International Journal of Secondary Metabolite

IJSM
International Journal of Secondary Metabolite

e-ISSN: 2148-6905

http://dergipark.org.tr/en/pub/ijsm

IJSM

Volume: 7
Number: 3
2020
September

International Journal of Secondary Metabolite (IJSM)
is a peer-reviewed online journal

e-ISSN: 2148-6905

IJSM
International Journal of
Secondary Metabolite

Volume 7, Issue 3, September 2020

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Publisher : İzzet KARA
Frequency : 4 issues per year (March, June, September, December)
Online ISSN : 2148-6905
Website : https://dergipark.org.tr/en/pub/ijsm

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International Journal of Secondary Metabolite

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ISSN:2148-6905

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 3, 139–159

https://doi.org/10.21448/ijsm.714310

Published at http://dergipark.gov.tr/en/pub/ijsm Review Article

Imidazole Antifungals: A Review of Their Action Mechanisms on Cancerous Cells

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Abstract: Imidazoles, together with triazoles, constitute azole sub-group of antifungal drugs which acts by inhibiting cytochrome P450-dependent enzyme, the lanosterol 14-α-demethylase. In addition to their primary use, when it comes to additional anti-cancer function, clotrimazole, econazole and ketoconazole have come to the fore among the imidazoles. Based on the findings up to now, although having different effects, disruption of the glycolytic pathway, blockage of Ca^{2+} influx and nonspecific inhibition of CYP450 enzymes can be regarded as the main ones responsible for the anti-neoplastic activities of the mentioned drugs, respectively. Considering the advantages of repurposing of drugs with known pharmacology compared to new drug development studies requiring labor, time and cost, it will be extremely important and valuable to continue the clarification of the different mechanisms of these antifungals on cancerous cells and benefit from them especially to increase drug efficacy and overcome drug resistance. In this review, the action mechanisms of imidazole antifungals on cancerous cells and consequently, their potential for use in cancer treatment alone or in combination with conventional therapeutics were discussed in detail.

ARTICLE HISTORY

Received: April 03, 2020
Revised: May 07, 2020
Accepted: May 29, 2020

KEYWORDS

Imidazole Antifungals,
Clotrimazole,
Econazole,
Ketoconazole,
Cancer

1. INTRODUCTION

As their names imply, antifungal drug is an agent which selectively kills fungal pathogens with minimal toxicity to the host and antifungal/antimitotic medication is to use these agents to treat and/or prevent serious systemic fungal infections [1]. According to their mode of action, there are seven known classes of antifungals including targeting ergosterol biosynthesis, fungal cell wall synthesis, sphingolipids biosynthesis, nucleic acid synthesis, protein biosynthesis, microtubules biosynthesis and disrupting fungal membrane. The antifungal class which acts by targeting biosynthesis of ergosterol, one of the main constituent of membranes, is called azoles. To put it more clearly, the azole class of antifungal drugs inhibits cytochrome P450 (CYP450)-dependent enzyme, the lanosterol 14-α-demethylase [2]. The inhibition of the so-called enzyme induces both the ergosterol consumption and sterol accumulation, the situation of which leads to loss of many functions of fungal membrane and afterwards fungal growth [3]. Various azoles have been used effectively for more than 35 years on the management of invasive fungal infections caused by the strains such as dermatophytes and *Candida* spp. Among the azoles, the

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ISSN-e: 2148-6905 /© IJSM 2020

imidazoles are the first developed ones, the usages of which were limited for superficial mycoses, the fungal infections invading the most superficial layer of the epidermis, because of gastrointestinal intolerability and neurologic adverse effects at high doses besides their low solubility in physiological solutions. However, triazoles were developed to meet a broad range of treatments and new-generation azole antifungals are under trial for more efficient medication, minimum side effects and, of course, ease of administration [4]. On the other hand, it should be emphasized that there has been also promising researches on using imidazoles against cancer besides their primary uses. From this point of view, it was aimed to collect the results of the studies, from past to present, assessing the effects of imidazoles on various cancerous cell or tissue types to make a general conclusion about the possible potentials of these antifungals in the treatment of cancer.

1.1. Imidazoles

Imidazole antifungal drugs have two atoms of nitrogen in their azole rings. Bifonazole, butoconazole, clotrimazole, ketoconazole, econazole, fenticonazole, isoconazole, miconazole and oxiconazole are some of the examples of this azole class. According to the literature survey, it can be clearly seen that among these imidazoles, clotrimazole (CTZ), econazole (ENZ) and ketoconazole (KTZ) are the most hitherto investigated ones for their potentials in cancer treatment.

1.1.1. Clotrimazole

CTZ (1-[(2-chlorophenyl)-diphenylmethyl]imidazole) has been known as a broad spectrum antimycotic or antifungal agent since its introduction to German markets in 1973 [5, 6]. It has long been known that the drug is effective on the strains of *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, *Candida albicans* and *Malassezia furfur* [7]. Apart from its routine antifungal function, through the studies trying to reveal the direct anti-inflammatory properties of the azole derivatives, CTZ was recognized as one of the antifungals having ability to antagonize calmodulin (CaM) activity with the IC_{50} value of 18.4±4.6 µM, which was shown based on the inhibition of CaM-dependent phosphodiesterase (PDE) [8]. CaM is multifunctional intermediate calcium (Ca^{2+})-binding messenger protein expressed in all eukaryotic cells [9] and so its antagonists play significant roles in many cellular processes [10]. It is not wrong to say that, with the discovery of CaM antagonist function, possible anticancer effects of CTZ started to be investigated. As known, the primary energy source of cancer cells is glycolysis that is controlled by allosteric regulators and by reversible attachment of the glycolytic enzymes to cytoskeleton and mitochondria [11-19]. Due to Ca^{2+} ions have impact on the increase of glycolytic enzyme attachment; CTZ could be an effective agent for inhibition of glycolysis. Indeed, the studies to date have indicated that CTZ shows its main action on cancerous cells by disrupting the glycolytic pathway (Table 1). According to literature survey, the primary study introducing the inhibition effect of CTZ on cell proliferation was conducted by Benzaquen and her group in 1995 [20]. In the so-called paper, it was stated that 10 µM CTZ completely inhibited growth factor-stimulated proliferation of Albino Swiss mouse embryo fibroblasts 3T3 (Swiss 3T3), bovine aortic endothelial cells (BAEC) and rat aortic smooth muscle (RASM) cells besides several human and mice cancers including lung, colon and melanoma with no apparent toxicity. The researchers based this anti-proliferative effect of CTZ on inhibition of Ca^{2+} movement across the plasma membrane but they did not investigate the possible alterations in glycolytic pathway. On the other hand, in the research conducted on B16 melanoma cells, it was firstly found that CTZ (5-50 µM) significantly reduced the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP, in a dose dependent manner within 1 h [21]. Following these findings, in other two studies carried out under the same conditions and with the same cell line, CTZ was shown to induce detachment of phosphofructokinase and aldolase from cytoskeleton, and hexokinase from mitochondria in

a dose dependent manner, which made this antifungal promising agent in treatment of melanoma [22, 23]. Afterwards, the disruptive effect of 0-500 µM CTZ on glycolytic pathway within 0-48 h was determined for various cancer cell lines including breast, kidney, lung, colon, glioblastoma and ovarian cancers [24-28]. In addition, it should be stated that CTZ induced detachment of glycolytic enzymes preceded the cell death that generally based on apoptosis. As is very well known, apoptosis is one of the programmed cell death types and it is intended to eliminate cancer cells by primarily this death pathway. Studies carried out until now have shown that CTZ induced apoptotic death in various cancer cell lines, characterized by phosphatidylserine externalization, DNA fragmentation, induction in cell-cycle arrest in G1- and M- phases, release of pro-apoptotic factor cytochrome c into the cytoplasm, down-regulation of the anti-apoptotic Bcl-2 protein, increase in pro-apoptotic Bax protein levels and induction in wild type p53 levels [26, 29-32]. Furthermore, maybe it could be supportive to state that CTZ significantly induced the activity of metacaspase Yca1, structural homolog to apoptotic human caspases, in yeast model organism [33]. Finally, it was demonstrated in a limited number of studies that some derivatives of CTZ or its combination with antineoplastic drugs exhibited more cytotoxicity on cancerous cells than its individual application [32, 34, 35].

Table 1. Action mode of clotrimazole-induced death on several cell lines and tissues

Mode of Action	Cell/Tissue Type	References
Inhibition of growth factor stimulated cell growth, Blockage in mitogen-induced intracellular Ca2+ increase in Swiss 3T3 cells, Depletion in intracellular Ca2+ stores in Swiss 3T3 cells	albino swiss mouse embryo fibroblast cell line (Swiss 3T3), bovine aortic endothelial cell line, rat aortic smooth muscle cell line, human lung and colon carcinomas, human and mice melanoma cells	[20]
Decrease in the levels of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP in a dose and time dependent manner, Detachment of phosphofructokinase and aldolase from cytoskeleton in a dose and time dependent manner, Detachment of hexokinase from mitochondria in a dose dependent manner	mouse melanoma cell line	[21-23]
Inhibition of basal and growth factor stimulated vascular endothelial cell proliferation in a dose dependent and non-toxic manner, Inhibition of the basal and bFGF-stimulated migration of bovine capillary endothelial cells, Inhibition of the angiogenesis both *in vitro* and *in vivo* models	human, bovine and porcine vascular endothelial cells stimulated with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), bFGF-injected male C57B16 mice	[129]
At higher doses (50 – 70 µM) Inhibition of cell growth in a dose-dependent and toxic manner *At lower doses (0 – 30 µM)* Inhibition of cell growth in a dose-dependent and non-toxic manner, Formation of small retracted cell bodies and thin elongated cytoplasmic processes, Cell cycle arrest at G0/G1 phase, Increase in p53 and glial fibrillary acidic protein (GFAP) expressions, Decrease in cellular c-myc and c-fos expression, Sensitization to antitumor effect of cisplatin	human glioblastoma cells (with wild or mutant types p53)	[130]

Suppression of cell recovery in a dose dependent manner, Cytotoxicity even against cells with multidrug-resistant phenotype and carrying genetic abnormalities, Depletion of intracellular Ca2+ stores, Apoptosis shown by Annexin V binding reactivity and induction of DNA fragmentation	human B- and T-lineages acute lymphoblastic leukemia (ALL) cell lines and human primary ALL cells	[30]
Decrease in the levels of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate in a dose- and time-dependent manner, Reduction in ATP levels and time-dependent reduction in the viability, Alteration in cytosolic ion concentrations, Induction of necrotic morphological changes	murine lewis lung carcinoma cell line, murine colon adenocarcinoma cell line	[24]
Decrease in cell viability in a dose- and time-dependent manner, Induction in morphological and functional alterations including nuclear condensation and loss of protrusion, Detachment of 6-phosphofructo-1-kinase and aldolase from cytoskeleton, Decrease in glucose consumption and lactate production rates	human breast cancer cell line	[25]
Cell cycle arrest at the G1 phase in a dose- and time-dependent manner, Overexpression of p27Kip and decreased expression of p21Cip, cyclin-dependent kinase 1, cyclin-dependent kinase 4, and cyclin D, Translocation of mitochondrial-bound hexokinase II to the cytoplasm, Release of cytochrome c into the cytoplasm, Sensitization to radiation-induced apoptosis evidenced by Annexin V-FITC staining	human glioblastoma cell line	[26]
Reduction in lactate production, Inhibition in phosphofructokinase activity, *In vitro* dissociation of phosphofructokinase from f-actin	human breast cancer tissue	[57]
Inhibition of migration in an aggressiveness dependent manner, Decrease in cell proliferation, Decrease in cell viability in an aggressiveness-dependent manner, Dose- and aggressiveness-dependent reduction in glucose uptake and mitochondrial activity, Decrease in cellular ATP levels in a dose dependent manner, Inhibition in hexokinase, phosphofructokinase-1, pyruvate kinase and glucose 6-phosphate dehydrogenase activities, Decrease in cell viability in an aggressiveness-dependent manner	human breast cancer cells (with different aggressiveness profiles)	[27]
Inhibition of cell viability in a dose- and time-dependent manner, Decrease in cell colony formation in a dose-dependent manner, Cell cycle arrest at the G0/G1 phase in a dose-dependent manner, Induction of apoptosis showed by Annexin V/PI staining in a dose-dependent manner,		

Down- and up-regulations of the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins, respectively both *in vitro* and *in vivo* models, Decrease in *in vivo* tumor volume and proliferation rate, Induction of apoptosis in *in vivo* model showed by cleaved caspase-3 expression	human oral squamous cell carcinoma (OSCC) cells, OSCC xenograft nude mouse model	[31]
Reduction in cell viability in a dose-dependent manner, Induction of mainly early stage apoptosis showed by Annexin V/7-AAD staining, Induction of internucleosomal DNA fragmentation and accumulation of histone-complexed DNA fragments in the cytoplasmic fraction, Absence of histone-complexed DNA fragments in the culture supernatant (the evidence of cell death without any involvement of necrosis), Induction of cell-cycle arrest at G1/S phase transition, Reduction in the expression and activity of hexokinase type-II	human cutaneous melanoma cell line	[131]
When combined with romidepsin (RDP) or belinostat (BNS), increased sensitivity to apoptosis showed by Annexin V-SYTOX staining, Requirement of caspase-3 activation and, Bak and Bax expression for RDP plus CLT induced apoptosis, Decrease in total hexokinase 2 (HK2) levels and decreased HK2 expression on the mitochondria after treatment with CLT	human breast, kidney, lung, colon and ovarian cancer cells	[132]

1.1.2. Econazole

Another imidazole that has exhibited a remarkable effect on cancer cells is ENZ (1-[2-[(4-chlorophenyl)methoxy]-2-(2,4-dichlorophenyl)ethyl]imidazole). This antifungal is widely used in the treatments of vulvovaginal candidiasis, superficial fungal infection, keratitis and athlete's foot [36-39]. Early findings about the other functions of this agent revealed that it inhibited lipopolysaccharide-inducible nitric oxide synthase (iNOS) activity and reduced prostaglandin E2 production, which were related with its anti-inflammatory and cyclooxygenase inhibition effects, respectively [40, 41]. In addition, its antagonistic impact on CaM was found about 3-fold more effective than CTZ [8]. Interestingly, we could not discover any study that has assessed the effect of ENZ on glycolysis pathway. It is maybe because of the least selectivity of this azole antifungal against CaM-dependent PDE [8]. On the other hand, as a Ca^{2+} influx blocker, the effectiveness of ENZ on various tumors by different action mechanisms has been demonstrated (Table 2). Zhang et al. stated that MCF-7 and MDA-MB-231 breast cancer cell lines lost their viability after treatment with ENZ for 24 h in a dose dependent manner (0-15 µM), the concentration range which was slightly higher in magnitude than those required to kill bone marrow derived murine mast or 32D-Kit leukemia cells [42, 43]. In the same studies, it was also showed that ENZ caused a sharp loss of cell clonogenicity almost above 10 µM in parental and doxorubicin-resistant MCF-7 and MDA-MB-231 breast cancers, and 32D-Kit leukemia cells. However, unlike mast, p815 mastocytoma or 32D-Kit leukemia, the so-called breast cancer cell lines did not exhibit typical apoptotic morphology. Importantly, it should be also stated that this imidazole plus epithelial growth factor combination was successfully utilized for the purpose of decontamination of hematopoietic

progenitor cell preparations from breast cancer cells, in other terms for *in vitro* purging [42]. A similar ENZ application was shown for *in vitro* purging of bone marrow from P815 leukemic cells [44]. Ho and his friends showed the molecular mechanisms of ENZ induced anti-proliferative effect on human colon cancer cells [45]. According to their results, while lower doses of ENZ (5-10 µM) suppressed COLO 205 cell proliferation, higher doses (>20 µM) triggered apoptotic cell death characterized by DNA ladder formation, caspase-3 and -9 activation, induction in Bax protein expression, translocation of cytochrome c and apoptosis inducing factor from mitochondria to cytosol. In an another study, it was found that overnight treatment of the human prostate cancer cell line PC3 with 1-30 µM ENZ resulted in a decrease in cell proliferation by 20–100% and 5 µM of the drug induced both extracellular Ca^{2+} influx and intracellular Ca^{2+} release in this cell line [46]. In this point it must be stated that although ENZ generally recognized as an inhibitor of capacitative Ca^{2+} entry through store-operated calcium channels, it has been found to increase intracellular Ca^{2+} levels in some other cell lines too [39, 47-53]. Yu et al. showed the modulator function of c-myc and hypoxia-inducible factor 1 transcription factors in regulating ENZ sensitivity of rat fibroblast and human myelomonocytic leukemia cells [54]. While Fang et al. showed ENZ induced caspase-12 and -7 dependent apoptosis in mouse leukemia [55], caspase-3, -9 and Bcl-2 dependent apoptotic death was demonstrated for MCF-7 breast cancer cells [56].

Table 2. Action mode of econazole-induced death on several cell lines and tissues ([a]*Carried out only on the human colon adenocarcinoma*)

Mode of Action	Cell/Tissue Type	References
Inhibition of the tumor weight and the tumor/body weight ratio in human colon cancer xenografts ***At lower doses (5 - 20 µM)*** Dose-dependently suppression of *in vitro* cell proliferation but in a less profound manner in healthy ones, Induction of cell cycle arrest at the G0/G1 phase in a dose-dependent manner [a], Induction in p53, p21/Cip1, p27/Kip1 and cyclin E protein levels in a dose-dependent manner [a], Decreases in the expression of phospho-CDK2 and phospho-Rb proteins in a dose-dependent manner [a] Suppression of CDK2 and CDK4 kinase activities [a] ***At higher doses (>20 µM)*** Induction of *in vitro* cell death but in a less profound manner in healthy ones, Arrestment at the sub-G1 peak phase [a], Downregulation in phospho-CDK2, CDK4, cyclins A2, D1 and D3 protein levels [a], Apoptosis induction evidenced by DNA ladder formation [a], Caspase-3 and -9 activation [a], Induction in Bax protein expression [a], Translocation of cytochrome c and apoptosis-inducing factor [a]	human normal colon epithelial cell line, human colon adenocarcinoma cell line, human colon cancer xenografts *in vivo*	[45]
Dose-dependently induction of intracellular Ca^{2+} concentration by stimulating Ca^{2+} influx into cells and Ca^{2+} release from the endoplasmic reticulum via a phospholipase C independent mechanism	human prostate adenocarcinoma cell line	[46]
Induction in intracellular Ca^{2+} concentration in a dose-dependent manner,	mouse leukemia cell line	[55]

Apoptosis induction evidenced by phosphatidylserine externalization in a dose-dependent manner, Induction in expression of caspase-12 and caspase-7 in a dose-dependent manner		
Cell resistance in terms of colony-forming ability in c-myc negative rat fibroblasts, Reduced level of apoptosis showed by Annexin V-Cy5 staining in c-myc knock-down leukemia cells compared with vector control-infected ones, Impaired ER Ca^{2+} release in c-myc-negative rat fibroblasts, Reduced ROS generation in c-myc-negative rat fibroblasts and c-myc knock-down leukemia cells related with decrease in mitochondrial content, Restoration of the sensitivity with the addition of H_2O_2 in terms of apoptosis in c-myc-negative rat fibroblasts and c-myc knock-down leukemia cells, Restoration of mitochondrial content, ROS generation and the sensitivity in terms of apoptosis in c-myc negative rat fibroblasts with HIF-1α knock-down	human leukemia cell line, rat fibroblast cells (c-myc-negative and -positive)	[54]
Dose-dependently induction of intracellular Ca^{2+} concentration by stimulating Ca^{2+} influx into cells and Ca^{2+} release from the endoplasmic reticulum via phospholipase A2 and C and protein kinase C dependent mechanisms, Induction of cell death regulated by ERK/MAPK pathway	human oral cancer cell line	[39]
Reduction in cell viability in a time- and dose-dependent manner, Reduction in cell growth in a dose-dependent manner, Decrease in cell number, Apoptosis induction evidenced by the induction of morphological changes including blebbing of nuclei and formation of granular apoptotic bodies, and the appearance of DNA ladder, Decrease in mitochondrial membrane potential in a dose-dependent manner, Decrease in Bcl-2, and increase in caspase-3 and -9 protein levels	human breast cancer cell line	[56]
Reduction in cell viability in a dose-dependent manner, Dose-dependently induction of apoptosis evidenced by Annexin V-FITC staining, and PARP and caspase-3 cleavages, Decrease in p-AKT and Bcl-2 protein levels, Synergistic and additive killing effects when combined with cisplatin	human lung cancer cells	[133]

1.1.3. Ketoconazole

Although both CTZ and ENZ have been accepted as antifungal drugs possessing profound anti-proliferative effects on tumorigenic and metastatic cells while having minimal effects on non-tumoral ones [27, 42, 57], there is no any (un)approved application or combination formulation, most probably because of the need of a more complete understanding of their action mechanisms and bioavailability limitations. However, another imidazole KTZ (1-[4-[[(2S,4R)-2-(2,4-dichlorophenyl)-2-(imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl

]piperazin-1-yl]ethanone) has found application in castration-resistant prostate cancer (CRPC) as an off-label and a second-line hormonal treatment, alone or in combination with other compounds and therapies, since the 1980s [58-71]. It should be noted that this is an officially unapproved treatment, generally without survival benefit. CRPC is defined as the lethal phenotype of prostate cancer and it can occur in most of the patients received long-term androgen deprivation therapy (ADT) which is the standard initial management for the disease. KTZ nonspecifically inhibits CYP450 enzymes including 17α-hydroxylase/17, 20 lyase (CYP17A1), the function of which is to catalyze significant reactions in the gonadal and adrenal steroidogenesis pathways [72, 73]. Based on these inhibitions, the drug suppresses both gonadal and adrenal androgen synthesis, while only gonadal androgen synthesis can be suppressed with ADT. Of course, nonspecific action of this antifungal on CYP450 enzyme family has given rise to concerns about its potential toxicity, the most common of which is hepatotoxicity. As a matter of fact, the usage of oral KTZ tablets in their primary target fungal infections has not been allowed due to its side effects in liver [74]. Nevertheless, the efficacy and safety of cancer treatment were enhanced by clinical dose-reduction studies. For example, Harris et al. indicated a regimen of low dose oral KTZ (200 mg 3 times daily) with replacement doses of hydrocortisone which can be well tolerated and has moderate activity in CRPC patients [75]. Again a significant improvement in terms of toxicity was achieved by clinical dose reduction (from 400 to 200 mg orally 3 times daily) not or in combination with steroids again in CRPC patients [76]. In a more recent study conducted with docetaxel pre-treated CRPC patients, low dose oral KTZ (200 mg 3 times daily) plus hydrocortisone regimen was suggested as well-tolerated, relatively inexpensive and clinically active treatment alternative [77]. Of course, the number of these examples can be increased. Moreover, similar applications have been suggested and extended for other tumors such as breast and colorectal carcinomas [78, 79]. From this point of view, KTZ yet offers alternative ways for the treatment of advanced cancers with its low-priced, a relatively favorable toxicity profile compared to chemotherapy and efficacy both before and after chemotherapy [80, 81] especially when newer and more specific options are not available [71, 82-84].

As can be seen in Table 3, KTZ also has cytotoxic effects on different tumors. In an old study conducted by Naftalovich et al., it was demonstrated that 4-7 µg/ml KTZ caused to 50% inhibition of DNA synthesis in radiation leukemia virus induced T-cell lymphomas, while at least 50 µg/ml of the drug was needed to induce a similar inhibition in bone-marrow and spleen cells prepared from healthy mice [85]. 25-50 µM KTZ was shown to have cytotoxic and apoptotic effects on both different human cancer and rat liver cells, but in a significantly more sensitive manner when wild-type p53 protein was present [86]. The same group demonstrated that wild type p53 dependency of KTZ- induced cytotoxicities in human colorectal and hepatocellular carcinoma cell lines were more pronounced at lower doses (2 µM) of the antifungal [87]. Additionally, it was stated that approximately 40 µM KTZ induced p53-mediated cell cycle arrest at the G0/G1 phase together with p21/Cip1 induction and decrease in Cdk activities, especially in Cdk4. Lin et al. exerted cytotoxic and apoptotic effects of KTZ above the concentration of 20 µM on human osteosarcoma cells [88]. While this observed cell death depended on KTZ-induced JNK phosphorylation, it was independent from the increase in intracellular Ca^{2+} concentrations. In a current work of Agnihotri et al., it was firstly found by means of drug-screening research that KTZ and posaconazole, a second-generation triazole agent, displayed the greatest inhibitory effect on glioblastoma both *in vitro* and *in vivo* by effecting hexokinase II-related gene expression signature [89]. This finding has special importance in terms of the lack of specific hexokinase II inhibitors, which makes this enzyme unactionable in cancer medicine. Considering the findings that azole antifungal drugs have given rise to mitochondrial loss [90, 91], Chen et al. put forward a novel impact of KTZ on mitophagy programme of hepatocellular carcinoma cells [92]. According to the data of the

researchers, 20 µM KTZ induced apoptosis of liver cancer cells by triggering aberrant mitophagy with the downregulation of cyclooxygenase-2. The same researchers also indicated the synergistic action of KTZ with clinically approved multi-kinase inhibitor sorafenib and rightfully suggested the use of KTZ especially in patients having high cyclooxygenase-2 expression levels.

Some other medically important *in vitro* effects of KTZ are the induction of the DT-Diaphorase (NQO1), modulation of the calcium-activated potassium channels, blockage of the activation of orphan nuclear receptors (NRs), and specific and reversible inhibition of CYP3A4 [79, 93-96]. NQO1 is one of the phase II enzymes that detoxificate xenobiotics. Accordingly, KTZ induction of the enzyme indicates the chemoprotection function of this antifungal. The associations between key aspects of cancer and potassium channel dysregulation are gradually growing, which makes potassium channels a valuable target [97]. On the other hand, when the literature is examined, it can be clearly seen that researchers especially have focused on the inhibition effects of KTZ on NRs and CYP3A4. As is well known, these receptors play role in the regulation of CYP3A4, which accounts for approximately half of the metabolism of most approved drugs [98]. Based on the KTZ induced inhibition of this enzyme and, therefore, the reduction in drug clearance, alternative and effective formulas benefiting from the so-called antifungal have been developed for advanced cancer patients [79, 99-101].

Table 3. Action mode of ketoconazole-induced death on several cell lines and tissues (*[a]Used to determine cytotoxicity; [b]Used to determine cytotoxicity and DNA fragmentation; [c]On hepatocellular carcinoma with wild p53; [d]On colon adenocarcinoma with mutant p53; [e]On hepatocellular carcinomas; [f]On colon adenocarcinomas; [g]On colon and liver cancers*)

Mode of Action	Cell/Tissue Type	References
Cytotoxicity in a dose and time dependent manner	human breast carcinomas, human pancreatic adenocarcinomas, human colon adenocarcinoma, human prostate carcinoma, rat pancreatic carcinoma, murine leukemia	[134]
Increased sensitivity to the cytotoxicity and apoptosis depend on carrying wild type p53 and being cancerous, Induction of apoptosis evidenced by the formation of DNA fragment ladder in all cell lines, Nuclear accumulation of p53 protein c, Bax protein induction e and bcl-2 protein inhibition g, Activation of caspase-3 c, Poly-(ADP ribose) polymerase and lamin A degradation c	normal human breast skin fibroblast cell line a, primary culture of rat liver cells b, human colon adenocarcinomas (with wild or mutant types p53), human hepatocellular carcinomas (with wild type or deleted p53)	[86]
Increased sensitivity to the cytotoxicity depend on carrying wild type p53, Increased sensitivity to cell cycle arrest at G0/G1 phase depend on carrying wild type p53 f, G0/G1 arrest mediated by induction in p21/Cip1, p27/Kip1 and inhibition in cyclin D3 and CDK4 expressions d	human colon adenocarcinomas (with wild or mutant types p53), human hepatocellular carcinomas (with wild type or deleted p53)	[87]

Induction of apoptosis evidenced by the sub-diploid nuclei and activation of caspase-3, Induction of the phosphorylation of ERK and JNK proteins, Reversed apoptosis by the selective JNK inhibitor SP600125, but not by the selective ERK inhibitor PD98059, Increase in intracellular Ca2+ concentration, Total inhibition of the increases in intracellular Ca2+ concentration by the chelator BAPTA, but without reversing cell death	human osteosarcoma cell line	[88]
Selective effect against tumoral cell lines, Increase in proportion of apoptotic cells evidenced by the detection of DNA breaks, Influence on genes and pathways regulated by hexokinase II	fetal normal human astrocytes immortalized with hTERT, human neural stem cell line, human glioblastoma cell lines, patient-derived glioma stem cells, mouse models of glioblastoma	[89]
Enhancement in Ganoderma microsporum immunomodulatory protein induced cytotoxicity and apoptosis of cancerous cells in a concentration-dependent manner, Enhancement in Ganoderma microsporum immunomodulatory protein induced migration inhibition of cancerous cells in a concentration-dependent manner, Reduction in the level of Ganoderma microsporum immunomodulatory protein induced phosphorylated-adenosine monophosphate-activated protein kinase (p-AMPK)-α and autophagy of cancerous cells, Reduction in the monocyte chemoattractant protein-1 (MCP-1) secretion of cancerous cells	human melanoma and skin fibroblast cell lines	[135]

1.1.4. Other Imidazoles

Apart from these three drugs, there are some studies on the possible anticancer effects of some other remaining imidazoles including bifonazole (BFZ) and miconazole (MCZ). As in the case of CTZ, BFZ has been known as calmodulin antagonist for more than two decades which can detach glycolytic enzymes from cytoskeleton or mitochondria in murine melanoma cells [23]. In a more recent study, Cheng et al. demonstrated the BFZ-induced intracellular Ca^{2+} increases in human prostate cancer cells through the induction of phospholipase C- and protein kinase C-dependent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} influx via non-store-operated pathways [102]. The researchers also recorded Ca^{2+}-independent apoptotic cell death in these cells. We finally came across the abstract paper of Robey et al. in which synergistic apoptotic effects of romidepsin, a histone deacetylase inhibitor, plus CTZ/BFZ were found on colon, lung, breast and renal cancer cell lines [103]. Surprisingly, no other studies on the effects

of BFZ or its combination formulations on cancer cells/tissues have been discovered and therefore it is thought that the action mechanism of this drug is still in need of investigation. Nevertheless, it would not be wrong to say that the action mechanism of MCZ on cancer cells is relatively better understood compared to BFZ. Literature review shows that this drug has an impact on human bladder, breast and colorectal cancers, human osteosarcoma cells, human hepatocellular carcinoma, human acute myelogenous leukemia, and mouse skin melanoma cells [104-108]. It is clearly seen that the main focus of these studies was to explore the relationship between MCZ-induced cell-cycle arrest and apoptosis via the determination of the levels of main regulatory proteins involved in the so-called processes. Unfortunately, this antifungal causes cardiotoxicity via generation of superoxide anion, inhibition of APE/Ref-1 and induction of apoptosis in cardiomyocytes and cardiomyoblast cell line [109, 110], which might make it unnecessary to focus on researches regarding with its anticancer potential.

1.2. Triazoles

As stated at the beginning of the paper, triazole antifungal drugs having three nitrogen atoms in their azole rings were developed to fulfill the need of a much broader spectrum of treatment options by comparison with the imidazoles. The first synthetic triazole antifungal to be discovered was fluconazole (FCZ) and it was introduced to the markets in the early 90s [111]. By the way, FCZ is different from other azoles in terms of the presence of two triazole rings making this compound less lipophilic and protein bound [112]. After FCZ, itraconazole (ICZ) is the second member of first generation triazoles. Between these two triazoles, while it has been shown that ICZ is toxic to various cancer types, no study on the subject has been found for FCZ. On the other hand, although it is off topic, it could be useful to say that some *Candida* cells, a kind of yeast species causing fungemia to which cancer patients are especially susceptible, are resistant to FCZ-induced death and this resistance can be broken by combined application of FCZ with certain phenolic compounds [113-115].

As mentioned above, ICZ has capability to inhibit the proliferation of distinct cell lines established from breast, gastric, glioma, pancreatic, melanoma, esophageal, gastrointestinal, non-small cell lung cancers and acute myeloid leukemia [116-123]. It can be reported that the prominent anticancer mechanism of this triazole is the inhibition of hedgehog pathway having important function in the embryonic development and tissue homeostasis. Moreover, recent findings have put forward that this signaling cascade is related with the neoplastic transformations, malignant tumors and drug resistance of a multitude of cancers [124]. The relationship with drug resistance may be especially remarkable given the findings suggesting that ICZ has a synergistic effect when combined with the known drugs as bevacizumab, doxorubicin and 5-fluorouracil on several cancer cell lines resisting these drugs when they are given alone [117, 121, 123]. In addition, the review paper conducted by Tsubamoto et al. can be browsed for more detailed information for both *in vitro* and ongoing *in vivo* trials with ICZ [125].

Approximately a decade later from the first generation triazoles, voriconazole, posaconazole, ravuconazole, albaconazole, isavuconazole and efinaconazole were developed as second-generation triazole antifungals for more efficiency and safety [126]. As with FCZ, there are no detailed researches dealing with the action mechanisms of these triazoles against cancer cells. However, due to the limited treatment strategies available because of the reasons including antifungal resistance, toxicity, drug interactions and expense; clinical studies are being conducted to evaluate the potentials of the so-called antifungals against invasive fungal infections in patients with cancer whose immune systems are severely suppressed [127, 128]. In short, when it comes to cancer, basic focus for triazoles is the modulation of fungal population aiming at increasing morbidity and mortality in cancer patients and ICZ is the only triazole whose effects on cancers have been broadly investigated.

2. CONCLUSION

As a conclusion, it is clear that imidazole antifungals have additional anti-proliferative effects against divergent solid and leukemia tumors. In this sense, CTZ, ENZ and KTZ can be said to be most striking imidazole antifungals with their significant *in vitro* effects that may be valuable in medicine. Despite above-mentioned remarkable data, to be sure, it would be appropriate to continue investigate the new biological effects of all imidazoles besides CTZ, ENZ and KTZ. Additionally, it should not be forgotten that although KTZ has already found a medical application, the same situation is not valid for other imidazoles for the time being. Accordingly, as with the usage of KTZ in especially prostate cancer, the significance and effectiveness of sequential applications or combined therapies in many diseases including cancer must be taken notice. It can be clearly said that application of dual drugs with known pharmacology and complementary to each other in terms of their targets has always been a valuable option for the scientists in the area due to difficulty of and requiring a long time for new drug developments. Eventually, considering the biochemical effects on cancer cells, the so-called antifungal drugs should be also taken into account in combined therapies, maybe particularly with new generation anticancer drugs that are target specific but against which cancer cells can resist.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 3, 160–173

https://doi.org/10.21448/ijsm.690926

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

A Pharmacognostical Comparative Investigation on *Valeriana alliariifolia* Adams

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Abstract: Free radicals are shown as the main reason of many chronic and degenerative diseases. Because of the toxicity and undesirable effects of synthetic antioxidants, finding of new antioxidant natural agents is very important, especially edible plants. As an edible plant, valerian is used for various aims. Also, *Valeriana* species are known with their antioxidative compounds. In our previous study, some biological activities of *Valeriana alliariifolia* Adams roots extracts, collected in 2012, and the chemical compositions of active samples were determined. For this study, after 5 years collected plant materials were investigated again to determine and compare the antioxidant activities, the total phenolic contents and the chemical composition profile of the extracts from different plant-parts and to compare obtained results with the previous data. While RWI, RWM1 and RWM1residue were found most active by DPPH method and AHM1 by FRAP method, REM1 showed the highest activity by CUPRAC method. The high activity of AHM1 is parallel to its phenolic content. It can be thought that the difference between the results of our two studies, is due to the change of plant content from year to year and various environmental factors. This is important for achieving standardization in the production of its preparations.

ARTICLE HISTORY

Received: February 18, 2020
Revised: April 27, 2020
Accepted: June 25, 2020

KEYWORDS

Valeriana alliariifolia,
Antioxidant,
Total Phenolic Contents,
Chemical Content,
Valerenic Acid

1. INTRODUCTION

Free radicals are shown as the main reason of many chronic and degenerative diseases, including coronary heart disease, stroke, inflammation, aging, cancer and diabetes mellitus. Reactive oxygen species (ROS), free radicals such as hydroxyl radical singlet oxygen, superoxide anion and hydrogen peroxide, can cause initiate peroxidation of polyunsaturated fatty acids and cellular injuries, which include DNA damage, protein damage, and important enzymes in human body. In this case, various free–radical-related diseases can be occured consequently. Therefore, investigations for finding of antioxidant agents were began to protect

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ISSN-e: 2148-6905 /© IJSM 2020

cells and organisms from oxidative damage and to reduce the risk of degenerative diseases arising from oxidative stress. The antioxidants can be classified in two groups: primary and secondary antioxidants. The primary antioxidants neutralize free radicals by a single electron transfer mechanism or by hydrogen atom transfer mechanism, and the secondary antioxidants passivate and deactivate prooxidant catalysts, including chelators of prooxidant metal ions (iron and copper etc.), exemplified by ethylenediaminetetraacetic acid (EDTA) and citric acid (CA) or reactive species such as singlet oxygen (beta-carotene etc.). Synthetic antioxidants -butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertbutylhydroquinone (TBHQ)- have been preferred as primary antioxidants against free radicals, oxidation and off-flavour development. They have been widely used in the food industry for the prevention of the oxidative deterioration, but they have also the toxicity and undesirable effects e.g. carcinogenic effects. For example, BHA and BHT are held responsible for liver damage and carcinogenesis. The consumers concern about foods without/with lower levels of chemical preservatives because of their harmful effects, and demand for the long shelf-life of food and for absence of risk of causing foodborne diseases. Because of these reasons, the researchers and also food industry focused on finding new antioxidant agents from natural sources such as plants, especially edible plants, to prevent or reduce the harmful effects of oxidative stress on cells. After the exploration of important roles of bioactive compounds in free radical chain reactions to exhibit the antioxidant activity, the investigations on these compounds, especially food phenolics, and plant extracts which are considered potential antioxidants, increased dramatically in the past 25 years. On the contrary, very few antioxidants from natural sources took place in the market because of various problems. Nevertheless, there are some commercial products of antioxidant plants or their phenolic compounds in the market, such as rosemary extract, green tea and mixture of tocopherols. The phenolic compounds, which have antioxidant activity, can be also added into wine from the wood barrels used in storage and aging [1-3].

The *Valeriana* genus (Caprifoliaceae), represented by more than 350 species worldwide, comprises about 17 species, 4 of which are endemic, in Turkey. In Turkish traditional medicine, *Valeriana officinalis* L. is preferred for the treatment of hysteria, neurasthenia, nervous insomnia and palpitations, and its infusions for the treatment of wounds [4-9]. *Valeriana* species contain over 150 chemical constituents such as sesquiterpenes, iridoids, flavonoids, alkaloids and other compounds (pinoresinol and its analogs). Many of the iridoids of *Valeriana* species were investigated for their biological activity e.g. the antispasmodic, sedative, antimycobacterial, antiviral, cytotoxic and anxiolytic effects. They contain valepotriates, which have an important place among the iridoids. Beside of this, 8-hydroxypinoresinol and prinsepiol exhibited powerful antioxidant activity in Trolox equivalent antioxidant activity (TEAC) and chemiluminescence (CL) tests [10-12]. Due to the knowledge of good antioxidant activity of *Valeriana* species and the valepotriates, there are numerous studies on antioxidant activity of *Valeriana* species exist in the literature [13]. In recent years, the investigations concentrated especially on the antioxidant activity of the extracts and its compounds, and also their effect mechanisms [13-19]. The usages of *V. officinalis* were recorded for its analgesic and sedative effects, and its infusion to treat neural diseases and for tranquilizer effect, in Turkey [20, 21]. Also, *V. alliariifolia* roots are in use traditionally. Its infusion is preferred with the sedative and antispasmodic purposes, in Turkish traditional medicine [22]. With the previous studies in Turkey, the contents of the essential oils of various *Valeriana* species were determined [20, 21, 23-25]. Also, the investigations on the chemical composition of the essential oil from *V. alliariifolia* exist in literature. 68 constituents were identified in the essential oil from the subterranean parts of *V. alliariifolia* by using capillary Gas Chromatography (GC) and GC/MS. Isovaleric acid (28.6 %), δ-guaiane (7.2 %), α-humulene (4.7 %), hexadecanoic acid (4.3 %), valeric acid (3.7 %) and humulene epoxide-II (3.6 %) were found as the major compounds [26].

Further this study, the extracts of *V. alliariifolia, V. sisymbriifolia* Vahl. and *V. officinalis*, collected in Azerbaijan were analysed for their valepotriates content by the isolation of the extracts from different plant parts through using TLC and UV-spectrophotometry [27]. Along with the identification the compounds of *V. sisymbriifolia*, *V. alliariifolia* and *V. officinalis* from Iran, limonene (3.53%) was found in *V. alliariifolia* as the main component [28]. In another study on the chemical composition of the *V. alliariifolia* essential oil, *trans*-caryophyllene (38.96 %) was the major compound, by following β-pinene (12.06 %), α-pinene (9.94 %), α-terpinene (9.49 %), isoterpinolene (7.15 %), 1,8-cineole (6.76 %) [7].

In light of these data, the plants, especially essential oil containing plants, have different contents according to the different collecting localities or time, and different contents also reveal different biological activities. As seen in previous studies, these content differences occurred in *V. alliariifolia*. In a previous study of our group, the antioxidant, cytotoxic and insecticidal activities and the chemical composition of the active samples were investigated. Besides of the identification of the compounds in active samples, their antioxidant activities by DPPH and ABTS methods and total phenolic contents were determined. Two methanol extracts, prepared with gradient and non-gradient maceration, exhibited higher antioxidant activity and total phenolic contents than other samples. It was appeared that, there is a correlation between the antioxidant activities and total phenolic contents, the polar compounds in EM1 have a role in the antioxidant activity, and finally the compounds with high polarity and also moderate polarity in this *Valeriana* species can have a role in the antioxidant activity [29]. 5 years after this study, the aerial parts and roots of *V. aliiariifolia* were collected again in Trabzon-Turkey for the present study. This present study aims to determine the antioxidant activities of the extracts from two different plant parts, the total phenolic contents and the chemical composition profile, also the presence of the valerenic acid and its derivatives, and to compare the results about the content and antioxidant activity with the previous data.

2. MATERIAL and METHODS

2.1. Plant Material

The aerial parts and roots of *Valeriana alliariifolia* were collected from Trabzon (Turkey), in July 2017 and the voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, University of Istanbul (ISTE 115520).

2.2. Preliminary Qualitative Phytochemical Analysis

2.2.1. Preparation of the Plant Material for the Preliminary Analysis

From 5 g of each air-dried and powdered plant parts, the infusions were prepared with 100 boiled water in 30 min. After cooling, the infusions were filtered. With these filtrates, following tests were performed for qualitative detection of phytochemicals e.g. flavonoids, anthracenes, saponins, tannins (catechic tannin and gallic tannin). In the tests alkaloids, the dried aerial parts and roots were used separately [30]. The results are expressed in the presence (+) and absence (−) of the phytochemicals.

2.2.2. Test for flavonoids

Over 5 mL infusion, 5 mL Shibata Reagent (1 part conc. HCL + 1 part water + 1 part ethanol) and a piece of Magnesium were added and it has been observed whether orange, red or purple color has occurred (Shibata Reaction = Cyanidin Reaction)

2.2.3. Test for Anthracene Compounds

Over 10 mL infusion, 5 drops concentrated H_2SO_4 was added. The mixture was warmed in boiling water for 15 min. and extracted with 5 mL toluene after cooling. Over the toluene

phase, 3 mL NH_3 solution (10%) was added and it has been observed whether rose pink to red color has occurred.

2.2.4. Test for Saponins

10 mL infusion was shaken vigorously in a graduated cylinder for 30 sec. It has been observed after 15 min. whether minimum 1 cm foam layer has occurred.

2.2.5. Test for Tannins

2.2.5.1. Gelatin Test (for the General Determination of Tannins)

Over 5 mL infusion, 2 mL Gelatin-salt Reagent (Gelatin solution (1%) saturated with NaCl) was added. It has been observed whether cream-beige precipitate has occurred.

2.2.5.2. Separation of The Type of Tannins

1. step: Ferric Chloride Test: Over 10 mL infusion, 3 drops $FeCl_3$ (5%) was added. It has been observed whether blue-black colour (gallic tannin) or dark olive green (catechic tannin) has occurred.

2. step: Stiasny Reaction (for the separation of tannin types): Over 10 mL infusion, 5 mL Stiasny Reagent (formol in water (30%) + conc. HCl) was added. The mixture was warmed in water (80°C) for 30 min. When the material contains catechic tannin, precipitates in potions appeared. After cooling of the mixture, it was filtered. 3mL filtrate was saturated with sodium acetate. After adding of 3 drops diluted $FeCl_3$ solution, it has been observed whether blue-black precipitate or colour has occurred.

2.2.6. Test for Alkaloids

1 g of each air-dried and powdered plant parts were extracted with 10mL H_2SO_4 solution (3%) in hot water. They were cooled and filtered. After adding 5 mL NH_3 solution (10%), it was stirred with 10 mL ether. The layers were allowed to separate. The etheric phase was evaporated to dryness. The residue was dissolved in 10 mL H_2SO_4 solution (3%). The alkaloid control reactions were made on this solution in 3 portions.

1. After adding of the Mayer Reagent (mercuric chloride + potassium iodide + water), it has been observed whether milk-white precipitate has occurred.
2. After adding of the Bouchardat Reagent (iodine + potassium iodide + water), it has been observed whether dark red precipitate has occurred.
3. After adding of the Dragendorff Reagent (bismuth carbonate + potassium iodide + water), it has been observed whether orange-red precipitate has occurred.

2.3. Determination of Total Moisture (Loss on Drying) and Total Ash Content

2.3.1. Total Moisture (Loss on Drying) Content

For the determination of the moisture content, two different methods in European and Turkish pharmacopoeia were used.

Method in European pharmacopoeia: Air-dried and powdered plant parts (weight: A) were put into a pre-dried and weighed (Wa1) crucible. The samples were dried in an oven at 100-105 °C for 2 hours and weighed (Wa2). The percent loss of weight of air-dried sample was calculated by equation: % = (Wa2-Wa1) x100 / drug weight

Method in Turkish pharmacopoeia: Air-dried and powdered plant parts (weight: A) were put into a pre-dried and weighed (Wb1) crucible. The samples were dried in an oven at 100-105 °C for 3 hours and reweighed (Wb2). The percent loss of weight of air-dried sample was calculated by equation: % = (Wb2-Wb1) x100 / drug weight

2.3.2. Total Ash Content

Air-dried and powdered plant parts (weight: A) were put into a pre-dried and weighed (Wc1) crucible. The samples were ignited gradually in an electrical muffle in 600 °C for 30 min. It was cooled in desiccators and reweighed (Wc2). Total ash content was calculated as in equation: % = (Wc2 – Wc1) x 100 / A

2.4. Preparation of Extracts

The dried and powdered roots, used in traditional medicine, were successively macerated with hexane (AHM1 for the aerial parts extract and RHM1 for the roots extract), chloroform (ACM1 for the aerial parts extract and RCM1 for the roots extract), and ethanol (AEM1 for the aerial parts extract and REM1 for the roots extract) and water (AWM1 for the aerial parts extract and RWM1 for the roots extract), with stirring for a day. Furthermore, two portions of the aerial parts were individually macerated with ethanol (AEM2 for the aerial parts extract and REM2 for the roots extract) and water (AWM2 for the aerial parts extract and RWM2 for the roots extract), with stirring for a day. An infusion was prepared from another portion with boiled water (AWI for the aerial parts infusion and RWI for the roots infusion) as in its traditional use. The organic extracts were evaporated to dryness and the aqueous extracts were lyophilized. The extracts were stored at ± 4°C till further used.

Due to the occurrence of precipitation in the AWM1 and RWM1 during the storage at ± 4°C, the extracts were filtered, and the filtrates (AWM1filtrate and RWM1filtrate) and residues (AWM1residue and RWM1residue) were studied separately.

2.5. Extract Yield Percentage

The extraction yield is a measure of the solvents efficiency to extract specific components from the aerial parts and roots. The percentage yield was calculated with: (A2 -A1 / A0) ×100

(A0 = weight of the initial dried, used plant part; A1 = weight of container; A2 = weight of container + extract)

2.6. Chromatographical Methods of Chemical Composition

2.6.1. Thin Layer Chromatography (TLC)

In order to estimate the chemical profile of the extract, thin layer chromatographic analyses were performed. As stationary phase, silica gel 60 F254 aluminium plates. For the development used mobile phases can be seen in the Table 1. Chromatograms were visualised by exposing to UV at 254 and 366 nm (Camag UV lamp) or by using derivatization agent, Anisaldehyde reagent (105°C; 5 min).

Anisaldehyde reagent:

Stock solution: 10 mL anisaldehyde + 90 mL ethanol

Dilution of stock: 20 mL stock solution + 2 mL conc. H_2SO_4

2.7. Qualitative Analyse of the Valepotriates

The qualitative analyses of two plant parts were performed by using the method in the investigation of Hassan et al. [31]. In this study, it was recorded that, the absorbance measurement at 212 nm (hydroxyvalerenic acid), 217 nm (acetoxyvalerenic acid) and 218 nm (valerenic acid). The evaluation of the present results were realized according of this absorbance values.

2.8. Determination of Total Phenolic Contents in Extract

Total phenolic content of plant extracts was determined with the Folin-Ciocalteu reagent (FCR) method [32]. Briefly, 0.1 mL of the extract was put in a plate and 4.5 mL of water was

added. Then, 0.1 mL of FCR (diluted with distilled water to the ratio 1:3) and 0.3 mL of 2 % sodium carbonate solution were added to the mixture. The mixture was left at room temperature for 2 h, and then absorbance was measured against the reference at 760 nm. Total phenolic content was expressed as mg of gallic acid equivalents per g of the extract.

Table 1. TLC mobile phases

System No.	Mobile phases	Solvent ratios	Aim
1	toluene : ethyl acetate : methyl ethyl ketone	80 : 15 : 5	To determine and compare the low, moderate and high polarity
2	hexane : ethyl acetate: glacial acetic acid	65 : 35 : 0.5	To determine and compare the low, moderate and high polarity
3	chloroform : methanol	20 : 1	To determine and compare the moderate and high polarity
4	chloroform : methanol	9 : 1	To determine and compare the moderate and high polarity
5	chloroform : methanol	1 : 20	To determine and compare the moderate and high polarity

2.9. Antioxidant Assays

2.9.1. Chemicals and Reagent for Antioxidant Activity

2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), Folin-Ciocalteu's phenol reagent 2N, gallic acid, and ascorbic acid, all of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO, US).

2.9.2. DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picryl-hydrazil) radical scavenging activity of different extracts were measured by the DPPH$^{\bullet}$ method proposed by Fu et al. [33]. Briefly, 240 µL of DPPH solution (0.1 mM) was added to 10 µL of extracts. Then the mixture was allowed to stand at room temperature for 30 min. The absorbance of the mixture was measured against the reference using a micro plate reader at 517 nm.

2.9.3. Ferric Reducing Antioxidant Power Activity (FRAP)

The FRAP reagent was prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of the TPTZ solution (10 mM TPTZ in 40 mM HCl) and 2.5 mL of 20 mM $FeCl_3.6H_2O$. Then the FRAP reagent was kept at 37°C for 30 minutes in incubator device (Nuve). 190 µL of the FRAP reagent was mixed with 10 µL of extract and after 4 minutes the absorbance of the mixture was measured against the reference at 593 nm. FRAP values of the extracts were expressed as mM Fe^{2+}/mg extract [34].

2.9.4. Cupric Reducing Antioxidant Capacity (CUPRAC)

Cupric reducing antioxidant capacity assay was carried out according to the method of Apak et al. [35]. 60 µL Cu(II)x$2H_2O$, 60 µL neocuproine and 60 µL, NH_4Ac (1 M) were mixed. Then 60 µL of the extract and 10 µL of ethanol were added to the mixture. After the duration time of 60 min, the mixture absorbance was spectrophotometrically measured at 450 nm. CUPRAC values of the extracts were given as mM trolox/mg extract.

2.10. Statistical Analysis

All the experiments were done in triplicates. The results of the antioxidant, experiments were demonstrated as mean ± SD. All the data was analysed by the Graphpad Prism 5 program.

3. RESULTS and DISCUSSION

3.1. Preliminary Qualitative Phytochemical Analysis

The results of the preliminary qualitative phytochemical analysis on aerial parts and roots of *V. alliariifolia* are shown in Table 2.

Table 2. The preliminary qualitative phytochemical analysis results to determine the contents of *V. alliariifolia* aerial parts and roots

Secondary metabolites		Aerial Parts	Roots
Alkaloids	Bouchardat Reaction	-	-
	Dragendorff Reaction	-	-
	Mayer Reaction	-	-
Anthracene analogs		-	-
Flavonoids		+ Flavones (Light orange)	+ Flavonols (Light red)
Saponins		-	-
Tannins	Gallic tannin	-	+
	Catechic tannin	+	+

3.2. Determination of Total Moisture (loss on drying) and Total Ash Content

3.2.1. Total Moisture (loss on drying) Content

The total moisture contents of the aerial parts and roots according to the European pharmacopoeia (EP) and Turkish pharmacopoeia (TP) methods are represented in Table 3.

Table 3. The values of the total moisture contents according to the European pharmacopoeia (EP) and Turkish pharmacopoeia (TP) methods.

Total Moisture Contents	Aerial Parts	Roots
with EP method (%)	14.44	10.85
with TP method (%)	11.62	11.28

3.2.2. Total Ash Content

The total ash contents according to the European pharmacopoeia (EP) and Turkish pharmacopoeia (TP) methods are given in Table 4.

Table 4. The values of the total ash contents.

	Total Ash Contents (%)
Aerial Parts	6.88
Roots	4.43

Both of the loss on drying values and total ash content of valerian roots should not exceed 12.0% (w/w) according to TP and EP. Since these values were below the maximum acceptable limit of TP and EP, the roots of *V. alliariifolia* were found to be suitable for TP and EP.

3.3. Extracts Yields Percentages

The yields of the extracts are shown in Table 5 and summarized in Figure 1.

Table 5. Extracts yields by prepared with different solvents and different methods from the aerial parts and roots of *V. alliariifolia*.

Yields (%)	Aerial Parts	Roots
Hexane extr. – gradient maceration (GM)	1.786	2.626
Chloroform extr. – GM	1.41	4.066
Ethanol extr. – GM	3.196	4.362
Water extr. filt. – GM	5.542	3.6
Water extr. residue – GM	2.6	2.2
Ethanol extr. – standard maceration (SM; maceration for 24h)	2.314	4.82
Water extr. – SM	16.680	17
Infusion	12.748	9.5

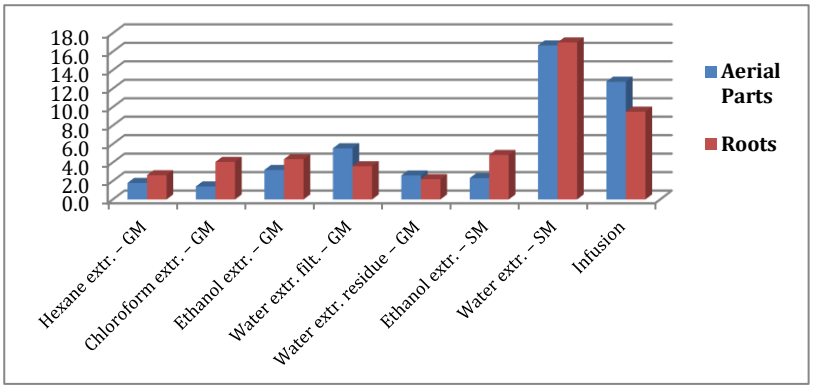

Figure 1. Summary of the extracts yields.

3.4. Chromatographical Methods of Chemical Composition

The mobile phases, mentioned mostly in literature, were used in thin layer chromatography (TLC) methods. However, when the chromatograms were examined, a different mobile phase (System 5) was also needed beside of the mobile phases System 3 and 4. Therefore, the extracts were applied on TLC additionally with the System 5.By comparing the chromatograms, the system 5 was found the best mobile phase for examination of the alcohol and water extracts. For the separation of the compounds in nonpolar extracts, it was seen that the systems 1 and 2 were more useful than other mobile phases. In various studies, the Rf values of valerenic acid analogs were found similar to slightly different depending on the polarity of TLC mobile phases. In a study on the quantification of valerenic acid and its derivatives in certain *Valeriana* species, valerenic acid appeared at Rf 0.49 on TLC chromatogram developed with hexane:ethyl acetate:glacial acetic acid (65:35:0.5) beside of the presence of acetoxyvalerenic and hydroxyvalerenic acids [31]. In a reference book, it was recorded that, the spots of valerenic, acetoxyvalerenic and hydroxyvalerenic acids could be monitored as violet after derivatization with anisaldeyde–sulphuric acid reagent by TLC

[hexane:ethyl acetate:glacial acetic acid (65:35:0.5); Rf values: 0.55, 0.34, 0.1]. [36]. Additionally, Caudal et al. used a mixture of cyclohexane, ethyl acetate and acetic acid (60:38:2) as mobile phase and designated acetoxyvalerenic acid at Rf 0.43 beside of valerenic and hydroxyvalerenic acids (Rf values: 0.61 and 0.25, respectively) [37]. Similarly in extracts prepared from the aerial parts (especially AEM2 and AEM1), the valerenic acid analogs (valerenic, acetoxyvalerenic and hydroxyvalerenic acids) showed a fluorescence quenching at 254 nm on the chromatogram (Mobile Phase System 2), which were seen as violet spots after derivatization with anisaldeyde–sulphuric acid reagent. Thus, the presences of valerenic, acetoxyvalerenic and hydroxyvalerenic acids were determined as like as in System 1 and 3. From the TLC chromatograms developed with mobile phase systems 1 and 3, the valerenic acid analogs content in root extracts were also indicated. Especially RCM1 contains higher amount of these compounds than other extracts prepared from the roots.

3.5. Qualitative Analyse of the Valerenic Acid and Its Derivatives

Following the method described by Hassan et al., the extrcats were prepared from the aerial parts and roots of *V. alliariifolia* and measured between the ranges of 190-1500 by UV-spectrophotometry. The absorbance values (Table 6) were evaluated in comparison to the absorbance values, which were specified for the valerenic acid analogs (valerenic, acetoxyvalerenic and hydroxyvalerenic acids) in the study of Hassan et al. [31]. The valerenic, acetoxyvalerenic and hydroxyvalerenic acids were detected in these extracts in parallel to the results of TLC.

Table 6. The absorbance values of the extracts from aerial parts and roots by UV-spectrophotometry.

Extracts	Absorbance values
Extract from aerial parts	219, 216.5, 214
Extract from roots	219, 216, 214

3.6. Antioxidant Assays and Determination of Total Phenolic Contents

The results are obtained as in Table 7. According to the antioxidant activity results by DPPH method, RWI, RWM1 and RWM1residue were found most active (67.67±0.38; 63.91±1.13 and 63.91±0.99, respectively). The antioxidant activities of polar compounds can be determined with this method. Also, it is known that, the phenolic compounds are related to the antioxidant activity. As a matter of fact, the active extracts have quite high phenolic content. The much higher activity of REM1 than AEM1 by DPPH can be indicated that, the roots of *V. alliariifolia* contain more polar compounds than its aerial parts.

Beside of DPPH method, the FRAP and CUPRAC methods are very important for testing of the reductive potantials of the compounds on heavy metals. While AHM1 was most active (0.269±0.012 mM Fe^{2+}/mg extract) by FRAP method, REM1 showed the highest activity by CUPRAC method (0.891±0.034 mM trolox/mg extract). The high activity of AHM1 is parallel to its phenolic content, but the total phenolic contents of other active extracts by FRAP were found fewer than expected amounts. It can be concluded that, the nonphenolic compounds in these extracts have a role in activity of extracts. Similarly, the phenolic content of REM1 (most active extract by CUPRAC) was found less than the content of less active extracts. In FRAP tests, exhibiting the higher activities of the nonpolar extracts than other extracts can be elucidated that nonpolar compounds of this species have a role in this activity. Due to the results of CUPRAC tests, it can be seen that these process in FRAP tests has proceeded for the nonpolar extracts of the roots differently and the polar extracts of the roots showed higher activity than its nonpolar extracts. But the nonpolar extracts of the aerial parts exhibited higher activity by CUPRAC, as expected.

Table 7. The absorbance values of the extracts from aerial parts and roots by UV-spectrophotometry.

Extracts	DPPH (%) (200 µg/mL)	FRAP (mM Fe^{2+}/mg extract)	CUPRAC (mM trolox/mg extract)	Total phenolic content (mg of GAE per g of extract)
AHM1	5.64±0.75	0.269±0.012	0.369±0.002	34±0.008
ACM1	4.89±1.13	0.210±0.028	0.629±0.179	26±0.014
AEM1	28.32±1.52	0.116±0.002	0.680±0.084	4±0.003
AWM1	43.61±0.75	0.044±0.008	0.464±0.042	11±0.002
AWM1residue	48.75±0.95	0.039±0.005	0.584±0.061	14±0.001
AEM2	30.08±1.59	0.136±0.005	0.571±0.007	15±0.009
AWM2	26.32±0.38	0.087±0.011	0.400±0.092	9±0.002
AWI	44.74±1.36	0.030±0.006	0.425±0.0638	6±0.002
RHM1	4.14±0.65	0.217±0.016	0.243±0.015	2±0.001
RCM1	10.53±0.99	0.232±0.017	0.223±0.002	10±0.005
REM1	53.13±1.78	0.070±0.002	0.891±0.034	17±0.001
RWM1	63.91±1.13	0.026±0.002	0.619±0.095	20±0.005
RWM1residue	63.91±0.99	0.044±0.004	0.549±0.031	16±0.005
REM2	41.48±0.78	0.143±0.004	0.720±0.030	14±0.002
RWM2	35.09±0.22	0.041±0.003	0.390±0.052	8±0.001
RWI	67.67±0.38	0.057±0.003	0.721±0.050	23±0.003
BHA	83.22±0.7	-	-	
BHT	-	1.1±0.12	5.78±0.07	

These values are the means of three replicates ± standard deviation. ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; BHT – butylated hydroxytoluene; DPPH – 2,2-diphenyl-1-picrylhydrazyl; GAE – gallic acid equivalents

In general, the total phenolic contents were not parallel to the activity results. In the study by Jugran et al., the correlation between phenolic content and the antioxidant activity of the extracts from 25 populations of *V. jatamansi* Jones. were exhibited obviously [38]. In the contrary of the data about this correlation in previous studies on *Valeriana* species, any correlation between phenolic content and antioxidant activity was found in this study as like as in our previous investigation on *V. alliariifolia* collected in 2012 [29]. In our previous study on *V. alliariifolia* roots, the activity of the extracts, evaluated in terms of DPPH radical scavenging activities, is as follows: EM1 (IC50: 17.69 µg / mL)> EM2 (IC50: 20 µg / mL) > W1 (IC50: 37 µg / mL). In this study, the DPPH radical scavenging ability of root extracts was ranked as: W1 (67.67%) >WM1=WM1residue (63.91%)> EM1(53.13%)> EM2(41.48%). According to these results, the activity of the EM1 and EM2 extracts was in parallel with the previous study results, but in this study, no parallelism was detected in the W1 extract and it was found that it showed higher activity than the other extracts. In comparison between the extracts from aerial parts and roots, it is seen that the root extracts exhibited higher DPPH radical scavenging activity than the extracts from aerial parts. In our previous study, it was found that the extracts, prepared from the roots, contain higher phenolic substance amounts than the total phenolic content results in this study. These differences between the results can be explained by the fact that the content of plants, especially the essential oil containing plants, can be variable depending on the collection of the plant in different locations and in different time periods. Thus, these content differences occurred also in *V. alliariifolia*, as seen in previous studies on the composition of its essential oil. From this point of view, besides polar and nonpolar compounds play a role in the activity together, phenolic substances as well as non-phenolic substances involve in eliciting antioxidant effect. Based on this, it can be concluded that both of the polar and nonpolar compounds of *V. alliariifolia* play a role in the activity together, and also its phenolic substances as well as non-phenolic substances involve in eliciting antioxidant effect.

4. CONCLUSION

When the antioxidant test results of the root extracts in our two studies were compared, it was observed that the results differed from each other. It can be thought that this difference is due to the change of plant content from year to year and various environmental factors. This is important for achieving standardization in the use of the plant as a medicine and in the production of its preparations. By increasing the studies in this direction, it can be concluded that this plant can be very useful in the treatment area by standardization in plant studies.

Acknowledgements

The authors declare that, this study was a graduation thesis of Pharm. Merve Cimsit (Advisor: Burcu Sen-Utsukarci).

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 3, 174–180

https://doi.org/10.21448/ijsm.722574

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Antimicrobial and Antiproliferative Activities of Chia (*Salvia hispanica* L.) Seeds

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Abstract: The genus *Salvia* L. (Lamiaceae) has been traditionally used for the treatment of various illnesses since ancient times. *Salvia hispanica* L., commonly known as Chia, is an annual herbaceous plant which was one of the most significant crops for pre-Columbian civilizations (Aztec and Maya) in America. Nutritional potential and beneficial effects of Chia seeds on human health have been previously reported. Therefore, this study aims to investigate anti(myco)bacterial, antifungal, and antiproliferative activities of Chia seeds. Ethanol extract of Chia seeds were tested against *Staphylococcus aureus* (ATCC 25925), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25923), *Acinetobacter baumannii* (ATCC 02026), *Aeromonas hydrophila* (ATCC 95080), *Candida albicans* (ATCC 14053), *Candida tropicalis* (ATCC 1369), and *Candida glabrata* (ATCC 15126) using broth microdilution method. Antimycobacterial activity was performed against *Mycobacterium tuberculosis* H37Rv using resazurin microtiter plate method. Ampicillin, Ethambutol, Isoniazid, and Fluconazole were chosen as reference drugs. Antiproliferative effect of the various concentrations (200, 100, 50, and 25 µg/mL) of ethanol extract was tested against A549 human lung cancer cell lines using MTT method. Ethanol extract was found to be more effective against *A. baumannii* (MIC: 62.5 µg/mL) than reference drug Ampicillin (MIC: 125 µg/mL). There was a correlation between increased doses and antiproliferative activity of extract against A549 human lung cancer cell lines (p <0.05).

ARTICLE HISTORY

Received: April 18, 2020
Revised: May 10, 2020
Accepted: June 25, 2020

KEYWORDS

Salvia hispanica L.,
Chia,
Seed,
Antimicrobial activity,
Antiproliferative activity

1. INTRODUCTION

Intake of dietary phytochemicals has been played important roles in the prevention of various illnesses including cancer, inflammatory, and cardiovascular diseases. Due to their medicinal properties plants and their metabolites are also used in different industries [1]. *Salvia* L. (sage) is the most species-rich genus of the family Lamiaceae (mint family) with approximately 1000 species [2]. It has been reported since ancient times that *Salvia* species have been traditionally used in the treatment of tuberculosis, bronchitis, and microbial infections [3]. Some species of the genus have been used worldwide on account of their beneficial effects on human health and nutritional properties [4].

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ISSN-e: 2148-6905 /© IJSM 2020

Salvia hispanica L., commonly named as Chia, is an annual herbaceous plant which is native to northern Guatemala and southern Mexico and is also cultivated in some countries including Mexico, Bolivia, Australia, Argentina, Colombia, Peru, and Guatemala [4]. In the recent years, Chia seeds which were one of the most significant crops for pre-Columbian civilizations (Aztec and Maya) in America [4,5], have been used in the food, animal feed, medical, cosmetics, and pharmaceutical industries [6]. Chia seeds have important roles as nutritional supplement and functional food. Moreover, seeds contain no toxic components and gluten, thus making Chia seeds a safe ingredient also gluten free diets [4].

According to the literature, antiproliferative activity of Chia seeds was studied against some cancer cell lines [7,8]; however, we didn't reach any available literature on antiproliferative effect of Chia seeds against A549 human lung cancer cell lines. Additionally, antimicrobial activity of Chia seeds has been investigated in few studies [9,10]. But some factors such as geographical origin and extraction procedure were changed composition of bioactive compounds in seeds. The consumption of Chia seeds has been increasing over the years due to their health benefits and uses in cooking [4]. Therefore, in this study, we aimed to investigate *in vitro* anti(myco)bacterial, antifungal, and antiproliferative activities of Bolivian Chia seeds.

2. MATERIAL and METHODS

2.1. Chemicals

Isoniazid, Fluconazole, Ethambutol, RPMI 1640 Medium, 3-(N-morpholino)-propanesulfonic acid, Resazurin sodium salt powder, Dulbecco's modified eagle's medium (DMEM), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] (MTT), and Fetal calf serum (FCS) were purchased from Sigma-Aldrich (St. Louis, MO, USA); ethanol and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany); and Middlebrook 7H9 broth, casitone, glycerol, and oleic acid-albumin-dextrose-catalase were purchased from Becton Dickinson (Sparks, MD, USA). All solutions were prepared with distilled water and freshly prepared solutions were used.

2.2. Plant Material and Extraction Procedure

Commercially available Chia seeds from Bolivia (2019 harvest) were purchased from a local market. Powdered seeds were extracted twice with ethanol (20 mL solvent per 1 g seed; 96%) by stirring overnight at room temperature then filtered using Whatman Grade No.1 filter paper. Solvent was evaporated via a vacuum evaporator (Heidolph Instruments, Germany) and obtained extract was kept in the dark at 4 °C.

2.3. Antimicrobial Activity

Gram-negative bacterial strains [*Acinetobacter baumannii* (ATCC 02026), *Escherichia coli* (ATCC 25923), *Aeromonas hydrophila* (ATCC 95080)]; gram-positive bacterial strains [*Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25925)]; *Mycobacterium tuberculosis* H37Rv; and fungal strains [*Candida albicans* (ATCC 14053), *Candida tropicalis* (ATCC 1369), *Candida glabrata* (ATCC 15126)] were procured from Refik Saydam Hıfzıssıhha Institute, Ankara, Turkey.

2.3.1. Antibacterial Activity

Antibacterial activity was studied using a broth microdilution method [11]. Ampicillin was used as a reference drug. Sample was dissolved in DMSO for preparing initial concentration (2000 µg/mL). The mixture was used for having stock solution which was diluted in Mueller-Hinton broth. Further dilutions of reference drug and extract were prepared (1000-0.9 µg/mL). Standard strain working suspensions were made in sterile tubes. Turbidity adjusted to match McFarland standard No: 0.5. Further dilutions (1:20) of suspensions were prepared in

distilled water and added to each plate (10 µL). Thus each plate's bacterial concentration was adjusted to $5x10^5$ CFU/mL. Effect of DMSO was tested. The minimal inhibitory concentration (MIC) values were determined in duplicate tests.

2.3.2. Antimycobacterial Activity

Antimycobacterial activity was tested using the resazurin microtiter assay [11]. Isoniazid and Ethambutol were used as reference drugs. Resazurin reagent was prepared using Resazurin sodium salt powder. Middlebrook 7H9 broth containing 0.1% casitone, 0.5% glycerol, and 10% oleic acid-albumin-dextrose-catalase and 7H9-S medium were used for preparing culture medium. A resazurin working solution (0.01% (w/v)) made in distilled water and stock solutions (1000 µg/mL) of extract and reference drugs prepared in DMSO were filtered through 0.22 µm membrane filter (Ministar, Goettingen, Germany). A two-fold dilution series were performed using 7H9-S medium (100 µL) in a 96-well microtiter plate. 0.12-250 µg/mL concentration ranges were detected. A growth control and a sterility control were added to each plate. The bacterial inoculum was prepared in a tube which was containing 7H9-S medium (5 mL) via resuspending a loopful of Lowenstein-Jensen culture medium. During 2 min the tube was mixed then waited to allow sediment. After supernatant was added in sterile tube, the turbidity adjusted to match McFarland standard No: 1. 7H9-S medium was used to prepare dilutions (1:20) of these suspensions. Plates were inoculated with diluted suspension (100 µL) then put into plastic bags. After incubation period (37 °C, 7 days) Resazurin working solution (30 µL) was added to each well then plates were incubated (37 °C, 24 h) and results were visually recorded. The lowest concentration that prevents complete color change of resazurin from blue to pink was determined as MIC value. Experiments were done in duplicate.

2.3.3. Antifungal Activity

Antifungal activity was studied using a broth microdilution method of NCCLS [12]'s standard document (M27-A2) with minor modifications [11]. RPMI 1640 medium which buffered to pH 7.0 with 0.165 M 3-(N-morpholino) propanesulfonic acid was used. Fluconazole was used as a reference drug. Working suspensions of standard strains were made as a 1:100 dilution followed by a 1:20 dilution of the stock suspensions using RPMI 1640 medium. Stock solutions (1000 µg/mL) of extract and reference drug dissolved in DMSO were filtered through membrane filters. Two-fold dilution series were added in a 96-well microtiter plate using RPMI 1640 medium (100 µL). 250-0.12 µg/mL concentration ranges were tested. A growth control and a sterility control were added to each plate. 100 µL of working inoculum suspension was added to each plate and plates were incubated (48 h, 35 °C). MIC values were visually determined in duplicate tests.

2.4. Antiproliferative Activity

Determination of cell viability was studied by MTT method. A549 human lung cancer cell lines were procured from ATCC (American Type Culture Collection, VA, USA). DMEM which was supplemented with FCS (10%) was used for cell cultivation. Cells were kept in suitable culture conditions (95% air; 5% CO_2; 37 °C). After reaching 70-80% confluency cells were detached with Trypsin-EDTA solution (3.0 mL) and settled to 96-well plates (10^4 cells per well). After 24 h, various concentrations (200, 100, 50, and 25 µg/mL) of ethanol extract dissolved in DMSO were applied and cells were incubated (24 h). Cells treated with growth medium containing no FCS were used as a control. After incubation, supernatants were replaced with MTT (1 mg/mL) dissolved in growth medium then incubated (37 °C) until purple precipitate was visually detected. The supernatants were removed; cells which absorbed MTT were dissolved in DMSO. Plates were detected using a spectrophotometer (Epoch, Winooski, USA) at a 550 nm. Effect of DMSO was tested. Experiments were done in four replicates [13].

2.5. Statistical Analysis

SPSS 25.0 (IBM, NY, USA) was used for statistical analyses. The data are provided as the mean ± SD. Kruskal Wallis H and one-way analysis of variance (ANOVA) with Tukey's post hoc test were used. P values < 0.05 were considered as significant.

3. RESULTS and DISCUSSION

In the present study antimicrobial and antiproliferative activities of ethanol extract (yield 20.67% (w/w)) of Chia seeds were evaluated. Antimicrobial activity results are provided in Table 1. When compared to reference drug Ampicillin (MIC: 125 µg/mL) seed extract had greater activity against *A. baumannii* (MIC: 62.5 µg/mL). Extract showed antimycobacterial activity against *M. tuberculosis* H37Rv (MIC: 62.5 µg/mL); however, the efficiency of the extract was not found as strong as Isoniazid and Ethambutol (MIC values: 0.97 µg/mL and 1.95 µg/mL, respectively). Extract showed the highest antifungal activity against *C. glabrata*; but result was not found as high as Fluconazole (MIC values: 31.25 µg/mL and 3.90 µg/mL, respectively).

Table 1. Minimum inhibitory concentrations of Chia seeds, and reference drugs against bacterial and fungal strains (µg/mL).

Microorganisms	*S. hispanica*	Reference drugs			
		A	I	E	F
Bacterial strains					
Staphylococcus aureus ATCC 25925	250	31.25	-	-	-
Bacillus subtilis ATCC 6633	250	0.9	-	-	-
Escherichia coli ATCC 25923	250	15.62	-	-	-
Acinetobacter baumannii ATCC 02026	62.5	125	-	-	-
Aeromonas hydrophila ATCC 95080	125	31.25	-	-	-
Mycobacterium tuberculosis H37Rv	62.5	-	0.97	1.95	-
Fungal strains					
Candida albicans ATCC 14053	62.5	-	-	-	31.25
Candida tropicalis ATCC 1369	62.5	-	-	-	15.62
Candida glabrata ATCC 15126	31.25	-	-	-	3.90

Values determined in duplicate with deviations within one two-fold dilution. -: Not tested. (A: Ampicillin; I: Isoniazid; E: Ethambutol; F: Fluconazol)

Antimicrobial effect of Chia seeds was investigated against several microorganisms including *E. coli, A. baumanii, S. aureus,* and *C. albicans*; and aqueous and aqueous-ethanol extracts exhibited antimicrobial activity against *E. coli* [10]. Despite these results, protein hydrolysates of Chia seeds were not showed antimicrobial activity against *E. coli, Salmonella typhi, Shigella flexneri, Klebsiella pneumoniae, S. aureus, B. subtilis*, and *Streptococcus agalactiae* [9]. In our study, seeds exhibited antibacterial activity against both tested gram-negative and gram-positive bacterial strains. According to the literature, composition and concentration of bioactive compounds in Chia seeds vary depending on some factors like geographical origin, climatic conditions, agricultural practices, and extraction procedures [4]. These factors also change the effectiveness of the tested extract. This may explain different results in our study and in previous studies. Due to their hydrophilic cell wall structure which contains lipopolysaccharides inhibits accumulation of hydrophobic oils and extracts, and penetrations of these substances through the target cell membrane, gram-negative bacteria are more resistant against natural components than gram-positive bacteria [14]. When compared to Ampicillin, seeds showed greater activity against gram-negative nosocomial pathogen *A.*

baumannii which is one of the important healthcare problems worldwide because of its ability to gain resistance to all classes of antimicrobial agents used against it [15]. According to the our results, Chia seeds might be promising sources in the development of novel therapeutic agents against infections caused by *A. baumannii*.

Antiproliferative activity results are shown in Table 2. Significantly lower cell viability levels were observed in 100 µg/mL concentration applied group than control group (0.963 ± 0.036 and 1.092 ± 0.012, respectively) and in 200 µg/mL concentration applied group than control and DMSO groups (0.936 ± 0.036; 1.092 ± 0.012; and 1.085 ± 0.009, respectively) ($p < 0.05$). However, there were no significantly differences found between the other groups ($p > 0.05$).

Table 2. Antiproliferative effect of Chia seeds on A549 human lung cancer cell lines in MTT cell viability assay.

Groups	Control	DMSO	*S. hispanica*			
			Studied concentrations of the extract (µg/mL)			
			25	50	100	200
Results	1.092 ±0.012 (1.083-1.109)	1.085 ± 0.009 (1.073-1.095)	1.021 ± 0.025 (1.000-1.057)	0.983 ± 0.031 (0.953-1.025)	0.963 ± 0.036[a] (0.938-0.974)	0.936 ± 0.036[ab] (0.896-0.971)

Measuring the average ± SD. Min-Max value intervals are in parenthesis. Kruskal Wallis H and ANOVA with Tukey's *post hoc* test were used. n = 4. A P value less than 0.05 was considered to be significant. [a] Significantly different from control group. [b] Significantly different from DMSO group. (Control: the group was not exposed any chemical, just incubated only with medium; DMSO: the group was treated medium with DMSO).

Chia oil reduced tumor growth, metastasis, and cell mitosis in neoplastic tissue and increased apoptosis. However; in Walker 256 model Chia flour supplementation didn't prevent tumor bearing effects [8]. Effects of mucilage compounds on some cancer lines (HeLa, HCT-15, HCT-116, MCF7, MDA-MB-231, MCF7/Vin, MCF7/Vin^+, MCF7/Vin^- cells, Vero, and HepG2 cells) were evaluated and significant inhibition on proliferation of MCF7, HeLa, and HepG2 cells with low toxicity were determined [7]. However, we didn't reach any available literature on antiproliferative effect of Chia seeds against A549 human lung cancer cell lines. The lung cancer which is the most common type of malignant tumors with high mortality is expected to cause over than 3 million deaths for the year 2035 [16]. A549 human lung cancer cell lines have been widely studied for cancer research since 1976 [17]. In our study, ethanol extract of Chia seeds were found to be more effective against A549 human lung cancer cell lines at a dose of 200 µg/mL and there was a correlation between increased doses and activity.

Phenolic acids (caffeic acid and its derivatives, ferulic acid, rosmarinic acid [4], chlorogenic acid [4, 6, 10]), flavonoids (myricetin, quercetin, kaempferol [4, 6, 10], rutin [10], daidzin, genistein, genistin, glycitein, glycitin [4]), fatty acids (α-linolenic acid, linoleic acid, palmitic acid, stearic acid, oleic acid), tocopherols (α-, δ-, γ-tocopherol), dietary fiber, proteins, carbohydrate, vitamins, amino acids [4, 6], minerals [4, 18], mucilage [6,7], and phytosterols [19] have been reported as chemical constituents of Chia seeds in previous studies. Ethanol was a suitable solvent for phenolics, sterols [20] and broad range of polar constituents [21]. Increased uses of phenolic compounds as antimicrobial agents, and food stabilizers in food technology have been reported. Because of food safety, ethanol and water are the most suitable solvents for extraction of phenolics [22]. Therefore, in the current study, ethanol was preferred as extraction solvent. Antimicrobial properties of flavonoids, phenolic acids [20], polysaccharides and sterols [14] and antiproliferative activity of phenolic compounds [16] have been reported previously.

4. CONCLUSION

The consumption of Chia seeds has been increasing over the years and its health benefits related to chronic diseases like cardiovascular diseases, obesity, cancer, and diabetes. In the current study, we investigated some biological properties of Bolivian Chia seeds. Chia seeds might be promising sources in the development of novel therapeutic agents against *A. baumannii*. Antiproliferative effect of Chia seeds against A549 human lung cancer cell lines was determined for the first time. In the future, this study may be the basis for further studies on the effects of seeds against lung cancer and in addition to its nutritional potential this might be a new topic to support Chia consumption.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 3, 181–191

https://doi.org/10.21448/ijsm.706514

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Antioxidant, Enzyme Inhibitory and Calcium Oxalate Anti-crystallization Activities of *Equisetum telmateia* Ehrn.

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Abstract: *Equisetum* L. is the only genus of the Equisetaceae family, which commonly known as horsetails, in English and atkuyruğu or kırkkilit in Turkish. In traditional medicine, *Equisetum telmateia* Ehrh. is used in diseases of the urinary system, such as pyelonephritis, prostatic hypertrophy, and cystitis. Besides, this species is known to be used by humans to treat kidney stones or kidney sand. The extracts were obtained from the aerial parts of the *E. telmateia* using three different extraction methods (maceration, Soxhlet, ultrasonic bath) and their antioxidant (ABTS, CUPRAC), anti-urease and anticholinesterase activities were examined. Also, calcium oxalate anti-crystallization activity of Soxhlet methanol extract showing strong antioxidant activity was determined. Soxhlet methanol extract exhibited stronger ABTS radical scavenging (0.0676 mM Trolox/mg extract) and cupric ion reducing/antioxidant (4.351 mM Trolox/mg extract) activity than other extracts. Soxhlet methanol (65.528%) and maceration methanol (61.965%) extracts showed the strongest anticholinesterase activity. In the anti-urease assay, it was found that Soxhlet petroleum ether extract (15.302%) had the highest anti-urease activity. Furthermore, the data obtained showed that the Soxhlet methanol extract had high efficacy in the nucleation and aggregation phase of calcium oxalate crystals. These results prove that Soxhlet methanol extract has antioxidant, anticholinesterase and anti-crystallization capabilities. Therefore, this extract can be used in the future as an antioxidant and anticholinesterase agent as well as the treatment and / or prevention of stone formation.

ARTICLE HISTORY

Received: March 21, 2020
Revised: April 18, 2020
Accepted: July 08, 2020

KEYWORDS

Equisetum telmateia,
Anti-urease,
Anticholinesterase,
Anti-crystallization

1. INTRODUCTION

Equisetum L. is the only genus of the Equisetaceae family, which commonly known as horsetails, in English and atkuyruğu or kırkkilit in Turkish. While there are approximately 32 known species in the world, there are 7 species in the Flora of Turkey. They are usually perennial plants rich in cell wall silica, growing in moist and wet places. *Equisetum telmateia* Ehrh. species has the widest distribution among *Equisetum* species in regions such as Europe, West Asia, Northwest Africa and North America [1]. *Equisetum* species is often used in traditional medicine for various ailments as a medicinal tea. They are very effective in the

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ISSN-e: 2148-6905 /© IJSM 2020

treatment of urinary tract infections, cardiovascular diseases, respiratory infections, and medical skin conditions. In traditional medicine, *E. telmateia* is used in diseases of the urinary system, such as pyelonephritis, prostatic hypertrophy, and cystitis. Besides, this species is known to be used by humans to treat kidney stones or kidney sand. The aerial parts of the *E. telmateia* are used in the treatment of stomach pain, abdominal pain, peptic ulcers, eczema, acne and rheumatism in Anatolia [2-4]. *E. telmateia* species contain flavonoids and phenolic acids. *E. telmateia* has been known neuroprotective, antimicrobial, anti-inflammatory, diuretic antiulcerogenic and potent antioxidant effects. Phenolic compounds are known to be effective in these biological activities of the plant [5].

Free radicals are compounds that contain one or more unpaired electrons. Since free radicals contain unpaired electrons, they can easily react with biological molecules in the organism, such as lipids, nucleic acid, protein, and carbohydrates. Therefore, these radicals are effective in the formation of various diseases formation of various diseases including cardiovascular diseases, tissue damage,ischemic heart diseases, cancer, atherosclerosis, central nervous system damage, nervous disorders, obesity, gastritis, arthritis and aging in humans [6-8]. As with other aerobic organisms, free radicals occur as a result of naturally occurring metabolic events in humans. These free radicals are eliminated by antioxidant defense mechanisms. Antioxidants inhibit lipid peroxidation by inhibiting the peroxidation chain reaction or by collecting reactive oxygen species. Therefore, antioxidants play an important role in both protection and treatment against these various ailments caused by free radicals, by preventing DNA damage, reducing the abnormal increase in cell division [9]. Increased intake of exogenous antioxidants reduces the effects caused by these radicals. Natural antioxidants are commonly found in food and medicinal plants, and they have anti-inflammatory, anti-aging, anti-atherosclerosis and anticancer effects [10-12].

Alzheimer's disease (AD) is a progressive neurodegenerative disease [13]. Some studies are promising for this disease, but unfortunately, there is currently no cure for the disease [14, 15]. *Helicobacter pylori (H. pylori)* infection is among the world's most common infections. *H. pylori* infection is a common worldwide infection that is an important cause of peptic ulcer and gastric cancer [16, 17]. Antibiotic therapy for the treatment of *H. pylori* infections has many disadvantages, such as lack of effectiveness, development of resistance, gastrointestinal side effects and possible recurrence of the disease. Among the difficulties for *H. pylori* eradication, increased resistance to antibiotic regimens currently used in therapy is a major concern, suggesting that therapeutic strategies need to be changed. As a result, there is a growing interest in the development of new antimicrobial therapeutic agents, preferably with a higher chance of natural treatment against *H. pylori* [18].

Stone formation in the kidney and urinary tract is a serious pathology that is important today and can lead to kidney failure if left untreated. It is known that it affects an average of 4-20% of the population. In recent years, the incidence of the disease has also been increasing [19]. A kidney stone is a total of crystallization stages consisting of nucleation, growth and aggregation stages of crystals in saturated urine in epithelial cells in renal papillae. These crystals are essentially available in three different forms: calcium oxalate monohydrate ($CaC_2O_4.H_2O$, COM or whewellite), calcium oxalate dihydrate ($CaC_2O_4.2H_2O$, COD or weddellite) or calcium oxalate trihydrate ($CaC_2O_4.3H_2O$, COT or caoxite). Calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) form the main structure of kidney stones. Calcium oxalate trihydrate (COT) is rarely found in kidney stones. Calcium oxalate monohydrate (COM) is the thermodynamically most stable form and has a high affinity for renal tubular cells. As a result, it has the effect of initiating stone formation in the urinary area and kidney. Therefore, the number of studies on the inhibition of calcium oxalate monohydrate crystals has been increasing in recent years [20-22]. Although there are important advances in

the pathophysiology and treatment of kidney stones, it is highly likely that kidney stones may occur again. The painful and high cost of surgical methods and drug treatments led to new searches in treatment. Studies on natural products have been focused on the prevention and recurrence of this disease [23, 24].

The purpose of this study was to evaluate *in vitro* antioxidant, anti-urease, anticholinesterase and calcium oxalate anti-crystallization activities of different extracts from *E. telmateia* aerial parts.

2. MATERIAL and METHODS

2.1. Identification of Plant Material

E. telmateia was taxonomically identified by Assist. Prof. Dr. Ahmet DOĞAN. The voucher specimens, representative samples of the plant material, were archived in the herbarium of the Faculty of Pharmacy, Marmara University and documented with the herbarium number of MARE:20465.

2.2. Preparation of *E. telmateia* Extracts

Aerial parts of *E.telmateia* were dried at 25ºC in the shade. Dried parts of the plant were treated with a mechanical grinder (Renas, RBT1250) for fine powder and proper weight. The three extraction methods were performed to gain crude extracts from the aerial parts of the plant.

Maceration: Plant powder (45 g) was extracted with the use of petroleum ether, chloroform and methanol until colorless.

Soxhlet extraction: 45 grams of plant powder was extracted in the Soxhlet apparatus with petroleum ether, chloroform and methanol.

Ultrasonic bath extraction: 45 grams of plant powder were extracted in an ultrasonic bath with petroleum ether, chloroform and methanol. The nine different extracts from the plant were concentrated by a rotary vacuum evaporator. All the extracts obtained were stored at 4°C for future analysis.

2.3. $ABTS^{\cdot+}$ Radical Scavenging Assay

50 µL of extracts prepared at different concentrations (1–5 mg/mL), 50 µL of $ABTS^{\cdot+}$ working solution and 150 µL distilled water were added on the prepared extracts. The mixture absorbance was determined against the reference at 734 nm for 6 min. The control sample was prepared under the same conditions with the use of 50 µL distilled water instead of experimental and standard materials. The control sample was daily measured. ABTS radical scavenging determination was applied to Trolox solutions prepared at different concentrations (0.2-1 mM). The results of this study were given as mM Trolox/mg extract [25].

2.4. Cupric Ion Reducing/Antioxidant Power (CUPRAC) Assay

60 µL Cu(II)x2H_2O, 60 µL neocuproine and 60 µL, NH_4Ac (1 M) were mixed. Then 60 µL of the extract and 10 µL of ethanol were added to the mixture. after 60 min duration time, the mixture absorbance was spectrophotometrically measured at 450 nm. CUPRAC values of the extracts were given as mM Trolox/mg extract [26].

2.5. Anti-Urease Activity Assay

Stock solutions were prepared from different extracts obtained from the plant and these solutions were diluted to prepare working solutions. A working solution (100 µL) was taken and urease (500 µL) was added on it. The mixture was incubated at 37ºC for 30 min. Then, 1100 µL of urea was added to this mixture and kept in the incubator at 37ºC for 30 min. R1 (1% phenol, 0.005% sodium nitroprusside) and R2 (0.5% NaOH, 0.1% sodium hypochlorite)

reagents were added to the mixture, respectively. After the incubation period at 37ºC for 2 h, the absorbance of samples was measured at 635 nm [27].

The % inhibition of urease was calculated by the formula:

% enzyme inhibition = $[(A_0 - A_1)/A_0] \times 100]$

A_0: The absorbance of the control solution

A_1: Absorbance of plant extracts and standard solutions.

2.6. Anticholinesterase Activity Assay

Inhibition activities of acetylcholinesterase (AchE) were measured using a microplate reader. Acetylcholinesterase is an enzyme source derived from electric fish, acetylthiokolin iodide was used as a substrate. Yellow-colored 5,5-dithiobis- (2-nitrobenzoic acid) (DTNB) was used for the measurement of activity. As a control, ethanol and galantamine, the alkaloid type drug isolated from the *Galanthus* plant, were used as controls. Briefly, the AchE (20 µL) and different concentrations of extracts (20 µL) were added to phosphate buffer solution (pH 8.2 0.1 M, 40 µL). This mixture was incubated at 25ºC for 10 min. After incubation, DTNB (100 µL) and AcI (20 µL) as substrate were added to the mixture. The same procedure was applied to the galantamine used as standard. 5-thio-2-nitrobenzoic acid was spectrophotometrically measured at 412 nm. Anticholinesterase activity of the extracts was calculated using the following equation as% inhibition relative to control [28].

$\%I = (A_{control} - A_{sample}/A_{control})x100$

2.7. Crystallization Assay

2.7.1. Nucleation Assay (Turbidity Method)

Solutions of calcium chloride (5 mM) and sodium oxalate(7.5 mM) were prepared, respectively, in a buffer containing Tris-HCI 0.05 M and NaCl 0.15 M at pH 6.5. 3 mL of calcium chloride and sodium oxalate solution was mixed with 1 mL of the extract at different concentrations. The mixture solution was mixed with vortex for 30 seconds. After the incubation period at 37ºC for 30 min. The absorbance of the solution was monitored at 620 nm [29].

2.7.2. Aggregation Assay

CaOx monohydrate (COM) crystals were prepared by mixing calcium chloride and sodium oxalate at 50 mM. Both solutions were equilibrated to 60 °C for 1 hour in a water bath. It was then kept in an incubator at 37 °C for 1 night and then placed in the desiccant. COM crystals were used at a final concentration of 0.8 mg/mL, buffered with buffer at pH 6.5. 3 mL of CaOx solution was mixed with 1 mL of the extract at different concentrations. The solution was mixed with vortex for 30 seconds. After the incubation period at 37ºC for 30 min the absorbance of solution was monitored at 620 nm [29].

2.7.3. Crystal Characterization

The number, size and morphology of CaOx crystals was observed by light microscopy (40x magnification) in the presence and absence of extracts after nucleation and aggregation experiments [29].

2.8. Statistical Analysis

All the experiments were done in triplicates. The results of the antioxidant, anticholinesterase and anti-urease experiments were demonstrated as mean ± SD. All the data were analyzed by the Graphpad Prism 5 program. Statistical differences between the study groups were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test and p-values less than 0.05 were considered statistically significant.

3. RESULTS and DISCUSSION

3.1. Antioxidant Activity

The results of the ABTS assay showed that Soxhlet methanol (0.0676 mM Trolox/ mg extract) and maceration methanol (0.0662 mM Trolox/mg extract) extracts exhibited the strongest ABTS radical cation scavenging activity. Also, it was found that petroleum ether extract obtained from ultrasonic bath and Soxhlet methods did not show ABTS activity. The Soxhlet methanol (4.351 mM Trolox/mg extract) and maceration methanol (3.826 mM Trolox/mg extract) extracts exhibited stronger cupric reducing antioxidant activity than other extracts. In this study, it was found that the Soxhlet extraction technique is the most suitable method to get the most powerful ABTS and CUPRAC antioxidant activity. In addition, it was determined that the solvent with strong antioxidant activity was methanol in these assays.

The antioxidant and antifungal activity of dichloromethane, ethyl acetate, methanol and water extracts of the sterile stem of the plant was investigated. The methanol extract (IC_{50} 12.92 µg/mL) was found to have stronger DPPH activity than dichloromethane (not detected), ethyl acetate (IC_{50} 50.90 µg/mL) and water (IC_{50} 20.32 µg/mL) extracts (Table 1). Also, antifungal activity of all extracts against *Rhizopus stolonifer* strain was not detected [30]. In our study, it was found that the Soxhlet (0.0676 mM Trolox) and maceration (0.0662 mM Trolox) methanol extracts showed strong radical scavenging (ABTS) activity in parallel with this study. In another study, antioxidant activities of water and ethyl acetate extracts obtained from the aerial parts of the plant were evaluated by DPPH and TEAC methods. Besides, as a result of analysis of these extracts with HPLC-PAD-ESI/MS, it was found that both extracts contain flavan-3-ol, kaempferol and phenolic acid derivatives as major phenolic compounds. In this study, it was observed that ethyl acetate extract have higher DPPH (IC_{50} 0.018 mg/mL) and TEAC (342.8 mg Trolox/mg extract) activity than water extract (IC_{50} 0.455 mg/mL; 11.00 mg Trolox/mg extract) [31]. When the TEAC values of the ethyl acetate (342.8 mg Trolox) and methanol (0.0676 mM Trolox) extracts were compared, it was observed that the ethyl acetate extract had higher TEAC value than methanol extract. The different TEAC values may be due to used of the different solvent and the extraction method. Besides, harvesting the plant from different times and regions may have been effective in showing different ABTS (TEAC values) radical scavenging activity.

The antioxidant activities of aqueous, methanol and ethanol extracts of the aerial parts of *E. telmateia* have been previously investigated by the CUPRAC assay. In this study, it was found that ethanol (Abs:0.6 nm) extract had higher cupper reducing antioxidant activity than methanol (Abs:0.5 nm) and aqueous (Abs:0.2 nm) extracts [32]. In our study, it was determined that methanol extracts [especially Soxhlet methanol (4.351 mM Trolox)] obtained by three extraction techniques showed strong cupper reducing antioxidant activity in parallel with the literature study (Table 1).

3.2. Urease Inhibitory Activity

The percentage of inhibition of urease enzyme at 12.5 µg/mL concentration of different extracts was determined by using the Indophenol method and the results were shown in Table 2. Soxhlet petroleum ether (15.302%) and maceration petroleum ether (8.815%) extracts exhibited stronger anti-urease activity than other extracts. In the ultrasonic bath, chloroform (4.531%) and methanol (3.498%) extracts showed the best anti-urease activity. Comparing the activity results of all the extracts, the Soxhlet petroleum ether extract had the strongest anti-urease activity and all extract showed lower activity than thiourea compounds (70.05%). The Soxhlet extraction technique is the most suitable method to obtain the strongest anti-urease activity. It was also found that the most suitable solvent for obtaining strong anti-urease activity was petroleum ether.

Table 1. The antioxidant activity of *E. telmateia* different extracts

Samples	ABTS (mM trolox/mg extract)			CUPRAC (mM trolox/mg extract)		
	Ultrasonic bath	Maceration	Soxhlet	Ultrasonic bath	Maceration	Soxhlet
Petroleum ether	NA	0.0063± 0.004[a]	NA	1.18± 0.0412[a]	1.086± 0.03[a]	1.515±0.068 [a]
Chloroform	0.0003± 0.001[a]	0.0082± 0.003[b]	0.0103± 0.0035[a]	1.587± 0.1513[b]	1.576± 0.03[b]	1.661±0.055 [b]
Methanol	0.0638± 0.0011[b]	0.0662± 0.0016[c]	0.0676± 0.0013[b]	2.791± 0.0849[c]	3.826± 0.20[c]	4.351±0.014 [c]
Ascorbic acid	0.013± 0.001[c]	00.013± 0.001[d]	0.013± 0.001[c]			
BHA				1.83± 0.0002[d]	1.83± 0.0002[d]	1.83± 0.0002[d]

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts (a-d) are significantly different, $p<0.05$; NA: not activity

Table 2. The anti-urease activity of different extracts of *E.telmateia*

Samples	Enzyme inhibition percentage (12.5 µg/mL)		
	Ultrasonic bath	Maceration	Soxhlet
Petroleum ether	2.62±2.3302[a]	8.815±0.52[a]	15.302±1.251[a]
Chloroform	4.531±1.196[b]	2.635±1.998[b]	3.76±2.001[b]
Methanol	3.498±3.57[c]	6.149±3.1732[c]	7.921±4.363[c]
Thiourea	70.05±0.007[d]	70.05±0.007[d]	70.05±0.007[d]

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts (a-d) are significantly different, $p<0.05$

3.3. Anticholinesterase Activity

The percentage of inhibition of acetylcholinesterase enzyme at 200 µg/mL concentration of different extracts was determined by using the Ellman method [28] (Table 3). The Soxhlet (65.528%), maceration (61.965%) and ultrasonic bath (32.179%) methanol extracts exhibited the highest percentage of inhibition of acetylcholinesterase enzyme. The Soxhlet petroleum ether and chloroform extracts obtained by three extraction techniques did not show acetylcholinesterase inhibitory activity.

The anti-urease activity of methanol extract from *Equisetum arvense* stem at a concentration of 10 mg/mL was examined. In this study, it was found that methanol extract (52.35%) had moderate activity compared to the hydroxyurea (100%) which used as standard [33]. In our study, unlike the above study, the petroleum ether extract (15.302%) of the *E. telmateia* aerial parts at a concentration of 12.5 µg/mL showed stronger anti-urease activity other other extracts. It was also found to have lower anti-urease activity compared to the

standard (70.05%). The anticholinesterase activity of ethanol extract from *E.arvense* aerial parts was examined by Ellman method [28]. According to the results, ethanol extract (IC_{50}:3.134 mg/mL) exhibited lower anticholinesterase activity than galantamine (IC_{50}: 0.003 mg/mL) which used as standard [34]. In our study, in parallel with the study above, it was observed that the Soxhlet (65.528%) and maceration (61.965%) methanol extracts of the *E. telmateia* aerial parts at a concentration of 200 µg/mL showed lower anticholinesterase activity than galantamine (85.289%). As far as we know, the present work was the first reporting on the anti-urease and anticholinesterase activity of different extracts from *E. telmateia* aerial parts.

Table 3. The anticholinesterase activity of different extracts of *E. telmateia*

Samples	Enzyme inhibition (%) (200 µg/mL)		
	Ultrasonic bath	Maceration	Soxhlet
Petroleum ether	16.719±2.6683[a]	33.489±1.7713[a]	NA
Chloroform	NA	NA	NA
Methanol	32.179±6.623[b]	61.965±3.0718[b]	65.528±2.8942[a]
Galantamine	85.289±0.8852[c]	85.289±0.8852[c]	85.289±0.8852[b]

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts (a-c) are significantly different, $p<0.05$; NA: not activity

3.4. Inhibition of the Calcium Oxalate's Crystallization

Antioxidants have the effect of preventing stone formation by protecting endothelial cells against oxidative damage [35]. Therefore, the inhibitory effect of Soxhlet methanol extract showing strong antioxidant activity against the crystallization of calcium oxalate was compared *in vitro* and presented in Table 4, Figure 1 and Figure 2. The data obtained showed high efficacy in preventing nucleation of calcium oxalate crystals of methanol extract at a concentration of 5 mg/mL (85.59%), but it did not show any effect at a concentration of 2 mg/mL. It was also found that potassium citrate used as positive control showed 98.25% and 93.56% inhibition values at concentrations of 5 mg/mL and 2 mg/mL, respectively in this study. Regarding the aggregation phase, although the methanol extract at a concentration of 5 mg/mL (35.31%) showed high efficacy, no effect at 2 mg/mL concentration was observed, similar to the nucleation assay. Potassium citrate has been studied at 5 mg/mL (85.14%) and 2 mg/mL (71.32%) concentrations and has been found to have higher efficacy than methanol extract at both concentrations. These findings showed that the plant's methanol extract had calcium oxalate anti-crystallization activity in parallel with traditional medicine.

3.5. Crystal Characterization

To confirm the results we obtained in the calcium oxalate anti-crystallization assay, we followed the presence of crystals nucleation and aggregates using a light microscope (Figure 1 and Figure 2). Light microscopy analysis of the images taken during the nucleation phase shows that the number of crystals is higher in the control (without extract) state and the number and size are become becomes less important in the presence of methanol extract at a concentration of 5 mg/mL (Figure 1). Furthermore, the analysis of the images taken at the aggregation phase showed the presence of larger and more aggregates without extract, while the size and number of crystals decreased in the presence of methanol (5 mg/mL) (Figure 2).

As far as we know, there are no reports on calcium oxalate anti-crystallization activity of *E. telmateia* aerial parts. The activity of calcium oxalate anti-crystallization of Soxhlet methanol extract from *E. telmateia* were evaluated for the first time by us.

Table 4. Calcium oxalate anti-crystallization activity of methanol extract from *E. telmateia*

Concentration (mg/mL)	Nucleation (%)		Aggregation (%)	
	Methanol	Potassium citrate	Methanol	Potassium citrate
5	85.59±0.12	98.25±0.65	35.31±0.25	85.14±0.92
2	NA	93.56±1.02	NA	71.32±0.89

NA: not activity

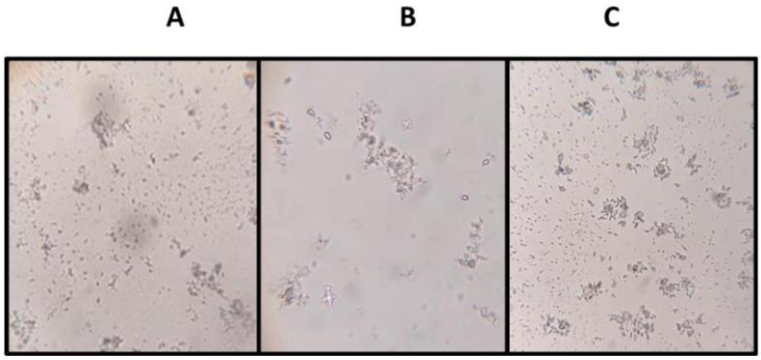

Figure 1. Calcium oxalate crystals in nucleation stages: A: Absence of inhibitor, B: methanol extract (5mg/mL), C: methanol extract (2 mg/mL)

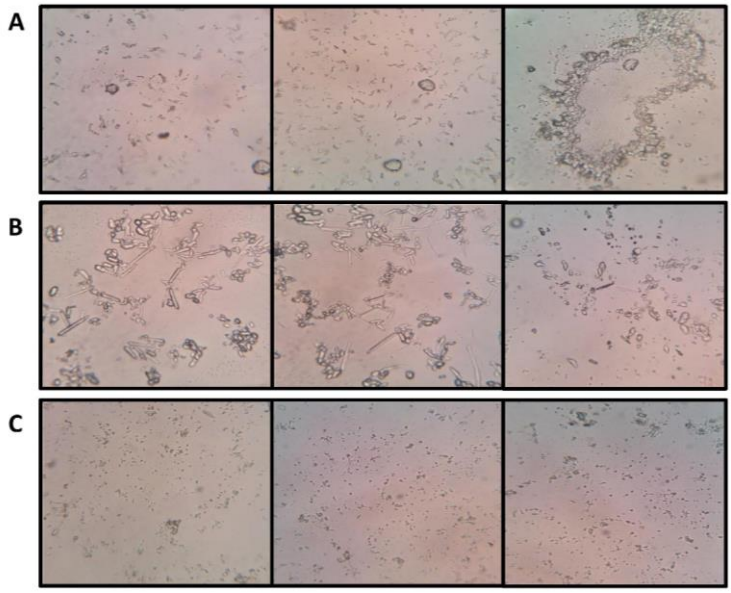

Figure 2. Calcium oxalate crystals in aggregation stages: A: Absence of inhibitor, B: methanol extract (2 mg/mL), C: methanol extract (5 mg/mL)

4. CONCLUSION

In this study, it was found that Soxhlet methanol extracts from the aerial parts of the plant showed higher antioxidant and anticholinesterase activity than other extracts. Soxhlet

petroleum ether extract exhibited the strongest anti-urease activity. Furthermore, it was found that Soxhlet methanol extract at a concentration of 5 mg/mL had strong inhibition of the calcium oxalate's crystallization. Therefore, methanol extract can be used in the future as an antioxidant and anticholinesterase agent as well as the treatment and /or prevention of stone formation.

Acknowledgements

This research was not received any grant or fund support from public or commercial sectors.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 3, 192–199

https://doi.org/10.21448/ijsm.732568

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Comparison of The Antimicrobial Activity of Some *Scutellaria orientalis* L. Taxa Growing in Turkey

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Abstract: *Scutellaria* species are commonly used in the treatment of various diseases in traditional medicine. One of the members of Lamiaceae, the genus *Scutellaria* L. has approximately 471 species on earth. The genus is represented by 39 taxa in Turkey which 17 of them are endemics. In this study, plant samples of 15 *S. orientalis* subspecies from different regions of our country were collected and methanol extracts were prepared from aerial parts. *In vitro* antimicrobial activity of these extracts against three gram positive and three gram negative bacteria and against a yeast using broth microdilution method. Methanol extracts of *S. orientalis* taxa were found to have moderate to low antimicrobial activity compared to the literature.

ARTICLE HISTORY

Received: May 06, 2020
Revised: June 17, 2020
Accepted: August 10, 2020

KEYWORDS

Scutellaria orientalis,
Lamiaceae,
Antimicrobial activity

1. INTRODUCTION

Due to increasing resistance to antimicrobials and slowdown in the exploration of new ones, combating with infectious diseases is getting harder nowadays. Studies on extracts or compounds from plants remain important to discover new sources as antimicrobial agents. The genus *Scutellaria* L. (Lamiaceae) contains 471 species throughout the world [1]. *Scutellaria* species are generally subcosmopolitan plants and are distributed especially in the central Iran-Turanian region of Asia [2]. In Turkey, the genus includes about 39 taxa and 17 of these are endemics (43.6%) [3-9].

Scutellaria orientalis L. consists 16 subspecies and 2 varieties in Turkey and most of them are endemic [3,7]. The plants have been dispersed among the East-West Anatolia and Iran-Turanian regions of Turkey. Many *Scutellaria* species have been used in traditional medicine for centuries. The genus has numerous biological activities such as anti-convulsant, anti-cancer, anti-diarrheal, anti-feedant, anti-hypertensive, anti-inflammatory, anti-microbial, anti-oxidant, anti-thrombotic, hepatoprotective and sedative activities [10-11]. *Scutellaria* species are known as “kaside, korku otu, sancı otu, şimşek otu” in Turkish. There are various uses in traditional medicine and the most common of them are as sedative in the form of

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ISSN-e: 2148-6905 /© IJSM 2020

hemostatic, wound healing agent and tonic in Turkey [7,12-13]. According to ethnobotanical studies in Anatolian traditional medicine, leaves of some subspecies of *S. orientalis* L. (subsp. *orientalis*, subsp. *sosnowskyi* (Takht.) Fed., subsp. *bicolor* (Hochst.) J.R.Edm., subsp. *pichleri* (Stapf) J.R.Edm. and subsp. *virens* (Boiss. & Kotschy) J.R.Edm.) were also used as wound healer, externally, as carminative, abdominal pain relief and also used for its astringent effects [14-21].

There are many studies on the phytochemical properties of *Scutellaria* species. Phenolic compounds, diterpenoids were isolated by Ersöz et al. and Rodríguez et al. from *S. pontica* K.Koch [16, 19]. Essential oil compositions of *S. albida* L., *S. diffusa* Benth., *S. heterophylla* Montbret & Aucher ex Benth, *S. salviifolia* Benth., *S. brevibracteata* Stapf, *S. galericulata* L. and *S. tortumensis* (Kit Tan & Sorger) A.P.Khokhr. were also investigated [20-23]. Cytotoxic activity of phenylethanoid glycosides isolated from *S. salviifolia* was determined [24]. The acetylcholinesterase, butyrylcholinesterase and tyrosinase inhibitory activities of methanol extracts and the antioxidant activity of methanol and ethyl acetate extracts were investigated by DPPH and FRAP experiments in a study by Şenol et al. [25]. In recent years, İçen et al. investigated the chemical composition of *S. orientalis* subsp. *virens* essential oil [26]; Yavuz et al. examined antibacterial effects of *S. salviifolia* [27]; Zengin et al. (2018) studied antioxidant activity, enzyme inhibitory activity and phenolic components of *S. orientalis* and *S. salviifolia* [28]; Arıtuluk et al. examined antibacterial and antifungal activity of *S. diffusa, S. pontica* K. Koch and *S. salviifolia* [11]; Bardakçı et al. performed flavonoid quantification of *S. albida, S. albida* L. subsp. *velenovskyi* (Rech.f.) Greuter & Burdet, *S. hastifolia* L. and *S. orientalis* from Turkey [29]. In this study, we planned to investigate the antibacterial and antifungal activity of methanol extracts of 15 subspecies of *S. orientalis* using broth microdilution method.

2. MATERIAL and METHODS

2.1. Plant Material

Aerial parts of fifteen subspecies of *S. orientalis* taxa (*S. orientalis* L. subsp. *virens* (Boiss. & Kotschy) J.R.Edm, *S. orientalis* L. subsp. *orientalis*, *S. orientalis* L. subsp. *sosnowskyi* (Takht.) Fed., *S. orientalis* L. subsp. *bicolor* (Hochst.) J.R.Edm., *S. orientalis* L. subsp. *macrostegia* (Hausskn. ex Bornm.) J.R.Edm., *S. orientalis* L. subsp. *cretacea* (Boiss. & Hausskn.) J.R.Edm., *S. orientalis* L. subsp. *pectinata* (Benth.) J.R.Edm., *S. orientalis* L. subsp. *pinnatifida* J.R.Edm., *S. orientalis* L. subsp. *alpina* (Boiss.) O.Schwarz var. *alpina*, *S. orientalis* L. subsp. *porphyrostegia* J.R.Edm., *S. orientalis* L. subsp. *carica* J.R.Edm., *S. orientalis* L. subsp. *santolinoides* (Hausskn. ex Bornm.) J.R.Edm., *S. orientalis* L. subsp. *sintenisii* (Hausskn. ex Bornm.) J.R.Edm., *S. orientalis* L. subsp. *haussknechtii* (Boiss.) J.R.Edm., *S. orientalis* L. subsp. *bornmuelleri* (Hausskn. ex Bornm.) J.R.Edm.) were collected from common territories of diverse localities in Turkey. All taxa were identified according to "*Flora of Turkey and the East Aegean Islands*" [3] by Mehmet Çiçek. The voucher specimen are kept in the Herbarium of Ankara University Faculty of Pharmacy, Ankara, Turkey (AEF). The species names, collection localities, dates and herbarium numbers of 15 taxa are given in Table 1.

2.2. Preparation of Extracts

Aerial parts of *S. orientalis* taxa were dried and then powdered. About 5 g powdered samples were extracted with methanol (2x200 ml) in a rotary shaker for 24 h. Extracts were filtered and further concentrated to dryness under reduced pressure at 37ºC using a rotary evaporator (Büchi, Switzerland). Methanol extracts were obtained and kept in the freezer +4°C until the experimental practices.

2.3. Antimicrobial Activity

2.3.1. Preparation of Bacterial and Fungal Suspensions

Microorganisms used in the experiment were gram negative bacteria (*Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922; gram positive bacteria *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and yeast *Candida albicans* ATCC 10231). Microorganisms were obtained from the culture collection of the Ankara University, Faculty of Pharmacy, Pharmaceutical Microbiology Department.

Antibacterial and antifungal activity tests proceeded similarly with CLSI recommendations by broth microdilution method with some modifications [30,31]. Glycerol stocks kept at -80°C were inoculated to Sabouraud Dextrose Agar (SDA, Oxoid) medium for yeast and Mueller-Hinton Agar (MHA, Merck) medium for bacteria, incubated for 20 h at 35±2°C. Isolated colonies from the second passage of overnight cultures transferred into 0.85% NaCl to obtain turbidity of 0.5 McFarland standards. Sabouraud Dextrose Broth (SDB) medium for yeast and Mueller-Hinton Broth (MHB, Merck) medium for bacteria were used to dilute these suspensions to give a final concentration 2.5×10^3 CFU/ml for yeast and 5×10^5 CFU/ml for bacteria.

2.3.2. Broth Microdilution Method

Firstly, two-fold 8 serial dilutions of *S. orientalis* methanol extracts (1000 to 7.81 µg/ml) were prepared in 96-well microplates using SDB media for yeast and MHB media for bacteria in 100 µl volume. After serial dilution, 100 µl concentration-adjusted microorganism suspensions were transferred to each well and microplates were left for incubation (24 h for yeast and 20 h for bacteria) at 35±2°C. Sterility and growth control wells were included. Ciprofloxacin and miconazole were used as positive controls. The well of lowest concentration with no growth was recorded as MIC (µg/ml) value, at the end of incubation time. All experiments conducted in two parallels.

3. RESULTS and DISCUSSION

3.1. Antimicrobial Activity Results

In this study, *in vitro* antibacterial and anti yeast activity of methanol extracts of fifteen *S. orientalis* taxa was evaluated using broth microdilution assay against the above mentioned panel of human pathogenic strains of three gram positive bacteria, three gram negative bacteria and a yeast. The results are given in Table 2 as Minimum Inhibitory Concentrations (MIC).

To the best of our knowledge and according to the literature survey, there is no report on comparative antimicrobial activity of *S. orientalis* subspecies growing naturally in Turkey. This study is the first to demonstrate that 15 subspecies of *S. orientalis* possessed *in vitro* antibacterial activity.

According to the results of our study, antibacterial activity values of all methanol extract of *S. orientalis* taxa were found to be between 250-62.5 µg/ml. Antimicrobial activity scale of MIC values 500 to 100 µg/ml were evaluated as moderate, and MIC values less than 100 µg/ml were considered to be good according to the concentration ranges stated by Morales et al. (2008) [32]. As seen in Table 2, it was found that methanolic extracts obtained from the aerial parts of *S. orientalis* taxa have moderate to good antimicrobial activity. All of the species (except *S. orientalis* subsp. *virens* (125 µg/ml MIC value) showed stronger activity (62.5 µg/ml MIC value) against Gram negative *P. aeruginosa* ATCC 27853. Other gram negative bacteria, *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922 were affected more than gram positive bacteria. Least affected bacteria were *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212.

Among the extracts, *S. orientalis* subsp. *orientalis*, *S. orientalis* subsp. *santolinoides* and *S. orientalis* subsp. *haussknechtii* were the most effective ones.

Table 1. The localities of studied the subspecies of *S. orientalis*

Scutellaria ssp.	Locality	Altitude	Collection Date
S. orientalis subsp. *alpina* var. *alpina*	Isparta: Yalvaç, between Akşehir and Isparta	1470 m	19.05.2005
S. orientalis subsp. *bicolor*	Elazığ: Maden to Elazığ	986 m	20.05.2006
S. orientalis subsp. *bornmuelleri*	Van: Hakkari to Van	1383 m	13.06.2006
S. orientalis subsp. *carica*	Aydın: Aydın to Karacasu	321 m	02.05.2006
S. orientalis subsp. *cretacea*	Malatya: Kayseri between Malatya	895 m	28.05.2005
S. orientalis subsp. *haussknechtii*	Mardin: south of Mardin, near Deyrulzafaran Monastery	927 m	30.05.2005
S. orientalis subsp. *macrostegia*	Malatya: 34 km from Kemaliye to Arapgir	1200 m	22.05.2007
S. orientalis subsp. *orientalis*	Erzincan: 45 km from Tercan to Erzincan	1291 m	28.06.2006
S. orientalis subsp. *pectinata*	Malatya: 2 km northwest of Darende	1214 m	28.05.2005
S. orientalis subsp. *pinnatifida*	Ankara: Gölbaşı, Beynam forest	1515 m	11.06.2006
S. orientalis subsp. *porphyrostegia*	Siirt: near Botan Çayı	530 m	19.05.2006
S. orientalis subsp. *santolinoides*	Erzincan: İliç, near Boyalık village	1160 m	23.05.2007
S. orientalis subsp. *sintenisii*	Sivas: Divriği to Gedikbaşı	1177 m	25.06.2006
S. orientalis subsp. *sosnowskyi*	Van: Güzelsu between Başkale, Güzeldere	2757 m	13.06.2006
S. orientalis subsp. *virens*	Van: 66 km from Tatvan to Van, Kuskunkıran pass	2245 m	22.06.2007

Also, all of the extracts showed good activity with a value of 62.5 µg/ml against *C. albicans* ATCC 10231, except for *S. orientalis* subsp. *virens, S. orientalis* subsp. *santolinoides* and *S. orientalis* subsp. *bornmuelleri* since their activities were moderate with a value of 125 µg/ml. According to these results, all methanol extracts of *S. orientalis* taxa showed higher antimicrobial activity against yeasts than bacteria.

Dereboylu et al. (2012) investigated antimicrobial activity of *Scutellaria cypria* var. *cypria, S. cypria* var. *elatior* and *S. sibthorpii* essential oils against *B. subtilis* ATCC 6633, *S. aureus* ATCC6538-P, *E. faecalis* ATCC 29212, *Salmonella typhimurium* CCM 5445, *K. pneumoniae* CCM 2318, *E. coli* ATCC 12228, *P. aeruginosa* ATCC 27853 and *C. albicans* ATCC 10239. They reported the antibacterial activity in the range of ≥20-10 mg/ml and antifungal activity ≥20 mg/ml. [33].

In a study conducted in 2017, antimicrobial activity of *S. salviifolia* methanol extract was tested with the disc diffusion and microdilution methods. MIC results of methanol extract varied between 12,5-25 mg/ml against *E. coli, K. pneumoniae, S. enteritidis P. aeruginosa, S. aureus* [27].

Table 2. Minimum inhibitory concentration results of methanol extracts of samples (in µg/ml)

Methanol extracts	Microorganisms						
	S. aureus ATCC 29213	*E. faecalis* ATCC 29212	*B. subtilis* ATCC 6633	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*K.pneumoniae* ATCC 13883	*C. albicans* ATCC 10231
S. orientalis subsp. *virens*	250	250	250	250	125	125	125
S. orientalis subsp. *orientalis*	125	250	125	125	62,5	125	62,5
S. orientalis subsp. *sosnowskyi*	250	250	250	250	62,5	125	62,5
S. orientalis subsp. *bicolor*	250	250	250	125	62,5	125	62,5
S. orientalis subsp. *macrostegia*	250	250	125	125	62,5	125	62,5
S. orientalis subsp. *cretacea*	250	250	250	125	62,5	125	62,5
S. orientalis subsp. *pectinata*	250	250	125	125	62,5	125	62,5
S. orientalis subsp. *pinnatifida*	250	250	125	250	62,5	125	62,5
S. orientalis subsp. *alpina* var. *alpina*	250	250	250	125	62,5	125	62,5
S. orientalis subsp. *porphyrostegia*	250	125	250	250	62,5	125	62,5
S. orientalis subsp. *carica*	250	250	125	125	62,5	125	62,5
S. orientalis subsp. *santolinoides*	250	125	125	125	62,5	125	125
S. orientalis subsp. *sintenisii*	250	125	125	250	62,5	125	62.5
S. orientalis subsp. *haussknechtii*	125	250	125	125	62,5	125	62,5
S. orientalis subsp. *bornmuelleri*	250	250	250	250	62,5	125	125
Ciprofloxacin	0,312	0,156	0,078	0,0097	0,625	0,039	-
Miconazole	-	-	-	-	-	-	1,56

Arıtuluk et al. (2019) found that aqueous, methanol and n-butanol extracts from roots and methanol and n-butanol extracts from aerial parts of *S. salviifolia, S. diffusa* and *S. pontica* and n-hexane extracts from root parts of *S. diffusa* showed low or no antibacterial activity (≥1024 µg/ml). Aqueous extracts from the aerial parts showed moderate activity with the values of 1024-512 µg/ml; chloroform, ethylacetate and n-hexane extracts of roots and aerial parts showed low to moderate or no activity with the values of ≥1024-256 µg/ml, ≥1024-256 µg/ml, ≥1024-256 µg/ml, respectively. Their anti yeast fungal activity results ranged between ≥1024-32 µg/ml. Chloroform extracts from roots and *n-hexane* extracts from aerial parts of *S. salviifolia* and chloroform and aqueous extracts from the aerial parts of *S. pontica* had good antifungal activity against some *Candida* spp. with values between 64-32 µg/ml. And totally they found higher antifungal activity than antibacterial, in accordance with our results [11].

4. CONCLUSION

This study is the first report on comparative antimicrobial activity of *S. orientalis* taxa from Turkey with methanol extracts by microdilution method to the best of our knowledge. It was determined that the methanol extracts of these 15 taxa had similar levels of activity with slightly different values, which ranged between 250-62.5 µg/ml against all microorganisms. In the future, we plan to investigate antibacterial and anti yeast tests of different *Scutellaria* species and compare their results.

Acknowledgements

This research did not receive any grant or funding support from public or commercial sectors.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 3, 200–212

https://doi.org/10.21448/ijsm.726140

Published at http://dergipark.gov.tr/en/pub/ijsm Review Article

Antioxidant, Pharmacological, Medical Properties and Chemical Content of *Rosa* L. Extracts

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Abstract: Currently, increased attention is paid to plant raw materials as a source of biologically active substances. As a consequence of this great scientific interest is dog rose (lat. *Rosa*), a genus of wild plants of the Rosaceae family (Rosales) and it is widely used as a medicinal, vitamin source and food raw material. The species of *Rosa* genus have rich vitamin content and different chemical structures. Dog rose has medicinal value as a multivitamin remedy. Many works have been devoted to the study of the dynamics of the accumulation of vitamins depending on the forms and types of dog rose, geographic location, meteorological conditions, soil, fertilizer application and other environmental factors. The most economically valuable part of the dog rose is the pulp of the fruit. *Rosa* is used in official and traditional medicine. They also have anti-inflammatory, choleretic, diuretic properties and a beneficial effect on carbohydrate metabolism and, they regulate the activity of the gastrointestinal tract, enhance tissue regeneration, the synthesis of hormones. In this review article, antioxidant, pharmacological, medical properties and chemical content of the *Rosa* genus has been discussed in detail.

ARTICLE HISTORY

Received: September 22, 2019
Revised: April 23, 2020
Accepted: June 18, 2020

KEYWORDS

Rosa L.,
Antioxidant,
Antibacterial,
Phytochemicals,
Medicine

1. INTRODUCTION

Rosa L. is an upright broadleaf shrub, and its height reaches up to 1-2 m [1, 2]. Stems and branches are usually spiked. There are more than 120 species of wild rose in the world and they are widespread in temperate and subtropical zones of the Northern Hemisphere, and occasionally in the mountainous regions of the tropical belt [3, 4]. Some species of wild rose penetrate northward to the Arctic Circle, and southward to Ethiopia, Arabia, Northern India, and the Philippine Islands, and from North America to Mexico. Especially favorable conditions for its growth are in the region from the Mediterranean to the Himalayas and further in East Asia (Figure 1). These plants are resistant to harsh environmental conditions (rocky and sloping terrain, poor soil, lack of water) [5].

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ISSN-e: 2148-6905 /© IJSM 2020

Pliny the Elder (23-79 AD) was the first person to describe the healing properties of *Rosa* when they saw and observed that French ethnic groups use for the treatment of dog bites. Because of this usage for treatment of dog bites, they gave "dog rose" name for it [6]. *Rosa* was also used by a German woman in Europe to make tea that could cure certain diseases. Because of their high content of vitamin C, “dog rose” became a main source of vitamin C in the UK during World War II, the government organized its enormous harvest [7]. Moreover, the great Iranian physician Avicenna (AD 980-1037) mentioned Rosa in his book “The Canon of Medicine” as alighol-kalb (most of the dog) and he indicated that it can treat ulcers, including mouth ulcers and strengthening of the gums [8]. An excellent source of vitamin C, rosehip is an effective medicinal plant for the treatment of gingivitis and swollen or bleeding gums [9-10], which are the main clinical manifestations of scurvy (vitamin C deficiency).

The value of *Rosa* was confirmed after the publishing of stamps with images of 23 species in 19 countries [11]. It should be noted that various parts of this plant have traditionally been used to treat various diseases. Its root, for example, has been used to treat cough *Rosa*, hemorrhoids, and dysuria. Its leaves are used in the treatment of colds, flu and cough, and its branches are useful in the treatment of urolithiasis. Also, its fruits were used to treat asthma, bronchitis and the common cold. Finally, its seeds have been used to treat osteoarthritis, rheumatism and gout [12].

A study conducted in 2010 on six plant species, including *Rosa* that was collected from Southern Europe, indicated that had excellent antioxidant properties, so it could be used as an alternative to synthetic antioxidants [13]. Since plants are among the rich sources of antioxidant compounds, the amount of antioxidant compounds in dietary plants, including various types of fruits, berries, vegetables, cereals, nuts and beans, has been investigated. The results showed that between different antioxidants in different food plants more than 1000 times *Rosa* hips, cherries, blackberries, strawberries, raspberries, sunflower seeds and pomegranates are the plants with the most antioxidants [14].

Figure 1. Habitats of all *Rosa* species around the world.

1.1. Plant Material and *In Vitro* Culture Conditions

The chemical composition of *Rosa* is well studied, including modern high-precision methods of analysis. Minerals of the plants are easily digestible form, they have high biological activity involving biochemical processes in the human body [15]. In the study of *Rosa* fruits by the mass spectrometry method revealed the presence of 16 mineral elements [16]. Shanina and Rubchevskaya [15] studied the mineral composition by the spectral method on a DFS-8 instrument and determined the presence of 28 elements. In the mineral composition of *Rosa* hips was determined by using the atomic absorption method [17, 18]. Data on the mineral composition of the wild rose fruits and leaves are presented in Table 1. It should be noted that, despite the different collection sites and different methods of analysis of plant raw materials, potassium and calcium are the prevailing mineral elements [15, 16, 18].

Table 1. Mineral elements of *Rosa* [40, 44].

Treatment (mM)	Content	
	In Fruits	In Leaves
	Macronutrients	
Potassium	7.05-12.17 g / kg [16]	5.80-5.82% [15]
Calcium	7.63-15.65% [15]	6.23-6.25% [15]
Magnesium	150-180 mg% [17]	11.60-11.61% [15]
Sodium	150-180 mg% [17]	4.64 * 4.69% [15]
Phosphorus	4.28-10.80 g / kg [16]	(1.71-1.711) 10-3% [15]
	Trace elements	
Silicon	1,20-11,60 % [15]	1,160-1,28 % [15]
Manganese	24,91-50,70 mg/kg [16]	(65,0-200,0)*10-3% [15]
	(122,0-239,0) 10-3 % [15]	
	124-189 mkg/100 g [17]	
	0,37-0,59 mg % [16]	
Copper	3,92-14,41 mg/kg [18]	(11,52-25,60)*10-3% [15]
	(22,87-47,80) 10-3 % [15]	
	0,1-0,15 mg/kg [17]	
	0,28-0,35 mg % [16]	
Zinc	3,92-14,41 mg/kg [18]	(6,50-25,70)*10-3 % [15]
	(22,87-47,80) 10-3 % [15]	
	0,1-0,15 mg/kg [17]	
	0,28-0,35 mg % [15]	
Nickel	1,01-2,42 mg/kg [16]	(0,70-1,80)*10-3 % [15]
	1,7-3,4 mkg/100 r [17]	
	(4,58-7,17) 10-3 % [15]	
Cobalt	<0,003 mg/kg % [18]	
	0,48*10-3 % [15]	
Aluminum	0,13-3,48% [15]	1,06-1,75% [15]

1.2. Plant Material and *In Vitro* Culture Conditions

Rosa is considered as a raw material of multivitamin (Table 2). It is rich in a natural source of vitamin C, the content of which exceeds currants 10 times and lemon fruits fifty times [1]. At the same time, the biological role of vitamin C is manifested in the presence of organic acids and P-active compounds, which include anthocyanins, catechins, leucoanthocyanins and flavonols, which differ in chemical composition, but have a similar effect on the human body

[28]. Flavonoids act as antioxidants and inactivate free radicals in the presence of metals [29]. In the fruits of the plant of the genus *Rosa*, they are represented in particular by quercetin, hyperoside, astragalin, rutin, kaempferol-3-arabinoside, etc. [30].

Table 2. Vitamins and biological active substances of *Rosa* hips and leaves [40, 44].

Substance	Content	Substance	Content
	Rosehips		Leaves
Vitamin C	1007.63 - 1901.47 mg% [2, 20]	Vitamin C	11,60-218,80 mg % [2]
	203.09-1082.69% mg [25]		
	498.96–947.69 mg% [12]		
	681–840% UHB * [3]		
	2483.65–3577.07 mg% DM ** [13]		
Vitamin P	184.0 mg / kg [10]	Vitamin P	0,72-1,30 mg % [2]
Vitamin B1	0.73-0.90 mg% [2,6]	Vitamin B1	0,20-1,67 mg % [2]
Vitamin K	1.40-2.00 mg% [2,6]	Vitamin K	0,15-1,40 mg % [2]
Flavonoids	78-102 mg of EGC *** / g CB [3]	Flavonoids	0,10-0,45 mg ER****/ml [14]
Polyphenolic	48.8 mg / kg [10]	Lycopene	0,03-0,005 mg/ml [14]
Substances	4.80-5.90% [10]	β-carotene	0,188-0,277 mg/ml [14]
Flavonols	3.28-4.20% [12]	Total amount	5,41-8,63 mg EGK /ml [14]
Catechins	62-76 mg% [6]	phenolic compounds	
Leucoanthocyanins	740-857 mg% [6]		
Anthocyanins	231-315 mg% [6]		
Tannins	877-1370 mg% [6]		
Chlorophyll	5.71-9.1% [20]		
	5.20-7.80 mg%		
	7.90-5.27 mg% SV [17]		

* FS - fresh substance; ** DS - dry substances; *** EGA - the equivalent of gallic acid, ER **** Equivalent routine.

Tocopherols [31] were found in the rosehips, the antioxidant properties of which are based on the ability to form stable little-reactive radicals as a result of the cleavage of a hydrogen atom from a hydroxyl group upon interaction with active radicals [32]. Carotenoids

are represented mainly by lycopene, lutein and β-carotene [33, 34]. Their role is to bind singlet oxygen and inhibit the formation of free radicals, which helps to prevent the negative effect of the latter on the body [33, 35]. The number of carotenoids increases during the growing season, while the number of chlorophylls decreases [36]. Vitamin and mineral compositions of *Rosa* depends on many environmental factors, but the main ones are ecological and genetic. The ecological factor is the leading one; it is explained by the composition of the water, the nature of the microorganisms and the soil structure, the quality and quantity of the fertilizers applied [37, 38]. A huge role in this process is played by the soil, which contains mobile forms of mineral substances that are successfully absorbed by the plant and contribute to the normal course of the synthesis of vitamins and other important organic compounds. With an increase in the height of the growth of shrubs above sea level, the content of ascorbic acid, carotene, catechins, leucoanthocyanins, anthocyanins, and flavonols increases, but the content of tannins in fruits decreases [39]. At the same time, in addition to the rosehips, *Rosa* leaves have a rich chemical composition (Table 2). *Rosa* leaves occupy the second place in the content of ascorbic acid concerning the vegetative part of the plant [36]. In the leaves of wild rose, the presence of such biologically active substances as carotenoids, chlorophylls, tocopherols and flavonoids has been revealed [35, 40]. Ascorbic acid is found in significant amounts in products of plant origin (*Rosa* hips, primrose leaves, etc.) and it plays an important role in the vital activity of the organism. Due to the presence of a diene group in the molecule, ascorbic acid has strongly pronounced reducing (antioxidant) properties [41].

As a result of a phytochemical study (Table 3) of *Rosa* roots growing in the North Caucasus, the presence and content of biologically active compounds were determined: organic acids, water-soluble polysaccharides, pectinaceous substances, ascorbic acid, tannins and easily oxidizable substances, tannin, triterpenic saponins, amino acids. Arginine, glutamic acid, aspartic acid, and lysine predominate in the amino acid composition of *Rosa* roots. Defined macro-and microelement composition. The results indicate the promise of further research of raw materials and drug production [27].

The advantages of natural origin food and non-toxic antioxidants are obvious when compared with synthetic ones. It is known that non-toxic antioxidants are found in vegetable oils, plant extracts and other plant products [43]. Therefore, along with the study of the chemical composition, the authors widely studied the effect of the vegetative part of *Rosa*, containing natural antioxidants, on the mechanism of inhibition of oxidative processes [44]. The authors also confirm that the sugar or ascorbic acid content in the extract is responsible for the antioxidant activity of rose hips [45].

1.3. Medical Meaning of *Rosa*

For hundreds of years, *Rosa* raw materials used in scientific and traditional medicine. *Rosa* is a pharmacopoeial raw material that is used as a vitamin remedy. The oils prepared from the rosehips have wound-healing and choleretic effects. A promising and important direction in the development of pharmacy is the comprehensive study and rational usage of the whole plant [46].

Ancient medicine defined the nature of *Rosa* as hot and dry in the II degree. It opens the blockage in the internal organs and cleans. The smell of *Rosa* strengthens the heart, brain, sense organs, the brain is hot, heals the cold of nerves. It kills worms in the ear and helps with ringing in the ears, and also it is good for toothache. When *Rosa* is applied to forehead, it can heals a headache [47].

Table 3. The content of biologically active compounds in *Rosa* genus roots [42].

Biologically active compounds	Analysis method	Metrological specifications
Free organic acids in terms of on apple acid	Alkalimetry	S = 0,0474342 SX = 0,0212138 X±ΔX = 8,06±0,047 ε = ±0,58%
Ascorbic Acid	Titrimetry	S = 0,0035637 SX = 0,0015938 ΔX = 0,0035417 X±ΔX = 0,262±0,0035
Tannin	HPLC	N=5 f=4 S=0,02645 SX = 0,011832 ΔX=0,0262942 X±ΔX =1,46±0,026 ε = ±1,80%.
Saponins	Gravimetry	S = 0,0238747 SX = 0,0106774 ΔX = 0,0237273 X±ΔX = 4,66±0,0237 ε = ±0,51%
Amino Acids in recalculated on alanine.	Spectrophotometry	S = 0,015379 SX = 0,006878 ΔX = 0,015284 X±ΔX = 3,788±0,015 ε = ±0,40%

Rosa is very popular in traditional medicine. Its boiled fruits are used as a choleretic and fortifying agent. When Rosa juice is drunk with honey, it can act as a diaphoretic and it can remedy colds, hypertension, liver diseases.The local population of Central Asia prepares preserves of wild rose petals andthey use it as a heart booster and a sedative. The boiled *Rosa* galls are used for treatment of gastric ulcer, duodenal ulcer, malaria, and pulmonary tuberculosis [48]. Jam of its flowers relaxes, heals the heartbeat. It is useful for tumors of the throat and tonsils. Treatment of wild rose flower petals eliminates the unpleasant smell of sweat in the bath. of the boiled rose hips treat stomach cancer. the boiled galls help to remedy the hemorrhoids, soothes pain and burning. Hippocrates used rosehip for treatment of gallbladder disease. Dioscorides also used it for abdominal pain. *Rosa* juice was used as a fixative and hemostatic agent [49, 50]. the boiled branches and leaves of *Rosa* are used for stomach pain, dysentery and boiled roots of it are drunk as a strong diuretic for urolithiasis. the grinded seeds of *Rosa*, with alum, are used for treatment of external wounds. The branches of the plant are burned, and a resinous substance is obtained. With this remedy, psoriasis is typically treated [51]. In Russian traditional medicine, *Rosa* tincture on vodka (1:10) is used in the treatment of diarrhea. *Rosa* liqueur (1 cup of fruit is drawn into the Sun with 1.5 cups of sugar in 3 cups of vodka, for 5 days) is drunk 15-20 grams each, after food as an anticonvulsant, painkiller [52]. In Chinese traditional medicine, the roots of the *Rosa* are used as an antihelminthic. In Tibetan traditional medicine, *Rosa* flowers are used for treatment of neurasthenia, atherosclerosis, and tuberculosis. In Mongolian traditional medicine, *Rosa* is used in the treatment of headaches,

dizziness, burning skin. In Bulgarian folk medicine, fruits, *Rosa* flowers are used as a choleretic, sedative [53].

In modern scientific medicine, *Rosa* has also very widely usage. At First, all of its fruits are used as a source of vitamin C. 5-6 rosehips, fully provide a daily dose of this vitamin. Thanks to vitamin C and polyphenols, the rosehips of its leaves have a pronounced antioxidant and anti-inflammatory effect [54-57].

Experimental studies have revealed the immunomodulatory properties of *Rosa* extract [58, 59]. *Rosa* has been successfully applied to prevent morbidity among frequently ill children [60]. Its positive effects on diseases of the liver and gallbladder are noted. *Rosa* preparations normalize the secretion of the digestive tract, have a sedative, hemostatic effect [61].

Rosa is widely used for treatment of type II diabetes [62, 63]. *Rosa* hips have a hypolipidemic and hypoglycemic effect in diabetes mellitus [64]. Plant extracts inhibit the enzyme α amylase [65]. Prolonged intake of *Rosa* prevents cognitive impairment in diabetic patients [66].

Experimental studies have shown that rosehip extracts inhibit lipid accumulation in white adipose tissue, increases fatty acid oxidation processes in the liver and skeletal muscle, thereby preventing the development of obesity [67].

Randomized placebo-controlled studies have shown that taking *Rosa* for 12 weeks reduces the fatty lining of the abdomen, the body mass index in patients prone to obesity, and prevents the development of obesity [68]. Clinical, randomized, controlled studies have shown that the fruits, rosehips and seeds improve the condition of patients with osteoarthritis [69- 75].

Quercetin, isolated from *Rosa*, inhibited the activity of melanogenesis in melanoma cells [76, 77]. *Rosa* leaf extracts have an antiproliferative effect on leukemia [78], colon tumors [79]. Thanks to flavonoids and vitamin C, *Rosa* extracts have an antitumor effect [80]. The marked stimulating effect of *Rosa* on the secretion of the adrenal gland and thyroid gland. *Rosa* preparations stimulate the immune system, and therefore, it is advisable to prescribe them in infectious diseases [61].

Hot infusions of *Rosa* can be presented as a functional food for individuals with a high level of urates, and as a therapeutic agent for hyperoremic patients [81]. *Rosa* leaf extracts have antidiarrheal properties [82]. *Rosa* extracts prevent hepatic from tetrachloride methane damage [83].

Fruits harvested from various types of *Rosa*, belonging to the three sections, are used as medicinal plant raw materials: Cinnamomeae sections (high vitamin types) - May rosehip (brown rosehip) (*R. canina* (L.), Acicular rosehip (*R. acicularis* Lindl.), Daurian rosehip (R. *davurica* Pall.), Begger's rosehip (*R. beggeriana* Schrenk), Fedchenko's rosehip (R. *fedtschenkoana* Regel), Kokand rosehip (*R. Kokanica* (Regel) Regel ex Juz.); Rugosae sections (high vitamin types) - wrinkled briar (*R. rugosa* Thunb.); and Caninae sections (low vitamin types) - canine briar (*R. canina* L.), briar shchitkon wasp (*R. corymbifera* Borkh.), dog rose small-flowered (*R. micrantha* Smith.), dog rose sand-loving (*R. psammophila* Chrshan.), dog rose felt (*R. tomentosa* Smith.), dog rose zangezura (*R. zangezura* P. Jarosch.) [84- 86].

The raw material is a whole, peeled from the sepals and peduncles false fruits of various shapes: from spherical, ovoid or oval to highly elongated spindle-shaped. The length of the fruit is 0.7 - 3 cm, diameter - 0.6 - 1.7 mm. At the top of the fruit there is a small round hole (in species of the Cinnamomeae section) or a pentagonal platform (in species of the Canina section). Fruits consist of overgrown, fleshy, when ripe juicy receptacle and numerous fruit-nuts enclosed in its cavity. The outer surface of the fruit is shiny, less often matte, more or less wrinkled. Inside, the fruit is covered with long, bristly hairs [87]. Nuts are small, oblong, with weakly expressed edges. The color of fruits is from orange-red to brownish-red, the nuts are

light yellow, sometimes brownish. The smell is absent. The taste is sour-sweet, slightly astringent [85]. There are differences in the preparation of high and low vitamin species. The types with high vitamin content are harvested in August-September. In the instructions for harvesting indicated that the harvesting of fruits should be completed before frost, since after frost during thawing, the content of ascorbic acid in the raw material is reduced. After harvesting fresh *Rosa* can be stored no more than 3 days [86]. When fruits of *Rosa* type with high vitamin content are dired, the various type deyers are used and the fruits exposure at 80-90 Cº. It is considered that at this temperature, the fruit dries quickly without significant loss of ascorbic acid [86, 88].

The collection of low vitamin A species of *Rosa* is carried out throughout the autumn - from the moment of their full reddening to frosts. Fruits should not be harvested until they are completely reddened, since the immature raw materials contain insufficient organic acids and carotenoids [86]. To dry low vitamin A species of *Rosa*, both artificial and natural air drying can be used [86].

2. CONCLUSION

The chemical composition of the *Rosa* is quite rich, so broths, medical extracts and syrups are prepared from its dried fruits. *Rosa* is rich in many vitamins, minerals and tannins. It also has pectin, organic acids and sugar, which is easily digested. Due to the chemical composition of the rosehips, they contain useful for the body bioflavonoids with antioxidant action. They also help protect the body from aging and detoxify. *Rosa* is extremely useful, they allow a person to replenish stocks of missing vitamins, trace elements, essential acids and antioxidants. The simplest use of *Rosa* for the prevention of beriberi is a drink from its dried fruits.

Rosa is an excellent therapeutic and prophylactic agent. It is widely used in medicine, cosmetology and cooking. But when it is used it is worth remembering that everything is good in moderation. It is also necessary to take into account that the use of *Rosa* has a number of contraindications, therefore, you must first consult with your doctor.

Acknowledgements

This research was not received any grant or fund support from public or commercial sectors.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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International Journal of Secondary Metabolite
2020, Vol. 7, No. 3, 213–228

https://doi.org/10.21448/ijsm.753739

Published at http://dergipark.gov.tr/en/pub/ijsm Research Article

Mineral Nutrition Status of Some Aromatic Plants Grown in Muğla, Determination of Their Soil Characteristics and Uses in the Kitchen

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Abstract: In this study, mineral nutrition status of some aromatic plants such as *Foeniculum vulgare*, *Cichorium intybus*, *Brassica oleracea* var. *capitata*, *Liquidambar orientalis* that are naturally spread in Muğla province and its locality and consumed by the local people, and the soil characteristics of the species were investigated. According to the results, it was determined that the species of plants spread between pH-mild acid and neutral in a loamy, salt-free and low-lime environment. In addition to this, while it was determined that plants were at high levels in terms of N, Ca and Fe contents, Zn value was found to be at a low level. In the second phase of the study, it was aimed to obtain detailed information for above mentioned local plants by using qualitative research method. It was found that there were differences in the nutrition habits of the societies due to different cultures. Local differences mostly stemmed from the yields grown in that region. One another aim is to investigate the food preparations in Muğla cuisine preferred by the local people regarding "arapsaçı, hindiba, cibez and günlük" (TR), which are among the medicinal aromatic plants found in Muğla, and the consumption preferences of these plants. The main purpose of this research was to determine the aromatic plant consumption habits of the people living in Muğla province and evaluate these consumptions in terms of health. As a result of the interviews, the recipes of these dishes, information on their preferability and their effects on health were obtained.

ARTICLE HISTORY

Received: June 16, 2020
Revised: August 17, 2020
Accepted: September 02, 2020

KEYWORDS

Aromatic plants,
Gastronomy,
Mineral nutrition,
Regional cuisine

1. INTRODUCTION

The number of plant species spread in Turkey is close to the number of plant species spread in the whole European continent. Our country has a very rich flora in terms of existing plant diversity. The flora of our country consists of approximately 12.006 taxa [1]. The main reasons of how our flora is so rich are that our country is in a location where three different phytogeography regions in terms of climate and vegetation cover, briefly floristic structure,

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ISSN-e: 2148-6905 /

intersect (which are: Europe-Siberia phytogeography in North Anatolia; Mediterranean phytogeography in West and South Anatolia; and Iran-Turan phytogeography in Central and South East Anatolia), that Anatolia is like a bridge between Europe and Asia continents and the diversity and reciprocal plant migration between the two continents increase accordingly, that Anatolia is the center of many different kinds and sections, and that edaphic (earth) factors are quite diverse [2]. In addition to this, with the rapid population growth and destruction of nature, some plants are endangered. Determining and protecting the life circuits, morphological and anatomical structures, habitat characteristics and soil plant diversity of these plants and culturing them is of great importance for the continuity of generations.

Taking certain minerals in proper portions in daily nutrition is one of the fundamental principles of healthy lifestyle. On the other hand, due to the fact that these elements are natural and used industrially, the existence of these elements over certain limit values may have negative effects on living beings [3]. Despite the fact that the insufficiency or high values of mineral nutrients cause serious health problems, sufficient amounts play an important role as micro/macro or beneficial elements in human metabolism [4].

Plants take many elements from the environment in which they grow so that they can continue their vital activities, grow and develop. However, some of these elements (about 20) are those that are extremely essential nutrients for plants. In addition to the parameters that are the subject of the study, selenium is an important nutrient for human and animal nutrition. Selenium is one of the fundamental trace elements involved in the 25 different selenoproteins with its various biological roles especially like antioxidant and anti-inflammatory agent [5]. Even if there are preliminary reports in the countries with high dietary selenium and rare selenium deficiency, it is possible that the effect of externally administered selenium on both cancer risk and cancer disease has been underestimated up until now [6]. Besides, due to its antioxidative effects, selenium contributes to plant growth and development, and yield quality [7]. Plants play a crucial role in Se transfer in the human food chain [8-10].

This study is of great importance in terms of examining the macro and micro nutrients and selenium levels of some edible plants that naturally spread in Muğla, which is deemed significant regarding human health and nutrition. Accordingly, the determination of the uses of these plants, the reasons of preferences by the local people and the detection of the mineral nutrients covered by these plants constituted the main subject of the study. In addition to this, with these plants which are intensively consumed in the region, it was aimed to form the source materials for future studies and contribute to the studies in such fields as health and gastronomy. Another purpose of this research was to determine the aromatic plant consumption habits of the people living in Muğla province and to evaluate this consumption in terms of health. Within the context of this purpose, the sub-problems of this research could be given as follows; (a) what kinds of dishes can be made with herbs, (b) whether their long-term uses are possible or not, (c) what benefits are expected from these products, (d) how often they are consumed, (e) what the effect of family members is in aromatic consumption habits, (f) what kind of plant nutrients exist in which plants, and (g) what the effects of the dishes and beverages made with these herbs are on health. It was also aimed to reveal the dishes made from the plants that were the subject of the research, record the forgotten cultural values, and draw attention to their importance in terms of both human health and gastronomy tourism.

2. MATERIAL and METHODS

2.1. The Collection of the Plants and Analyses

The localities of the plants in the research were determined by reviewing the data obtained from the preliminary examinations made in the book “Flora of Turkey” [11, 12] and the

previous flora studies conducted in Muğla province. The scientific name, local name, locality and herbarium codes of the plant species presented in the study were given in Table 1 below.

Table 1. The botanization localities of plant materials

The name of the species	Local name	Locality	Code name	Herbarium Code
Foeniculum vulgare Mill.	Arapsaçı, fennel, rakı otu (TR)	Muğla, Menteşe	Fv	MUH 549
Cichorium intybus L.	Hindiba, hindibağ, hindibahar, güneğik, aslan pençesi, tavuk ayağı, radika, (TR)	Muğla, Marmaris	Ci	MUH 309
Liquidambar orientalis Mill.	Sığla, günlük (TR)	Muğla, Köyceğiz	Lo	MUH 586
Brassica oleracea var. *capitata* L.	Cibez, lahana patlağı, lahana filizi, lahana keli, azman (TR)	Muğla, Ortaca	Boc	MUH 1241

2.2. The Soil Characteristics of the Localities in which the Species Are Spread and Plant Nutrient Determination Studies

In soil samples; the structure (sand, clay, silt ratio) was determined by hydrometer method; lime was determined calcimetrically by organic matter quantity method [13], receivable Zn, Fe, Mn and Cu were determined according to the DTPA method (diethylene-triamine-penta-acetic-acid) [14], receivable K, Ca and Mg content [15] and receivable Na content [16] were determined in the extracts obtained with 1N neutral ammonium acetate solution on atomic absorption spectrophotometer. Besides, Water Soluble Phosphorus content was determined in a calorimetrically on the spectrophotometer according to the Bingham [17] method. Receivable B by the plant in the soil was made according to 0,01 M mannitol+CaCl2 extract method and the amount of B was determined in the ICP-OES device [18], while pH and EC were determined by the combined pH-EC meter. The total nitrogen in soil samples was determined by Kjeldahl method.

Macro and micro element analyses of the plant nutrient contents were performed in the ICP-OES device by using a separate wavelength specific for each element according to the method determined by Kacar [19]. The results were given as %, ppm and ppb according to dry matter principle.

2.3. The Use of the Plants in the Kitchen and Local Dishes

As a result of the interviews with the local people in Menteşe, Bodrum, Marmaris, Fethiye, Ortaca, Datça, Dalaman, Köyceğiz, Seydikemer, Milas, Ula, Yatağan and Kavaklıdere districts of Muğla province, the women having been living there for many years or since birth and who were experienced in making local dishes and getting to know herbs from nature, were determined by random sampling. Face-to-face interviews were made with these women between the dates of 16 May 2018 and 30 June 2018 within the framework of the semi-structured interview form prepared previously. 59 interviews were made in total; 6 of which were in Bodrum, 3 were in Dalaman, 3 were in Datça, 6 were in Fethiye, 3 were in Kavaklıdere, 3 were in Köyceğiz, 5 were in Marmaris, 7 were in Menteşe and in the center of Muğla, 6 were in Milas, 6 were in Ortaca, 3 were in Seydikemer, 2 were in Ula and 6 were in Yatağan. Each interview lasted for about 50-60 minutes. In the interview form, there were questions regarding the use and cooking method of the herbs determined for the research, their frequency and period of use, the place from which they were obtained, their effects on health that were known, and whether the women used a storage method that would ensure them to be used outside the season. The photos of these herbs were taken during the study, and while some of them were cooked by the participants, some others were tried to be cooked by the researcher in accordance with

the given recipes. Descriptive analysis, which is one of the qualitative research methods and which enables the findings to be presented by summarizing and interpreting, was preferred as the research method.

3. RESULTS and DISCUSSION

3.1. The Determination of the Soil Characteristics on Which the Species Are Spread

As a result of the analyses performed, some of the physical and chemical characteristics of the localities on which the species spread was given in Table 2. When the soil characteristics of the localities on which the species were spread was evaluated in general, it was determined by analyses performed that all the plant species were spread between pH-mild acid and neutral, in loamy, salt-free environment. When the lime contents of the locations were examined, it could be seen that all the plant species in the research preferred low-lime soils. It was also determined as a result of the soil analyses of the plants in the research that the soil of *Brassica oleracea* var. *capitata* species had the lowest organic matter content with 1.94%, while the soil of *Cichorium intybus* species had the highest organic matter content with 3.24% (Table 2).

Table 2. The findings regarding the soil analysis of the localities on which the species are spread

Parameters		Fv	Ci	Lo	Boc
Saturation		42.00	50.00	45.00	40.00
EC (dS m^{-1})		0.81	1.19	0.28	0.80
% Salt		0.02	0.04	0.01	0.02
pH		6.81	6.56	6.73	6.84
Lime (%)		0.73	1.36	12.10	0.64
Organic Matter (%)		3.04	3.24	2.35	1.94
Nitrogen (N) (%)		0.15	0.16	0.12	0.10
Macro Elements (ppm)	Phosphorus (P)	28.00	11.00	12.00	19.00
	Potassium (K)	58.78	420.87	248.51	82.54
	Calcium (Ca)	2883.70	11960.93	13580.01	2604.52
	Magnesium (Mg)	2048.17	396.02	307.95	2291.39
	Sodium (Na)	36.67	20.97	75.66	148.34
Micro Elements (ppm)	Iron (Fe)	42.88	47.57	51.42	42.91
	Manganese (Mn)	30.32	98.54	84.26	32.26
	Zinc (Zn)	0.40	1.04	0.48	0.38
	Copper (Cu)	2.34	2.93	2.16	2.86

*Fv: *Foeniculum vulgare*, Ci: *Cichorium intybus*, Lo: *Liquidambar orientalis*, Boc: *Brassica oleracea* var. *capitata*

In addition to this, the total nitrogen content of the soils of the plants in the research was the lowest in *Brassica oleracea* var. *capitata* species with 0.10%, while it was the highest in *Cichorium intybus* species with 0.35% in capitate type (Table 2). Chapman [20] reported that the N limit value recommended to be present in the soil in terms of plant development should be between 0.11–0.15%. Considering the reference ranges, when the total nitrogen contents of the soils on which all the research plants were grown were evaluated, it could be seen that they were between the reference values.

When the other soil macro element contents of the localities on which all the species mentioned were gathered were considered, it was determined by the analyses that P content

(11-28ppm) was among K (58.78-420.87 ppm), Ca (2604.52-13580.01 ppm), Mg (307.95-2291.39 ppm) and Na (20.97-148.34 ppm) (Table 2). It was revealed that soil P contents on which the plant species other than *Foeniculum vulgare* species, which was one of the species in the research, were grown were sufficient. When the adaptable Potassium contents of the soils of the plants in the research were analyzed, it was determined that the lowest ratio was in the soil of *Foeniculum vulgare* species, whereas the highest ratio was in the soil of *Cichorium intybus*. According to literature, the K values accepted in the soils are between 201 and 250 ppm [21]. In our study, the Potassium contents in the soils of *Cichorium intybus* species were found to be higher when compared to the other plants. Considering the limit values, while the Ca values of the soils of *Foeniculum vulgare* and *Brassica oleracea* var. *capitata* species analyzed were found to be sufficient, the soil contents of the other plant species in our study were found to be at very high levels. When the limit values were taken into consideration, it was determined that 50% of the plant soils analyzed were sufficient, while 50% contained too much magnesium [21]. When the sodium contents of the soils were analyzed in general, it was revealed by the analyses that they were below the recommended limit values.

In the soils of the plants in the research, it was found out that the useful Iron (Fe) contents of the microelements were the lowest in the soil of *Foeniculum vulgare* species (42.88 ppm), while they were the highest in the soil of *Liquidambar orientalis* species (51.42 ppm). According to Lindsay and Norvell [14], the useful Fe value recommended was between 6 and 10 ppm, and when this value range was examined, it was revealed by the analyses that the iron concentration was high in all the soils analyzed in the research. While the receivable Manganese (Mn) content varied between 30.32-98.54 ppm, the mean value was found as 61.35 ppm (Table 2). According to these limit values, it could be said that receivable Mn element was sufficient in the soil with a 90% ratio and high in the soil with a 10% ratio. When the Zinc (Zn) concentrations were examined, it was found that they were the lowest in the soil of *Foeniculum vulgare* species with 0.40 ppm value and the highest in the oil of *Cichorium intybus* species with 1.04 ppm (Table 2). When the soils analyzed were compared with the determined Zn sufficient level (0.8-2.5 ppm), it was revealed that there was no insufficiency in the soils of *Cichorium intybus* species in terms of receivable Zn, whereas all the other soils were insufficient in terms of Zn content. Finally, it was reported that the sufficiency level of the receivable Copper (Cu) content, which was another micro element in the soil, was >0.2 ppm and therefore, it was determined according to this recommended value that all the soils were sufficient in terms of Cu content [14].

3.2. The Determination of the Species' Plant Nutrient Element Contents

In order to reveal the nutritional status of the 4 naturally spreading edible plant species in Muğla province, mineral substance contents of the plant parts used in gastronomy were investigated. The changes in the macro element contents of the species were shown in Table 3.

When the nitrogen content of the edible parts of the plants in our study was considered, it was determined that the % N content was the lowest in *Liquidambar orientalis* plant (2.27%), while the % N content was the highest in *Foeniculum vulgare* species (5.37%). The average N ratio of the edible parts of the samples was determined as 3.74% (Table 3). The % N reference value determined by Kacar and Katkat [22] was between 1.5-5 and it was observed in the analyses within the scope of the research that the nitrogen content of *Foeniculum vulgare* plant sample was at a high level, whereas the nitrogen contents of the other plants in the study were between the recommended reference intervals. Similar to our study, Korkmaz et al. [23] reported that the nitrogen contents in some medicinal and aromatic plants (laurel, chamomile, nettle, yarrow, centaury and linden) ranged from 1.41% to 3.78%. In another study, the nitrogen contents of 29 medicinal and aromatic plants that were mostly consumed in Greece ranged from 0.14% to 3.24% [24]. In the light of the literature reviewed, it could be noted that there was a

quite wide variation in medicinal and aromatic plants in terms of the nitrogen content and that it was compatible with the nitrogen contents obtained in this study.

Table 3. Macro and micro element contents of the species' edible parts

Parameters		Fv	Ci	Lo	Boc
Macro Elements (%)	Nitrogen (N)	5.37	4.51	2.27	2.80
	Phosphorus (P)	0.41	0.36	0.22	0.38
	Potassium (K)	3.39	2.13	1.10	2.48
	Calcium (Ca)	2.61	1.92	0.46	9.07
	Magnesium (Mg)	0.36	0.18	0.45	0.58
Micro Elements (ppm)	Iron (Fe)	509.29	158.95	247.71	13621.24
	Manganese (Mn)	24.84	30.61	36.95	47.16
	Zinc (Zn)	58.06	33.05	49.12	260.35
	Copper (Cu)	47.34	26.81	28.79	67.82
	Boron (B)	32.12	27.20	12.93	20.17

*Fv: *Foeniculum vulgare*, Ci: *Cichorium intybus*, Lo: *Liquidambar orientalis*, Boc: *Brassica oleracea* var. *capitata*

The % P scopes revealed in this study seemed to vary between 0.22 (*Liquidambar orientalis*) and 0.41 (*Foeniculum vulgare*) (Table 3). Since the % P value determined in the plant was between the limit values (0.20-0.75%), there were no drawbacks [22]. In a study conducted, the phosphorus content in 8 medicinal and aromatic plants widely used by public in South West Nigeria exhibited a wide variation and that it varied between 100-3500 ppm [25]. In another study, it was stated that The P content of some members of the *Lamiaceae* family (thyme, rosemary, lavender, sage, basil and melissa) ranged from 0.07% to 0.34% [26]. Considering the P content in our study, it could be stated that it was supported by the literature mentioned above. The K content was measured the lowest in *Liquidambar orientalis* plant (1.10%) and the highest in Foeniculum vulgare plant (3.39%) (Table 3). In a study conducted, it was stated that the amount of potassium in mallow, chicory, wild asparagus, worm seed, fennel, poppy and crook, which were consumed as vegetables in the Aegean Region, varied between 0.29-0.58 mg $100g^{-1}$ [27]. In another study, it was determined that the amount of potassium of the wild plants in the region of Kilis and Gaziantep varied between 2.5-3% [28]. These results coincide with the results of our study. While the Ca content of the species in our study was the lowest in *Liquidambar orientalis* species with a value of 0.46%, it was highest in *Brassica oleracea* var. *capitata* species with a value of 9.07% (Table 3). Low and high values were determined according to the % Ca reference interval (1-3%) determined by Kacar and Katkat [22]. This is thought to be due to the climatic and topographic characteristics of the region. Of the studies with similar findings; Ashraf, Hayat, and Mumtaz [29] found that calcium content of the 17 endemic species they studied in their research from Artemisia to Pakistan was between 0.057-1.069%. In another study, the calcium content of 10 medicinal plants grown in the north-east of India was examined and it was reported that there was a wide variation with the limits between 0.166-3.20% [30]. Finally, considering the Mg content of the edible parts of the plants in our study, it was determined that the lowest Mg content was in *Cichorium intybus* species (0.18%), while the highest Mg content was in the edible parts of *Brassica oleracea* var. *capitata* species (0.58%) (Table 3). Considering the results of the analysis we performed, it was determined that the Mg contents were close to the % Mg reference interval (0.25-1%) determined by Kacar and Katkat [22]. It was also found that the magnesium content was between 0.0117-0.114% in some of the spices and medicinal plants [31], and between 0.03-2.293% in some wild vegetables [32].

In addition to this, together with the fact that the microelements are the mineral elements that have a wide range of biochemical functions in living organisms and are vital for human health, they can have harmful effects when taken in high concentrations [33].

In this study, the lowest Fe content of the plant samples was determined in *Cichorium intybus* species with 158.95 ppm, while the highest Fe content was determined in *Brassica oleracea* var. *capitata* species with 13621.24 ppm. (Table 3). The reference level for iron in the plants was reported as 50-250 ppm [22]. Considering the limit values, the Fe content determined for *Cichorium intybus* and *Liquidambar orientalis* plant species were between the recommended values, whereas the Fe content of the other plants were quite high. In a similar study, Baydar and Erdal [34] examined the effects of plant growth regulators on the leaf quality of Izmir thyme (*Origanum onites* L.) (Fe: 47.25-97.50 ppm) and reported that the plants had high iron contents. In another study, it was also reported that the iron content in the 12 medicinal and aromatic plants obtained from the herbalists in Ordu, Samsun and Giresun provinces in the Black Sea Region of Turkey varied between 93 ppm (yarrow) and 105.3 ppm (basil) [35]. The literature studies given above seem to support our findings. Considering the Mn contents, it was determined that *Foeniculum vulgare* was 24.84 ppm and *Brassica oleracea* var. *capitata* was 47.16 ppm (Table 3). The reference level for manganese in the plants was reported as 25-200 ppm [22]. Meraler [36] noted that the mahaleb plant had differences in terms of manganese content compared to the parts of the plants investigated. The highest Mn content was found in the leaves of the mahaleb (36 ppm), while the lowest Mn content was found in the resin part of the mahaleb (8 ppm). As a result of the research on the plants growing naturally in Ordu province and its region and consumed as vegetables, it was determined that the manganese content of the plants was between 21.40-77.40 ppm [37]. When the Zn content was examined, it was revealed that the lowest was in *Cichorium intybus* plant with 33.05 ppm and the highest was in *Brassica oleracea* var. *capitata* with 260.35 ppm. (Table 3). The reference level for zinc in the plants was reported as 25-150 ppm [22]. The plants were also reported to have normal values in terms of zinc other than *Brassica oleracea* var. *capitata* plant samples. In the study where the mineral contents of some plants used as spices in Turkey were examined, it was found that *O. vulgare* contained 19.3 ppm of Zn [38]. In the study examining some biological and ecological properties of *Origanum* (*Lamiaceae*) species endemic for Antalya province, it was reported that the annual average zinc contents of *O. solymicum* P.H. Davis, *O. husnucanbaseri* H. Duman, *O. bilgeri* P.H. Davis, *O. minutiflorum* O. Schwarz & P.H. Davis plants that belonged to *Origanum* species were 14.5 ppm, 31.75 ppm, 38.75 ppm and 32.75 ppm, respectively [39]. It was also determined as a result of the analyses performed on the plants obtained in the research areas that the Cu content was the lowest in the edible parts of *Cichorium intybus* plant (26.81 ppm), whereas it was the highest in the edible parts of *Brassica oleracea* var. *capitata* plant (67.82 ppm) (Table 3). In the study that examined 30 medicinal plants in Kayseri [40], it was determined that there was a very wide variation between copper content 3.32 (jujube)-30.2 μg g^{-1} (basil). A similar result was obtained by Şekeroğlu et al. [41] in terms of the copper content in the wild plants consumed as vegetables in Ordu province and it was determined that copper ratios ranged from 2.7 to 21.3 ppm. The reference level for copper in plants was reported as 6-30 ppm [22]. It was stated that toxic effects were detected when the copper content in plants was more than 20 ppm [42]. In this respect, our study is in accordance with the literature. When the boron (B) contents were examined, it was noted that *Liquidambar orientalis* (12.93 ppm) was below the recommended limit values (20-60 ppm) [22]. In the study by Turan [43], it was reported that avocado (78 ppm), golden rod (39 ppm), comfrey (33 ppm) had the highest mean boron ratios in their plant leaves, whereas pilewort (1.55 ppm), bistort (0.25 ppm) and green tea (0.07 ppm) had the lowest mean boron ratios in their plant leaves, respectively. In another study, it was reported that the mean boron ratio was the highest in alcea (16 ppm), sage (16 ppm) and hop (14 ppm) plants, whereas it was the lowest in chamomile (7.5

ppm), yarrow (6.9 ppm) and cassidony (5.8 ppm) plants. Among the root/rhizome plants examined, the highest mean boron ratio was found in mullein (13.156 ppm), nip (11.2 ppm) and cocklebur (7.9 ppm) plants, whereas the lowest boron ratio was found in least galangal (0.95 ppm), turmeric (0.7 ppm) and ginger (0.6 ppm) plants [44].

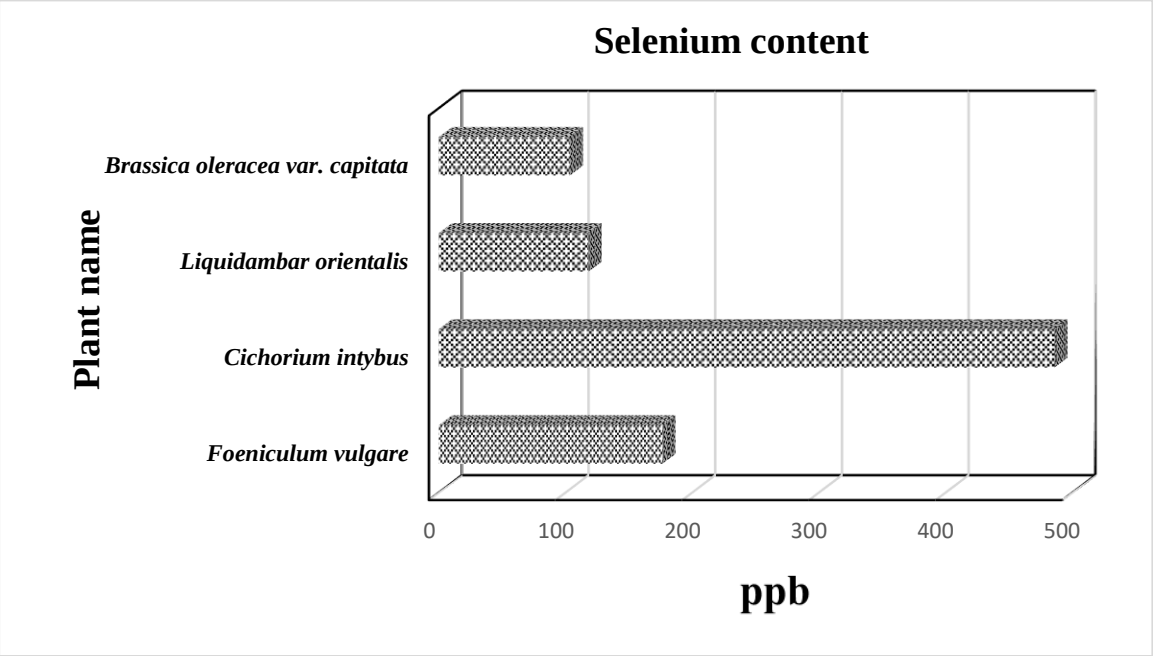

Figure 1. Se contents of the edible parts of the species

Selenium, whose certain necessity in plants is a matter of discussion, is an absolutely necessary element for animals and humans [45, 46]. Selenium, which is vital for human health, is an essential mineral for the proper functioning of the thyroid gland and also plays an important role as in the antioxidant defense as an enzyme cofactor. The recommended daily amount of Se intake for humans has been reported as 55 µg per day^{-1} [47]. When Se contents of the edible parts of the species were examined, it was determined that *Brassica oleracea* var. *capitata* species had the lowest Se content (103.42 ppb), whereas there was approximately 5.5 times more Se content in the leaves of *Cichorium intybus* species (485.82 ppb) (Figure 1). In the study by Şimşek [48], it was reported that the Se content of the 4 wild plant species (nettle, kaldir (TR), cat brier, sakarca (TR)) obtained from 10 different locations in Ordu province and its region varied between 0-76 ppb. Besides, in the study on the nutritional status of the 7 edible plants in Niger, it was suggested that the lowest Se content was found in *Corchorus tridens* L. plant (14.9 µg g^{-1}), while the highest Se content was found in *Maerua crassifolia* Forssk. plant (33.9 µg g^{-1}) obtained from Maradi region [49]. In the light of these data, it was determined that the Se contents in our samples were high and low according to the literature.

3.3. The Findings Obtained from the Interviews with the Local People in Terms of Gastronomy

In the interviews, the participants were asked about the use and cooking method of each plant in the research, how often they were used, whether they had a local name, where they were obtained from, whether anything was done in order to keep them for a long time, and their known benefits for health. The data obtained were evaluated and recorded in the written form.

3.3.1. The Recipes of Arapsaçı Herb and Related Research Findings

In the interviews made in all the districts of Muğla province, the participants expressed that the smell of arapsaçı, whose local name was called "sıra", was like aniseed and those who did not prefer a very intense aroma stated that they did not use it alone. It is used in artichokes with olive oil, in broad bean dishes by chopping in order to give flavor, and by adding into the herb mixtures prepared for pastries. In addition to this, the participants who enjoyed the taste and smell of it stated that they consumed it by cooking with lamb meat, and with onions and eggs, or by mixing with dry cottage cheese at breakfast. It was also stated that the best time of arapsaçı, which grew naturally in the fields and gardens, was in March-April, and that it was good for urinary tract disorders and cancer. Since its seeds were good for gas pains, it was expressed that the plant and its seeds were used by drying when it was not the season. In the interviews made in Fethiye district, the participants also stated that they prevented gas pains by using it in dry beans. It was believed that it was good for the heart, lowered cholesterol and was said to be good for insomnia and feel relieved if it was boiled and its water was drunk. "Sıra", which was mentioned as the indispensable part of herb dishes, was also said to go with the dishes with olive oil, stuffed grape leaves and stuffed vegetables. Arapsaçı grows naturally in the mountains, water-lands and gardens especially in February-March-April, and can always be found in the street markets. The plant, whose kinds are called "wise" and "crazy" can also be obtained in nature or by planting in the garden. One interviewee stated that they knew it as "sıra", "fennel" or "rakı otu".

In accordance with the interviews, it can be said that arapsaçı is used in many dishes as a fresh spice due to its aromatic effect. It was observed that it was used while preparing the mixtures of pastries and cooking olive oil dishes like broad beans, and in local herb frying dishes. Apart from being a subsidiary aromatic ingredient, some of the examples of the recipes obtained as a result of the research are as follows;

Arapsaçı with Lamb Meat

Ingredients: 1/2 kg arapsaçı (2 bunches), 250 g lamb meat (chopped), 1 onion, 1 glass (small tea glass) of olive oil, salt, 1 tablespoon flour, juice of 1 lemon

Preparation: The meat is fried until it releases its water and then boils down. Olive oil and chopped onion are added. After arapsaçı is washed, its stem and leaves are cut separately. Chopped stems are added into the meat and onion, and it continues to be fried. Then, the leaves are added and salt is added with enough hot water over the mixture. When it boils, the sauce prepared in a separate bowl with flour, lemon juice and water of the dish is added gradually by stirring. It is cooked on medium heat for about ten minutes with the lid closed.

PS: The same dish can also be made with the stems and tomato paste only. Sauce is not used in the sauced one.

Arapsaçı with Dry Cottage Cheese (for Breakfast)

Ingredients: 1 bunch of arapsaçı (leaves only), 1 large bowl of dry cottage cheese, salt, pepper, powdered red pepper, 2-3 spoons of olive oil

Preparation: Arapsaçı is washed and chopped. All the ingredients are added in it and mixed. It is consumed at breakfast.

Arapsaçı with Milk

Ingredients: 1 onion, 1/2 tea glass of olive oil, 1/2 kg arapsaçı, 1 tea glass of bulghur, salt, powdered red pepper, 1/2 kg milk

Preparation: Onion is chopped and fried. Chopped arapsaçı, powdered red pepper (optional) and bulghur is added in it, and it continues to be fried. Bulghur is cooked by adding a little hot water and boiling down. Until this stage, salt is not added as it is not desired that salt

sours the milk. When hot milk is added and it is boiled once again, salt is added. It is rested with the lid closed.

3.3.2. The Recipes of Hindiba Herb and Related Research Findings

It is known that the plant whose leaves are consumed is especially rich in vitamin C [27, 50]. Hindiba, which is known and used as salad, is also consumed by frying with onions and eggs after boiling. It was stated that it was good for the stomach and it prevented stomach bloating, it was diuretic and good for the liver and cancer. It was also noted that hindiba, which was also known as "redshank herb" in Milas and Yatağan districts, was boiled and served as salad with syrup and garlic. It was said to be good for stomach and gallbladder and melted the stones in the gallbladder. In Kavaklıdere, together with the name "redshank herb", it was also called as "tavukayağı" and "radika". In central Muğla, it was seen that both entitling and uses differed. In Menteşe, it is known with many names like "hindibağ", "hindibahar", "güneğik" and "aslanpençesi". Though it was stated to be mostly boiled and served as salad with syrup, it was also fried with onions or eggs and cooked as a dish with bulghur. It was also said that the sour orange was ideal for the syrup and that by crunching (frying) dry pepper, it was put on it. The recipes and research findings regarding the herb that is said to be one of the herbs always found in the street markets are as follows;

Hindiba Salad

Ingredients: 1/2 kg of hindiba, 1/2 tea glass of olive oil, 1-2 cloves of garlic, salt, lemon juice (or pomegranate syrup)

Preparation*:* Hindiba is boiled in the boiling water for 3-4 minutes. It is drained in cold water and then, chopped up. It is flavored with lemon juice or pomegranate syrup, olive oil and crushed garlic and salt.

Hindiba with Syrup

Ingredients: 1/2 kg hindiba, 1 tea glass of olive oil, 2-3 spoons of pomegranate syrup (or 1/2 lemon), 2-3 cloves of garlic, salt

Preparation: Washed and sorted hindiba is put in the boiling water as a whole. 3-4 minutes later, it is drained, cooled and chopped. A sauce is prepared with crushed garlic, pomegranate syrup, salt and olive oil. Hindiba is added into the sauce and rested until it is flavored. It is served cold.

3.3.3. The Recipes of Cibez and Related Research Findings

In the region, it is known as "lahana patlağı" or "azman". It is cooked by boiling and frying it with a lot of onions and consumed fresh, which is caused by the fact that it can be easily found in the gardens. In Bodrum, their photos were shown to the participants when they did not know it by the name. However, some of them expressed that they did not know what cibez was, and some others stated that they did not know it as cibez, but as "lahana patlağı", "lahana filizi" or "lahana keli". They also stated that they boiled and fried it, made stuffed dishes and salads by boiling. It was said to be good for gas pains, diabetes and edema. It was noted that it was found easily in January, February and March, and also in spring. It was also said to be found in the gardens and greenhouses, and therefore, consumed fresh. It was expressed that it was also known as "azman" in Fethiye district.

Cibez, which is among the most commonly used herbs in Cretan cuisine, is known as the new shoots that are formed in the cut after the harvest of cabbage or broccoli [51]. The recipes and research findings regarding Cibez are as follows;

Cibez Salad

Ingredients: 1/2 kg of cibez, 1/2 tea glass of olive oil, 1-2 cloves of garlic, salt, lemon juice (or pomegranate syrup)

Preparation:Cibez is boiled in the boiling water for about 3-4 minutes. It is drained in cold water and then, chopped up. It is flavored with lemon juice or pomegranate syrup, olive oil and crushed garlic and salt.

Fried Cibez (with or without Eggs)

Ingredients: 1/2 kg cibez, 2 onions, 2 cloves of garlic, 1/2 teaspoon of olive oil, 1 teaspoon of butter, 2 eggs, salt, pepper, powdered red pepper

Preparation: Onions and garlic are chopped up and fried with olive oil and butter. Boiled and chopped up cibez are added in it. It is flavored with salt, pepper and powdered red pepper. Eggs are added and cooked by stirring.

Cibez Stew

Ingredients: 1/2 kg of cibez, 2 onions, 1 spoon of tomato paste (red pepper and tomato together), 1 teaspoon of powdered red pepper, 1/2 cup of bulghur (rice may also be preferred), 1 teaspoon of olive oil, salt.

Preparation: The diced onion is sauted in olive oil. It is fried with tomato paste and powdered red pepper. Boiled cibez is added. After stirring, a glass of hot water is added. Then, bulghur and salt are added and cooked on low heat until bulghur softens up.

3.3.4. The Recipes of Günlük Tree (Sığla) Leaves and Related Research Findings

It was mentioned especially in Köyceğiz, Ortaca, Dalaman, Fethiye districts that the tree, which was named as “günlük” or “sığla” by the local people, was used to cook a dish from its fresh shoot leaves. Anatolian Sweetgum Tree (*Liquidambar orientalis* Mill.), which spreads endemically in Muğla province, is a perennial outdoor ornamental plant with a woody structure that sheds its leaves in winter [52, 53]. It was the only plant among the plants in the research which was generally known by all the districts and expressed with a single cooking technique. As its leaves are thought to be beneficial due to its unique aroma and the benefits of its oil, it is thought to be cooked as a dish. The recipe of the “günlük” leaf cooked with bulghur in Muğla is as follows;

Günlük (Sığla) with Yoghurt

Ingredients: 1 bunch of günlük tree leaves, 2 leeks, 4-5 branches of “sıra” herb, 1 bunch of mixed herbs, salt, 1/2 cup of bulghur, 2 cloves of garlic, 2 glasses of yoghurt, 1 spoon of butter, 1/2 tea glass of olive oil, powdered red pepper, dried hot pepper (optional).

Preparation:All the herbs are washed and boiled with a little water in such a way that they still remain fresh and then, chopped. The leeks are chopped up and fried with butter and a little olive oil. Boiled and chopped herbs are added into the fried leeks. Bulghur and salt are added and then, the mixture is stirred. 1 tea glass of water is added and it is cooked until bulghur softens up and boils down. Garlic is crushed in a bowl and mixed with yogurt. Herbs cooked with the garlic yogurt are mixed. Powdered red pepper or dried pepper is fried with oil in a separate pan and put over. It is served warm or cold.

4. CONCLUSION

Today, the use of herbal medicines in the treatment of diseases is increasing day by day, and therefore, related scientific fields and research have gained great importance. While new possibilities are being investigated in the treatment of diseases, intensive studies are carried out in order to prevent diseases and lead a healthy life. The most intensive studies in this regard are

on nutrition. As a result of the examination of the macro and micro nutrients in the leaves of the plants, it was observed that the values obtained were generally in line with and in contrast to the literature. Together with the fact that a general nutritional status of the species investigated was revealed as a result of the study, it was also stipulated that more detailed researches should be conducted because the fact that the micro elements or heavy metals were above a certain limit value had a toxic effect on the plants and human health was revealed by various researches. In this regard, it was revealed by the literature research carried out and the data obtained that determining the mineral element contents in the plants could be beneficial for human health and nutrition.

It is possible to find the plants given in the study from nature in the districts of Muğla province as well as in the street markets brought by the sellers or cultivated. These herbs, which can be obtained from local markets, are generally cooked with similar cooking techniques. These techniques are souring, milking, frying with or without eggs, preparing like salad with syrup sauce, frying with bulghur, making it with yoghurt. These cooking styles are the unique cooking techniques of this region. The common pre-treatment is boiling with little water in such a way that it stays fresh without darkening.

It has similarities to the Cretan cuisine with its such properties as boiling herbs fresh, wishing to cook without darkening, using olive oil intensively. As well as the characteristics of its own local dishes, it is possible to explain this situation as that it is influenced by the Cretan cuisine due to the migrations experienced years ago.

It can be said that the herbs, which are well known and available in the street markets, are still being cooked but the herbs that do not have a culture or need to be collected are consumed less and even about to be forgotten. Indeed, the fact that the wild peas were not known by the participants in the interviews conducted in the places other than Bodrum is a proof for this situation.

The interviewees stated that hindiba was good for the stomach, prevented stomach bloating, diuretic, and was good for the liver and cancer disease. When the literature was examined, it could be noted that this finding coincided with the results of the study by Gül and Dinler [54] who stated that it was good for diabetes, liver and bladder stones. It was believed by the interviewees that arapsaçı was good for the heart and lowered cholesterol, and also said to relieve and be good for insomnia if it was drunk after boiling. In their study, Karaca, Yıldırım, and Çakıcı [55] expressed the benefits of arapsaçı as "good for the kidney stone, diabetes, bronchitis, chronic cough, gas pains and eye-strengthening, as well as facilitating digestion, soothing, preventing heart throbs, enhancing breast milk in the mothers breastfeeding their babies, and relieving stomachaches in the children". Therefore, it could be said to show similarities with what the participants said in terms of the effects of arapsaçı on health. All these results coincide with the data we obtained as a result of the interviews. It was also stated that arapsaçı, which was locally called "sıra" was good for urinary tract disorders and cancer, and its seeds were good for gas pain. This finding could be said to be in line with the result obtained in the study by Kaya, İncekara, and Nemli [27], who expressed that the plant facilitated digestion, relieved the stomach and had a soothing effect as it contained nonpersistent gas and oil. The fact that it was used in all herb dishes and in the dishes made with olive oil just like a fresh spice rather than being used alone due to being an aromatic herb and having a sharp aroma was also mentioned in the study by Kaya, İncekara, and Nemli [27] as it was used for the same purpose in fish dishes and in "rakı" (a famous Turkish alcohol drink) production.

Unlike other cooking techniques, as the cooking technique called milking was not seen in the places other than Fethiye district and its region, and also in other studies, it could be considered to be new information and deemed necessary to be recorded.

One of the mixtures they used as a sauce was the coloured sauce that they usually prepared with onions and tomato paste or powdered red pepper, or sometimes concentrated with flour and cooked by adding the main ingredients. The other was the sauce served by putting the boiled herbs prepared with olive oil, garlic and syrup (mostly pomegranate syrup, and also lemon juice, bitter orange juice or lemon salt), which they called syrup sauce. Another sauce was yoghurt with garlic. Besides, cooking with bulghur by frying with plenty of onions, which they could consume not only with the sauce but also without the sauce, was one of the most common techniques used. The other cooking technique preferred at least as much as cooking with bulghur was frying with onions and eggs. Olive oil was used in all the herb dishes.

Local people generally preferred to collect and consume the herbs freshly and believed that it should be consumed in the season as there were such a lot of herbs that they did not feel the necessity of storing them for a long time. The herbs growing naturally and obtained from the mountains or natural environments are thought to have a positive effect on health. However, due to the fact that most of these herbs are not available or are rare in the street markets, it is required to collect them, which lead to cooking these herb dishes less. The fact that they were cooked more in the past was also a finding obtained in many other studies. Obtaining these herbs from nature by providing their sustainability under controlled conditions and delivering them to people will enable the continuity of the culture of herb dishes.

Acknowledgements

This study has been granted by the Muğla Sıtkı Koçman University Research Projects Coordination Office through Project Grant Number: (BAP-17/160).

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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