An-Najah National University
Faculty of Engineering and Information Technology

Chemical Engineering Department
Graduation Project 2

"Drying Rates of *Calendula* Herb and Antioxidant Test of Ascorbic Acid"

Prepared by:

Dalal Salah

Leena Khaled

Mahmoud Fashafsha

Malik Mahmoud

Shahd Odeh

Supervised By:

Dr. Husni Odeh

Dr. Majd Shhadi

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Abstract

Calendula officinalis L. (Pot marigold) is one of the commonly used medicinal plants in India, China, Europe and US. This plant can be planted easily and successfully in Palestine in winter. The plant species has been reported to contain a variety of phytochemicals, including carbohydrates, phenolic compounds, lipids, steroids, tocopherols, terpenoids, quinones and carotenoids.

The *Calendula officinalis* extracts possess a wide range of pharmacological effects and are used as antiseptic, stimulant, diaphoretic, anti-spasmodic and anti-pyretic agents. The flower extracts of the plant have anti-viral effects on HIV. In-*vitro*, *Calendula officinalis* plant extracts show anti-cancerous activity on various tumor cell lines derived from leukemia's, melanomas, breast, cervix, prostate, pancreas and lung. It has also been internally used for the treatment of gastritis, colitis and bleeding of duodenal ulcers.

The main objective is to examine the present of antioxidant agents in both of *Calendula* leaves and flowers, and then compare it with the antioxidant activity of Ascorbic acid. The ethanolic extracts are prepared from *Calendula* powders obtained from dried *Calendula* leaves and flowers. The chosen antioxidant test is 1,1-diphenyl-2-picrylhydrazyl (DPPH). DPPH experiments have been started only for the Ascorbic acid, but not completed due to COVID-19 disease outbreak.

The Graduation Project work is completed by carrying out theoretical analysis of experimental drying data conducted by Dr. Husni Odeh at room temperature and at 55-60 °C for *Calendula* flower. The drying rate equations and drying characteristic curves for *Calendula* herb flowers are derived and presented. The increasing drying condition from 20- 22 ºC to 55-60 ºC shows increasing the rate of drying about 150 times.

Contents

List of Tables

List of Figures

List of Figures from Appendices

Abbreviations

Calendula Officinalis. L.: *Calendula officinalis Linn.*

HIV: Human Immunodeficiency Virus.

GC-MS: Gas chromatography–mass spectrometry.

m/z: Mass-to-charge ratio.

ROS: Reactive oxygen species.

SO: Superoxide dismutase.

UV–Vis: Ultraviolet–Visible Spectrophotometry.

Cis and Trans: Are from Latin "this side of" and "the other side of", respectively.

w or wt %: Weight percentage.

OS: Organic solvent extraction.

h: Hour.

min: Minute.

g: Gram.

mg: Milligram.

mmol: Millimol.

µg: Microgram.

v/v: Volume ratio.

HECO: Hydroethanolic extract of *Calendula officinalis.*

et al.: For the Latin phrase et alia which means "and others."

A: Area.

H: Height.

DPPH: 1,1-diphenyl-2-picrylhydrazyl.

ABTS: 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid.

FRAP: Ferric Reducing Antioxidant Power.

EDTA: Ethylenediaminetetraacetic acid.

BHT: Butylated hydroxytoluene.

IC: Inhibitory concentration.

EC: Effective concentration.

A_0: Absorbance of the control reaction.

A_1: Absorbance in presence of all of the extract samples and reference.

nm: Nanometer.

R: Rate of drying.

X: Moisture content.

FD: Freeze drying.

HA: Hot air drying.

FIR-HA: Combined far-infrared radiation with hot air convection.

Chapter One: Introduction

1.1: Background

Calendula officinalis is also known as garden marigold. It is a member of the Asteraceae family. Other members of this plant family include daisies, arnica, chamomile, and yarrow. This bright, flowering herb opens its gold blossoms in the morning and closes them at dusk, or when rain threatens. *Calendula* is native to Asia and southern and central Europe. It is cultivated throughout the world and valued for its culinary and medicinal uses. The first name, *Calendula*, is from the Latin kalendae, the word Romans used to indicate that it bloomed throughout the year in their area. The second name *officinalis* indicates that *Calendula* was included in official lists of medicinal herbs. The common name marigold refers to the blossoms' association with the Virgin Mary (Health Benefits, 2020).

Calendula plant likes sun and will re-seed from year to year, even in poor soil. The erect, square and branching stems emerge from a taproot to grow up to 60 cm high. The bushy herb blooms continuously throughout the summer. Seeds are crescent to horseshoe shaped with a rough exterior (Health Benefits, 2020).

Calendula flower was grown as an ornamental plant for long times until its medicinal effects were discovered and thus, it began to be used as a medicinal plant. In Europe, the growth of this plant began in 12th century. Its flowers have been introduced as drugs in some pharmacopoeia, to cure stomach and intestine diseases. This plant has bitter taste and has been used as an efficient pesticide, and the plant is used among other plants to kill insects as well. On the other hand, the pot marigold plant grown by Egyptians, Arabs, Indians, Greeks and European has been used for medicinal purposes from 12th century (Kashani and Mohammad, 2012).

This plant is grown as a medical drug in Germany, Australia, Czech, Austria, Switzer land, Hungary and recently in Egypt and Syria as well. It is also grown in the Mediterranean countries such as the Balkan states, the east of Europe, north of America and also in Germany (Kashani and Mohammad, 2012).

Calendula officinalis is globally known for its medicinal importance containing various phytochemicals including carbohydrates, amino acids, lipids, fatty acids, carotenoids, terpenoids, flavonoids, quinones, coumarins and other constituents, showing some important biological activities like wound healing, immuno-stimulant, spasmogenic and spasmolytic, hepatoprotective, genotoxic and antigenotoxic, anti-amylase, anti-inflammatory, anti oedematous, anti-bacterial and anti-fungal, antioxidant, antidiabetic, anti-HIV and anti-cancerous, nephron-protective, prevention of oropharyngeal mucositis, hypoglycemic and gastroprotective activities with no toxic effect (Raina *et al*., 2014). Phytochemicals in this project will assay by the GC-MS.

Distribution in Palestine: Mediterranean Woodlands and Shrublands, Semi-steppe shrublands, Shrub-steppes, Deserts and extreme deserts, Mediterranean coast, Nablus mountains, Jerusalem and Dead Sea valley (Mahmiyat.ps, 2020).

1.1.1: Introduction to drying of herbs

Requirements of worldwide progress in cultivation of medicinal and aromatic herbs are necessary for their processing and safety storage. The need for high quality herb raw material is increasing. This phenomenon is proven by number of cultivation results and registration procedures, concerning medicinal and aromatic plant cultivars, recently reported from different countries. In pharmacy, plant raw materials are important sources of new medicines and their substitutes.

Natural medicines of plant origin have a wider therapeutic spectrum, milder action and less frequent side effects compared with synthetic substances. According to the data of the World Health Organization, about 70 000 plant species are currently used for medicinal purposes; about 1000 species are used in the European pharmaceutical industry (Bernáth, 2002).

Preservation of production is a very important problem to be solved by producers of these products. One of the ways of preservation of products is drying. Medicinal plants can be dried in a number of ways: in the open air (shaded from direct sunlight); placed in thin layers on drying frames, wire-screened rooms or buildings; by dielectric source as microwave; or infrared devices (Raila *et al*., 2009). When possible, temperature and humidity should be controlled to avoid damage to the active chemical constituents. The method and temperature

used for drying may have a considerable impact on the quality of the resulting medicinal plant materials.

Chemical changes are the most important in the post-harvest of medicinal herbs that can be influenced by drying. Moreover, drying can promote changes in the product appearance (color) and smell, modifying the final quality (Aboltins and Kic, 2016).

The drying process is characterized by the existence of transport mechanisms such as surface diffusion, pure diffusion, capillary flow, evaporation, thermo-diffusion, etc. Many studies were done to process medical plant drying by small heated air (Aboltins and Kic, 2016).

1.1.2: Free Radicals and Antioxidants

Free radicals are atoms, molecules or ions with at least one unpaired electron in the outermost shell. Free radicals are highly reactive due to the presence of unpaired electron(s). Free radicals and oxidants play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body. They are produced either from normal cell metabolisms in situ for example, blood cells destroy parasites, bacteria and viruses by using oxidants such as nitric oxide, superoxide and hydrogen peroxide, another example is cellular components called peroxisomes produce hydrogen peroxide as a byproduct of the degradation of fatty acids and other molecules. Or from external sources (pollution, cigarette smoke, radiation, medication). Free radicals that can be formed within the body include: the superoxide anion (O_2^-), the hydroxyl radical ($OH^\cdot$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) (Lien Ai Pham-Huy, 2008).

Oxidative stress is defined as the state in which the level of toxic reactive free radicals overcome the endogenous antioxidant defenses of the host. This state results in an excess of free radicals, which can react with cellular lipids, proteins, and nucleic acids, leading to local injury and eventual organ dysfunction. Lipids are probably the most susceptible biomolecule to free radical attack. So, free radicals have been implicated as playing a role in the etiology of cardiovascular disease, cancer, Alzheimer's disease and Parkinson's disease. (This is why free radical damage is also called “oxidative damage.”) (Liou, 2011).

The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ known as endogenous antioxidants, including superoxide dismutase (SOD), catalase, many vitamins (vitamin C, vitamin E, beta-carotene), minerals and glutathione peroxidase that neutralize many types of free radicals. Or externally called exogenous antioxidant supplied through foods and/or supplements Antioxidants that come from outside the body like vitamins and herbs. Herbs, such as bilberry, turmeric (curcumin), grape seed or pine bark extracts, ginkgo and calendula can provide powerful antioxidant protection for the body (Biofizyka.p.lodz.pl, 2014).

In this project, antioxidant capacity of calendula extract will evaluate by DPPH method. The results will compare with data found in previous study. Radicals percent will be scavenging will measure by ultraviolet–visible spectrophotometry (UV–Vis) device.

1.1.3: Ultraviolet–Visible Spectrophotometry (UV–Vis)

UV-Vis Spectroscopy (or Spectrophotometry) is a quantitative technique used to measure how much a chemical substance absorbs light. This is done by measuring the intensity of light that passes through a sample with respect to the intensity of light through a reference sample or blank. This technique can be used for multiple sample types including liquids, solids, thin-films and glass. Spectrophotometry is a quantitative measurement of the absorption/transmission or reflection of a material as a function of wavelength (Instruments Ltd., 2012).

Using a spectrophotometer and carrying out absorption/transmission measurements we can determine the amount (or concentration) of a known chemical substance simply, by studying the number of photons (light intensity) that reach the detector. The more a material absorbs light at a specific wavelength, the higher the concentration of the known substance (Instruments Ltd., 2012).

1.2: Problem Statement

The medical aspect is one of the areas that fall within the field of chemical engineer work, and since the manufactured chemicals showed side effects and high cost during manufacture. Resorting to medicinal herbs seemed to be an appropriate solution in treating many diseases at a lower cost and without possible toxic effects. *Calendula* herb was used to extract its compounds in order to work on dermatology formulation.

Phytochemicals in *Calendula* including carbohydrates, amino acids, carotenoids, terpenoids, flavonoids, and other constituents, show some important biological activities like wound healing, anti-inflammatory, anti-bacterial, anti-fungal, and antioxidant.

Different chemical tests are needed to ensure the safety of the bioactive compounds of *Calendula* healing effects. Therefore, the antioxidant tests are necessary tools to make the road map of solving the uncertainty of *Calendula* healing effects.

Moreover, all of analytical tests are carried on dried herb powders. Therefore, it is necessary to examine the behavior of the herb through drying by deriving the plant drying characteristic curves at different temperatures.

1.3: Objectives

The main objective of this project is to evaluate the antioxidant activity of these extracts by DPPH method. Which could be used in dermatological treatments, since the antioxidants may protect the body cells against the effects of free radicals. The results will be compared with data found in the previous studies.

The preparation of medicinal product of natural ingredients for the treatment of skin diseases is another objective of this project. This enhance the world's orientation towards the exploitation of natural antioxidants in plants and less reliance on manufactured antioxidants; that meets the human and environmental interest.

It is known that plant must be dried and grinded before any test. So, the drying rates of herb flowers at different temperatures is presented and characterized.

1.4: Scope of the Work

The scope of this project is to evaluate the antioxidant capacity of *Calendula* extract by DPPH method. The experimental work is stopped or in another word the antioxidant experiments are halted, and only the ascorbic acid was tested for its antioxidant activity, due to the COVID-19 disease outbreak. And instead theoretical study of drying data of *Calendula* flowers is completed and presented.

1.5: Significance

Herbs/medicinal plant/homemade remedies are less expensive than the synthetic drugs and majority peoples in rural/backward area have blind faith on them. They are right because they can treat any disease by using them without any lethal side effects. Homemade remedies are not only useful for the treatment of different diseases but are also widely used for enhancing beauty and for curing skin related issues. On the other hand, synthetic drugs synthesized by employing different methodologies are expensive with some side effects. Although herbal medicines are less potent in comparison to synthetic drugs in some cases but still these are considered less toxic or having less side effect in contrast to synthetic drugs. The ultimate norm for any medicine (human made or natural) is their nontoxicity, effectiveness, specificity, stability and potency. Now many chemists switching theirs field from synthetic to natural side in order to explore nature more and more (Anbudhasan *et al*., 2014).

The consumer concern regarding the safety of using synthetic antioxidants in convenient food products has forced and motivated the food processors to seek for natural alternatives. This leads to a situation where the application of synthetic antioxidants started to decrease drastically in food products. Hence there has been an increasing global trend towards the use of natural antioxidants present in fruits and green leafy vegetables. The effects of these

natural antioxidants in scavenging the free radicals are well discussed and reported in the earlier studies. The factors that encourage the use of natural antioxidants are its low cost, compatibility with diet and less harmful effect in the human body. The strong H-donating capacities of various phytochemicals make them as effective natural antioxidants. Phenols present in plant extracts acts as a potential antioxidant by inhibiting the free radical formation and prevent auto oxidation. Phenolic acids, flavonoids and volatile oils possess higher antioxidant activity and also acts as the essential part of diet and these claims were supported by various scientific evidence. The health promoting capacity of these natural antioxidants help in eradicating chronic diseases such as cancer. Hence, in this review the action of antioxidants of calendula extract on free radicals was discussed (Nisar, Sultan and Rubab, 2018).

In spite of importance of conduction of antioxidant tests. The knowledge of the rate of drying of *Calendula* flowers is still attract the attention of researchers. The rate of drying equations are derived at different safe temperature. This means that you can get dry powder safely through three hours instead of one week at ambient temperature.

1.6: Organization of the Report

This report consists of seven main chapters; the first introduces the *Calendula* plant with general information about drying herbs and antioxidants. The second chapter presents some of the difficulties and constraints, the third chapter shows the previous researches and studies published literature on this subject. The next chapter includes the methodology of our work, summarizes what has been done. Chapter five shows the results and analysis. Finally, chapters six and seven show the conclusion, and future work & recommendation respectively.

Chapter Two: Difficulties and Challenges

It was impossible to continue the prescribed stages of our experimental program due outbreak of pandemic COVID-19 virus. The research direction is changed towards theoretical calculation of experimental drying data of *Calendula* flowers.

The rate of drying section was added and prepared during the ban period, as it was not among the project objectives, and the experimental data are provided by Dr. Husni.

Chapter Three: Literature Review

3.1: C*alendula* general uses

Calendula leaves and petals are edible, it can be used for culinary purposes, the leaves are added to salads dishes and soups, the petals of this plant are used for decoration, the Greeks and Romans used it as a culinary garnish (Brush Creek Wool Works, 2019).

Colored substances extracted from the *Calendula* flowers are used in coloring food products; it can be used as a saffron substitute for coloring and flavoring rice. *Calendula* also used in dying fabrics, cosmetics. In cosmetics, the flowers of this plant are used for washing hair, soften the skin, and to cure skin spots, Ancient Egyptians used it to rejuvenate their skin (Brush Creek Wool Works, 2019).

An essential oil from plant used in perfume-making, also *Calendula* has many medicinal uses, calendula has been revered as a magical medicinal for centuries, The flower is widely used as a medicinal plant whose anti-inflammatory, antifungal, antibacterial, antioxidant, antimicrobial activities, anti HIV activities, anticancer activities properties make it a strong ingredient for healing (All Good Products, 2019).

The food and drug administration (FDA) has approved *Calendula* for use as a spice and as an ingredient in cosmetics, soaps and shampoos, body creams, and wound treatment (All Good Products, 2019).

One medieval author named Macer described Marigold in his volume on herbs he thought that merely to look upon the blooms would improve eyesight and draw evil “humors” from the head (Indigo Herbs, 2019).

Figures 1 and 2 show some kinds of *Calendula* plants.

Figure 1: *Calendula* plant.

Figure 2: Different colors of *Calendula* flowers.

3.2: Therapeutic Potential of *Calendula officinalis*

The use of plants to treat disease is as old as the human. *Calendula officinalis* has many therapeutic potential and medicinal uses, such as:

3.2.1: Antidiabetic and anti hyperlipidemic activities

Hydro alcoholic extract of *Calendula officinalis* has both antidiabetic and antihyperlipidemic effects. Because the extract increases the total hemoglobin level and the extract is similar to that of insulin (Ashwlayan *et al*., 2018).

3.2.2: Cardiovascular activities

Calendula officinalis could be cardioprotective. *Calendula* achieved cardio protection; by encouraging left ventricular developed pressure and aortic flow, also by reducing myocardial infarct size and cardiomyocytes apoptosis (Ashwlayan *et al*., 2018).

3.2.3: Hepatoprotective activities

Calendula flower hot water showed anti hepatoma activity (25-26% inhibition) against five human liver cancer cells (John and Jan, 2017).

3.2.4: Antioxidant activities

Most of phytochemicals presence in *Calendula officinalis* have free radical scavenging activity (John and Jan, 2017). The leaves and petals of *Calendula officinalis* are a potential source of natural antioxidants (Ashwlayan *et al*., 2018).

3.2.5: Anthelmintic activities

Anthelmintic activity presence in flowers and leaves of *Calendula officinalis* in dried phase. The aqueous extract of dried flowers and leaves of *Calendula officinalis* were prepared by decoction method. Anthelmintic activity due to it contain saponins (Ashwlayan *et al*., 2018).

3.2.6: Anti inflammatory activities

Calendula is highly effective for the prevention of acute dermatitis of grade 2 or higher and should be proposed for patients undergoing postoperative irradiation for breast cancer (Ashwlayan *et al*., 2018).

3.2.7: Wound healing activities

The extract of *Calendula* flower has important healing activity by increasing hexosamine and collagen hydroxyproline content. Wound healing activities due to the antioxidant and antimicrobial activities of *Calendula* (Ashwlayan *et al*., 2018).

3.2.8: Anticancer activities

Calendula extracts has a direct mitogenic effect on human lymphocytes or thymocytes and inhibitory effect on the proliferation of lymphocytes in the presence of polyhydroxyalkanoates (Ashwlayan *et al.*, 2018).

3.2.10: Antibacterial activities

The growth of the bacteria inhibition by ethanolic and aqueous extracts of *Calendula officinalis*, at concentrations ranging from 125 µg/ml to 64 mg/ml. Methanolic extract inhibited the growth of both *Staphylococcus aureus* and *Escaheriachia coli* at 64 mg/ml (Ashwlayan *et al*., 2018).

3.2.11: Sedative drugs

Ingested of *Calendula* considered sedatives (Ashwlayan *et al*., 2018).

3.2.12: Antihypertensive drugs

High doses of *Calendula* possess hypertensive effects (Ashwlayan *et al*., 2018).

3.2.13: Hypoglycemic drugs

The activity of hypoglycemic medications or insulin increased by *Calendula* (Ashwlayan *et al.*, 2018).

3.2.14: Cholesterol lowering drugs

Calendula decrease lipids and triglyceride (Ashwlayan *et al.*, 2018).

3.3: Phytochemistry of *Calendula officinalis*

A number of phytochemical studies have well reported about the presence of several classes of chemical compounds, the main ones being terpenoids, flavonoids, coumarin, quinines, volatile oil, carotenoids and amino acids in the plant.

3.3.1: Terpenoids

They include sitosterols, stigmasterols, diesters of diols, oleanolic acid saponins, and other constituents (John and Jan, 2017).

3.3.2: Flavonoids

They include quercetin, isorhamnetin, and isoquercetin (Ashwlayan *et al*., 2018).

3.3.3: Coumarins

Contain coumarins-scopoletin, umbelliferone and esculetin (Ashwlayan *et al*., 2018).

3.3.4: Quinones

Some quinones were reported in the chloroplast, mitochondria, and in the leaves (Ashwlayan *et al.*, 2018).

3.3.5: Volatile oil

Calendula officinalis flowers contain maximum volatile oil at full flowering stage (0.97 wt %) and minimum during the pre-flowering stage (0.13 wt. %). Various monoterpenes and sesquiterpenes have been reported in the volatile oil (John and Jan, 2017).

3.3.6: Carotenoids

Total carotenoid (mg/g dry weight) was 7.71% for petals and 1.61% for pollens, and the total carotenoids (mg/g dry weight) for the leaves is 0.85% and for stems 0.18% (Ashwlayan *et al.*, 2018).

3.3.7: Amino acids

Amino acid content of the leaves is about 5%, stems 3.5% and flowers 4.5%, some reported amino acids: aspartic acid, glutamic acid, serine, and phenylalanine (Ashwlayan *et al*., 2018).

3.3.8: Carbohydrates

Contain polysaccharides (Ashwlayan *et al*., 2018).

3.3.9: Lipids

The amount of neutra lipids in the seeds was15.7%, phospholipids 0.6% and glycolipids 0.9%. Some fatty acids reported in flowers were: lauric, stearic, oleic, and linolenic acid (Ashwlayan *et al*., 2018).

3.3.10: Other constituents

Other phytochemicals include the bitter constituent, loliolide (calendin), calendulin and paraffins (Ashwlayan *et al*., 2018).

3.4: Extraction

The importance of choosing the extraction method comes from that it influences the yield of needed compounds in the plant and the quality of extract.

- Extraction technique: Organic solvent extraction.

The organic solvent extraction (OS) when a solid material is in contact with a solvent, the soluble components in the solid material are transferred to the solvent. When applying this extraction method, the active soluble mass is non-selectively transferred to the solvent due to a concentration gradient (Salomé-Abarca *et al*., 2015).

Different methods were done for the extraction. Sana Fatima *et al*., 2018, have separated the petals and leaves from the plant, dried it at room temperature, and grinded it using grinding machine. Ethanol solvent was used for the extraction. It was then carried out using Soxhlet apparatus. 15 grams of grinded dried (petals/leaves) were added to 300mL of (ethanol) then conducted for 20 cycles at 70 °C. The extract was then concentrated in rotary evaporator to recover the solvent and then the extract was kept at refrigerator for further drying (Sana Fatima *et al*., 2018).

Other method: Ten grams of dried grinded (flowers/leaves) have been transferred into a flask containing 150 mL of the solvents (ethanol). The materials were stirred at 350 rpm, 35°C for 24 h using an orbital shaker. The sample was filtered and then evaporated under vacuum using the rotary evaporator. Then finally the extract was transferred into a small vial and stored at room temperature (Efstratiou *et al*., 2012).

3.5: Antioxidant Capacity of *Calendula*

Antioxidants are compounds produced in the body and found in foods. They help defend cells from damage caused by potentially harmful molecules known as free radicals. When free radicals accumulate, they may cause a state known as oxidative stress. This may damage your DNA and other important structures in your cells (Raman, 2018).

Sadly, chronic oxidative stress can increase your risk of chronic diseases such as heart disease, type 2 diabetes and cancer (Raman, 2018).

Fortunately, eating a diet rich in antioxidants can help increase your blood antioxidant levels to fight oxidative stress and reduce the risk of these diseases. *Calendula* plant is one of these foods (Raman, 2018).

This section will present several studies about antioxidant capacity of calendula. The topics will be covered are

1. Some methods to evaluating the antioxidant.
2. Antioxidant evaluation of *Calendula* extract by DPPH and other methods.
3. Comparison between antioxidant activity of *Calendula* extract and another antioxidant.
4. The relationship between antioxidant activity and phenolics compounds.
5. Conclusion.
6. DPPH method.

3.5.1: Some methods for evaluating the antioxidant.

ABTS Assay

The 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay that uses $ABTS^{\cdot+}$ radicals performed by oxidation of ABTS with potassium persulphate. Thus, this assay

becomes time consuming in terms of waiting for the ABTS radicals to be generated as it takes around 12-16 hours for the reaction of ABTS with potassium persulphate, unlike the DPPH assay where one does not have to wait for it to be generated. However, once the radicals are generated, it is a very simple assay in terms of performing the assay. The ABTS radical is soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds/samples. One major drawback of this assay is that the radicals formed are not very stable and the results are not reproducible (Shah and Modi, 2015).

FRAP Assay

Ferric Reducing Antioxidant Power (**FRAP)** method is based on the reduction of colorless ferric complex (Fe^{3+} tripyridyltriazine) to blue-colored ferrous complex (Fe^{2+} tripyridyltriazine) by the action of electron donating antioxidants at low pH. The FRAP assay is more tedious and time consuming in terms of preparing the chemicals of the working solution. It is a simple and inexpensive method and does not require the use of any exclusive chemicals (Shah and Modi, 2015). According to Shah and Modi (2015), the results obtained in FRAP are found to be reproducible for all the concentrations. Hence, FRAP is a suitable method for the determination of antioxidant activity. In another study done by Thaipong *et al.* (2006) for estimating antioxidant activity from guava fruit extracts, they concluded that the FRAP assay showed high reproducibility, was simple and could be rapidly performed. FRAP method, however, has its own limitations, especially for measurements below non-physiological pH values i.e. at pH 3.6. Physiologic-ph approximately 7.4 (Moniruzzaman *et al.*, 2012). Physiologic-pH of *Calendula* ranges between 4.5-8.3, depends on the pH of the soil (Plants for a Future, 2007).

DPPH Assay

DPPH is one of the few stable and commercially available organic nitrogen radicals. It is one of the most widely reported methods for the determination of antioxidant activity (Alam *et al.*, 2013). DPPH is not a very tedious assay in terms of preparation of chemicals and also in

terms of performing the assay and hence can be used for its operational simplicity. DPPH showed high reproducibility according to Shah and Modi (2015). In one study done by Katalinic *et al.* (2006), they suggested that from the methodological point of view, the DPPH method is easy and accurate with regard to measuring the antioxidant activity of the extracts and also the results are highly reproducible. According to Shah and Modi (2015), DPPH was found to be the most suitable method for the determination of antioxidant activity of mycelia of the three mushroom species because it could be rapidly performed and showed high reproducibility. Similar comment was also obtained by Kedare and Singh (2011), DPPH assay is considered rapid, simple and inexpensive. The advantage of this method is that DPPH may be utilized in aqueous, polar, and nonpolar organic solvents and can be used to examine both hydrophilic and lipophilic antioxidants compounds for their DPPH scavenging capacities under the same experimental conditions without use of solubilizing agents such as the b-cyclodextrin that are necessary in other assays (Kedare and Singh, 2011). In the DPPH method, the radicals did not have to be generated before the assay which turned out to be the biggest disadvantage of the ABTS method (Moniruzzaman *et al*., 2012).

Antioxidants react with free radicals by different mechanisms, hydrogen atom transfer (HAT) or single electron transfer mechanism (SET); or the combination of both HAT and SET mechanisms. The HAT reaction is a concerted movement of a proton and an electron in a single kinetic step, as shown in Figure 3. In HAT mechanisms, the free radical removes one hydrogen atom of antioxidant, and the antioxidant itself becomes a radical. In this mechanism, the bond dissociation enthalpy (BDE) is an important parameter in evaluating the antioxidant action (Mader *et al*., 2007). The lower the BDE of the H-donating group in the potential antioxidant, the easier it will be for the reaction of free radical inactivation. In SET mechanisms, the antioxidant provides an electron to the free radical and itself then becomes a radical cation. In this mechanism, the ionization potential (IP) of the antioxidant is the most important energetic factor in evaluating the antioxidant action. The lower the ionization potential, the easier is the electron abstraction. It is very difficult to distinguish between HAT and SET reactions. In most situations, these two reactions take place simultaneously, and the mechanism of the reaction is determined by the antioxidant's structure and solubility, the partition coefficient and solvent polarity. In the DPPH assay, an odd electron displays a strong absorption band at a wavelength of 519 nm, which loses

absorption once the odd electron is paired off by a hydrogen or electron-donating antioxidant (Figure 3). DPPH radical react with antioxidants by SET or HAT mechanism. Examples of HAT-based assays include ABTS. Examples of SET-based assays include DPPH and FRAP (Liang and Kitts, 2014).

The DPPH radical is a dark-colored crystalline powder. The reaction between a sample and a DPPH radical is performed in a solvent such as ethanol/methanol, which facilitates the extraction of antioxidant compounds from the sample. The DPPH radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized (Kedare and Singh, 2011).

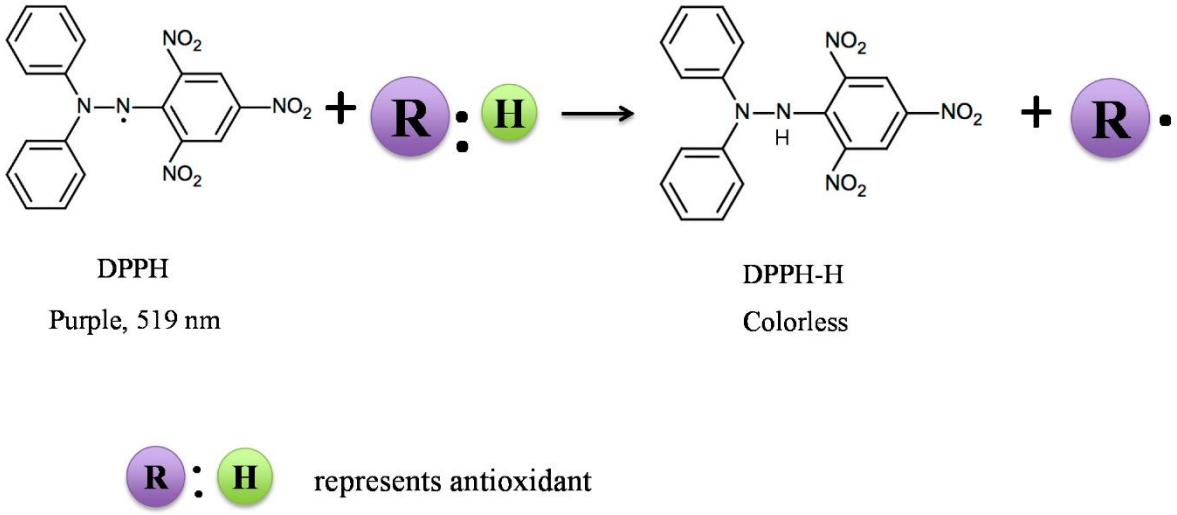

Figure 3: Reaction mechanism of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant. R: H = antioxidant radical scavenger; R = antioxidant radical.

Phosphomolybdenum method

The total antioxidant assay based on reduction of Phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V) by the sample analyte and subsequent formation of a green phosphate Mo (V) complex at acidic pH. Sample solution is combined with reagent (sulfuric acid, sodium phosphate and ammonium molybdate) (Untea *et al*., 2018).

3.5.2: Antioxidant evaluation of *Calendula* extract by DPPH and other methods

There are many methods to measure the antioxidant capacity of *Calendula*. This part will show some research that used different methods to evaluate Antioxidant of *Calendula* extract and the results they have achieved. Researchers have conducted various studies on antioxidant capacity of *Calendula* by several method. One of these studies is for Untea *et al*. (2018), the aim of their research were to calculate the antioxidant activity of *Calendula* extract by DPPH, ABTS and phosphomolybdenum method. The results showed that, Calendula leaves proved to have an important antioxidant activity determined by two different methods, DPPH and phosphomolybdenum. Antioxidant activity of marigold by DPPH was 225.14 mmol eq trolox/kg. By phosphomolybdenum method was 135.74 mmol eq ascorbic acid/kg. On the other hand, by ABTS method the antioxidant activity was lower than two methods 65.69 mmol eq trolox/kg.

One study has shown that *Calendula* extract has a biological activity. According to Preethi *et al*. (2006), *Calendula officinalis* extract effectively scavenged superoxide, hydroxyl, and nitric oxide radicals in vitro. These radicals are generated inside the body during the normal metabolism. The concentration of the extract needed for 50% scavenging of superoxide generated by photoreduction of riboflavin (IC_{50}) was 500 µg of extract/mL of superoxide whereas for the inhibition of hydroxyl radicals generated by Fe^{+3} /ascorbate/EDTA/H_2O_2 system it was 480 µg/mL. The IC_{50} for nitric oxide scavenging was 575 µg/mL. Preethi *et. al* (2006), also conducted a study in *vivo*. They found that *Calendula officinalis* scavenged the superoxide generated in vivo in the mice. Moreover, administration of *Calendula officinalis* significantly increased the *catalase* and glutathione levels in blood and liver. Glutathione reductase was increased in liver of treated groups. These results show that *Calendula officinalis* has a profound effect on the antioxidant defense system both in *vitro* and in *vivo* (Preethi *et al*, 2006).

3.5.3: Comparison between antioxidant activity of *Calendula* extract and another antioxidant

When an overload of free radicals caused by internal and external condition cannot gradually be destroyed by antioxidant produced in the body, external antioxidant must be supplied either by natural antioxidant or by synthetic. Comparison between external synthetic and natural antioxidant activity is so important. Marinescu *et al.* (2018) compared the antioxidant activity of ethanol extracts of *Calendula* with antioxidant activity of synthetic butylated hydroxytoluene (BHT). The results showed that Antioxidant activity of ethanol extracts tenfold higher than that of a 0.05 mg/ml BHT solution (Marinescu *et al*., 2018).

Deuschle *et al.* (2015), conducted another study, this study compared between antioxidant activity of Ascorbic acid (vitamin C) and rutin with hydroethanolic extract (HECO) from the leaves of *Calendula officinalis L*. The results show that the HECO at lowest concentration assayed (7.81 µg/mL) gave a DPPH inhibition of 79.84 %, exhibited an excellent in *vitro* antioxidant capacity. With the same concentration, ascorbic acid showed an inhibition of 14.93%, and rutin showed an inhibition of 62.44 %. The percentage of antioxidant capacity for this method is proportional to the amount consumed by DPPH, and the greater this percentage, the greater the ability of the sample in scavenging free radicals. The inhibitory concentration (IC_{50}) also was determined in this study, the inhibitory concentration (IC_{50}) is the concentration that will inhibit 50% of free radicals. The IC_{50} values were 16.57 µg/mL for ascorbic acid, 6.25 µg/mL for rutin, and 5.86 µg/mL for HECO. From these results can be concluded that antioxidant of *Calendula* extract is very effective and useful (Deuschle *et al*., 2015).

3.5.4: The relationship between antioxidant activity and phenolic compounds

Phenolic compounds are considered the most important antioxidants of plant materials due to their chemical structures, and that they play an important role in the capture and neutralization of free radicals (Kumar Verma *et al*., 2014). Kumar Verma *et al*. (2014) conducted a study on ethanolic extract and aqueous extract of *Calendula*, they found that

ethanolic extract have significant high total antioxidant activity as compared to aqueous extract of *Calendula officinalis*. This is because ethanolic floral extract has significantly high total phenolics, flavonoids tannin, β-carotene lycopene and chlorophylls contents as compared to aqueous extract of *Calendula officinalis* so, there is a positive correlation between phenolic content and total antioxidant activity of extracts (Raina, Kumar and Sultana, 2014). Similarly, *in vitro* ABTS radical scavenging potential of floral extract of *Calendula officinalis* and their relationship with total polyphenolic contents have been also reported (Habila *et al*., 2010). On the other hand, some authors showed no correlation between total phenolic content and antioxidant activity (Sulaiman *et al*., 2011). The low correlations might be explained that total antioxidant activity is not due to only one contributor, the presence of non-phenolic antioxidants (vitamin C, vitamin E and carotenoids) having accountable antioxidant activity (Kumar Verma *et al*., 2014).

3.6: *Calendula* side effects and special precautions

Natural products are not always safe, and it is important to know if they have any side effects before medical use. It has been shown from the long-term use throughout the ages and transcontinental of *Calendula* as a medicinal plant, that it has no negative effect mentioned, and it is likely safe when taken by mouth or applied to the skin for most people (WebMD, 2015).

However, pregnant women advised to avoid taking *Calendula* by mouth, or topical use, because there are some concerns of miscarriage, and for more precautions; breast-feeding women are advised to avoid it as well, since there is not enough information yet indicating the safety of using *Calendula*. Also, people who are sensitive to the Asteraceae/Compositae family are advised to follow the doctor's instructions before taking Calendula, because it may cause an allergic reaction to them. The use of *Calendula* should be discontinued if it is taken in conjunction with the medications taken during and after surgery because in this case, it causes much drowsiness (WebMD, 2015).

3.7: Drying of herbs

The researchers investigated the influence of some process parameters (temperature, sample thickness, layer thickness, air flow rate, etc.). The effect of the used airflow and drying air temperature on the drying kinetics was studied in [Raila *et al*., 2009; Čipliene, 2015].

Drying is the most common and fundamental method for post – harvest preservation of medicinal plants. Natural drying can be considered only for drying of small quantities. In case of mass production, the use of technical drying applications is indispensable. For preservation of active ingredients of plant material low drying temperature is recommended (less than 60°C). It means long drying duration. Drying represents 30-50 % of total costs in medicinal plant productions. Energy demand of drying represents is a significant cost factor. It is largely due to the high moisture content of the leaves and flowers, to be dried. Different parts of the plant and their drying aspects were considered in (Müller and Heindl, 2006).

For indoor drying, the duration of drying, drying temperature, humidity and other conditions should be determined on the basis of the plant part concerned (root, leaf, stem, bark, flower, etc.) and volatile natural constituents, such as essential oils.

Figure 4 below, shows calculation was made per 500 g of material in order to compare the drying dynamics of different types and weights of herbs (Aboltins and Kic, 2016).

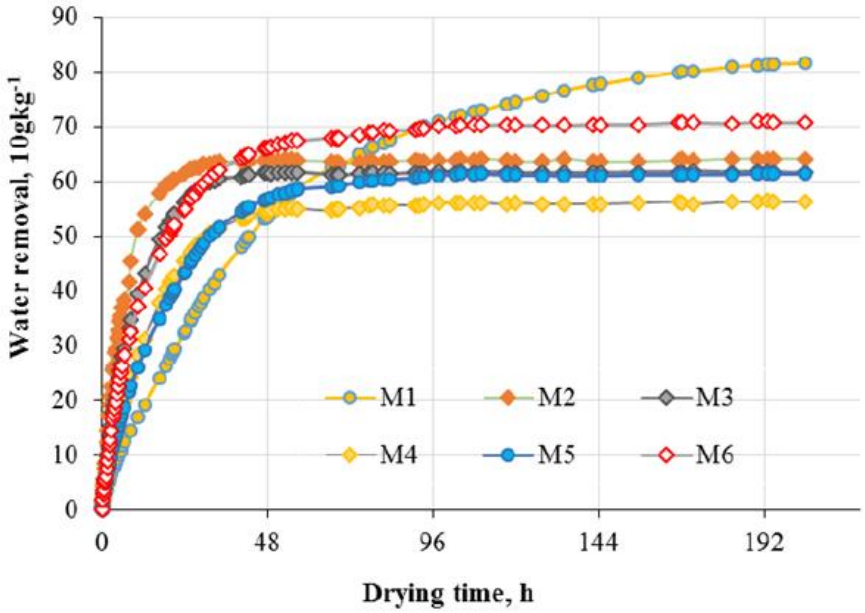

Figure 4: Different plant drying dynamics: M1- marigold, M2- lemon balm, M3- origanum, M4- common agrimony, M5- common lavender, and M6- common sage (Aboltins and Kic, 2016).

Looking at the results it is seen that drying dynamics of all samples are similar except for garden marigold, where longer sample drying is observed. Other examples dry for five days, but *Calendula* flowers dries up to 6-7 days at the same conditions. The difference between garden marigold and other plant drying can be explained by the fact that flowers of garden marigold are thicker and inside moisture diffusion affects the drying process more.

3.7.1: Effect of different drying methods on antioxidant activities and color of *Calendula* herb.

Drying is one of the most important processes for producing marigold powder. Therefore the effects of different drying processes, namely freeze drying (FD), hot air drying (HA) and combined far-infrared radiation with hot air convection (FIR-HA), on the color and antioxidant activities of marigold flowers were evaluated by (Siriamornpun *et al*., 2012).

The results presented in this study suggest that the changes in the color of FIR-HA dried were smaller as compared to FD and HA dried. As the color after FIR-HA drying appeared to be more like fresh petals, this may imply that this drying method can better preserve their

bioactive compounds and activities than do the FD and HA methods. As for the antioxidant activities, the results were shown Combined FIR-HA drying gave higher values of DPPH radical scavenging (85%) than did FD drying or fresh petals (67% and 65%, respectively), while HA drying had the lowest DPPH radical scavenging (52.4%). This indicated that the increase was induced by the FIR-HA treatment, thereby supporting a previous study that FIR radiation increases the antioxidant activity of rice-hull extracts (Lee *et al*., 2003), and mulberry tea (Wanyo *et al*., 2011). An increase of antioxidant activities may be explained by the fact that since FIR creates internal heating with molecular vibrations of materials, it may have the capability to breaking down the covalent complex molecular structures and release some antioxidant compound such as flavonoids, carotene, lycopene, tannin, ascorbate, flavoprotein or polyphenols from polymers. Moreover, the mechanism of far infrared drying is different from hot air drying. FIR is thought to liberate and activate low-molecular-weight natural antioxidant compounds, because it heats materials without degrading the constitutive molecules of the surface and contributes to an even transfer of heat to the center of the material (Niwa *et al*., 1988).

3.7.2: Effect of drying on concentrations of several phytochemicals and chemical components of essential oils.

Many studies have examined the effect of drying on chemical components. Okoh O.O. *et al.* (2007) Conducted a study on effect of drying on chemical components of essential oils of *Calendula*, the results showed a difference in the proportions of the compounds in fresh and dried sample at different method. The same results appeared with Siriamornpun *et al.* (2012). The results from this study suggest that drying methods differ in their effects on bioactive compounds. For example, HA gave the highest content of b-carotene (15.5 mg/100 g DW), while FIR-HA and FD provided the highest levels of lutein and lycopene. Hence, we recommend that each drying method could be suitable for different products depending on the type of compounds considered to be the most important.

Martinov, M., and coworkers have carried out drying experiments at different drying heights and five drying experiments. The experiments include five different drying temperatures and plant material layer heights, total carotenoid content and total microbial count were

measured. The significant differences in moisture content between lower and upper layer of dried plant material were evident, pointing out the necessity of turning plant material during drying procedure. The content of total carotenoids was higher in lower layer, in all the experimental variants and lower by natural drying, pointing out that higher temperatures positively affected the total carotenoid content. The lowest total microbial count was obtained with the lower plant material layer while higher values were recorded in natural dried material. The highest values were recorded in fresh samples (Martinov *et al*., 2009).

Dorozko, J., and coworkers 2019 have analyzed the influence of various drying methods on the quality of edible flower petals. The study was carried out using different drying methods such as hot air-drying, microwave drying, and freeze-drying. Edible petals of garden marigold (*Calendula* officinalis *L*.), and true lavender (*Lavandula* angustifolia *L.).* Total phenolic, total flavonoid content and antioxidant activity were determined in their research. All three drying methods had adverse effects on biologically active compounds of the analyzed edible flowers petals. Despite the fact that freeze-drying is the most popular method, microwave drying had the most positive effect in terms of bioactive component content in this study (Dorozko *et al*., 2019).

Freeze-drying would be the best method of water removal, but it is also expensive method. This method is based on the dehydration by sublimation of frozen sample and the major advantages are protection of bioactive compounds and original shape, color and flavor of flowers (Zheng *et al.*, 2015). Microwave drying is alternative to the conventional drying method that allows the product to preserve its useful qualities and is suitable for almost at home. Very important that the heat not only on the surface but also inside the food products or plants. Very high speed of drying gives the quality of the final food product. Shi *et al.* (2017) reported that microwave drying helps to remain higher content of flavone, vitamin C and soluble sugars in medicinal chrysanthemum flowers (Shi *et al*., 2017).

Chapter Four: Methodology

4.1: Extraction

Based on the findings of the literature survey, the antioxidant tests will be performed on the ethanolic extract of *Calendula* flowers and leaves using *1,1-diphenyl-2-picrylhydrazyl (*DPPH) method (Do *et al*., 2014).

Solvent for *Calendula* extraction: Ethanol was used for the extraction process, according to the FDA (Food and Drug Administration), ethanol considered as an effective, efficient, safe, reliable, and consistently producing potent extractions with minimal fuss (High Purity Extractions, 2016). Furthermore, ethanol can dissolve both polar and non-polar substances (EasyChem Australia, 2013). The ethanol extract showed total antioxidant activity by using DPPH higher than water and methanol (Do *et al*., 2014).

Extraction procedure:

- The supervisor Dr. Husni, planted and harvested *Calendula* plants. He provided the GP group of fresh leaves and yellow flowers.
- Leaves and flowers have been separated from the plant, the green leaves weight 113 gm., and the fresh flower weight is 37 gm., then the leaves were dried at room temperature for approximately a week, in the shadow place.
- After drying they were grinded using grinding machine.
- The grinded leaves were weighed, and it is recorded 17 gm., only 8.4 gm. was taken and dissolved in 80 ml ethanol. Where 6 gm of grinded flowers weighed and dissolved in 75 ml ethanol.
- Then, the two flasks after tightly closed were put into a water shaker bath for 72 hours, at 100 rpm and 40°C. The purpose of the shaker is to steadily shake and mix samples while maintaining a constant temperature.
- After the shaker, the samples were transferred from the flasks into two bottles and kept for a day, before filtration.
- Vacuum filtration was done on both samples for solid-liquid separation, and the outcome of filtration was kept in bottles for a day.

- The extract samples were concentrated in a rotary evaporator attached with a vacuum pump for solvent recovery, the solvent (more volatile) evaporated at reduced pressure and temperature and then condensate and trapped in a flask for recovery. It needed around 2 hours for each sample for complete evaporating.
- The extracted material will next be scraped from the flask and will be weighed, to be ready for the DPPH test.
- Figures A 1 to A 7 from Appendices show some of the previous procedures.

4.2: UV-Test for Absorbance

The free radical scavenging activity of the reference and extracts will be evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to Shen *et al*., 2010 method.

According to Shen *et al*., 2010, a 0.004% (W/V) solution of DPPH in ethanol was prepared and 3 ml of this solution was added to 3 ml of the solution of all extracts/reference in ethanol at different concentration (50,100, 200, 400 & 800 µg/mL). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical can be calculated by using the following formula.

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{\text{A0} - \text{A1}}{\text{A0}} * 100\%$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and/or reference (Shen *et al*., 2010).

Ascorbic acid was used as a reference to compare its antioxidant activity with the antioxidant of the *Calendula* plant extract. According to literature, the ascorbic acid stock solution

concentration used for the test was 800 μg/ml, however, in practical experience, this concentration was found to be high, and the absorbance readings at 517 nm for several diluted concentrations showed relatively close results. This concentration was then diluted to 50 μg/ml.

Test for the absorbance wavelength:

- The wavelength of DPPH sample was found equal to 517 nm using ethanol as a blank. Figure A 8 from Appendices shows the absorbance wavelength.

DPPH preparation:

- 4 mg of DPPH was dissolved in 100 ml of ethanol, then covered by aluminum foil, and kept in cool conditions, because DPPH radical is sensitive to light. According to Ozcelik *et al*. (2003), the light effect on the absorbance of DPPH.

Ascorbic acid stock solution (10 ml) preparation:

- 4 mg of ascorbic acid was dissolved in 5 ml of deionized water, this gives a concentration of 800 μg/ml.
- Dilute the stock solution to 50 μg/ml according to the following:

$$M1 \times V1 = M2 \times V2$$

Where:

M1: Concentration of the ascorbic solution (before dilution) (μg/ml).

V1: Volume needed from the ascorbic solution to achieve a diluted solution (ml).

M2: Concentration of the diluted ascorbic solution (μg/ml).

V2: Volume of the diluted ascorbic solution (ml).

$$800 \times V1 = 50 \times 10$$

$$V1 = 0.625\ ml = 625\ \mu ml$$

- 625 μml of ascorbic acid solution was withdrawn by a micropipette and then deionized water was added until having 10 ml of diluted ascorbic solution.

- For serial dilution of the ascorbic acid (10, 20, 30, 40 & 50 µg/ml) using the same dilution equation for total 3 ml solution, 5 clean test tubes were needed:
  - ✓ For a concentration of 10 µg/ml:

$$M1 \times V1 = M2 \times V2$$

$$50 \times V1 = 10 \times 3$$

$V1 = 0.6\,ml = 600\,\mu ml$ of ascorbic solution was withdrawn by a micropipette, and then ethanol was added in a test tube until having 3 ml solution.

$$Volume\ of\ ethanol\ needed = 3 - 0.6 = 2.4\,ml$$

Table 1: The amount needed from each solution to achieve a certain diluted concentration of ascorbic acid.

Conc. of diluted ascorbic acid µg/ml	Volume of ascorbic acid solution (ml)	Volume of ethanol needed (ml)
0	0	3
10	0.6	2.4
20	1.2	1.8
30	1.8	1.2
40	2.4	0.6
50	3	0

- The remaining calculations are included in the Appendices page **F,** and the summary results shown in Table 1.

Preparation of the control solution:

- 3 ml of DPPH solution (0.004% W/V) was added to each one of the five previous test tubes that containing the Ascorbic acid at different concentrations (10, 20, 30, 40 & 50 μg/ml).
- Then the test tubes were kept at room temperature for 30 minutes in the dark (covered by aluminium foil).

Measuring the absorbance at 517 nm:

- The blank solution contains ethanol only was inserted in the UV-VIS spectrophotometer and the test was run.
- Then DPPH solution sample, and the 5 other samples (ascorbic acid at different concentration with DPPH), were inserted individually in the UV-VIS for running the test.
- Absorbance values were measured for each sample individually.

4.3: Drying herbs

Materials and method

The laboratory measurements were carried out at the Laboratory of the chemical engineering department. The studied and measured material samples were put in plastic perforated sheets used in Aluminum window frame.

Two drying experiments are carried out at different temperature one is at room (ambient) temperature on 20-22 ºC. The other drying experiment is carried out at 55-60 ºC.

Drying at ambient temperature: During the experiment, the average room temperature was 20-22 ºC. The dimension of drying samples are presented in Table 2. The average relative humidity in Nablus area is about 60%.

Table 2: Start parameters of dried *Calendula* flowers.

Temperature °C	Start weight (g)	Drying surface area (cm^2)	The average dimension of the drying plate (cm)
room (20-22)	500	900	30x30x3
55-60	200	177	D=15cm, height 2-3 cm

The air temperature and humidity were measured by thermometer and humidity sensor. The moisture content was identified by gravimetric measurement in regular time intervals. The samples were weighed on the digital laboratory balance with maximum load weight 500 g and with resolution 0.01 g.

The total drying time was adapted to the need for determination of the final moisture content.

Experimental data of *Calendula* at room temperature is shown in Table 3.

Table 3: Drying data obtained at room temperature.

Time (h)	Weight (g)
0	500
10	450
24	360
30	340
40	283
50	224
60	176
70	136
80	114
90	98
100	80
120	74
140	73
160	74

Drying at 55-60 ºC. Here the sample is placed on a circular tray on a pan, the diameter of tray is 15 cm. the heat is provided by hair drier that placed and hanged on about 25- 30 cm from a sample. The weight is continuously and directly measured. The temperature is measured by a digital thermometer. The start weight is taken 200 g. Experimental data of *Calendula* flowers at 55-60° C is shown in Table 4.

Table 4: Drying data obtained at 55-60° C

Time (min)	Weight (g)
0	200
10	179
20	141
30	122
40	83
50	66
60	50
70	40
80	35
90	32
100	29
120	27
140	25
150	26

Chapter Five: Results and Discussion

5.1: Drying of *Calendula* flowers

The aim of drying experiments was to investigate and compare principal theoretical problems of drying by free convections for medical plants as flowers of garden marigold (*Calendula Officinalis)*. Determining the drying rates of *Calendula* flowers at constant and at falling rate periods, drying equation constants at falling rate periods (R=aX+b) experimentally. The experiment is carried at room temperature and 55-60 °C.

The falling rate of drying will be given by a linear straight line equation. (R=aX+b). Where R is the rate of drying, X is free moisture content, a and b are constants that experimentally determined.

Generally, the medicinal plants should not be collected in wet and rainy weather, but when they are dry. The plants should be collected as the whole, if possible, and cut after drying. Most plants should be dried in the shade, where they will not be soiled by dust, birds, insects, etc. The fresh plants must not be added to dried plants since the herb gets wet again and may degrade by mold.

Drying aims to inhibit micro-organism growth and prevent its deteriorations and spoilage, by reducing the water content of the plant, which has a negative effect on the action of enzymes (Honório *et al*., 2016). Moreover, it reduces weight and volume so it will minimize packaging and storage costs. Using drying induces quantitative and qualitative differences in the components of fresh and dry plant materials (Okoh *et al*., 2008).

The dried herbs color remained unchanged, but the bulk density decreased significantly during drying. It can be expected that the drying process was under conditions, which were adequate for the *Calendula* flowers.

The demonstration of experiment data is shown at different forms as presented in drying literature. This show at the following Figures 5, 6 & 7. These present how a given property change with time.

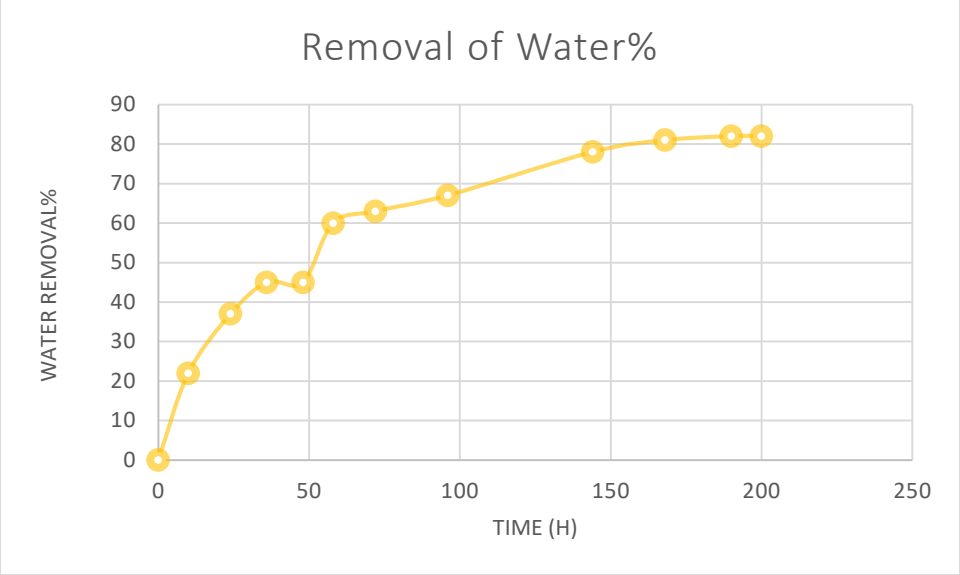

Figure 5: Drying dynamics of *Calendula* flowers.

In order to find the equations of constant rate and falling rate, a plot of free moisture X vs. time needed; to find the tangent lines that fit the most possible points in order to get the range where constant and falling rate periods occurred.

X (Free moisture) determined by:

$$X = \frac{Weight\ (g) - Lowest\ Weight}{Lowest\ Weight} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

Where weight (g) is the weight at any time (t).

For example: at room temperature, the lowest weight is 73 g, for time equals 140 hr. the value of X is :

$$X = \frac{450-73}{73} = 5.16$$

It is important to remind that the lowest weight still has about 12% moisture (equilibrium moisture content) at drying condition. So, it is more exact to take the lowest weight as (73*0.88=64 g). So, the bone dry is 64 g and not 73 g.

Same calculations were done on all weights, and the obtained results shown in Table 5 and Table 6.

Table 5: The value of X at room temperature.

Time (h)	X
0	5.84932
10	5.16438
24	3.93151
30	3.65753
40	2.87671
50	2.06849
60	1.41096
70	0.86301
80	0.56164
90	0.34247
100	0.09589
120	0.0137
140	0
160	0.0137

Table 6: The value of X at 55-60 °C.

Time (min)	X
0	7
10	6.16
20	4.64
30	3.88
40	2.32
50	1.64
60	1
70	0.6
80	0.4
90	0.28
100	0.16
120	0.08
140	0
150	0.04

Excel was used to plot the data, and to determine the tangent lines as shown in Figure 6 & Figure 7.

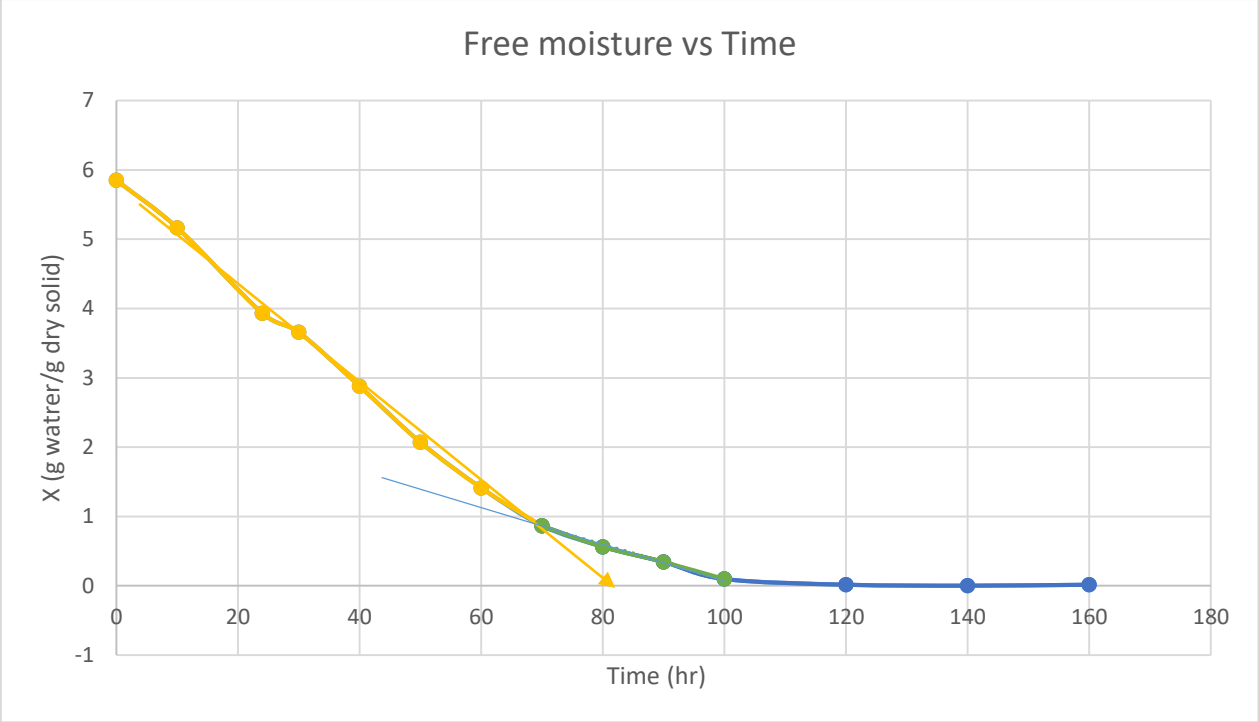

Figure 6: Plot of X vs. time at room temperature.

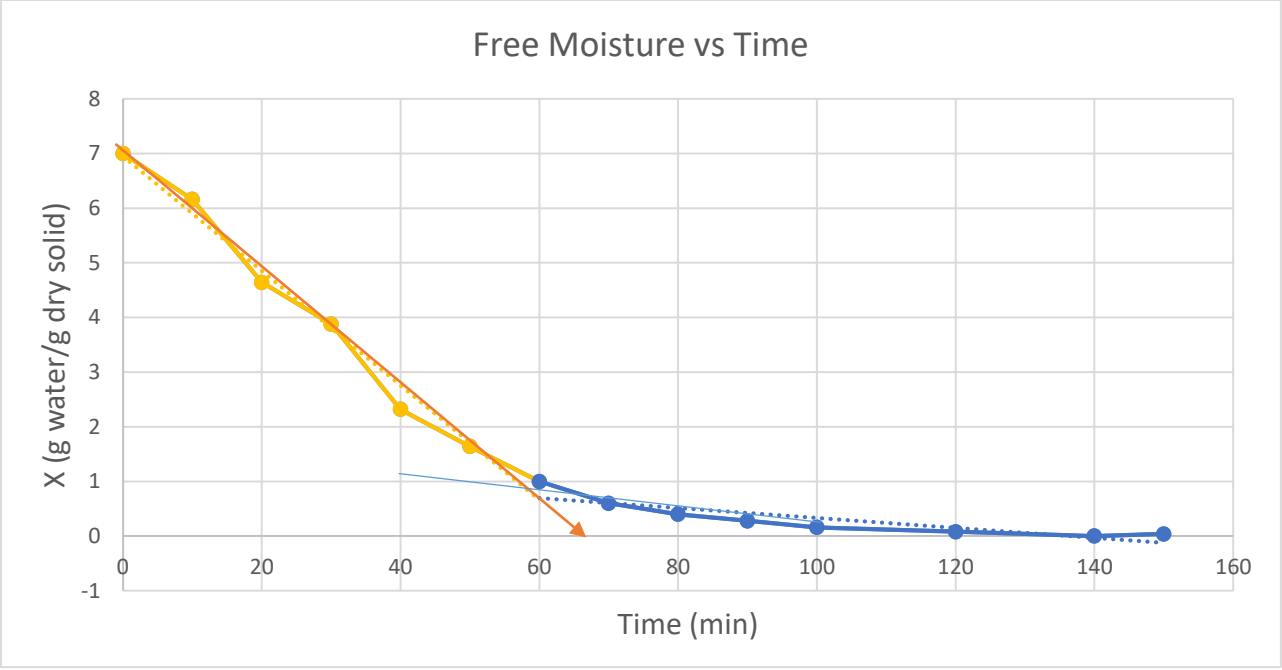

Figure 7: Plot of X vs. time at 55-60 °C.

The orange arrows show the period where the constant rate of drying, while the blue lines show the period of the falling rate of drying. The intercept between the arrow and the line determines the X critical (Xc). From the intercept shown that Xc in Figure 6 is at 70 hr., and it is 60 min (1 hr.) in Figure 7.

To find the constant rate of drying: (McCabe *et al.*, 2001)

$$R = -\frac{Ls}{A} \times \frac{\Delta x}{\Delta t} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 2$$

Where:

Ls: Weight of the dried flowers used (Kg).

A: The exposed area m^2.

R: Constant rate of drying (Kg water/ $h.m^2$).

At ambient temperature, the weight of the dried flowers was 73 g, the moisture content in this stage must be considered, and it was assumed to be approximately 12%, so:

$$Ls = 73\ g \times 0.88 \times 0.001 = 0.0642\ Kg$$

$$A = 30\ cm \times 30\ cm \times (10^{-2})^2 = 0.09\ m^2$$

From Figure 6, the period of the constant rate is at Δt = 70 hr. and

so Δx = 5.8493 - 0.8630 = 4.9863, the rate constant is:

$$R = -\frac{0.0642}{0.09} \times \frac{4.9863}{70} = 0.0508\ (\text{Kg water}/\ \text{h.}\ \text{m}^2)$$

And this value is constant for the whole period of constant rate, and it will be used to the plot of R (rate of drying) vs. X (free water) as in Figure 8, to determine the equation of the falling rate, an arrow was added manually reached between the end of the constant line and the zero point.

Table 7: Experimental data for the plot of drying rate vs. moisture at room temperature.

Time (h)	X	R
0	5.84932	0.0508
10	5.16438	0.0508
24	3.93151	0.0508
30	3.65753	0.0508
40	2.87671	0.0508
50	2.06849	0.0508
60	1.41096	0.0508
70	0.86301	0.0508

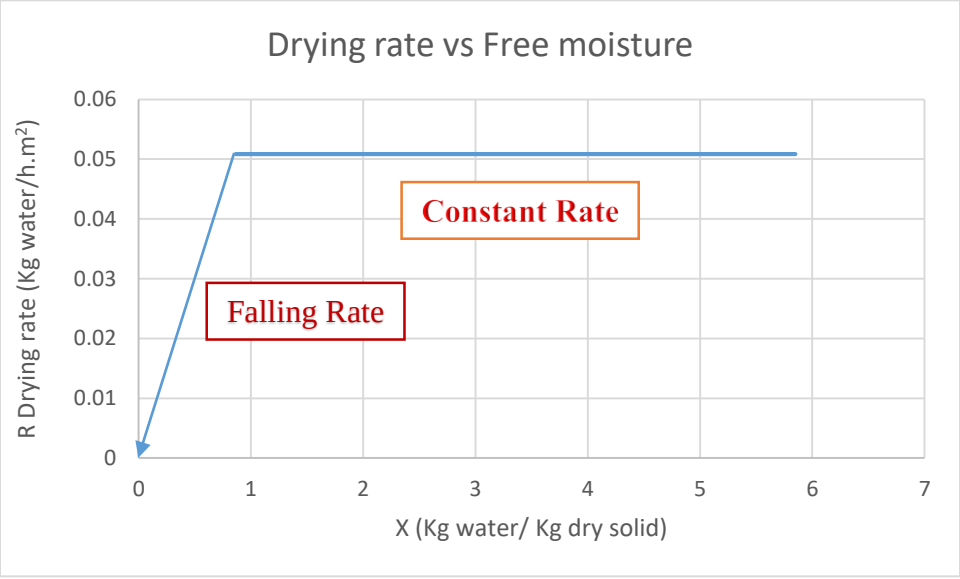

Figure 8: Drying rate vs. free moisture at room temperature.

Equation of the falling rate: $R = aX + b$

Where:

a: is the slope.

b = 0

To find the slope: $a = \frac{\Delta R}{\Delta X} = \frac{(0.0508-0)}{(0.8630-0)} = 0.0589$

Then, R = 0.0589X

Table 8: Summary results for data at 20-22 °C drying.

Start weight (g)	Dimension of drying plate (cm)	Ls (Kg of solid used)	A exposed area m^2	Ls /A (Kg/m^2)	R (Rate constant) Kg $H2O/h.m^2$	Slope of falling
500	30x30x 1	0.0642	0.09	0.7138	0.0508	0.05891

Same calculations and procedures were made for the data at 55-60 °C drying temperature, and the results obtained shown in the following Table 9 and Figure 9.

Table 9: Summary results for data at 55-60 °C drying.

Start weight (g)	Diameter of circular pan (cm)	Ls (kg of solid used)	A exposed area m^2	Ls /A (Kg/m^2)	R (Rate constant) kg $H_2O/h.m^2$	Slope of falling
200	15	0.022	0.0177	1.25	7.47	7.47

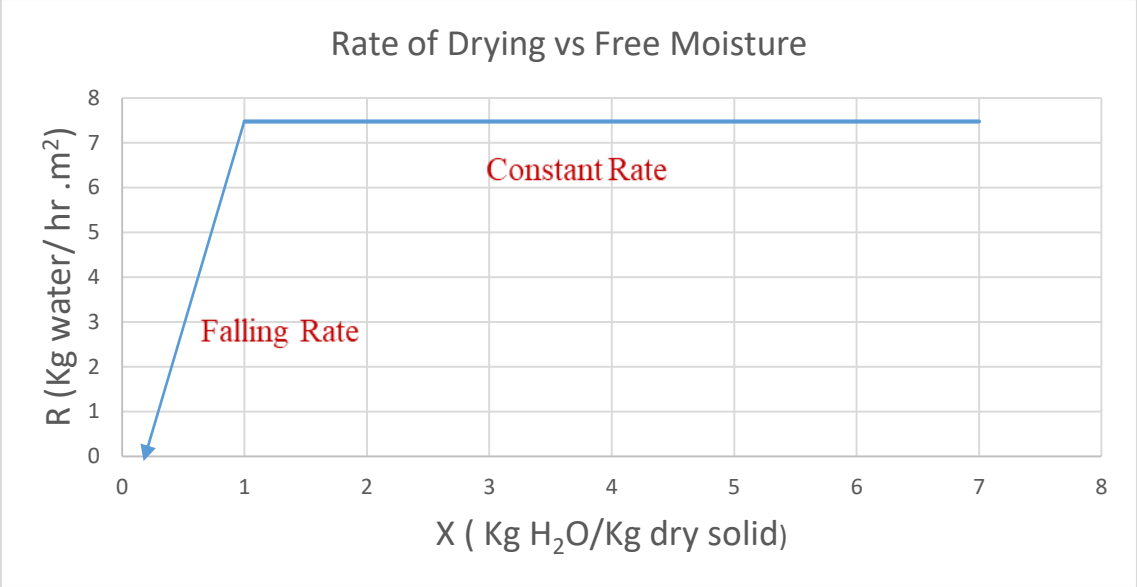

Figure9 : Rate of drying vs free moisture at 55-60 °C.

The tendency of drying curves earned at room temperature 20-22 ºC is similar to that obtained at 55-60 ºC. The only difference is the drying time is reduced for about 2.5-3 h. While the drying time at ambient temperature takes few days around 6 to 7 days.

The drying rate at 55-60 ºC is obviously higher than at ambient temperature at both constant drying rate period and the falling rate of the drying period. At ambient temperature, the constant drying rate is 0.05 kg water/ (m^2.h). While the rate of drying at falling rate period is calculated from R=0.0508X. The rate of drying at a constant rate period is about 7.5 kg water (m^2.h) at higher temperature or at 55-60 ºC. It means 150 times is faster the drying at 55-60 ºC. The straight-line drying equation for the falling rate of drying at 55-60 °C is read out directly from R vs. X Figure 9. Which is about R=7.47X.

It is clear that the shape of R vs. X drying curves are the same at different temperature. But the values of the rate of drying at higher temperature is very high or $R_{55\text{-}60^oC} = 150\ R_{20\text{-}22^oC}$

An investigation was completed to assess the drying conduct of *Calendula* flowers utilizing a laboratory scale dryer with controlled air temperature and relative humidity (Matouk *et al.*, 2016).

The considered parameters included four distinct degrees of drying air temperature (55, 60, 65, and 70 °C) and three levels of air relative humidity (40, 50, and 60%). All test runs were

directed at a steady airspeed of (0.6 m/sec) (Matouk *et al.*, 2016). The results are shown in the following both Figure 10 and Figure 11 (Matouk *et al.*, 2016).

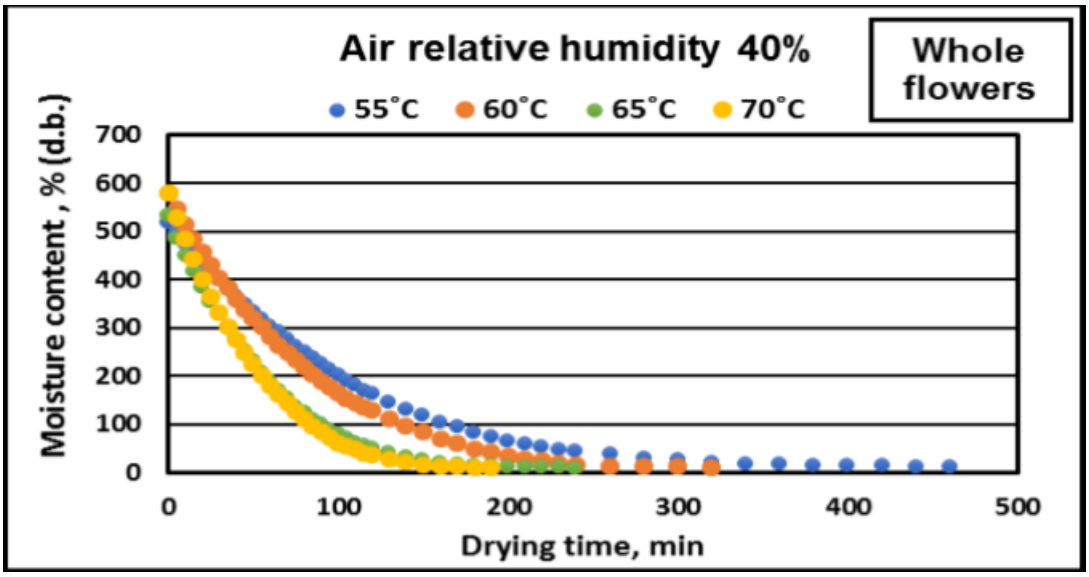

Figure 10: Changes in the moisture content of *Calendula* flowers as related to drying time at different degrees of drying air temperature and relative humidity of 40%.

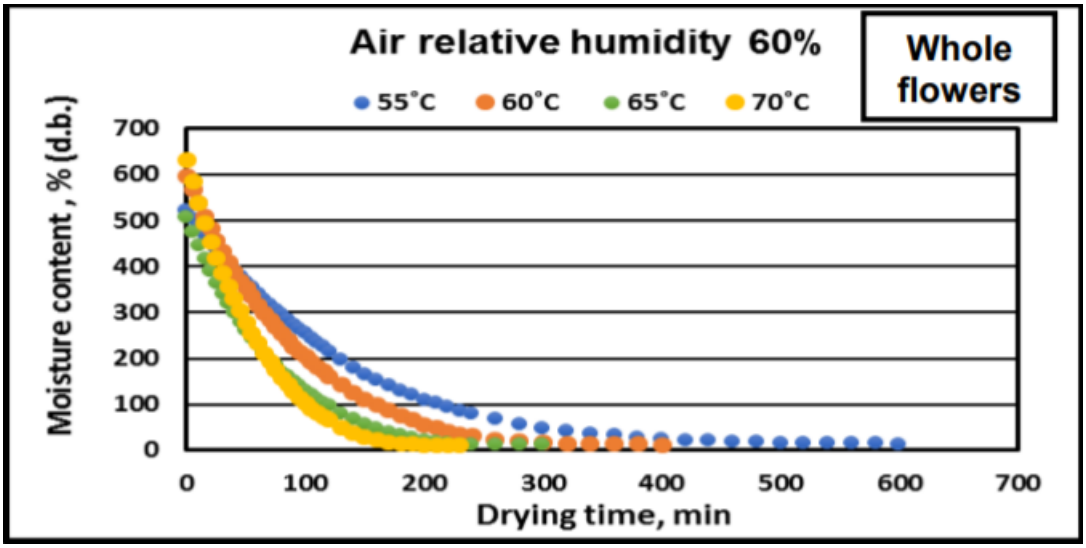

Figure 11: Changes in the moisture content of *Calendula* flowers as related to drying time at different degrees of drying air temperature and relative humidity of 60%.

It is obvious from Figure 10 and Figure 11 that as the temperature increases the time of drying decreases. However, when the humidity is increasing the time of drying will increase too.

The moisture content of the *Calendula* flowers ranged between 83.5 and 87.3 w% (Matouk *et al.*, 2016), and it was between 85.2 and 87 w% in our experimental work. At the same conditions of 60% humidity and temperatures ranges between 55-60 ºC, the time of drying in our experiment was ranged between 2-2.5 h, where in the literature (Matouk *et al.*, 2016), it was ranged 6.5-10 h, due to different initial weight for drying, the moisture content and the drying method.

Although the drying rate increased when the heat was raised, some studies showed that drying by hot drying at high temperatures may cause damage to the chemical compound present in the plant. There are other drying methods such as far infrared ray combined with air convection (FIR-HA) mentioned in previous studies, which reduces the drying time or increases the drying rate, and doesn't cause damage to the chemical compounds in the herbs, which may be an alternative to drying hot air.

5.2: Absorbance and %inhibition for Ascorbic acid solution

Table 10 shows the readings of absorbance from UV-Spectrophotometry, with %inhibition for the ascorbic acid:

Table 10: Absorbance readings and inhibition calculation for ascorbic acid.

Concentration µg/ml	Absorbance	%inhibition ratio	IC50
0	0.272	75	
10	0.214	80	
20	0.184	83	
30	0.188	83	
40	0.185	83	
50	0.238	78	
Control	1.096		

- A Control sample contains 0.004% W/V DPPH solution.

READINGS, AND THUS RESULTS DON'T MAKE SENSE, some offered causes: A reaction may have occurred between DPPH and light, or there may be a human error in preparing concentrations of the solutions, or in using the UV spectrophotometry.

Sample of calculation

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{A0 - A1}{A0} * 100\%$$

$$(\%\ \text{inhibition}) = \frac{1.096 - 0.272}{1.096} * 100\% = 75\%$$

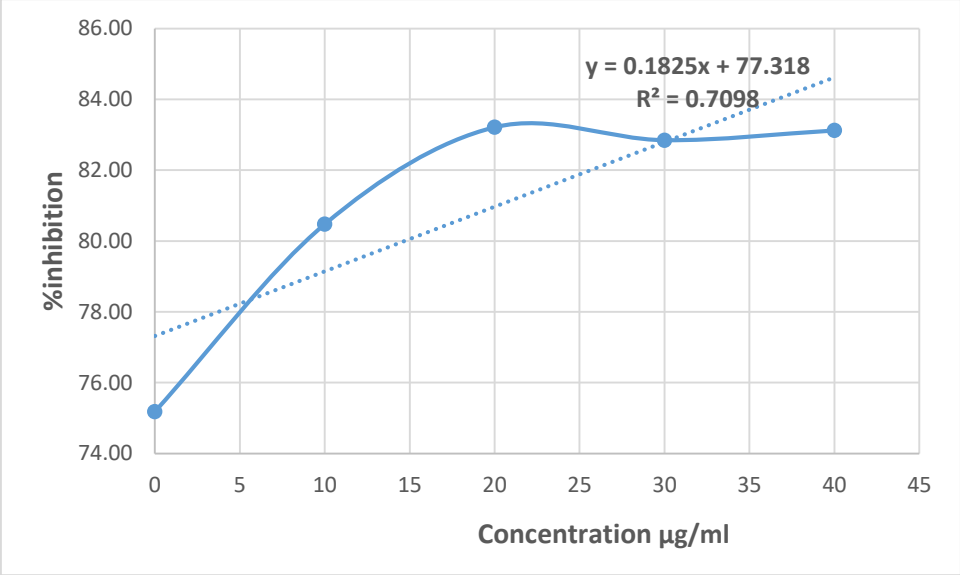

Figure 12: Setting %inhibition vs. Concentration.

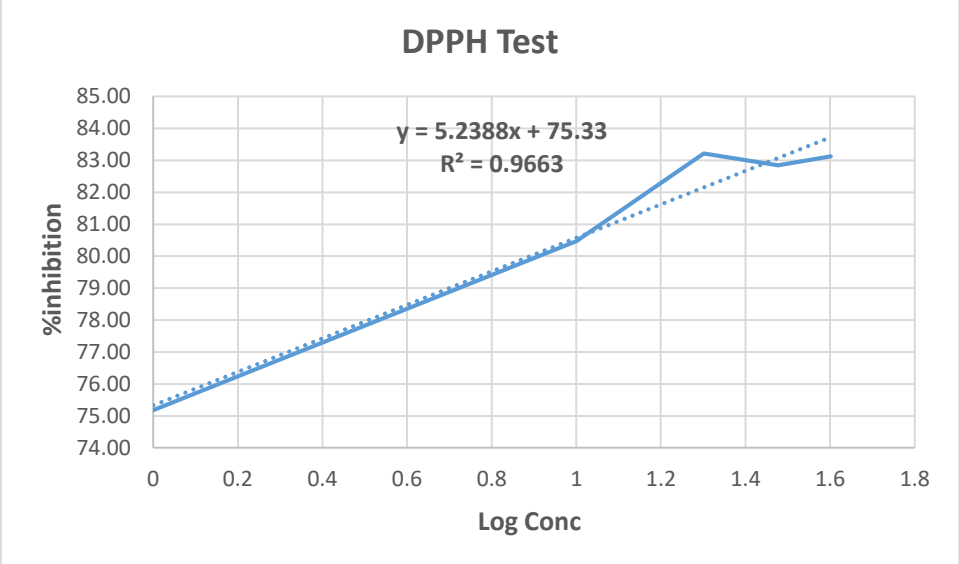

Figure 13: DPPH test by setting %inhibition vs. Log concentration.

To find IC50, the equation of the trend line from Figure 13 will be used **(y = 5.2388x + 75.33),** where y must = 50, and x is the needed concentration for the 50% inhibition. These results were intended to be compared and calibrated with the results of the extract inhibition.

Chapter Six: Conclusion

Since there were no complete results for the antioxidant experiment, an analytical study of drying flowers at two different temperatures and safe conditions was included as part of this project. It is suggested to dry the herb flower soon at cover transparent plastic sheets to conserve energy and to dry fast. The drying rates at constant drying period and at falling drying period are calculated. The drying characterization curves (X vs. time, R vs. X) are presented as well. The increasing drying condition from 20-22 ºC to 55-60 ºC increases the rate of drying about 150 times. The characteristic drying curves calculated by our group and that are taken by literature are the same.

The antioxidant test has not been accomplished as mentioned before, and so no reliable results could be mentioned.

Chapter Seven: Future Work and Recommendation

Since the desired results to complete the main project goal of testing the antioxidant of ethanolic extract of *Calendula* wasn't accomplished, due to the COVID-19 pandemic, it is possible in the future to complete the experimental work after the end of the pandemic, using the following recommendations:

- Making sure all tools are perfectly cleaned to avoid turbidity.
- Trying to provide as much of *Calendula* herb as possible for the purpose of experiments.
- For the calibration curve using ascorbic acid as a reference, each working concentration should be performed in triplicate.
- Continue the work on *Calendula* extracts (leaves and flowers) to measure the absorption and antioxidant scavenging, using the modified method of Shen *et al*., 2010, and compare the results with the reference's results.
- Screening and scouring method could be applied to test the most appropriate solvent and the antioxidant test method, either through laboratory experience or by inferring from the literature survey.
- Conduct the inhibition concentration IC_{50} and the effective concentration EC_{50} calculations.
- Studying the components of the herb, which are most affected by drying, and those which have the highest antioxidant effect such as phenols. If there is an intention to make a marketing product for a certain disease.
- Literature survey experiences several drying methods, it is recommended to test the best method of drying to achieve a certain purpose. Such as far-infrared ray combined with air convection (FIR-HA) drying method, according to Niwa *et al*., 1988, it showed that it could be a suitable alternative to hot drying, by increasing the rate of drying without causing damage to the plant compounds, also microwave drying showed preserving the quality of the components of the plant after drying, besides it

has a positive effect in terms of bioactive component, according to Dorozko *et al*., 2019.

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Appendices

Figure A 1: Dried *Calendula* flowers.

Figure A 2: Grinded *Calendula* flowers.

Figure A3 : Water shaker bath.

Figure A 4: The two samples of *Calendula* flowers and leaves in the shaker.

Figure A 5: Vacuum filtration of the flowers.

Figure A 6: During the process of vacuum filtration of the flowers.

Figure A 7: After collecting the samples from the vacuum filtration.

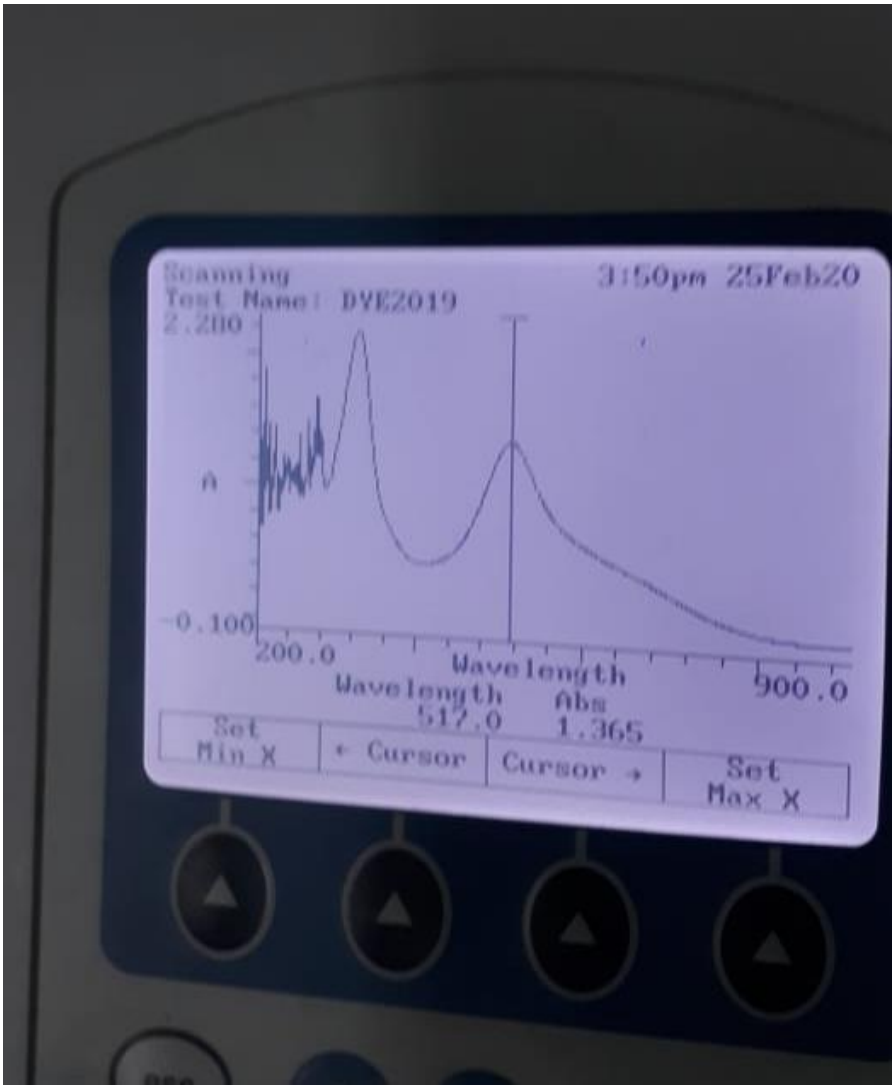

Figure A 8: Scanning of the wavelength of the blank.

- Sample of calculation for diluted concentrations of ascorbic acid solution:

✓ For a concentration of 20 µg/ml:

$$M1 \times V1 = M2 \times V2$$

$$50 \times V1 = 20 \times 3$$

$V1 = 1.2\,ml = 1200\ \mu ml$ of ascorbic solution was withdrawn by a micropipette, and then ethanol was added in a test tube until having 3 ml solution.

$$Volume\ of\ ethanol\ needed = 3 - 1.2 = 1.8\,ml$$

✓ For a concentration of 30 µg/ml:

$$M1 \times V1 = M2 \times V2$$

$$50 \times V1 = 30 \times 3$$

$V1 = 1.8\,ml = 1800\ \mu ml$ of ascorbic solution was withdrawn by a micropipette, and then ethanol was added in a test tube until having 3 ml solution.

$$Volume\ of\ ethanol\ needed = 3 - 1.8 = 1.2\,ml$$

✓ For a concentration of 40 µg/ml:

$$M1 \times V1 = M2 \times V2$$

$$50 \times V1 = 40 \times 3$$

$V1 = 2.4\,ml = 2400\ \mu ml$ of ascorbic solution was withdrawn by a micropipette, and then ethanol was added in a test tube until having 3 ml solution.

$$Volume\ of\ ethanol\ needed = 3 - 2.4 = 0.6\,ml$$

✓ For a concentration of 50 µg/ml:

3 ml of ascorbic solution was taken only in the test tube, no need for ethanol.