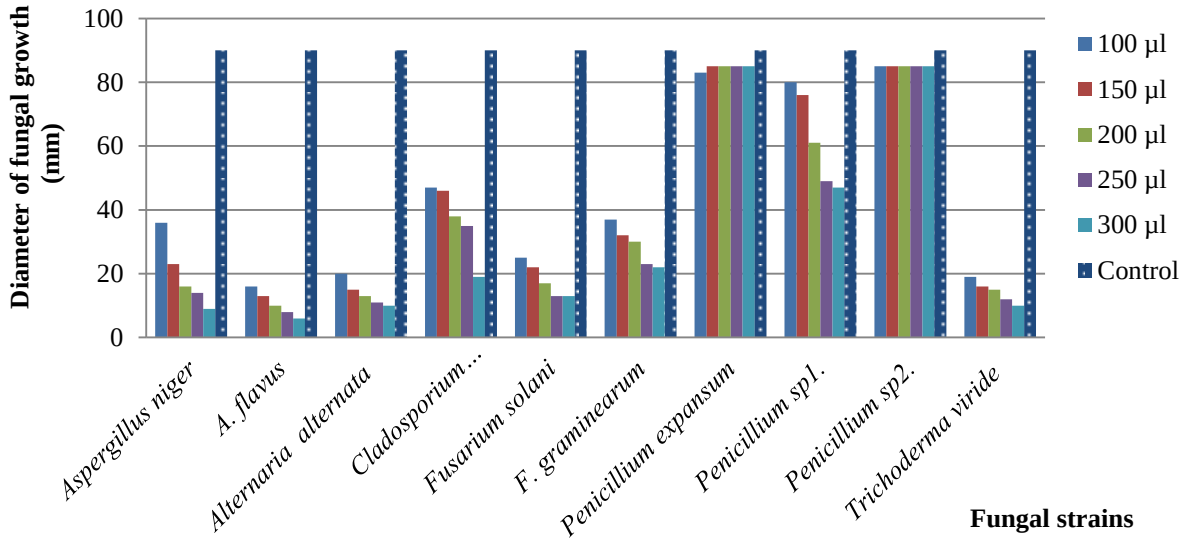


Figure 20. Antifungal activity of Lantana EO

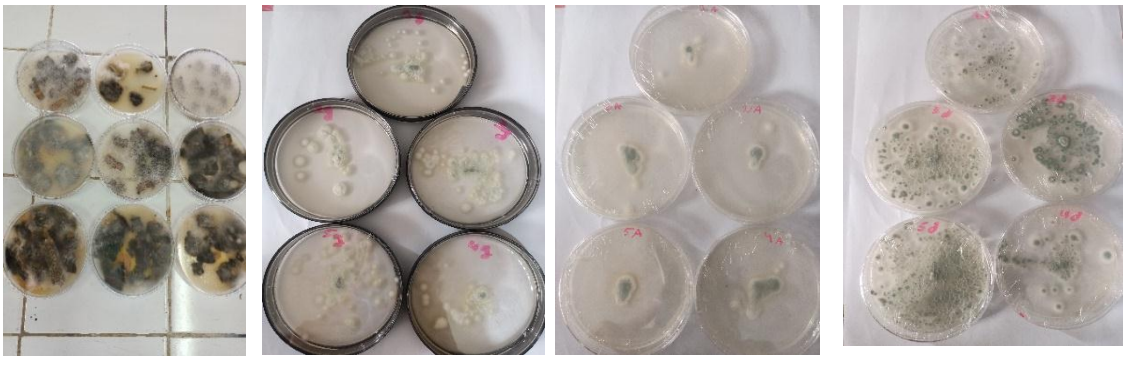


Figure 21. Antifungal activity of EOs

7.5.2. Antifungal effect of crude Extracts

The antifungal potential of the extracts was evaluated in terms of mycelial growth area of the fungi compared to the control. In addition, the experiment carried out also focused on the changes in the morphology of fungi under the influence of plant extracts. The most common were changes in the mycelial structure and coloring of the obverse and reverse of the colony. With increasing concentration of extract in the medium, changes in colony structure intensify. However the sensitivity of different fungal species to these extracts varied which may be due to the difference in intrinsic tolerance of these isolates. It is important to note that certain extracts even tend to accelerate the mycelial growth of certain species. Literature studies have demonstrated the richness of medicinal plant leaves in different biomolecules such as tannins, flavonoids and phenolic acids which have a very direct effect on pathogenic fungi. (Kursa *et al*., 2022). Variations in the sensitivity level of fungal species could be due to the inherent tolerance of fungi (distinctive cell wall) and the mixtures of bioactive molecules present in the extracts (Ali et Abbas, 2023). Additionally, Satish *et al.* (2007) claimed that the antifungal activity of plant extracts prepared in various solvents is considerable. According to Lagrouh *et al.* (2017), plant extracts and essential oils can affect pathogenic fungi through six mechanisms of action: inhibition of electron transport in mitochondria, inhibition of cell

division, interference with nucleic acids synthesis and/or inhibition of protein synthesis, and inhibition of efflux pumps.

- ***M. alba* aqueous leaf extract**

The aqueous extract of *Morus alba* leaves exerted moderate to weak antifungal activity on the strains tested (Fig22). Each fungal species reacted differently to the addition of plant extracts to the substrate and to their concentration. The strongest antifungal effect was recorded at 300µl for *A. terreus* with a mycelial diameter of 43±8.62 mm, followed by *F. solani* with 46±10.26 mm. Regarding the treatments; the weakest effect of the extract was recorded for all concentrations, especially against *F. graminearum* and *R. oryzae,* where the fungal growth diameter was 85 ±0.00 mm. Our results concerning *Rhizopus oryzae* are far from those of Viloria *et al.* (2017), who reported an inhibition zone with an interesting diameter of 19.83 mm. A similar result was also reported by Niratker *et al.* (2015), whose methanolic extract of mulberry (*Morus indica*) has maximum antifungal activity. Indeed, among the three species of Aspergillus tested in this study, the antifungal activity of the aqueous extract of medicinal plant with leaves selected on *A. terreus* was moderate and varied between 53 and 43 mm. On the contrary, Serrano *et al.* (2018) showed that with a hot water extract of *M.alba,* the antifungal activity against *A. niger* gave an inhibition zone with a diameter of 17.60 mm. Similarly, Hussein *et al.* (2021) using a distilled water plant extract on the same species, reported 1.33 ± 0.44 cm of growth, while Ghareeb *et al.* (2016) affirmed the absence of effect of this extract against *A. niger*. In another study on the effect of 0,8% of *Morus nigra* aqueous leaf extract on both *A. flavus* and *A.terreus* was 26.3 and 22 mm respectively. Landero-Valenzuela *et al*. (2017) mentions the significant effect of plant aqueous extracts, including *Morus alba* leaf extracts, against *Penicillium expansum*, where the diameters of inhibition of radial growth were 79, 66 and 62 mm under 4, 8 and 12% of extract, which is closer to our findings. Another study by Omidiran *et al.* (2012) highlights the antifungal activity of the aqueous extract of white mulberry leaves (*Morus alba* L.) on fungal species including *A. niger, A. tamari, Fusarium oxysporum* and *Penicillium oxalicum*, where their results were more powerful in suppressing fungal growth than ethanolic extract.

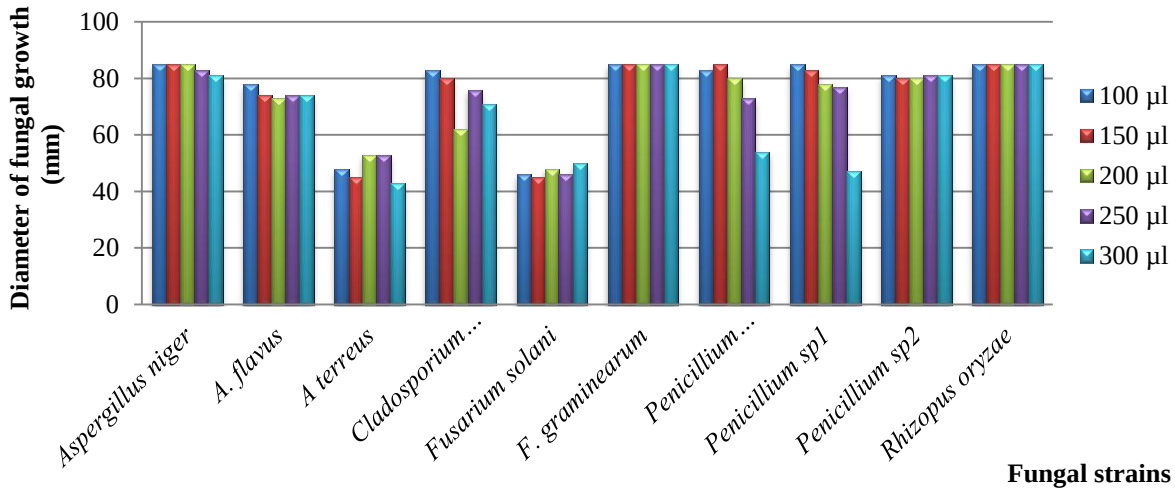


Figure 22. Effect of *M. alba* aqueous leaf extract on fungal species

- ***M. alba* methanolic leaf extract**

In the present study we studied the antifungal activity of the methanolic extract of white mulberry leaves, which seems very interesting given that the extract exerted a very strong antifungal activity on half of the strains tested.It is important to note that, as reported by Kwon *et al*. (2019), the methanolic extract of mulberry leaves even tended to accelerate the mycelial growth of some species. This is the case for *A. terreus*, *C.herbarum* and *F. graminearum* (Fig 23). While for the rest, mycelial growth decreases with increasing doses of extracts. *Rhizopus oryzae* appears to be the most sensitive species and its mycelial growth at the highest dose had a diameter of 8 mm. The extract used at a rate of 300 µl showed also a very strong effect on *A. niger* and *A. flavus*, where their growth diameter was 11 and 16 mm respectively. Our results are consistent with the findings of Anwar *et al.* (2015) because the extract showed very high activity for both species with a diameter of 13.5 and 14.9 mm respectively. On the contrary, Hussain *et al.* (2021) reported moderate activity against *A. niger* with a fungal diameter of 4.50 ± 0.29 cm. Interestingly, Ghareeb *et al*. (2016) demonstrated no effect of this extract against *A. niger*. However, Niratker *et al*. (2015) observed that the methanolic extract of *Morus alba* leaves was most effective against the two fungal species Aspergillus and Penicillium with the largest inhibition zone diameter of 30 mm and 20 mm, respectively. On the other hand, the methanolic extract of *M.alba* showed very strong activity on *Penicillium expansum* with a zone of 20 mm, while Landero-Valenzuela *et al.* (2017) reported a fungal diameter of 53 mm at 12%. Additionally, *Fusarium solani* and *F. graminearum* showed sensitivity to the plant extract at 300 µl and their growth was 29 and 12, respectively. In contrast, Kwon *et al.* (2019) revealed that methanolic extract of mulberry

leaves did not suppress the mycelial growth of *Fusarium sp* and *Alternaria sp*. but tended to accelerate their mycelial growth.

The findings of the present study postulate that the methanol extract is more potent compared to the aqueous extract in terms of fungal activity. Methanol is a good solvent for extraction and it is frequently used in biology because of its polarity. It is capable of extracting both lipophilic and hydrophilic substances. *M. alba* leaves were rich of bioactive compounds, such as phenolic acids, flavonoids, flavonols, anthocyanins, and others biomolecules (Chen *et al*., 2021).

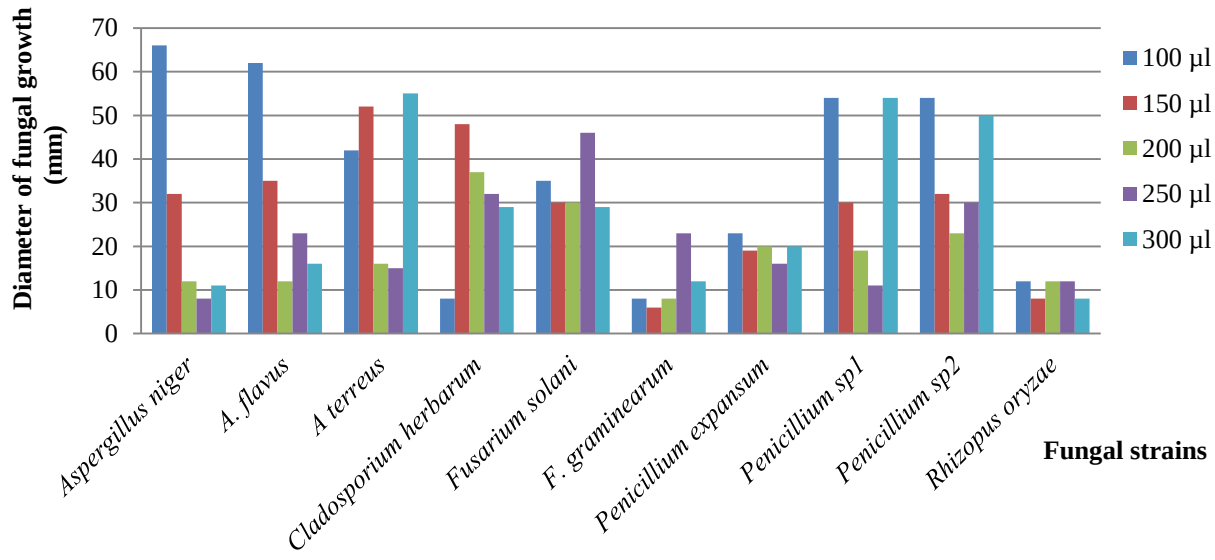


Figure 23. Effect of *M. alba* methanolic leaf extract on fungal species

- ***Rubus ulmifolius* aqueous leaf extract**

The antimicrobial effects of Rubus species are rarely evaluated, especially with regard to fungal strains. Therefore, the antifungal activity could in most cases be attributed to phenolic acids (da Silva *et al*., 2019) and also the presence of tannins, saponins, cardiacglycosides found in phytochemical screening in *R ulmifolius* (Chabane et Saidi, 2014). Rubus extracts were tested for antimicrobial activity against various fungi and bacteria. The leaves of *Rubus idaeus* L. and *Rubus fruticosus* L. were found to possess antimicrobial activities against a range of microorganisms. However, no activity was observed against *Penicillium ciclopsis*, *Aspergillus niger*, *Mucor sp*., and *Trichothecium roseum* (Kucharski *et al*., 2022). It is also important to note that the results are limited to the strains tested

In the present study, the **aqueous extract of *R.ulmifolius*** leaves evaluated against fungal strains appears to have low activity against the vast majority of species (Fig 24). This

is the case for *A.niger, F. graminearum, P. expansum, P. sp1., P. sp2.* and *Rhizopus oryzae*, whose growth diameter varies between 81 and 85 mm. Conversely, *F. solani* seems to be sensitive (34 mm). However, our results are close to those of Chabane and Saidi (2014) who reported zero activity of this extract on *A. niger*. A study conducted by Sisti *et al.* (2008) against Geotrichum, revealed that the aqueous extract of *Rubus ulmifolius* exhibited antifungal activity in vitro. Moreover, plant extracts of wild blackberry (*Rubus ulmifolius*) completely inhibit mycelial growth of *Geotrichum candidum* fungus, which causes the rot pathogen of citrus plants in roots, both *in vitro* and *in vivo* (Jiménez-Reyes *et al.*, 2018)

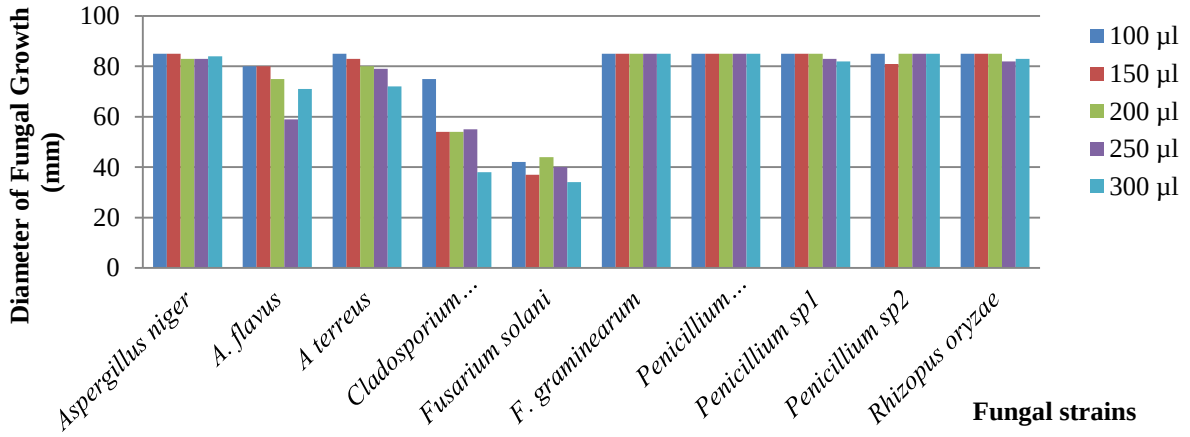


Figure 24. Effect of *R. ulmifolius* aqueous leaf extract on fungal species

➢ ***Rubus ulmifolius* methanolic leaf extract**

The methanolic extract of *R.ulmifolius* leaves was also evaluated against fungal strains and it was noted that this extract had better antifungal activity than that of the aqueous extract, which is supported by the work of Castillo *et al.* (2019). Therefore, our results show that *Fusarium graminearum, Penicillium expansum, P. sp1* and *Rhizopus oryzae* were resistant and their mycelial growth at 300 µl ranged from 80 to 85 mm. On the other hand, the activity of the extract seems to be strong at 300 µl for *A. niger, A. flavus, Cladosporium herbarum* and *Fusarium solani*. The smallest diameter of fungal growth was 25 mm, while very high activity was recorded against *A. terreus* with a diameter of 16 mm (Fig25). The influence of solvent polarity on the antimicrobial activity of *R. ulmifolius* extracts was previously observed by Panizzi *et al.* (2002) as well as Veličković *et al.* (2021) with the extract of the fruit of *R. discolor*. They found that antimicrobial effectiveness increased with solvent polarity, which is in agreement with our results. In fact, Boy *et al.* (2023) report resistance of two strains of aflatoxigenic mold *A. flavus*, Cq103 and Cq8 to the phenolic extract at 150 and 250 mg/L, which also supports our results, despite the fact that Mingo *et al.* (2016) suggested that the

phenolic compounds composition was also strongly involved in the antimicrobial effect. For instance, methanolic extract of dried *Rubus ulmifolius* plantlets has been found to control isolates of Fusarium species (Fraternale *et al.*, 2007). Additionally, Kucharski *et al.* (2022) showed that the leaves of *Rubus idaeus* and *Rubus fruticosus* exhibit antifungal properties against a variety of microorganisms, including Fusarium species. A study of Chabane and Saidi (2014) contradicts our results and reported the resistance of *A. niger*. Based on the findings of Castillo *et al.* (2019), the particularly high activity of methanol extracts was attributed to phenolic acids (gallic, ferulic, and possibly caffeoylquinic acid) and tannins. According to Fraternale *et al.* (2007), most of the antifungal effect of Rubus extract comes from an additive effect of different endogenous tannins.

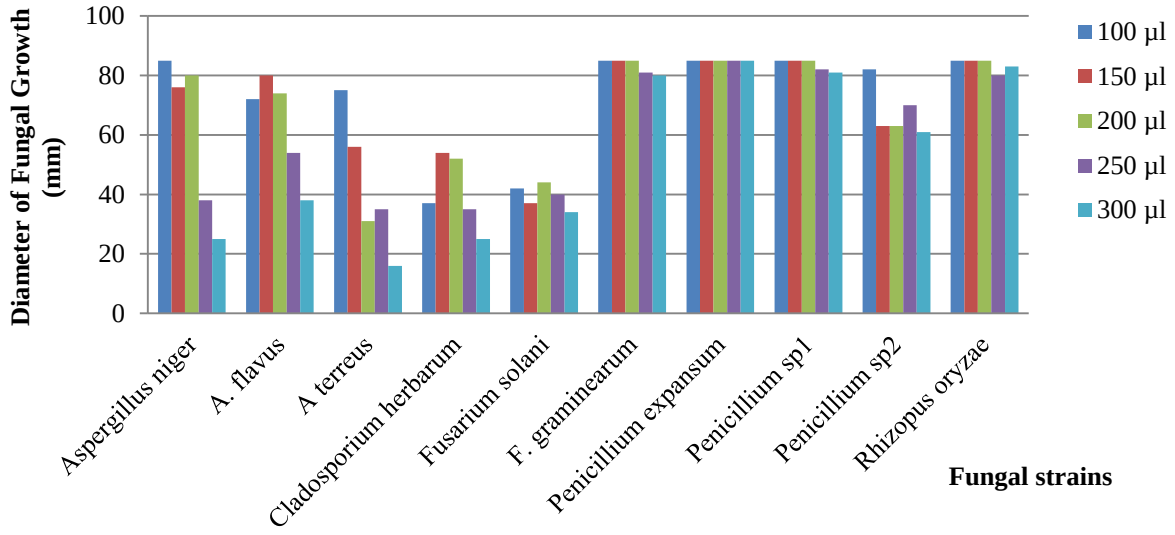


Figure 25. Effect of *R. ulmifolius* methanolic leaf extract on fungal species

➢ **Effect of the combination of methanolic extracts of leaves of *R. ulmifolius* and *M. alba***

This study investigated the antifungal activities of single and combined methanolic extracts of leaves of *R. ulmifolius* and *M. alba* against the selected fungal pathogens. Since the methanolic extracts of leaves of *R. ulmifolius* and *M. alba* provide very powerful antifungal properties, the combination (50/50, v/v) between them also gave a relevant result. Our results showed that effect of the combination of the two methanolic extracts against fungal strains was found to be less effective than that of the methanolic extract of *M.alba* alone. However the sensitivity of different species to the combinaison varied. We note that *Rhizopus oryzae* and *P. sp2*. were the most resistant (85 mm), followed by *P. expansum* and *P. sp1*. with low fungal activity. The combined activity of the two extracts is described as “strong” against *F.*

solani, *F.graminearum* and *A. terreus* with a growth diameter of between 34 and 40 mm, while it is very strong against *A. niger* and *A. flavus* (Fig 26).

The combination between extracts could produce an effect that could be synergistic, antagonistic or additive against the fungi tested. In case of additive, the effect is observed when the combined effect is not greater than the individual effect of the most active plant extract. In this case, even lower concentrations than those obtained with the individual extracts alone also produce the same effect. For the synergistic effects, the concentration of each extract in combinations need not be equal to that obtained when it is used separately. If reduced amounts of each interacting extract or a particular component alone can react, they will still produce the required synergy. Sometimes the effect observed may indicate that the activities of the extract have decreased and this effect is called subadditive, which reflects our results. These findings might be due to some components which are antagonists and might neutralize each other and weaken their antifungal activity (Masoumian et Zandi, 2017).

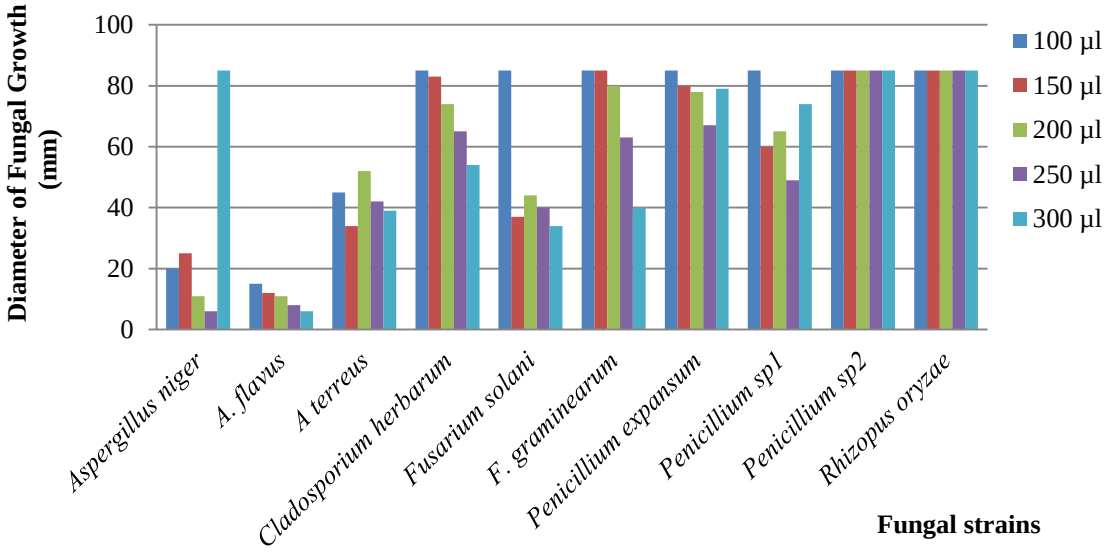


Figure 26. Effect of the combinaison of methanolic extracts of leaves of *R. ulmifolius* and*M. alba* on fungal species

- **Lantana ethanolic leaf extract**

The ethanol extract of *L. camara* was found to have strong inhibitory activity against all the tested strains. This agrees with the report of Ezebo *et al.* (2021) who attributed this to the fact that ethanol is an organic solvent and will dissolve organic compounds better, hence liberate the active compounds needed for antifungal activity. The inhibitory activity of the ethanol leaf extracts increased with concentration. This agrees with the report that more active ingredients in plants are dissolved in solution at higher concentrations. So far, researchers

reported different activities based on the type of the extract and the plant organ used (Zare, 2021). In the present study, the ethanolic extract of leaves of this plant at 300 µl has very strong activity against *Aspergillus flavus* (12mm) and *F.solani* (0 mm), strong activity against *A. niger*, *P. sp.1*, *A. terreus* and *R. oryzae* (respectively 30, 23, 32 and 29 mm) and low activity against *Penicillium expansum* and *P. sp*.2 (Fig 27). Kulkarni *et al.* (2019) support our results, reporting that the ethanolic extract of this plant exhibited marked antifungal properties against *A. niger* and *Rhizopus oryzae*. Also, Zare (2021) reported very high activity against *A. niger* with a growth diameter between 9 and 1.66 mm for doses of 6.25 to 50%. For their part, Oladoye *et al.* (2021) partly support our results for *A. niger* and *A. flavus* (11 and 8.5 mm), but are far from ours for *F. solani* (26 mm); where our extract completely inhibited its growth from 150 µl. On the other hand, Abdel-Hafez *et al.* (2021) recorded a closer result of 6 mm growth of *F. solani*. Specific compounds, such as lantadene A and boswellic acid, have been identified from *L. camara* that have the potential to control phytopathogenic Fusarium species (Seepe *et al*., 2022). It should be noted that *L. camara* leaf extracts have been found to exhibit antifungal activity against *Fusarium solani* that causes rhizome rot of ginger (Joshi *et al*., 2023). Furthermore, Kumar (2017) revealed low antifungal activity of Lantana leaf extracts of petroleum ether, diethyl ether, chloroform and acetone against *Penicillium sp*. The report of Saraf *et al.* (2011) revealed that *A. niger* (FGCC 492), *A. flavus* (FGCC 133), *Penicillium sp* (FGCC 124) and *Alternaria alternata* (FGCC 418) exhibited maximum percentage growth inhibition at 1000 µg/ml concentration. Bashir *et al.* (2019) alluded to the presence of saponins, tannins, phlobatannins, flavonoids, terpenoids, and steroids as phytochemical constituents responsible for the antifungal activity of the leaf extract of *Lantana camara*. In addition, extracts of the plant have been found to be effective in controlling green mould disease caused by *Trichoderma harzianum* in cultivation (Gaur *et al*., 2023).

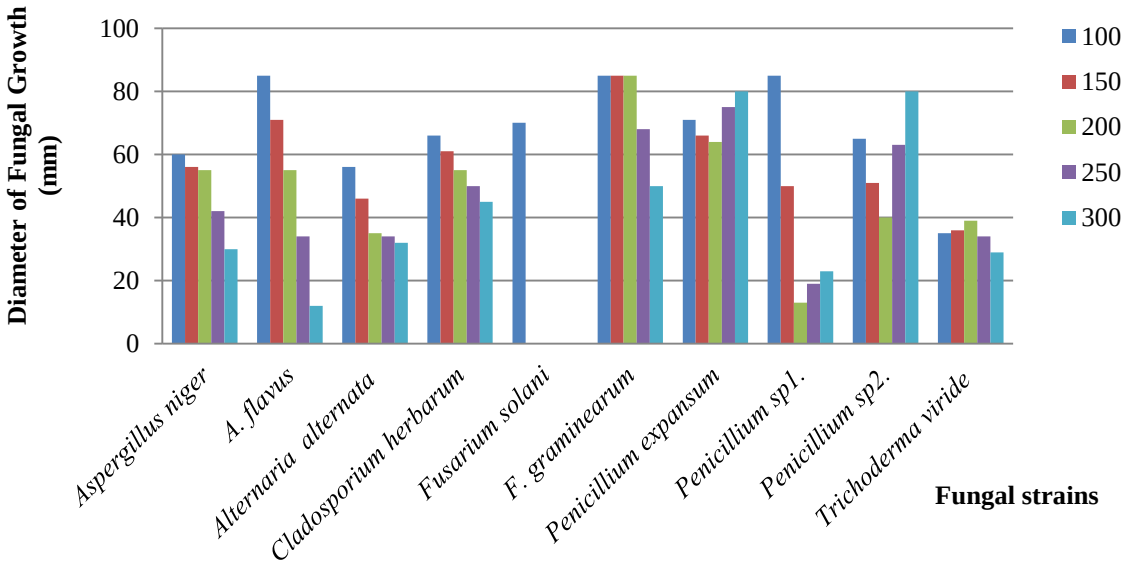


Figure 27. Effect of *L. camara* ethanolic leaf extract on fungal species

➢ ***Platycladus orientalis* ethanolic cones extract**

Mycelia inhibition method used in the evaluation of the antifungal potential of the ethanolic extract of *P. orientalis* cones shows low activity against all fungal strains tested, whereas *P.expansum*, *P.sp*.1, *P.sp*.2 and *Trichoderma viride* appear to be resistant. Overall, the data indicate that this activity seem to be dose dependant (Fig 28). First of all, the results of our studies are contradictory with those of Ali (2017) who observed that the addition of alcoholic extract of foliage and fruit of *Thuja orientalis* in culture medium (PDA) at a rate of 0.2, 0.5 and 1% induced a strong reduction in the growth of *A. flavus* with inhibition percentages of 70.25, 45.40 and 100% for the three concentrations. Also, Ain *et al.* (2021) recorded against *Fusarium oxysporum f. sp. cubense* an inhibition rate of 50.24 and 65.83% for respectively 5% and 10% of the same plant cone extract. Furthermore, for the same fungal species, the ethanolic extract of *Thuja orientalis* leaves presented close results with those of the ethanolic extract of the cones with 47.74 and 62.38%. It can be attribuated to the high amount of phenolics and flavonoids present in ethanolic leaf extract of *T. orientali.*

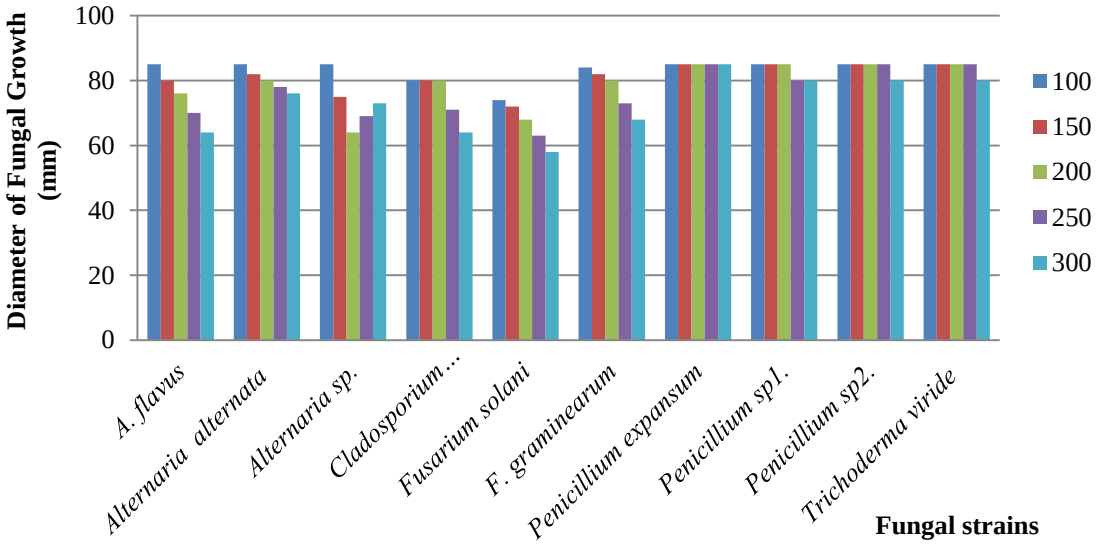


Figure 28. Effect of *P. orientalis* ethanolic cones extract on fungal species

➢ **Effect of *Dittrichia viscosa* aqueous leaves extract**

Overall, this extract showed high antifungal activity against all fungal strains tested, except resistant of *F. solani* (Fig29). According to Pane *et al.* (2023), the extract of dried green parts and fresh inflorescences of this plant at the highest concentration significantly reduced the growth of the fungal pathogen *Alternaria alternata* to 54%. Our results are in line with findings reported in Abdel Rahman *et al*. (2022), that the antifungal activities of Inula, onion, basil, and quinoa extracts were evaluated using the food poison method, shows that the Inula aqueous extract was the most potent among the tested plant extracts, and it was found to inhibit fungal growth by 63.52%, 70.35%, and 65.48% for *Aspergillus sp.1*, *Aspergillus sp.2* and *Fusarium sp*., respectively. While Gueribis (2020) observed resistance in the strains *Fusarium culmorum, F. graminearum, F. solani, Alternaria sp., F. oxysporum fsp. cicero, Botrytis cineria, Phoma medicaginis pinodella* and *Didymella pinodes* in the presence of aqueous extract of this plant. According to Abdel Rahman *et al*. (2022), the major classes of phytochemicals present in the tested aqueous extract of Inula were alkaloids, glycosides, phenols, tannins, and terpenoids, particularly caffeic acid derivatives and flavonoids were mainly related to the inhibition of bacterial and fungal growth (Grauso *et al.*, 2020).

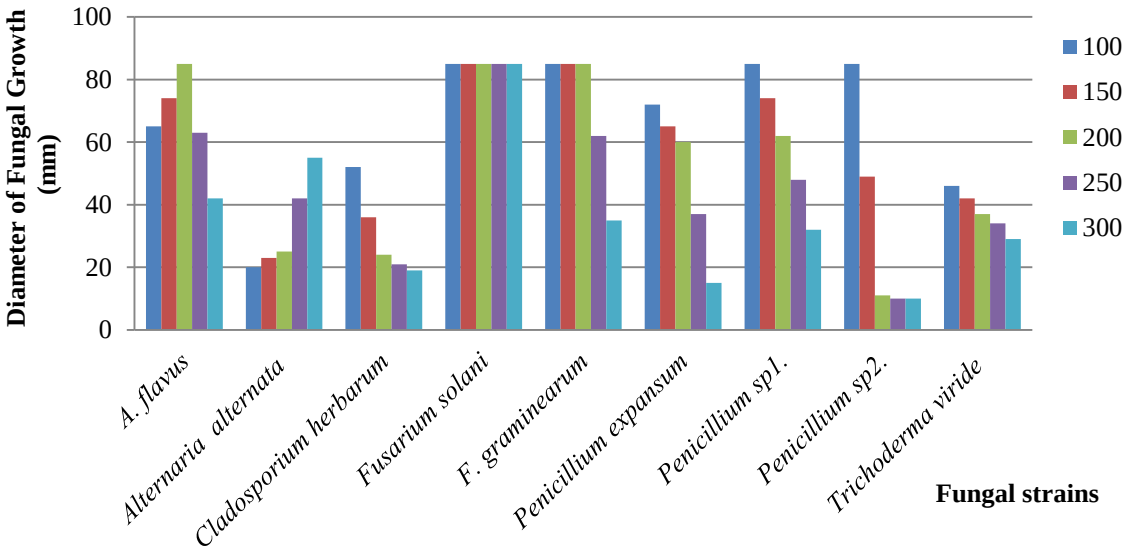


Figure 29. Effect of *D. viscosa* aqueous leaves extract on fungal species

7.5.3. Effect of sodium bicarbonate and chemical fungicides

The extensive and prolonged use of the fungicides has resulted in the development of resistance to fungal diseases. To this effect are added the residual effects on crops and environmental pollution. In recently, the use of natural compounds, such as oils, salts and plant extracts either alone or in combination with other control methods, appears to represent one of the best alternatives to synthetic fungicides.

The effect of sodium bicarbonate (SBC), a food additive particularly preservative, on mycelial growth of stored wheat fungi varied greatly (Fig30). Assays showed that, SBC provided a significant inhibition of mycelial growth of *A. flavus*, *Alternaria alternata* and *Fusarium solani* with a diameter of fungal growth of 38, 31 and 42 mm, respectively, while, *F.graminearum* was slightly higher (47 mm) than *F. solani*. It seems that the growth of the 3 species of Penicillium, *Cladosporium herbarum* and *Trichoderma viride* was not affected; the diameter of fungal growth was 80 mm. The growth of the pathogenic fungus decreases with increasing concentration. *Aspergillus* species produce various life-threatening biotoxins such as aflatoxins (AFTs), ochratoxins (OTA), patulin (PAT), citrinin (CIT), aflatrem (AT) and other characteristic molecules (Ráduly *et al*., 2020). Various studies have been carried out to control *Aspergillus spp*. However, the results of the current study do not support previous research about assessing Aspergillus species. Mohammed and Saadon (2023) revealed a relationship between the concentration (10, 20 and 30 mg/ml) of SBC and its inhibitory capacity of *A.niger* compared to the control and also founded that at a concentration of 30% of SBC, the fungus got a diameter of 1.13 ± 0.01 cm As for the effect of SBC on the radial

growth of *A.flavus* on PDA medium, Nakrani's study (2020), to find the best seed sterilizing chemical to reduce *A. flavus* infection. The seeds of groundnut variety GG-20 (healthy) were dipped into different chemicals viz., sodium hypo chloride, sodium bicarbonate, calcium chloride, sodium chloride, boric acid, propionic acid and neem leaf extract at different concentrations for 5 minutes. These were then treated with 5 ml of standardized spore suspension of *A. flavus* (10^6 spores/ml) and were placed on blotter paper. The data on per cent seed infection recorded after 10 days of incubation revealed that there was a significant difference among different treatments, whereas SBC recorded a hignt percentage of seed infection (83,33% at 0,5% of SBC), while, our data recorded adverse effect with SBC at 300 mg/ml on *A. flavus* (38 mm). It has been reported that SBC could inhibit growth of numerous fungal species such as *Candida sp., Pythium sp., Rhizopus stolonifer, Aspergillus niger* (Kareem *et al*., 2018), *Fusarium oxysporum* (Turkkan et Erper, 2014), and *Penicillium digitatum* (Zamani *et al*., 2009). In the case of Penicillium, our findings are supported by the results of Soto-Muñoz and Martínez-Peniche (2009), who revealed the ineffectiveness of SBC (0, 2 and 4%) against *P. expansum* in two apple varieties in postharvest (immersed in BCS solution during 2 min at 20 ± 1 °C). Their results coincide with those of Yao *et al*. (2004) which found that SBC at 2% was not effective in controlling the blue rot of pear trees, due to the low susceptibility of *P. expansion* to this compound. In contrary, *In vivo* and *in vitro* studies have demonstrated the effectiveness of salts such as SBC against *P. digitatum* and *P. italicum* decay in oranges, mandarins and lemons (Askarne *et al*., 2013), (Youssef *et al*., 2014). In order to investigate the effect of different concentrations of SBC (3, 6 and 9 ppm) on growth inhibition isolate *F. solani* (PotatoFusariun dry rot), on PDA medium containing mineral salt, the inhibition rate of growth was 42,33% after 7 days of incubation at 25°C (Ghadiri *et al*., 2013). Similarly, fungal growth of *F. solani* and *F. graminearum* in this study were moderately inhibited. On the other hand, anterior studies reported thet SBC enhances efficacy of *Trichoderma harzianum* DGA01 in controlling crown rot of banana (Alvindia, 2013) and also *T. viride* for minimizing the rhizome rot of turmeric caused by *Sclerotium rolfsii* (Jagtap *et al.,* 2013). In generally, some researchers speculated that pH may be involved in the inhibitory activity of SBC since it could change the pH of the growth environment of microbes (Lyousfi *et al*., 2022). Poschenrieder *et al*. (2018) reported that SBC plays a role in controlling cell pH of all organisms, while Lai *et al*. (2015) founded that pH could induce different expressions of some genes, resulting in the inhibition of the growth of *P. expansum*. Youssef *et al.* (2014) reported that SBC were been able to induce defense mechanism by producing increasing the activity of -1,3-glucanase, peroxidase, and

phenylalanine ammonia-lyase (PAL) enzymes. Additionally, SBC can inactivate fungal extracellular enzymes since they can act directly on cell membranes and lead to an alteration of cellular physiology (Castro-Ríos *et al.*, 2021). In addition, bicarbonate salts could reduce the intense pressure of fungal cells by increasing osmotic stress and causing the collapse and contraction of hyphae and spores (Alvindia, 2013).

Susceptibility of plant cultivars and the conduciveness of environmental conditions before, during, and after fungicide application will affect disease development and ultimately, the performance of the fungicide. Furthermore, the sensitivity of the pathogen to the fungicide may change with usage over time through adaptation or genetic resistance and thus, this greatly influences the success or failure of any fungicide product used under field conditions. Synthetic fungicides used have not yet been reported, to the best of our knowledge, because they have various trade names. Our data (Fig 29) suggested that among the two fungicides (belonging to the TRIAZOLE family) conventionally used in agricultural field, Agriconazole had moderate to strong inhibitory activity against tested fungi. It appears that increasing the concentration of the fungicide results in decreased fungal growth. It was found to be strongly effective against *Aspergillus niger*, *Penicillium expansum*, *P. sp1*. and *P.sp2*. with total growth inhibitions. Difenoconazole (DIF) (the active ingredient of Agriconazole) is a new demethylation inhibitor (DMI) fungicide, which has a systemic activity and broad-spectrum antifungal potency (Ali et Amiri, 2018). DMI have been used for years to control *P. digitatum* and *P. italicum* and other citrus pathogens (Ali et Amiri, 2018). However, difenoconazole (DIF) is the first DMI recorded for the management of *P. expansum* and other postharvest diseases of pome fruits (Ali et Amiri, 2018), where the *P. expansum* population was highly sensitive to DIF, as shown by low EC50 values (<0.5 µg/ml) for all growth stages, i.e. germination, germinal tube elongation and mycelial growth, in vitro as well than by the ability of DIF to fully control blue mold infections on apples for at least 6 months in cold storage. Specifically, Vidan had the worst inhibitory perform. Fungal strains that are sensitive to the first fungicide are recognized as resistant to the second. In another hand, Vidan exhibit very strong inhibition of *A.flavus* and *Cladosporium herbarum* with a fungal growth of respectively 0 and 2 mm. Triazoles includes several classes of excellent systemic fungicides, such as Vidan and Agriconazole; they are applied as foliar sprays and as seed and soil treatments. The metabolite triadimenol is also active and is registered separately for use as seed treatment. Triazoles: demethylation (ergosterol or sterol biosynthesis) inhibitors are inhibitors of the enzyme lanosterol 14α-demethylase, which is essential for the biosynthesis of

ergosterol, a key fungal cell membrane component, thus inhibiting fungal growth (Cycon *et al*., 2006). A fungicide depends on the chemical characteristics of each chemical group to which it belongs. Generally, its maximum activity period vary according to its intrinsic characteristics and the stage of development of the pathogenic agent. It has been demonstrated that certain fungicides tested on stored wheat must be both functional and effective (those which controlled the fungi also killed the seed).

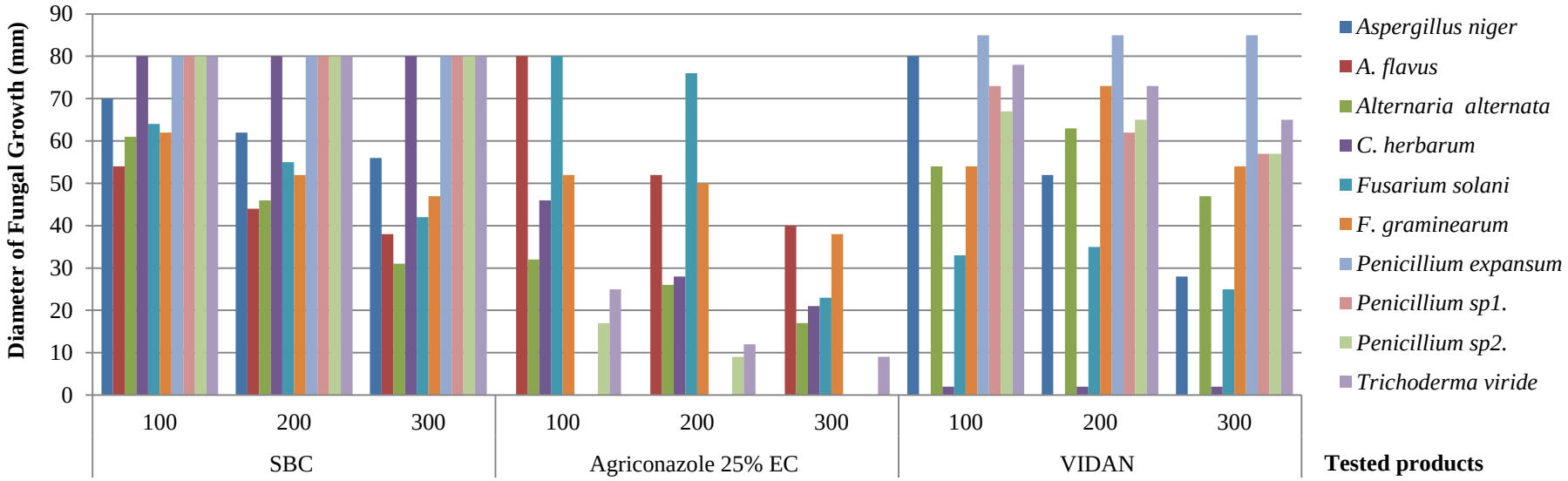


Figure 30. Inhibition of mycelial growth at increasing concentrations of Sodium bicarbonate (SBC), Agriconazole 25% EC and VIDAN

Conclusion

In order to contribute to the valorization of Algerian aromatic and medicinal plants, we propose, in this study, to describe the chemical composition and biological activities of plant extracts of *Cupressus sempervirens, Salvia rosmarinus, Eucalyptus polybractea, Lantana camara, Morus alba, Rubus ulmifolius, Dittrichia viscosa* and *Thuja orientalis*. At first, the results of antibacterial activity of four EOs of *C. sempervirens, S. rosmarinus, E. polybractea* and *L. camara* against six pathogenic bacteria as *Pseudomonas aeruginosa* ATCC27853, *Staphylococus aureus* ATCC25923, *Echerichia coli* ATCC25922, *Salmonella sp, S. aureus, P. aeruginosa* and one yeast *Candida albicans* were able to effectively inhibit mycelia growth and alter fungal morphology. Based on our findings, EOs can be classified according to their efficiency *E. polybractea* EO, Lantana EO, Cypress EO and Rosemary EO. Secondly, our results confirm the antifungal activity of these plant extracts of *Lantana camara, Morus alba, Rubus ulmifolius, Dittrichia viscosa* and *Thuja orientalis* against fungi of stored wheat grains including *Aspergillus niger, A. flavus, A. terreus, Alternaria alternata, Cladosporium herbarum, Fusarium solani, F. graminearum, Penicillium expansum, P.sp1., P.sp2.* and *Trichoderma viride*. This does not exclude the resistance of certain strains. While the methanolic extract of *M. alba* leaves and the aqueous extract of *Dittrichia viscosa* leaves showed a very promising antifungal effect, that of the methanolic combination between Morus and Rubus is the weakest. However, the study also examined the antifungal effectiveness of sodium bicarbonate ($NaHCO_3$) compared to two chemical fungicides (Agriconazole and Vidan) on the same fungi tested. Considering this alternative, sodium bicarbonate should be highlighted due to the positive results obtained. In addition, our best weapon for controlling pathogenic fungus is fungicides, but growing resistance is leading us to discover new antifungal compounds. Indeed, the biological effects of different concentrations of these plant-extracts or EOs showed a satisfactory biological activity and strong inhibitory effect on microorganisms, particularly antifungal activity compared to sodium bicarbonate and chemical fungicides. Therefore, these plant-extracts and sodium bicarbonate can be utilized as a promising alternative to synthetic fungicides in the fungal control of stored wheat grains. However, additional research is needed to understand the significant compounds responsible for the biological activity, to evaluate the in vivo effectiveness of these EOs and plant-extracts and understand their effects.

CHAPTER 3:
INSECTICIDAL ACTIVITY OF PLANT EXTRACTS AGAINST TRIBOLIUM

Chapter 3: Insecticidal activity of plant extracts against Tribolium

Introduction

Tribolium castaneum has a long history as a model species in many distinct fields. It was the first beetle whose genome was sequenced, which has facilitated its increased use in a wide range of functional genomics research (Campbell *et al.*, 2022). This insect has also been a model in the development of pest monitoring and management tactics, including the evaluation of insecticide resistance mechanisms (Campbell *et al*., 2022). The storage pest *T. castaneum* is the primary insect pests that cause significant quantitative and qualitative deterioration of agrifood commodities during postharvest storage. Integrated pest management involves the use of several tactics. One of those strategies is the utilization of plant extracts or essential oils (green pesticides or bio pesticides), which are more environmentally friendly and sustainable than the commercial synthetic insecticides widely used in agriculture. There are reports in the literature regarding the influence of various plant extracts or essential oils on mortality of *T. castaneum* and other insects (Boukouvala *et al*., 2023).

1. Generalities

Beetles (*Coleoptera*) constitute the largest order in the animal kingdom and are certainly one of the major orders comprising pest species, responsible for agricultural losses (Rösner *et al.*, 2020). The main primary insect-plague of storage grain is *Rhyzopertha dominica, Sitophilus oryzae,* and *S. zeamais*, and, among secondary insect-plague, the following species are highlighted: *Oryzaephilus surinamensis* and *Tribolium castaneum* (Garcia *et al*., 2017). *Tribolium* is a genus of small Tenebrionid beetles, two of which, the red flour beetle, *T. castaneum* (Herbst 1797), and the confused flour beetle, *T. confusum* (Jacquelin du Val) (*Coleoptera: Tenebrionidae*) are international spread insects that attacks stored grains and foods (El-Aziz, 2011), (Campbell *et al.*, 2015). The red flour beetle (*T. castaneum* Herbst) is one of the common and polyphagous post-harvest pests of cereal grains, with a nearly worldwide distribution (Campbell *et al*., 2022). It's an important secondary storage pest because of its broad host range and its abilities to find and infest commodities and to rapidly increase in population size after establishment (Gurdasani *et al*., 2018). In addition to being a global insect pest of stored products, especially flour and grain, in silos, warehouses, bakeries,

and grocery stores, *T. castaneum* is a powerful model organism for developmental, physiological and applied entomological research on beetle species (Rösner *et al*., 2020). As part of the most species-rich order (Stork *et al*., 2015), it occupies a relatively basal position among the Holometabola (transforming insects) and is less highly derived than Drosophila (the main insect model), Tribolium beetles have many representatives (Brown *et al.,* 2003). *T. castaneum* was the first agricultural beetle and pest whose genome was sequenced (Tribolium Genome Sequencing Consortium *et al.,* 2008) and the annotations continue to be updated (Herndon *et al*., 2020).

2. Description of beetles of the family *Tenebrionidae*

Tenebrionidae (*Insecta*: *Coleoptera*) have a worldwide distribution and are one of the larger beetle families with more than 30,000 described species (Bouchard *et al*., 2021). Commonly known as darkling beetles because they have a characteristic dark body color, the Tenebrionidae are a conspicuous component of desert fauna worldwide. They have developed numerous morphological, physiological and behavioural adaptations to cope with extremely arid conditions and are therefore largely responsible for most of the nutrient cycling in deserts (Cheli *et al.,* 2022). Currently, 11 subfamilies, 106 tribes and 2,307 genera of *Tenebrionidae* are recognized (Bouchard *et al.,* 2021), mainly based on the morphological characters. Most species are generalistic omnivores, and feed on decaying leaves, rotting wood, fresh plant matter, dead insects, and fungi as larvae and adults. The larvae, known as mealworms or false wireworms, are usually fossorial heavily sclerotized and nocturnal. Adults of many species have chemical defenses and are relatively protected against predators. Adults of most species, except grain pests, have slow metabolisms, and live long lives compared to other insects, ranging from approximately six months to two years. Darkling beetles are generally stocky insects with a rather dull livery; the antennae, filiform, moniliform or slightly clubbed, are inserted in front of the eyes, under the anterior edge of the head. They are of size included between 2 mm and 80 mm, of very varied form, with teguments generally rigid, thick, black matt or shining, of dark color, coloured or "metallic". The antennae of 11 articles, more rarely 10.apterous or winged, with wing venation of the primitive type, 5 abdominal sternites, long legs or on the contrary, contracted, often burrowing. Some species of *Tenebrionidae* have been reported as crop pests and others attack stored or preserved food. Of these, the genus *Tribolium* includes two main common and pest species: *T. castaneum* Herbst. and *T. confusum* Duv. Like many other tenebrionids, *Tribolium* beetles are saprophagous, phytophagous, or mycophagous. The genus *Tribolium* has 36 species, four of which are

cosmopolitan (Angelini *et al*, 2008). For the identification of the genus *Tribolium*, according to Ferrer (1995), two characters are essential (i) the existence of a carinated suture, (ii) the meso tibia and meta tibia are simple.

3. Presentation of *T. castaneum*, Herbst (1797)

The red flour beetle (*T. castaneum*, Herbst (1797) is an easy to rear insect of 3-4 mm in length (Photo 15), with a short development cycle of 30 days, a longevity of six months to four years, which is exceptional for an insect, and high fecundity (Bonneton, 2008). This insect, considered as a strict secondary pest, is a cosmopolitan and polyphagous pest whose stain corrupts many starchy commodities, especially cereal flours (Bonneton, 2010). In nature, this insect lives under the bark of trees, but laboratory lines come from flour mills and silos. *Tribolium* is now so cosmopolitan and commensal to humans that its origin is uncertain. It would come from regions of southern Asia with a hot and dry climate, perhaps from India. The genus *Tribolium* includes 36 species, four of which are cosmopolitan (Angelini and Jockusch, 2008). This insect is a member of the huge family Tenebrionidae, whose 17000 species are distributed worldwide, preferably in arid areas (Bonneton, 2010).

According to Weidner and Rack (1984), the red flour Tribolium belongs to the following classification:

Table 27. Taxonomic classification of *T. castaneum*

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Coleoptera
Suborder	Polyphaga
Family	Tenebrionidae
Subfamily	Ulominae
Genus	Tribolium
Species	*T. castaneum* (Herbst)

Photo 15. An adult Tribolium beetle. (Khan *et al.*, 2016b)

In a recent review of post-harvest issues, it is stated that the confused flour beetle *T. confusum* and the red flour beetle *T. castaneum* are "the two most common secondary pests of all plant commodities (Sallam, 2008). Both *T. castaneum* and *T. confusum* are small, about 3–6 mm in 9 lengths, and reddish-brown in colour. The main distinguishing physical difference is the shape of their antennae: antennae of the confused flour beetle increase gradually in size and have four clubs, while red flour beetle antennae have only three (Photo 16). Furthermore,

T. castaneum has been known to fly short distances, while *T. confusum* does not. Various secretions produced by the pest can also cause serious contamination effects on the product infested, causing the damaged grain to agglomerate, develop mildew, and smell. However, most researchers agree that the main volatile compounds of *T. castaneum* are 1-pentadecene, 2-methyl-p-benzoquinone and 2-ethyl-p-benzoquinone (Han *et al*., 2023).

Photo 16. Comparison of the head and thorax (pronotum) of the confused flour beetle (A) and red flour beetle (B). Note the more distinct antennal club and convex sides of the thorax in the red flour beetle (In Crawley et Bertone, 2022).

4. Life cycle of *T. castaneum*

The potential of an insect population to increase through time and space relates closely to some life history traits such as survival, development, and fecundity (Papanikolaou *et al*., 2019). The growth of insects is done by a succession of moults. As a typical holometabolous insect, *T. castaneum* is characterized by having three distinct stages: larvae, pupa, and adult, which have little or no resemblance to each other (Suzuki *et al*., 2009). This pest develops through several larval stages usually 7, but 5 or 6 when starved (Chafino *et al*., 2019), followed by metamorphosis (Fig 31). Eggs, white or transparent, are laid into the substrate (flour) and embryonic development takes 3 days at 32 °C. They are 0.61 mm to 0.77 mm long and 0.35 mm to 0.4 mm wide. They emit fluorescence below 365 nm (ultraviolet radiation) (Leelaja *et al*., 2007). Because its integument is rigid, the larva consumes several times its own weight in food and moults on a regular basis, allowing it to grow larger. When mature, the larva is pale yellow with a few short yellow setae on the sides. The dorsal surface and the head capsule are slightly reddish. The nymph is formed after the last larval molt, it does not feed and has a cylindrical shape. It is whitish in color turning to yellow. The terminal part of the abdomen has two spines. The *pupa libera* allows visual inspection of external structures facilitating phenotypic studies of metamorphosis. Female beetles need a few days after hatching until they start laying eggs, which they continue for 3–4 months. Likewise, development from egg to adult speeds up with temperature from 74 days at 22.5 °C to about 23 days at 32 °C, which is short enough for large scale genetic experiments. The adult

T.castaneum has a smooth and elongated body. The antennae end in a clearly distinct club. It is an insect characterized by sexual dimorphism; the male can be distinguished from the female by the presence of a rounded piliferous tubercle at the base of the anterior femur (Delobel and Tran, 1993). The red flour beetle can fly very well (Ridley, 2011), it has poorly developed wings. Growth from larvae to pupa and the adult form takes up to 13 weeks (Islam, 2017).

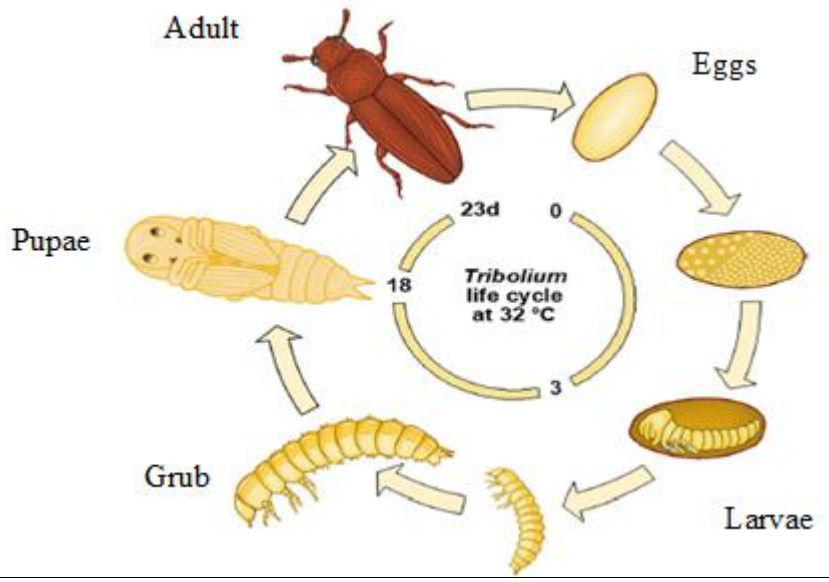


Figure 31. The life cycle of *T. castaneum*
(Klingler and Bucher, 2022)

5. Pest management approaches

The presence of insects in stored grains always leads to significant losses in both quality and quantity of food; a decrease in nutritional value; and a loss of marketing value due to the contamination with insects' body waste. Coleopteran arthropod insect pests are the major causal agents of grain loss during storage, worldwide. Chemical fumigants and contact insecticides are still the main method for storage seed protection. Alternative pest control methods such as mechanical control, planned crop rotation, biological control, cultural control and the use of biopesticides could help minimize the use of chemical pesticides, leading to improved food security, human health and environmental conservation, as well as the risk of pests resistance evolution (Dar *et al*., 2020) has forced the search for alternative management tools. In the same context, many strategies require an approach combining sanitation, identification, surveillance and prevention practices. In general, there are many methods and control measures taken against storage beetles (Fig 32).

Figure 32. Pest management practices for stored grain pests (Hajam et Kumar, 2022)

5.1. Physical methods of protection

The most effective way to control stored grain pests is to avoid them. The goal of sanitation is to eliminate insect eggs, pupae and adults. All stored areas must be cleaned. Even a small old grain or fines left in a place where new grains should be stored can harbor insects that can infest the whole grain. However, it is less well understood how sanitation not only affects overall pest abundance, but interacts with various other pest control and monitoring techniques, either decreasing or increasing their effectiveness depending on the level of sanitation in the anthropogenic environment. Physical factors such as oxygen, moisture, relative humidity, and temperature have a major impact on the storability of grain; they influence the conditions for insect multiplication and mold growth during grain storage, which eventually affects the storability of the grain. Controlled storage atmosphere with inert gases such as CO_2 is an alternative non-chemical method for controlling cereal pests, however, it requires long exposure times (more than 10 days) and, in combination with other fumigants such as phosphine are deadly for stored grain pests (Constantin *et al.*, 2020). In the same context, the effectiveness of O_3 in killing a varied range of stored-grain pests, including internal and external feeders, was established (Subramanyam *et al.*, 2017). According to Mishra *et al.* (2019), O_3 was lethal to different stages of *Rhyzopertha dominica* and *T.*

castaneum. Although O_3 has been shown to be an effective fumigant, the selection of products for which exposure to O_3 must be considered, as it is a strong oxidant. Studies related to the design and development of compatible storage structures for O_3 processing with closed-loop components, pilot-scale studies and large-scale applications are still to be carried out. It should also be noted that heat or cold (including dielectric heating) significantly affects the growth and development of insects. Grain disinfestation by thermal treatments was traditionally practiced using hot/cold-air/water combinations, under controlled conditions (Macana *et al.*, 2018). Also the radiations are not only lethal to insects but also cause sterility in the survived populations (Paul *et al.*, 2020). In recent years, many researchers have demonstrated that ionizing irradiation, especially X-rays and gamma irradiation can effectively control stored grain pests without detrimental effects on the commodity (Salim *et al.*, 2018). They proved that 0.50 kGy (kiloGray) dose of gamma radiation can kill 100% of *T. confusum* adults and larvae in 22 days. Sileem *et al.* (2019) studied the effect of gamma-irradiation in combination with food-grade diatomaceous earth (DE) against *Sitophilus granarius, T. castaneum, and R. dominica*. Among their treatments, the combination of 100 Gy gamma-radiation exposures with DE at 1g/kg was found significant with complete mortality.

5.2. Chemical management

The chemicals used in this control may be natural products, synthesized mimics of natural products, or completely synthetic materials. Repellents, confusions and irritants are generally not toxic to insects, but interfere with their normal behavior and thereby keep the insects from causing damage. The majority of research studies on the protection of stored grain in the 20th century were based on chemical applications, particularly surface/preventative sprays and fumigation. Fumigation is the way to control detected infestations, but spraying is carried out regularly to prevent infestation. However, increased reliance on chemicals has created health and environmental health concerns. In practice, most gases have been eliminated due to their unfavorable properties (chemical instability) such as methyl bromide. Phosphine is the widely used fumigant, but its continued use has led to the evolution of resistant populations and environmental contamination (Nayak *et al*., 2020). The pesticides are most commonly classified on the basis of the target pests; mode of action; and chemical nature (Fig33).

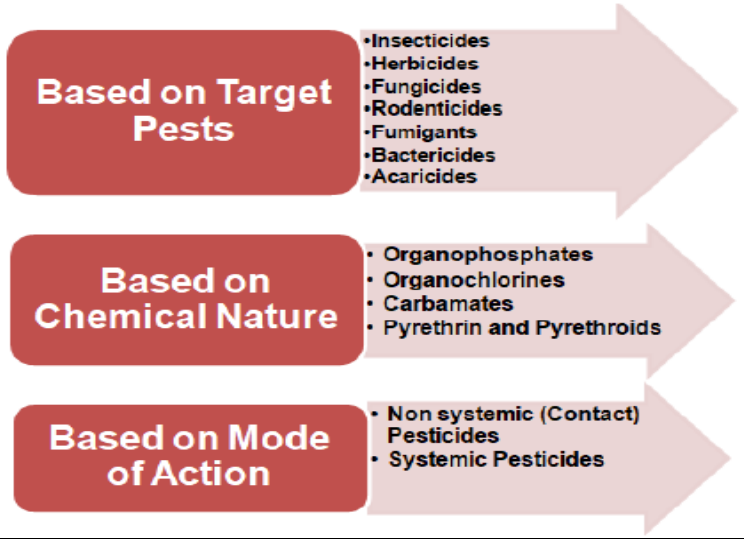


Figure 33. Classification of Pesticides (Laxmishree et Nandita, 2017)

Table 28 lists the names of the active substances and the expiry date of the authorization, as well as the types of application. Protectants are insecticides that are applied directly to the grain of cereals, and are important because the treatment is most often conducted at the beginning of storage at the entrance of the grain after cleaning and when there is the least dust, which reduces the efficiency. In situations where a grain insect population is present, fumigation is more important and effective. To moderate pests damages liquid insecticides are usually pulverised on the stored grains such as organophosphorus compounds (pirimiphos methyl, chloropyrifos etc.) or pyrethrinoides (such as deltamethrin or cypermethrin, etc.). Stored grain protection can be achieved through the direct mix with insecticides like Malathion powder, dusting the outer surfaces of grain bags to prevent new infestations, and fogging around the pile of grains to prevent crawling of insects. Malathion is widely used in empty stores or as a protective insecticide to combat stored grain pests (Mutlu *et al*., 2019). However, chemical controls have many disadvantages: most have biological activity against many life forms and therefore can affect non-target organisms; for the same reason, they present various levels of hazard to humans; most are highly toxic to beneficial insects, such as pollinators, predatory and parasitic natural enemies; both target and non-target insects can develop resistance to insecticides, sometimes very rapidly. Over-reliance on chemicals and less reliance on other control methods have helped push agriculture away from a more natural, balanced state.

Table 28. Active substances and dates of expiration of approval and type of treatment (European Commission, 2020)

Active substance	Expiration of approval	Type of treatment
Dichlorvos	1998	Protectant
Pirimiphos-methyl	31 July 2021	Empty storage
Methyl-bromide	2011	Fumigant
Carbon dioxide	31 August 2020	Fumigant for grains
Aluminium phosphide	31 August 2022	Fumigant for grains and empty storage
Magnesium phosphide	31 August 2022	Fumigant for grains and empty storage
Sulphuryl fluoride	31 October 2023	Fumigant for empty storage

5.3. Biological methods

There are many different source species which are used for the biological control of invertebrate insects. These can be grouped into predators, parasites and pathogens and taxonomically into mites, nematodes, insects and micro-organisms such as bacteria, viruses, and fungi (Hajek, 2018). Pathogens which are responsible for disease in insects are called entomopathogens, such as entomopathogenic fungi or bacteria, especially *Beauveria bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii*, have been used to control pest insects for many years. Martynov (2017) lists 28 natural enemies for *Tribolium castaneum* and 43 other species for totals of 11 bacterium, 15 fungi, 13 protozoan, 10 nematodes, 7 acarina, 1 coleopteran, 8 hemipteran and 6 hymenopteran species. Several species of beneficial insects that attack major insect pests in stored grain, including granary weevil, rice weevil, rusty grain beetle, lesser grain borer, confused flour beetle, sawtooth grain beetle, Angoumois grain moth and Indianmeal moth are commercially available. Limitations of this approach include expense, potential negative effects of grain protectants on biocontrol agents, and the presence of live beneficial insects and insect parts in the grain (Schöller *et al*., 2018). Natural enemies (predators and parasitoids) to manage stored pests have been widely studied. Interest in parasitoid-based biological control of pests of stored products has grown considerably in recent decades and has mostly focused on Bethilidae, Braconidae, Ichneumonidae and Pteromalidae parasitoids (Schöller *et al.*, 2018). A previous study using small containers (2 kg) of brown rice showed that *Anisopteromalus calandrae* (a solitary ectoparasitoid) significantly limited *Sitophilus zeamais* population growth (up to 99% compared to the control treatment) and associated damage to rice grains (insect-damaged

kernels, frass production, and mold presence), at 23 °C and 28 °C (Solá *et al.,* 2020). *Amblyseius swirskii*, another parasitoid is able to prey on the eggs of stored pests such as *Ephestia kuehniella* Zeller (*Lepidoptera: Pyralidae*) and *Callosobruchus chinensis* L. (*Coleoptera: Chrysomelidae*) in the laboratory, and the predator *Neoseiulus cucumeris* is capable of consuming the eggs of *E. kuehniella* (Iturralde-García *et al*., 2020).

Semiochemicals are chemical signals produced by one insect/organism, which evokes behavioral or physiological responses in the receiving insect/organism. They are commonly classified into "*pheromones*": intra-specific chemical signals and "*allelochemicals*"- inter-specific chemical signals (Abd El-Ghany, 2019). They are used to manipulate insect behaviour by affecting the survival and/or reproduction of insect pests for controlling their infestations on crops. The semiochemicals belong to various chemical groups, including aldehydes, alcohols, sulfur-containing compounds, esters, terpenes, alkanes, heterocyclic aromatic compounds, proteins, amino acids, triglycerides, and salts. They include insect attractants or stimulants, arrestants, repellents and deterrents. Moreover, successful pheromonal studies under laboratory conditions were done in stored-grain moths (*Ephestia* spp., *Plodia interpunctella, Sitotroga cerealella*) and stored-grain beetles *(Lasioderma serricorne, Trogoderma granarium, Tribolium* spp., *Stegobium paniceum*). Although biological control has a limited scope in stored grain management, it becomes an important element of an integrated control strategy. The main disadvantages of this method are that it is very expensive and that maintenance of the crop is essential to combat harmful insects.

5.4. Botanical contrôl

Botanical pesticides or natural insecticides were widely used for millennia in agriculture before the development of synthetic pesticides. Botanical pesticide ingredients are natural chemical derivatives of plants (plant secondary metabolites) that act as repellents, attractants, antifeedants and growth inhibitors (Stankovic *et al*., 2020) (Fig 34), they can also inhibit egg-laying activities, or even directly kill the insects by irreversible inhibition of one or more essential reactions in their metabolism. Biopesticides work by denaturing protein, causing metabolic disorder and paralysis, activating target-poisoning mechanisms, exhibiting multisite inhibitory actions, and releasing neuromuscular toxins and bioactive compounds (Dar *et al.,* 2021). Essential oil from *Coleus aromaticus* Benth., *Hyptis suaveolens* (L.), *Azadirachta indica, Ageratum conyzoides* L., and *Achillea sp*., have been reported to control the infestation

of *Tribolium castaneum* (Herbst), a red flour beetle that destroys many crop species (Upadhyay *et al*., 2018).

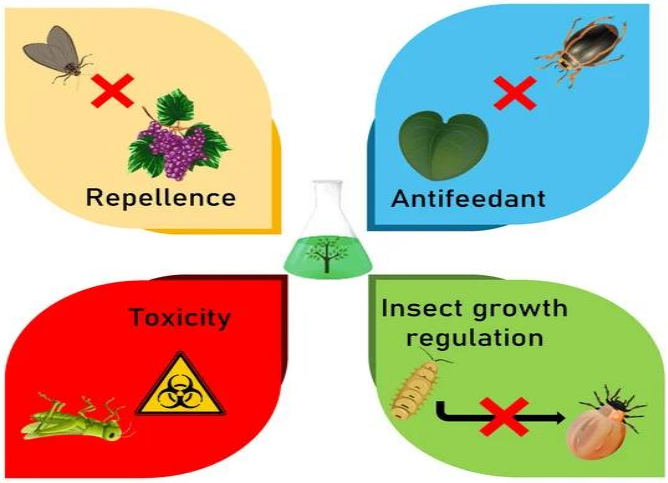


Figure 34. Mode of action of phyto insecticides (Acheuk *et al*., 2022)

Plant families that have been reported to contain bioactive compounds with activity against important crop pests include *Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae* and others (Tab29). They are readily available, making them inexpensive and can be easily integrated into agricultural production and conservation systems. Essential oils (EOs) and plant extracts are the botanical products most frequently used as biopesticides. The active principles of botanical pesticides, especially the unique structural motifs of secondary metabolites, e.g., alkaloids, essential oils including terpenes, flavonoids, phenolics, phytosterols and polyketides as well as resins, are qualified to confer antibacterial, antifungal, herbicidal and insecticidal action (Lengai *et al.,* 2020). However, biopesticides compounds including terpenes, such as 1,8-cineole (eucalyptol), β-caryophyllene, linalool, D-limonene, α-pinene, α-terpineol, thymol, carvacrol, and α-thujone exhibit insecticidal properties.

Table 29. Plant and organic compounds used against stored grain pests (Singh *et al.*, 2021)

Plant species	**Family**	**Active ingredient**	**Target pest**
Acorus calamus	Acoraceae	β-Asarone	*Sitophilus zeamais*
Aloysia citriodora	Verbenaceae	Citronellal and sabinene	*T. castaneum, T. confusum*
A. polystachya	Verbenaceae	Carvone and limonene	*T. castaneum, T. confusum*
Artemisia annua	Asteraceae	1, 8-cineole	*T. castaneum*
Baccharis salicifolia	Asteraceae	3-Carene	*T. castaneum, S. zeamais*
B. salicifolia	Asteraceae	β-Pinene	*T. castaneum, S. zeamais.*
Brugmansia suaveolens	Solanaceae	β-Pinene	*Zabrotes subfasciatus*
Carum carvi	Apiaceae	Carvone, Limonene,	*Rhyzopertha dominica, S.*

Plant species	Family	Active ingredient	Target pest
		(E)-Anethole	*oryzae, S. zeamais*
Chamaecyparis obtusa	Cupressaceae	Bornyl acetate	*S. oryzae, C. chinensis*
Chenopodium ambrosioides	Amaranthaceae	Hexadecane	*T. castaneum, S. granarius*
Cinnamomum aromaticum	Lauraceae	Cinnamaldehyde	*T. castaneum, S. zeamais*
Citrus	Rutaceae	Limonene Eugenol	*T. castaneum, S. oryzae*
Colocasia esculenta	Araceae	2, 3-Dimethylmaleic anhydride	*S. oryzae, T. castaneum, C. chinensis*
Convolvulus arvensis	Convolvulaceae	Hexadecanoic acid	*R. dominica, S. oryzae*
Conyza dioscordis	Asteraceae	Dicotlyhexanedioate	*T. castaneum, S. granarius*
Coriander sativum	Apiaceae	Linalool	*S. oryzae, R. dominica and C. pusillus*
Cupressus lusitanica	Cupressaceae	Umbellulone and α-pinene	*T. castaneum, A. obtectus, S. cerealella and S. zeamais*
Duguetia lanceolata	Annonaceae	2,4,5-trimethoxystyrene	*Z. subfasciatus*
Eucalyptus spp.	Myrtaceae	α-Terpinene; 1, 8-Cineole; α-pinene	*S. oryzae*
Eucalyptus saligna	Myrtaceae	p-Cymene	*T. castaneum, S. oryzae*
Evodia ruticarpa	Rutaceae	Triterpenes	*T. castaneum, S. zeamais*
Feoniculum vulgare	Apiaceae	Phenylpropenes (E)-anethole Estragole (þ)-Fenchone	*S. oryzae, Lasioderma serricorne*
Juniperus foestidissima	Cupressaceae	Citronellol	*Trogoderma granarium*
Lantana camara	Verbanaceae	Coumaran	*S. oryzae, T. castaneum, R. dominica*
Melaleuca cajuputi	Myrtaceae	Terpine-4-ol Terpiniolene γ-Terpinene	*T. castaneum, S. oryzae, E. kuehniella, R. dominica*
Mentha citrate	Lamiaceae	Carvone, menthol, linalool, linalyl acetate	*T. castaneum, C. maculatus*
Nardostachys jatamansi	Caprifoliaceae	Aristolone	*T. castaneum, S. oryzae*
Ocimum canum	Lamiaceae	Linalool	*T. castaneum, S. granarius*
Ocimum kilimandscharium	Lamiaceae	Camphor	*S. oryzae*
Pimenta racemose	Myrtaceae	Linalool	*S. zeamais*
Rosmarinus officinalis	Lamiaceae	Camphor	*S. oryzae*
Spent hops	Lamiaceae	Xanthohumol	*S. granarius L., T. confusum and T. granarium*
Tagetes filifolia	Asteraceae	(E)-anethole and estragole	*T. castaneum*
Thespesia populnea	Malvaceae	Phenol	*C. maculatus*
Zingiber officinale	Zingiberaceae	1, 8-cineole	*T. castaneum, S. zeamais*
Z. officinale	Zingiberaceae	β-Zingiberene	*T. castaneum*

5.5. Nanotechnology in stored-grain protection

Nanotechnology experiments are rising in basic and applied scientific fields, including stored-grain protection. Nanobiopesticides are formulated from nanomaterials and applied specially fixed on a hybrid substrate, encapsulated in a matrix or functionalized nanocarriers for external stimuli or enzyme-mediated triggers (Pan *et al.*, 2023). The nanopesticide formulations can increase water solubility, bioavailability and protect agrochemicals against environmental degradation, revolutionizing the control of pathogens, weeds, and insects in the crops (Yadav *et al.*, 2020). Encapsulation is a process of surrounding one biologically active ingredient with the intention that the core confined material or into capsule walls can be released to the environment under specific conditions over a predetermined time or when external stimuli activate the capsule walls to break, melt or dissolve slowly. For instance, pesticides from nanomaterials, such as magnesium oxide, magnesium hydroxide, copper oxide, and zinc oxide derived from aqueous extracts of *Chamaemelum nobile* flowers, *Punica granatum* peels, green peach aphid (GPA) and *Olea europaea* leaves have been reported in the control of insects (Grillo *et al.*, 2021). The ability of copper oxide nanoparticles and zinc oxide nanoparticles to control *Alternaria citri*, a causative agent of citrus black rot disease in the plant has as well been reported (Lasso-Robledo *et al.*, 2022). The fungal and insecticidal effects of copper nanoparticles have been demonstrated against *Tribolium castaneum*, a pest that affects grain (El-Saadony *et al.*, 2020). Green nanotechnology enters as a sustainable approach with three main aims: use of biocompatible and non-toxic solvents; use of natural raw materials; and use of energy efficient processes (Campos *et al*., 2019).

6. Insecticide efficacy and resistance

Very serious problems can arise from the extensive use of chemical pesticides when insects develop resistance to them. Insecticides are commonly used in pest management programs, but there is increasing emphasis on making applications more targeted and using reduced-risk materials. It is well known that tenebrionids can develop resistance to several insecticides (Cui *et al*., 2021). Therefore, new insecticidal formulations are necessary to be developed (Petrović *et al*., 2019).

Tribolium castaneum has been widely used in research evaluating the efficacy of traditional and novel insecticides not only because of its importance as a pest, but also because it is often a good indicator species, given that it tends to have lower susceptibility to many insecticides (Campbell *et al*., 2022). Insecticides can be applied as a gas (fumigant),

aerosol, or spray or incorporated into materials such as packaging or netting. Resistance to insecticides has evolved frequently in *T. castaneum* (Collins et Schlipalius, 2018). More recently, multiple resistances to organophosphates and pyrethroids in *T. castaneum* has been reported, which complicates further the use of traditional insecticides for the control of this species (Kalsi et Palli, 2017). As such, several types of mechanisms are involved in insecticide resistance. Populations of *T. castaneum* have been characterized as strongly resistant to phosphine (Collins et Schlipalius, 2018). Their mechanisms may be behavioral (behavior of different insects in the presence of insecticides), physiological resistance, behavioral resistance, biochemical resistance, and metabolic resistance (Louat, 2013). Whalon *et al*. (2008) ranked *T. castaneum* 19th among the 20 most insecticide-resistant arthropods with 132 reported cases of insecticide resistance. Adults of this species mainly excrete a mixture of 1,4-benzoquinone, methyl-1,4-benzoquinone, and ethyl-1,4-benzoquinone compounds (Han *et al*., 2023). These cuticular secretions play a defensive role against predators and microbes and a putative regulatory effect on their own population growth (Rafaluk-Mohra *et al*., 2018). This insect has shown resistance to most classes of insecticides, an observation that can be attributed in part to its ability to produce detoxification enzymes that are encoded by insecticide resistance genes such as cytochrome P450 (Agrafioti *et al*., 2020). Recently, it has been shown that resistance involves two genes, *rph*1 and *rph*2, with the latter being responsible for the high level of resistance (Jagadeesan *et al*., 2013).

7. Material and methods

This part of the study evaluates the insecticidal activity (fumigant and repellent proprieties) of EOs and plant-extracts (previously used) as control alternatives against the stored products beetle *T. castaneum*. Considering the lack of information regarding the insecticidal activity of *E. polybracteae*, *M. alba* and *R. ulmifolius*, we have carried out laboratory bioassays to examine their extracts effects for the control of a major stored-product beetle specie *Tribolium castaneum*.

7.1. Biological material

T. castaneum adults were reared on wheat semolina at 25 ± 1 °C. Adult insects of mixed sex, 7–14 days old, were used for bioassays tests (Photo 17).

Photo 17. Rearing of *T. castaneum* in wheat semolina (a) and single adult (b) (Chibi, 2022)

7.2. Plant-based extracts

Plant extracts and essential oils come from plants already mentioned in the previous chapter. It consists of 4 EOs of Rosemary (*Salvia rosmarinus*), Cypress (*Cupressus sempervensis*), Eucalyptus (*Eucalyptus polybractea*) and Lantana (*Lantana camara*) as well as six extracts: two methanolic extracts of *Morus alba* and *Rubus ulmifolius*, two ethanolic extracts of *L. camara* and *Thuya orientalis*, an aqueous extract of *Dittrichia viscosa*, also a methanolic combination of Morus and Rubus.

7.3. Insecticidal activity

7.3.1. Repellency bioassay

Repellency assays were carried out according to the experimental method described by Jilani and Saxena (1990) at 25 ± 1 °C. Whatman filter papers (diameter 8 cm) were cut in half. On a half disc of filter paper were applied as evenly as possible with a micropipette:

- 10, 20, 30 and 40 µl of EOs,
- 50, 100 and 150 µl of methanolic extracts and 50, 80 and 100 µl of ethanolic and aqueous extracts.

The other half of the filter paper was treated with acetone alone as a control. The treated and control half discs were air-dried under a fan to evaporate the solvent completely. Treated and untreated halves were attached to their opposites using adhesive tape and placed in Petri dishes. Ten adult (7–14 days old) beetles of mixed sex were released at the centre of each filter paper disc. The dishes were then covered and sealed with Parafilm. Four replications were used for each concentration. Observations on the number of insects present on both the

treated and untreated halves were recorded after 15, 30 and 45 min. Percentage Repellency (PR) was calculated according to Nerio *et al*. (2009) as follows: PR=[Nc−Nt/(Nc+Nt)]×100. Nc was the number of insects on the untreated area after the exposure interval and Nt was the number of insects on the treated area after the exposure interval. The average percentage of repulsion for each oil is calculated and assigned to one of the different repulsive classes ranging from 0 to V according to McDonald *et al*. (1970) (Tab30).

Table 30. Percentage repellency scale (McDonald *et al.*,1970).

Class	Repulsion interval	Propriety
0	PR ≤ 0,1%)	Very low repellent
I	0,1 % <PR ≤ 20%	Low repellent
II	20%< PR ≤ 40 %	Moderately repellent
III	40 %< PR ≤ 60 %	Medium repellent
IV	60% < PR≤ 80 %	Repellent
V	80 %< PR≤ 100 %	Very repellent

7.3.2. Fumigant activity bioassay

This test consists of studying the in vitro effect of plant essential oils on the mortality rate of *T. castaneum* adults by inhalation using the method described by Papachristos and Stampoulos (2002) (modified):

- In 50 ml centrifuge tubes, a dose of
  - 0, 0.5, 1, 2, 4, 6, 8 and 10 µl of EOs
  - 0, 10, 20 and 30 µl of plant-extracts

were placed on 1 cm diameter filter paper glued to the inside of the lid;

- Ten adult weevils are introduced into each tube which is tightly closed;
- Fumigation mortality was assessed in four replications per volume, for each exposure time (24, 48 and 72 hours). When no leg or antennal movements were observed, insects were considered as dead.
- The Mortality was calculated and corrected according to Abbott's (1925) formula, taking into account the natural mortality (Mt) observed on the control: Mc (%) = (Mt-Mo)/Mo × 100

Mc: corrected mortality; Mo: mortality of the tested sample and Mt: mortality in the untreated control.

7.4. Statistical analysis

Statistical analysis of the test results of insecticidal fumigant toxicity was carried out using Graph Prism software version 9, data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. The differences between treatments were considered significant if p-value was less than 0.05.

8. Results and discussion

8.1. Repellency bioassay

8.1.1. Repellency bioassay of EOs

Repellency is a behavioral measure describing the extent of the aversive action of the tested substance on the tested organism. Stronger the repellent action the further the extent to which the animal would avoid the space treated with the tested substance. The rellepency bioassay of 4 EOs was reported in Table 31. Repellent action was highly dependent upon EOs concentration (10, 20 30, and 40 µl) and exposure time (15, 30 and 45 minutes). The results show that mortality increases with increasing exposure time. In filter paper tests and after 45 minutes of exposure at 40 µl, the order of insecticidal activity of EO was: *Eucalyptus polybractea* EO (EPEO) showed the highest repellent activity (86%), followed by *Lantana camara* EO (LCEO) (76%), *rosemary* EO (REO) (66%) and finally *Cupressus sempervirens* EO (CSEO) (63%). Sabbour and Abd-El-Aziz (2018) stated that EOs have a great repellent effect on insects from stored products during the storage period. In this study, the effectiveness of different EOs could be due to some factors which contribute to enhancing the repellent activity, such as the odour, chemical compositions, main components or fractions of EOs. The results are supported with the findings of Jayakumar *et al*. (2017) who stated that repellency of essential oils increases with increase in concentration and exposure time when tested the repellent activity of 10 essential oils against the adults of rice weevil, *Sytophilus oryzae*. In addition, the chemical constituents of essential oils do not only repel the insects but also affect the insect's respiration rate, impair muscle activity and disrupt the nervous system and physiological processes which leads to paralysis or eventually death (Germinara *et al*., 2015). Eugenol, α-terpineol and L-carvol cause hyperactivity in insects at first, stretching the legs and numbness precede the insect’s death (Govindarajan *et al*., 2016). Interestingly, the degree of toxicity depends on the bioassay method and the target insect. Essential oils, in particular monoterpenes, are volatile and lipophilic, which allows them to quickly penetrate the integument of insects, interfering with physiological parameters and causing an alteration

of all vital functions especially the neurotoxic action attributed to the inhibition of AChE (Nenaah *et al*., 2022), (Isman, 2020). Plant EOs can interfere with other protein targets, disrupting the insect's nervous system, such as nicotinic acetylcholine receptors (nAChR) and octopamine or γ-aminobutyric acid (GABA), neurotransmitter inhibitor. EOs have been found to inhibit enzyme biosystems (superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and glutathione reductase (GR)), peroxidases (POx) and non-enzymatic substances (glutathione (GSH)) antioxidant defense biosystems (Isman, 2020).

It should be noted that there is until now little bibliographic data on the repellent activity of ***Eucalyptus polybractea*** **EO** (Tab 31). However, exposure of the red flour beetle (*T. castaneum*) to 0.5 ml of *Eucalyptus sp* oil showed complete repellency (100%) 36 hours after application (Alsudani *et al*., 2021). The proven rellepent activity of EPEO may be attributed to the active chemical constituents of these oils and the chemo-receptors on insect body wall (Tyagi, 2016). It is interesting to observe that a high class of repulsion (Class III, IV and V) effect was found in the doses of 40 µl, after an exposure of respectively 15, 30 and 45 minutes, compared to those of lower doses of EPEO. In similar study of Elnabawy *et al*. (2021) with *E. camaldulensis* EO; the repellency assay showed 77.77% repelled adult red flour beetle after 180 min of exposure at 15%. Abdelrazik *et al*. (2018) assessed the repellent activity of *E. globulus* EO against *T. castaneum*. Their result showed high effectiveness of this oil with 66.67±18.9% and 90.0±10.0% after 3 and 72 hours. Our results indicated that Eucalyptol was one of the major chemical compositions in the eucalyptus essential oil, which made up (34.87%) of the total chemical composition of the oil. Eucalyptol has insecticidal activities against many insects (Gao *et al*., 2023).

Studies indicate that ***L. camara*** **EO** (LCEO) possess insecticidal properties against various pests found in agricultural and domestic settings, making it comparable to modern pesticides in effectiveness and economically viable (Liambila *et al.,* 2021). The results of Sabbour (2020) are in accordance with the findings of present study where LCEO gave the highest mortality percentage of *T. castaneum* after seven days with 83.5% repellency. In similar studies by Nea *et al.* (2020), the repellent property of LCEO (leaf and flower) against adults of *Sitophilus granarius* increases with their concentration. At the concentration of 42 µL/mL, these two EOs exhibited high activity, with repellency rates of 86.66 ± 0.57% and 93.33 ± 0.57%, respectively. Karahacane and Kaci (2021), in their study, reported the effectiveness of LCEO against adult red flour beetle, where maximum mortality was 93.33%

at 120 hours of exposure at 200 Jil. It is interesting to notice the high class of repulsion (Class IV) effect was revealed at the doses of 30 and 40 µl, after an exposure of 45 minutes (Tab 31).

Furthermore, the repellent activity of ***Salvia rosmarinus* EO** (SREO), in the present study, reached 66% after 45min at the dose of 40 µl (Tab 31), comparatively; Boukraa *et al*. (2022) reported that the repellency percentage of this oil against *T. castaneum* was equal to 41.67 ± 10.41%. In a similar study, Khalil *et al*. (2015) revealed a percentage of SREO repulsion (wild and cultivated) of 65% and 45% respectively against *Tribolium castaneum* adults recorded after 120 min. Particular attention to the results of Mahfuz *et al*. (2023) who observed that the highest effect of repellency of rosemary EO was found in the doses of 62.87 and 31.44 µg/cm^2, on the other hand, the highest doses (i.e., 251.49 and 125.75) have a weak effect. In the research of Mahfuz *et al*. (2023), active compounds of rosemary essential oil also showed effective repulsion, which agrees with our current research, they also reported a class III of repulsion of rosemary oil against *T. castaneum*, which was founded repellent (class IV) in this study. Whereas, Rosemary oil was found to be more toxic towards *Callosobruchus maculatus* as compared to *Tribolium castaneum*, because only 22% mortality was achieved with the highest concentration to *T. castaneum* giving at the LC_{50} (35µl/m) compared to 100% mortality of *C. maculatus* (Singh, 2016). Another finding that stands out from the results previously reported by Shawer *et al.* (2022) reported that the two lowest concentrations of SREO (10 and 20 ppm/cm^2) were not able to cause a reduction in the number of *T. castaneum* adults at 1, 2 or 3 days, neither showing no mortality, while the highest concentration (200 ppm/cm^2) showed significant activity against mortality of 40, 53.3 and 67%, respectively, at 1, 2 and 3 days. It is also reported that linalool (one of SROE componments) has an acetylcholinesterase inhibition and repellence effect against some insects (Elnabawy *et al*., 2021).

In the present investigation, the repellent and toxic potentials of ***Cupressus sempervirens* EO** (CSEO) and its major component (2-Pinene: 20.44% and 3-Carene: 11.98%), were evaluated at 63% against adult *T. castaneum* for a period of 45 mins under a dose of 40 µl and reached the class IV of repellent activity (Tab 31). In the current study red flour beetle showed greater sensitivity to CSEO. Our results are in accordance with a study investigated by Ncibi *et al*. (2019) showing that CSEO caused 100% repellency after 6 hours of exposure to the dose 0.15µl/cm². Similarly, Amini *et al*. (2023), revealed that higher concentrations of essential oils of different Cupressus species (*C. sempervirens* L. and *C. arizonica* Greene (Sarv - e -simin) collected from Tehran, *C. sempervirens* L. var. horizontalis

(Mil.) Gord (Zarbin) and *C. sempervirens* L. var. stricta (Sarve -Shirazi) were gathered Mazandaran, Zarbin pure essential oil (France) against the flour weevil (*Tribolium castaneum* Herbst). Specifically, the EO from *C. sempervirens* (France) exhibited a significantly higher repellent effect (87.5 %) at low concentration (10µl/70 cm^2) on *T. castaneum* compared to the other essential oils of *C. sempervirens* (Tehran), *C. sempervirens* (Mazandaran) and *C. arizonica* had 74.11, 73.75 and 74.5% repellent effect, respectively on *T. castaneum*. The result also showed that *C. Sempervirens* essential oil (France) (357 µL/L of air) had the highest mortality percentage 90 % after 24 h and 100 % after 48 h. In the chemical analysis α-Pinene was the major constituent in five species of the *Cupressaceae* family, which is consistent with other research results (Louni *et al.*, 2019), (Sriti *et al.*, 2023) et (Almadiy et Nenaah, 2022). Our results of the contact test are also in agreement with those of Saad *et al*. (2013) who evaluated the effectiveness of CSEO at different concentrations (50, 100, 200, 300 and 400 µg/mL) against *T. castaneum*, recording efficiencies lower than ours, ranging from 42% to 20%.

Table 31. Repellency of adult *T.castaneum* with OEs and their classification

EOs	Time (min)	Doses (µl/cm^2) and Repellency class			
		10 µl	**20 µl**	**30 µl**	**40 µl**
E. polybractea	15	36±1.52 (II)	50±1.00 (III)	53±1.52 (III)	60±2.00 (III)
	30	43±1.15 (III)	56±2.08 (III)	66±1.15 (IV)	63±1.15 (IV)
	45	56±1.52 (III)	60±2.00 (III)	76±0.57 (IV)	86±0.57 (V)
L. camara	15	16±0.57 (I)	23±0.57 (II)	40±1.00 (II)	30±1.00 (II)
	30	30±1.00 (II)	26±1.52 (II)	60±1.00 (III)	50±2.00 (III)
	45	43±0.57 (III)	53±0.57 (III)	70±1.00 (IV)	76±1.52 (IV)
S. rosmarinus	15	13±0.57 (I)	26±1.15 (II)	30±1.00 (II)	40±2.00 (II)
	30	20±1.00 (I)	30±1.00 (II)	46±0.57 (III)	60±1.00 (III)
	45	26±0.57 (II)	33±1.52 (II)	60±1.00 (III)	66±0.57 (IV)
C. sempervirens	15	10±1.00 (I)	26±1.15 (II)	43±1.52 (III)	50±2.00 (III)
	30	16±1.15 (I)	33±1.52 (II)	50±0.00 (III)	56±0.57 (III)
	45	23±1.52 (II)	46±1.15 (III)	56±1.15 (III)	63±1.15 (IV)

8.1.2. Repellency bioassay of plant-extracts

Repelling is the system that tends to deter pests from a susceptible crop, which can be called a push approach. However, plants are a natural source of this repulsive approach, reported in numerous ethnobotanical informations. Indeed, due to the different extraction and presentation procedures of the findings, it is difficult to compare the results with previous studies and under laboratory conditions. The different concentrations of methanolic plant extracts used for the evaluation are 50, 100 and 150 µl. These values are selected after a series

of tests, which led us to define another range of concentrations for the rest of the extracts (50, 80 and 100 µl). Many studies showed that polar solvents, especially methanol and ethanol, are more efficient in extracting phenolic compounds because they have high polarity and good solubility. It should be also noted that the bibliographic query shows a lack of specific information on the repellent activity of extracts of *Morus alba* and *Rubus ulmifolius* on *Tribolium castaneum*. The present study demonstrated that plant-extracts used with different concentrations for each observation period showed significantly higher differences in *T. castaneum* mortality. Control (acetone) had no effect on this stored product pest.

Repellent effect of leaves methanolic extract of ***Morus alba*** showed very high repellent power among other plant extracts tested, and resulted in 100% repellency 45 minutes after application of a dose of 150 µl (Tab 32). It seems that this plant extract has gone from repellent class III at 10 µl, to class IV at 100 µl to reach class V at the highest dose. However, biotest results support the present conclusion of the repulsive nature of white mulberry extracts against stored product pests (Sabuj *et al*., 2017a; Sabuj *et al*., 2017b). Due to its high polarity, methanol was found to be more effective in extracting various polar phytol compounds from the leaves of this plant.

The repellent potential of the methanolic extract of ***R. umlifolius*** leaves against *T. castaneum* was medium (class III) at 150 µl after 45 minutes (Tab 32). Akkari *et al.* (2016) showed that the methanolic extract from *Rubus ulmifolius* possesses *in vitro* anthelmintic and inhibited 95.66% of egg hatching at 4 mg/ml. Ghabbari *et al*. (2018) revealed that *R. ulmifolius* extract elicited a significant insecticidal activity against fruit fly *Ceratitis capitata* (a major pest of fruit orchards).

The topic of **combination effects** between extracts has gained popularity in many scientific disciplines in order to evaluate the interactions between several bioactive constituents. The combination ratio between plant extracts does not fully describe these botanical extracts because other important factors influence the composition of the final extracts, such as the quality of the raw material, the extraction solvent(s) used (s), the duration and temperature of extraction, the percentage and type of excipients present (Monagas *et al.*, 2022). Botanical extracts may contain hundreds or even thousands of individual constituents at varying abundance. Too often it is assumed that the behavior of a mixture can have a synergistic, additive or antagonistic effect. Given the significant insecticidal effect of Morus methanolic extract alone, his combination with Rubus methanolic extracts at ratio of 50:50, has led to an antagonist insecticidal effect (Tab 32).

Table 32. Repellency of adult *T.castaneum* with plant methanolic extracts and their classification

Methanolic extracts	Time (min)	Doses (µl/cm^2) and Repellency class			
		50 µl	100 µl	150 µl	Acetone
Morus	**15**	36±1.52 (II)	53±0.57 (III)	66±1.15 (IV)	0.00
	30	53±0.57 (III)	66±1.15 (IV)	76±0.57 (IV)	0.00
	45	60±2.00 (III)	76±0.57 (IV)	100±0.00 (V)	0.00
Rubus	**15**	13± 0.57 (I)	23±0.57 (II)	36±1.52 (II)	0.00
	30	16±0.57 (I)	33±1.52 (II)	43±0.57 (III)	0.00
	45	56±1.52 (III)	60±2.00 (III)	60±2.00 (III)	0.00
***Morus*+*Rubus* (50 :50)**	**15**	13± 0.57 (I)	16±0.57 (I)	23±0.57 (II)	0.00
	30	23±1.52 (II)	36±1.52 (II)	43±0.57 (III)	0.00
	45	43±0.57 (III)	53±0.57 (III)	60±1.00 (III)	0.00

This study revealed the effect of aqueous extracts obtained from ***Dittrichia viscosa*** leaves, on the adults of *T. castaneum*. Therefore, it is the most recommended method for the extraction of the bioactive compounds. Plants are known to produce a wide variety of bioactive compounds and substances characterized as natural defence molecules. The concentration of each compound in the plant is influenced by several factors including plant physiology, growing conditions and geographic location. The repellent effect increases with increasing exposure duration and dose used. At high dose (100 µl), the effect reached the class IV as medium repellent (Tab 33). The results of our study confirm those of Al-Joary *et al.* (2021) who observed that *D. viscosa* causes a low repellent rate against adults of *Tribolium confusum*. Furthermore, our results are also consistent with what Rotundo *et al.* (2019) observed by studying the biological effectiveness of three different extracts (N-hexane, methanol and distilled water) of the aerial part of *D. viscosa* against adults of *Sitophilus granarius* L, using ingestion and contact methods, where they found that the ingestion method caused low mortality rates and a repellent effect, while only a slight decrease was recorded in some nutritional parameters for the aqueous extract. In the biological assays of Lampiri *et al.* (2020), the water-soluble extract powder of lyophilized epicuticular material of this plant was applied in four doses to wheat: 0 (control), 1,000, 3,000 and 5,000 ppm and the mortality of exposed individuals was measured after 1, 3 , 7, 14 and 21 days of exposure. It turned out that among these species tested, *Oryzaephilus surinamensis* was found to be the most sensitive, followed by *Tribolium confusum* and *Sitophilus oryzae*, while *Rhyzopertha dominica* was not practically affected.

The results showed that the ethanolic extract of ***L. camara*** leaves demonstrated different levels of repellency, with the greatest effectiveness (classified repellent) being demonstrated at 100 µl with 86% after 45 minutes (Tab 33). There is no previous report about the toxicity of ethanol extract of *L. camara* to *T. castaneum.* Generally, the effectiveness of *L.camara* extract can be explained by the presence of a highly repellent bio-pesticide in the leaves of this plant. This is consistent with the study of Mvumi et Maunga (2018) who indicated that Lantana leaves have some toxic properties and may be a potential source of biopesticides such as dodecanol, 1-eicosano, piperidine and ethoxy (Ayalew, 2020) for use in pest control strategies against weevils with economic and environmental benefits. In the recent study of Ayalew (2020), the extracted oil and powder from the leaves of *L. camara* was found to be effective in controlling maize grain weevils (*Sitophilus zeamais*) with extracted by methanol fraction showed highest percentage of mortality rate (74%). Asiry et Zaitoun (2020), under laboratory conditions, found that ethanolic extract of *L. camara* leaves was the most effective, where it caused mortality rate of 73.3% of Khapra beetles larvae *Trogoderma granarium* Everts (Coleoptera: Dermestidae) at 400 ppm after 2 d, and 86.7 % mortalities after 6 days.

The results in Table (33) showed that the ethanolic extract of ***P.orientalis*** cones did not cause a strong repellent effect on *T. castaneum*, with the highest repellency percentage being 76% at 100 µl after 45 minutes of exposure. Repellency class reported for this plant extract was from weakly repellent (class 1) at doses of 50 and 80 µl after 15 min to medium repellent at 80 and 100 µl after 45 min of exposure. Generally previous reports on the repellency of *P. orientalis* extracts against weevils cannot be compared, given the difference in plant part, method and solvent used and specifically the difference in taxonomic ranks of the insects tested. However, the results of our study contradict those of Al-Joary *et al.* (2021) who observed that *P. orientalis* causes a low repellent rate against adults of *Tribolium confusum* less than 1. Adeyemo Moses et Ileke (2019) revealed that *T. orientalis* leaves and fruit oils evoked 80%, 95%, 100%, 100%, 100%, and 100% mortality of adult *Sitophilus zeamais* at rates of 0.1ml/20g, 0.2ml/20g, 0.3ml/20g, 0.4ml/20g, 0.5ml/20g, and 1.0ml/20g of maize grain after 24hours of post treatment respectively. According to Adeyemo Moses and Ileke (2019), limonene was among the main components of *P. orientalis* EOs which have insecticidal and repellent bioactivities for *T. castaneum*.

Table 33. Repellency of adult *T.castaneum* with plant extracts and their classification

Extracts	Time (min)	Doses (µl/cm^2) and Repellency class			
		50 µl	80 µl	100 µl	Acetone
Aqueous extract of *D. viscosa* leaves	**15**	10±1.00 (I)	13± 0.57 (I)	16±0.57 (I)	0.00
	30	36±1.52 (II)	43±1.15 (III)	53±0.57 (III)	0.00
	45	46±1.15 (III)	56±2.08 (III)	63±1.15 (IV)	0.00
Ethanolic extract of *L. camara* leaves	**15**	3± 0.57 (I)	6±0.57 (I)	13± 0.57 (I)	0.00
	30	46±0.57 (III)	53±0.57 (III)	66±1.15 (IV)	0.00
	45	63±1.15 (IV)	76±0.57 (IV)	86±0.57 (V)	0.00
Ethanolic extract of *P.orientalis* cones	**15**	3± 0.57 (I)	6±0.57 (I)	10±1.00 (I)	0.00
	30	36±1.52 (II)	43±0.57 (III)	60±1.00 (III)	0.00
	45	43±0.57 (III)	63±1.15 (IV)	76±0.57 (IV)	0.00

8.2. Fumigant activity bioassay

8.2.1. Fumigant activity of EOs

Insecticidal fumigation is one of the most widely adopted control methods to protect stored products from insect infestations. This study showed that EOs have variable significant toxicity against adult *Tribolium castaneum*. Since all oils tested showed a mortality rate greater than 50% at the highest dose tested (10 µl), *Eucalyptus polybractea* EO exhibited the greatest fumigant toxicity, followed bythe EOs of *Lantana camara*, Rosemary and finally Cypress. Interestingly, the toxicity of the fumigating EOs tested varied depending on exposure time and treatment doses (Panezai *et al*., 2019) (Mahfuz *et al.,* 2023). Furthermore, it's not acceptable to compare the similarity of the results of different studies on the fumigation activity of EOs because these studies are carried out at different doses and exposure durations. According to Khalil *et al.* (2015), it seems that EO from wild rosemary turned out to be more toxic on *T. castaneum* than that from cultivated ones. Overall, all concentrations tended to increase the mortality effect on *T. castenum* as the concentration of used EOs increased, which is supported by the results of several studies (Panezai *et al*., 2019); (Eman, 2018); (Alghamdi, 2018). These results can be explained by the relationship that may exist between the mode of action of EOs and their chemical composition. Furthermore, in the present investigation, all EOs studied had a large effect (>40%) at 2µl. Similarly, Juan *et al.* (2011) discovered that plant EO components influence insect toxicity. Linalool present in most tested EOs (Lantana, Rosemary and Cypress EO) was considered as an acetylcholinesterase inhibitor and it has been demonstrated as a potent contributor to the repellent and insecticidal activities (Jiang *et al.,* 2016). Moreover, Amy *et al*. (2020) reported that the linalool component inhibits both c-aminobutyric acid type A receptors and nicotinic

acetylcholine receptors. However, *T. castaneum* seems to be more resistant to the fumigant activity than other weevils such as *Rhyzopertha dominica* (Ncibi *et al*., 2019), *Trogoderma granarium* (Khalil *et al*., 2015), *T. confusum* (Eman, 2018). In this current study, the mortality of the control was 0.

The result of a previous research study may be analogous to the present finding, as we found that ***E. polybractea*** EO was the most effective against adult red flour weevil with total mortality (100%) at 8µl after 72 h and also at 10 µl after 48h. The fumigant activity showed that LC_{50} was 4.12 µl/l air (Fig 35). However, there is limited information available on the activity of fumigation bioassays of *E. polybractea* EO against stored food pests. Similar findings were obtained whith Abdelrazik *et al*. (2018), with a fumigant activity of 7 EOs including *Eucalyptus globulus* EO against adult *T.castaneum* where the highest mortality effects were recorded as 90% at higher concentration of 25% after 72 hrs, and 100% mortality of larvae at 12.5% after 72 hrs and also at 25% after 48 hrs. Cluster analysis was carried out by Siddique *et al*. (2017) with EOs of 10 selected species of *Myrtaceae* including *Eucalyptus crebra, E. kitsoniana, E. melanophloia, E. microtheca, E. pruinosa, E. rudi* and *E. tereticornis* from Pakistan, to assess the fumigant bioassay activity against red flour weevil. The essential oils from Eucalyptus species caused different mortalities in *T. castaneum*, their LC_{50} values ranged from the most lethal fumigant *E. rudis* EO (LC50 = 146.3 µl/L), while *E. pruinosa* EO was the least toxic (LC_{50} = 1046.1 µl/L). In another study (Lucia *et al.*, 2012), among 15 species of the genus *Eucalyptus*: the EO of *E. polybractea* was the most effective adulticide in terms of fumigant activity against *Aedes aegypti* with a KT_{50} (min) value of 3.86, which can be related to the presence of large amounts of 1,8-cineole component and their relative concentration in the EO.

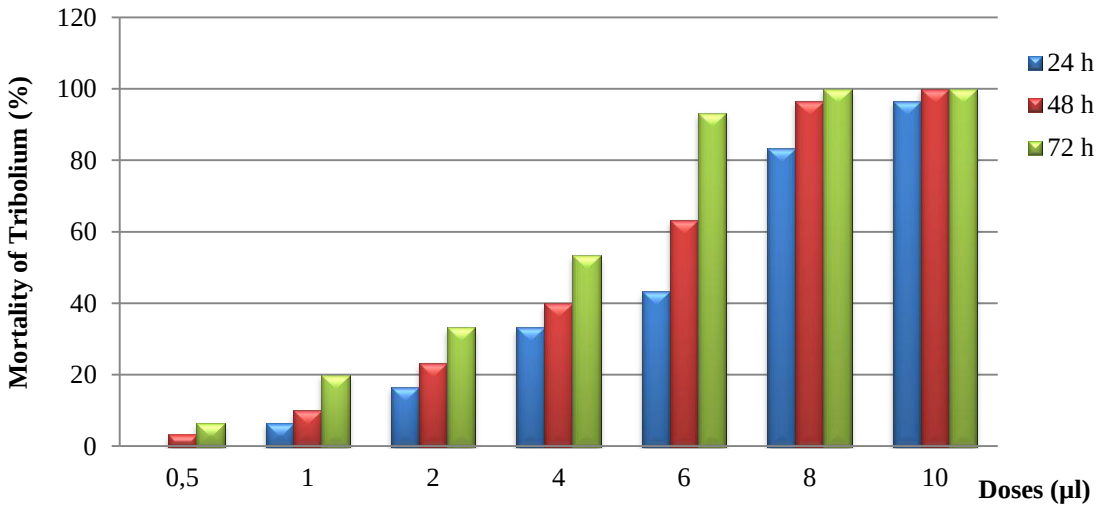


Figure 35. Mortality (%) of *T. castaneum* using *E. polybractea* EO

In the present investigation, the fumigantl activity of ***Lantana camara*** EO was evaluated against adult *T. castaneum* at different exposure time and treatment doses. Experimence shows great effectiveness of EO becauce it causes total mortality of adult castaneum at high dose of 10µl after 72 hours. The results also shows that at 2 µl, the mortality effect exceeded 50% after 48 hrs and LC_{50} = 5.27 µl/l of air (Fig36). This outcome is different from those described by Kumar and Pandey (2021). They reported the high effectiveness (mortality of 100%) of 9 EOs including the EO of *L. camara* at 0.1, 0.2, 0.3 and 0.4% against *Sitophilus oryzae*, *Rhyzopertha dominica* and *T. castaneum* in wheat stored after 24 hours of treatment for up to fifteen days. In the same context, previous study of Henagamage (2023) reported very promising fumigant toxicity (76.7%) of 20 µL of *L. camara* ethyl acetate extract against *T. castaneum* over the treatments (48 hours). Our study confirmed the dose dependent manner of the mortality with the time and the extract concentration, which supported the findings of Alghamdi (2018). In another study of Kumar *et al*. (2022), fumigant toxicity tests of 4 plants EOs including *L.camara* and their combinaison against three stored product beetles (*Sitophilus oryzae*, *Rhyzopertha dominica* and *Tribolium castaneum*) in wheat stored in superbags produced strong effect (100% mortality) after 10 and 12 months of storage.

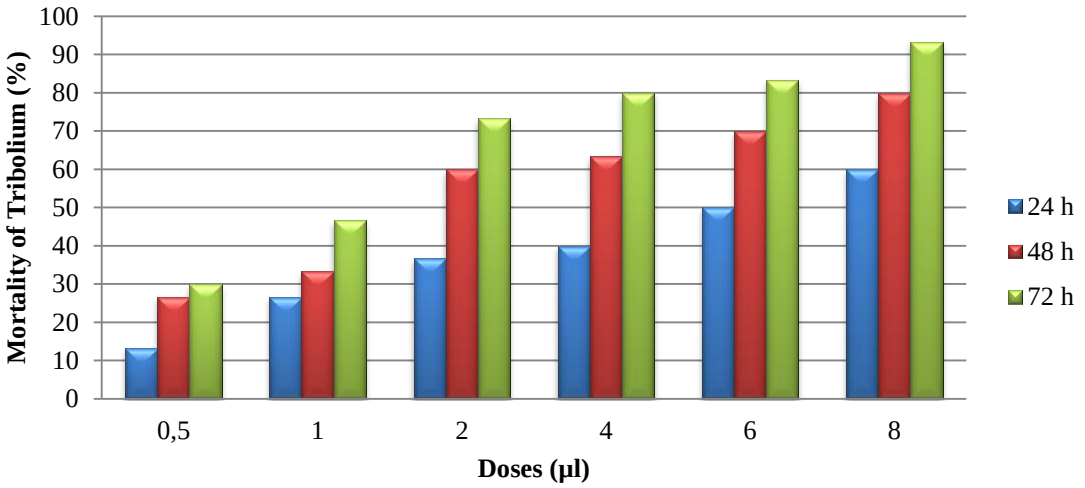


Figure 36. Mortality (%) of *T. castaneum* using *Lantana camara* EO

In fact, as shown in Figure 37, **rosemary** leaf EO was toxic towards *T. castaneum*, as the highest concentration (6, 8 and 10 µl) led to mortality percentage >50% after 24 hours and had increased up to 90% at 10 µl after 72 h. Insecticide fumigation showed that LC_{50} was 5.27 µl/l of air. In most cases, *T.castaneum* mortality percentages increased with the concentration and exposure periode. Comparatively, our results are in consistent with those of Khalil *et al*. (2015), who showed that for wild and cultivated rosemary essential oils, respectively, LC_{50} values were 65.5 µl/l air and 180 µl/l air and also percentage mortality of 92 and 56%, 24 hours after treatment at concentrations of 200 µl/l air. In another investigation of Panezai *et al*. (2019), rosemary ethanolic extract had a significant and similar effect *T. castaneum* larvae and adult, as the mean percent mortality was 58.67%. In the same context, Teke et Mutlu (2021) founded that Rosemary oil exhibited 58.41% fumigant activity against red flour weevil. One of the most widely recognized hypotheses that rosemary EO is responsible for the inhibition of acetylcholinesterase (AChE) due to the presence of terpenes and monoterpenes, which are the main constituents of Rosemary EO, 1,8-cineol (monoterpenoid), camphor (terpene) and α-pinene (monoterpenoid) (Bajalan *et al*., 2017). Another study investigated by Ncibi *et al*. (2019) exhibited that Rosemary EO showed over 50% of mortality of *T. castaneum* after 24 hours.

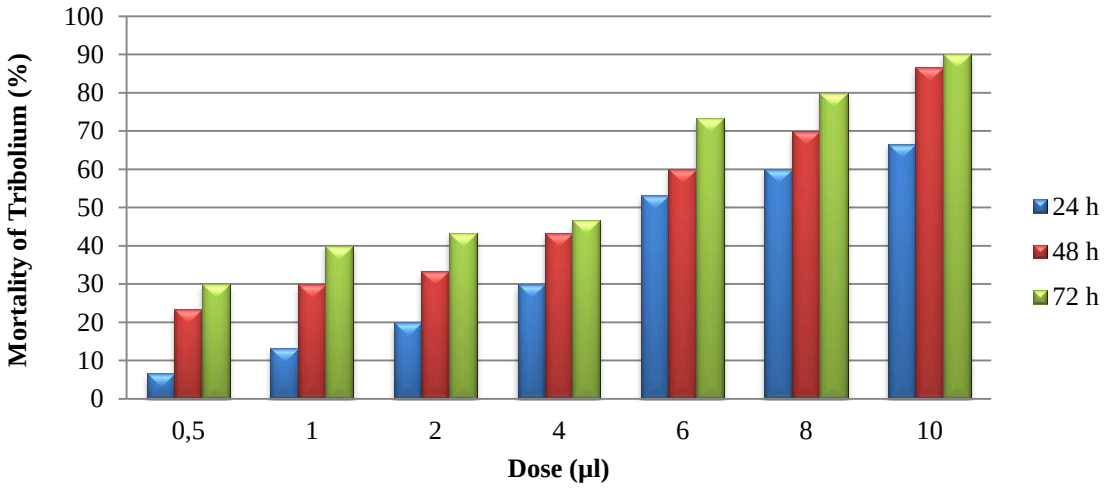


Figure 37. Mortality (%) of *T. castaneum* using *Rosemary* EO

Insecticidal bioactivity by fumigation, studied with **Cypress** EO, revealed that after 1µl, the mortality effect reached more than 50%. The highest value of 83% was recorded at 10 µl after 72 hours and the LC_{50} scored 1.67 (Fig 38). The results of mortality percentage showed that in the studied EO with increasing concentration and time, the mortality percentage on adult *T. castaneum* increases (Amini *et al.,* 2023). Similar supportive studies by Amini *et al*. (2023) have explored fumigant efficacy of 4 EOs of different Cupressus species (*C. arizonica* Greene collected from Tehran, *C. sempervirens* L. var. *horizontalis* (Mil.) and *C. sempervirens* L. var. stricta were gathered from Mazandaran (Chalus valley) and *C. sempervirens* L. (France), with fumigant properties against the flour weevil (*Tribolium castaneum* Herbst). High mortality percentage was related to *C. sempervirens* EO (France), which at a dose of 357 µL/L of air, after 24 and 48 hours were 90 and 100%, but lowest mortality percentage was related to *C. sempervirens var. stricta* EO, which at a dose of 428.5 µL/L of air after 48 h had 59 % of mortality. This shows the difference in effectiveness between species of the same genus. However, *C. sempervirens* EO exhibited the strongest fumigant bioactivity of *Sitophilus oryzae* exposed to (10 µL/L air) caused 100% adult mortality after 4 days (Almadiy *et al.*, 2023). The bioactivity of plant EOs is attributed to several components, although a synergy with other minor constituents is common where each oil component participates in penetration, fixation, and distribution into biomembranes (Nenaah *et al.,* 2022). A synergy between the components of an EO might be occurring between several components contained in the same EO, or between different EOs with known biological activities (Nenaah *et al.*, 2022).

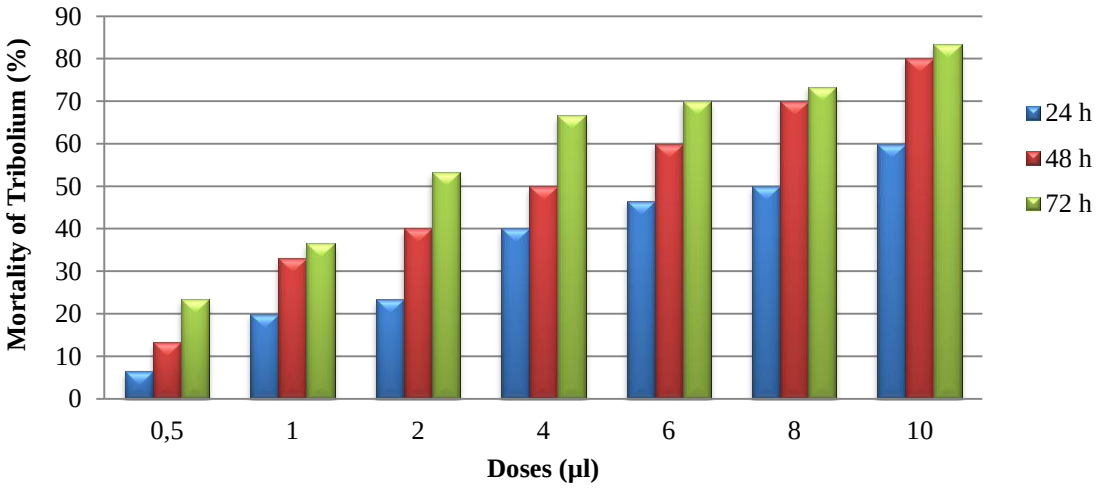


Figure 38. Mortality (%) of *T. castaneum* using Cypress EO

8.2.2. Fumigant activity of plant-extracts

The fumigant action of different plant extracts was evaluated using different plants against many weevils. Their effectiveness depends on several factors, such as the dosage of the treatment, the development stage of the insect, the part of the plant and the solvent used. It is also important to note that the cuticle composition of storage-produced insect species is dynamic and changes depending on the age, developmental stage and metabolic state of the insect (Grigoraki *et al*., 2020), exhibiting its essential function in the adaptation capacity and survival of the insect in the face of changing environmental factors (Balabanidou *et al*., 2018). It has therefore been demonstrated that variations in exposure reflect changes in the cuticular components of stored product insects (Claudio-Piedras *et al*., 2021). To our knowledge, this study is the first that examined the insecticidal effect of **mulberry** methanolic extract for the control of stored-grain insect *T. castaneum*. Generally, the weevil mortality percentages increase with concentration and exposure period. In fact, as shown in Figure 39, methanolic extract of Morus leaves was toxic towards *T. castaneum*, as the highest concentration 30 µl led to mortality percentage of 76,6% after 72 h.

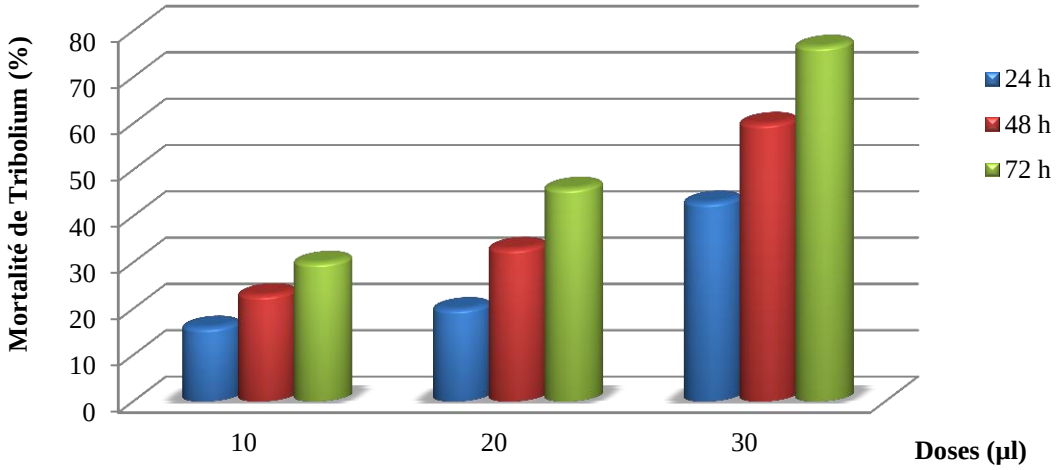


Figure 39. Fumigant effect of methanolic extract of Morus leaves on *T. castaneum*

Rubus ulmifolius has been the subject of many studies concerning the antimicrobial effects of plant extracts, but there is still inadequate information regarding its insecticidal effects on stored product insects. Like Morus, the methanolic extract of the leaves of this plant gradually increase in the percentage of mortality of *T. castaneum* in parallel with the increase in doses and periods of exposure, knowing that 30µl marked 56.66% of mortality after 72h (Fig 40).

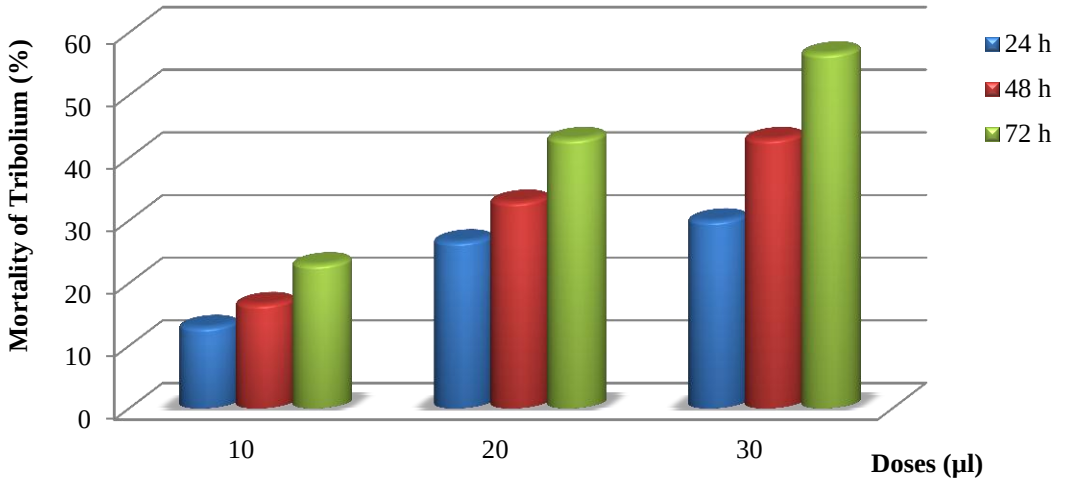


Figure 40. Fumigant effect of methanolic extract of Rubus leaves on *T. castaneum*

The combination of the methanolic extracts of Morus and Rubus at a ratio of 50:50 led to a synergistic insecticidal effect, as the mortality rate increased to 80% compared to 76.66 and 56.66% recorded for the Morus and Rubus respectively at high dose after 72 hours. Synergy between extracts is preferable for the control of weevils in stored products, as plant extracts in combination provide more benefits than are typically available alone (Atta *et al.*,

2023). The combined use of plant extracts could improve the effectiveness of insecticidal action by achieving synergy, acting simultaneously on multiple targets, reducing doses of individual extracts and minimizing side effects (Soulaimani *et al.*, 2021).

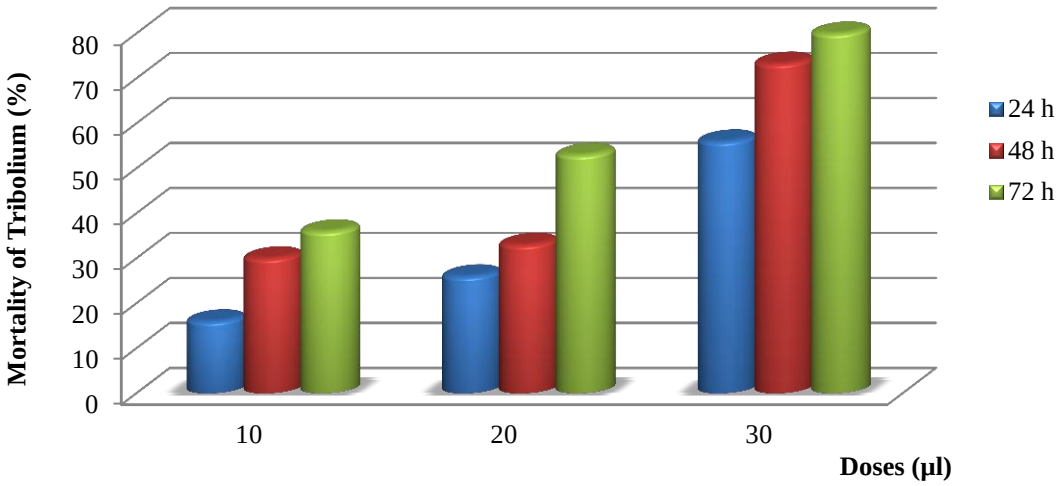


Figure 41. Fumigant effect of methanolic extract of (Rubus+Morus) leaves

The average mortalities corrected for the different concentrations of ***Dittrichia viscosa*** aqueous extract used as a function of exposure time are illustrated in Figure 42. However, there is little information about the false yellowhead, *Dittrichia viscosa* (L.) Greuter, despite some work showing potential insecticidal value (Rotundo *et al.*, 2019). The results show a proportional action between the exposure time and the dose used. After 72 hours, the mortality rate for a high concentration of 30 µl is 30%. In a recent study by Lampiri (2020), the toxicity of lyophilized epicuticular material of *D. viscosa* on four major stored product beetle species was found to be most sensitive on *Oryzaephilus surinamensis*, followed by *Tribolium confusum* and *Sitophilus oryzae*, while *Rhyzopertha dominica* was practically unaffected. However, Rotundo *et al.* (2019) found that aqueous extract of Inula does not affect *Sitophilus granarius* (0% mortality).

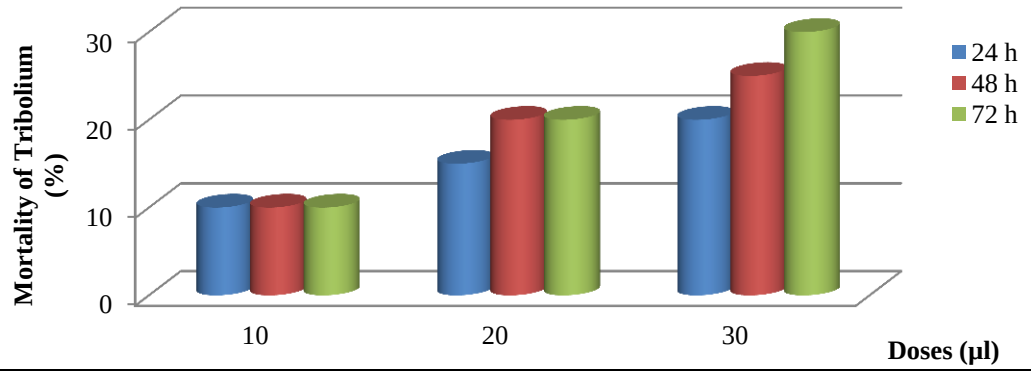


Figure 42. Fumigant effect of aqueous extract of Dittrichia leaves

Fumigant toxicity of ethanolic extract of ***Thuja orientalis*** cones against *T. castaneum* show that an increase in doses is followed by an increase in mortality (%) (Fig43). We note that after 72 hours, the mortality in Tribolium was 52, 56 and 64% for doses of 10, 20 and 30 µl. Through literature reports, it should be noted that there is limited information available specifically on the fumigant toxicity of *Thuja orientalis* extracts on *Tribolium castaneum*. Generally, the fumigant toxicity of plant extracts can vary depending on the specific compounds present in the extract and the concentration used. Different insects may also react differently to these compounds. Hashemi et Safavi (2012) reported that oils extracted from leaves and fruits of *Platycladus orientalis* (L.) Franco contain α-pinene as a major component (35.2%, 50.7%), respectively, and were most effective against *T. castaneum*.

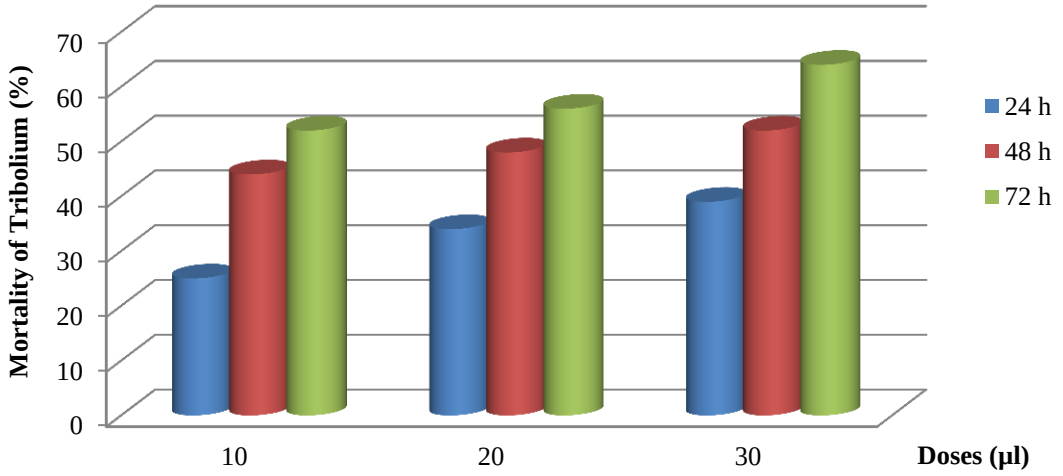


Figure 43. Fumigant effect of ethanolic extract of Thuja cones

It should be noted that there are very few previous reports on the toxicity of ethanolic extract of ***L. camara*** leaves to *T. castaneum*. Our results clearly showed a result similar to that of Henagamage (2023) and Rajashekar *et al.* (2014) confirming that the effectiveness of this plant extract increases by increasing the dose and exposure time, which indicate that the effect of the plant extract was dose-dependent. In addition, this plant-extract exhibited a fumigant activity between 42 and 52% at 72 h (Fig 44). Rajashekar *et al.* (2014) affirmed that the methanol extract from leaves of *L. camara* has fumigant toxicity against adults of *T. castaneum*, where 80 % mortality of the insects was achieved only at the higher dose of 500 mg/L[1] in 7 day exposures. Similarly, Asiry et Al Nasser (2022) showed that the highest adult mortality of *T. castaneum* was 97.8% with the use of the methanolic extract of *L. camara* at 500 ppm after 6 days exposure. In a study of Asiry et Zaitoun (2020), the ethanolic extract of *L. camara* caused mortality rates of Khapra beetle *Trogoderma granarium* Everts

(Coleoptera: Dermestidae) under laboratory conditions at 400 ppm of 73.3% after 2 days and 86.7% after 6 days. However, gas chromatography mass spectrometry analysis of *L. camara* extract indicated the existence of effective fumigating molecules.

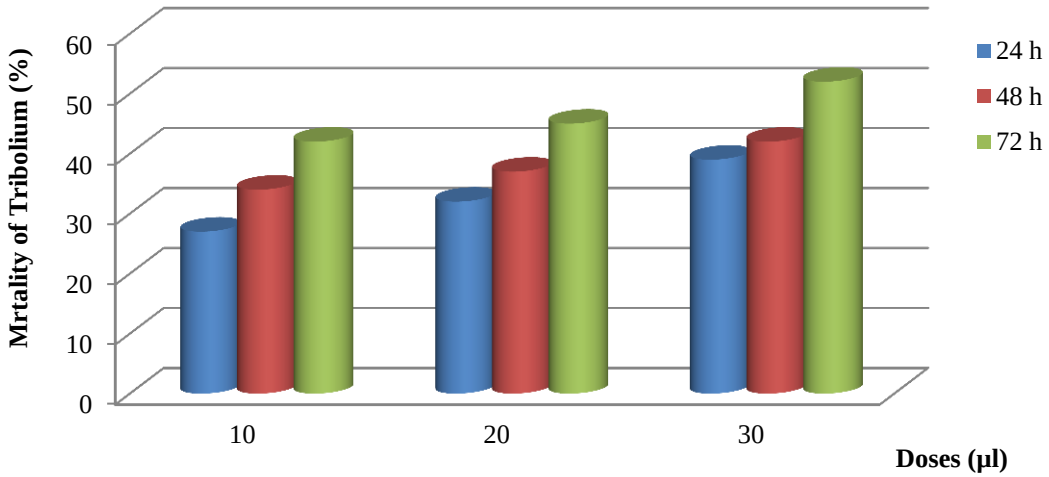


Figure 44. Fumigant effect of ethanolic extract of Lantana leaves

Conclusion

Stored grain insects cause significant damage to stored wheat. Therefore, in the present investigation, all EOs and plant-extracts tested showed relatively strong insecticidal activity and confirmed high repellent and fumigant capacity against the red flour beetle *T. castaneum*. In addition, the results revealed that the effectiveness of the insecticidal activity strongly depended on the concentration and the exposure time. It is worth noting that fumigation yielded better results than repellent tests. However, it is important to know what therapeutic or even toxic response leads to the combined effect of plant extracts and to optimize the appropriate proportion that produces a more effective effect. To summarize, the biological fumigation activity of EOs was very remarkable, with the highest concentration tested showing 100% insecticidal activity. Depending on the strength of the fumigant effect, the order of activity of the EOs was as follows: *Eucalyptus polybractea* > *Lantana camara* > *Salvia rosmarinus* > *Cupressus sempervirens*. On the other hand, the preliminary examination showed a strong repellent activity of the EOs with significant differences between them. Likewise, if we want to classify these EOs according to their effectiveness, we have: *E. polybractea* > *L. camara* > *S. rosmarinus* > *Cupressus sempervirens*.

In parallel and through this study, we have tested the fumigant and repellent efficacy of six different plant extracts from five commonly grown plants of Algeria against *T.*

castaneum infesting stored wheat. Concerning the fumigant effect, the results suggest the following classification starting from the most effective extract which is the methanolic extract of the combination between *Morus alba* and *Rubus ulmifolius* followed by the methanolic extract of *M. alba* leaves, then the methanolic extract of *R. ulmifolius*, the ethanolic extract of *Thuja orientalis* cones, the ethanolic extract of *Lantana camara* leaves and finally the aqueous extract of *Dittrichia viscosa* leaves. However, the repellency test revealed the effectiveness of methanolic extract of *M. alba* leaves, followed successively by the ethanolic extract of *Lantana camara* leaves, the ethanolic extract of *Thuja orientalis* cones, the aqueous extract of *Dittrichia viscosa* leaves and finally the methanolic extract of *R. ulmifolius* followed by methanolic extract of the association between *M. alba* and *R. ulmifolius.*

From the current vantage point, the use of the different plant extracts and EOs as insecticides (fumigant and repellent) is problematic in many respects. Some relate to chemical characteristics, such as their volatility, quality and in particular their chemical compositions (the presence of major and active biological components); others are linked to environmental nature and geographical affiliation, without forgetting their rapid penetration into insects. Solvents play also an important role in the extraction process by significantly affecting the quantity and nature of plant secondary metabolites. Generally, under laboratory conditions, the dose used, duration of treatment, parts extracted and extraction techniques also have a major influence. The current investigation found that the relatively strong insecticidal activity that explore fumigant and repellent toxicity offers the possibility of future use of these plant-extracts and EOs in the control of harmful insects. Additionally, consideration of alternative methods used and targeted biological agents can produce positive results. Finally, these plant-extracts and EOs can be used as a safe and effective substitute for hazardous chemical pesticides used to control *Tribolium castaneum*.

GENERAL CONCLUSION
&
PERSPECTIVES

GENERAL CONCLUSION

One of the main management challenges associated with grain storage is preventing fungal growth on stored wheat. Our first objective was to increase knowledge of fungal species contaminating stored wheat grains; we identified several species in Algerian stored wheat for 1 year. The condition of wheat grains depending on the variety and the conditions for regulating fungal development in these stored grains. The study of the fungal procession associated with wheat revealed 80 strains were discovered, including 53 identified strains belonging to 18 different fungal genera. *Fusarium, Alternaria, Aspergillus, Penicillium, Cladosporium, Rhizopus, Absidia, Rhizoctonia, Trichoderma, Microdochium, Purpureocillium, Acremonium, Rhizomucor, Helminthosprium, Geotrichum, Sclerotina, Ulocladium et Chaetomium*. When grains are colonized by moulds there is a significant risk of contamination with mycotoxins, which are toxic chemical products, formed as secondary metabolites by these fungi. Many species of *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* are sources of important mycotoxins of concern in relation to animal and human health. Timely assessment of these contaminants and identification of major fungal species (toxicogenic or not) are important, not only for assessing food quality, but also for the development of control strategies to ensure food safety. Monitoring and control of mycotoxigenic fungi and mycotoxins becomes essential to maintaining high quality of grain and grain products in indoor storage facilities.

The second aim was to point out the biological activities of some plant resources of Eastern Numidia: 8 natural plants were used to prepare 8 extracts and 4 EOs and their valorization in the conservation of wheat *Triticum durum* Defs. We propose, in this study, to describe the chemical composition and biological activities of plant extracts of different plants *Cupressus sempervirens, Salvia rosmarinus, Eucalyptus polybractea, Lantana camara, Morus alba, Rubus ulmifolius, Dittrichia viscosa* and *Thuja orientalis*. Natural products, safe for the environment and low toxicity to living organisms, are attracting increasing interest as important sources for the development of biofungicides. Firstly, the extraction of the EOs of *C. sempervirens, L. camara, S. rosmarinus* and *E. polybractea* by hydrodistillation gave a yield of 0.66%, 0.76%, 0.68% and 0.80% respectively. The results of their analysis identified 65 components for *C. sempervirens*, with the main constituents that 2-Pinene, 3-Carene, Cedrol, alpha.- Terpinyl acetate , 4-Isopropylidene-1-cyclohexe, icyclo[3.1.1]heptane, 6,6-dimethyl. 65 constituents for *L. camara*, with the major constituents being

Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl) (10.22%), Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4- (7. 89%), 5-Hepten-3-one, 2-(5-ethenyltetrahydro-5-methyl-2-fur (7.83%), 1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z- (6. 93%),(-)-Germacrene D(5.49%), (1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0] undec (5.02%), Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate. The results of the analysis of *S. rosmarinus* EO identified 45 compounds, the main constituents of which are Phenol, 2-methyl-5-(1-methylethyl)- (35.47%), Eucalyptol (17.04%), Thymol (14. 36%), 1,7,7-trimethylbicyclo[2.2.1]heptan-2-one (5.04%), (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (4.74%) and 45 compounds for *E. polybractea* The most prominent compounds were Eucalyptol (34.87%), alpha.- phellandrene (13.10%), alpha.-Terpineol (12.17%), (1R)-2,6,6-Trimethylbicyc Trimethylbicyclo[3.1.1]hept-2-ene (5.49%) and Terpinen-4-ol (5.18%).

Secondly, the antimicrobial activity results, acquired through the disc diffusion method (aromatogram), against six pathogenic bacteria including *Pseudomonas aeruginosa* ATCC27853, *Salmonella sp, S. aureus, P. aeruginosa* and the yeast *Candida albicans*, indicate that the EOs possesses significant potential. *C. Sempervensis* exhibits weak activity against the bacterial strain *E. coli ATCC 25922*. The oil *S.rosmarinus*, on the other hand, displays weak activity against *Salmonella sp*. *S.rosmarinus* also show weak activity against the yeast *candida calbicans*. However, all oils tested demonstrate excellent activity against the tested strains. MIC (Minimum Inhibitory Concentration) values ranging from 0.91 to 8.7 (mg/ml) were obtained through micro-dilution testing. According to our findings, these EOs have demonstrated efficacy against various fungi, including *Aspergillus niger, A. flavus, Alternaria alternata, Cladosporium herbarum, Fusarium solani, F. graminearum, Penicillium expansum, P. sp1, P. sp2*, and *Trichoderma viride*. The potential for resistance in certain strains cannot be dismissed.

Furthermore, under laboratory conditions, the antifungal activity of plant extracts appears in the form of modifications in the morphology of the fungi and a reduction in mycelial development. However the sensitivity of different fungal species to these extracts (methanolic and aqueous extracts of both *Morus alba* and *Rubus ulmifolius* leaves, ethanolic extract of both *Lantana camara* leaves and *Thuja orientalis* cones aqueous extract of *Dittrichia viscosa* and methanolic extract of the combinaison *M. alba* and *R. ulmifolius*) varied which may be due to the difference in intrinsic tolerance of these isolates. While the methanolic extract of *M. alba* leaves and the aqueous extract of *D. viscosa* leaves showed a

very promising antifungal effect, that of the methanolic combination between Morus and Rubus is the weakest.

However, the investigation assessed how effective sodium bicarbonate ($NaHCO_3$) is at restraining the growth of two chemical fungicides, Vidan and Agriconazole, against the same fungi that were tested. Due to the favourable results, sodium bicarbonate should be prioritised when considering this substitute. Fungicides are currently the most effective method of combating harmful fungi; however, the rise of resistance to these products has necessitated the pursuit of new antifungal substances. Various doses of EOs or plant-extracts have exhibited substantial inhibitory effects on microorganisms as well as good biological activity, including antifungal activity, when compared to chemical fungicides and sodium bicarbonate. Therefore, plant-extracts, EOs and sodium bicarbonate show potential as an alternative to synthetic fungicides for managing fungal growth on stored wheat grains.

Moreover, the insecticidal effects on *Tribolium castaneum* were assessed through fumigation and repellency assays. In one hand, the repellent effect of EOs was noticeable at a concentration of 40µl. Nevertheless, these plant EOs proved to be highly effective following three days of fumigation treatment. At a concentration of 10 µl, *L. camara* and *E. polybractea* exhibited an overall mortality of three days. Meanwhile, at a concentration of 10 µl/cm^3, the percentage of mortality reached 90, and 83% for *S. rosmarinus*, and *C. sempervensis*, with respective LC50s of 5.27 and 1.67 mg/L. Our findings suggest that the use of Eucalyptus and Lantana EOs was the most effective. In the other one, we have tested also the fumigant and repellent efficacy of six different plant extracts from five plants of Algeria against *T. castaneum* infesting stored wheat. Concerning the fumigant effect, the results suggest the methanolic extract of the combination between *M. alba* and *R. ulmifolius* as the most effective followed by the methanolic extract of *M. alba* leaves, then ethanolic extract of *Thuja orientalis* cones. However, the aqueous extract of *D. viscosa* leaves was the weakest. The repellency test revealed the effectiveness of methanolic extract of *M. alba* leaves (showed totle mortality), followed by the ethanolic extract of *Lantana camara* leaves (86%), the ethanolic extract of *Thuja orientalis* cones (76%), the aqueous extract of *Dittrichia viscosa* leaves (63%) and finally the methanolic extract of *R. ulmifolius* followed by methanolic extract of the association between *M. alba* and *R. ulmifolius.* It is worth noting that fumigation produced superior results compared to the repellent tests. This suggests that the tested plant-extracts exhibit potential as a bioinsecticide source and are suitable for further investigation in the realm of biological control.

Finally, the present study represent a great advancement to elucidate the use of plants as biocontrol agents against bacteria, fungi and weevils in stored wheat to contribute to a sustainable and greener lifestyle. There is consensus on the need for new pesticides that are affordable, easily biodegradable, environmentally friendly and sustainable. Pesticides of plant origin are excellent candidates because they possess a wide range of biological activities.

In Perspectives

This study requires supplementation with a number of other studies:

- The large biodiversity of plants provides a great exploration field for research on bioactive compounds and their biological properties;
- Varying the concentrations used would better determine the effect of EOs or plant-extracts;
- Changing the experimental conditions would improve understanding of the differences in results;
- Fractionating these EOs or plant-extracts would help to identify the molecules responsible for these biological activities and to understand their mode of action;
- Evaluate the toxicity of combinations between EOs or plant-extracts and increasing the combination of two to three EOs or plant-extracts will result in a marked effect;
- Investigate additional biological properties of EOs or plant-extracts;
- Extend the exposure period for insecticidal activity, do other tests (antifeedant, sterilization) and also test the different stages of development of *T. castaneum* weevil (larvae and adults) to find the most sensitive stage;
- The huge amount of information generated by *in vitro* assays must be confirmed by *in vivo* assays and large-scale investigations.
- Molecular approaches, such as PCR, can serve as good alternatives to conventional methods for detection of mycotoxigenic fungi and mycotoxins in wheat grains;
- A good surveillance and proper hygienic measures should be implemented to reduce the likelihood of pathogenic contaminants in stored wheat.

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Original Research Article

Essential Oil of *Eucalyptus polybractea* (L.): Chemical Composition, Antifungal, Insect Repellent and Insecticidal Activities

Asma Chibi[1]* and Amina Hassaine[2]

[1]Faculty of Sciences, Department of Biology, Badji Mokhtar University Annaba Algeria. Plant Genetic Improvement Research Laboratory.
[2]Faculty of Sciences, Department of Biology, Badji Mokhtar University Annaba Algeria. Plant Biology and Environment Laboratory

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ABSTRACT

Eucalyptus essential oil has a wide range of bioactivity, but research on the activity of *Eucalyptus polybractea* is limited. Due to the great need for sustainable pesticides, this study was carried out to assess the antifungal and insecticidal activities of essential oil (EO) of *E. polybractea*. EO was extracted from the leaves by hydrodistillation and the composition of the essential oil was determined by gas chromatography-mass spectrometry (GC-MS). The antifungal activity was tested against the following organisms; *Aspergilus niger*, *Fusarium graminearum*, *Penicillium sp1*, *Penicillium expansum*, *Cladosporium herbarum*, *Penicillium sp2*, *Fusarium sp*, *Aspergilus flavus*, *Alternaria alternata* and *Trichoderma viride* which were isolated from wheat grain. The insecticidal and repellant activity was tested against stored product pests; *Tribolium castaneum*. GC-MS analysis revealed a significant number of monoterpenes in the essential oil with Eucalyptol (34.87%) being the major component. The highest antifungal activity was observed against *Fusarium graminearum*, *Penicillium sp2*, *Aspergilus flavus* and *Trichoderma viride*. EO showed repellent activity to *T. castaneum* (PR = 65%, after 45 min) and highly toxic with 100% mortality after 72 hours of exposure. The study therefore revealed significant intra-specific changes in EO quality, which is reflected in the different rates of antifungal and insecticidal activity.

Keywords: Essential oil, *Tribolium castaneum*, *Eucalyptus polybractea*, Antifungal activity.

Introduction

Wheat (*Triticum durum* L.) is one of the most important staple foods and cereal crops in the world. Its proper storage is essential, and more importantly, the storage conditions should be such that it is unfavorable for pests and moulds. During storage, wheat is affected by fungal, bacterial and viral pathogens, which cause diseases of varying severity. Various insect pests attack wheat and cause significant loss of grain yield. About thirty-nine (39) pest species have be found to attack stored grain and grain products.[1] Amongst the different insect pests that affect wheat, *Tribolium* species are known to infest stored wheat. As a secondary pest, *T. castaneum (Herbst) (Coleoptera: Tenebrionidae)* is the species that feeds mostly on processed or damaged stored products.[2] The major fungi associated with stored wheat grain, are those belonging to the genera of *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* and are responsible for production of mycotoxins that are harmful to animals and humans.[3,4] The establishment and spread of these pests on stored wheat has two consequences: changes in grain quality, which affects the nutritional value of the derived products, and mycotoxins production. As a result, grain stock pests can obstruct any production attempt if no protective measures are applied.[5] Due to their high concentration of bioactive components which readily breakdown into harmless products, plant essential oils (EOs) may be an alternative source of pest control agents and are therefore suited for use in integrated management programs.

*Corresponding author. E-mail: chibiasma@gmail.com
Tel: 213 555879927

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The *Myrtaceae* family is a potentially attractive choice for biocontrol agents in this context.[6] The volatile chemical components present in Eucalyptus essential oil are abundant in the plant's flowers, bark, seeds, roots, fruits, and wood. The genus Eucalyptus has over 900 species, 300 of which have volatile essential oils in their leaves.[7] Eucalyptus essential oils have several commercial and medicinal applications. Insecticidal, herbicidal, anti-microbial, antiviral, and fungicidal effects are found in the oils.[8] The most abundant component of eucalyptus leaf essential oils are monoterpenes and sesquiterpenes. However, depending on the species and variation, their relative numbers or ratios may differ. Even within the same variety, the chemical composition might vary depending on its geographical origin.[7] There is little research on the antimicrobial activity of *E. polybractea* essential oil against grain pests and moulds. The present study therefore seek to determine the chemical composition of Algerian *Eucalyptus polybractea* essential oils, their antifungal activity against ten stored wheat moulds as well as their repellent and insecticidal properties against *T. castaneum*.

Materials and Methods

Plant materials and EO extraction

The leaves of *E. polybractea* were collected in January 2021 in Algiers forest (Northern Algeria). The essential oil was obtained by hydrodistillation of 100 g of dry leaves using Clevenger-type apparatus for 3 hours. The water vapour laden with essential oils condensed in a refrigerant were collected in a separatory funnel, the less dense oil were collected by simple decantation and dried on anhydrous sodium sulphate (Na_2SO_4) before analysis. The essential oil was stored in a refrigerator at 4°C.

Gas Chromatography-Mass Spectrometric (GC-MS) analysis

GC-MS analysis was performed on a Hewlett Packard 5890 II/MSD 5973 system outfitted with a DB-5MS column (30 m 0.25 mm, film thickness 0.5 m; J&W) using the above operative column and conditions; helium flow was set to 1 mL/minute. It was in electron

impact (EI) mode at 70eV, 300A, with a 220°C ion-source temperature and a 250°C quadrupole temperature. In splitless mode, samples were injected. The mass spectra were scanned in the 33-500 m/z range.

Insect Breeding
Strain of *Tribolium castaneum* (Coleoptera: Tenebrionidae), originated from stored durum wheat was used in the study. A group of 20 adult insects of indeterminate sex was placed in 500 mL glass jars with mesh lid filled with 250 g of healthy durum wheat for the breeding of *T. castaneum*. The assembly was placed in a laboratory chamber with appropriate temperature and humidity conditions of 27 ± 1°C and 70 ± 5%, respectively. After two or three weeks of infestation, the adult insects were removed from the breeding environment.

Fungal isolates
Ten fungal isolates: *Aspergilus niger, Fusarium graminearum, Penicillium sp1, Penicillium expansum, Cladosporium herbarum, Penicillium sp2, Fusarium sp, Aspergilus flavus, Alternaria alternate*, and *Trichoderma viride* were isolated directly from stored durum wheat grain for one year on Potato Dextrose Agar (PDA) medium.

In vitro antifungal activity assay
The antifungal activity of essential oils was tested using the radial growth technique.[9] The concentrations prepared were 0.089, 0.133, 0.178, 0.222 and 0.267 mg/L. To accomplish this, appropriate volumes of essential oils were dissolved in Dimethyl sulfoxide (DMSO) and immediately added to PDA medium before being poured into 9.0 cm diameter Petri dishes. The controls were made with DMSO mixed with PDA (no essential oil was used). The isolated fungi were loaded into mycelial, then transferred aseptically to the center of the Petri dishes and incubated. This procedure was carried out three times. The percentage inhibition of mycelial growth was expressed by the antifungal index according to the following formula:

IA (%) = [1-(D-test /D-control)] x 100

Where, IA(%) = Inhibition rate expressed as a percentage
D-test = test colony diameter in mm
D-control = diameter of control colonies in mm

Insect Repellent assay
Insect repellent assay of *E. polybractea* EO was performed on filter paper using the preferred area method, as described by Jemˆaa *et al*. (2012).[10] The 9 cm diameter filter paper discs used for this purpose were cut in halves. The EO was diluted in acetone to make four doses (2, 4, 6, and 8 µL/L). Then, each dose was uniformly spread over one half of the disc, while the other half received only acetone, the two halves of the discs were re-welded using adhesive tape. In a Petri dish, a reconstituted filter paper disc was placed, and 10 adult non-sexed insects were placed in the center of each disc. Each dose was subjected to three repetitions. The number of insects present on the part of the filter paper treated with the EO (Nt) and those present on the untreated area (Nc) were counted after 15, 30 and 45 minutes. The following formula was used to calculate the percentage repellency (PR):

PR = [Nc – Nt / (Nc + Nt)] x 100
The average repellency rate for essential oil was calculated and assigned according to ranking of McDonald *et al*. (1970),[11] to one of several repellent classes ranging from 0 to 5. Results were presented as the mean of percentage repellency ± the standard error.

Insecticidal activity assay
To assess the insecticidal activity of *E. polybractea* essential oils, 10 insects were placed in a glass jar with a capacity of 100 mL as an exposure chamber to test the toxicity of the essential oil against adults of *T. castaneum*, in which a single load of EO was spread on a Whatman paper disc 5 cm in diameter and then attached to the inner face of the lid. Thereafter, the device was sealed and left at room temperature. Seven doses of essential oil (0.5, 1, 2, 4, 6, 8 and 10 µL/L) were tested. Control insects were kept in the same conditions as the experimental insects but were not given any essential oil. The number of dead insects in each jar was counted every 24, 48, and 72 hours of exposure. Each concentration was replicated four times. Insects were considered dead when no leg or antennal movement was observed.[10] The percentage insect mortality was calculated using Abbott's (1925) correction formula.[12,13]

Mc = Mo – Mt/ 100 – Mt [(M0 - Mt) / (100 - Mt)] x 100
Where, Mc = percentage corrected mortality;
Mt = mortality of the tested sample;
Mo = mortality in the untreated control.

Statistical analysis
Statistical analysis of the test results of insecticidal fumigant toxicity was carried out using Graph Prism software version 9, data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test. The differences between treatments were considered significant if p-value was less than 0.05.

Results and Discussion

Chemical composition of *E. polybractea* Essential oil
The chemical composition of the EO of *E. polybractea* analyzed by GC-MS revealed 45 compounds (Table 1, Figure 1). The most prominent compounds were Eucalyptol (34.87%), alpha-phellandrene (13.10%), alpha-Terpineol (12.17%), (1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (5.49%) and Terpinen-4-ol (5.18%). The chemical composition analysis showed that *E. polybractea* EO is dominated by monoterpenoids. Previous studies on *E. polybractea* EO revealed the presence of different principal constituents. The results from the present study are different from that of Poli *et al* (2018)[14] who showed the domination of p-cymene (25.5%) and cryptone (11.42%) in *E. polybractea* EO. In some works, other chemotypes have been found with the predominant compound being 1,8-Cineole.[15-17]

In vitro antifungal activity
The majority of the detected fungi in stored wheat grain such as *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* are common mycotoxigenic fungi. The growth of test fungi treated with essential oil varied between 9 mm and 88 mm. The high concentration of EO (0.267 mg/L) showed higher antifungal activity compared to the control. *Fusarium graminearum, Penicillium sp2, Aspergilus flavus* and *Trichoderma viride* were significantly more sensitive. The inhibition zone diameter (IZD) of mycelium growth by the EO at low concentration (0.089 mg/L) was 87 mm and at high concentration (0.267 mg/L) was 38 mm. *Penicillium sp2* had IZD of 71 mm at low concentrations and 9 mm at high concentrations of the EO (Table 2). *Aspergilus flavus* at low concentrations of EO had IZD of 86 mm and at high concentrations had IZD of 38 mm. *Trichoderma viride* had IZD of 87 mm and 17 mm at low and high concentrations of EO, respectively.

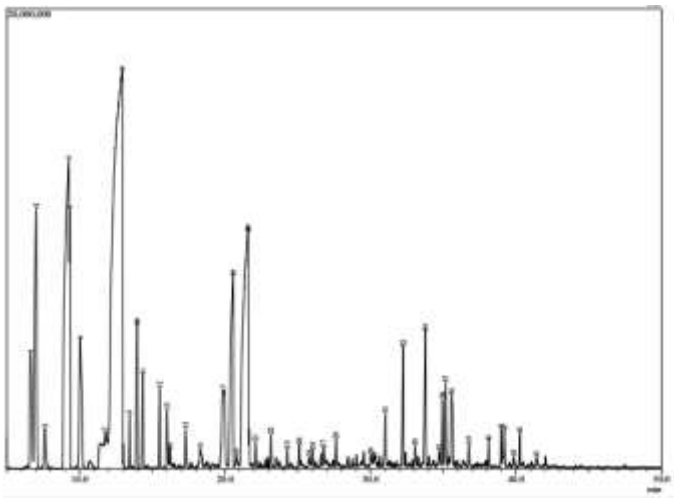

Figure 1: GC Chromatogram of leaf essential oil of *E. polybractea*

Table 1: Chemical composition of *E. polybractea* Essential oil

Peak	RT (min)	Area%	Compound Name
1	6.664	2.33	alpha.-phellandrene (only name in Wiley6)
2	7.033	5.49	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
3	7.619	0.61	Camphene
4	9.268	13.10	alpha.-phellandrene (only name in Wiley6)
5	9.344	1.83	2(10)-Pinene
6	10.052	3.11	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-
7	11.784	0.29	Benzene, 1-methyl-4-(1-methylethyl)-
8	12.943	34.87	Eucalyptol
9	13.440	0.42	1,3,6-Octatriene, 3,7-dimethyl-, (E)-
10	13.952	1.69	gamma.-Terpinene
11	14.350	1.00	5-Isopropyl-2-methylbicyclo[3.1.0]hexan-2-ol
12	15.510	0.80	4-Isopropylidene-1-cyclohexene
13	16.016	0.78	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-
14	16.240	0.23	Linalool
15	17.291	0.49	2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, cis-
16	18.305	0.45	p-Menth-2-en-1-ol
17	19.888	2.75	alpha.-Terpineol
18	20.550	5.18	Terpinen-4-ol
19	20.808	0.21	2-Cyclohexen-1-one, 4-(1-methylethyl)-
20	21.568	12.17	.alpha.-Terpineol
21	22.105	0.27	2-Cyclohexen-1-ol, 3-methyl-6-(1-methylethyl)-, trans-
22	23.154	0.33	2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-
23	24.278	0.20	p-Menthane-1,2,3-triol
24	25.105	0.32	trans-Ascaridol glycol
25	25.799	0.18	Bicyclo[2.2.1]hept-2-ene, 2,3-dimethyl-
26	26.012	0.22	trans-Ascaridol glycol
27	26.782	0.29	2H-Pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl-
28	27.635	0.29	Cyclohexene, 1-(1,1-dimethylethoxy)-2-methyl-
29	30.032	0.20	Ylangene
30	30.265	0.27	Copaene
31	31.020	0.63	1-Methyl-1-ethenyl-2,4-bis(1'-methylethenyl)cyclohexane
32	32.246	1.45	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]-
33	33.082	0.24	Neoalloocimene
34	33.776	1.96	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-
35	34.727	0.28	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR
36	34.954	0.73	(1R,2S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]
37	35.178	0.94	beta.-Selinene
38	35.583	1.42	1.beta.,4.beta.H,10.beta.H-Guaia-5,11-diene
39	36.768	0.27	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-
40	38.132	0.24	1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)-
41	38.991	0.39	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.
42	39.203	0.45	5-Oxatricyclo[8.2.0.04,6]dodecane, 4,12,12-trimethyl-9-methylene-, (1R,4R,6R,
43	39.835	0.17	Guaiol
44	40.266	0.31	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene

45	41.432	0.17	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.

RT = Retention time

Table 2: Antifungal activity of *E. polybractea* EO

Strains	Concentrations (mg/L)					
	0.089	**0.133**	**0.178**	**0.222**	**0.267**	**Control**
Aspergilus niger	$87^{a} \pm 2.51$	$84^{a} \pm 6.11$	$88^{a} \pm 2.86$	$87^{a} \pm 3.00$	$84^{ad} \pm 4.00$	$90^{a} \pm 00$
Fusarium graminearum	$87^{a} \pm 2.51$	$60^{b} \pm 3.05$	$54^{b} \pm 2.51$	$34^{b} \pm 25.42$	$38^{b} \pm 3.51$	$90^{a} \pm 00$
Penicillium sp 1	$87^{a} \pm 2.51$	$87^{a} \pm 4.61$	$85^{a} \pm 5.13$	$79^{a} \pm 9.64$	$65^{c} \pm 4.00$	$90^{a} \pm 00$
Penicillium expansum	$85^{a} \pm 4.04$	$78^{ac} \pm 2.30$	$77^{a} \pm 2.51$	$72^{c} \pm 3.51$	$72^{ac} \pm 3.00$	$90^{a} \pm 00$
Cladosporium herbarum	$90^{a} \pm 0.00$	$88^{a} \pm 1.52$	$89^{a} \pm 0.57$	$86^{a} \pm 3.05$	$85^{d} \pm 4.04$	$90^{a} \pm 00$
Penicillium sp2	$71^{b} \pm 3.21$	$66^{bc} \pm 8.50$	$15^{c} \pm 3.05$	$12^{d} \pm 2.00$	$9^{e} \pm 1.15$	$90^{a} \pm 00$
Fusarium sp	$90^{a} \pm 0.00$	$87^{a} \pm 3.00$	$86^{a} \pm 3.60$	$83^{ac} \pm 5.68$	$56^{f} \pm 7.76$	$90^{a} \pm 00$
Aspergilus flavus	$86^{a} \pm 3.51$	$83^{a} \pm 6.24$	$77^{a} \pm 12.05$	$55^{b} \pm 5.00$	$38^{b} \pm 3.60$	$90^{a} \pm 00$
Alternaria alternata	$87^{a} \pm 2.51$	$83^{a} \pm 6.02$	$79^{a} \pm 9.0$	$78^{ac} \pm 10.81$	$59^{cf} \pm 5.50$	$90^{a} \pm 00$
Trichoderma viride	$87^{a} \pm 2.51$	$84^{a} \pm 5.03$	$55^{b} \pm 4.04$	$30^{e} \pm 7.00$	$17^{e} \pm 4.16$	$90^{a} \pm 00$

Data are Mean ± SD. Values followed by the same letter within each column are not significantly different

The essential oil of *E. polybractea* showed antifungal activity against *Fusarium graminearum, Penicilium sp2, Aspergilus flavus* and *Trichoderma viride*. However, for *Aspergilus niger, Penicillium sp1, Penicillium expansum, Cladosporium herbarum, Fusarium sp, Alternaria alternate*, it showed the lowest activity (Table 2). The strains studied showed different responses to eucalyptus essential oils. With inhibition rates of 57%, 57%, 81%, and 90% for *F. graminearum, Aspergillus flavus, Trichoderma viride*, and *Penicillium sp2*, respectively appear to be by far the most susceptible strains to different doses of eucalyptus oil. In the presence of EO, the inhibition rates of *Penicillium expansum, Penicillium sp1, Alternaria alternata*, and *Fusarium sp* were less than 50%. The lowest inhibition rates were shown by *Aspergillus niger* and *Cladosporium herbarum* with 6% and 5% inhibition, respectively (Table 3). Currently, very little data are available in the literature on *E. polybractea* EO antifungal activity. The present study showed the effect of the essential oil of this plant on ten species of fungi, six of which (*Aspergilus niger, Penicillium sp1, Penicillium expansum, Cladosporium herbarum, Fusarium sp* and *Alternaria alternate*) proved insensitive, while four strains (*Fusarium graminearum, Penicillium sp2, Aspergillus flavus* and *Trichoderma viride*) were susceptible. The efficacy of *E. polybractea* EOs against microorganisms could in part be attributed to their volatile bioactive components.[18] Previous research on the antifungal activity of eucalyptus oil indicated the resistance of *Penicillium sp* and *Aspergillus sp* against eucalyptus oil.[19] In contrast to our study, the results of Pedrotti *et al*. (2022)[20] showed that *Eucalyptus staigeriana* oil was effective in reducing the incidence and severity of black rot caused by *Alternaria alternata* in preventive and curative treatments at different concentrations, indicating susceptibility of *Alternaria alternata*. In general, the composition of the fungal microbiota of wheat grains found in the current study agree with previous studies.[3,21] The antifungal activity of *E. polybractea* essential oil may be attributed to Eucalyptol a major bioactive terpenoid constituent of the plant whose antifungal properties have been explored previously.[22]

Insect repellent activity

The repellent effect of *E. polybractea* essential oil was tested on adult *T. castaneum*. Table 4 shows the repellent capacity of *E. polybractea* EO at concentrations of 2, 4, 6 and 8 µL/L of air at different times after treatment. The results showed that the essential oil repels *T. castaneum*. The repellent effect of the essential oil against *T. castaneum* showed that the higher the concentration, the longer the repulsion time. *E. polybractea*'s EO repulsion time was 15 min, 30 min, 45 min, and 65 min at concentrations of 2, 4, 6 and 8 µl/L air, respectively. This finding agrees with the work of Mangang and Manickam (2022)[23] who demonstrated that the active components of eucalyptus oil formulated as insect repellent pellets (IRPs) showed repellent effect against adult *T. castaneum*, and the work of Alsudani *et al*. (2021)[24] who showed that the rellepent effect of Eucalyptus EO against this pest is about 46.66% 12 h post treatment.

Insecticidal activity

After 24 h of exposure to EO, insect mortality was 43% and 93% at 0.5 and 10 µL/L air, respectively. After 48 h, the mortality was 100% at 10 µL/L air (Table 5). This shows that essential oil of *E. polybractea* demonstrated significant insecticidal activity against adult *T. castaneum* after 48 h exposure. The mortality of adults *T. castaneum* was less than 50% after 48 h of exposure to the essential oil at 0.5 and 1 µL/L air. Insecticidal activity tests of *E. polybractea* essential oil showed significant insecticidal activity after 72 h of treatment (Table 5). The results showed that the insecticidal activity of *E. polybractea* EO against *T. castaneum* varies with the concentration and duration of treatment, allowing for maximum mortality after 72 h of exposure to a dose of 10 µl/L air. The insecticidal properties of essential oils are most likely due to the main constituent of the essential oil. Some reports have indicated that *E. polybractea* essential oil has insecticidal activity against *Haematobia irritans* adults,[25] insecticidal and larvicidal activities against *Aedes aegypti*.[26] The study of Yeom *et al*. (2013)[27] showed that *E. polybractea* oil at 15 mg/L air and 7.5 mg/L air demonstrated 100% mortality against adult male German cockroaches. The calculated 50% lethal concentration (LC_{50}) of 0.00014 µL/L air after 72 h of exposure indicated that adults *T. castaneum* are extremely sensitive to this oil.

Conclusion

In the present study, the antifungal potential of *Eucalyptus polybractea* EO was investigated against ten (10) fungal strains of stored wheat grains and the insecticidal and repellent activity against *Tribolium castaneum* was also tested. The results showed that *Eucalyptus polybractea* EO has enhanced activity against *Fusarium graminearum, Aspergillus flavus, Trichoderma viride, Penicillium sp2.* The insecticidal and repellent activities reveal a strong effect of eucalyptus oil against *T. castaneum.* From this, it is clear that *E. polybractea* EO possess a wide spectrum of activity against stored wheat grain moulds and insect pest. However, the leaves essential oil, composed predominantly of eucalyptol, may be an excellent choice as a natural pesticide. These findings will serve as a basis for further research into this plant species with a view to the discovery of the bioactive compounds.

Table 3: Inhibition of fungal mycelial growth at different concentrations of EO

Concentration (mg/L)	**0.089**	**0.133**	**0.178**	**0.222**	**0.267**
Strains	**Inhibition (%)**				
Aspergillus niger	3.33	6.66	2.22	3.33	6.66
Fusarium graminearum	3.33	33.33	40	62.22	57.77
Penicillium sp 1	3.33	3.33	5.55	12.22	27.77
Penicillium expansum	5.55	13.33	14.44	20	20
Cladosporium herbarum	0.00	2.22	1.11	4.44	5.55
Penicillium sp2	21.11	26.66	83.33	86.66	90
Fusarium sp	0.00	3.33	4.44	7.77	37.77
Aspergillus flavus	4.44	7.77	14.44	38.88	57.77
Alternaria alternata	3.33	7.77	12.22	13.33	34.44
Trichoderma viride	3.33	6.66	38.88	66.66	81.11

Table 4: Mean repellence time (min) against *T. castaneum* at different concentrations of *E. polybractea* EO

Essential oil	**Concentrations (µL/L air)**			
	2	**4**	**6**	**8**
E. polybractea	14.00 ± 6.92	30.00 ± 11.94	45.00 ± 19.14	65.00 ± 34.15

Data are Mean ± SD

Table 5: Mortality (%) of *T. castaneum* on exposure to *E. polybractea* EO

Concentration	**Time (h)**		
(µl L air)	**24**	**48**	**72**
0.5	13 ± 5.77	26 ± 5.77	43 ± 5.77
1	*20* ± 0.00	36 ± 5.77	56 ± 5.77
2	26 ± 5.77	50 ± 10.00	73 ± 5.77
4	56 ± 5.77	63 ± 5.77	65 ± 5.77
6	56 ± 11.54	63 ± 5.77	73 ± 11.54
8	70 ± 10.00	76 ± 5.77	83 ± 11.54
10	93 ± 5.77	100 ± 0.00	100 ± 0.00

Data are Mean ± SD

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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