An-Najah National University

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Antioxidant and anticholinesterase potentials of essential oils of selected aromatic plants under secondary treated effluent irrigation

By

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Dedication

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents whose words of encouragement and push for tenacity ring in my ears.

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الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Antioxidant and anticholinesterase potentials of essential oils of selected aromatic plants under secondary treated effluent irrigation

اقر بان ما اشتملت عليه هذه الرسالة إنما هو من نتاج جهدي الخاص، و ان هذه الرسالة ككل أو أي جزء منها لم يقدم من قبل لنيل أية درجه علميه أو بحث علمي أو بحثي لدى أية مؤسسه تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's name: **اسم الطالبة:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

AD	Alzheimer's disease
Ach	Acetylcholine
AChE	Acetylcholinestrase
AChEI	Acetylcholinestrase inhibitor
BuCh, BCh	Butyrylcholine
BuChE, BChE	Butyrylcholinesterase
BuChEI, BChEI	Butyrylcholinesterase inhibitor
FR	Free radical
ROS	Reactive oxygen species
TAPHM	Traditional Arabic Palestinian herbal medicine
NA-FB	Naphthyl Acetate- Fast Blue B
MM	Millimolar
M	Molar
µL	Microliter
Hr	Hour
DPPH	2,2-Diphenyl-1-picrylhydrazyl
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
EO	Essential oil
HPLC	High-performance liquid chromatography
BERC	Biodiversity and environmental research center
%I	Percentage of inhibition
A_{sample}	Absorbance of the Sample
$A_{control}$	Absorbance of the control
IC_{50}	Inhibition concentration
EC_{50}	Effective concentration
DM	Dry matter
Spp	Species
FDA	Food and Drug Administration
NMDA	N- methyl- D-Aspartate
pH	Potency of hydrogen
EC	Electrical conductivity
SAR	Sodium Absorption Ratio
PW	Potable water
TE	Treated Effluent

Antioxidant and anticholinesterase potentials of essential oils of selected aromatic plants under secondary treated effluent irrigation

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Abstract

Background: The shortage of water throughout the world dictates application of marginal water for irrigation. Secondary treated municipal effluent (TE) is a common alternative water source for irrigation in arid and semiarid areas. Aromatic herbs are cultivated as industrial crops for herb essential oil (EO) production. The EOs can play a role as natural antioxidants and enzyme inhibitors targeting human diseases, e.g., Alzheimer's disease (AD). Such agents can prevent oxidative deterioration of foods, minimize oxidative injury of living cells, treat AD and enhance memory. Replacement of potable water (PW) with (TE) for irrigation of aromatic plants could encourage the expansion of large-scale agro-industrial systems for aromatic crops, and EOs in Palestine. This study was aimed at (1) evaluating the applicability of TE for agricultural crops, (2) assessing the effects of continuous use of treated water on soil and crops, and analyzing the antioxidant and anticholinesterase activities of these plants and their essential oils under irrigation with TE.

Materials and Methods: To compare responses of selected aromatic plants to irrigation with PW and TE an experimental field has been established, each treatment consisted of 4 replicated plots. The plants were exposed to the water treatments for two consecutive seasons. EO from fresh plant areal parts of both treatments was obtained during the two seasons (summer and fall 2016) by hydrodistillation. The extracted EOs and methanolic leaf extracts were tested for their antioxidant activity using DPPH scavenging of free radicals and reductive potential (RP), and for acetyl and butyryl cholinesterase inhibitory activity (AChEI and BuChEI) using the NA-FB method.

Results: Regardless of the differences in the quality of water, the TE did not affect fresh and dry biomass production, spices output and yield of EO quantity in the crops except for *Salvia fruticosa* which was statistically significant and increased for all previous yield parameters. Also, water quality did not affect the cholinesterase inhibitory activity of the plants (EOs or extracts). *Mentha spicata* plant extract showed the highest AChE and BuChE inhibitory activities with selectivity index (SI) of 2.93 and 3.85 in PW and STE, respectively. Whereas it's EO showed the lowest inhibitory activity against both enzymes. Overall, the tested methanolic extracts were found to be more selective inhibitors of AChE than BuChE, while their EOs were more selective inhibitor on BuChE. The antioxidant activity of the extracts also was not affected by effluent- irrigation, *M. spicata*, and *R. officinalis* exhibited the best RP and DPPH-scavenger,

whereas the only EOs from *O. syriacum* and *R. officinalis* exhibited strong to weak activity as antioxidants.

Conclusion: The data demonstrate that TE can be effectively utilized for irrigation of industrial crops for EO, natural antioxidants, and anticholinestrase agents production because the yield and bioactivity were not affected.

Chapter one
General Introduction

1.1 Overview of non-conventional water resources - Secondary treated effluent

In many arid and semi-arid regions, natural fresh water resources are limited, whereas the demand is constantly increasing due to industrial and population growth. The greater challenge to meet water demands and manage its limited natural resources has led to the use of alternative irrigation sources (Hassanli et al., 2009; Khaskhoussy et al., 2015). One of these alternatives is to use non-conventional water resources, such as treated waste water. The largest source of marginal water for agriculture is secondary treated municipal sewage water (Haytham et al., 2004). Treated waste water can be used to help in reducing natural water consumption, in restoring and preserving degraded land and in aiding the growth of vegetation (Aggeli et al., 2009; Khaskhoussy et al., 2015).

The TE contains higher levels of micronutrients (Fe, Zn, Mn, Cu, Mo) and macronutrients (N, P, K, S, Ca, and Mg), salinity sources (Na, Cl), HCO_3^{-1}, NH_4^{+1}, NO_3^{-1}, and B than the local PW and is characterized by higher values of electrical conductivity (EC), pH, and sodium absorption ratio (SAR) (Jeison et al., 2003). These nutrients may serve to replace part of the requirements for nutrients (macronutrients), or accumulate in the plant tissue and may reduce plant performance and yield quality (salts and heavy metals) (Jeison et al., 2003; Maheswari et al., 2012).

Effluent-based agriculture therefore depends on identification of crops that are able to maintain high performance under the suboptimal conditions imposed by this low-quality TE water.

Perennial aromatic plants are cultivated as cash-crops for fresh or dry herb production, or as a source of essential oils and natural antioxidants. These mainly summer crops require substantial amounts of water, up to $9000 m^3 ha^{-1}$ $Dunum^{-1}$ throughout the growing season, to satisfy their potential for intensive biomass production (Putievsky et al., 1990; Dudai, 2005: Ali-Shtayeh et al., 2018a). Thousands of dunums of these crops are required to facilitate an economically viable industrial production system. Therefore, shortage of fresh water for irrigation in arid and semiarid regions restricts utilization of aromatic plants as industrial crops. Replacement of fresh water with treated effluent for irrigation of these plants could promote development of large-scale production systems for biomass, essential oil, and natural antioxidants in these regions (Ali-Shtayeh et al., 2018a).

Cultivation of aromatic plants for essential oils is thought to be suitable for irrigation with TEs because the heat applied during oil extraction eliminates human bacterial pathogens originating in the effluents and alleviates health concerns. Additionally, the essential oil, which is extracted mainly by steam distillation, will be free of inorganic ion contaminants such as heavy metals originating from the effluents, which may accumulate in the plant tissues and the soil (Ali-Shtayeh et al., 2018a).

The essential oil of perennial members of the Lamiaceae family including *Origanum syriacum, Rosmarinus officinalis, Mentha spicata, Salvia fruticosa* and *Micromeria fruticusa* is considered to be a good source for natural phenolic antioxidants (Putievsky et al., 1988; Ravid et al., 1997; Munné-Bosch and Alegre, 2000; Chun et al., 2005; Skerget et al., 2005). Previous studies on *Origanum syriacum, Rosmarinus officinalis* and *Micromeria fruticusa* reported that irrigation with TE as compared to PW did not change antioxidant activity of the plant tissue. Total phenolic compounds content was also not affected by irrigation with the effluent (Bernstein et al., 2009; Ali-Shtayeh et al., 2018 a,b). Several essential oils are reputed to enhance memory. Of the most essential oils tested for improving study skills and test taking ability are the Lemon and Rosemary essential oils (Sanchez and Elamrani, 2014). The most acceptable theory for their memory enhancing/antidementia activities is their ability to enhance the cholinergic function by inhibiting cholinesterase (Murray et al., 2013). Interestingly, new naturally occurring cholinesterase inhibitors continue to be identified in numerous plant species suitable for irrigation with TE.

Plants subjected to environmental stresses, including salinity, produce reactive oxygen species (ROS). To diminish the excess of ROS, plants have developed an antioxidant defense system, which comprises enzymatic and nonenzymatic components. The antioxidants directly react with and scavenge ROS. The effects of salt stress on antioxidant responses have been studied in a range of plant species, and salinity-induced changes in

activities of antioxidant enzymes are well documented (Bernstein et al., 2009; de Azevedo Neto et al. 2006). Salinity induces oxidative-stress in leaves, which results in increased activity of ROS-scavenging enzymes along with a decrease in ROS-producing enzymes in the growing cells. Indeed, it has been demonstrated that improved salt tolerance could be brought about by improved resistance to oxidative stress (Bandeoglu et al., 2004; Bernstein et al., 2009; de Azevedo Neto et al., 2006).

1.2 Overview of Alzheimer's disease (AD)

Alzheimer's disease (AD) is a neurodegenerative disease that primarily affects the elderly population over 65 years of age. AD is estimated to account for about 70% of the dementia cases, and now affects approximately 24 million people worldwide (Huang et al., 2013; Ali-Shtayeh et al., 2014). It is a slowly progressive disease of the brain that is characterized by memory loss, difficulty performing familiar tasks, problems with language, disorientation of time and place, poor or decreased judgment, problems with abstract thinking, misplacing things, changes in mood or behavior, changes in personality, and loss of initiative.

Accumulation of the protein amyloid plays a significant role in the development of Alzheimer's disease, which is a characteristic hallmark, besides the protein tau in the patients with AD (Howes and Perry, 2011). However, AD is diagnosed when a person has sufficient cognitive decline to meet the criteria for dementia or the clinical course is consistent with that of AD.

Two types of cholinesterases are present in the healthy human body, acetyl- and butyryl-cholinesterases. In healthy human brain, acetylcholinesterase (AChE) is mainly located within cholinergic axons and the cell bodies of neurons whereas butyryl-cholinesterases (BChE or BuChE) predominantly appears to have a neuroglial distribution and considered to play a minor role in regulating brain acetylcholine (ACh) levels. However, both enzymes are also found in neuritic plaques and tangles in patients with AD (Wright et al., 1993, Topcu and Kusman 2014). The exact cause of AD is not well understood yet.

Butyrylcholine (BCh or BuCh) is not a physiological substrate in the human brain, and the only chemical difference from ACh is the presence of two additional methylene (CH_2) groups in BCh (Fig1.1). Due to its predominantly neuronal distribution, AChE activity is higher than BChE activity in the human brain. Depending on the region, human brain AChE activity is 1.5-fold to 60 fold higher than BChE activity (Giacobini, 2001).

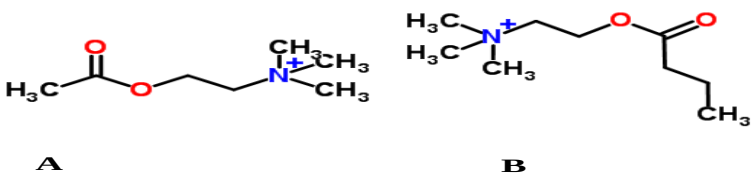

Figure 1.1: the chemical structure of A. Acetylcholine (ACh) and B. Butyrylcholine (BCh).

The loss of memory is considered to be the result of a shortage of the nerve transmitter ACh. It is possible to increase the level of this transmitter in the brain by inhibiting the activity of the enzyme AChE, which splits or

breaks down the transmitter substance. Drugs that inhibit the breakdown of the messenger or transmitter ACh delay the development of the disease (Singhal et al., 2012), which explains the cholinesterase cascade in AD (Fig 1.2).

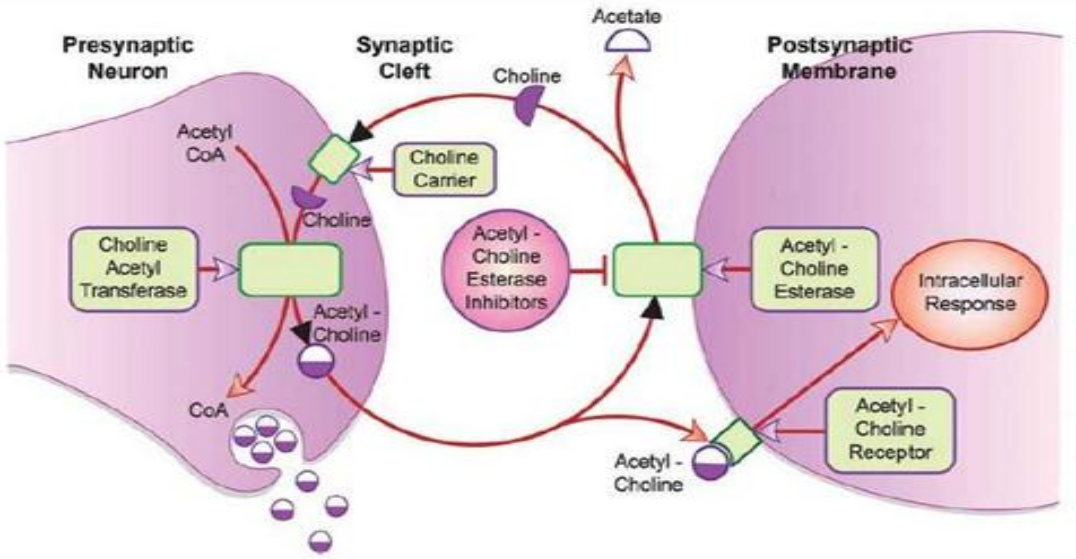

Figure 1.2: Acetylcholine inhibitors (AChEIs) in cholinergic nerve transmission. Acetylcholine is produced in the presynaptic neuron by the enzyme choline acetyltransferase from acetyl-coenzyme A and choline, and later released in the synaptic cleft where it binds to the acetylcholine receptor on the postsynaptic membrane, triggering an intracellular response. The enzyme acetylcholinesterase hydrolyses acetylcholine into acetate and choline in order to terminate synaptic transmission. Choline is transported into the presynaptic neuron by the choline carrier and serves as a substrate for the described production of acetylcholine. AChEIs inhibit the enzyme acetylcholinesterase, which in turn inhibits the breakdown of acetylcholine into acetate and choline and prolongs its duration of action (Eur Heart J. © 2013 Oxford University Press)

The brains of those with mild-to-moderate AD, a progressive type of dementia, have abnormally low ACh concentrations. This means that any compound that enhances the cholinergic system in the brain may be useful in treating AD and similar brain malfunctions. However, BChE activity progressively increases in patients with AD, while AChE activity remains unchanged or declines. The two enzymes differ in substrate specificity,

kinetics, and activity in different brain regions. Recent evidence suggests that both AChE and BChE may have roles in the etiology and progression of AD beyond regulation of synaptic acetylcholine levels (Howes and Perry, 2011, Greig et al., 2002).

In addition to vitamin E, vitamin C and beta carotene, as well as many natural compounds, may help in scavenging free radicals generated during the initiation and progression of this disease. Several natural substances with neuroprotective effects have been widely studied by a number of researchers. These substances have remarkable antioxidant properties, mainly by scavenging free radical species. Some of them increase cell survival and improve cognition by directly affecting amyloidogenesis and programmed cell death pathways (Ansari and Khodagholi, 2013). However, there is still an immediate requirement for both the diagnosis for patients with AD at the early stage and exploration of more efficient drugs than the ones prescribed by physicians at present, as known cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) glutamate receptor antagonists cannot provide a fully satisfactory cure for AD.

In order to find new natural agents for the treatment of AD based on the cholinesterase inhibitory mechanism, many research groups in the world study different plants from various families, including the Amaryllidaceae, Fumariaceae, Papaveraceae, and Lamiaceae families

(Jayaprakasam et al., 2010; Ali-shtayeh et al 2014; Ali-shtayeh et al., 2018 a, b).

At present, two types of medications in AD treatment are approved by the FDA; these are cholinesterase inhibitors were originally isolated from plants or are derived from templates of compounds isolated from plants. The two major synthetic AD therapeutics available on the market are tacrine and donepezil as acetyl cholinesterase inhibitors, besides a natural drug, galantamine, and a naturally derived compound, rivastigmine (Palmer, 2002) and a glutamate NMDA receptor antagonist such as Memantine. However, these drugs are known to have limitations due to their short-half-lives and/or unfavorable side effects (including gastrointestinal disturbances) and problems associated with bioavailability (Wszelaki et al., 2010), which necessitates the interest in finding better AChEIs from natural resources (Adewusi et al., 2011; Ali et al., 2013; Amessis-Ouchemoukh et al., 2014; Benamar et al., 2010; Feitosa et al., 2011).

1.3 Oxidative Stress in the AD

It is highly impossible to consider a biological life without oxygen and this valuable oxygen is metabolized and produce free radicals (FR) in human body by oxidative process having an extensive effects on human health (Roy et al., 2011, Ayaz et al., 2014). FR and its by-product reactive oxygen species (ROS) are continuously produced in human body (Moniruzzaman et al., 2015). When natural enzyme controls fail, free

radicals in the body attack lipids, proteins, and nucleic acids. Oxidative stress is one of the first steps of AD, which may play a pathogenic role in its progress. Plants and their constituents with pharmacological activities may be relevant for the treatment of cognitive disorders, including enhancement of cholinergic function in the central nervous system and anti-inflammatory and antioxidant activities (Beckman & Ames, 1998).

Generally, the physiological role of antioxidant compounds is to attenuate the oxidation chain reactions by removing free-radical intermediates (Liu & Nair, 2010). Consequently, the use of antioxidants has been explored in an attempt to slow AD progression and neuronal degeneration (Howes et al., 2003).

1.4 Plant Extracts and Phytochemicals as Potential Therapeutic Agents in Alzheimer's Disease

Strong experimental evidences have indicated that plants with antioxidant and anticholinestrase activities have potential to be used as a novel template for the development of effective drugs for the treatment of AD that have been reported in Traditional Arabic Palestinian Herbal Medicine (TAPHM) (Ali-Shtayeh et al., 2014). Some herbs have anti-inflammatory, antioxidant, cognitive-enhancing, neuroprotective, and anti-aging effects that may reduce inflammation of the brain tissue and used in the treatment of AD (Howes et al., 2003).

In order to find new natural agents for the treatment of AD many research groups in the world study different plants from various families, including the Amaryllidaceae, Fumariaceae, Papaveraceae, and Lamiaceae families (Jayaprakasam et al., 2010).

1.4.1 Lamiaceae (*Labiatae)* family plants and their importance

The Lamiaceae or Labiatae (the mint family) is one of the largest families, with around 7000 species worldwide. Lamiaceae plants are widely used in TAPHM since antiquity, especially the aromatic and culinary herbs, such as mint, rosemary, sage, savory, marjoram, oregano, thyme, and lavender. The phenol-containing species, *Origanum syriacum*, *Rosmarinus officinalis, Mentha spicata,* and leaves of *Salvia fruticosa* and *Micromeria fruticosa* are used in herbal and medicinal tea preparations (Ravid and Putievsky,1983).

1.4.2 Phytochemistry of Lamiaceae family

This family contains a wide variety of chemicals. A wide range of compounds such as terpenoids, iridiods, phenolic compounds and flavonoides have been reported from the members of the family (Richardson, 1992; Zegorka and Gloiwniak, 2001). The family is also a rich source of plant species containing large amounts of phenolic acids. For example, rosmarinic acid, this compound has anti-bacterial, antiviral, antioxidant and anti-inflammatory properties. More and more studies carried out in numerous research centers show that the complex

pharmacological activity of some medicinal plants of the family and their essential oil is strictly connected with the presence of phenolics (Zegorka and Glowniak, 2001). Flavonoides also occur in the *Labiatae* in a variety of structural forms including flavones, flavonols, flavanones, dihydroflavonols and chalcones (Tomas-Barberan and Gil, 1992) having a number of biological activities, including antimicrobial, anti-inflammatory, antioxidant, antiviral, cytotoxic, wound healing, neuroprotective, and anticholinesterase.

Members of the Lamiaceae family and their phytochemicals have been studied for pharmacological and some clinical effects relevant to dementia (Perry & Howes, 2011). Among Lamiaceae plants, especially *Salvia* and *Rosmarinus* species, have been known to have neuroprotective properties for many years (Tuncer, 1978). Also Lamiaceae family plants have been used in Traditional Arabic Palestinian Herbal Medicine (TAPHM) to treat various disorders, including improvement of memory, AD and old age related diseases (Ali-Shtayeh & Jamous, 2006, 2008).

As *Lamiaceae* herbs considered to be rich sources of phenolic phytochemicals and antioxidants, plants belonging to the family *Lamiaceae* are great sources of natural antioxidants, often used as spices and aromatic herbs. The antioxidant activity of phenolic compounds may not be mainly due to phenolic content, but may be due to the redox properties, physicochemical structure and nature of the individual phenolics (Kähkönen et al., 1999; Chun et al., 2005).

1.4.3 Literature review of some Lamiaceae plants having potential as anticholinesterase, antioxidant and/or other related activities in the treatment of Alzheimer's disease

Many aromatic culinary herbs of Lamiaceae plants, especially *Origanum, Rosmarinus, Salvia, Mentha* and *Micromeria* species contain several phenolic compounds (Topcu and Kusman, 2014). Several non-volatile compounds such as carnosol, quercetine, caffeic acid and rosmarinic acid are well known to be good scavengers of free radicals. It has been established that the antioxidant effects are mainly due to phenolic compounds. The main phenolic compounds identified in Lamiaceae plants are rosmarinic acid, carnosic acid, carnosol (Figure 1.3), methyl carnosate, rosmanol, epirosmanol and rosmadial. Carnosol and carnosic acids possess good peroxyl and hydroxyl radical scavenging activity, since they inhibit the formation of hydroxyl radicals and chelate metals, while only carnosic acid appears to scavenge H_2O_2 (Pizzale et al., 2002).

Origanum species

Origanum species (known as Marjoram) have been referred to as one of the memory-enhancing plants. They also have antioxidant, antibacterial, anti-inflammatory, and antispasmodic effects (Başer, 2002). *O. syriacum* L. oil was evaluated for its anticholinesterase and antioxidant activities (Topcu and Kusman, 2014). *O. syriacum* oil exhibited strong activity against both cholinesterases. In addition, essential oil of *O. syriacum* moderately inhibited oxidation of linoleic acid after incubation. Thus, it

had a significant potential as a natural antioxidant and anti-ChE agent (Ali-Shtayeh et al., 2018a).

***Rosmarinus officinalis* L.**

R. officinalis (known as Rosemary) is a dietary herb that possesses high antioxidant activity and was first marketed as a source of natural antioxidants (Papageorgiou et al., 2008). It has been used as a medicinal herb since early times, and it has received increasing attention due to its antimicrobial, anti-inflammatory, and antioxidative constituents. Rosemary contains a large number of compounds consisting of phenolics and abietane diterpenes, which are responsible for its antioxidant and cytotoxic activity, such as rosmarinic acid carnosic acid, and carnosol (Figure 1.3).

R. officinalis is used in connection with AD and dementia for general symptoms of old age, debility, and fatigue. The anticholinesterase activity of the essential oil of *R. officinals* most likely depends on a synergistic mechanism of oil components. In contrast to the essential oil, the major compound rosmarinic acid in the methanolic extract of the plant was considered to be responsible for strong anti BChE activity(Orhan et al., 2008).

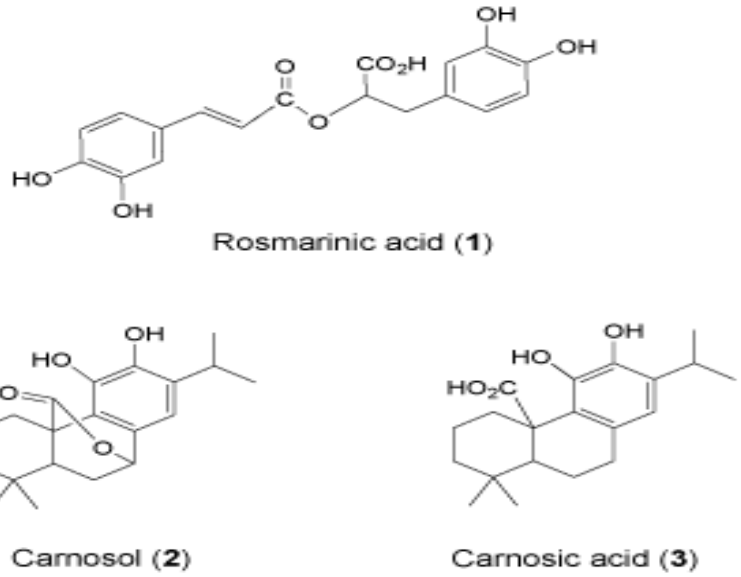

Figure1.3: Chemical structure of Rosmarinic acid, Carnosol and Carnosic acid.

The plant was found to protect neuronal cells against hydrogen peroxide-induced injury (Fregozo et al., 2012), and it is suggested that *R. officinalis* might potentially serve as an agent for prevention of several human neurodegenerative diseases caused by oxidative stress and apoptosis due to the presence of antioxidant compounds and flavonoids (Park et al., 2010).

Activated glutathione metabolism participates in protective effects of carnosic acid against oxidative stress in neuronal HT22 cells (Tamaki et al., 2010). Carnosic acid is also involved in the synthesis of nerve growth factor, which is necessary for the growth and maintenance of nerve tissue. Carnosic acid and carnosol have the potential to protect cortical neuronal cells by activation of the Keap1/Nrf2 pathway (Kayashima and Matsubara, 2012).

Some *Salvia* species, particularly *S. officinalis* L. and *S. triloba* L., showed similar chemical composition to *R. officinalis*. However, *R. officinalis* leaves are especially much more rich in carnosic acid and carnosol, which are the main abietane diterpenes responsible for prevention of membrane damage and vascular brain circulation, with high antioxidant and neuroprotective properties.

Salvia fruticosa **Mill. (*S. triloba* L.)**

One of the most well-known Lamiaceae family plants is the genus *Salvia,* which has wide distribution, with nearly 1000 species in the world. *Salvia* (sage) species have been used since ancient times in folk medicine for cognitive brain function, along with various biological activities. Sage extracts possess antioxidant, estrogenic, and anti-inflammatory properties, which may help strong anticholinesterase effects by inhibiting both butyryl- and acetyl-cholinesterases (Topçu, 2006).

The genus *Salvia* is a huge and important source, rich in terpenoids and flavonoids and other phenolics with antioxidant, anti-tuberculous, anti-inflammatory, neuroprotective, and anticholinesterase properties (Wu et al., 2012).

S. fruticosa (synonym; *S. triloba*) tea, the leaves of the plant is commonly used to cure colds, stomach ache, memory enhancing and neuroprotecting (Şenol et al., 2011). Many studies on the essential oil of *S. fruticosa* reported 1,8-cineole to be the main component, followed by

camphor, α-thujone, β-thujone , and β-caryophyllene . The species is known to contain biologically active sesquiterpenes and diterpenes, besides the high content of oxygenated monoterpenes (Topçu et al., 2013).

In an evaluation of cholinesterase inhibitory and antioxidant properties of wild and cultivated samples of *S. fruticosa* by activity-guided fractionation, *S. fruticosa* extracts showed moderate anti-cholinesterase activity (Şenol et al., 2011). A more recent study on the plant however, found that a methanol extract of the plant showed strong activity against both AChE and BChE as well as strong antioxidant activity (Topçu et al., 2013; Topçu and Kusman, 2014).

Salvia fruticosa Mill. extracts afforded six abietane diterpenoids (carnosol, carnosic acid, carnosic acid 12-methyl ether, rosmadial, isorosmanol, ferruginol); a labdane diterpene manool; four triterpenoids, oleanolic acid, ursolic acid, erythrodiol, and α-amyryltetracosanoate; a steroid (3-acetylsitosterol; and salvigenin, which is a characteristic flavone of *Salvia* species (Topçu et al., 2013).

The oil of the plant exhibited high anticholinesterase activity, particularly against BChE (Topçu and Kusman, 2014). The essential oil, consisting mainly of 1.8-cineol, obtained from the aerial parts of the plant, exhibited high AChE inhibitory activity. The antioxidant activity and anticholinesterase potential of the methanol extract and the triterpenoids α-amyryltetracosanoate, oleanolic acid, ursolic acid, and 3-acetylsitosterol were also investigated, and the methanol extract exhibited the highest

antioxidant and anticholinesterase activity, surpassing those of pure compounds, probably due to synergistic effects of all components together (Topçu and Kusman, 2014).

Micromeria fruticosa (L.)

Micromeria fruticosa (known as White micromeria) is generally consumed as herbal tea and for folk medicinal purposes in colds. *M. fruticosa* is the well-known subspecies of the genus micromeria growing on the eastern coast of the Mediterranean, and some of its uses are documented in various reports (Ali-Shtayeh et al., 2018b). It is believed in the Palestinian society that drinking an infusion of *M. fruticosa* leaves and stalks helps in curing different types of paralysis, nervous system disorders and it has calming effect (Azab, 2016). The use for treating nervous system disorders is mentioned in traditional Palestinian medicine along with other uses: treatments of diabetes, illnesses of respiratory system, especially cough, urinary diseases, headaches and fever (Ali-Shtayeh & Jamous, 2008). As a result of recent studies, it has been shown that the essential oil of *Micromeria* species has biological activity such as antimicrobial, antibacterial, antifungal, and antioxidant (Gulluce et al., 2004). The ethanolic extract of *Micromeria* is rich in phenolic acids like chlorogenic acid and flavonoids like hesperitin, myrcetin and quercetin. These phenolic compounds were shown to be effective against a wide range of diseases as they possess antimutagenic, anti -cancer and antioxidative activities (Topçu and Kusman, 2014).

***Mentha spicata* L.**

M. spicata L. (known as spearmint), is commonly cultivated in the world for essential oil production that is used extensively in the liquor and confectionary industries, flavoring, perfume production and medicinal purposes. Leaves, flowers and the stem of *Mentha* spp are frequently used in herbal tea or as additives in commercial spice mixtures for many foods to offer aroma and flavor (Moreno et al., 2002). In addition, *Mentha* spp. have been used as a folk remedy for treatment of nausea, bronchitis, and liver complaints due to its antinflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant, emmenagogue, and anticatharrhal activities (Moreno et al., 2002). Furthermore, it is well-documented that the essential oils or/and extracts from some *M. spicata* possess antimicrobial and antioxidant properties (Gulluce et al.,2007). *Mentha* species are favorable free radical scavengers as well as primary antioxidants that may react with free radicals and limit ROS attack on biological and food systems (Nickavar et al., 2008).

Mint species are used widely throughout the world as an important medicinal plant. Their oils are one of the most popular and widely used essential oils, mostly because of its main components such as menthol and carvone. (+) Menthol , neomenthol and cineole were the main components in the oil of *M. piperita*, whereas carvone and D-Limonene in *M. spicata* essential oil. The essential oils of mint species showed strong antimicrobial and free radical scavenging activity (Dinis Pedro et al., 2013). In addition,

Mentha species have the capacity to inhibit acetylcholinesterase, due to the presence of rosmarinic acid, eriocitrin and eriodictyol.

1.5 Problem hypothesis

Salinity and heavy metals contained in TEs may increase antioxidant activity and reactive oxygen species (ROS) production in plants. Increased antioxidant content and antioxidant activity were demonstrated in many plants in response to environmental stresses (Mittler, 2002). In rosemary, water stress induced changes in antioxidants which were suggested to be involved in prevention of plant tissues damage (Munné-Bosch and Alegre, 2000). Therefore, irrigation of antioxidant producer crops with TE containing high levels of salts and heavy metals may induce stress on the plants, increase antioxidant phenolic compound production, and may lead to an economic advantage over regular water irrigation (Bernstein et al., 2009).

Studies have demonstrated that antioxidant rich plant preparations can prevent cancer, as well as cardiovascular-, neurodegenerative-, inflammation- and other aging-related diseases (Duan et al., 2006). Currently, there is global interest in finding new and safe antioxidants and anticholinesterase agents from natural sources, to prevent oxidative deterioration of foods and to minimize oxidative injury of living cells and to treat Alzheimer’s disease and enhance memory (Ali-Shtayeh et al., 2014, Ali-Shtayeh et al., 2018a,b).

1.6 Objectives

- Evaluating the effect of irrigation with secondary TE as compared with potable water irrigation, over two convictive seasons, on yield components (including fresh biomass, dry herb yield and essential oil yield), antioxidant and anticholinesterase activities of methanolic extracts and essential oil from the five selected aromatic plants (*Rosmarinus officinalis*, *Origanum syriacum, Mentha spicata, Micromeria fruticosa* and *Salvia fruticosa*).

- Explore newly potent and safe natural therapeutic agents for the treatment of AD from aromatic plants suitable for irrigation with TE.

- Investigating the effects of secondary treated water irrigation on plant and soil properties and its macro- and micro-nutrients, and heavy metal content, in order to establish the basis for safe treated waste water agricultural reuse.

Chapter Two
Materials and Methods

2.1 Plant Material

Five aromatic plants suitable for production of essential oils, antioxidants and anticholinesterase agents were evaluated for their responses to irrigation with PW and TE. The crops that tested were selected based on potential for marketing, demographic interest and traditional usage. Crops analyzed are: *Rosmarinus officinalis*, *Origanum syriacum*, *Mentha spicata*, *Micromeria fruticosa* and *Salvia fruticosa*. All are used commercially in Palestine for herb production.

2.2 Growing Conditions

The experiment was carried out in an experimental aromatic plants field constructed on 26 th of June 2014 by BERC Aromatic Plants Research Group at the Biodiversity & Environmental Research Center (BERC), Til, Nablus, Palestine (Figure 2.1) in an open field. In this field a factorial design with 5 plant species and two water qualities (potable water and treated effluent) were planted as a random design with four replicate plots to compare the responses of plants to irrigation with TE, and PW. The experimental design was completely randomized blocks, five species with two water qualities (PW and TE), and 4 replicated blocks. Each treatment will hence consist of 4 replicated plots per each species, 3 m long and 0.6 m wide each. The experimental field therefore comprised 40 plots (Figure 2.2). The plants were irrigated with PW (control) or TE. Sampling

was conducted only from the center bed, leaving the margins on each side, to ensure root exposure only to the appropriate water quality. Plants were collected in 2016 after 2 years of plant exposure to irrigation with the two water qualities during two consecutive seasons (Summer and Fall 2016) (Figure 2.3).

Figure 2.1: Constructed field at BERC, Til, Nablus, Palestine. Plots were established to compare responses of the plants to irrigation with TE and PW.

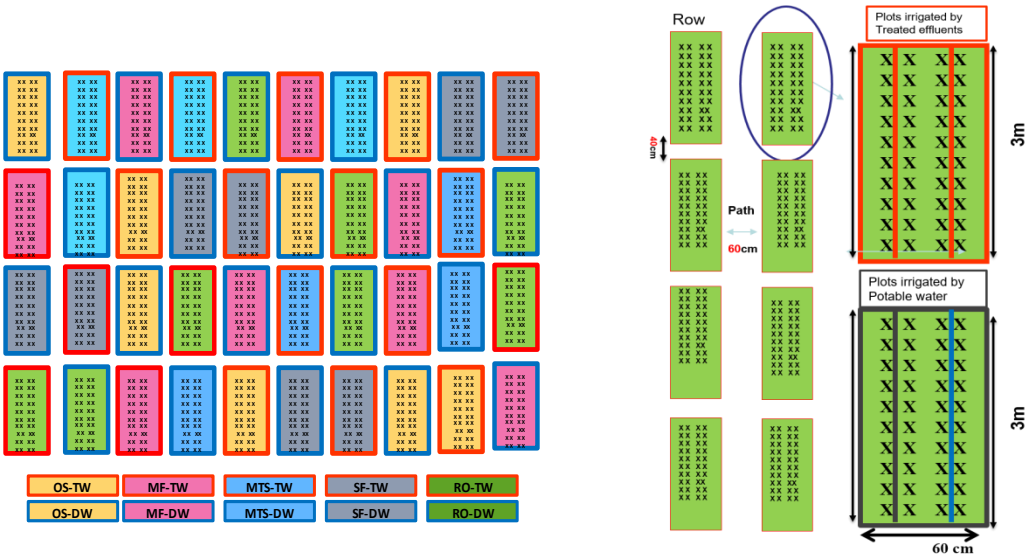

Figure 2.2: Experiment and plot design, there were ten columns, four plant plots in each column, each plant has eight plots irrigated with two water treatments, forty plants per each plot.

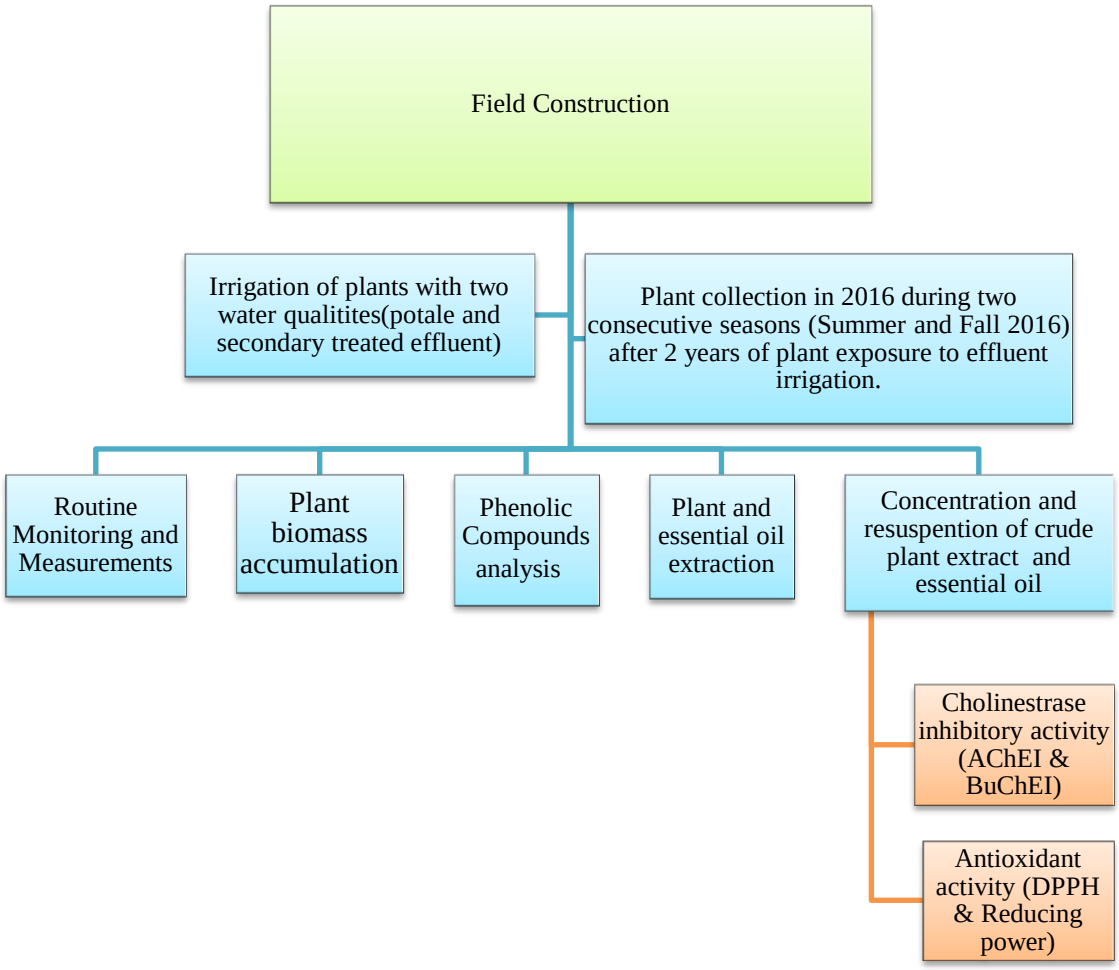

Figure 2.3: Diagram showing the layout of field establishment, irrigation, plant collection, plant extracts preparation, anticholinestrase and antioxidant activity evaluation.

2.3 Water quality

The secondary treated effluent TE used in the study was kindly provided by Nablus Western Wastewater Treatment Plant- Deir-Sharaf , the chemical composition of both water qualities used for irrigation is detailed in table 2.1, the field was irrigated by drip irrigation , a total of 5000 m^2 ha^{-1} water was applied.

2.4 Monitoring and measurements

Throughout the experiment, pH, EC, Cl, NO_3-N, SO_4-S, Na, K, Ca+Mg, B, Cu, Cd, Co, Cr, and Fe in the source water were analyzed (Siraj & Kitte, 2013) (APPENDIX A).

Table 2.1: Quality of PW and TE used in the study.

Parameter	Potable water	Treated effluent
PH	7.07-7.6	6.93-7.95
EC (dS/cm)	200-554	900-1551
Cl (ppm)	35.45-39	177.25-338
Na (ppm)	12	87
Ca+Mg (meq/l)	5-7.5	6.05-15
B (ppm)	0	0.2-0.5
NO_3-N(ppm)	0.8-1.6	0.9-1.7
SO_4-S(ppm)	1.6-1.9	21.67-24.3
K (ppm)	2.1-3	52-100.6
Fe (ppm)	0.07	0.19
Cr (ppm)	0.007	0.009
Co (ppm)	0.015	0.023
Cd (ppm)	0.012	0.02
Cu (ppm)	0.04	0.03

The plants were collected at each season, and the quantity and quality of the yield were assessed for each harvest. The plants were cut 10–20 cm above ground, and sampled for contents of inorganic elements. For each plant, biomass of the four replicates was combined, mixed, and ground together and a 5-g subsample was used for the analysis.

Two different procedures were used for the extraction of various mineral elements from the plant tissue. The method of Plank (1992) was used for the analysis of P by colorimetry and S by turbidimetry. For the

analysis of Na, K and heavy metals Association of Official Analytical Chemists (AOAC) methods 1995 was used (see APPENDIX A).

Soil mineral content was evaluated on soil samples collected from the experimental field established by BERC research team in Til, Nablus, before the beginning of irrigation of plants with effluents (June 2014), after one and two years of exposure to irrigation effluent. Soil samples were taken separately from 0 to 30 cm depth. Soil from four replicates was combined, mixed and ground together and a subsample was used for the analysis. The pH, EC, and the concentrations of Cl, Ca, Ca+Mg, CO_3, HCO_3, P, S-SO_4, N-NO_3, Al^{+3} were determined in the soil from extracts of saturated soil paste (Estefan et al., 2013). The EC of the saturated soil paste extracts and the irrigation source water was measured with a conductivity meter, Mg+Ca and Ca by titration. In addition, Cu, Cd, Fe, Mg, Mn, Zn, Ni, and Pb were determined (APPENDIX A).

2.5 Plant biomass accumulation

Plant samplings were used for evaluation of fresh and dry biomass production of the plants. The harvested plants were evaluated for fresh biomass production and percentage of dry weight by separating twenty five grams of fresh plant material, washed and dried in an oven at 68ºC for 48 hr, the dry weight was recorded and percent dry matter (DM%) was calculated for each plot in addition to the percentage of dry leaves weight from total fresh weight biomass was done by collecting forty grams from the fresh plant material, leaves were isolated, weighted,

washed and dried in the oven at 40 ºC for one week (Bernstein et al., 2009). The dried leaves weight was recorded and the percentage of dry leaves weight from total fresh weight biomass was calculated using the following equations:

$$\textbf{Fresh biomass production}(\textbf{g/m2}) = \text{Fresh biomass production (g)}/1.8\text{m2}$$

$$\textbf{Percent dry matter}(\textbf{DM}\%) = \text{dry weight (g)}/\text{fresh weight (25 g)}\text{X}100\%$$

$$\textbf{Percentage of dry leaves weight from total fresh weight biomass}\ \% = \frac{\text{leaves fresh weight} * \text{leaves dry weigt}}{\text{whole plant weight} * \text{leaves fresh weight}}\text{X}100\%$$

2.6 Phenolic compounds analysis

Many aromatic plants produce antioxidants which may have a commercial value as well. Irrigation with effluents, which imposes stress on the plants, may increase the antioxidant activity of the plant cells. Total phenol and flavonoids analysis in the plant material were performed in each season (Bernstein et al., 2009). Ten grams of plant tissues were ground using electrical blender. Frozen ground tissue was lyophilized at 0.02 (Pressure) and -50^{o} C for three hours. Then the lyophilized plant material was sieved and kept in tightly closed container at -20 ºC to be used for plant extract preparation. For the extraction of phenolic compounds 100 mg of ground dried leaves were mixed with 10 mL of 80% MeOH, mixture was incubated at 25 C over night and spun for 5 min to eliminate particles before use for the analysis. The remaining extract was kept at -20°C for further investigation.

2.6.1 Total phenolic compounds determination

This was done following the Chun et al. (2005) method with some modifications. Briefly, 50 µl of plant extract were added to a test vial, mixed with 7 mL water, and 0.5 ml 1*M* Folin–Ciocalteu reagent was added. One ml 5% Na_2CO_3 was added after 3 min, and the reaction mix was allowed to stand for 30 min then absorbance measured at 760 nm. A standard curve was constructed using (5-1000 µg/ml) gallic acid in water. Phenol concentrations were calculated in terms of gallic acid equivalents (GAE) mass basis (µg/mg of dry mass).

2.6.2 Total flavonoids determination

The method of Pourmorad et al. (2006) was used for flavonoids determination. Each MeOH extract (500 µl) was separately mixed with 1.5 ml of MeOH, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1 *M* CH3COOK, and 2.8 ml of distilled water. This solution was kept at room temperature for 30 minutes; the absorbance of the reaction mixture was then measured at 415 nm using spectrophotometer. A calibration curve was constructed by preparing eight quercetin solutions at concentrations (12.5 to 200 µg/ml) in MeOH. Total flavonoid values were expressed as quercetin equivalent (µg/mg of dry mass).

2.6.3 HPLC analysis of phenolic compounds

High performance liquid chromatography (HPLC) (Agilent Technologies 1260 Infinity HPLC) was used for the evaluation of carnosic acid and carnosol in grinded, lyophilized and sieved plant materials. A reversed phase C18 Purospher STAR RP-18 endcapped 5 μm pore size (Merck, Darmstadt, Germany) using a C18 guard column. 20uL of the MeOH plant extract were injected (Bernstein et al., 2009). The mobile phase were programmed with a linear gradient from 90% A (840 ml of deionized water with 8.5mL of CH3COOH and 150 ml of acetonitrile), 10% B (MeOH), to 100% B in 40 min, with a flow rate of 1.0mL min^{-1}, and 100% B to 45 min. The compounds were identified by comparison with the relative retention time of authentic samples. The absorbance at 284 nm of carnosic acid and carnosol were determined based on a calibration curve of these compounds.

For carnosic and carnosol acids extraction, 100 mg of each sample were weighed and placed in a 100 ml Erlenmeyer flask (weighing range: 0.1000 to 0.1020). In the hood, 100 ml of the extraction solution (900 ml EtOH, 50 ml MeOH, 50 ml 2-Propanol and 1.2 ml H_3PO_4) were added, samples were arranged in a sonicator, filled with water and ice to a height of about 1 cm below the extraction liquid. Sonicator was operated for 60 minutes. Using a disposable syringe, 1 ml of each sample were filtered through a Teflon filter to an HPLC vial.

2.7 Extraction of plant material and essential oil

2.7.1 Plant material extraction

Each plant was air dried in the dark, and grounded into fine powder using an electric mill. The powdered materials (20 g) were extracted with 200 ml of absolute methanol for 24 hr at room temperature (Gholamhoseinian et al., 2009). The suspensions were then filtered and concentrated to dryness under reduced pressure in a rotary evaporator (45◦C) (Stuart) (Figure 2.4). The extracts were stored at -20 °C until used.

Figure 2.4: Rotary evaporator used for drying plant extracts under reduced pressure.

2.7.2 Essential oil extraction

Hydrodistillation was used for the extraction of EO from fresh plant material. 250g-samples of fresh plant material were extracted for 90 min using a modified Clevenger apparatus (Figure 2.5). The EO was cooled and

separated from the cohobated water and stored in dark bottles at 4 °C until used (Bernstein et al., 2009).

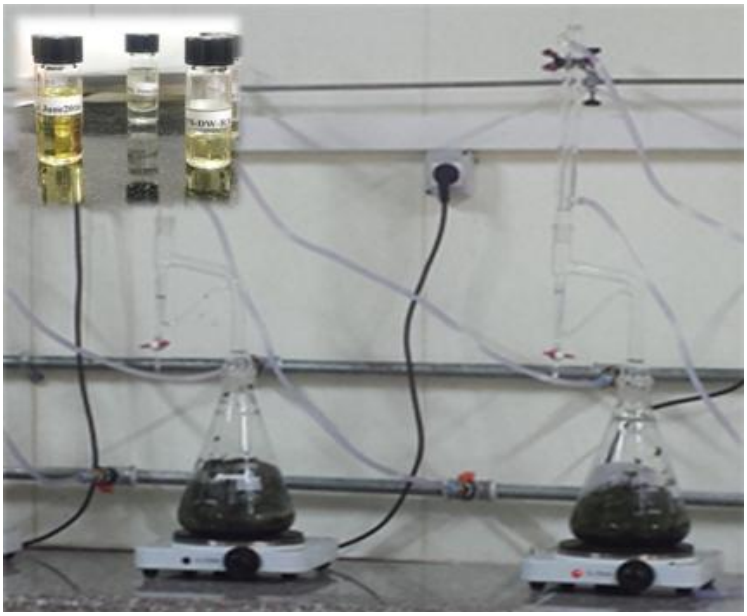

Figure 2.5: Essential oil extraction by hydro distillation using modified Clevenger apparatus.

2.8 Analysis of plant extract and essential oil for antioxidant activity

2.8.1 Preparation of the extracts and solutions

The evaporated methanolic plant extracts were dissolved in absolute methanol to obtain a concentration of 1mg/ml and essential oil were dissolved in absolute methanol to obtain 100µl/ml**.**

2.8.2 Determination of antioxidant activity using DPPH radical

Free radical scavenging activity of extracts was evaluated using the stable free radical DPPH (1,1-diphenly-2-picrylhydrazyl-hydrate) (Sharma & Bhat, 2009). An antioxidant can donate an electron to DPPH, and the purple color formed would indicate free DPPH radical decay and the

change in absorbance at 517 nm can be measured spectrophotometerically. The method of Moniruzzaman et al. (2015) was used to estimate the effect of the plant extracts and EO on DPPH radical. Two ml of methanolic solution of plant extract (1mg/ml), essential oil (100µl/ml) or standard (Trolox) at 800ug/ml was mixed with 3ml (0.02 %) of MeOH solution of DPPH. After incubation for 30 min in the dark the absorbance was measured at 517 nm against MeOH as blank by using spectrophotometer (6800UV/vis, GENWAY).

The radical-scavenging activity of samples, was expressed as percentage inhibition of DPPH (I %), and it was calculated according to the formula:

$$\%\, I \;=\; [(Acontrol - Asample)/Acontrol]x100$$

Where $A_{control}$ is the absorbance of DPPH radical with methanol and A_{sample} is the absorbance of DPPH radical with sample extract/standard.

IC_{50} of extracts, oil and standards were evaluated using linear regression analysis of the dose-%I relationship.

2.8.3 Determination of antioxidant activity using reducing power

The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. Reducing power (RP) was measured by direct electron donation in the reduction of $Fe^{3+}(CN^{-})_6$–$Fe^{2+}(CN^{-})_6$ (Yen & Chen, 1995). The product was visualized by forming the intense Prussian blue color complex and then measured at λ700nm. The RP was determined following the method of Ferreira et al. (2007). Same concentrations of

methanolic plant extracts, essential oil or standards used for DPPH scavenging activity (2.5 ml) were mixed with 2.5 ml of 200 mM Na_3PO_4 buffer (pH 6.6) and 2.5 ml of 1% $K_3[Fe(CN)_6]$. The mixture was incubated at 50 C for 20 minutes. After that 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 650 rpm for 10 minutes. Five mL of the upper layer were mixed with 5 ml deionized water and 1 ml of 0.1% of $FeCl_3$, and the absorbance was measured at 700 nm.

Higher absorbance indicates higher reducing power. The extract effective concentration (EC_{50}) providing 0.5 of absorbance was calculated from the graph of absorbance at 700 nm against extract concentration. Trolox was used as standard.

2.9 Analysis of plant extract and essential oil for anticholinesterase activity using NA-FB method

2.9.1 Principle of the reaction

Cholinesterase inhibitory activity including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was determined spectrophotometrically using (NA-FB) following Ali-Shtayeh et al. (2014). The chemical principle of the reaction is shown in Fig 2.6. The substrate β-naphthyl acetate is hydrolyzed by the enzyme to naphthol and acetate. Naphthol is permitted to react with fast blue B, with the reaction resulting in the development of a purple color. The intensity of the color is measured at 600 nm, and it is proportional to the activity of enzyme.

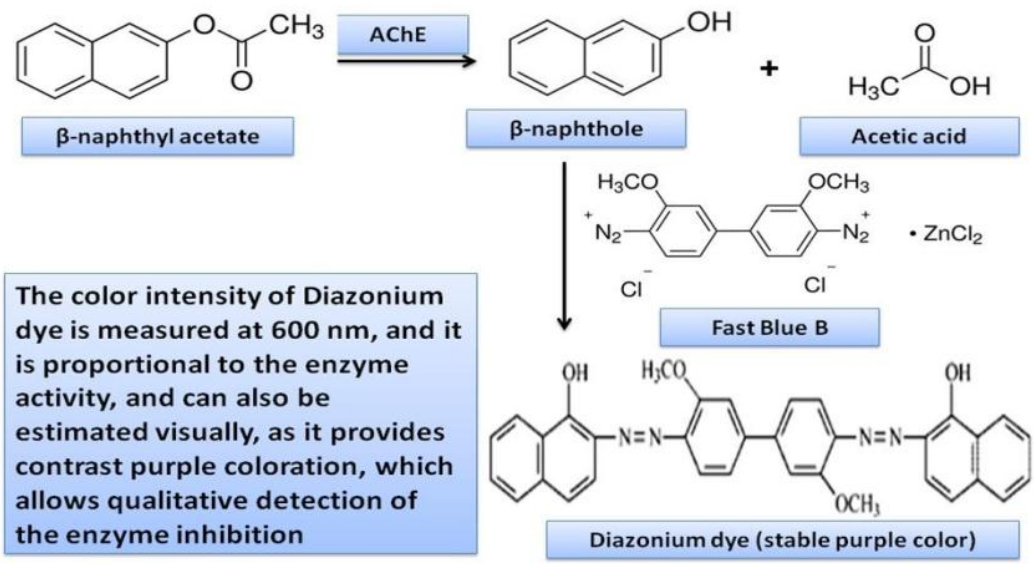

Figure 2.6: Chemical mechanism of (NA-FB) method, Ali-Shtayeh et al. (2014).

2.9.2 Preparation of the extracts, enzymes and solutions

The enzymes (AChE and BuChE) were prepared in buffer (Tris HCl, 20 mM, pH 7.5) to get 1000 U/ml and 500 U/ml respectively stock solutions as described in Ali-Shtayeh et al. (2014). These enzyme stock solutions were kept in -20 C° freezer and further diluted to obtain 3.3 U/ml and 1 U/ml AChE and BuChE, respectively with 20 mM Tris HCl, pH 7.5 buffer containing 0.1% BSA, β-naphtyl acetate (0.25mg/ml) was dissolved in absolute methanol and fast blue B salt (2.5mg/ml) was dissolved in distilled water. Galanthamin (500µg/ml) was extracted from tablets and prepared in distilled water and used as a reference standard in addition to neostigmine bromide. The evaporated methanolic plant extracts were dissolved in water to obtain 10 mg/ml and essential oil were dissolved in 50% DMSO so obtain the required concentration of 50 µl/ml.

2.9.3 Evaluation of AChEI and BuChEI activity

According to Ali-Shtayeh et al. (2014), 30 µl of plant extract or essential oil was placed in Eppendorf tube with 150 µl (0.25mg/ml) of β-naphthyl acetate, and 600 µl of enzyme solution (3.33U/ml for AChE and 1U/ml for BuChE), the mixture was incubated at 4°C for 40 minutes. Later, 30µl (2.5mg/ml) of fast blue B was added to the mixture and the absorbance measured at 600 nm. Blank samples were used to correct the absorption of the mixture, in which the enzyme was replaced with buffer solution.

For the control, 30 µl of water or 50% DMSO was added instead of plant extract or essential oil respectively.

2.9.4 Calculation of Enzyme Activity

The enzyme activity was calculated for each reading as the color changes; purple color indicates negative cholinesterase inhibitory activity while the absence of purple color indicates positive cholinesterase inhibitory activity. Any increase in the absorbance due to substrate spontaneous hydrolysis was corrected by subtracting the absorbance before and after adding the enzyme. The % inhibition of the enzyme activity for each test solution was calculated as described in Ali-Shtayeh et al. (2014) using the following equation:

$$\%\, I \;=\; [(Acontrol - Asample)/Acontrol]x100$$

Where A_{sample} and $A_{control}$ represent the change in the absorbencies of sample and control.

2.9.5 Estimation of IC_{50} Values.

The IC_{50} value (concentration of compounds that inhibits the substrate hydrolysis by 50 %) was determined spectrophotometriclly of the effect of increasing concentrations of test compounds (plant extracts, essential oil and positive controls) on AChE and BuChE activity. To evaluate the IC_{50} values, each plant extract was assayed at seven concentrations starts from 10 mg/ml while essential oil begins from 50 mg/ml Figure 2.7. IC_{50} values were obtained from dose-effect curves by linear regression.

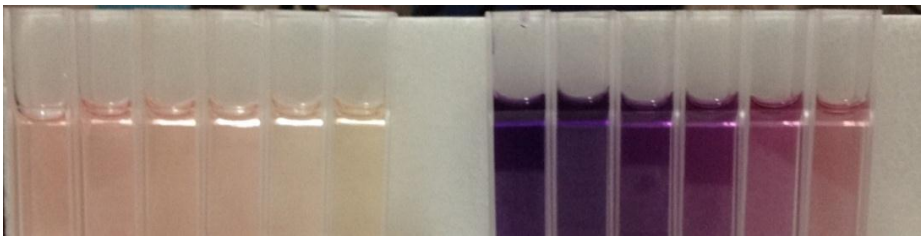

Figure 2.7: Estimation of IC_{50} values using cuvits with different concentrations (mg/ml) of plant extracts, essential oil and their blanks.

2.10 Data analysis

Results of yield biomass quantity and quality parameters were expressed as average (two seasons *four replicates) ± standard deviation (SD). Statistical analysis (ANOVA) by means of the IBM SPSS Statistics for Windows, Version 23.0 (2015) was applied to the data to Determine differences (p = 0.05) between water treatments and between different

tested plant species, in case of significant differences between plant species comparison of means was performed by means of the Tukey-Kramer test at significance level of 0.05.

Chapter three
Results

3.1. Effect of the effluent irrigation on chemical properties of soil and plants

3.1.1 Soil chemical properties

Soil samples were obtained following two years of irrigation with the TE and PW to evaluate cumulative effects on some chemical soil characteristics compared with soil characteristics before planting (Table 3.1). With the exception of EC and Na content, 2-year period of TE irrigation did not affect chemical characteristics of the experimental field soil.

The soil conductivity values EC (micro-Siemens per centimeter, µS/cm) increased significantly compared to the control (935 µS/cm) in soil irrigated with PW and soil under TE irrigation, to reach a maximum value of 1855 and 3620, respectively. Also, Na levels increased significantly in the top 30-cm layer of plots irrigated with TE (0.4-1.04 meq L^{-1}) compared to soil irrigated with PW (0.27-0.58 meq L^{-1}).

Table 3.1: Main parameters measured on soil samples collected at 30-cm soil depth in the test field irrigated with PW and effluent for two consecutive years. Data are ranges (n=5), the treatment effect was estimated using the Tukey-Kramer test at alpha =0.05.

Parameter	Prior irrigation treatment	Potable	Effluent	p-value
EC (µS/cm)	935	1170 -1855	2060-3620	0.03
pH	8.12	7.93-8.17	7.92-8.18	0.33
Cl (ppm)	40	90-220	165-570	0.07
Na (meq/L)	0.17	0.27-0.58	0.4-1.04	0.03
K(meq/L)	0.92	0.46-1.05	0.79-1.18	0.69
Ca (meq/L)	6.5	4.4-6.9	5-12.8	0.60
Ca+Mg (meq/L)	7.5	5.5-7.9	5.9-13.7	0.67
SAR	0.09	0.14-0.32	0.18-0.49	0.05
Cd (ppm)	0.16	0.11-0.12	0.1-0.12	0.01
Fe (ppm)	0.52	0.08-0.24	0-0	0.01
Pb(ppm)	0.73	0.83-0.98	0.84-0.98	0.06
Ni (ppm)	0.3	0.22-0.33	0.09-0.25	0.11
Mn (ppm)	0.72	0.38-4.29	0.39-2.65	0.66
Zn (ppm)	1.72	2.1-2.88	2.29-2.6	0.06
Cu (ppm)	1.49	0.6-2.13	0.41-1.3	0.16

3.1.2 Plant chemical properties

Plant mineral content including Primary (macro) nutrients (phosphorus potassium), secondary nutrients (Magnesium and Sulphur), and micronutrients (cooper, iron, manganese, and zinc) were estimated for the 5 aromatic plants under both irrigation types. The analysis for heavy metals (Fe, Mn, Zn, Cu, Pb, Ni, Cd) were conducted in plant tissues before and after essential oil extraction aiming to evaluate the effect of TE irrigation on the accumulation of heavy metals in plant tissues of the test plants.

Macroelement (primary nutrients) contents were not affected by the treatment (Table 3.2). Contents of K, P in leaves of plants irrigated with PWs were similar to those irrigated with TE. The five plants have similar contents of K and P, with K content ranging between 0.06-0.12 % and the P content ranging between 0.22-0.35 %.

Table 3.2: Plant chemical properties under both irrigation systems

	O. syriacum		*M. fruticosa*		*M. spicata*		*R. officinalis*		*S. fruticosa*	
	Potable water	**Treated effluent**	**Potable water**	**Treated effluent**	**Potable water**	**Treated effluent**	**Potable water**	**Treated effluent**	**Potable water**	**Treated effluent**
K (%)	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
P (%)	0.3±0.0	0.4±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.2±0.1	0.2±0.1	0.3±0.1	0.3±0.1
S (%)	0.3±0.1	0.4±0.1	0.3±0.1	0.3±0.1	0.4±0.1	0.5±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.4±0.1
Mg (%)	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Heavy metals (before essential oil extraction) (ppm)										
Fe	215.8±21.6	195.3±47.4	209.8±57.5	174.6±74.7	156.3±52	182.7±30	157.7±20	133.2±15	154.6±21.6	180.8±16.6
Mn	21.9±5.5	28.4±6.1	25.6±9.9	27.1±9.9	30.9±7.5	28.9±3.9	21.4±1.1	18.7±3.8	18.9±7.7	14±2.4
Zn	28.9±1.7	22.1±3.3	33.1±2.5	28.3±2.2	20.2±4.4	20.6±6.9	16.4±4.5	18.1±1.5	19.8±4.0	21.4±7.6
Cu	14.38±3.9	11.8±10.0	9.5±6.1	7.7±5.4	8.2±2.2	11.8±3.2	8.2±7.0	9.8±3.1	11.5±6.4	10.6±0.3
Pb	0.9±0.3	0.8±0.4	0. 9±0.3	0.8±0.2	0.8±0.3	0.8±0.2	0.6±0.1	0.7±0.1	0.7±0.3	0.8±0.3
Ni	1.8±0.3	1.5±0.7	2.1±0.7	2.3±1.2	0.9±0.1	1.4±0.3	1.1±0.5	0.9±0.5	0.8±0.3	0.8±0.3
Cd	0.1±0	0.1±0.01	0.1±0	0.1±0.01	0.1±0.01	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
Heavy metals (after essential oil extraction) (ppm)										
Fe	211.7	48.3	110.5	99.9	128.0	159.9	101.6	143.7	102.6	76.6
Mn	16.5	2.0	12.8	9.5	19.4	16.2	6.8	15.3	15.9	5.8
Zn	52.4	5.8	34.2	19.9	24.0	31.5	14.3	35.0	36.0	16.0
Cu	12.1	1.3	4.5	2.5	6.5	4.0	3.5	6.0	6.6	4.8
Pb	0.8	0.6	0.8	0.5	0.6	0.6	0.6	0.8	0.7	0.7
Ni	0.7	0.3	0.4	0.4	0.4	0.1	0.3	0.4	0.3	0.2
Cd	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1

Under both irrigation systems, the five plants have similar contents of Mg and S. The Mg levels in the five plants ranged between 0.05-0.13 %.The maximum level of 0.13% was detected in the leaves of *S. fruticosa*. The S levels were ranged between 0.26-0.47 (Table 3.2).

Contents of microelements Zn, Mn, Cu, and Fe did not differ in any of the test crops irrigated with treated effluent or potable water (Table 3.2). Contents of Fe, Mn, Zn, Cu, Pb, Ni and Cd were not affected in any of the tested crops or its plant tissue residue following EO extraction (Table 3.2).

3.2. Effect of irrigation with treated effluent on plants yield parameters

3.2.1 Fresh biomass production

Parameters of biomass yield accumulated through the period from April to October 2016 results are presented in Table 3.3. Fresh biomass production, which is an indicator of aromatic plants yield for the fresh-herb market was evaluated for the five plants, among the five studied crops, *S. fruticosa* showed the highest accumulated fresh biomass (accumulated fresh biomass of 7.71 and 13.99 kg/m^2) compared to *R. officinalis. M. spicata, M. fruticosa and O. syriacum* in which accumulated fresh biomass ranged between 2.72 and 5.08 kg/m^2.

TE has led to an increase in the fresh biomass yields (Table 3.3). The increases were consistent for the five plants. However, this effect of irrigation with TE was only statistically significant (P-value=0.018) in case of *S.*

fruticosa; irrigation with TE increased the fresh biomass 1.8 fold compared to fresh biomass in PW irrigation (fresh biomass in PW =7.71 and in TE 13.99).

The increase in fresh biomass production was not statistically significant in *R. officinalis. M. spicata, M. fruticosa and O. syriacum* (p-values =0.091, 0.462, 0.067, and 0.54, respectively).

Table 3.3: Effect of irrigation with TE on fresh biomass production of selected aromatic plants.

	Origanum syriacum	***Micromeria fruticosa***	***Mentha Spicata***	***Rosmarinus officinalis***	***Salvia fruticosa***
	Fresh biomass (Kg/m^2)				
Potable	4.35±1.93^{b}	2.79±1.05 ^{b}	4.24±0.65 ^{b}	2.72±0.25^{b}	7.71±1.13 ^{a}
Treated effluent	5.08±1.18 ^{b}	4.60±1.23 ^{b}	4.99±1.79 ^{b}	3.40±0.63 ^{b}	13.99±0.92 ^{a}
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.54	n.s., p=0.07	n.s., p=0.05	n.s., p=0.09	p=0.02

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; p≤0.05**.** Means followed by different letters within a row are significantly different according to Tukey-Kramer test at p≤0.05.

3.2.2 Dry Biomass production

The total dry biomass yield and dry matter were evaluated in the fresh biomass after oven desiccation at 64°C for 48 h. (Table 3.4).

The highest dry matter percentage of 35 % was obtained from *O. syriacum* and *M. fruticosa* irrigated with TE, while lowest percentage of 20 % was obtained from *R. officinalis* irrigated with TE (Table 3.4).

The produced dry biomass yield (kg/m^2) was ranged between 0.7 and 3.5*; S. fruticosa* produced the highest dry biomass of 2.04 and 3.5 kg/m^2 whereas minimal dry yield of 0.7 kg/m^2 was produced by *R. officinalis.* However the five aromatic plants dry matter percentages were not significantly affected by irrigation with the TE (Table 3.4).

Table 3.4: Effect of irrigation with TE on dry matter percentage and dry biomass production of selected aromatic plants.

	Origanum syriacum	***Micromeria fruticose***	***Mentha Spicata***	***Rosmarinus officinalis***	***Salvia fruticosa***
Dry matter (%)					
Potable	32.27±3.23^a	31.84±0.82^a	24.31±2.67^b	25.84±2.05^b	26.95±2.29^b
Treated effluent	35.11±3.67^a	35.82±4.99^a	25.69±1.98^b	20.56±1.91^b	26.40±2.54^b
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.29	n.s., p=0.17	n.s., p=0.44	n.s., p=0.09	n.s., p =0.46
	Total dry matter (Kg/m^2)				
Potable	1.38±0.58^b	0.89±0.34^b	1.02±0.1^b	0.70±0.10^b	2.04±0.22^a
Treated effluent	1.77±0.41^b	1.69±0.65^b	1.27±0.43^b	0.71±0.19^b	3.53±0.22^a
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.31	n.s., p=0.07	n.s., p=0.29	n.s., p=0.98	p=0.02

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; p≤0.05**.** Means followed by different letters within a row are significantly different according to Tukey-Kramer test at p≤0.05.

3.2.3 Dry leaf biomass

Leaf dry weight was evaluated after separation from stems, as an indicator of marketable dry herb yield. The % dry weight biomass of leaves was evaluated after desiccation at 40°C**.**

Percentage of dry leaves from total weight of fresh biomass was calculated for the five aromatic plants, the percentage of dry leaves was ranged between 13.66 and 22.74 % (Table 3.5). Under irrigation types, *M. fruticosa* and *M. spicata* produced the lowest percentage of dry leaves whereas *S. fruticosa*, *R. officinalis* and *O. syriacum* produced relatively higher percentages (18.3-22.74%).

S. fruticosa produced the highest amount of dry leaves (1.36 and 2.63 kg/m^2) whereas *R. officinalis, M. spicata*, *M. fruticosa and O. syriacum* produced comparable amount of dry leaves. However irrigation with TE has significant effect on *S. fruticusa* leaves dry yield (p-value=0.018).

Table 3.5: Effect of irrigation with TE on percentage of dry leaves from total fresh weight biomass of selected aromatic plants.

	Origanum syriacum	***Micromeria fruticosa***	***Mentha spicata***	***Rosmarinus officinalis***	***Salvia fruticosa***
	Percentage of dry leaves from total fresh weight biomass (%)				
Potable	19.72±2.71^{a}	14.67±0.24^{b}	14.87±1.15^{b}	22.65±0.95^{a}	18.90±3.65^{ab}
Treated effluent	18.84±2.1^{a}	13.66±0.99^{b}	13.75±1.8^{b}	22.74±2.99^{a}	18.30±2.74^{a}
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.68	n.s., p=0.09	n.s., p=0.33	n.s., p=0.95	n.s., p=0.80
	Total dry leaves biomass Kg/m^2				
Potable	0.78±0.31^{b}	0.41±0.15^{b}	0.63±0.12^{b}	0.62±0.08^{b}	1.36±0.36^{a}
Treated effluent	0.96±0.25^{b}	0.62±0.15^{b}	0.66±0.16^{b}	0.78±0.22^{b}	2.63±0.30^{a}
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.42	n.s., p=0.09	n.s., p=0.77	n.s., p=0.20	p=0.02

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; p≤0.05**.** Means followed by different letters within a row are significantly different according to Tukey-Kramer test at p≤0.05.

3.2.4. Essential oil yield

Essential oil yield was determined by hydrodistillation of fresh aerial parts of plants, under both types of irrigation. Comparable essential oil yields were produced by the test plants (Table 3.6). The extracted essential oil yields ranged between 5.85 and 12.93 ml/kg. The highest essential oil yields were obtained from *O. syriacum* (12.15, 12.93 ml/kg) and *M. fruticosa* (11.85 and 12.52 ml/kg), whereas the lowest yields were extracted from *M. spicata* (5.85 and 6.85 ml/kg).

Production of EO yield was not affected by irrigation with the TE; oil-yield in plants under irrigation with TE was comparable to those under PW irrigation (Table 3.6).

Table 3.6: Effect of irrigation with TE on the EO yield from fresh areal parts of the aromatics plants.

	Origanum syriacum	***Micromeria fruticose***	***Mentha Spicata***	***Rosmarinus officinalis***	***Salvia Fruticose***
	Essential oil yield ml/Kg				
Potable	12.93±0.46^{a}	11.85±0.75^{a}	6.85±0.44^{c}	8.60±0.95^{b}	8.80±0.85^{b}
Treated Effluent	12.15±1.10^{a}	12.52±0.79^{a}	5.85±0.50^{c}	8.85±0.81^{b}	8.40±0.89^{b}
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.24	n.s., p=0.26	n.s., p=0.06	n.s., p=0.70	n.s., p=0.56
	Total essential oil yield ml/m^2				
Potable	55.86±24.35^{b}	33.50±14.62^{bc}	28.88±3.54^{bc}	23.25±1.25^{c}	69.93±5.03^{a}
Treated Effluent	61.26±12.77^{b}	57.15±13.63^{b}	28.50±7.91^{c}	30.45±8.38^{c}	118.71±9.69^{a}
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.71	n.s., p=0.06	n.s., p=0.93	n.s., p=0.14	p=0.01

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; $p \leq 0.05$. Means followed by different letters within a row are significantly different according to Tukey-Kramer test at $p \leq 0.05$.

3.3 Effect of irrigation with treated effluent on antioxidant activity and phenolic compounds content

3.3.1 Antioxidant activity

Extracts and essential oils were tested for their antioxidant potential using two different methods including DPPH and Reducing Power assay.

3.3.1.1 Methanolic extracts DPPH Free radical scavenging capacity

Aromatic plants methanolic extracts were tested in a preliminary assay at a single concentration of 1 mg/mL compared to standard antioxidants (BHA, BHT, Torolox, chlorogenic acid and ascorbic acid) at a concentration of 0.8 mg/mL. At a concentration of 1mg/mL, all methanolic extracts had antioxidant activities more than 50 %. Therefore, the IC_{50} values were determined for these plants extracts (Table 3.7). All the extracts evaluated had dose-dependent inhibition. The best scavenging free radical effect was recorded for methanolic extracts of *M. spicata* (IC_{50}=0.13 mg/mL) and *R. officinalis* (IC_{50}=0.15 mg/mL).

Table 3.7: Antioxidant activity of methanolic extracts under irrigation with treated effluent. Analysis of variance at P = 0.05.

	IC50 (mg/ml)		ANOVA
Plant species	Potable	Effluent	
Origanum syriacum	0.53±0.01[c]	0.45±0.04[c]	n.s., *p*=0.16
Micromeria fruticosa	0.48±0.05[c]	0.54±0.04[d]	n.s., *p*=0.10
Mentha spicata	0.13±0.02[a]	0.13±0.01[a]	n.s., *p*=0.96
Rosmarinus officinalis	0.15±0.01[a]	0.15±0.00[a]	n.s., *p*=0.85
Salvia fruticosa	0.25±0.03[b]	0.28±0.00[b]	n.s., *p*=0.08
Standard antioxidants			
BHA			
BHT			
Trolox			
Chlorogenic acid			
Ascorbic acid			

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; p≤0.05**.** Means followed by different letters within a column are significantly different according to Tukey-Kramer test at p≤0.05.

3.3.1.2 Essential oil DPPH Free radical scavenging capacity

Free radical scavenging capacities were studied using DPPH free radical.

Essential oils were tested in a preliminary assay at a single concentration of 100 µl/mL (Figure 3.1). The essential oil from *O. syriacum* had the highest DPPH antioxidant activity (with 100 % inhibition) followed by *R. officinalis* (71%). *S. fruticose* and *M. spicata* had the lowest DPPH antioxidant activity with 35 - 37 % inhibition.

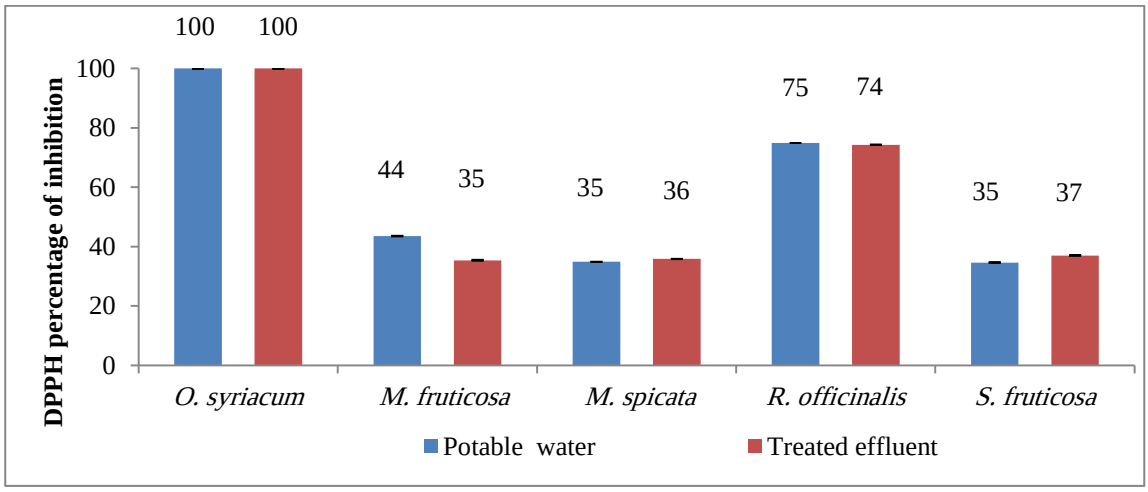

Figure 3.1: Scavenging activity (%) on DPPH radicals of essential oil from selected aromatic plants.

Only *O. syriacum* and *R. officinalis* essential oils were active enough to determine the IC_{50} values (Figure 3.2). Both had dose-dependent inhibition. Regardless of irrigation type, essential oils from PW and TE *O. syriacum* plants showed the highest DPPH radical scavenging capacity (IC_{50} of 0.17-0.36 µl /ml).

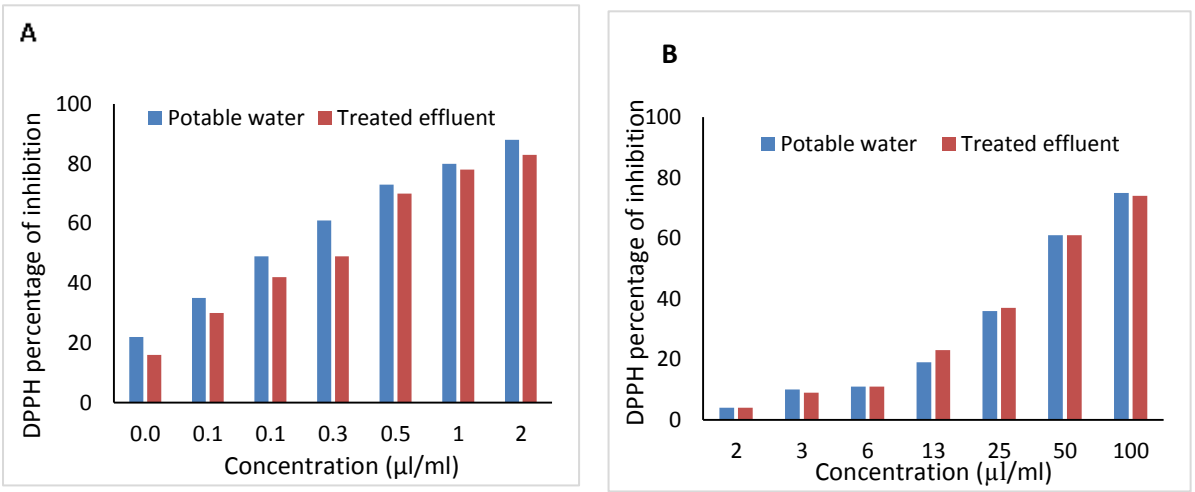

Figure 3.2: Dose- dependent DPPH antioxidant activity of *O. syriacum* (a) and *R. officinalis* (b) EO under both irrigation types (PW and TE).

3.3.2. Reductive potential

3.3.2.1 Methanolic extracts reductive potential activity

All evaluated methanolic extracts exhibited high reductive potential activities with RP_{50} ranged between 0.16 and 0.65 mg/ml (Table 3.8, Figure 3.3). The best reductive potential effect was recorded with methanolic extracts of *M. spicata* (RP_{50}=0.16-0.18 mg/mL) and *R. officinalis* (RP_{50}=0.2 and 0.21 mg/mL).

Table 3.8: Reductive potentials of methanolic extracts under irrigation with treated effluent. Analysis of variance at P = 0.05.

	RP_{50} (mg/ml)		ANOVA
Plant species	Potable	Treated Effluent	
Origanum syriacum	0.35±0.02[b]	0.34±0.05[b]	n.s., *p*=0.70
Micromeria fruticosa	0.65±0.12[c]	0.64±0.05[c]	n.s., *p*=0.91
Mentha spicata	0.16±0.06[a]	0.18±0.05[a]	n.s., *p*=0.52
Rosmarinus officinalis	0.20±0.01[a]	0.21±0.02[a]	n.s., *p*=0.51
Salvia fruticosa	0.27±0.04[ab]	0.33±0.02[b]	n.s., *p*=0.05
Standard antioxidants			
BHA			
BHT			
Trolox			
Chlorogenic acid			
Ascorbic acid			

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; p≤0.05**.** Means followed by different letters within a column are significantly different according to Tukey-Kramer test at p≤0.05.

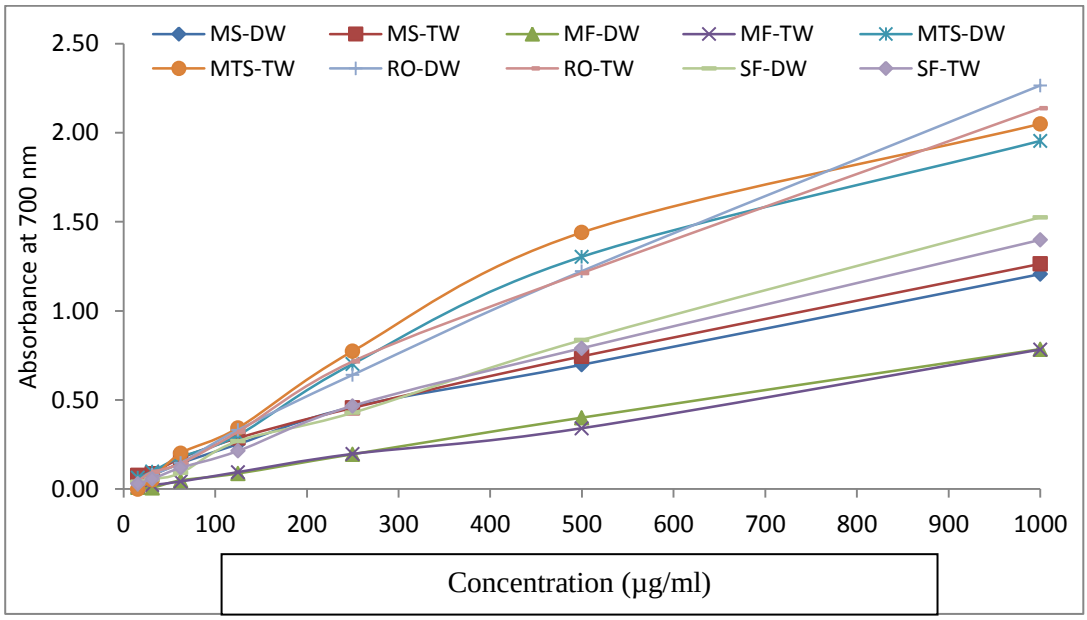

Figure 3.3: Reductive potential of the methanolic extracts under irrigation with PW and TE.

3.3.2.2 Essential oil Reductive Potential

Fe (III) reduction can be used as an indicator of electron-donating capacity and therefore reflects an antioxidant action. In this study, the reducing power was evaluated by monitoring the ferric-ferrous transformation at 700 nm. The reducing ability generally increased with increasing sample concentration.

O. syriacum oil had the best significant reductive potential (Figure 3.4), in contrast to that of other essential oil (RP_{50}=0.48-0.51 µl/ml) followed by *R. officinalis* (RP_{50}=70 and 71 µl/ml).

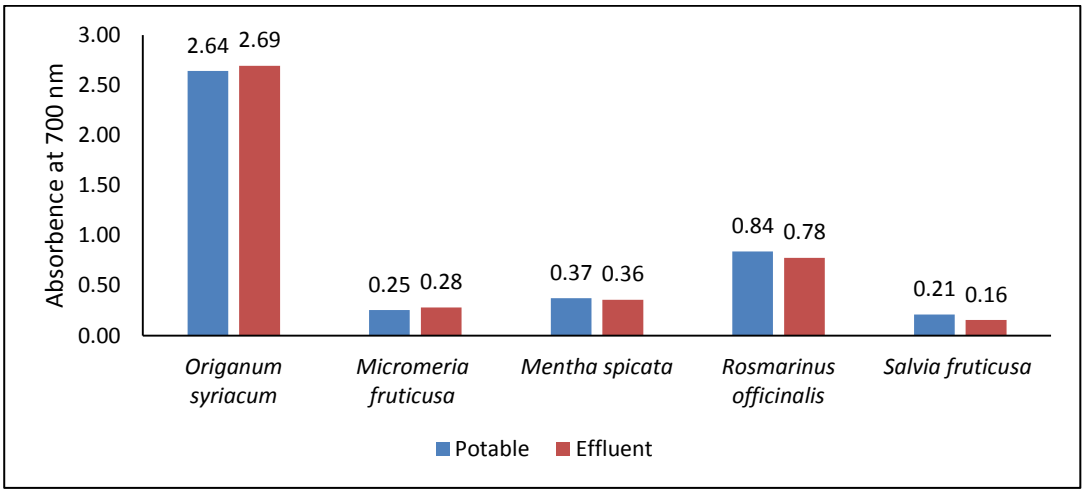

Figure 3.4: Reductive potential of the essential oils under irrigation with treated effluent.

Nevertheless it is noteworthy to stress the great difference between the oil of *O. syriacum* and the remaining essential oils, as already seen for the other method used (DPPH). There was also a dose-dependent behavior as seen in Figure 3.5.

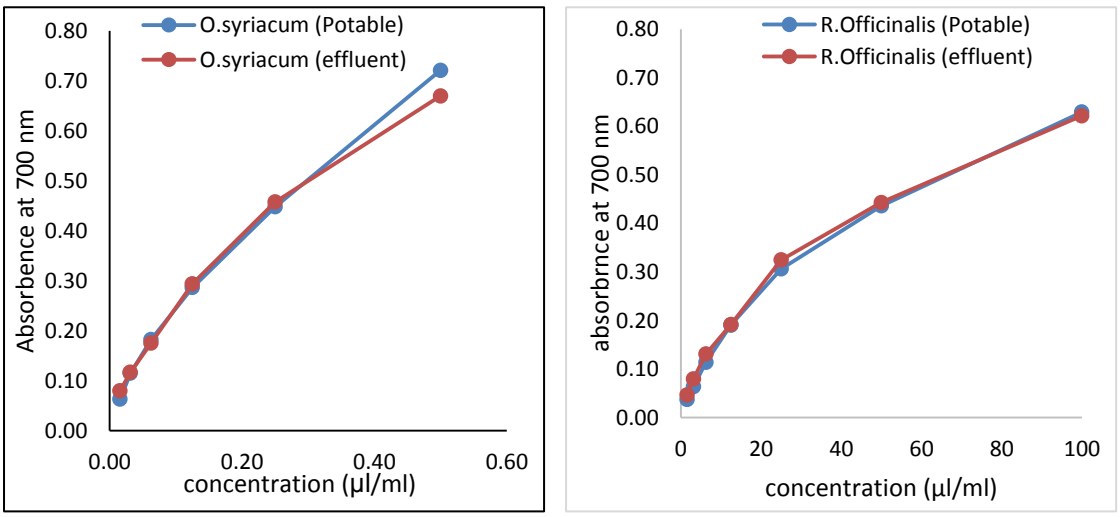

Figure 3.5: Reductive potential of the *O. syriacum* EO (a) and *R. officinalis* EO (b) under both irrigation types.

O. syriacum and *R.officinalis* essential oil results showed an evident positive correlation between DPPH radical scavenging ability and the reducing power assay, which indicated that the reducing ability of the oils contributed in part to the antioxidant activity.

3.3.3 Determination of total phenolic content

The total phenolic contents in the plant extracts using the Folin-Ciocalteu's reagent was expressed as gallic acid equivalent (Y= 0.0004X+0.019, R^2= 0.9977). The values of total phenols concentrations were expressed as mg of GA/g of extract (Table 3.9). The amount of total phenolics varied widely in plant materials and ranged from 4.09 to 26.6 mg GAE/g dry material (Table 3.9). Among the five aromatic plants low levels were found in *M. fruticosa* (4.09-4.86 mg/g GAE) whereas *R. officinalis* contained relatively high amounts of phenolics (23.84-26.6 mg/g GAE). Moderate levels were found in *O.syriacum* (19.29-21.2 mg/g GAE), *M.spicata* (16.83-19.4 mg/g GAE) and *S. fruticosa* (14.9-15.56 mg/g GAE).

Table 3.9: Effect of irrigation with TE on total content of phenolic compounds expressed as Gallic acid equivalents.

	Origanum syriacum	***Micromeria fruticose***	***Mentha Spicata***	***Rosmarinus officinalis***	***Salvia Fruticose***
	Total phenolic content (mg/gm)				
Potable	19.29±3.09^{b}	4.09±1.38^{c}	14.42±5.82^{bc}	23.84±3.47^{a}	14.90±4.87^{bc}
Treated Effluent	21.22±2.57^{b}	4.86±1.52^{c}	16.83±8.62^{bc}	26.60±3.42^{a}	15.56±4.23^{bc}
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.20	n.s., p=0.31	n.s., p=0.49	n.s., p=0.31	n.s., p =0.78

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; p≤0.05**.** Means followed by different letters within a row are significantly different according to Tukey-Kramer test at p≤0.05.

3.3.4. Determination of total flavonoids content

The concentration of flavonoids in various plant species was determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalent ($Y=0.0061X+0.0523$, $R^2= 0.9974$), mg of Q/g of extract (Table 3.10). The concentration of flavonoids in plant extracts ranged from 8.78 to 16.67 mg/g. *M. spicata* extract contains the highest flavonoid concentration (16mg Q/g). The concentration of flavonoids in *S.fruticosa* was 13mg Q/g, which was very similar to the value of *M. fruticosa* extract concentration. The lowest flavonoid concentration was measured in *O. syriacum* extract.

Table 3.10: Effect of irrigation with TE on total content of flavonoids expressed as quercetin equivalents.

	Origanum syriacum	***Micromeria fruticose***	***Mentha Spicata***	***Rosmarinus officinalis***	***Salvia Fruticose***
	Total flavonoid content (mg/gm)				
Potable	8.78±2.06 [c]	12.91±1.03 [b]	16.67±1.39 [a]	10.53±1.39 [ab]	12.72±3 [b]
Treated Effluent	9.42±1.45 [c]	12.26±1.31 [b]	15.69±1.61 [a]	10.95±0.71 [ab]	12.7±1.24 [b]
ANOVA					
Source of variation					
Water treatment	n.s., p= 0.48	n.s., p=0.28	n.s., p=0.22	n.s., p=0.25	n.s., p =0.83

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; $p \leq 0.05$**.** Means followed by different letters within a row are significantly different according to Tukey-Kramer test at $p \leq 0.05$.

3.3.5 HPLC analysis of the polyphenolic compounds

In all plant species, the content of the carnosic acid and carnosol were not affected by the TE irrigation except for *S. fruticosa* and *R. officinalis* that

shown the highest content of carnosic acid and carnosol, respectively comparable to other plant species (Table 3.11).

Table 3.11: Effect of irrigation with TE on plant content of carnosic acid and carnosol. Data are averages ± SD (n=8).

	Carnosic acid		**Carnosol**	
Species	Potable	Treated Effluent	Potable	Treated Effluent
	% of dry weight			
Mentha spicata	0.05±0.0	0.04±0.0	0.04±0.0	0.04±0.0
Micromeria fruticosa	n.d	n.d	0.20±0.0	0.21±0.0
Origanum syriacum	0.06±0.0	0.04±0.0	0.09±0.0	0.07±0.0
Rosmarinus officinalis	0.06±0.0	0.06±0.0	0.37±0.0	0.44±0.0
Salvia fruticosa	0.72±0.0	0.56±0.0	0.05±0.0	0.05±0.0
n.d: not determined				

3.4 Effect of irrigation with treated effluent on anticholinestrase activities

The inhibitory activity of the plants essential oil and methanolic extracts against AChE and BuChE was measured according to the colorimetric assay of Ali-Shtayeh et al 2014. Neostigmine bromide and galanthamin were used as the reference compounds.

The IC_{50} values of all tested extracts and essential oil and their selectivity index for AChE and BuChE are summarized in Tables 3.12 and 3.13. In general, under both irrigation systems aromatic plants essential oil and methanolic extracts showed moderate to good inhibitory activity on AChE and BuChE with IC_{50} ranged between 1.99 and 52 µl/ml and 0.6 and 3.7 µl/ml respectively.

3.4.1. Methanolic extracts Anticholinestrase activities

The methanolic extracts of *M. spicata* showed the highest AChE inhibitor (IC_{50}=0.6 and 0.6) and exhibited the strongest inhibitor to BuChE, with an IC_{50} values 1.93 and 2.34 mg/L. Over all, the tested methanolic extracts were found to be more selective inhibitors on AChE than BuChE with selectivity index (SI) values ranged between 1.23 and 3.85 (Table 3.12).

Table 3.12: Anticholinesterase activity of methanolic extracts under irrigation with treated effluent. Analysis of variance at P = 0.05.

	Origanum syriacum	***Micromeria fruticosa***	***Mentha spicata***	***Rosmarinus officinalis***	***Salvia fruticosa***	***Galanthamin***	***Neostigmine bromide***
	AChE IC50(mg/ml)						
Potable	3.12 ± 1.55^{b}	2.91 ± 0.52^{ab}	0.64 ± 0.11^{a}	3.24 ± 0.60^{b}	1.46 ± 0.46^{ab}	0.0076	0.0008
Treated Effluent	2.29 ± 1.41^{ab}	3.54 ± 0.46^{b}	0.6 ± 0.07^{a}	3.69 ± 0.57^{b}	1.68 ± 0.71^{ab}		
ANOVA							
Source of variation							
Water treatment	n.s., p= 0.46	n.s., p=0.14	n.s., p=0.59	n.s., p=0.51	n.s., p =0.79		
	BuChE IC50(mg/ml)						
Potable	5.56 ± 0.43^{c}	4.4 ± 0.24^{b}	1.93 ± 0.82^{a}	3.86 ± 0.21^{b}	3.9 ± 0.72^{b}	0.229	0.104
Treated Effluent	5 ± 0.84^{b}	4.44 ± 0.27^{b}	2.34 ± 0.53^{a}	4.45 ± 1.24^{b}	4.44 ± 0.88^{b}		
ANOVA							
Source of variation							
Water treatment	n.s., p= 0.28	n.s., p=0.83	n.s., p=0.44	n.s., p=0.41	n.s., p =0.38		
	Selectivity index(SI) (BuChE/AChE)						
Potable	2.13±0.94	1.67±0.39	2.93±0.86	1.23±0.29	2.50±0.37	30.01	131.6
Treated Effluent	3.23±2.29	1.3±0.25	3.85±0.45	1.39±0.78	2.95±1.3		

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; p≤0.05**.** Means followed by different letters within a row are significantly different according to Tukey-Kramer test at p≤0.05.

3.4.2. Essential oil anticholinestrase activities

The anticholinesterase activities were dose-dependent. Lower concentrations of *O.syriacum* essential oil were needed for inhibiting at least 50% the AChE activity, whereas the remaining oils needed more elevated concentrations. This was also confirmed when IC_{50} values were determined: 2.14 and 1.99 mg/mL for *O. syriacum*, significantly inferior to the remaining oils (Table 3.13) The poorest AChEI & BuChE activities were obtained with the oils of *M spicata* (IC_{50}=50 and 52 µl/ml).

O. syriacum, M. fruticosa, R. officinalis, and S. fruticosa essential oils were found to have comparable activities on BuChE, with an IC_{50} values ranged between 2.04 and 4.88. Whereas *M. spicata* has significantly lower activity with IC_{50}=8.13 and 8.19 mg/ml.

Over all the essential oils were more selective inhibitor on BuChE with selectivity index values ranged between 0.12-1.82 (Table 3.13).

Table 3.13: Anticholinesterase activity of EOs under irrigation with treated effluent. Analysis of variance at P = 0.05.

	Origanum syriacum	***Micromeria fruticosa***	***Mentha spicata***	***Rosmarinus officinalis***	***Salvia fruticosa***	***Galanthamin***	***Neostigmine bromide***
	AChE IC50(µl/ml)						
Potable	2.14±1.34[a]	13.37±3.33[b]	50±1.02[b]	19.11±2.65[b]	16.26±4.74[b]	0.0076	0.0008
Treated Effluent	1.99±0.29[a]	24.27±9.02[b]	52±2.26 [c]	19.23±8.33[b]	20.07±6.97[b]		
ANOVA							
Source of variation							
Water treatment	n.s., *p*= 0.84	n.s., *p*=0.09	n.s., *p*=0.11	n.s., *p*=0.98	n.s., *p* =0.41		
	BuChE IC50(*µl/ml*)						
Potable	2.77±1.88[a]	2.73±0.44[a]	8.19±2.17[b]	4.35±1.8[a]	2.04±1.41[a]	0.229	0.104
Treated Effluent	3.63±2.21[a]	3.42±0.66[a]	8.13±1.69[b]	4.88±3.05[ab]	2.43±0.85[a]		
ANOVA							
Source of variation							
Water treatment	n.s., *p*= 0.57	n.s., *p*=0.13	n.s., *p*=0.97	n.s., *p*=0.57	n.s., *p* =0.65		
	Selectivity index(SI) (BuChE/AChE)						
Potable	1.26±0.41	0.21±0.06	0.16±0.05	0.22±0.08	0.13±0.11	30.01	131.6
Treated Effluent	1.82±0.27	0.15±0.04	0.16±0.03	0.24±0.06	0.12±0.04		

Data are averages ± SD, (n=8). The significance of treatment effect is given as n.s.: not significant; p≤0.05**.** Means followed by different letters within a row are significantly different according to Tukey-Kramer test at p≤0.05.

Chapter four
Discussion

Perennial medicinal and aromatic plants (MAPs) are grown as agro-industrial crops for medicinal products. Such crops, however, require large quantities of irrigation water, up to 9000m^3ha^{-1} $Dunum^{-1}$per growing season, to meet their capacity for industrial biomass production (Dudai, 2005). Large areas of these crops are needed to develop a commercially feasible agro-industrial production scheme. So, scarcity of PW for irrigation in Palestine limits application of aromatics as agro-industrial crops. Replacement of PW with TE for irrigation of aromatics could encourage the expansion of large-scale agro-industrial production systems for EOs, dry and fresh biomass, antioxidants and other bioactive compounds and drugs in Palestine. However, the quality of TE is thought to affect produced secondary metabolites in plants and hence the composition and activity of EOs and other medicinal products of aromatic plants (Bernstein et al., 2009).

Antioxidant and Anticholinesterase potentials, and Phenolic Compounds Content

Plants produce ROS as a byproduct of the aerobic metabolism, also exposure of plants to stresses (e.g., salinity, heavy metals) induces the formation of these toxic byproducts (Apel and Hirt, 2004; Mittler, 2002). To decrease the elevated levels of ROS during oxidative stress conditions, some plants rise antioxidant activity by producing antioxidants. In such

plants, encouraged production of antioxidants indicates the exposure to less than optimal conditions. The phenolics present in plants have manifold biological properties, including antioxidant potentials (Kahkonen et al., 1999).

Phenolic compounds are mainly found in aromatic plants. The antioxidant potential of phenolic compounds can be attributed to their oxidoreduction activities and chemical compounds, which can perform an essential role in the removal of reactive oxygen species (e.g., free radicals, singlet and O_3, peroxides (Zheng & Wang, 2001). Due to the carcinogenic potential of manufactured antioxidants, natural phenolic antioxidants are being recommended as food and diet additives (Shetty, 1997; Botsoglou et al., 2002).

Aromatic plants are considered a natural source of antioxidants (Almela et al., 2006). Among the five aromatics tested in the present study, *O. syriacum* showed the highest DPPH and reductive potential antioxidant activity. This can be attributed to both the plants EO and soluble phenolics (Engleberger et al., 1988). In rosemary plants, the major antioxidant active phenolic compounds were reported to be rosmarinic and carnosic acid (Almela et al., 2006). Chloroplasts are protected from oxidative stress by carnosic acid in vivo by following an extremely regulated compartmentation of oxidation products (Munné-Bosch and Alegre, 2001).

Various studies were performed concerning anticholinesterase activity of extracts or extracted compounds from plants; however, up to

now, many studies were focused on anti-AChE activity, not BChE. Some of them are used to reduce the symptoms of neurodegenerative disorders, such as AD (Jung & Park, 2007). However, BChE plays an important role as AChE, and the inhibition of just AChE can lead to a compensation mechanism and increased activity of the other one (Houghton et al., 2006, Mukherjee et al., 2007). Therefore, there is a need to search for inhibitors not only of AChE, but also of BChE (Ali-Shtayeh et al., 2018 a,b).

In general, all tested methanolic plant extracts in this study had significant inhibition to AChE more than BChE with selectivity index (SI) values ranged between 1.23 and 3.85 (Table 3.12). While their EOs were more selective inhibitor on BChE with selectivity index values ranged between 0.12-1.82 (Table 3.13). Among results, *M. spicata* extract shows the highest flavonoid content therefore highest AChEI and BChEI activity. Meanwhile, the antioxidant activity also showed positive correlation with anti-cholinesterase activity of extracts. However, the strength of correlation is based on the antioxidant assay used. Previous study by Ferreira et al. (2006), showed that most of the medicinal plants from Portugal have positive correlation between the antioxidant and acetylcholinesterase inhibitory activity.

Effect of irrigation with TE on yield parameters

The applied TE was characterisedcharacterized by higher electrical conductivity (i.e., salinity) and pH values than PW. It contained higher levels of probably harmful salts (Na^{+1}, Cl^{-1}), heavy metals, and macro- and

microelements (K, N-NO_3, SO4-S, Ca+Mg, B and Fe) than the PW (Table2.1). However irrigation with TE did not affect plant growth and crop production in any of the tested plants. Thus, no effects on any economically important yield parameters for the aromatic plants including fresh and dry herbs, and EOs (Tables 3.3 - 3.6).

Feigin et al., 1991 showed that high B content and salinity found in TE may have effects on plant development and yield characteristics. The absence of harmful effects on the aromatics in the present study due to irrigation with TE may indicate their endurance to the levels of the minerals found in the TE.

Treated effluent usually has pH higher than PW from which they were obtained, and ranges between 7.5 - 8.5 (Feigin et al., 1991). TE used for this study has a pH ranging between 6.93 and 7.95 which was approximately one unit more than local PW (Table 2.1). At pH 7.6, many plant species exposed to root injury (Zieslin and Abolitz, 1994). High pH causes modifications in the solubility of compounds in the fertilization and soil leading to changes in the availability of nutrients to the plant roots and ion adsorption. The level of pH of saturated soil pastes were identical in the two water qualities, 7.93-8.17 for soil irrigated with PW and 7.92-8.18 for soil irrigated with TE at 0 to 30 cm depth (Table 3.1). So, the absence of negative effects on the plants due the higher pH value of the effluent (up to 7.95) can be attributed to short period of contact to the high pH, during irrigation, as well as plant resistance (Bernstein et al., 2009).

Effect of the effluent irrigation on chemical properties of soil and plants

The pH value, K, Cl, Ca, levels in soil were not affected by the irrigation qualities, the maximum value of SAR (which indicates sodium content relative to Ca + Mg) was 0.49, which is less than the level expected to induce damage to soils. Heavy metals concentration (Ni, Mn, Zn, Pb, and Cu) were not affected by either irrigation with potable or treated water (Table 3.1). However, small amounts of salts present in the TE was accumulated in the soil (Table 3.1).

This is manifested in measurements of ion contents in the plant tissues (Table 3.2). Effluent did not affect the inorganic mineral contents (Table 3.2), also the contents of Mg, S, P and K did not vary in plants irrigated with TE or PW (Table 3.2) and neither their heavy metals content: Ni, Cd, Pb, Cu, Zn, Mn and Fe. Under both irrigation systems, the five plants have similar contents of Mg, S, P, K and heavy metals. Previous study by Bernstein et al., 2009 showed that Oregano and Rosemary were not affected by the effluent.

Previous studies have also investigated the effect of high pH levels of irrigation water on aromatic plants. Gönüz et al. (1999) found pot marjoram (*Origanum onites*) to be abundant in soils with pH 7.99. Dagar et al. (2004) also demonstrated that palmarosa and East Indian lemongrass grew effectively on alkaline soils (pH up to 9.2). Gupta et al. (2002) found

that field mint (*Mentha arvensis*), to grow in soil with pH of 8.2 with no injury symptoms on the plants.

High levels of salinity is knowgn to constrain plant growth and development (Bernstein et al., 1995; Bernstein and Kafaf , 2000). In this study, during the summer months the concentrations of Cl and Na in the TE reached 338 and 87 mg/l, respectively. The EC value of 1551 dS/ cm, about two-fold higher than in PW (Table 2.1); yet no signs of salinity damage were seeming on the TE-irrigated plants (Tables 3.3, 3.4 and 3.5). Plant production and oil quantity did not differ after 2 seasons of exposure to the TE in comparison to PW (Tables 3.3 and 3.6).

This is an indication that the threshold for damage of all species due to salinity demonstrated in this study was higher than 1551 dS/ cm. Other aromatics showed modest tolerance to salinity. For example, mentha plants have survived at high levels of salinity, with about 65% of the yield obtained under 1 M sodium chloride (El-Keltawi & Croteau, 1987). Ozturk et al. (2004) showed that Lemon *Melissa officinalis* was modestly tolerant to salinity (optimal growth ranged between 1 -2 dS /m, and threshold level death of 6 dS/ m salinity in irrigation water.).

Conclusions

In the present study, we assessed the effects of irrigation with TE on EO yield quality and quantity and on the plants antioxidant and cholinesterase inhibitory activities. The results for quantity and quality of the EO yield, phenolic antioxidants and anticholinesterases exhibit the potential of these plants for rigorous high-quality industrial crop systems. The EOs produced by the five species, 0.6 to 1.3% of the fresh biomass, are well within the acceptable range for an intensive oil, antioxidants and cholinesterase inhibitors industrial production.

The results indicated that among the test MAPs can be considered as potent sources of natural inhibitors of AChEIs (e.g., MeOH extracts of *M. spicata, S. fruticosa,* and *O. syriacum*), and BChEIs (e.g., MeOH extract of *M. spicata,* and especially EOs of *S. fruticosa, Micromeria fruticosa,* and *O. syriacum*; with the exception of *O. syriacum* EO which shown to be AChEI selective, all other EOs tested were BChEI selective), with powerful antioxidant properties that might be used in the food stabilization and the prevention of Alzheimer's disease as a complementary pharmacological drug.

Appendix

APPENDIX A: Plant, soil and water analysis

- The procedure used for plant analysis followed AOAC 1995 for Sodium, Potassium and heavy metals determination. Five grams of dried plant samples were transferred to cold temperature-controlled furnace and slowly raise temperature 500°C set control and check for maintenance of 500°C ash for 2hrs remove sample, let cool to room temperature, cautiously add 2ml HNO3 and swirl, evaporate carefully just to dryness on warm hot plate then transfer to cooled furnace and slowly raise temperature to 500°C and hold at this temperature 1h remove dish and cool, add 10ml 1N HCL and dissolve ash by heating cautiously on hot plate , transfer to 25 ml volumetric flask , heated ash residue again successively, cool, filter by what man #42, dilute to volume with 1N HCL, and mix. The extract analyzed for Na, K by Flame Photometer and heavy metals using Flame Atomic Absorption Spectrophotometer.

- For soil analysis extracts were prepared by placing 5g of an air- dried , ground and sieved sample in an Erlenmeyer flask, adding 20ml of extracting solution (0.05N HCL + 0.025N H2SO4) place in a mechanical shaker for 15 min, and then filter through what man #42 filter paper into a 50-ml volumetric flask and dilute to 50 ml with extracting solution and measured by Flame Atomic Absorption Spectrophotometer.

- For water analysis, water samples were digested according to Siraj and Kitte, 2013.

  To ensure the removal of organic impurities from the samples and thus prevent the interference in analysis, the samples were digested with concentrated Nitric acid. 5 mL of conc. HNO3 was added to 100 mL of sampling water into the 250 mL conical flask then heated on a hot plate and evaporated till 20 mL was left. After cooling the flask

  again 5 mL of conc. HNO3 was added and heated the flask on the hot plate. The digestion was continued till 10 mL was left and finally filtered and diluted with distilled water into 100 mL of volumetric flask and stored in the refrigerator.

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جامعة النجاح الوطنية

كليه الدراسات العليا

الأنشطة المضادة للأكسدة والكولين ايستريز للزيوت الأساسية من نباتات عطرية تروى بمياه عادمة معالجة

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قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم الحياتية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

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الملخص

خلفية: تملي ندرة المياه في جميع أنحاء العالم استخدام المياه الهامشية لأغراض الري. تعتبر مياه الصرف البلدية المعالجة الثانوية مصدر مياه بديلة شائعة للري في المناطق الجافة وشبه الجافة. تزرع النباتات العطرية المعمرة كمحاصيل لإنتاج أعشاب طازجة أو جافة، أو كمصدر للزيوت العطرية. إن زراعة النباتات العطرية بهدف الحصول على الزيوت الأساسية مناسبة للري مع المياه المعالجة لأن الحرارة المستخدمة في أثناء استخراج الزيت تقضي على مسببات الأمراض البكتيرية البشرية التي تنشأ في الفضلات السائلة وتقلل من المخاوف الصحية. وتلعب الزيوت الأساسية دورًا مهما كمضادات للأكسدة الطبيعية ومثبطات الإنزيم التي تستهدف الأمراض البشرية ، مثل مرض الزهايمر. مثل هذه العوامل يمكن أن تمنع التدهور التأكسدي للأطعمة، وتقليل الإصابة التأكسدية للخلايا الحية، وعلاج الزهايمر وتعزيز الذاكرة. ويمكن أن يؤدي استبدال المياه العذبة بالمياه العادمة المعالجة لري هذه المحاصيل إلى تعزيز تطوير نظم الإنتاج على نطاق واسع للكتلة الحيوية، والزيوت العطرية. في هذه المناطق. في هذه الدراسة، قمنا بتقييم مدى قابلية استخدام مياه الصرف المعالجة ثانويا في ري محاصيل بعض النباتات العطرية الصناعية، وتقييم آثار الاستخدام المتواصل للمياه المعالجة على التربة والمحاصيل، وتحليل النشاط البيولوجي لهذه النباتات وزيوتها الأساسية بالمقارنة بالري بمياه الشرب.

الطرق: للمقارنة بين استجابات النباتات العطرية المختارة والري بالمياه الصالحة للشرب، تم إنشاء حقل تجريبي للنباتات العطرية التالية: (نعنع *Mentha spicata*)، (مريمية *Salvia fruticosa*)، (اكليل الجبل *Rosmarinus officinalis*)، (زعتر بلاط *Micromeria fruticosa*) و(زعتر عادي*Origanum syriacum*).

اشتملت كل معاملة على 4 قطع مكررة. تعرضت النباتات للري بالتنقيط لمدة موسمين متتاليين. تم استخراج الزيت العطري من أوراق النباتات الطازجة من كلتا المعاملتين خلال موسمين متعاقبين (الصيف والخريف 2016) بواسطة التقطير المائي باستخدام جهاز Clevenger معدل. تم اختبار الزيوت الأساسية المستخلصة ومستخلصات كحولية من الأوراق لنشاطها المضاد للأكسدة باستخدام DPPH- scavenging للجذور الحرة والقدرة المختزلة (RP)، وللنشاط المثبط لإنزيمي ايستيراز (AChEI) و (BuChEI) باستخدام طريقة NA-FB.

النتائج: على الرغم من الاختلافات في جودة المياه ، لم تؤثر المياه العادمة المعالجة على إنتاج الكتلة الحيوية الطازجة والجافة ، وكمية التوابل وحاصل كمية الزيوت الأساسية في المحاصيل باستثناء نبات الميرمية*S.) (fruticosa* التي كانت ذات دلالة إحصائية وزادت لجميع معايير الإنتاجية السابقة. كما أن نوعية المياه لم تؤثر على نشاط مثبطات إنزيم الكولينستراز لهذه النباتات (الزيوت العطرية أومستخلصات النبات الكحولية).

أظهر مستخلص نبات النعنع(*M. spicata*) أعلى تثبيط ل AchE و BuChE مع مؤشر الانتقائية (SI) من 2.93 و 3.85 في المياه الصالحة للشرب والمياه العادمة المعالجة ثانويا ، على التوالي. في حين أن أظهر الزيت العطري أقل نشاط مثبط ضد كل من الإنزيمين. بشكل عام ، تم تحديد المستخلصات الكحولية التي تم اختبارها لتكون مثبطات انتقائية لـ AChE أكثر من BuChE، في حين كانت الزيوت العطرية مثبط أكثر انتقائية في BuChE. لم يتأثر النشاط المضاد للأكسدة في المستخلصات أيضا بالري بالمياه المعالجة، النعنع(*M. spicata*)، و(*R. officinalis*) أظهر أفضل RP و DPPH-scavenger، في حين أظهرت الزيوت العطرية من

الزعتر(*O. syriacum*) واكليل الجبل(*R. officinalis*) نشاط قوي الى ضعيف كمضادات للأكسدة.

الاستنتاج: توضح النتائج أنه يمكن استخدام مياه الصرف البلدية المعالجة الثانوية بنجاح في ري محاصيل النباتات العطرية الصناعية لإنتاج الزيوت العطرية، ومضادات الأكسدة الطبيعية، وإنتاج العوامل المضادة للكولين ايستريز لأن العائد والنشاط الحيوي لهذه المحاصيل لم يتأثران بالمقارنة بالري بمياه الشرب.