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MEMORY

Presented by

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In order to obtain the

Master's Degree

In Microbiology and Quality Control

Theme

Evaluation of antimicrobial activity of different extracts of saffron petals (*Crocus sativus*)

Defended on Saturday 02/07/2022, before the jury composed of:

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الملخص:

بشكل عام، يتم إستخدام مياسيم زهرة الزعفران (*Crocus sativus*) فقط في إنتاج الزعفران التجاري، ويتم إهدار البتلات أو التخلص منها.

كنا مهتمين بدراسة وتقييم النشاط المضاد للميكروبات للمستخلصات المختلفة من بتلات زهرة الزعفران (المستخلص المائي، الميثانولي، مستخلص الأسيتات، بيتانوليك، الأسوتينيك و ثنائي الكلورو ميثان) على الكائنات الحية الدقيقة من خلال طريقة نشر القرص، وتحديد MIC و CMB.

وفقًا لطريقة CMI ، تم تثبيط نمو جميع الكائنات الحية الدقيقة المسببة للأمراض ، ومع ذلك ، عند التركيز العالي للمستخلص الميثانولي شبه الجاف 184 مجم / مل، لم يلاحظ أي نشاط مضاد للجراثيم ضد *P. aeruginosa*. تم تسجيله بواسطة هذا طريقة.

كان مستخلص الأسيتات شبه الجاف الوحيد القادر على ممارسة نشاط مبيد للفطريات على *Candida albicans CIP 444*.

وفقًا لنتائجنا، نعتبر أن أزهار الزعفران هي مصدر طبيعي للمركبات المضادة للميكروبات مع إمكانات كبيرة و مبشرة حتى على الكائنات الحية الدقيقة شديدة العدوى والمقاومة مثل *Pseudomonas aeruginosa*.

الكلمات المفتاحية: *زعفران*، *Crocus sativus*، البتلات، الكائنات الحية الدقيقة، النشاط المضاد للميكروبات، مستخلص.

Resumé:

Généralement, seuls les stigmates du safran (*Crocus sativus*) sont utilisés dans la production de safran commercial, et les pétales sont gaspillés ou jetés.

Nous nous sommes intéressées à étudier et à évaluer l'activité antimicrobienne des petales de différents extraits de pétales de la fleur de safran (Extrait aqueux, méthanol, acétate, butanol, acétone et dichlorométhane) sur des microorganismes par la méthode de diffusion sur disque, la détermination de la CMI et de la CMB.

Selon la méthode de la CMI, la croissance de tous les microorganismes pathogènes a été inhibée, cependant, à une concentration élevée de l'extrait méthanolique demi-séche de l'ordre de 184mg/mL, aucune activité antibactérienne sur *P. aeruginosa* n'a été enregistrée par cette méthode.

L'extrait d'acétate demi-séche était le seul capable d'exercer une activité fongicide sur *Candida albicans CIP 444*.

Selon nos résultats, nous considérons que les fleurs du safran est une source naturelle en composés antimicrobiens avec un potentiel antimicrobien considérable même sur le microorganisme hautement pathogène et résistant *Pseudomonas aeruginosa.*

Mots clés : *Safran, Crocus sativus*, fleurs, extrait, microorganismes, activité antimicrobienne.

Abstract:

Generally, only the stigmas of saffron (*Crocus sativus*) are used in the production of commercial saffron, and the petals are wasted or discarded.

We were interested in studying and evaluating the antimicrobial activity of different extracts of saffron flowers petals (Aqueous, methanol, acetate, butanol, acetone, dichloromethane) on different microorganisms by the disc diffusion method, the determination of the MIC and the CMB.

According to the CMI method, the growth of all pathogenic microorganisms was inhibited, however, at a high concentration of the semi-dry methanolic extract 184mg/mL, no antibacterial activity against *P. aeruginosa* was observed by this method.

The semi-dry acetate extract was the only one capable of exerting fungicidal activity on *Candida albicans CIP 444.*

According to our results, we consider that saffron flowers are a natural source of antimicrobial compounds with considerable antimicrobial potential even on the highly pathogenic and resistant microorganism like *Pseudomonas aeruginosa.*

Key words: *Crocus sativus,* petals, microorganisms, antimicrobial activity, extract.

Summary

Acknowledgements

First of all thanks for our parents for supporting us throughout our career

Our supervisors ***Dr. BELYAGOUBI Larbi*** *and* ***Prof. LOUKIDI Bouchra*** *which were always by our side to guide us and help us and we really appreciate their good humor and kindness.*

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Also ***Ms Meliani Nouria*** *for providing us with the extracts and supporting us.*

Also, ***Mr. Habi Salim*** *Thank you for leading us throughout our path.*

My collegues that worked with us in the laboratory.

Dedication

First, i dedicate this humble work to my dear parents who have always encouraged me throughout my entire life.

Too my whole Family

Special thanks to my Dad for all his sacrifices at work to provide all the capabilities and resources for me to continue my study at the University.

Too most of my teachers throughout this educational path.

I also dedicate this study too all my friends that have been with me

We worked together and we will always be together.

To my friend ***Mouad*** *that passed away twi months ago.*

To my homies ***Oussama*** *&* ***Nassro*** *that we choice together to study biology*

To all my Primary school mates where it all started.

To my middle & high school friends, thanks for all the good days.

And finally to all my University mates, special acknowledgement for my Lab colleagues I appreciate the good energy and the good vibes.

BELARBI ABDELLAH

Dedication

I would have the immense pleasure of dedicating this work to my very dear parents who have always supported me

It is thanks to them that I am now at the final stage of my formation.

i would thank my father for his encouragement, his advices and his sacrifices.

All my family for the love and respect they have always given me, all my friends that I meet on my way.

BENSAID ABDELLATIF

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Abbreviations

SAE: saffron aqueous extract

SE: saffron extract

FAE: fresh aqueous extract

SDAE: semi-dry aqueous extract

DAE: dry aqueous extract

FME: fresh methanolic extract

SDME: semi-dry methanolic extract

DME: dry methanolic extract

FACE: fresh acetonic extract

SDACE: semi-dry acetonic extract

DACE: dry acetonic extract

FBE: fresh butanolic extract

SDBE: semi-dry butanolic extact

DBE: dry butanolic extract

DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate

Pa: Pseudomonas aeruginosa

Ef: Enterococcus faecalis

Ec: Escherichia coli

Bc: Bacillis cereus

Ca: Candida albicans

ROS: reactive oxygen species

EFS: Elecrical field stimulation

ATCC: American Type Culture Collection

CIP: “Collection de l'Institut de Pasteur”

Introduction

Dried red stigmas coming from the flowers of *Crocus sativus L* are the main constituant of saffron spice, which is an autumn-flowering geophyte. The major saffron producing countries in the world are iran, Spain, Morocco, India, Greece, and Italy. It's annual production exceeds 220,000 kg and circa 110,000–165,000 flowers are needed to produce 1kg of dried stigmas. The intensive hand labour required for flower picking and stigma separation make the saffron the world's highest-priced spice, and for this reason its named "red gold".

Saffron flower induction is a complicated mechanism directly related to pedoclimatic conditions and field management. As in most geophyte plants, both seasonal and daily thermoperiodism are involved as the main environmental factors. Flower induction requires an incubation of the corms at high temperature (23–27°C), followed by a period of exposure at circa 17°C for flower emergence. In Mediterranean environments, flower induction occurs from early spring to midsummer, while flower emergence occurs from early- to late-autumn. Flowering is followed by a vegetative stage throughout the winter and formation of replacement corms at the base of shoots. At the end of spring, the leaves reach their highest length, start to Senescence wither, and the bulbs go into dormancy.

In the last decade, there has been an icreased interest in saffron for using it as an alternative crop for the diversification of agricultural production and as an important new source of income. Indeed for manyfarms, economic diversification has become a keystone for obtaining an adequate income and consequently, continuing business. This trend is particularly evident in mountain areas such as in the north western Italian Alps, where saffron production has been recently started.

There's three main components that their concentration control the quality of this product: crocin, picrocrocin, and safranal, which provide respectively the unique color, bitter taste and aroma, the concentration of these constituents concurs to determine the saffron quality as defined by the International Organization for Standardization

This spice has been used as a natural ingredient in food products. In addition, saffron has long been considered a medicinal plant for its therapeutic properties. Recently, studies related to the quality of saffron have revealed the properties of several compounds present in the spice and their positive influence on human health. Phenolic and anthocyanin contents of plant material play a main role in preventing oxidative damage caused by free radicals, which are both responsible for degradation of dietary lipids, hence of nutritional value, and the cause for various human diseases. Saffron active constituents such as carotenoids (i.e., crocins),

polyphenols and vitamins show significant antioxidant activity and could enhance the memory capability, and have antitumor and cancer-preventive properties **(Caser, 2020)**.

Nowadays, increasing antibiotic resistance of bacteria has provided the opportunity to replace herbal remedies with fewer side effects than conventional medicines.

In the production of commercial saffron usually they use only Stigmas, so the large amount of petals is being squandered, tepals have been revealing their potential as a rich source of bioactive compounds, so the main purpose of our study is to evaluate the antimicrobial activity of *Crocus sativus* petals extract (Aqueous, methanol, acetate, butanol, acetone, dichloromethane).

So many researchs have been done before on the plant of saffron as a very rich plant on bioactive compounds, we saw studies about the phytochemical compostion by **Shoib A et al** in 2015, **Lage M et al** in 2009 on the Maroccan saffron, same as a comparison between the chemical composition of saffron obtained from 5 different countries. Bilogical proporties and medicinal use of saffron by **Abdullaev F** in 2004, antidiabetic activity by **Saeed S et al. 2016**, and lately has been a lot of intrest in the antioxidant/antimicrobial activities of *Crocus sativus* stigma and petals, the like of the study conducted in Marocco by **Jadouali S et al. 2020** and the research made in our university of Tlemcen by **Belyagoubi L et al** last year in 2021.

Bibliographic research

I. Botanical description habitat and distribution

The genus *Crocus* comprises 85–100 species having an old world distribution, primarily in the Mediterranean – Europe and Western Asia. The limits of the entire genus lie within the longitude 10°W to 80°E and latitude 30°N to 50°N. These areas caracterised by cool to cold winters with autumn–winter–spring precipitation and warm summers with very little rainfall (**Palomares, 2015**).

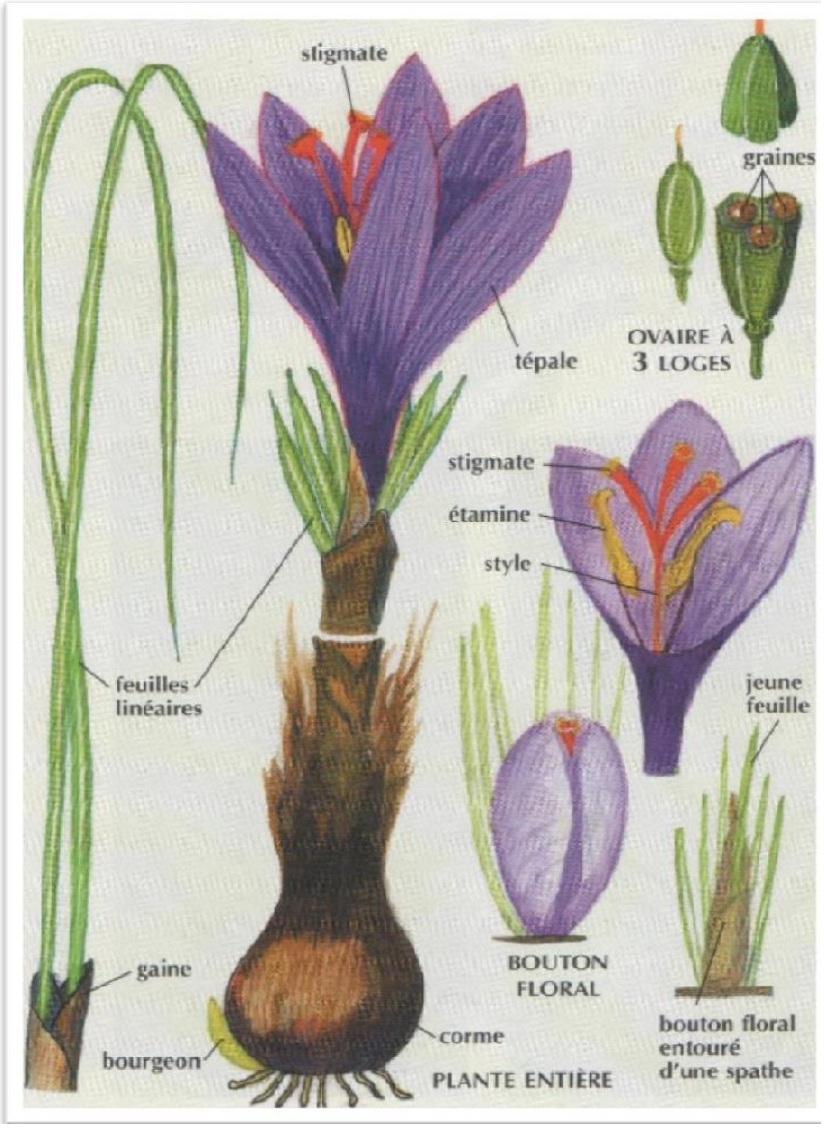
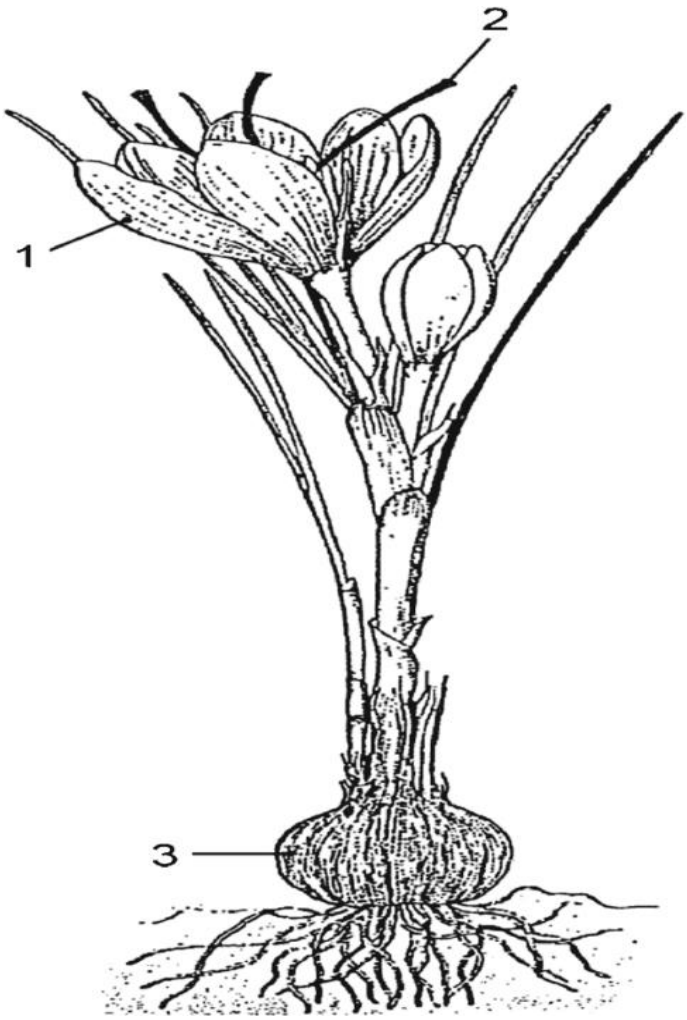


Fig1: General aspect of *Crocus Sativus* **(Palomares, 2015)**

II. Nomenclature and systematics

The taxonomic classification of *C. sativus* series is as follows:

1. Division: *Spermatophyta*
2. Sub-division: *Angiospermae*
3. Class: *Monocotyledonae*
4. Sub-class: *Liliidae*
5. Order: *Liliales*
6. Family*: Iridaceae*
7. Genus: *Crocus*
    a. Sub-genus: Anthers with extrose dehiscence
    b. Section *Crocus*: Scape subtended by a membranous prophyll
    c. Series *Crocus*: Corm tunics finely fibrous, usually reticulate, flowers autumnal, leaves rather numerous (Fig2), usually 5–30, appearing with the flowers or shortly after, bracts flaccid, usually not closely sheathing the perianth-tube, membranous, white or transparent with no marking, anther yellow, style branches 3, usually red and often expended at the apex, entire or at most fimbriate, seed coats covered with dense mat of papillae. 2*n* = 12, 14, 16, 26.
8. Speccy : *sativus* **(Saxena, 2010)**.



1-Flower
2-Stigma
3-Corm

Fig 2: Diagram of the organogram of *Crocus sativus* **(Rahaiee, 2015)**

III. Pharmacological and therapeutic study

Saffron has been recognized for its therapeutic virtues for centuries. While being a highly sought-after spice for its culinary qualities, it has always been used for its medicinal properties (Table1). It has the reputation of soothing many affections, bringing joy and cheerfulness. It has traditionally been used for cramps, asthma and whooping cough, menstrual disorders, liver disease, dental pain, and was used as an aphrodisiac **(Palomares, 2015)**.

Table1: Saffron properties and health effects **(Palomares, 2015)**

Properties	Indication
Antidepressant	• Nervous breakdown • Emotional fragility • Stress • Anxiety • Anguish
Satiety regulator in case of overload weight	• Overweight
Stimulant	• Overwork • Memory loss • General fatigue • Physical and mental • Asthenia
Tonic	• Lack of energy • Sports practice (training, competition, recovery)
Conditioner	• Infectious ground
Aphrodisiac	• Male impotence • Feminine frigidity
Antispasmodic	• Nervous tension
Analgesic	• Menstrual pain
Anti-inflammatory	• Articular pain
Degestive tonic	• Degestive laziness
Liver tonic	• Hepatic laziness
Immunostimulant	• Immonosuppression
Hypoglycemic	• Non-insulinodependant diabetes
Cholesterol lowering	• Excess of cholesterol
Antioxidant Anti-radical	• Premature and accelerated aging of the body • Smoking cessation

As a therapeutic products we have “topical spray” used for treatment of rhematoid and skin scars, “topical treatment for breast cancer”, “traditional Chinese medicine”, “cold and flu symptomatic relief composition”, “satiation agent for the treatment of obesity”, its also used as an antimicrobial composition that can treat bacterial infections such as acne, pseudofolliculitis, localized redness, and localized odor **(Mohajeri, 2020)**.

IV. Food products made from saffron (*Crocus sativus*)

- Vegetable drinks :

Healthy vegetable-based drinks contain raw materials such as saffron, jasmine, theanine, [gamma]-aminobutyric acid, and wolfberry, which lead to stress relief, improved quality of sleep, boosted immune system, gastrointestinal motility and regulation of endocrine functions **(Mohajeri, 2020)**.

- Healthy drink prepared from saffron pollen:

This healthy saffron drink provides many benefits by increasing the absorption of nutrients, supplying oxygen-rich blood in circulation, restoring skin health and beauty, regulating endocrine functions, and reducing stains caused by endocrine disorders **(Mohajeri, 2020)**.

V. Antimicrobial activity

Microorganism is an organism that can be seen only with the aid of a microscope and that usually consists of only a single cell. Microorganisms include bacteria, protozoans, and certain algae and fungi, sometimes we call viruses microbes too.

The antimicrobial activity can be defined as the ability of active molecules to inhibit the growth of bacteria (bacteriostatic) or the ability to destroy bacteria cells (bactericidal).

In the study made by **Parry J et al. (2015)**, the methanol extracts of *Crocus sativus* callus and stigma showed varying degrees of inhibitory effects on the pathogenic bacterial strains (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Shigella flexneri*)

Based on the results obtained by **Cenci-Goga B et al. (2018)**, it is possible to conclude that aqueous extracts of saffron stigmas had a moderate antimicrobial activity and can be a potential anti-microbial agent in various food products.

In the study conducted by **Belyagoubi L et al. (2021)**, both stigma and flower hydro-alcoholic extract showed a real potential antimicrobial activity against gram-positive bacteria (*M.luteus, B.cereus,B.subtilis*).

VI. Concept of pathogenicity

Pathogenicity is the capacity of a microbe to cause damage in a host **(Casadevall, 1999)**, and its also the quality or state of being pathogenic.

VII. Chemical compounds of petals

Saffron petal contain protein (10.20%), fat (5.3%), ash (7.00%), fiber (8.80%) its also composed of flavonoids (kaempferol, 12.6%w/w) carotenoids (crocin, 0.6%w/w and crocetin) anthocyanins, phenolic compounds as well as terpenoids and alkaloids **(Hosseini, 2018)**.

VIII. Phytochemical composition

Various analytical studies have been conducted to characterize a large number of biologically active compounds found in Saffron. The four main biologically active compounds are: crocin and crocetin, which are two carotenoid pigments responsible for the yellow-orange color of the spice, picrocrocin, giving saffron its flavor and bitter taste, and safranal which is a volatile compound responsible for the aroma and smell that is specific to Saffron.

Thus, these main constituents contribute not only to the sensory profil of saffron (color, taste, aroma) but also to the properties of interest to health **(Palomares, 2015)**.

Table2: Mineral compounds in saffron petals **(Hosseini, 2018)**

Mineral	Proportion(mg/100g)	Mineral	Proportion(mg/100g)
Sodium	25.75	Iron	17.99
Potassium	542.13	Magnesium	2.93
Calcium	486.25	Zinc	1.80
Copper	0.87	Phosphorus	209.90

Phytochemical content:

- Flavonoids:

Flavonoids are polyphenolic compounds with antioxidant properties, a lot of studies have shown that a high intake of flavonoids has been correlated to a decrease in heart disease **(Montoro, 2008)**.

In the study put together by **Belyagoubi L et al. (2021)**, flavonoid content was determined based on the technique of Zhishen et al, the results were as follow: 5.967±0.042 mg CE/g DM for stigma extarct and 4.322±0.185 mg CE/g DM for flowers extract

- Phenolics:

Alike the flavonoids, the phenolic compounds are also molecules that can play the role of an antioxidant to prevent heart disease as well as reducing inflammation, lower the chances of getting cancer and diabetes diseases besides the reducing in rates of mutagenesis in human cells **(Khoddami, 2013)**.

Using the folin-ciocalteu method, they evaluated the phenolic content by spectrophotometry and found that stigmas extract has more phenolics compounds with 97.993±10.548 mg GAE/g DM compare to 69.187±2.255 mg GAE/g DM for the flowers **(Belyagoubi, 2021)**.

- Crocin:

Crocins are glycosyl esters of crocetin, formed by esterification of crocetin with different glycosides, being the geometric isomers trans the majority and cis isomers the minority. Crocetin molecule is modified by the activity of glucosyltransferases, adding different numbers of glycosidic molecules to produce crocins, the major components of the stigmas of saffron and which confer solubility in water. The content of crocetin esters in saffron represents 16–28%, it is also the most important and studied due to its antioxidant activity, protecting against oxidative stress **(Cerda-Bernad, 2022)**.

- Crocetin:

Known as carotenoids, but it does not have a pro-vitamin function. The constituents of this class of small molecule compounds are mostly polyunsaturated hydrocarbons are the main constituant of this class (the formula is C40H56) or their oxygenated derivatives. There are small groups of caretonoids that are carboxylic acids. Among those groups there is crocetin, 8,8-Diapocarotenedioic acid, characterized by a diterpenic and symmetrical structure with alternating double bonds and four methyl groups. The chain is stabilized in the terminal parts by two carboxylic groups. Its elementary composition is C20H24O4 and its molecular weight

is 328.4. It is slightly soluble in aqueous solution (20 μM at pH 8.0) and it is soluble in organic bases, such as, pyridine. Crocetin is an amphiphilic low molecular weight carotenoid compound and consists of a C-20 carbon chain with multiple double bonds, and a carboxylic acidgroup at each end of the molecule **(Cerda-Bernad, 2022)**.

IX. Definition of antioxidant activity

The reactive oxygen species lead to different diseases via formation of superoxide anion radical, hydroxyl radical, and hydrogen peroxide, which damage cell membrane and attack molecules such as DNA, proteins, lipids and small cellular molecules. Most of herbal medicines are containing antioxidant compounds which scavenge free radicals and reduce cellular damage. A study showed antioxidant activity of saffron petal in lambs using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free-radical method **(Hosseini, 2018)**.

X. Antioxidant activity of saffron

Some compounds known as antioxidants are able to delay or inhibit the initiation or propagation of oxidative chain reaction and thus prevent or repair oxidative damage done to the body's cells by oxygen **(Sariri, 2011).**

The antioxidant property of *C. sativus* stigma could be credited to its phenolic content as well as to its active ingredients such as safranal, crocin, crocetin and carotene, so they are frequently used as antioxidant food supplements **(Rahaiee, 2015).**

XI. Reactive oxygen species

ROS are produced in the cells by cellular metabolism. In vivo, some of these play positive roles such as energy production, phagocytosis, regulation of cell growth and intercellular signaling, or synthesis of biologically important compounds. However, ROS may also be very damaging as they can induce oxidation of lipids, causing membrane damage, decreasing membrane fluidity, and leading to cancer via DNA mutation.

Crocin is a carotenoid pigment isolated from *Crocus sativus* and has a DPPH radical scavenging capacity of 0.1% of the total crocin showed 29.36% DPPH radical scavenging activity that can inhibit the reactive oxygen species ROS **(Hamid, 2009).**

XII. Anticancer activity

1. Gastric cancer: In the study undertaken by **Bathaie R et al. (2015)**, the beneficial effect of saffron aqueous extract (SAE) was investigated. SAE administration inhibited the progression of cancer of the gastric tissue.

2. Colorectal cancer: **Aung et al** suggested that Crocus sativus L. extract and its major constituent, crocin, significantly inhibited the growth of colorectal cancer cells while not affecting normal cells. So, Crocus sativus extract should be investigated further as a viable option in the treatment of colorectal cancer.

3. Prostate cancer: In a study conducted by **D'Alessandro et al**, the antiproliferative activity of saffron extract (SE) and its main constituent crocin on five different malignant and two nonmalignant prostate cancer cell lines were determined. Both SE and crocin decreased cell proliferation in all malignant cell lines in a time- and concentration-dependent manner. Nonmalignant cells were not affected.

4. Lung cancer: **Samarghandian et al** investigated the potential of saffron to induce cytotoxic and apoptotic effects in lung cancer cells (A549). The investigators demonstrated that the proliferation of the A549 cells were reduced following saffron administration in a dose- and time-dependent manner.

5. Leukemia: Results of the study conducted by **Sun et al** demonstrated that crocin suppressed HL-60 cell proliferation and stimulated apoptosis and cell cycle arrest at G0/G1 phase, in a concentration and time dependent manner.

6. Hepatic cancer.

7. Cervical, ovarian, and breast cancer **(Bhandari, 2015).**

XIII. Antidiabetic activity

The ability of saffron to decreas blood sugar levels by increasing insulin sensitivity alongside an improvement in β-cell function (islets of Langerhans) **(Yaribeygi, 2019).**

In the study made by **Farkhondeh, T., & Samarghandian. (2014)**, the radical scavenging activity of the saffron methanol extract and its components (crocin, crocetin, safranal) has been shown to be likely important because these atoms donate hydrogen atoms for the radical stability of DPPH. Saffron components modulate antioxidant gene expression and regulate

mitochondrial antioxidant genes, resulting in mitochondrial oxygen radical generation, which can slightly be incharge of enhancing hyperglycemia response and oxidative stress in an experimental diabetes model.

XIV. Anti-inflammatory activity

- Saffron extracts have shown anti-inflammatory potential by blocking inflammatory responses in various tissues and inhibit pro cytokines and inflammatory mediator expression at the mRNA**(Yaribeygi, 2019).**
- In the study constructed by Brijesh Rathore et al while they were seeking a cure for a Arthritis. they found out that the *Crocus sativus* extract have the ability to reduce the pro-inflammatory cytokines such as TNF-α and IL-1β in adjuvant induced arthritic mice. they also found that CSE exerts antioxidant effect during the disease. We can then conclude that CSE possess anti-inflammatory as well as antioxidant property due to presence of active constituents **(Rathore, 2015).**

XV. Antihypertensive activity

The effect of *Crocus sativus* (aqueous and ethanolic) petal extract on blood pressure has been studied as hypotension was observed at doses of 50 mg/100g **(Hosseini, 2018).**

XVI. Antispasmodic effects

They investegated the effect of saffron petals on tonicity of smooth muscle in rats and pigs. Elecrical field stimulation (EFS) was reduced by petal extracts, and lower responses to epinephrine. However, it has shown that the petal extract antagonizes adrenergic receptor mice, in addition it has reduced the petal extract of deflation caused by EFS by inhibiting muscarinic receptors **(Hosseini, 2018).**

XVII. Some microorganisms and their pathogenic power

- *Pseudomonas aeruginosa* is a gram-negative, strict aerobic bacteria and its also an important part of the psychrotrophic flora. This microorganism is able to proliferate in several environments such as water and soil, thanks to its very versatile metabolism, it is able to use a wide variety of compounds, including toxic wastes and several sources of carbon and nitrate as electron acceptors. In addition, its great capacity to adapt to a

hostile environment and its numerous virulence factors allow it to take advantage of certain situations to infect many types of hosts such as plants, insects and animals.

P. aeruginosa can be implicated in food-born illnesses. It is highly pathogenic for weakened or immunocompromised subjects, causing a high rate of morbidity and mortality. Thus, P. aeruginosa can be found in skin infections, the most serious of which concern burn victims **(NE, 2009).**

- *Bacillus cereus* are Gram-positive, sporogenic, facultative aero-anerobic, ciliatur-motile, telluric, ubiquitous bacilli found in soil, water, dust, plants and human and animal faeces. *B. cereus* infections can be classified into three categories: digestive infections occurring in the context of collective food infections, local infections and systemic infections **(Teyssou, 1998)**
- *Enterococcus faecalis* is a gram positive bacteria formerly classified as part of the group D *Streptococcus* system, it is a part of the digestive flora of humans and animals. It can colonize the skin, particularly the perineal area and the vagina, through contamination of the surrounding area. Like *Enterococci*, this species can be found in the environment, mainly responsible for urinary tract infections, most often secondary after urological explorations, endocarditis evolving in a subacute mode, on native valves or prostheses, and occurring after digestive or urological explorations, intra abdominal infections (biliary...) of various suppurations. The polymicrobial character of Enterococcus superinfections is frequent (enterobacteria and anaerobes). Like all Enterococcus, this species can be selected by certain antibiotic treatments (3rd generation cephalosporins, quinolones). Infections can have a nosocomial character **(ARCHAMBAUD, 2007).**
- *Escherichia coli* is a gram-negative bacteria and a member of the bacterial family of *Enterobacteriaceae* it belong to the gut microflora as it's also may be pathogene. As a commensal it lives in a mutually beneficial association with hosts, and rarely causes disease, its an aero-anaerobic microorganism, it is the most used microorganism in the field of recombinant DNA technology. It cause diarrhoea primarily in children, particularly under conditions of poor hygiene, as well as in animals, it is also frequently associated with nosocomial and community-associated infections **(Allocati, 2013)**
- *Candida albicans* is the most important and best-known species of yeast in the *Candida* genus, its a versatile organism living as a commensal of the gastro-intestinal

tract and capable of invading host tissues and to initiate serious diseases under the appropriate environmental conditions. The molecular basis for adherence, invasion, interactions with specific and non-specific immune factors have been studied in parallel to structural characteristics of the yeast. The main parasitologic features are closely linked to phenotypic variations. In this respect, mannoproteins are strongly involved in the cell wall variations **(Poulain, 1990)**.

Material and Method

I. Preparation of *Crocus Sativus* extracts

Crocus sativus petals were collected from the Djebel Zaafran of Ain Fezza, Tlemcen (VQH8+G5X) from the 15th October to the 15th November 2021, the extraction was made by **Ms *MELIANI NOURIA, PhD(c)*** using the maceration method to give us six different extracts (Fig3), Aqueous, Methanolic, Acetonic, Butanolic, Acetate and Dichloromethane.

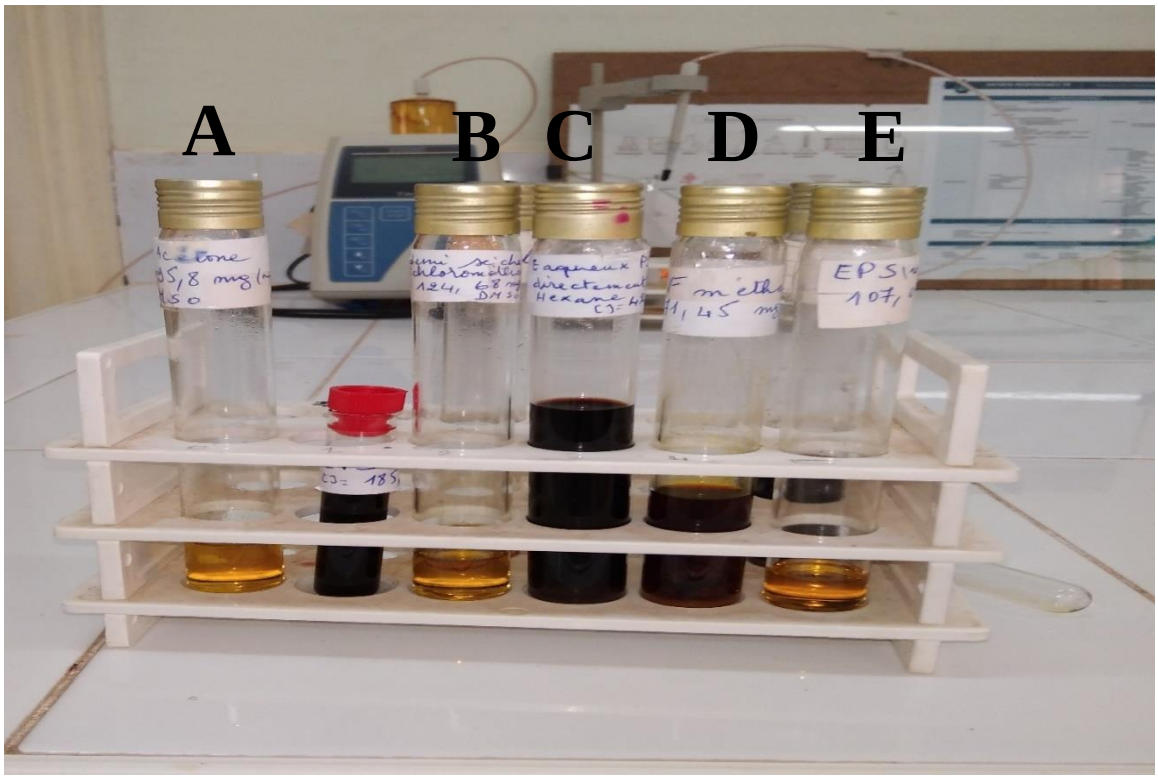


Fig3: Petals extracts

A: Acetonic extract, **B**: Dichloromethanic extract, **C**: Aqueous extract, **D**: Methanolic extract, **E**: Butanolic extract

II. Preparation of biologic material

The antimicrobial activity of *Crocus Sativus* was tested against four bacteria species and two strains of yeasts.

Table3: The microorganisms used

Microorganism	Gram	Code	Source
Escherichia coli	Negative	ATCC 25922	***LAMAABE***
Pseudomonas aeruginosa	Negative	ATCC 27853	
Enterococcus faecalis	Positive	ATCC 29212	***LAPSAB***
Bacillus cereus	Positive	ATCC 11778	***LAPRONA***
Candida albicans ATCC		ATCC 10231	
Candida albicans CIP		*CIP 444*	**LAPSAB**

- ***LAMAABE***: Ms BELLIFA Samia laboratory: Laboratory of microbiology applied to food, biomedical and the environment.
- ***LAPSAB***: Ms BOUCHERITE-OTMANI Zahia laboratory: Antibiotic, Antifongic laboratory.
- ***LAPRONA****:* Mr. BELYAGOUBI Larbi laboratory*:* Natural products laboratory (University of Tlemcen).

✓ We prepared Mueller Hinton Agar/Broth for the bacterial species and Sabouraud Agar/Broth for *Candida albicans*, the following table represente the extracts used in the MIC method.

Microorganisms	Extracts Used
Pseudomonas aeruginosa	Aqueous extracts, methanolic extracts and Fresh butanolic extract
Bacillus cereus	Aqueous fresh extract
Candida albicans CIP	Butanolic fresh and semi-dry acetate extract
Candida albican ATCC	Methanolic extracts
Enterococcus faecalis	Methanolic extracts and semi-dry acetate extract

III. Antibiogram

1. A preculture of microbial strains: with a platinum loop we took a colony from the strains of each microorganisms and we inoculated it in the test tubes which contains 10 mL of Mueller Hinton or sabouraud broth, after agitation we incubated them in 37°C for 18- 24 hours.
2. After the incubation we stoped the microbial growth using ice cubes.
3. Poured Petri Dichs with Mueller Hinton and Sabouraud Agar at a depth of 4mm.
4. Mesured the Optical Density (OD) using Colorimetry ≈ 10^8 UFC/mL (OD = 0.08 to 0.1/ λ 625nm)
5. We Soaked the swab in the bacterial suspension then rubbed it over the entire dry agar surface with tight and close streaks and we rotated the swab on itself, repeated for four times with turning the petri dish 30° each time, finishing the inoculation by passing the swab over the periphery of the agar and putting the swaped petri dishs in the fridge for 1h-2h.
6. We putted 6 discs of antibiotic (Ampicillin 10µg) in each petri dish of the four strains of bacteria and 6 discs filled with 100µg of the antifungic Nystatin Dihydrate in the two petri dishs inoculated previously with *Candida albicans* (Fig4 & Fig5).
7. The petri dishs were incubated at 37°C for 24 hours. The results were observed by measuring the inhibition zones diametre in mm, and readed using a table (Fig6).

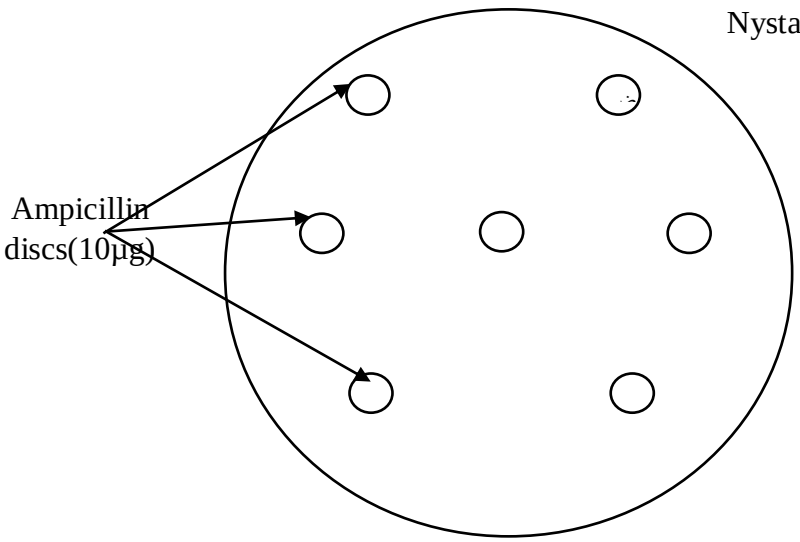


Fig4: Antibiogram method for bacteria

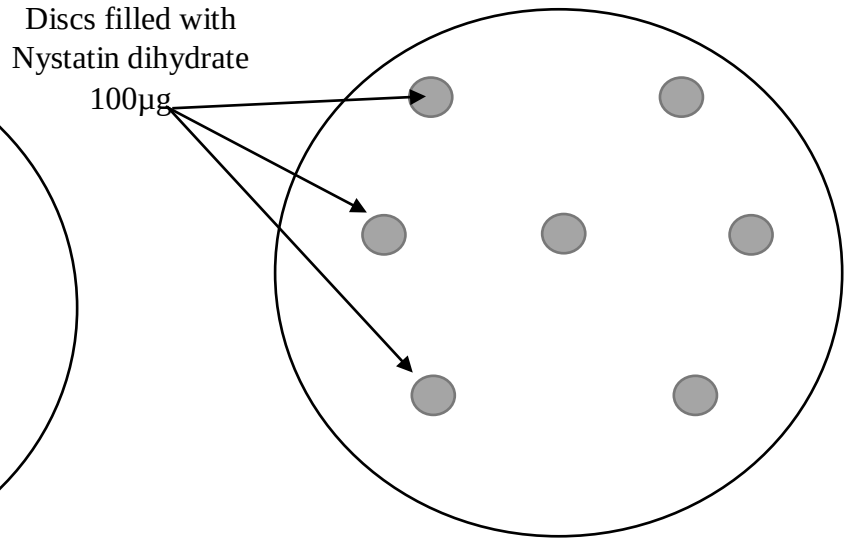
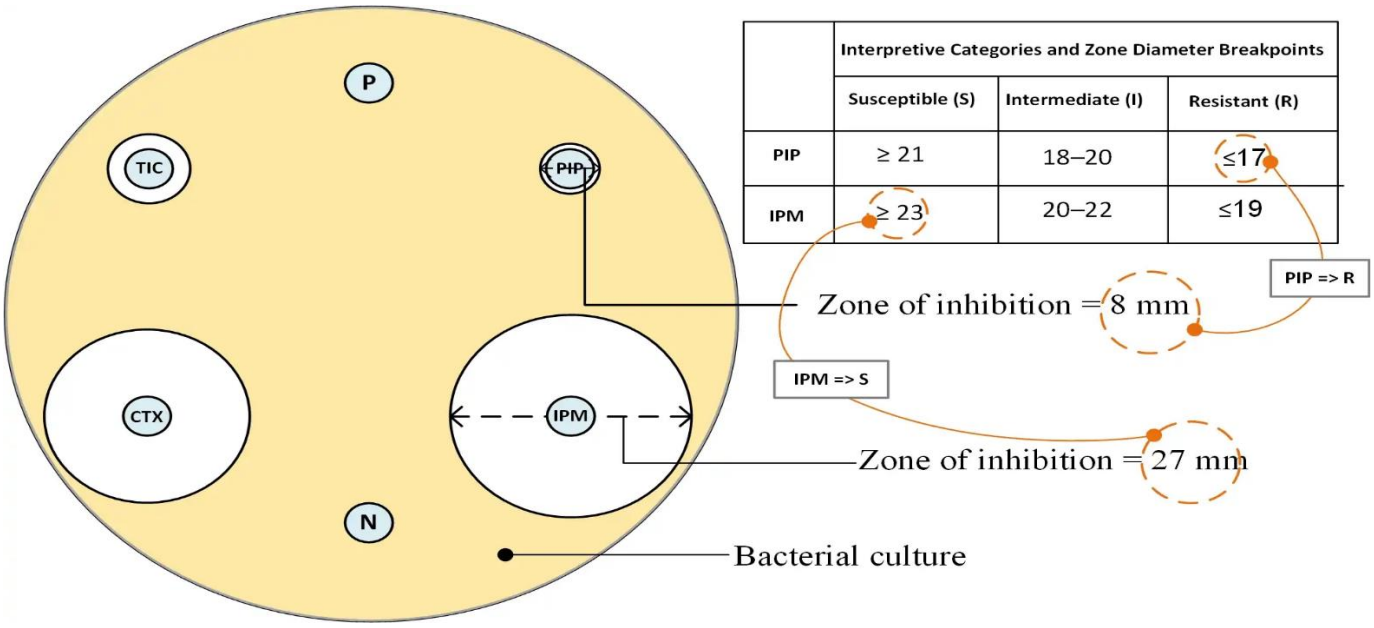


Fig5: Antibiogram method for *Candida*



	Interpretive Categories and Zone Diameter Breakpoints		
	Susceptible (S)	Intermediate (I)	Resistant (R)
PIP	≥ 21	18–20	≤17
IPM	≥ 23	20–22	≤19

Fig6: Antibiogram reading from microbiologie-clinique weebsite

IV. Disc diffusion assay

1. The first 5 steps are the same as the antibiogram from the preperation of the preculture to the rub of petri dishs
2. Preparation of extracts dilutions.
3. 6 mm diameter filter paper discs are sterilized in an autoclave and then impregnated with 10/7.5 µL of the *Crocus sativus* extracts. These discs are placed on the surface of M.H/SB agar previously inoculated with the microbial suspension, three discs are performed for each extract through all the six microorganisms, a negative control is also carried out using a paper disc loaded with 10µL/7.5µL of sterile distilled water for aqueous extracts and DMSO 5% for the other extracts (Fig7). Afterwards, the petri dishs were placed at 4°C for about 1 hour to allow pre-diffusion of the bioactive molecules containing in the extracts.

4. The petri dishs were incubated at 37°C for 24 hours. The results were read by measuring the diameter of the inhibition zones in mm.

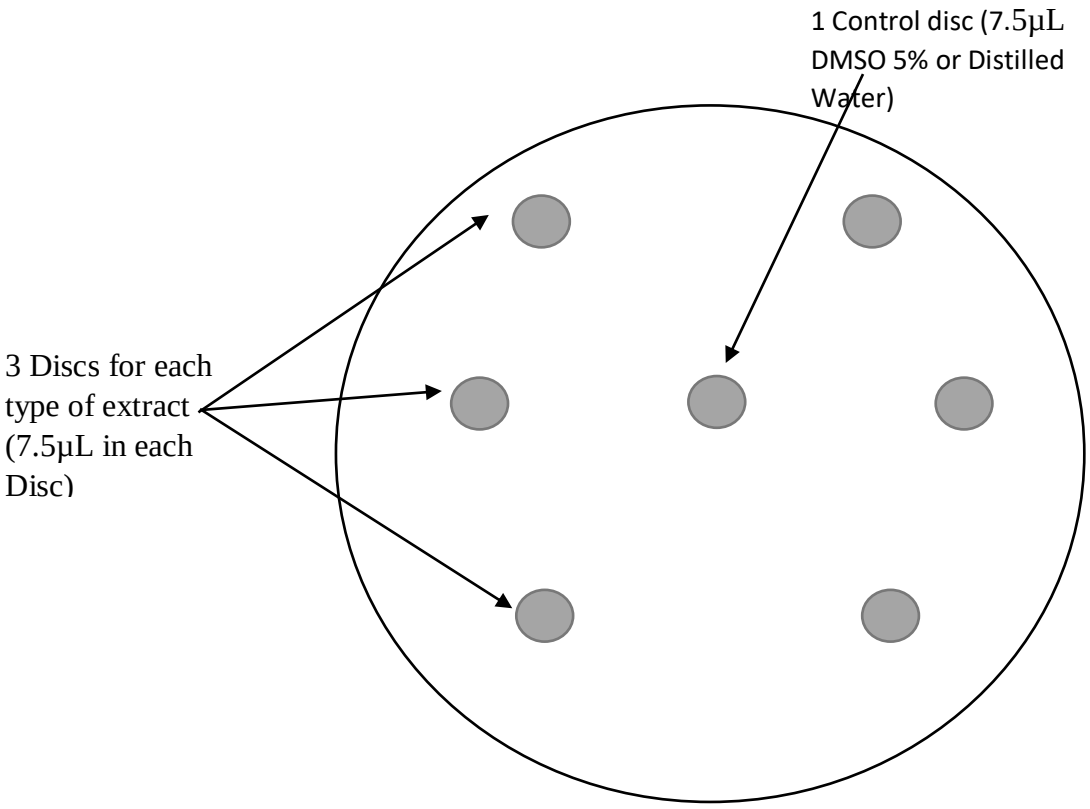


Fig7: Disk diffusion method

V. Minimum inhibitory concentration assay (MIC):

Based on the results of the discs diffusion method the minimum inhibitory concentration (MIC) was determined by microbroth dilution method (sterile microtiter plate) according to the National Committee for Clinical Laboratory Standards **(NCCLS,2001).**

We inoculated the bacteria in 10 ml of Mueller Hinton broth and the yeast in 10 ml of Sabouraud broth, after the incubation at 37°C for 24 hours, we calibrated the Optical Density (OD) at wave length of 625nm between 0.08 and 0.1 (10^8 UFC/ml), then we diluted the bacterial suspension to 10^6 UFC/ml.

In a 96 wells microplate, first we filled every two lines with 100µl of the broth used (MH/Sb) (we left an empty line between each extract to avoid contamination) then we added 100µl of extract in wells number 2 and 3 then we carry out successive dilutions (after agitation we took

100µl from well N03 and we putted in well N04 and so on until the well N22). Finally, we added 100µl of the bacteria suspension to all wells except the second well and we incubated the microplate at 37°C/24h (Fig8).

After the 24h incubation of the microplates, to read results we needed a specific reagent call β-Tetrazolium that dye the wells in the presence of a bacterial growth, due to the unavailability of that reagent in the laboratory, the results were determined visually based on the wells turbidity, we took the concentration of the last clear well with no bacterial growth as our MIC.

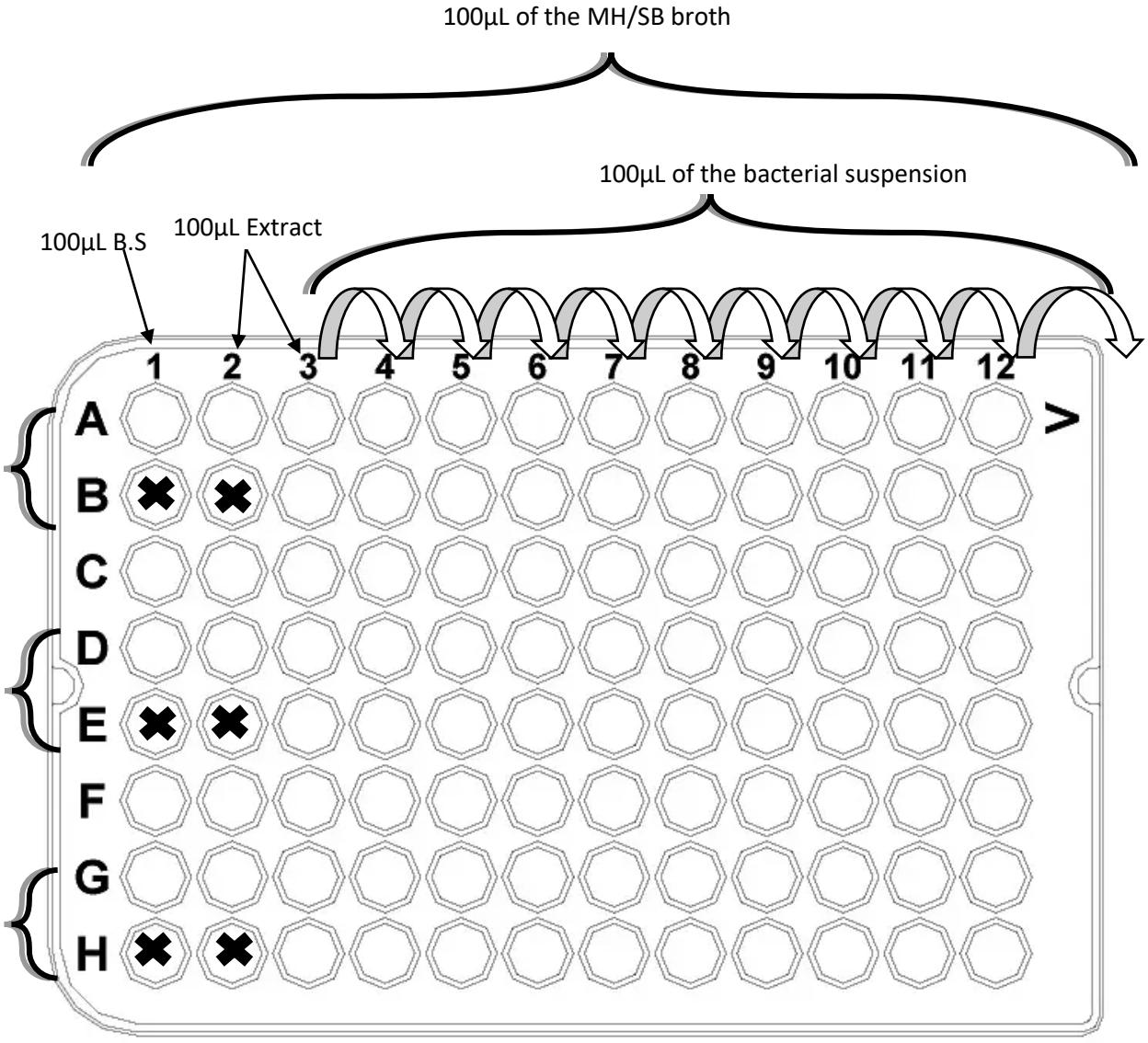


Fig8: Filling method of a 96 well microplate to determine MIC

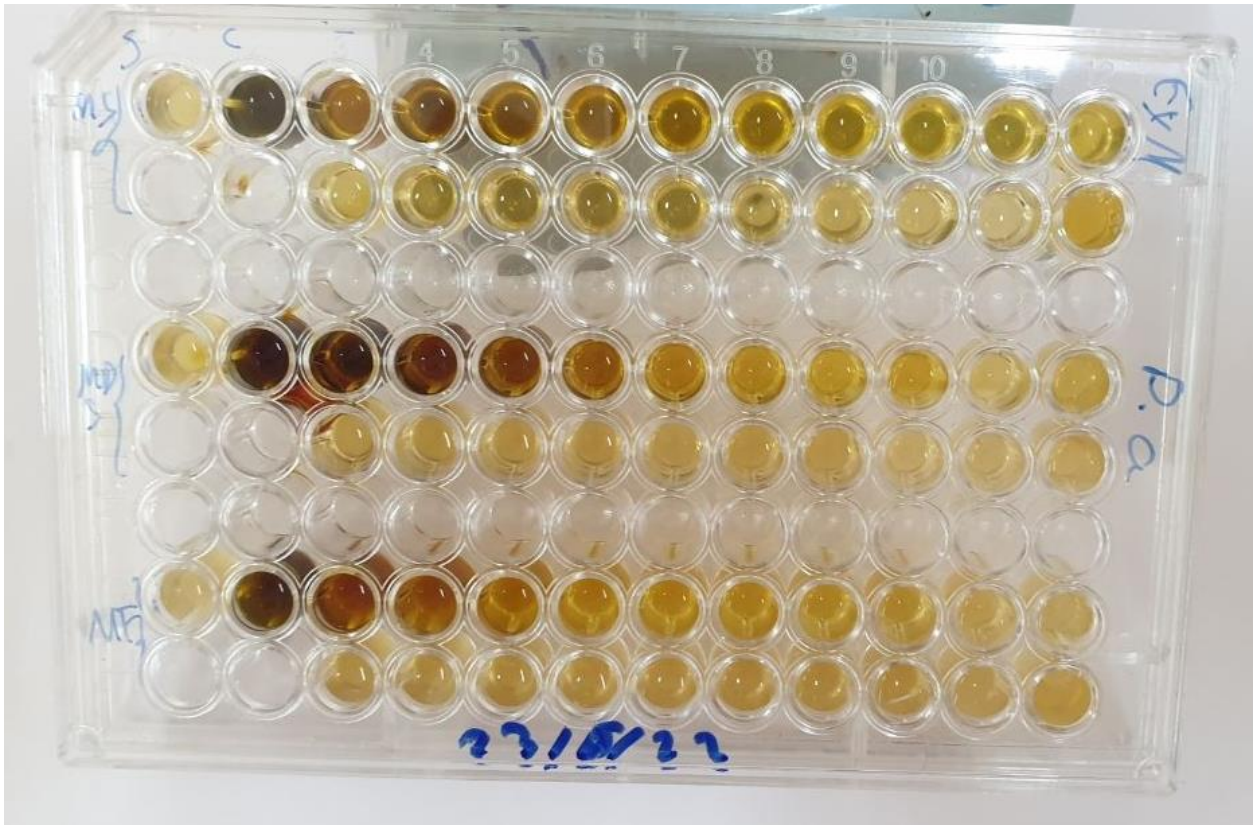


Fig9: A microplate for detemining the MIC of methanolic extract for *Pseudomonas aeruginosa* before the incubation.

VI. Minimum bactericidal concentration (MBC)

- We inoculated 10µl from each clear well without turbidity in a petri dish (MH/Sb agar) (Fig10).

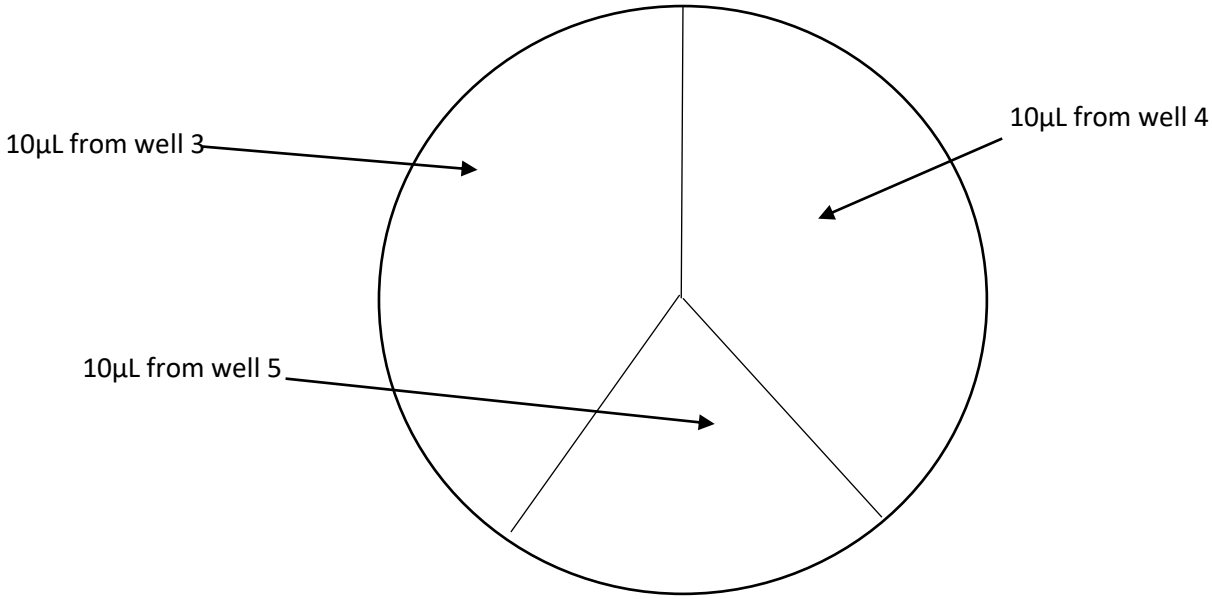


Fig10: Petri dish of MBC

- After the 24h incubation, the results were noted based on the bacterial growth from 0 to +++++

✓ 0 : No bacterial growth
✓ + : Low bacteria growth
✓ ++ : Moderate growth
✓ +++ : Medium growth
✓ ++++ : Superior bacterial growth
✓ +++++: the bacterial growth took all the agar space (bacterial lawn)

Results and discussion

a. Antiobiogram results

For the test of the molecules of reference (Ampicillin, Nystatin dihydrate) we got the following results presented by the inhibition zones diameter in mm (Table4).

Table4: Antibiogram inhibition zones

Type of molecule / Microorganisms	Ampiciline 10µg	Nystatin Dihydrate
E. coli	11±1.41 (R)	/
E. faecalis	8±4.41 (R)	/
P. aeruginosa	00±0 (R)	/
B. cereus	00±0 (R)	/
C. albicans CIP	/	39±4.24 (S)
C. albicans ATCC	/	32.5±0.70 (S)

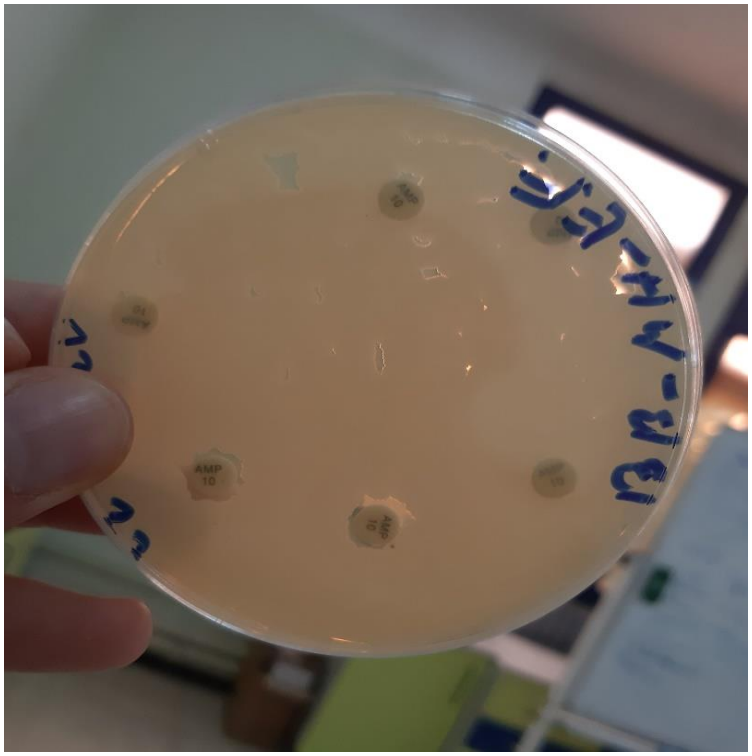


Fig11: *E.f* Antibiogram result

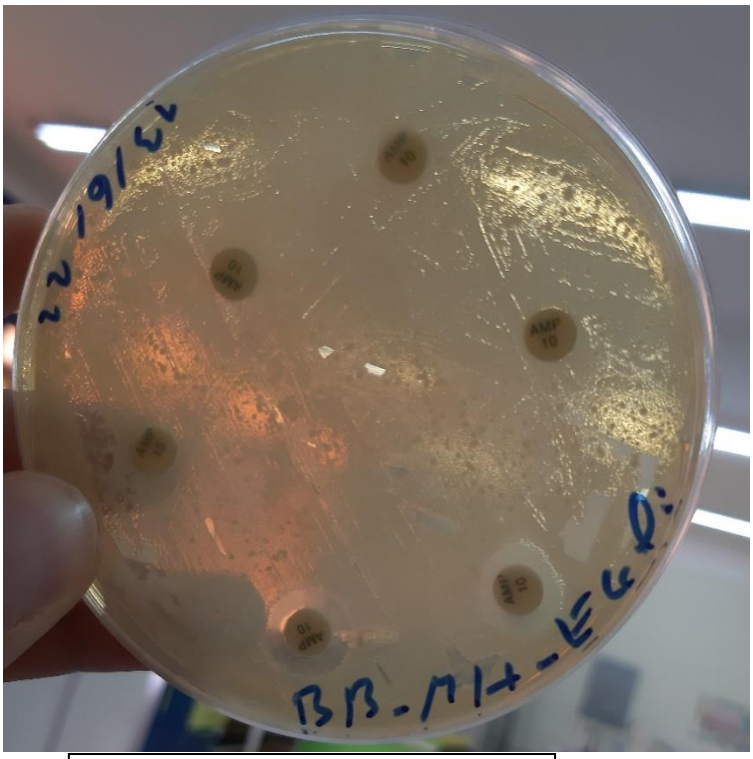


Fig12: *E.coli* Antibiogram result

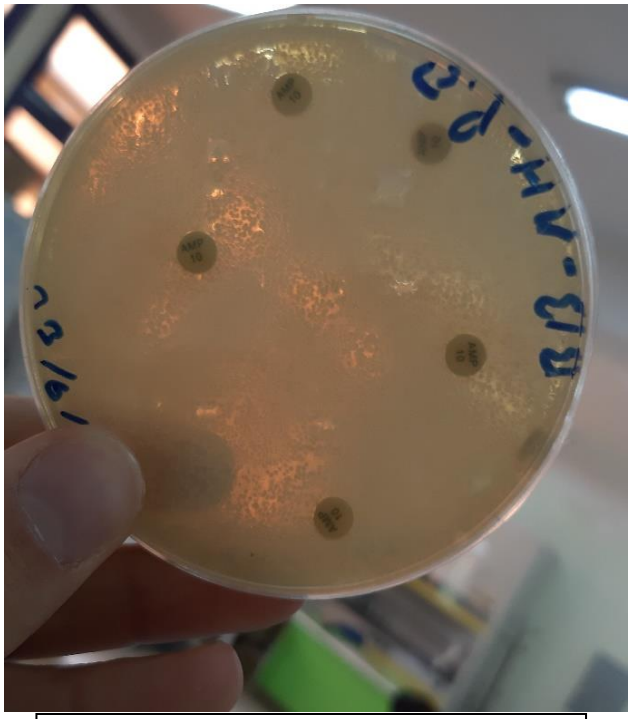


Fig13: *P.aeruginosa* antibiogram result

Fig14: *B.cereus* antibiogram result

- ✓ Ampicillin 10µg had no antibacterial effect on both *Pseudomonas aeruginosa* and *Bacillus cereus* since they are resistance to this antibiotic (Fig13 & Fig14).

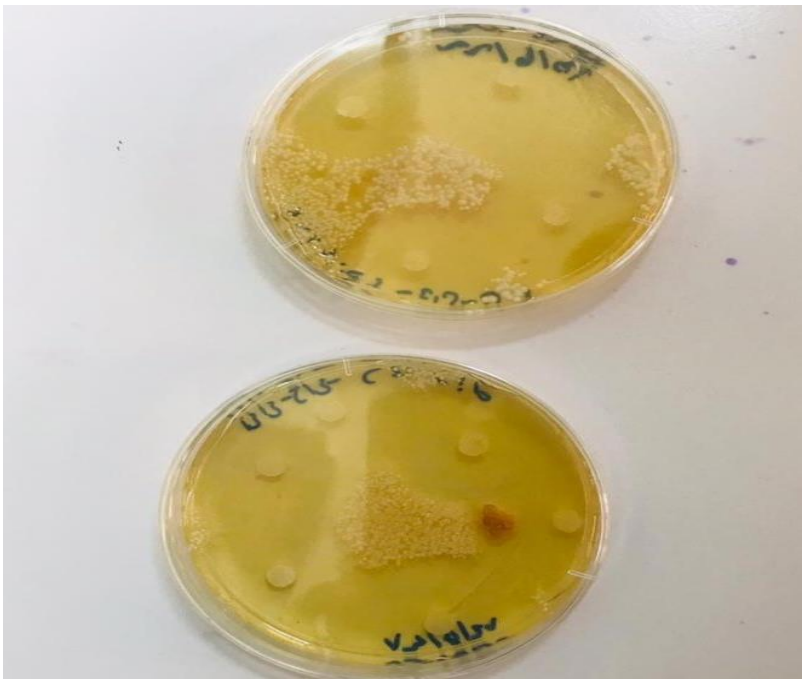


Fig15: *Candida albicans* antibiogram results

- ✓ Both the strains of *Candida albicans CIP* (Average of 39mm inhibition zone diameter) and ATCC (Average of 32.5mm inhibition zone diameter) (Table 4 & Fig15) are sensitive too the antifungic Nystatin Dihydrate, in the study made by **Bonouman-Ira** et al they found that *Candida* genus is sensitive to this antifungic **(Bonouman-Ira, 2011)**.

b. Discs assay results

After 24h from putting the extract discs we got the following results of each extract and their effects on six strains of bacteria/yeast (Table5).

1) **Aqueous extract 1000 µg/disc**

Table5: Average inhibition zones(mm) of different types of aqueous extract on several microorganisms

Extract / Microorganisms	Fresh petals extract	Semi-dry petals extract	Dry petal extract (10 days)	Dry petal extract (straight after hexan)
E. coli	0±0	0±0	0±0	0±0
E. faecalis	0±0	0±0	0±0	0±0
P. aeruginosa	9.5±0.70	0±0	0±0	0±0
B. cereus	0±0	0±0	0±0	0±0
C. albicans CIP	0±0	0±0	0±0	0±0
C. albicans ATCC	0±0	0±0	0±0	0±0

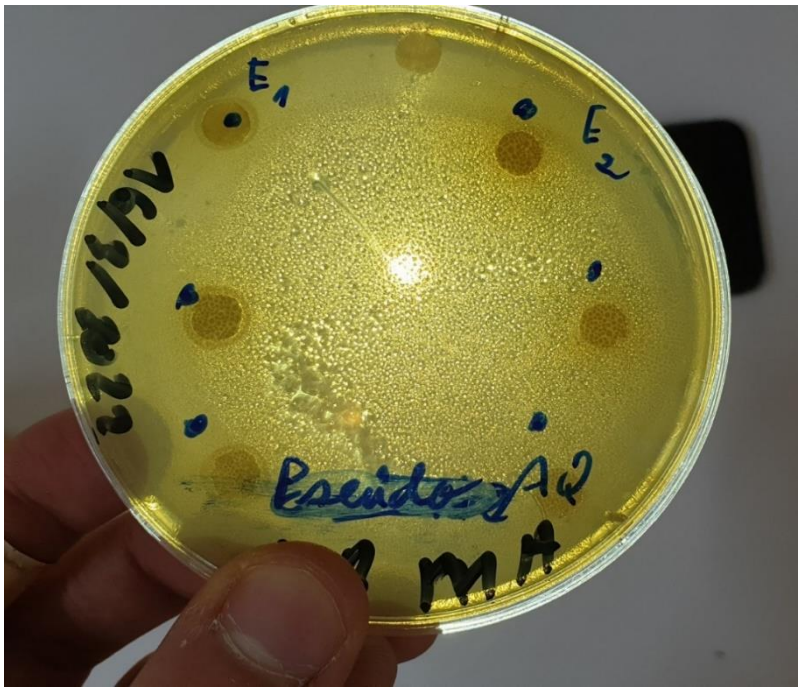


Fig16: The results of the E1(Aqueous Fresh petals extract) and E2(Aqueous semi-dry petals extract) on *P.aeruginosa*

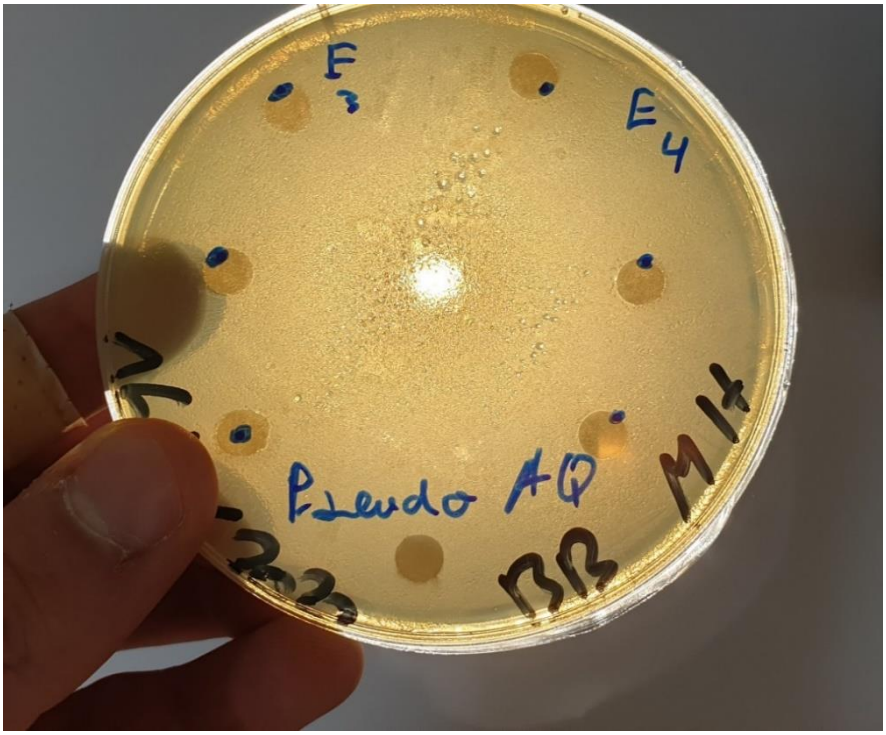


Fig17: The inhibition zones of the E3(aqueous dry petals extract) and E4(aqueous semi-dry petals with hexane extract) on *P.aeruginosa*

- ✓ The growth of *Pseudomonas aeruginosa* is inhibited at some degrees by the aqueous Fresh petals extract, in the other hand this strain of bacteria is resisted to aqueous dry/semi-dry petals extract and semi-dry petals with hexane extract (Fig15 & Fig16).

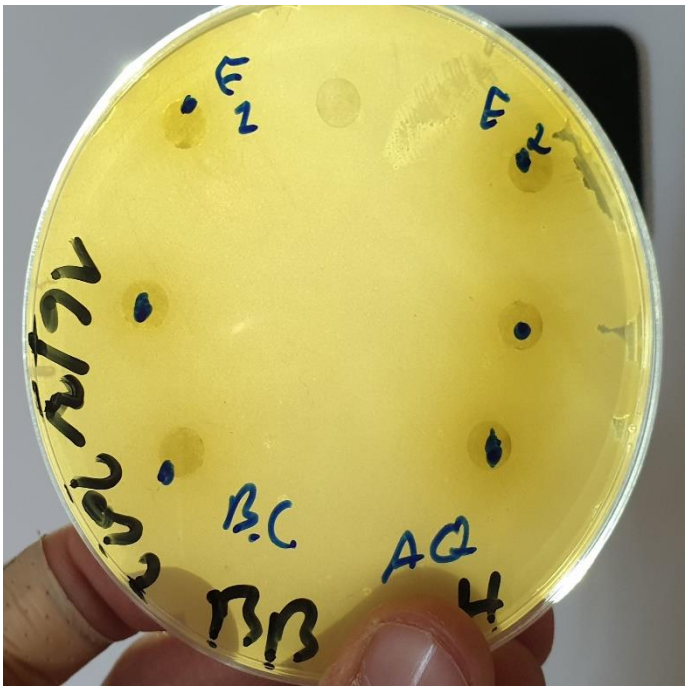


Fig18: The results of the E1(aqueous Fresh petals extract) and E2(aqueous semi-dry petals extract) on *B.cereus*

- ✓ The fresh petals extract have no antibacterial activity on *Bacillus cereus*.
- ✓ In the study made by **Bagherzade** in 2017 using the MIC method they also found that the aqueous saffron wastage extract had an insignificant antibacterial activity on the strains of *Pseudomonas aeruginosa* (ATCC 27853), but also on *Bacillus cereus* (ATCC 6633) which goes against our results **(Bagherzade, 2017).**

- ✓ Aqueous extract shows non activity on the strains of *E.coli* and *E.faecalis* as has been revealed before in the study of **Beniamino T. Cenci-Goga.**

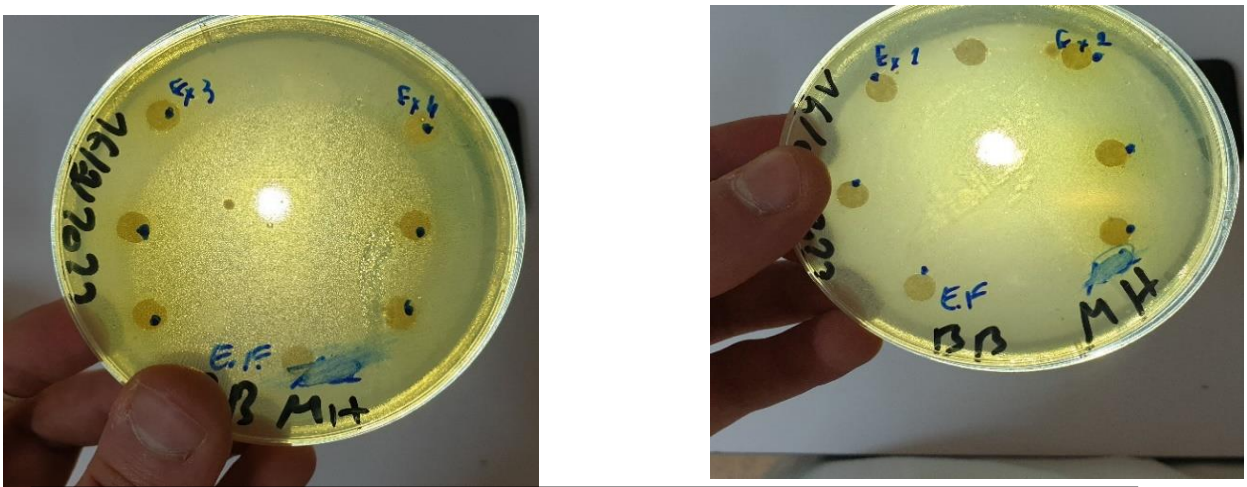


Fig19: The results of aqueous extract on *E.faecalis*

After the 24H incubation of the petri dishs inoculated with the 6 different microorganisms and contain 6discs filled with different types of methanolic extracts we got the following results:

2) **Methanolic extract 500µg/disc**:

Table6: Average inhibition zones(mm) of different types of methanolic extracts on several microorganisms

Extract / Microorganisms	Fresh methanolic extract	Semi-dry methanolic extract	Dry methanolic extract
E. coli	0±0	0±0	0±0
E. faecalis	9±0	8±2.12	10.5±0.70
P. aeruginosa	11±0	10.5±0.70	11±0
B. cereus	0±0	0±0	0±0
C. albicans CIP	0±0	0±0	0±0
C. albicans ATCC	0±0	7.75±1.76	0±0

Fig20: Inhibition zones of Ex03(methanolic semi-dry) and Ex4(methanolic dry) on *P.aeruginosa*

- ✓ All of the three methanolic extracts shows an antibacterial activity on the strain of *Pseudomonas aeruginosa* (Table6) as also has been revealed in the study made by **JAFARI-SALES, A., & PASHAZADEH, M** in 2020 where they found an inhibition zone of 7.8±1.14 mm at a concetration of 50mg/mL and 14.5±0.83mm at 400mg/mL on *Pseudomonas aeruginosa*.
- ✓ *Bacillus cereus* is resistant to all types of methanolic extracts unlike the results of **JAFARI-SALES, A., & PASHAZADEH, M** where they concluded that *B.cereus* was sensitive even at a low concentration (20mg/ml).
- ✓ The same can be said for *E.coli* but at a higher concentration(50mg/ml) **(JAFARI-SALES, 2020).**

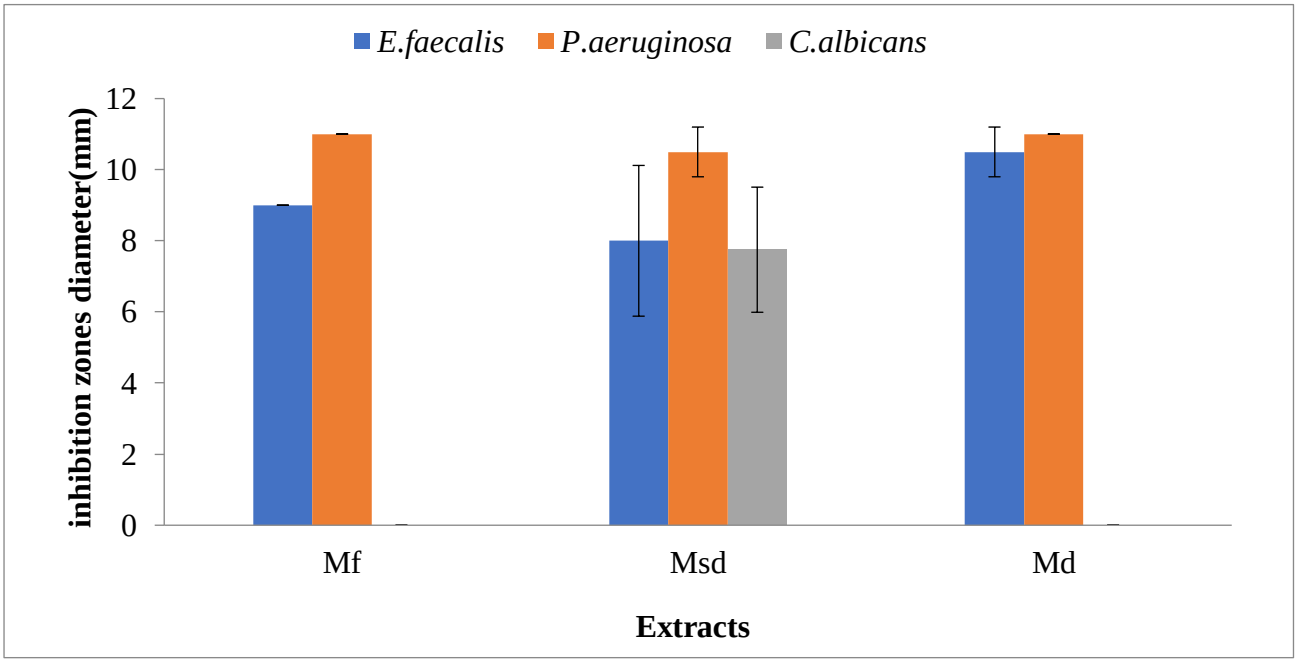


Chart1: Average inhibtion zones diameter of the methanolic extracts on several microorganisms

- ➢ Mf : fresh methanolic extract
- ➢ Msd: semi-dry methanolic extract
- ➢ Md: dry methanolic extract

- ✓ The three types of the methanolics extract shown a variant antibacterial activity on *Enterococcus faecalis* (Chart1).
- ✓ *Candida albicans* has been only affected by the semi-dry methanolic extract (Chart1).

After the 24H incubation of the petri dishs inoculated with the 6 different microorganisms and contain 6discs filled with different types of acetonic extracts we got the following results:

Table7: Average inhibition zones of different types of acetonic extract on several microorganisms

3) **Acetonic extract 500µg/disc**:

Extract / Microorganisms	Fresh acetonic extract	Semi-dry acetonic extract	Dry acetonic extract
E. coli	0±0	0±0	0±0
E. faecalis	0±0	0±0	0±0
P. aeruginosa	6.66±0.28	7±0	0±0
B. cereus	0±0	0±0	0±0
C. albicans CIP	0±0	0±0	0±0
C. albicans ATCC	0±0	0±0	0±0

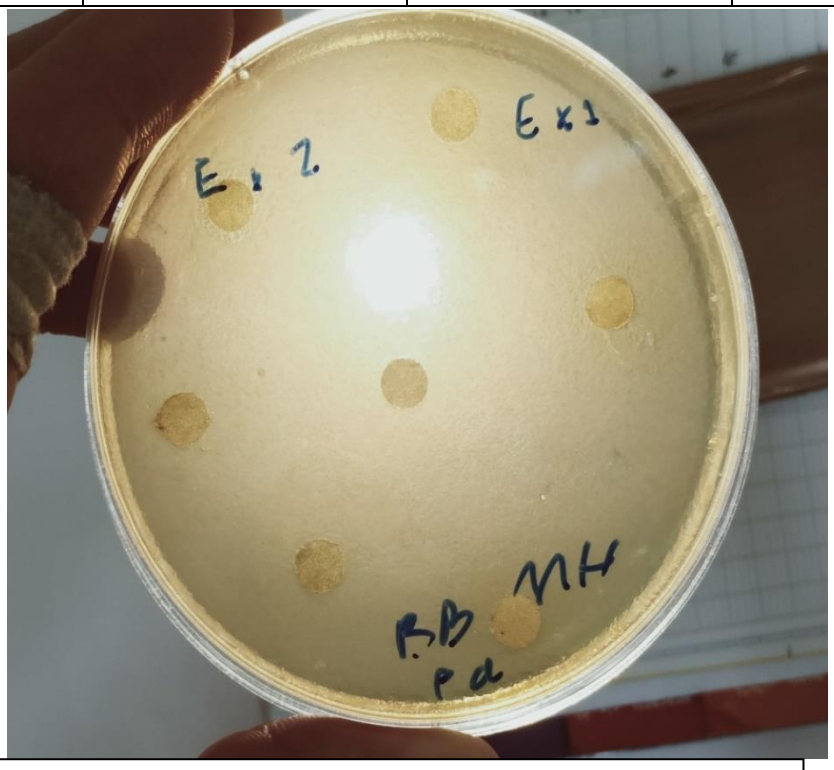


Fig21: Results of the Ex2(fresh acetonic extract) on *P.aeruginosa*

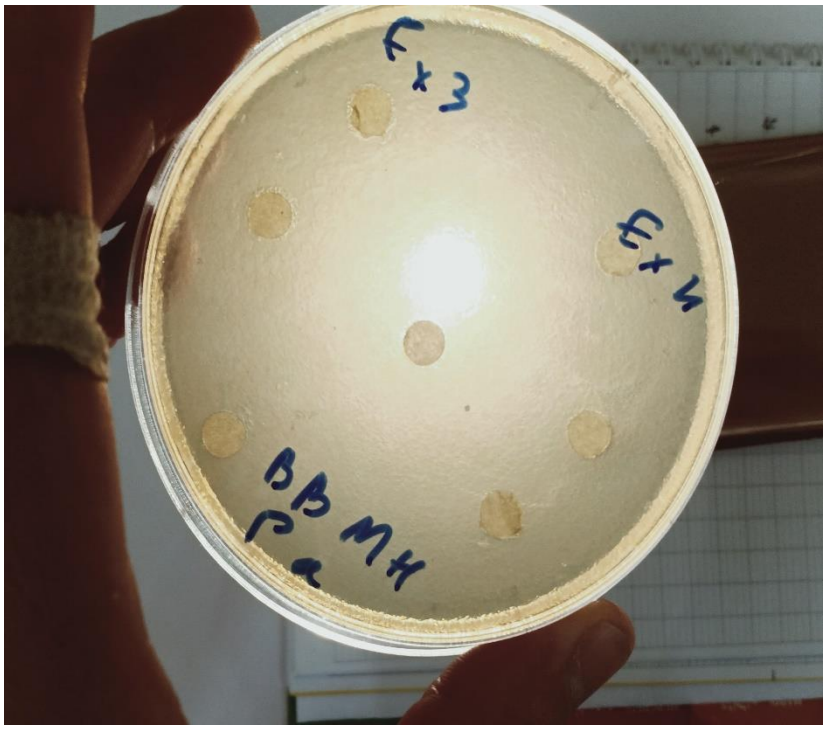


Fig22: Results of Ex3(semi-dry acetonic extract) and Ex4(dry acetonic extract)

- ✓ Acetone extract didnt affect any bacteria except *Pseudomonas aeruginosa*, which was marginally affected (Table7).

4) **Butanolic extract 500 µg/disc:**

Table8: Average inhibition zones of different types of butanolic extract on several microorganisms

Extracts / Microorganisms	Fresh butanolic extract	Semi-dry butanolic extract	Dry butanolic extract
E. coli	0±0	0±0	0±0
E. faecalis	0±0	0±0	0±0
P. aeruginosa	7±0	0±0	8±1.41
B. cereus	0±0	0±0	0±0
C. albicans CIP	8.5±0.70	0±0	0±0
C. albicans ATCC	0±0	0±0	0±0

✓ The fresh butanolic extract have a modurate effect on *Candida albicans* CIP (Fig23).

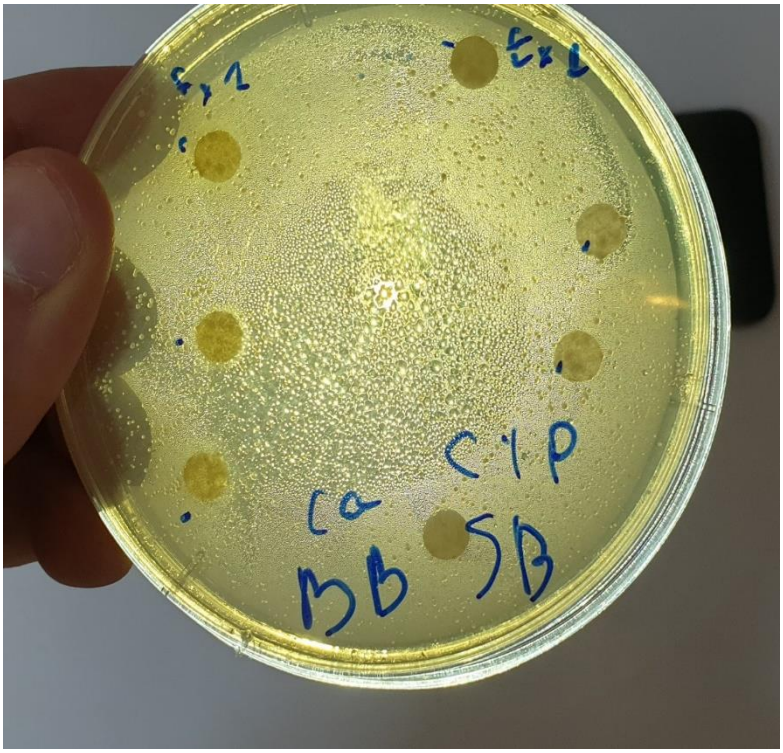


Fig23: Inhibition zones of Ex1 (fresh butanolic extract) on *Candida albicans* CIP

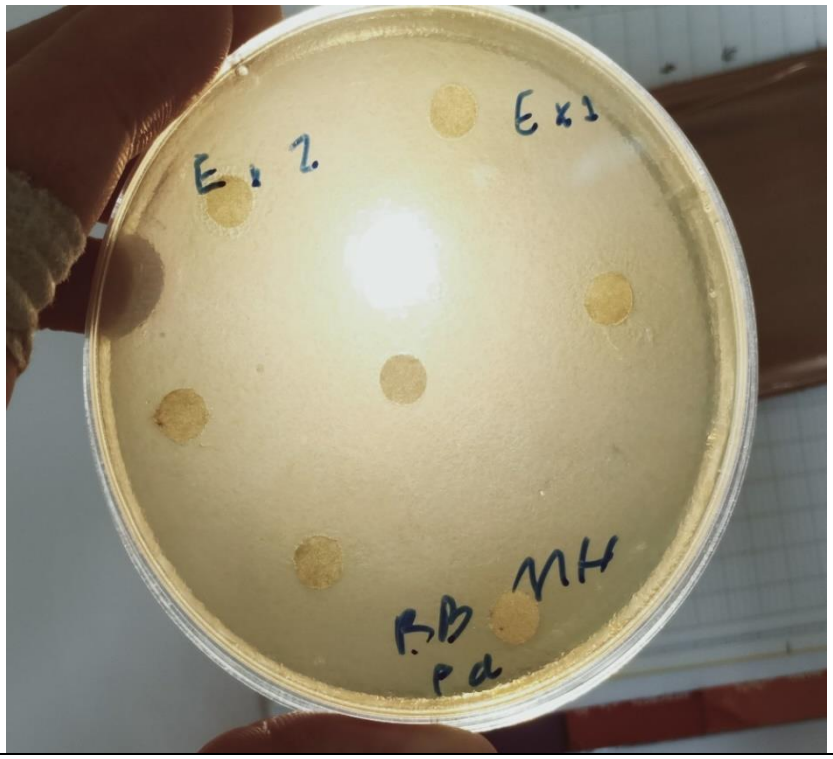


Fig24: Inhibition zones of Ex1 (semi-dry butanolic extract) on *P.aeruginosa*

- ✓ Both the fresh and dry butanolic extracts have varying effects on *Pseudomonas aeruginosa* (Table8).

5) Acetate extract 500 µg/disc

Table9: Average inhibition zones of different types of acetate extract on several microorganisms

Extract / Microorganisms	Semi-dry extract
E. coli	0±0
E. faecalis	8±0
P. aeruginosa	0±0
B. cereus	0±0
C. albicans CIP	8±1
C. albicans ATCC	0±0

- ✓ The semi-dry acetate extract did not affect any microorganism except *E.faecalis* and *C.albicans CIP* (Table9).

Table10: Average inhibition zones of different types of dichloromethane extract on several microorganisms

Extract / Microorganisms	Fresh extract	Semi-dry extract
E. coli	0±0	0±0
E. faecalis	0±0	0±0
P. aeruginosa	0±0	0±0
B. cereus	7.5±0.70	0±0
C. albicans CIP	9±0	0±0
C. albicans ATCC	0±0	0±0

- ✓ *B. cereus* and *C. albicans CIP* were the only ones affected by the fresh dichloromethane extract (Table10).

c. MIC/MBC results

After 24h incubation of the 96 wells microplate we got the following results:

Pseudomonas aeruginosa

Table11: Results of *P.aeruginosa* with the aqueous extracts and the butanolic extract

Extracts concentrations	MIC	MBC
[Fresh aqueous extract]= 321mg/mL	W3 W4 [W5]=15.07mg/mL	+++ +++ +++ >241.25mg/mL
[Semi-dry aqueous extract]= 233mg/mL	W3 [W4]=18.2mg/mL	++++ ++++ >58.25mg/mL
Butanolic extract		
[Fresh butanolic extract]= 249mg/mL	[W3]=62.25mg/mL	++++ >62.25mg/mL

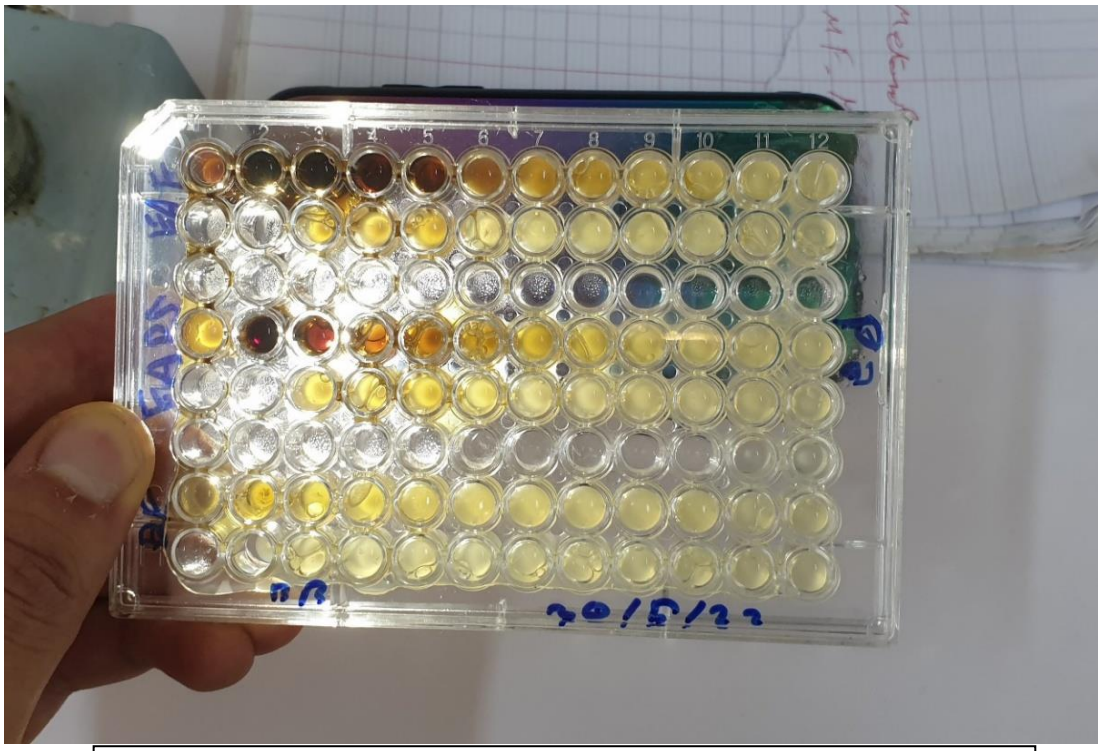


Fig25: The results of *P. aeruginosa* microplate to determine the MIC

Fig26: The MBC results for *P. aeruginosa*

- ✓ The growth of *Pseudomonas aeruginosa* is inhibited even at a low concentration of 15.07mg/mL and 18.2mg/mL of the aqueous extract, the same can be said for the butanolic extract but at a higher concentration of 62.25mg/mL, but both types of these extract were unable to make any bactericidal activity on this strain of bacteria (Fig26).

After the 24h of the microplate incubation and with a calculation we got the following results:

Table12: Results of *P. aeruginosa* with the methanolic extract

Methanolic extracts		
Extracts concentrations	MIC	MBC
[Fresh methanolic extract]= 243mg/mL	[W3]=60.75mg/mL	+++++ >60.75mg/mL
[Dry methanolic extract]= 420m/mL	[W3]=105mg/mL	+++++ >105mg/mL
[Semi-dry methanolic extract]=	No bacterial activity even at 184mg/mL in W3	No test needed >>184mg/mL

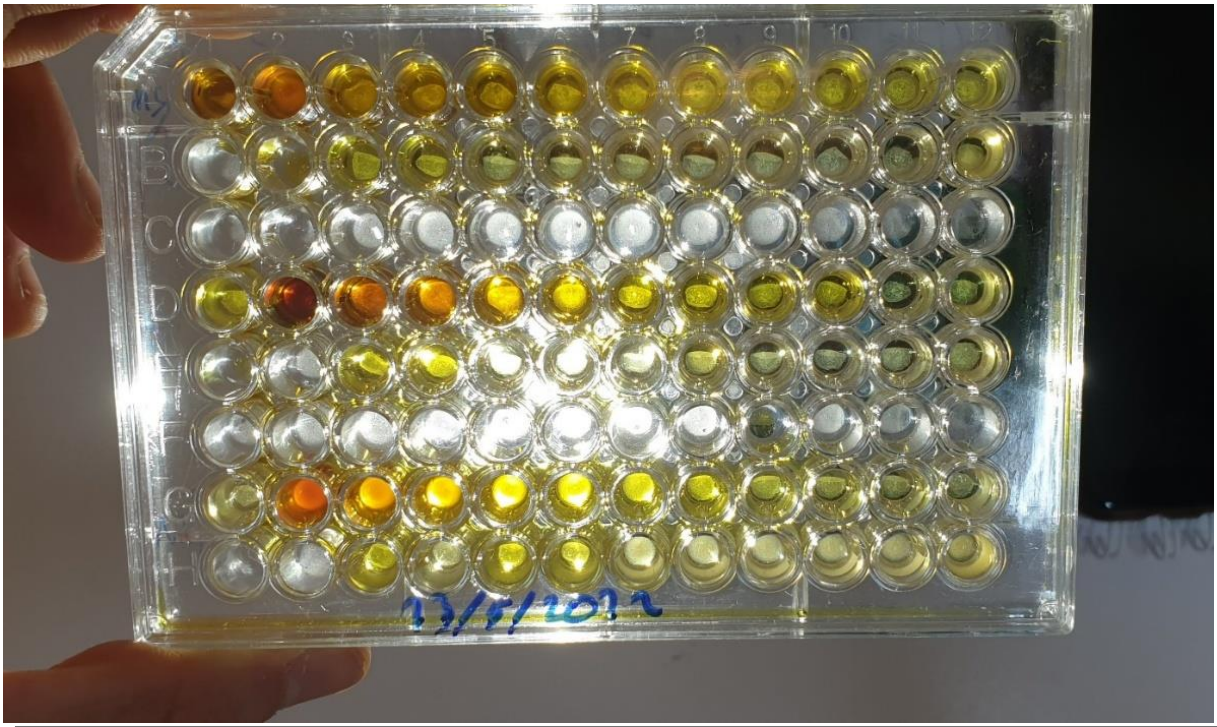


Fig27: The results of *P. aeruginosa* microplate to determine Mic of methanolic extract

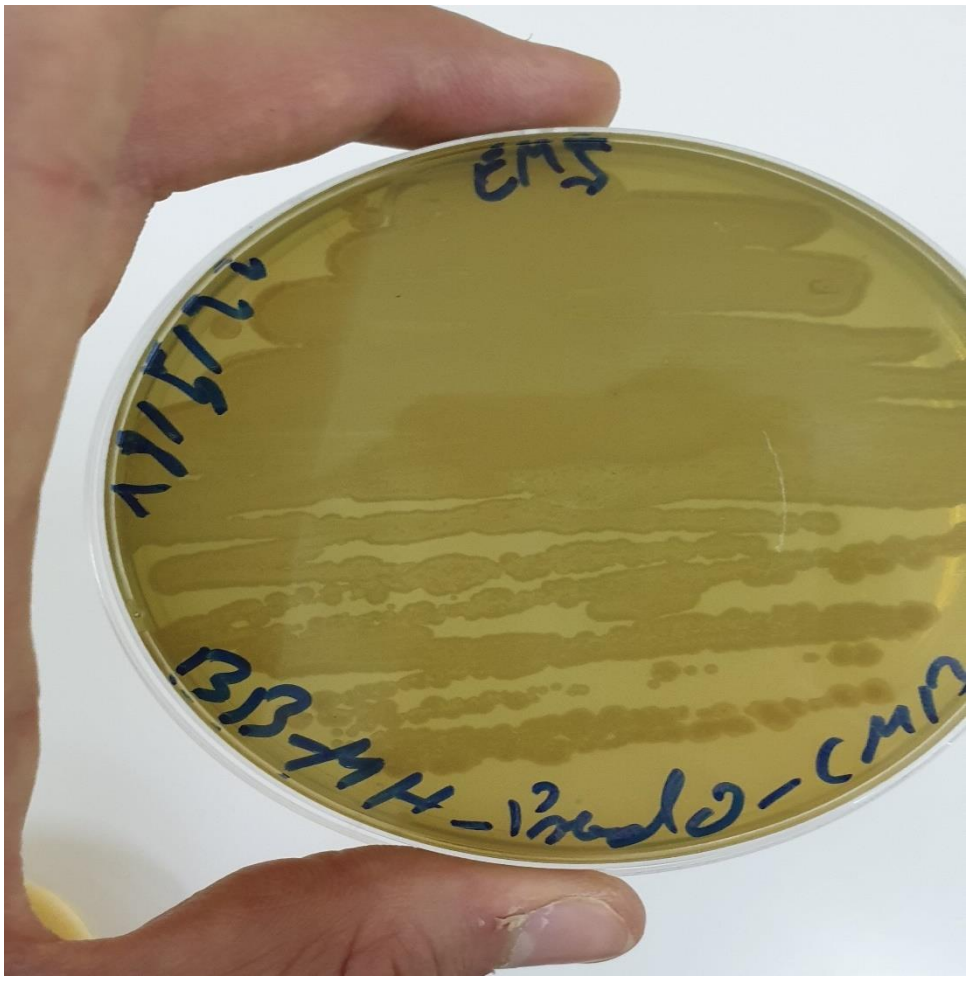


Fig28: The MBC results of dry methanolic extract for *P. aeruginosa*

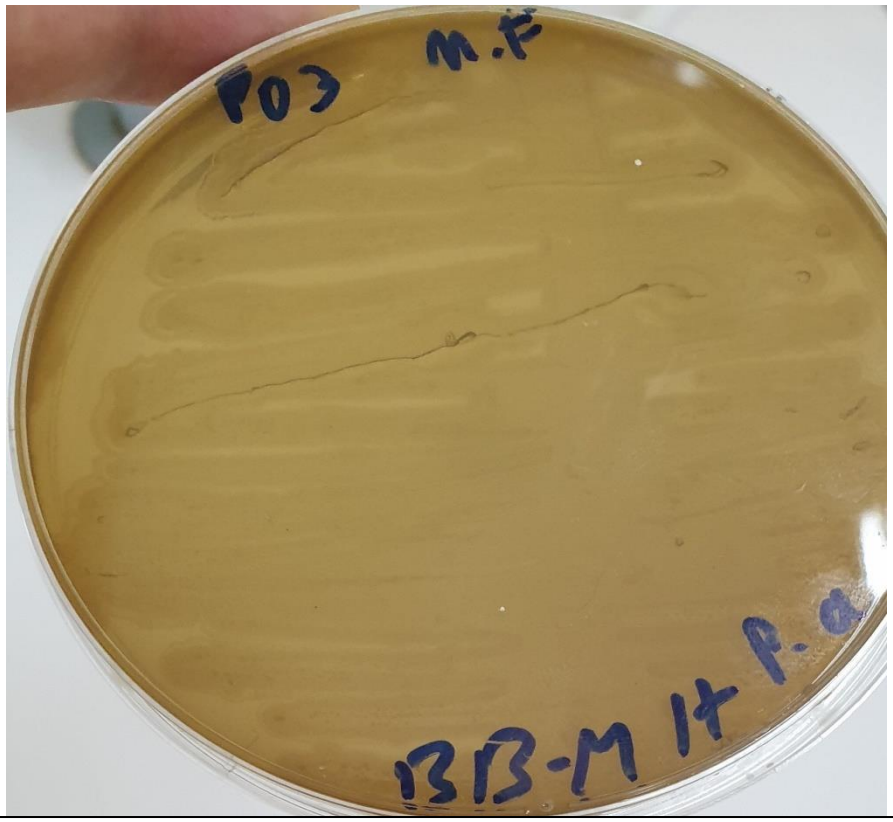


Fig29: The MBC results of fresh methanolic extract for *P. aeruginosa*

- ✓ We manage to inhibit the growth of *Pseudomonas aeruginosa* with the fresh and dry methanolic extract at 60.75mg/mL & 105mg/mL respectively but we were unable to inhibit this bacterium even at a high concentration of 184mg/mL at the well 3 of the semi-dry methanolic extract (Table12), thus for the MBC test we can see a bacterial lawn in the petri dishs (Fig28 & Fig29) which means that the bacteria is still alive.

Table13: Results of *B. cereus* with the aqueous extract

Aqueous extract		
Extract concentration	MIC	MBC
[Aqueous fresh extract] =321mg/mL	W3 [W4]=20.06mg/mL	++ +++ <80.25mg/mL

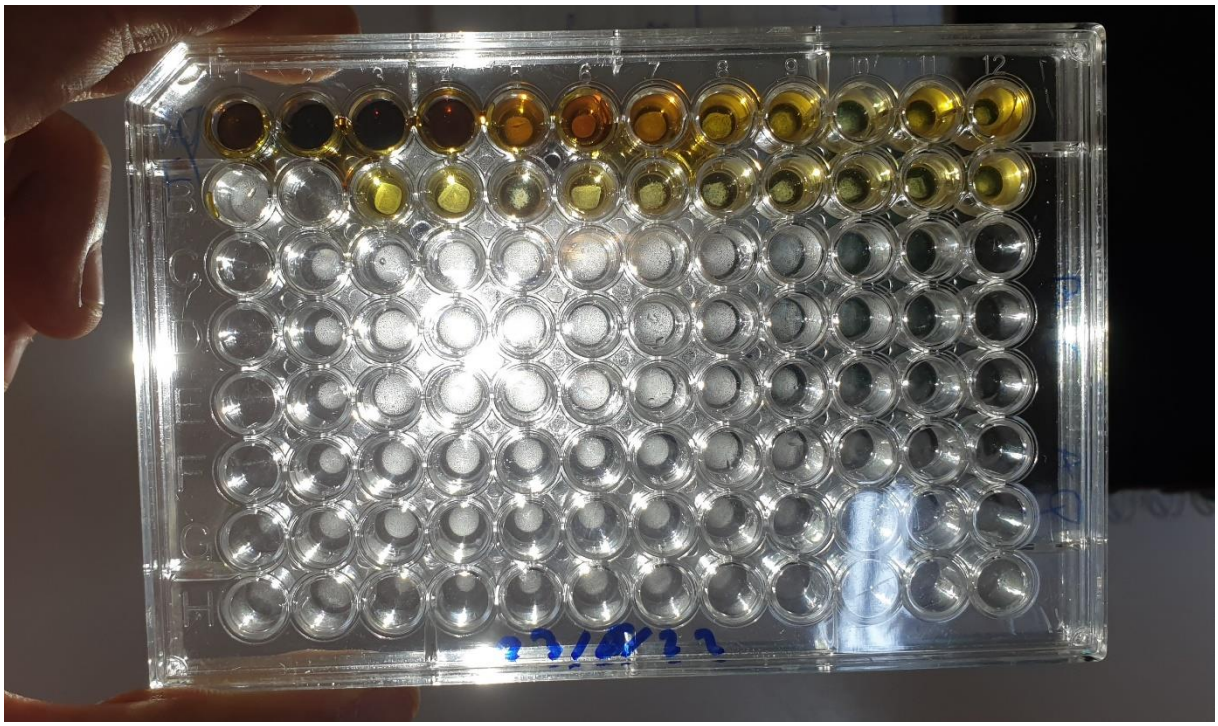


Fig30: The MIC results of fresh aqueous extract for *B. cereus*

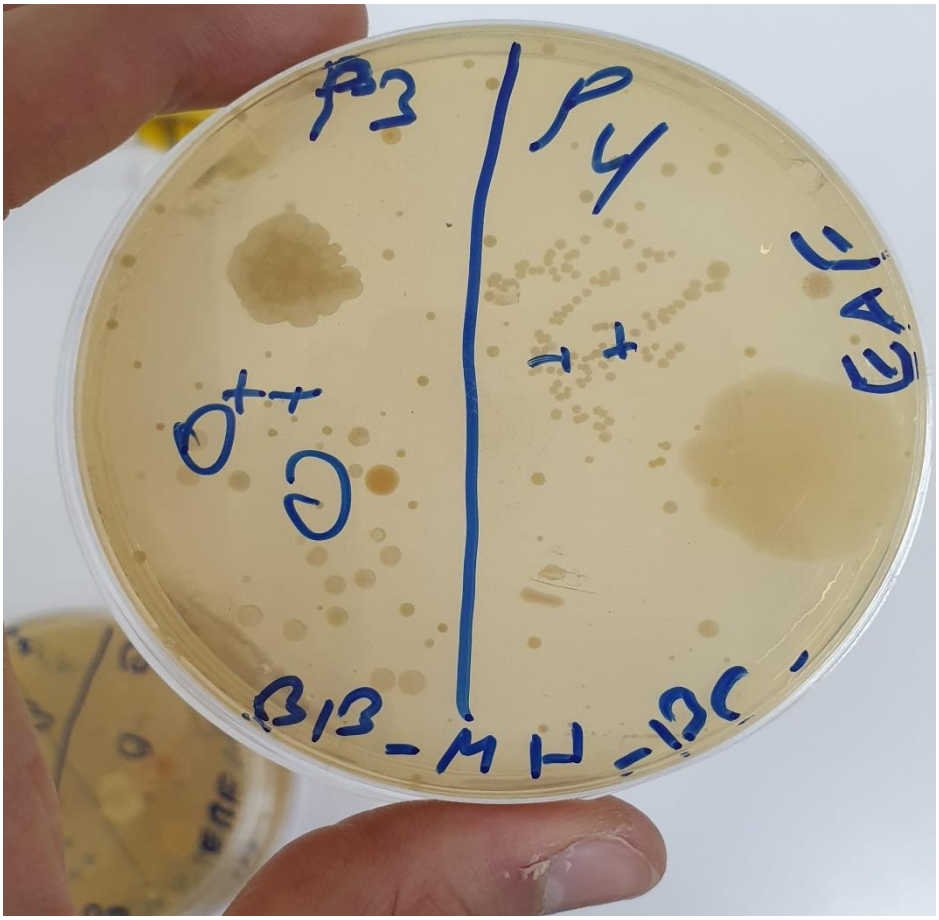


Fig31: The MBC results of fresh aqueous extract for *B. Cereus*

- ✓ The proliferation of *B. cereus* was inhibited at a low concentration of 20.06mg/mL in the fourth well (Table13 & Fig30), but we can see that the bacteria is still active at some extent in the MBC test (Fig31).

Table14: Results of *C. albicans* CIP with the butanolic extract

Butanolic extract		
Extract concentration	MIC	MBC
[Butanolic fresh extract]=80mg/mL	W3 [W4]=5mg/mL	++++ ++++ >20mg/mL

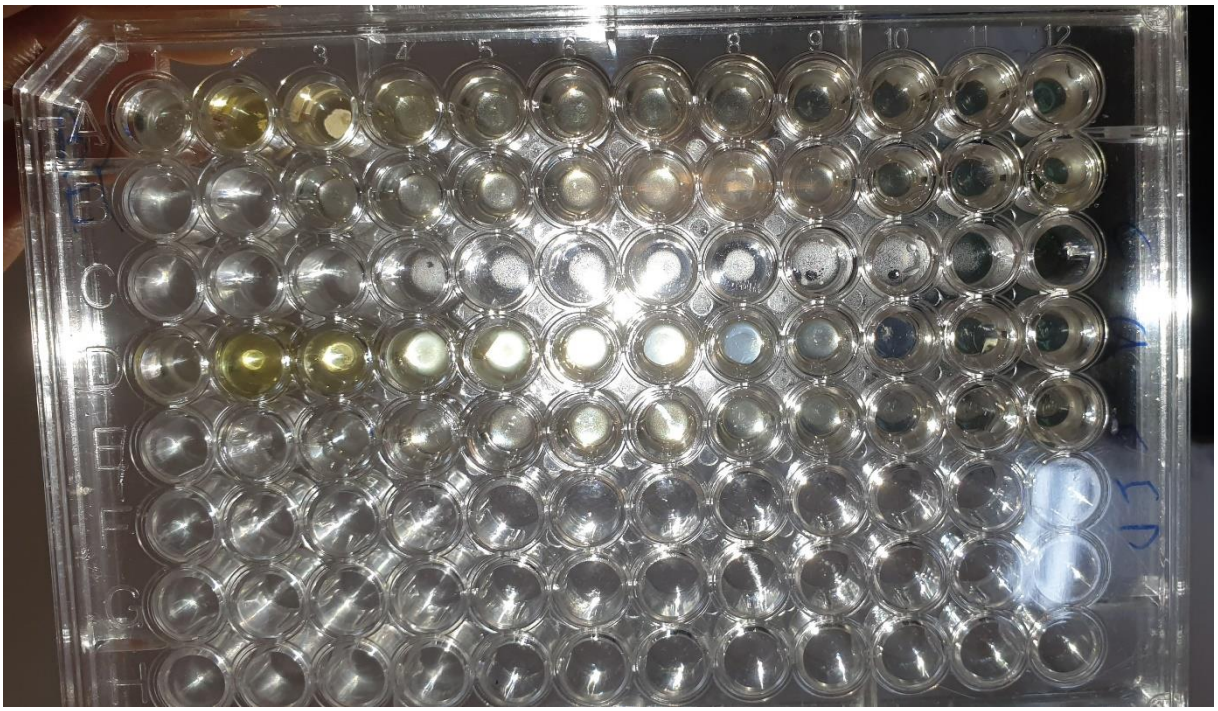


Fig32: The MIC results of fresh butanolic extract for *C. albicans*

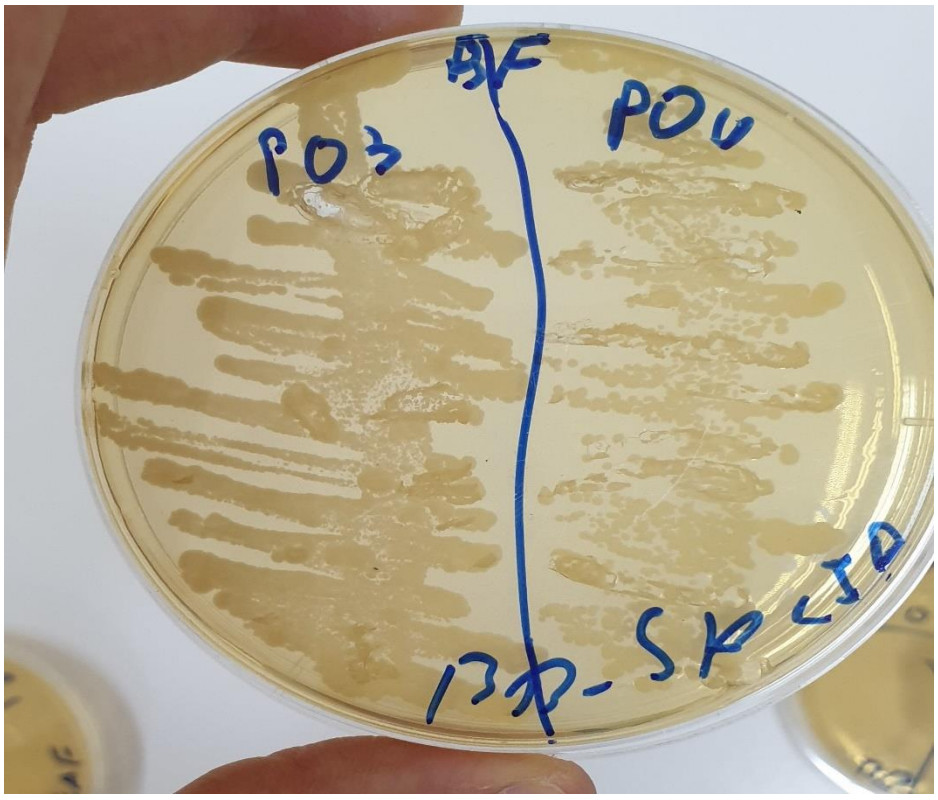


Fig33: The MBC results of fresh butanolic extract for *C. albicans*

- ✓ The growth of this yeast was inhibited by the fresh butanolic extract at a very low concentration of 5mg/mL in well 4 (Table14 & Fig33), but the 20mg/mL in well 3 wasn't enough to kill this yeast (Fig32).

Table15: Results of *C. albicans CIP* with acetate extract

Acetate extract		
Extract concentration	MIC	MBC
[Semi-dry acetate extract]=180mg/mL	W3 [W4]=11.25mg/mL	0 0 ≤ 45mg/mL

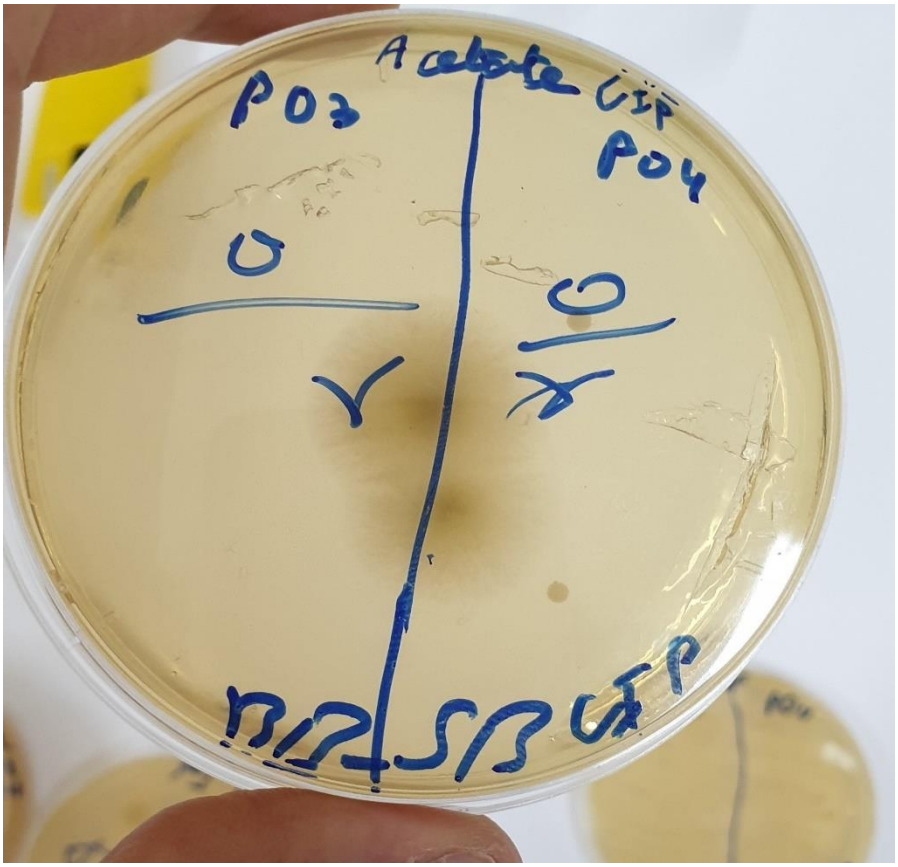


Fig34: The MBC results of semi-dry acetate extract for *C.albicans*

- ✓ The semi dry acetate extract manage to make a bacteriostatic activity at a concentration of 11.25mg/mL (Table15 & Fig34) and a bactericidal activity at 45mg/mL.

Table16: Results of *C. albicans ATCC* with the metanolic extracts

Methanolic extract		
Extracts concentrations	MIC	MBC
[Fresh methanolic extract]= 243mg/mL	W3 W4 [W5]=3.79mg/mL	++ ++ ++ >60.75mg/mL
[Semi-dry methanolic extract]= 736mg/mL	W3 W4 [W5]=11.5mg/mL	++ ++ ++ >184mg/mL
[Dry methanolic extract]= 420mg/mL	none	none

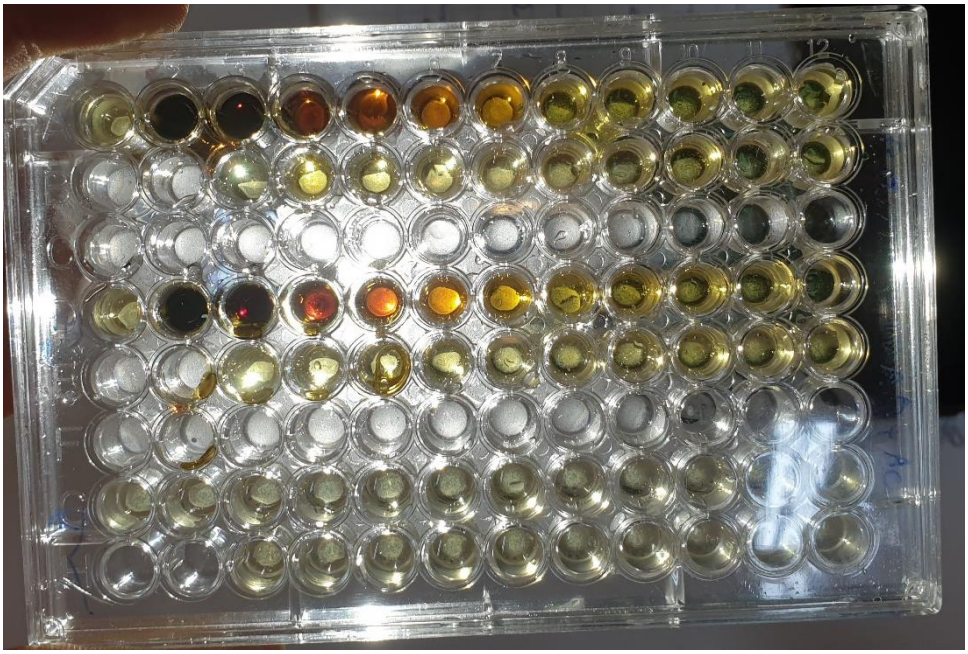


Figure 35: The MIC results of methanolic extracts for C. alibcans ATCC

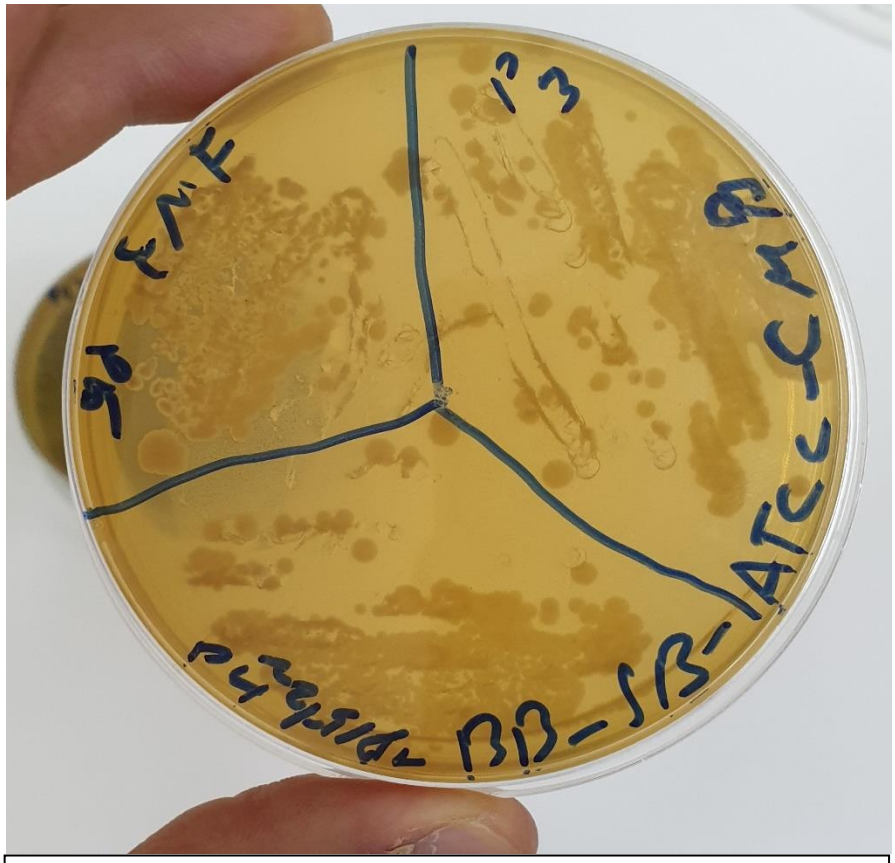


Fig36: The MBC results of the fresh methanolic extract for *C. albicans ATCC*

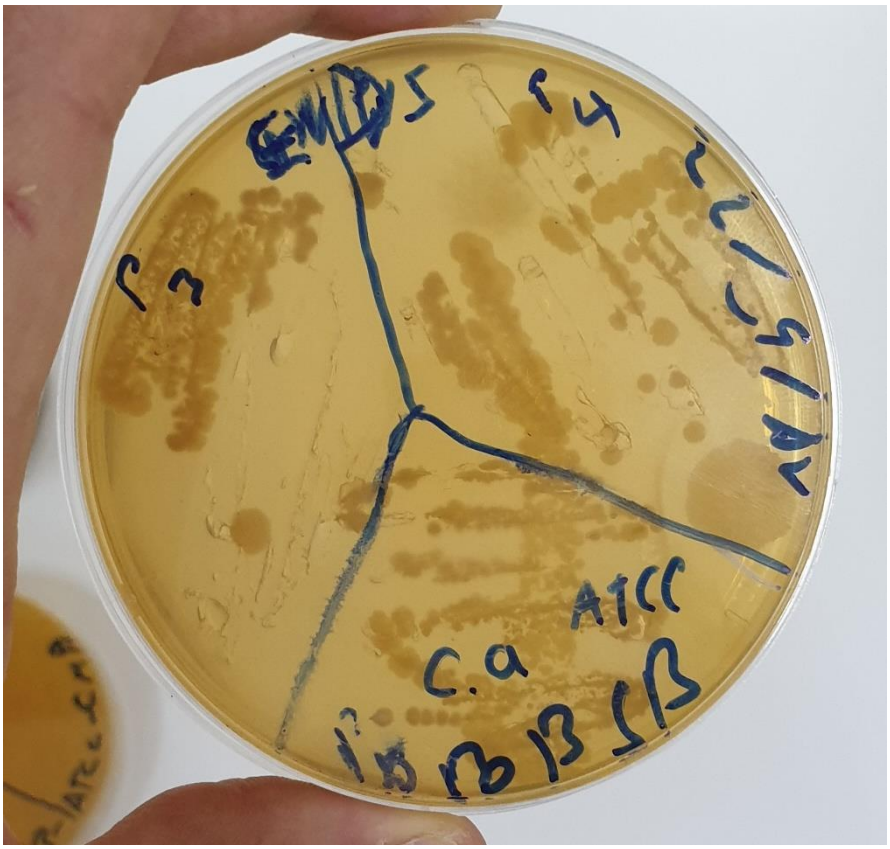


Fig37: The MBC results of the semi-dry methanolic extract for *C.albicans ATCC*

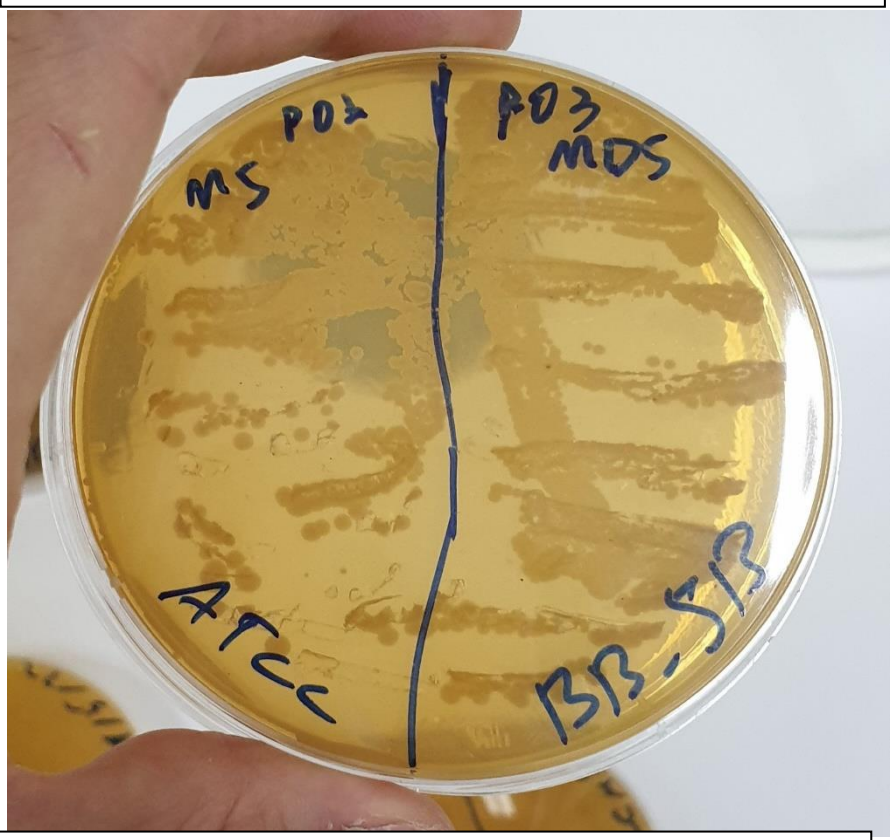


Fig38: The MBC results of the dry methanolic extract for *C.albicans ATCC*

- ✓ The 3.79mg/mL and 11.5mg/mL concentration of the methanolic extracts were enough to inhbit the growth of the *Candida albicans* ATCC (Table 16 & Fig35), but even 60.75mg/mL & 184mg/mL weren't sufficient to kill the yeast (Fig36 & Fig37).

Enterococcus faecalis

Table17: Results of *E. faecalis* with the metanolic extracts

Methanolic extract		
Extracts concentrations	MIC	MBC
[Fresh methanolic extract]= 422mg/mL	[W3]=105.5mg/mL	++++ >105.5mg/mL
[Semi-dry methanolic extract]= 592mg/mL	W3 [W4]=37mg/mL	+++ +++ >37mg/mL
[Dry methanolic extract]= 545mg/mL	[W3]=136.25mg/mL	+++ >136.25mg/mL

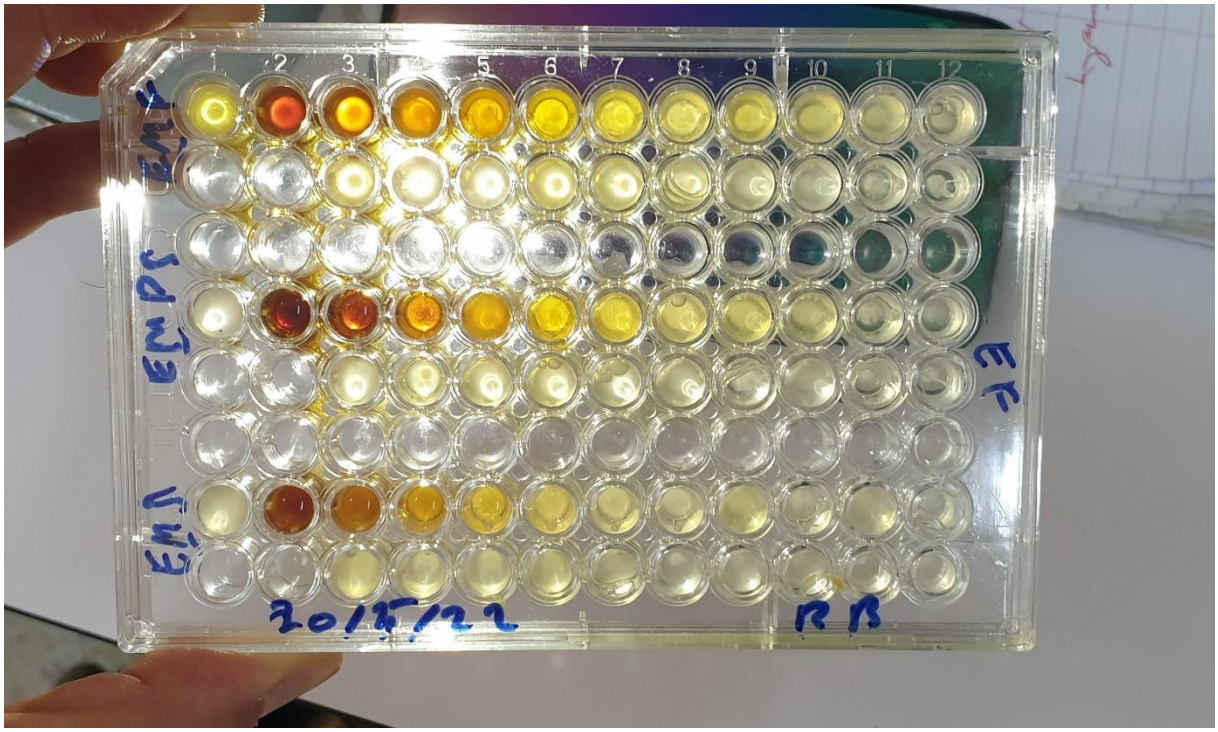


Fig39: The MIC results of methanolic extracts for *E. faecalis*

- ✓ A bacteriostatic activity for all types of methanolic extracts at a variant concentrations against *Enterococcus faecalis* (Fig39) but no bactericidal activity (Fig41), an increase in the concentrations is probably needed to get there.

Table18: Results of *E. faecalis* with acetate extract

Acetate extract		
Extract concentration	MIC	MBC
[Acetate semi-dry extract]=180mg/mL	[W3]=45mg/mL	+++ >45mg/mL

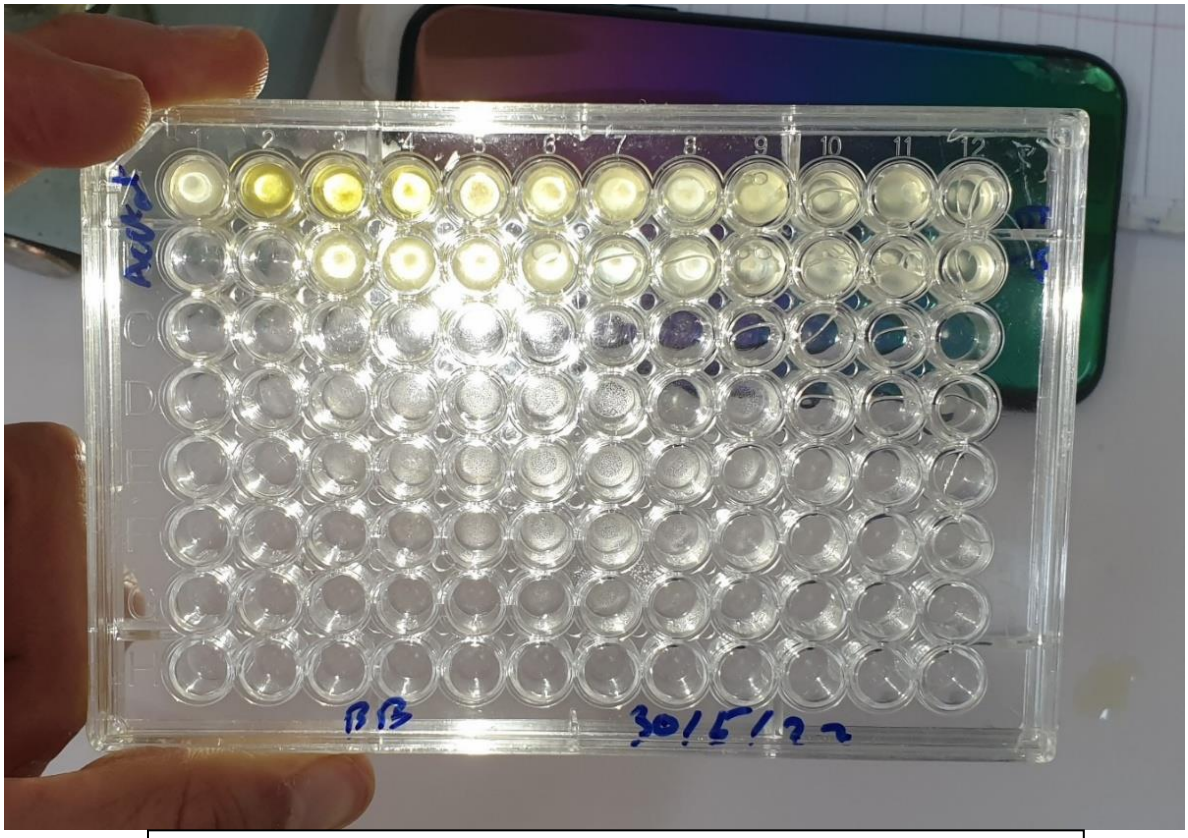


Fig40: The MIC results of semi-dry acetate extract for *E. faecalis*

- ✓ The groth of *E. faecalis* is inhibited at a minimal concentration of 45mg/mL of the acetate semi-dry extract (Fig40) without being able to kill the bacteria (Fig41).

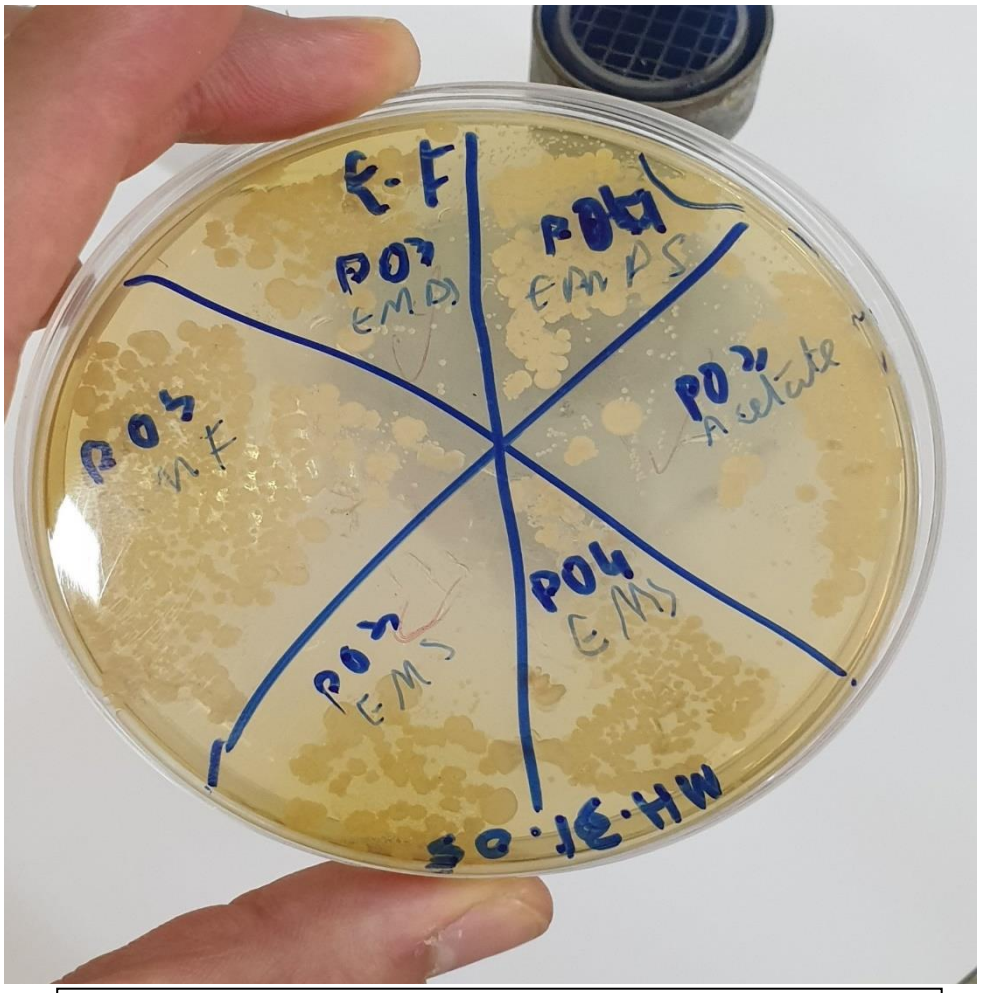


Fig41: The MBC results of the methanolic extracts and the acetate extract for *E. faecalis*

- ✓ Over-all in this present study, we were able to inhibit the growth of all strains of microorganisms at variance concentrations (5-136mg/mL) except the strain of *Pseudomonas aeruginosa* with the semi dry methanolic extract even at a high concentration of 184mg/mL.
- ✓ The only extract that was able to do a bactericidal activity was the semi dry acetate extract on the yeast strain *Candida albicans* at a concentration of 45mg/mL, which means that the saffron extracts need to go to a very high concentrations to get to that bactericidal activity, this theory can be confirmed by the study made on the moroccan *Crocus sativus* by **olfazl JAFARI SALES1** & **Mehrdad PASHAZADEH** in 2020 where they found that the minimal bactericidal concentration MBC was double the concentration of the minimal inhibitric concentration MIC.

Conclusion

There has been increasing interest in saffron in the past years for being an economical source as well as a medicinal plant for its healing properties which lie in its components such as crocin, picrocrocin and crocitin, they generally use only stigmas in its production and throw away a large amount of petals. The main purpose of our study was to evaluate the antimicrobial activity of petals.

We used the disc diffusion method to determine which extract had an inhibition effect on six different microorganisms. After we chose the extracts, we passed to MIC method to find the minimal concentration that is able to inhibit microorganisms growth and then the MBC method to find the lowest concentration that is able to kill the microorganisms entirely.

For the strain of *Pseudomonas aeruginosa* the aqueous and acetone extracts showed insignificant effect in contrast to the methanolic extract with satisfactory results, and also the butanolic extract has shown promising results. The methanolic extract showed a negligible effect on the yeast *Candida albicans ATCC* but strong effect on the strain of *Enterococcus faecalis* compare to the acetate that was less effective. None of the extracts used in this study had any effects on *Escherichia coli*. For *Candida albicans CIP* it had the same positive results against the 3 extracts butanol, acetate and dichloromethane. The inhibition of the fungi (*Candida albicans*) was with a minimal concentrations compared to the bacterial strains. The use of different solvent on extraction (butanolic, methanolic, aqueous...) leads to different results and also the sensitivity of different bacteria to different extracts is different.

Considering that tepals from the saffron (*Crocus sativus*) flower are considered as wastage in the past years, this study showed more potential on the bioactive compounds in petals as a food and cosmetics preservatives against pathogenic microorganisms or a source of an antimicrobial agent in the medical sector. However, a clinical-trials on patients is required to categorize saffron tepals as a medicinal plant. Further investigations are needed on these bioactive compounds (safranal, crocin and crocitin) and which specific phytochemical composition play a major role on the antimicrobial activity. Also, the region, submerged soil, harvest season, type of extraction and the solvent used may play a part on these tepals phytochemical content wish will directly impact the antioxidant and antimicrobial activity.

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Appendix

Annex01: Mueller Hinton: Mueller–Hinton agar is a microbiological growth medium that is commonly used for antibiotic and antimicrobial activity testing, specifically disk diffusion tests. It is also used to isolate and maintain Neisseria and Moraxella species.

- Mueller hinton agar compositions:

Acid hydrolyzate of casein (peptone)	17.5g
meat extract	2.0g
Starch	1.5g
Calcium	20 to 25mg
Magnesium	10 to 12.5mg
Agar	15.0g
pH = 7.4 +/- 0.2	
Distilled water	qsp 1 L

- ✓ We putted 21g of Mueller Hinto with 17g of agar in 1L of distilled water, PH= 7.3

Annex02: Sabouraud: Sabouraud agar or Sabouraud dextrose agar is a type of agar growth

Ingredients	**grams/litre**	**Ingredients**	**grams/litre**
Peptic digestion of animal tissues	5.0g	Pancreatic casein digestion	5.0g
Dextrose	40.0g/L	Agar	15.0g

Sabourad agar compostions

- ✓ We putted 30g of Sabouraud with 17g of agar in 1L of distilled water, pH=5.6

الملخص:

بشكل عام، يتم إستخدام مياسيم زهرة الزعفران (*Crocus sativus*) فقط في إنتاج الزعفران التجاري، ويتم إهدار البتلات أو التخلص منها.

كنا مهتمين بدراسة وتقييم النشاط المضاد للميكروبات للمستخلصات المختلفة من بتلات زهرة الزعفران (المستخلص المائي، الميثانولي، مستخلص الأسيتات، بيتانوليك، الأسوتينيك و ثنائي الكلورو ميثان) على الكائنات الحية الدقيقة من خلال طريقة نشر القرص، وتحديد MIC و CMB.

وفقًا لطريقة CMI ، تم تثبيط نمو جميع الكائنات الحية الدقيقة المسببة للأمراض ، ومع ذلك ، عند التركيز العالي للمستخلص الميثانولي شبه الجاف 184 مجم / مل، لم يلاحظ أي نشاط مضاد للجراثيم ضد *P. aeruginosa*. تم تسجيله بواسطة هذا طريقة.

كان مستخلص الأسيتات شبه الجاف الوحيد القادر على ممارسة نشاط مبيد للفطريات على *Candida albicans CIP 444*.

وفقًا لنتائجنا، نعتبر أن أزهار الزعفران هي مصدر طبيعي للمركبات المضادة للميكروبات مع إمكانات كبيرة و مبشرة حتى على الكائنات الحية الدقيقة شديدة العدوى والمقاومة مثل *Pseudomonas aeruginosa*.

الكلمات المفتاحية: *زعفران، Crocus sativus*، البتلات، الكائنات الحية الدقيقة، النشاط المضاد للميكروبات، مستخلص.

Resumé:

Généralement, seuls les stigmates du safran (*Crocus sativus*) sont utilisés dans la production de safran commercial, et les pétales sont gaspillés ou jetés.

Nous nous sommes intéressées à étudier et à évaluer l'activité antimicrobienne des petales de différents extraits de pétales de la fleur de safran (Extrait aqueux, méthanol, acétate, butanol, acétone et dichlorométhane) sur des microorganismes par la méthode de diffusion sur disque, la détermination de la CMI et de la CMB.

Selon la méthode de la CMI, la croissance de tous les microorganismes pathogènes a été inhibée, cependant, à une concentration élevée de l'extrait méthanolique demi-séche de l'ordre de 184mg/mL, aucune activité antibactérienne sur *P. aeruginosa* n'a été enregistrée par cette méthode.

L'extrait d'acétate demi-séche était le seul capable d'exercer une activité fongicide sur *Candida albicans CIP 444*.

Selon nos résultats, nous considérons que les fleurs du safran est une source naturelle en composés antimicrobiens avec un potentiel antimicrobien considérable même sur le microorganisme hautement pathogène et résistant *Pseudomonas aeruginosa.*

Mots clés : *Safran, Crocus sativus,* fleurs, extrait, microorganismes, activité antimicrobienne.

Abstract:

Generally, only the stigmas of saffron (*Crocus sativus*) are used in the production of commercial saffron, and the petals are wasted or discarded.

We were interested in studying and evaluating the antimicrobial activity of different extracts of saffron flowers petals (Aqueous, methanol, acetate, butanol, acetone, dichloromethane) on different microorganisms by the disc diffusion method, the determination of the MIC and the CMB.

According to the CMI method, the growth of all pathogenic microorganisms was inhibited, however, at a high concentration of the semi-dry methanolic extract 184mg/mL, no antibacterial activity against *P. aeruginosa* was observed by this method.

The semi-dry acetate extract was the only one capable of exerting fungicidal activity on *Candida albicans CIP 444.*

According to our results, we consider that saffron flowers are a natural source of antimicrobial compounds with considerable antimicrobial potential even on the highly pathogenic and resistant microorganism like *Pseudomonas aeruginosa.*

Key words: *Crocus sativus,* petals, microorganisms, antimicrobial activity, extract.