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International Journal of Secondary Metabolite (IJSM) purposes the publication of articles related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). IJSM is open-access, peer-reviewed academic journal published electronically and quarterly. The journal is the goal to improve the research culture and help knowledge spread rapidly in the academic world by providing a common academic platform in the scope. The journal is published in English.

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2. Research notes: These include articles such as preliminary notes on a study or manuscripts new records on secondary metabolites.

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4. Letters to the editor: These include opinions, comments relating to the publishing policy of the International Journal of Secondary Metabolite, news, and suggestions. Letters are not to exceed one journal page.

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Research Article

Effects of glutathione on mitochondrial DNA and antioxidant enzyme activities in *Drosophila melanogaster*

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Abstract: The free radical theory in aging assumes that the accumulation of macromolecular damage induced by toxic reactive oxygen species plays a central role in the aging process. The intake of nutritional antioxidants can prevent this damage by neutralizing reactive oxygen derivatives. Glutathione (GSH; en-L-Glutamyl-L-cysteinyl glycine) is the lowest molecular weight thiol in the cells and as a cofactor of many enzymes and a potent antioxidant plays an important role in maintaining normal cell functions by destroying toxic oxygen radicals. In this study, the effects of GSH on SOD, GST and catalase enzymes and mtDNA damage were investigated at various time intervals by giving reduced glutathione to Drosophila. It was observed that 3-week GSH administration did not have a statistically significant effect on SOD and GST activities whereas GSH application decreased the catalase enzyme activities significantly. Although the decrease in antioxidant capacity with age was observed in SOD and catalase enzymes, such a situation was not observed in GST enzyme activities. There was no statistically significant difference between the control and GSH groups in mtDNA copy number values, while in the GSH group, oxidative mtDNA damage was high. These results may be due to the prooxidant effect of GSH at the dose used in this study.

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1. INTRODUCTION

Reactive oxygen species (ROS) are by-products of exposure to cellular metabolism or xenobiotics. ROS production can be harmful because it may cause oxidative changes in cellular lipids, proteins, and DNA, or it may be beneficial by participating in intracellular signalling or cell regulation (El-Osta & Circu, 2016). Mitochondria, the centre of cellular respiration, is a quantitatively important source of ROS as a result of oxygen reduction. In addition, mitochondria are exposed to harmful reactive oxygen species that inhibit cellular antioxidant mechanisms such as SOD and glutathione (El-Osta & Circu, 2016; Collins, 2016).

Superoxide (O_2^-) is one of the free radicals that can exert oxidative effects on the cell. Superoxide is reduced by superoxide dismutase (SOD) enzyme to hydrogen peroxide (Abreu & Cabelli, 2010; Sheshadri & Kumar, 2016). There is evidence that ROS such as superoxide

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anion radical, hydrogen peroxide, carbon monoxide, and nitric oxide play an important role in various physiological processes such as cellular proliferation and differentiation, gene regulation, and anti-bacterial defense (Rebrin & Sohal, 2008). SOD can be considered as the first line of defense in ROS homeostasis (Abreu & Cabelli, 2010; Sheshadri & Kumar, 2016).

Catalase (CAT) is a common antioxidant enzyme found in almost all tissues using oxygen. This enzyme catalyzes the reduction of hydrogen peroxide (H_2O_2) to water and molecular oxygen to complete the detoxification process carried out by the SOD. The catalase is present in the cell in the peroxisome, not in the mitochondria, which shows that the separation of hydrogen peroxide to water and oxygen is carried out by another enzyme known as glutathione peroxidase in mammalian cell mitochondria (Ighodaro & Akinloye, 2018). Approximately 0.4% to 4% of the molecular oxygen metabolized in the mitochondrial electron transport chain is converted into ROS (Lim *et al*., 2006). Mitochondrial DNA (mtDNA) is a circular double helix DNA arranged in nucleotides. There is no histone in the mitochondrial genome and the genome is located very close to the mitochondrial electron transport system (ETS); therefore, mtDNA is very sensitive to oxidative damage (Circu & Aw, 2012).

Glutathione (GSH; γ-L-Glutamyl-L-cysteinyl glycine, the lowest molecular weight thiol in the cells, is composed of L-glutamate, L-cysteine, and glycine amino acids and is one of the most important antioxidant compounds in body fluids (Giustarini *et al*. 2016; Wu *et al*, 2004). GSH production is catalyzed by Gamma-glutamylcysteine synthetase (Gsh1p) and glutathione synthetase (Gsh2p) and synthesized in two steps dependent on ATP (Mannarino, 2008). Glutathione, an essential molecule and primary cellular redox buffer that participates in various biological processes, is often found in cells at millimolar concentrations (1-10 mM). Many processes in cells can use GSH as a cofactor and can be affected by changes in GSH level. The consequences of suboptimum GSH levels on all these processes are not yet clear (Ayer *et al.,* 2010). Glutathione is synthesized intracellularly and can be transferred from the cell. It then tends to be hydrolyzed to amino acids to be taken back by the cells and re-synthesized glutathione in the cell (Meister, 1991). When glutathione flows out of the cell as a tripeptide, a large amount cannot be absorbed back into the cell (Kern *et al*, 2011), which leads to basal glutathione content in serum with a normal range of 3.8-5.5μM and its half-life is reported as 14.1 ± 9.2 minutes. Cells that can absorb glutathione as a tripeptide are hepatocytes (HepG2), intestinal mucosal cells, and retinal cells (Aebi *et al*, 1991; Sze *et al*, 1993; Benard *et al*, 1993; Kannan *et al*, 1996). GSH is also an important source of cysteine and performs many physiological functions, including proliferation, cell cycle regulation, apoptosis, catabolism of xenobiotics, glutathionylation of proteins, and the production of some steroids, lipid compound, and deoxyribonucleotides (Bajic *et al*, 2019). Glutathione is present in a reduced form known as reduced glutathione (GSH) and an oxidized form (GSSG) after administration of antioxidant effects on targets. These two forms determine a ratio known as the GSH / GSSG ratio, and changes in this ratio indicate changes in cellular oxidative balance (Owen & Butterfield, 2010).

GSH, a cofactor of many enzymes and a potent antioxidant, plays an important role in maintaining normal cell functions by destroying toxic oxygen radicals. On the other hand, GSH level decreases in circulatory and tissue levels in chronic diseases such as diabetes and aging (Jain *et al*, 2016). The free radical theory in aging assumes that the accumulation of macromolecular damage induced by toxic reactive oxygen derivatives (ROS) plays a central role in the aging process (Lim *et al*, 2006).

Mitochondrial dysfunction in cells is closely related to formation of reactive oxygen species (ROS) and oxidative stress. Although ROS homeostasis and antioxidant enzymes are modulated by cellular mtDNA, the modulation of the cellular antioxidant defense system by changes in mitochondrial DNA (mtDNA) content is largely unknown (Min & Lee, 2019). The mitochondrial glutathione (mtGSH) pool is 10-15% of cellular glutathione and is derived from

the transport of cytosolic glutathione along mitochondrial membranes (Collins, 2016). Mitochondrial redox systems such as glutathione, thioredoxin, and pyridine nucleotide redox pairs participate in antioxidant defense by modulating mitochondrial functions, including apoptotic cell death. The imbalance between ROS and antioxidant defense causes oxidative stress and oxidative changes in cellular biomolecules. Increased oxidative stress also leads to loss of GSH (Choi *et al*, 2016). Furthermore, mitochondrial ROS can oxidize mitochondrial glutathione and cause the loss of intramitochondrial redox homeostasis since mitochondrial macromolecules including mitochondrial DNA (mtDNA) are exposed to irreversible oxidative modifications (El-Osta & Circu, 2016). In Min and Lee's studies (2019), expression and activity of glutathione peroxidase (GPx) and catalase were inversely proportional to mtDNA content in myoblasts. While the depletion of mtDNA slightly lowered both reduced glutathione (GSH) and oxidized glutathione (GSSG), the cellular redox status assessed by the GSH / GSSG ratio was similar to that of the control group. In their study, Min and Lee (2019) reported that ROS homeostasis and antioxidant enzymes are modulated by cellular mtDNA content and that increased Glutathione peroxidase (GPx) and catalase expression and activity through mtDNA depletion are closely related to the reduction of oxidative stress in myoblasts.

Reactive oxygen and nitrogen species can oxidize cellular glutathione or induce glutathione out of the cell, thereby reducing intracellular redox homeostasis and inhibiting the activation of the apoptotic signal cascade (Circu & Aw, 2012). Many defence mechanisms in the organism evolve reducing reactive oxidants and their damage (Ames *et al*, 1993). Apoptosis is a well-organized and important cell death pattern for tissue homeostasis, organ development, and aging. To date, exogenous (receptor-mediated) and intrinsic (mitochondrial-derived) apoptotic pathways have been characterized in mammalian cells. Reduced glutathione plays an important role in protecting the reduced intracellular environment. The mechanism in which oxidative mtDNA damage induces apoptotic signalling is unclear (Circu & Aw, 2012). Enzymes such as superoxide dismutase (SOD), catalase, and glutathione S-transferase (GST) neutralize reactive electrophilic mutagens. In addition to the protective effects of intrinsic enzymatic antioxidant defence mechanisms, studies suggest that antioxidant consumption in diet is of great importance for health. Consumption of fruits and vegetables as the main source of diet antioxidants reduces the risk of degenerative diseases. However, there are many arguments suggesting that antioxidant contents of fruits and vegetables have the greatest effect on their protective effects (Ames *et al*, 1993).

Studies on aging in Drosophila continue to provide new insights into the understanding of this complex process. Drosophila is well suited for experimental studies with its short life cycle, suitability for genetic manipulation, and its functionally preserved physiology (Shaw *et al*, 2008). In this article, however, the effects of glutathione on some antioxidant enzyme activities and mtDNA were investigated in *Drosophila melanogaster*.

2. MATERIAL and METHODS

Flies were produced in standard corn flour medium in an incubator at 25 °C. Flies of 1-3 days of age were included in experimental media. The control group included the standard Drosophila corn meal medium (corn meal, yeast, agar, sugar, water, and antifungal acid): in GSH group, GSH (Reduced L Glutathione 0.5%) was added in addition to this standard food medium.

Flies were evaluated once a week for enzyme activities. In each measurement, 12 flies were used from each group (GSH group and control group). Flies were homogenized in PBS buffer (pH: 7.4, with protease inhibitor) and then homogenized in ultrasonic homogenizer. The supernatant, which was centrifuged for 20 minutes at 20.000 g of homogenate, was used for enzyme activation measurement. GST activity was performed with CAYMAN GST activity assay kit and SOD analyzes were performed with CAYMAN SOD assay kit according to kit

instructions. BSA standards and total protein amount of samples were measured by Bradford method (SIGMA B6916 Bradford Reagent).

Oxidative mtDNA damage and mitochondrial copy number were measured by quantitative PCR method. The principle of this analysis is that a lesion in the template DNA prevents the progression of the thermostable polymerase. Thus, amplification in the damaged DNA template are reduced compared to that of the undamaged DNA. Considering that the number of mtDNA copies may be different in each sample, a short fragment of 100 bp was replicated for each sample to normalize the results (Yakes & Van Houten, 1997; Santos *et al*, 2002; Venkatraman *et al*, 2004). The ratio of long fragment results to that of short fragment results gives a relative amount of damage. In our study, SIGMA G1N350 Genomic DNA purification kit was used for total DNA isolation; Pico Green dsDNA quantitation dye and QUBIT 2.0 fluorometer were used for the quantitative analysis of template DNA and PCR products; 5 ng of template DNA was added to each tube; and Thermo Phire hot start II DNA polymerase was used for PCR processing, and 4% DMSO was added to the PCR mix.

For short fragment (100 bp) primers are as follows (Mutlu 2012a; Mutlu 2012b; Mutlu 2013):

11426 5'- TAAGAAAATTCCGAGGGATTCA - 3'

11525 5'- GGTCGAGCTCCAATTCAAGTTA - 3'

For long fragment (10629 bp) primers are as follows (Mutlu 2012a; Mutlu 2012b; Mutlu 2013):

1880 5'- ATGGTGGAGCTTCAGTTGATTT - 3'

12508 5'- CAACCTTTTTGTGATGCGATTA - 3'

Thermal conditions for long fragment amplification: At 98 °C for 1 minute and then for 21 cycles at 98 °C for 10 sec, at 52 °C for 45 sec and at 68 °C for 5 min, for a final elongation at 68 °C for 5 min. Thermal conditions for short fragment amplification: At 98 °C for 1 minute and then for 21 cycles at 98 °C for 10 sec, at 55 °C for 45 sec and 72 °C for 10 sec, for final elongation at 72 °C for 2 min. The statistical analysis of the data was performed using the Kruskal Wallis test in Minitab Release 13.0 statistics program. * Marked groups are statistically different from other groups (p<0.05).

3. RESULTS

In our study, it was observed that 3-week GSH administration did not have a statistically significant effect on SOD and GST activities (Table 1) whereas GSH application decreased the catalase enzyme activity significantly (p<0.05). This decrease in catalase enzyme activities may be due to the prooxidant effect of GSH at the dose used in this study. Although the decrease in antioxidant capacity with age was observed in SOD and catalase enzymes, such a situation was not observed in GST enzyme activities.

Table 1. Results of Cat, GST and SOD enzyme analysis.

Groups	Cat (IU SA /mg pro ± SE)	GST (IU SA /mg pro ± SE)	SOD (IU SA /mg pro ± SE)
GSH 8 days	115.2±8.41	4.6±0.8	16.72±4.66
Control 8 days	161.14±9.20*	4.34±0.81	20.91±3.94
GSH 15 days	106.63±6.06	4.42±0.71	17.27±3.71
Control 15days	146.62±5.54*	4.20±0.76	15.09±4.65
GSH 22 days	99.21±5.92	4.71±0.79	14.6±3.01
Control 22days	125.46±6.95	4.39±0.69	13.91±2.28

*Marked groups are statistically different from other groups (p<0.05). (Cat: Catalase, GST: Glutathione S-transferase, GSH: Glutathione, SOD: Superoxide dismutase, IU:International unit, SA: Specific activity, mg pro: miligram protein, SE: standard error).

There was no statistically significant difference between the control and GSH groups in mtDNA copy number values, but in the GSH group oxidative mtDNA damage was relatively high (Table 2). This may be due to the prooxidant effect of GSH at the dose used in this study.

Table 2. Results of oxidative mtDNA damage and mtDNA copy number analysis (Low relative amplification indicates high mtDNA damage)

Groups	Relative Amplification	mtDNA copy number
GSH 22 days	0.996±0.26	245.56±21.36
Control 22days	1.214±0.33	251.11±22.28

4. DISCUSSION and CONCLUSION

The level of glutathione (GSH) decreases with age or in chronic diseases such as diabetes (Jain *et al*, 2016). The presence of mitochondrial GSH (mtGSH) is necessary for the protection of mitochondrial DNA. In mouse embryonic fibroblasts, aging mice and rats have been reported to have a direct relationship between decreasing mtGSH and increased mtDNA damage in brain and kidneys (Circu & Aw, 2012). In their study, Dannenman *et al*, (2015) reported that GSH depletion significantly increased mtDNA lesions induced by H_2O_2 in fibroblasts.

Since glutathione is a small peptide molecule, it is exposed to hydrolysis with ɤ-glutamyltransferase in the small intestine (Garvey *et al*, 1976). However, it can also be absorbed as a tripeptide. Although the glutathione given orally to the rats causes an increase in serum and tissue glutathione, the general glutathione activity in humans does not correlate with dietary glutathione (Iantomasi *et al*, 1997; Hagen *et al*, 1990; Aw *et al*, 1991). Witschi *et al*, (1992) reported that oral intake of 3g glutathione (0.15 mM/kg) in healthy subjects failed to increase circulating glutathione concentrations for 270 minutes.

In most foods, glutathione is present in a wide range of 13-110 mg. More than half of the nutritional glutathione comes from fruits and vegetables, and less from meats. However, glutathione content of diet does not correlate with systemic glutathione activity in humans (Flag *et al*, 1994).

The GSH: GSSG ratio, which is the primary determinant of the cellular redox state, shifts more and more to oxidation in the aging process due to an increase in GSSG content or a decrease in de novo GSH biosynthesis. The Km of Glutamate-Cysteine Ligase (GCL), a speed-limiting enzyme in de novo GSH biosynthesis, increases significantly during aging. Particularly under stressful conditions, the speed of GSH biosynthesis is adversely affected. Experimental studies suggest that the accumulation of homocysteine, an intermediate in the trans-sulfation pathway, may cause loss of affinity between the GCL and its substrates. Over-expression of GCL has been shown to prolong the life of Drosophila by up to 50%, suggesting that irregularities in glutathione metabolism play a causative role in the aging process (Rebrin & Sohal, 2008).

Although GSH has an antioxidant effect, it can provide a pro-oxidant effect in some cases. During GSH catabolism, removal of γ-glutamate residue from the cysteine residue causes a prooxidant effect and may induce lipid peroxidation of the plasma membrane and some cellular damage associated with it (Bajic *et al*, 2019). It is thought that anti-oxidant defenses should be developed in the aging process or ROS production will be reduced. In addition, ROS can be useful as well as dangerous. For this reason, it has become quite problematic to establish a causal and effective relationship between ROS and its participation in the aging process. For example, although mitochondrial hydrogen peroxide production decreases with overexpression of Mn superoxide dismutase and ectopic catalase in mitochondrial matrix, Drosophila's life span is shortened instead of elongation. However, it is a fact that antioxidant defenses decrease

with aging. The balance between ROS and anti-oxidants increasingly shifts to a pro-oxidant state (Rebrin & Sohal, 2008).

There are scientific data showing that calorie restriction (CR) delays the onset and progression of age-related changes based on oxidative stress. Rebrin *et al*, (2003) reported that calorie restriction did not affect the concentration of GSH in mitochondria except for the increase in heart and eye, the GSSG concentration was significantly reduced in all tissues except the brain and the GSH and GSSG ratio increased significantly in all tissues.

Glutathione was generally considered to be safe for use as a dietary supplement, but in an oral acute toxicity study of GSH in mice, fatal dose 50 (LD50) was observed to be 5 g/kg (Weschawalit *et al*, 2017). Richie *et al*, (2014) reported that the increase in blood was dose and time dependent, and that levels returned to baseline after a period of 1 month in oral Glutathione intake. Glutathione is used orally or intravenously to whiten skin in various countries. Studies have shown that supplements in reduced and oxidized glutathione forms have skin lightening efficacy in humans; however, in humans, very good absorption in oral use does not occur. Furthermore, side effects, including colds, stomach disorders, headache, back pain, hot flashes, soft stool, eye twitching, ear infection, urinary tract infection, and constipation may be observed while intravenous applications are prohibited in some countries because of serious side effects including anaphylaxis (Arjınpathana & Asawanonda, 2012; Richie *et al*, 2014; Weschawalit *et al*, 2017).

There is a direct relationship between glutathione oxidation and mtDNA damage in apoptosis. However, one of the earliest and most noticeable events during apoptosis is a decrease in the concentration of GSH (Esteve *et al*, 1999; Franco *et al*, 2007). Buthionine sulfoximine (BSO) is a specific inhibitor of GSH biosynthesis (Pallardó *et al*, 2009). Marengo *et al*, (2008) demonstrated that acute treatment with BSO induces a significant GSH depletion that causes excessive production of radical oxygen species (ROS) and DNA damage and thus apoptosis. It is known that there is a decrease in cellular and mitochondrial glutathione during apoptosis, and this decrease is known to induce apoptosis inducing permeability transition pores (Armstrong & Jones 2002).

Allen and Bradley (2011) did not observe any significant changes in their study with oral glutathione supplementation, in healthy individuals, lipid peroxidation biomarkers, DNA, and glutathione. Although GSH is a very important antioxidant that plays a role in the elimination of oxygen radicals in the body, it has been demonstrated in some studies that it has prooxidant properties (Solov'eva *et al*, 2007). Glutathione may act as a pro-oxidant that damages DNA although it is present as a cleanser in millimolar levels (Fucassi *et al*, 2006).

In our study, the prooxidant properties of GSH were observed. In this case, it should be considered that intake of GSH and its derivatives as nutritional supplements in the form of pills or capsules may be harmful. The complexity of antioxidants or the ability of antioxidants such as glutathione to have prooxidant activity depending on the dose is a complex situation called "antioxidant paradox" by Halliwell (2013). In fact, the redox balance can be the cause or the result of a disease, and in some cases it is difficult to know the level at which an antioxidant becomes a prooxidant. Therefore, there is still much to understand about the role of glutathione levels in health (Minich & Brown, 2019).

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Hulya Yildiz: Investigation, Resources, Formal Analysis, Writing. **Duygu Dus:** Investigation, Resources, Formal Analysis. **Ayse Gul Mutlu:** Investigation, Resources, Formal Analysis, Statistics, Supervision.

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REFERENCES

Abreu, I.A., & Cabelli, D.E. (2010). Superoxide dismutases-a review of the metal-associated mechanistic variations. *Biochimica Biophysica Acta*, *1804*(2), 263-274. https://doi.org/10.1016/j.bbapap.2009.11.005

Aebi, S., Assereto, R., & Lauterburg, B.H. (1991). High-dose intravenous glutathione in man. Pharmacokinetics and effects on cyst(e)ine in plasma and urine. *European Journal of Clinical Investigation*, *21*(1), 103-110. https://doi.org/10.1111/j.1365-2362.1991.tb01366.x

Allen, J., & Bradley, R.D. (2011). Effects of oral glutathione supplementation on systemic oxidative stress biomarkers in human volunteers. *The Journal of Alternative and Complementary Medicine*, *17*(9), 827–833. https://doi.org/10.1089/acm.2010.0716

Ames, B.N., Shigenaga, M.K., & Hagen, T.M. (1993). Oxidants, antioxidants and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences*, *90*(1), 7 915-7922. https://doi.org/10.1073/pnas.90.17.7915

Arjinpathana, N., & Asawanonda, P. (2012). Glutathione as an oral whitening agent: A randomized, double-blind, placebo-controlled study. *Journal of Dermatological Treatment*, *23*, 97–102. https://doi.org/10.3109/09546631003801619

Armstrong, J.S., & Jones, D.P. (2002). Glutathione depletion enforces the mitochondrial permeability transition and causes cell death in Bcl-2 overexpressing HL60 cells. *Federation of American Socities for Experimental Biology*, *16*, 1263-1265. https://doi.org/10.1096/fj.02-0097fje

Ayer, A., Tan, S.X., Grant, C.M., Meyer, A.J., Dawes, I.W. & Perrone, G.G. (2010). The critical role of glutathione in maintenance of the mitochondrial genome. *Free Radical Biology & Medicine*, *49*(12), 1956-1968. https://doi.org/10.1016/J.Freeradbiomed.2010.09.023

Aw, T.Y., Wıerbızcka, G., & Jones, D.P. (1991). Oral Glutathione increases tissue glutathione in vivo. *Chemico-Biological Interactions*, *80*, 89-97. https://doi.org/10.1016/0009-2797(91)90033-4

Bajic, V.P. Van Neste, C., Obradovic, M., Zafirovic, S., Radak, D., Bajic, V.B., Essack, M., & Isenovic, E.R. (2019). Glutathione "Redox Homeostasis" and Its Relation to Cardiovascular Disease. *Oxidative Medicine Cellular Longevity*, Article ID 5028181. 14p. https://doi.org/10.1155/2019/5028181

Benard, O., & Balasubramanian, K.A. (1993). Effect of oxidant exposure on thiol status in the intestinal mucosa. *Biochemical Pharmacology*, *45*, 2011- 2015.https://doi.org/10.1016/0006-2952(93)90011-K

Choi, I.Y., Lee, P., & Hughes, A.J. (2016). Longitudinal changes of cerebral glutathione (GSH) levels associated with the clinical course of disease progression in patients with secondary progressive multiple sclerosis. *Multiple Sclerosis Journal*, *23*(1), 956-962. https://doi.org/10.1177/1352458516669441

Circu, M.L., & Aw T.Y. (2012). Glutathione and modulation of cell apoptosis. *Biochimica et Biophysica Acta*, *1823*, 1767–1777. https://doi.org/10.1016/j.bbamcr.2012.06.019

Collins, K. (2016). Determining the role of Mtm1 in Glutathione metabolism. *South Carolina Junior Academy of Science*. 9. https://scholarexchange.furman.edu/scjas/2016/all/9

Danneman, B., Lehle, S., Hildebrand, D.G., Kübler, A., Grodona, P., Schmid, V., Holzer, K., Fröschl, M., Essmann, F., Rothfuss, O., & Osthoff, K.S. (2015). High glutathione and glutathione peroxidase-2 levels mediate cell-type-specific DNA damage protection in human iInduced pluripotent stem cells. *Stem Cell Reports*, *4*(5), 886–898. https://doi.org/10.1016/j.stemcr.2015.04.004

El-Osta, H. & Circu, M.L. (2016). Mitochondrial ROS and apoptosis. *Mitochondrial Mechani sms of Degeneration and Repair in Parkinson's Disease*, 1-23. https://doi.org/10.1007/978-3-319-42139-1_1

Esteve, J.M., Mompo J., García de la Asunción, J., Sastre J., Asensi, M., Boix, J., Viña, J.R., Viña, J., Pallardo, F.V. (1999). Oxidative damage to mitochondrial DNA and glutathione oxidation in apoptosis: studies in vivo and in vitro. *Federation of American Socities for Experimental Biology*, *13*, 1055–1064. https://doi.org/10.1096/fasebj.13.9.1055

Flagg, E.W., Coates, R.J., Eley, J.W., & Jones, D.P., Gunter, E.W., Byers, T.E., Block, G.S., Greenberg, R.S. (1994). Dietary glutathione intake in humans and the relationship between intake and plasma total glutathione level. *Nutrition and Cancer*, *21*(1), 33-46. https://doi.org/10.1080/01635589409514302

Franco, R., Panayiotidis, M.I., & Cidlowski, J.A. (2007). Glutathione depletion is necessary for apoptosis in lymphoid cells independent of reactive oxygen species formation. *Journal of Biological Chemistry*, *282*, 30452–30465. https://doi.org/10.1074/jbc.M703091200

Fucassi, F., Lowe, J.E., Pavey, K.D., Shah, S., Faragher, R.G.A., Green, M.H.L., Paul, F., O'Hare, D., & Cragg, P.J. (2006). Alpha-ipoic acid and glutathione protect against the prooxidant activity of SOD/catalase mimetic manganese salen derivatives. *Journal of Inorganic Biochemistry*, *101*, 225–232. https://doi.org/10.1016/j.jinorgbio.2006.09.023

Garvey, T.Q., Hyman, P.E. & Isselbacher, K.J. (1976). γ-Glutamyl transpeptidase of rat intestine: Localization and possible role in amino acid transport. *Gastroenterology*, *71*(5), 778–785. https://doi.org/10.1016/S0016-5085(76)80360-5

Giustarindi, D., Tsikas, D., Colombo, G., Milzani, A., Donne, I.D., Fanti, P., & Rossi, R. (2016). Pitfalls the analysis of the physiological antioxidant glutathione (GSH) and its disulfide (GSSG) in biological samples: An elephant in the room. *Journal of Chromatography B, 1019*, 21-28. https://doi.org/10.1016/j.jchromb.2016.02.015

Hagen, T.M., Wierzbicka, G.T., Sillau, A.H., Bowman, B.B., & Jones, D.P. (1990). Bioavailability of dietary glutathione: effect on plasma concentration. *American Journal of Physiological*, *259*(4), 524-9. https://doi.org/10.1152/ajpgi.1990.259.4.g524

Halliwell, B. (2013). The antioxidant paradox: Less paradoxical now? *British Journal of Clinical Pharmacology*, *75*(3), 637-644. https://doi.org/10.1111/J.1365-2125.2012.04272.X

Iantomasi, T., Favilli, F., Marraccini, P., Magaldi, T., Bruni, P., & Vincenzini, M.T. (1997). Glutathione transport system in human small intestine epithelial cells. *Biochimica et Biophysica Acta*, *1330*(2), 274-83. https://doi.org/10.1016/s0005-2736(97)00097-7

Ighodaro, O.M., & Akinloye, O.A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, *54*(4), 287-93. https://doi.org/10.1016/j.ajme.2017.09.001

Jain, S.K., Marie, P.K., Warden, C., & Micinski, D. (2016). L-cysteine supplementation upregulates glutathione (GSH) and vitamin D binding protein (VDBP) in hepatocytes cultured in high glucose and in vivo in liver, and increases blood levels of GSH, VDBP, and 25-hydroxy-vitamin D in Zucker diabetic fatty rats. *Molecular Nutrition Food Research, 60*, 1090–1098. https://doi.org/10.1002/mnfr.201500667

Kannan, R., Yi, J.R., Tang, D., Zlokovic, B.V., & Kaplowitz, N. (1996). Identification of a novel, sodium-dependent, reduced glutathione transporter in the rat lens epithelium. *Investigative Ophthalmol and Visual Science*, *37*(11), 2269-2275. https://iovs.arvojournals.org/article.aspx?articleid=2180347

Kern, J.K., Geier, D.A., Adams, J.B., Garver, C.R., Audhya, T., & Geier, M.R. (2011). A clinical trial of glutathione supplementation in autism spectrum disorders. *Medical Science Monitor*, *17*(12), 677-682. https://doi.org/10.12659/msm.882125

Lim, H, Bodmer, R., & Perrin, L. (2006). Drosophila aging 2005/06. *Experimental Gerontology*, *41*, 1213-1216. https://doi.org/10.1016/j.exger.2006.10.013

Mannarino, S.C., Amorim, M.A., Pereira, M.D., Moradas-Ferreira, P., Panek, A.D., Costa, V., & Eleutherio, E.C.A. (2008). Glutathione is necessary to ensure benefits of calorie restriction during ageing in *Saccharomyces cerevisiae. Mechanisms of Ageing and Development, 129*, 700–705. https://doi.org/10.1016/j.mad.2008.09.001

Marengo, B., De Ciucis, C., Verzola, D., Pistoia, V., Raffaghello, L., Patriarca, S., Balbis, E., Traverso, N., Cottalasso, D., Pronzato, M.A., Marinari, U.M., & Domenicotti, C. (2008). Mechanisms of BSO (L-buthionine-S, R-sulfoximine)-induced cytotoxic effects in neuroblastoma. *Free Radical Biology and Medicine, 44*, 474-482. https://doi.org/10.1016/j.freeradbiomed.2007.10.031

Meister, A. (1991). Glutathione deficiency produced by inhibition of its synthesis, and its reversal; applications in research and therapy. *Pharmacology and Therapeutics*, *51*(2), 155-194. https://doi.org/10.1016/0163-7258(91)90076-x

Min, K.H, & Lee, W. (2019). Alteration of mitochondrial DNA content modulates antioxidant enzyme expressions and oxidative stress in myoblasts. *Korean Journal of Physiology Pharmacology*, *23*, 519-528. https://doi.org/10.4196/kjpp.2019.23.6.519

Minich, D.M., & Brown, B.I. (2019). A review of dietary (Phyto) nutrients for glutathione support. *Nutrients*, *11*(9), E2073. https://doi.org/10.3390/nu11092073

Mutlu, A.G. (2012a). Increase in mitochondrial DNA copy number in response to ochratoxin A and methanol-induced mitochondrial DNA damage in drosophila. *Bulletin of Environmental Contamination and Toxicology*, *89*, 1129-1132. https://doi.org/10.1007/s00128-012-0826-1

Mutlu, A.G. (2012b). Measuring of DNA damage by quantitative PCR, pp. 283-294. Polymerase Chain Reaction, Patricia Hernandez-Rodriguez, In tech, Rijeka, Croatia. https://doi.org/10.5772/38159

Mutlu, A.G. **(**2013**).** The effects of a wheat germ rich diet on oxidative mtDNA damage, mtDNA copy number and antioxidant enzyme activities in aging Drosophila. *Acta Biologica Hungarica*, *64*(1),1-9. https://doi.org/10.1556/abiol.64.2013.1.1

Owen, J.B., & Butterfield, D.A. (2010). Measurement of oxidized/reduced glutathione ratio. *Methods in Molecular Biology*, *648*, 269-277. https://doi.org/10.1007/978-1-60761-756-3_18

Pallardó, F.V., Markovic, J., García, J.L., & Viña, J. (2009). Role of nuclear glutathione as a key regulator of cell proliferation. *Molecular Aspects of Medicine*, *30*, 77–85. https://doi.org/10.1016/j.mam.2009.01.001

Rebrin, I., Kamzalov, S., & Sohal, R.S., (2003). Effects of age and caloric restriction on glutathione redox state in mice. *Free Radical Biology and Medicine*, *35*(6), 626–635. https://doi.org/10.1016/S0891-5849(03)00388-5

Rebrin, I., & Sohal, R. S. (2008). Pro-oxidant shift in glutathione redox state during aging. *Advanced Drug Delivery Reviews*, *60*(13-14), 1545-1552. https://doi.org/10.1016/J.ADDR.2008.06.001

Richie, J.J., Nichenametla, S., Neidig, W., Calcagnotto, A., Haley, J.S., Schell, T.D., & Muscat, J.E. (2014). Randomized controlled trial of oral glutathione supplementation on

body stores of glutathione. *European Journal of Nutrition, 54*(2), 251-263. https://doi.org/10.1007/s00394-015-0874-5

Santos, J.H., Mandavilli, B.S., & Van Houten, B. (2002). Measuring oxidative mtDNA damage and repair using QPCR. In: Copeland WC (ed) *Mitochondrial DNA methods and protocols.* Humana Press, Totawa, pp 159–176. https://doi.org/10.1385/1-59259-284-8:159

Shaw, P., Ocorr, K., Bodmer, R., Oldham, S. (2008). *Drosophila* aging 2006/2007. *Experimental Gerontology, 43*, 5-10. https://doi.org/10.1016/j.exger.2007.10.008

Sheshadri, P., & Kumar A., (2016). Managing odds in stem cells: insights into the role of mitochondrial antioxidant enzyme MnSOD. *Free Radical Research, 50*(5), 570-84. https://doi.org/10.3109/10715762.2016.1155708

Solov'eva, M.E., Solov'ev, V.V., Faskhutdinova, A.A., Kudryavtsev, A.A., & Akatov, V.S. (2007). Prooxidant and cytotoxic action of N-Acetylcysteine and Glutathione in combinations with Vit B12. *Cell and Tissue Biology, 1*, 40-49. https://doi.org/10.1134/S1990519X07010063

Sze, G., Kaplowitz, N., Ookhtens, M., & Lu, S.C. (1993). Bidirectional membrane transport of intact glutathione in Hep G2 cells. *American Journal of Physiology, 265*(6), 1128-34. https://doi.org/10.1152/ajpgi.1993.265.6.g1128

Venkatraman, A., Landar, A., Davis, A.J., Chamlee, L., Sandersoni, T., Kim, H., Page, G., Pompilius, M., Ballinger, S., Darley-Usmar, V., & Bailey, S.M. (2004). Modification of the mitochondrial proteome in response to the stress of ethanol-dependent hepatoxicity. *Journal of Biological Chemistry, 279*, 22092–101. https://doi.org/10.1074/jbc.m402245200

Weschawalit, S., Thongthip, S., Phutrakool, P., & Asawanonda, P. (2017). Glutathione and its antiaging and antimelanogenic effects. *Clinical Cosmetic Investigional Dermatology, 10*, 147–153. https://doi.org/10.2147/ccid.s128339

Witschi, A., Reddy, S., Stofer, B., & Lauterburg, B.H. (1992). The systemic availability of or al glutathione. *European Journal of Clinical Pharmacology, 43*(6), 667-669. https://doi.org/10.1007/BF02284971

Wu, G., Fang, Y.Z., Yang, S., Lupton, J.R., & Turner, N.D. (2004). Glutathione metabolism and its implications for health. *Journal of Nutrition, 134*(3), 489-492. https://doi.org/10.1093/jn/134.3.489

Yakes, F.M., & Van Houten, B. (1997). Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proceedings of the National Academy Sciences of the United States of America, 94*, 514-519. https://doi.org/10.1073/pnas.94.2.514

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Research Article

Composition analysis and antibacterial activity evaluation of different crude extracts of *Mentha piperita* (Lamiaceae)

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Abstract: Globally, the main target of scientists is to examine new medicinally beneficial plants for the preparation of herbal drugs as well as to explore possible uses in the food industry. In this regard, the target of our current study is to evaluate the antibacterial activity and biochemical analyses of the leaf extracts of Omani *Mentha piperita* L (*M. piperita*). The selected plant leaves coarse powder samples were extracted by using the Soxhlet extraction process with methanol at 65ºC for a period of 72 hours. The evaporation of methanol was done by a rotary evaporator under reduced pressure and temperature. The hydro alcoholic extract was fractionated with the various polarities of solvents with an increasing pattern. The biochemical evaluation and pharmacological activity of the prepared local plant extracts were completed by using established methods. The different polarities of leaves extracts showed positive biochemical tests of alkaloids, flavonoids, glycosides, saponins, steroids, tannins and triterpenoids. The microbial activity of the fractioned plant extracts was tested with improved agar gel method. The different polarity extracts at different concentrations did not display any activity against the tested Gram (+) *Staphylococcus aureus* (*S. aureus*) and Gram (-) *Escherichia coli* (*E. coli*) and *Pseudomonus aeruginosa* (*P. aeruginosa*) bacterial strains. Based on the biochemical and pharmacological evaluation results, the selected whole plant and its potential extracts might be used traditionally as natural antibiotics to treat infectious diseases.

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1. INTRODUCTION

Mentha piperita L is known as a hybrid of two species watermint and spearmint belonging to the Lamiaceae family. This plant is native to Oman including European countries, but it is cultivated globally due to its medicinal values (Georgiev & Stoyanova, 2006). Several other varieties/species like apple mint, water mint, horsemint, pennyroyal, and spearmint belonging to the same family are also available in Oman. The height of this plant is about 30–90 cm. The stems are smooth and square. The cross-section and its rhizomes are wide spreading. The size of the leaves is about 1.5-3.5 inches long and 2.5 inches wide with an oval shape. They are dark green with reddish veins (Figure 1).

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Figure 1. Appearance of extracted organs (leaves) of *M. piperita*

The selected plant is used as a traditional folk remedy including complementary and alternative herbal therapy for the treatment of different disorders. In addition, it is widely used in Chinese traditional healing medicine for different ailments. Since ancient times, the Egyptian people have been using the powder of this plant in pyramids to kill bacteria (Georgiev & Stoyanova, 2006). Also, the leaf crude extracts of the selected plant are used to prepare various modern medicines. Due to their aromatic qualities, nowadays, the essential oil and leaves crude extracts are commercially used to produce foods, and medicines (Georgiev & Topalova 1989). In addition, the plant oil is used to prepare commercial products like toothpaste, chewing gum, mouthwash, soaps, sweets, balms or creams, and cough medicine (Georgiev & Topalova 1989; Stanev & Jelyazkov, 2004; Nedkov *et al.*, 2005; McKay & Blumberg, 2006). People use this plant mainly for its oil. The main ingredients in the oil are composed of menthol and menthone along with a high percentage of other biologically active minor compounds like pulegone, menthofuran and limonene (Georgiev & Topalova 1989; Sivropoulou *et al.*, 1995). Several scientific data are available in the literature that the selected plant species showed different pharmacological activities including scavenging activity, anticancer, antispasmodic, antiviral and antimicrobial activities (Spirling & Daniels, 2001; Aswatha *et al.*, 2008; Hossain *et al.*, 2008; Sun *et al.*, 2014). The pharmacological activity of the plant extracts and oil are directly linked to the bioactive compounds. Most researchers have detected anti-inflammatory, anti-bacterial and anti-cancer properties of this plant (Hills *et al.*, 2005; Tabari *et al.*, 2012; Sun *et al.*, 2014). Previously, several researchers have worked on leaves (Tabari *et al.*, 2012; Sun *et al.*, 2014) and roots of this plant (Hills *et al.*, 2005) and studies have exposed significant biological activity against several Gram (+ and -) bacteria strains (Hills *et al.*, 2005; Stepan *et al.*, 2016**)**. However, so far, no detailed information related to chemical analyses and the antibacterial activity of different polarity crude extracts has been published on the Omani *M. piperita* species. Keeping this in mind, the current study design is to determine the antimicrobial activity and chemical analyses of different extracts of locally collected *M. piperita leaves*.

2. MATERIAL and METHODS

2.1. Materials and Chemicals

Solvents with different polarities such as hexane, methanol, chloroform, and butanol (analytical grade, BDH, Germany) were used in this present study. Most of the reagents for biochemical analyses were of analytical grade. Antibiotic levofloxacin (positive control) and dimethyl sulphoxide (DMSO, negative control) were obtained from BDH, Germany. A blender machine (Jaipan, Super Deluxe, India) was used for the grinding of plant samples. The rotary evaporator (Model Yamato, R.E801, Japan) and Soxhlet extractor (MEDIZLAB) were from Borosil, India.

2.2. Microbial Strains

Both Gram (+) and Gram (-) bacterial strains were obtained from the Department of Microbiology, of Nizwa Hospital in December 2012. The bacterial strains were maintained on nutrient agar at 4°C. Then, the bacterial strains were cultured into a nutrient broth by an established technique at the Department of Biology, University of Nizwa, the Sultanate of Oman.

2.3. Plant Materials

The selected Omani *M. piperita* species were obtained from Hay Al Thurat, Nizwa, Oman. The sample was collected in February 2012 and after collecting the leaves samples, the morphological identification was accomplished by https://en.wikipedia.org/wiki/ peppermint. The leaves were separated from the plant, packed in a bag, and kept at 4°C until the preparation of crude extracts.

2.4. Preparation of the Samples

The leaf samples were processed and dried at normal temperature. The dried leaves (100 gm) were crushed into coarse powder and kept in an amber colour bottle for extraction.

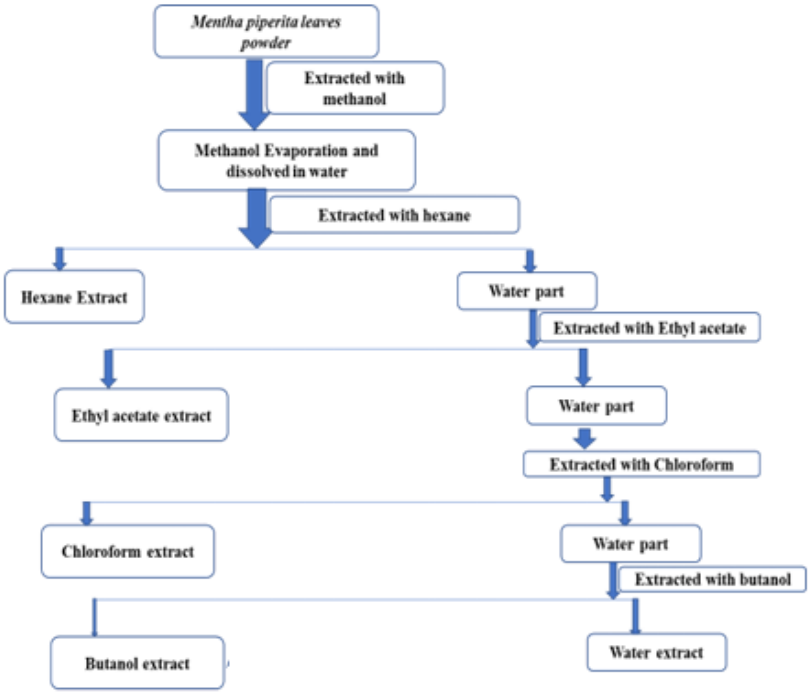
2.5. Extraction Procedure

About 80.33 gm of the powdered leaves samples were used for extraction by using a Soxhlet extractor with methanol (360 ml) at 68°C for a period of 72 hours (Asma *et al*., 2017; Raqiya *et al.*, 2017). After complete extraction and filtration, a rotary evaporator was used to remove methanol and give viscous semi-solid masses (7.99 gm, yield 9.94 %). The semi-solid extract (6.68 gm) was liquefied in water (75 ml) and then the sample was fractionated by various polarities of solvents: hexane (1.88 gm, yield 28.14%), ethyl acetate (1.65 gm, yield 24.7%), chloroform (0.12 gm, yield 1.79%) and butanol (1.51 gm, yield 22.60%) and the residual fraction was (0.90 gm, 1.34%) (Asma *et al.*, 2017; Raqiya *et al.*, 2017). The extraction process was repeated twice and then the solvent was evaporated under reduced pressure and temperature (Figure 2).

2.6. Biochemical Analyses

The plant powder materials, crude extracts and stock solutions were used for chemical analyses by the methods described by Tahiya *et al.*, 2014; and; Ali and Hossain, 2014). Each polarity plant leaf extracts (1 mg) was liquefied in it mother solvent (100 ml) to give the stock solution (1%, v/v).

Figure 2. Flow chart for extraction



2.6.1. *Identification of alkaloids*

The plant coarse powder (1 gm) was taken into a small beaker and dil NH_3 solution (3 ml) was added and it was kept for a few minutes with constant stirring. Chloroform (10 ml) was added to the same beaker and the mixture was shaken and filtered. The filtrate was evaporated to 50% by using heat and then Mayer's reagent was added drop by drop. A creamy colour precipitate appeared indicating the existence of alkaloids.

2.6.2. *Identification of flavonoids*

The stock solution of each plant extract (1 ml) was taken into an appropriate test tube. A few drops of dilute NaOH were added to each test tube. A deep yellow colour was found with the addition of dilute HCl that gradually becomes colourless. It showed the existence of flavonoids.

2.6.3. *Identification of saponins*

The stock solution of each plant extract (1 ml) was taken into six separate test tubes. The stock solution was diluted with the addition of H_2O and the whole solution was shaken for a few minutes. A foam layer appeared in the upper part of the test tube that showed the existence of saponins.

2.6.4. *Identification of steroids*

The stock solution of each plant extract (1 ml) was taken into a 20 ml beaker and was dissolved in $CHCl_3$ solvent (10 ml). Concentrated H_2SO_4 (1 ml) was added to the beaker dropwise. In the beaker, two layers were formed. The top layer turned red and the bottom layer turned deep yellow which indicated the existence of steroids.

2.6.5. *Identification of tannins*

The stock solution of each plant extract (3 ml) was taken into a beaker and was diluted with $CHCl_3$ solvent. Then, one millilitre $(CH_3CO)_2O$ was added to the beaker and finally Conc. H_2SO_4 was added carefully drop by drop. A green colour was obtained that showed the existence of tannins.

2.6.6. *Identification of triterpenoids*

The stock solution of each plant extract (5 mg) was taken in a beaker and dissolved in $CHCl_3$ solvent (2 ml) by shaking. One milliliter of $(CH_3CO)_2O$ and concentrate H_2SO_4 (1 ml) were added to the beaker drop by drop. It gave a violet colour that showed the existence of triterpenoids.

2.7. Antibacterial Activity Assay

The prepared leaf crude extracts of *M. piperita* were assessed for their *in-vitro* antibacterial activity by the agar diffusion method described by Fatma and Hossain (2016). Four different concentrations of each crude extract (2, 1, 0.5 and 0.25 µg/ml) were prepared by using a DMSO solution followed by a dilution method. The activity of the crude extracts at each concentration was determined against *S. aureus*, *E. coli* and *P. aeruginosa* bacterial strains. Approximately 5 mm diameter filter paper was used as a disc in the present experiment. Levofloxacin antibiotic and DMSO solvent were used as positive and negative controls. All discs were soaked in different concentrations of each plant crude extract along with the positive and negative controls. All the socked discs were placed on an inoculated agar plate and kept in an incubator at 37°C for 24 hours. The diameter of the inhibition zone of each incubated disc was manually measured. All the data were replicated three times and averaged.

3. RESULTS

Since ancient times, plant-based medicine has been used for the treatment of the majority of diseases. About 1200 plants have been considered medicinal plants all over the world due to their pharmacological activity. Specific plant species originate and grow well in certain regions with suitable environmental conditions and neighboring fauna and flora (Adamu *et al.*, 2004). The viscous semi-solid masses were obtained from the powdered leaves by the extraction of methanol and evaporation. The solvent-free methanol plant extract was liquefied by water and then extracted sequentially with various polarity of solvents to give hexane, ethyl acetate, chloroform, butanol and residual water fractions, respectively (Ali & Hossain, 2014; Tahiya *et al.*, 2014) (Figure 2).

3.1. Biochemical Analyses

The biochemical analysis of all prepared extracts and powders of *M. piperita* indicated the presence of alkaloids, flavonoids, saponins, steroids, tannins, and triterpenoids. However, chloroform leave extracts did not show any positive tests for tannin and steroids. Similarly, hexane plant crude extracts also did not show any positive tests for steroids (Table 1).

Table 1. Biochemical analysis of the hexane, ethyl acetate, chloroform, butanol and methanol crude extracts of *M. piperita*

Biochemicals	Inference				
	Hexane	Ethyl acetate	Chloroform	Butanol	Methanol
Alkaloids	+	+	+	+	+
Flavonoids	+	+	+	+	+
Saponins	+	+	+	+	+
Tannins	+	+	-	+	+
Triterpenoids	+	+	+	+	+
Steroids	-	+	-	+	+

+ = presence; - = absence

3.2. Antibacterial Activity

The antibacterial activity of different polarity crude extracts at different concentrations was qualitatively assessed against the employed bacteria strains in the existence or nonexistence of inhibition zones (Al-Rashdi *et al.*, 2021). The various polarity extracts at different concentrations (2, 1, 0.5 and 0.25 µg/ml) gave no significant activity against one gram (+) (*S. aureus)* and two gram (-) bacteria strains *(E. coli* and *P. aeruginosa)* at the range of 0-10 mm. Most of the crude extract of *M. piperita* did not show any impressive activity against *E. coli, P. aeruginosa* and *S. aureus* bacteria at the concentrations of 2, 1, 0.5 and 0.25 µg/ml is as presented in Table 2. The maximum antibacterial activity against *E. coli* was found in chloroform extract at the concentration of 2 mg/ml.

Table 2. Antibacterial activity of different crude extracts of *M. piperita* against *E. coli*, *P. aeruginosa* and *S. aureus*

Extract	Concentration (mg/ml)	*E. coli* (mm)	*P. aeruginosa* (mm)	*S. aureus* (mm)
Hexane	2	8±0.67	9±0.67	nd
	1	8±0.67	nd	nd
	0.5	8±0.67	nd	nd
	0.25	nd	nd	nd
	Control	nd	nd	nd
Ethyl acetate	2	8±0.67	nd	nd
	1	7±0.67	nd	nd
	0.5	7±0.67	nd	nd
	0.25	nd	nd	nd
	Control	nd	nd	nd
Chloroform	2	10±0.67	8±0.67	7±0.67
	1	8±0.67	7±0.67	nd
	0.5	nd	nd	nd
	0.25	nd	nd	Nd
	Control	nd	nd	Nd
Butanol	2	7±0.67	nd	7±0.67
	1	7±0.67	nd	6±0.67
	0.5	nd	nd	6±0.67
	0.25	nd	nd	6±0.67
	Control	nd	nd	Nd
Methanol	2	7±0.67	7±0.67	Nd
	1	6±0.67	7±0.67	Nd
	0.5	nd	7±0.67	Nd
	0.25	nd	nd	Nd
	Control	nd	nd	Nd

nd=not detectable

4. DISCUSSION

Nature has been an important source of medicines for a long time. The plant materials and their derivative products have been used as medicine in different traditional medicine systems to treat various diseases since old times. WHO estimated that approximately 50-60% of the world's population depend on plant derived medicines for their basic health care system. Based on their medical use in the traditional medicine system a good number of therapeutic agents have been extracted/isolated from natural resources (Frombi, 2003).

Treating various bacterial infection is growing difficult due to the development and spread of microbial resistance and the deficiency of the expansion of new antibacterial principles (Aishwarya, 2015). The natural product is one of the main sources that have provided the

pharmaceutical, agricultural and cosmetics industry with some of its natural sources of principal products in the search for new antibacterial drugs (Basheer & Abdullah, 2019). A huge number of local medicinal plants with a significant activity can represent a source of new antibacterial agents for the treatment of various infectious diseases (Aishwarya, 2015; Basheer & Abdullah, 2019). An active plant oils and extracts are used for food preservation, in finished pharmaceuticals products, and in therapies of alternative and complementary medicine systems (Bupesh *et al*., 2007).

M. piperita is a plant that grows well in all climates and exists all over the world including Oman. The oil from the selected plant is used to prepare toothpaste, chewing gum, mouthwash, soaps, sweets, balms or creams, and cough medicine (Spirling & Daniels, 2001; Aswatha *et al*., 2008; Hossain *et al.*, 2008; Sun *et al.*, 2014). A significant percentage of menthol and menthone are present in the plant. Menthol is a chemical ingredient used to treat a variety of gastrointestinal problems. In addition, it is also used for gastrointestinal problems, cramps, dyspepsia, nausea, the common cold, sore throat, colic, itching, inflammation and headaches (Hills *et al.*, 2005; Tabari *et al.*, 2012; Sun *et al.*, 2014).

4.1. Biochemical Analyses

The qualitative biochemical analysis of different polarity leaves extracts of *M. piperita* proved the presence of saponins, flavonoids, tannins, alkaloids, triterpenoids, and steroids. However, the hexane and ethyl acetate crude extracts did not show the presence of steroids. Similar chemical analysis results were reported on various extracts of the selected plant by other authors (Sujana *et al.*, 2013).

4.2. Antibacterial Activity

The antibacterial activity of different extracts of *M. piperita* was determined against the available three bacteria strains. Most leaf extracts from *M. piperita* showed no potentially significant activity against the selected Gram (+) and Gram (-) bacteria strains at the concentrations of 2, 1, 0.5 and 0.25 µg/ml (Abdulsattar & Hossain, 2020). Their calculated diameter zones of inhibition were within the range of 0-10 mm (Table 2). Among the extracts, the methanol extract only gave a low activity against *E. coli* and *P. aeruginosa* bacteria strains at the concentration of 2 and 1 mg/ml (Table 2). However, the methanol extract did not display activity against *S. aureus* at any applied concentrations. The same extract also failed to display any activity against *E. coli* and *P. aeruginosa* bacteria strains at low concentrations of 0.5 and 0.25 mg/ml. The butanol extract exhibited moderate antibacterial activity against *S. aureus* at all concentrations. But, less activity was obtained against *E. coli* at the concentration of 2 µg/ml. However, the other concentrations (1, 0.5 and 0.25 mg/ml) of butanol leaves extract did not give any activity against *P. aeruginosa*. In addition, hexane and ethyl acetate extracts from these leaves did not show any antibacterial activities against most of the tested bacteria strains. However, hexane and ethyl acetate extract crude extracts showed moderate activities at the concentrations of 2, 1 and 0.5µg/ml against *E. coli*. The chloroform extract of the selected plant also showed some activities at the high concentrations against all applied bacterial strains but at low concentrations did not show any activity. In our present study, the water extract also did not show any activity against all the tested bacterial strains (data not shown). It is very interesting that the negative and positive controls also did not inhibit the growth of the tested bacterial strains. Previous results of other authors showed that most of the polarities extracts give significant activities against the Gram (+ and -) bacterial strains (Hills *et al.*, 2005; Bupesh *et al.*, 2007; Tabari *et al*., 2012; Sujana *et al.*, 2013; Sun *et al.*, 2014; Aishwarya, 2015; Basheer & Abdullah, 2019). Our experimental results are different from the reported values may be due to the chemical composition, concentration of ingredients, plant maturity, geographical region and processing conditions (Zainab & Hossain, 2016; Doha *et al.*, 2020). Therefore, further studies will be needed for the antibacterial activity of the Omani species by other standard

screening methods against *S. aureus* and *E. coli* and *S. aeruginosa*. In addition, further studies will be needed to separate the bioactive ingredients from the leaves of *M. piperita* species.

5. CONCLUSION

The antibacterial and chemical composition of the selected plant's various extracts were determined by well recognized methods. Our current report related to chemical analyses and antibacterial activity will help the isolation of new products/drugs with significant activity from the selected plant species. Based on our results, it could be concluded that the leaves extract of *M. piperita* has been moderate against the applied Gram (+) and Gram (-) bacterial strains. However, the experiment could be repeated to check the antibacterial activity of different polarity extracts at different concentrations against a large number of bacterial strains that will be useful for the preparation of antibiotics.

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Declaration of Conflicting Interests and Ethics

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

Authorship contribution statement

Zahra Mohammed Al-Hajri: Investigation, Data curation, Analysis, and Draft writing. **Mohammad Amzad Hossain**: Study design, Supervision, Data analysis, Edit manuscript. **Salim Said Al-Touby:** Logistic support, Data analysis, Final edit.

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REFERENCES

Abdulsattar, A.M., & Hossain, M.A. (2020). Antibacterial and antioxidant potential of *Tetraena simplex* extracts of various polarities. *Toxicology Reports, 7*, 925–929.

Adamu, H.M., Abayeh, O.J., Agho, M.O., Abdullahi, A.L., Uba, A., Dukku, H.U., & Wufem, B.M. (2004). An ethnobotanical survey of Bauchi State herbal plants and their antimicrobial activity. *Journal of Ethnopharmacology, 99*, 1-4.

Aishwarya, B. (2015). Therapeutic Uses of Peppermint–A Review. *Journal of Pharmaceutical Sciences, 7*(7), 474-476.

Ali, S.H.J., & Hossain, M.A. (2015). Phytochemical studies of various polarities leave crude extracts of Omani *Datura metel* L. and evaluation of their antimicrobial potential. *Journal of Coastal Life Medicine, 3*(3), 214-219.

Al-Rashdi, R.S.Y., Hossain. M.A., & Al-Touby, S.S.J. (2021). Antioxidant and antibacterial activities of leaves crude extracts of *Adenium obesum* grown in Oman National Botanical Garden. *Advances in Biomarker Sciences and Technology, 3*, 8-14.

Asma, H. S., Moza, T. H. G., & Hossain, M. A. (2017). Brine shrimp toxicity of various polarities leaves and fruits crude fractions of *Ziziphus jujuba* native to Oman and their antimicrobial potency. *Sustainable Chemistry and Pharmacy, 5*, 122-125.

Aswatha, R.H.N., Shreedhara, C.S., Falguni, P.G., & Sachin, B.Z. (2008). *In vitro* free radical scavenging potential of methanol extract of entire plant of *Phyllanthus reticulatus* Poir. *Pharmacologyonline, 2*, 440-451.

Basheer A.S., & Abdullah A.A. (2013). Antimicrobial activity of the aqueous extract of mint plant. *Science Journal of Clinical Medicine, 2*(3), 110-113

Bupesh, G., Amutha, C., Nandagopal, G., Ganeshkumar, A., Sureshkumar, P., & Saravana, M. K. (2007). Antibacterial activity of *Mentha piperita* L. (peppermint) from leaf extracts-a medicinal plant. *Acta Agriculturae Slovenica, 89*(1), 73-79.

Chowdhury, J.A., Islam, M.S., Asifuzzaman, S., & Islam, M.K. (2009). Antibacterial and cytotoxic activity screening of leaf extracts of *Vitex negundo* (Fam: Verbenaceae). *Journal of Pharmaceutical Sciences and Research, 1*, 103-108.

Doha, M. A., Afaf M. W., Hossain, M. A., & Al Touby, A. (2020). Toxicological study of various crude extracts of *Hyoscyamus gallagheri* native to Oman. *Toxicology Reports, 7*, 680-684.

Frombi, E.O. (2003). African indigenous plants with chemotherapeutic potential and bioactive prophylactic agents. *African Journal of Biotechnology, 2*, 662-671.

Fatma, R. S., & Hossain, M. A. (2016). In vitro antimicrobial potential of crude extracts and chemical compositions of essential oils of leaves of *Mentha piperita* L native to the Sultanate of Oman. *Pacific Science Review A: Natural Science and Engineering, 18*, 103-106.

Georgiev, E., & Stoyanova, A. (2006). *Peppermint oil. In: A guide for the specialist in aromatic industry*, Dimitrov D (ed), UFT Academic Publishing House, Plovdiv, p. 219-232.

Georgiev, S., & Topalova, V. (1989). Chemical composition and antimicrobial activity of Bulgarian peppermint oils. *Plant Science, 26*, 33-37.

Hossain, M.A., Ismail, Z., Rahman, A., & Kang, S.K. (2008). Chemical composition and antifungal properties of the essential oil and crude extracts of *Orthosiphon stamineu. Industrial Crops and Products, 27*, 328-334.

Hills, J. M., & Aaronson, P.I. (2005). Beecham, The mechanism of action of peppermint oil on gastrointestinal smooth muscle. An analysis using patch clamp electrophysiology and isolated tissue pharmacology in rabbit and guinea pig. *Journal of Gastroenterology, 32*(6), 765-768.

Kumar, S., Sharma, S., Kumar, D., Kumar, T., Arya, R. & Kumar, K. (2012). Pharmacognostic study and anti-inflammatory activity of *Phyllanthus reticulatus*. *Asian Pacific Journal of Tropical Disease*, S332- 335.

McKay, D.L., & Blumberg, J.B. (2006). A review of the bioactivity and potential health benefits of peppermint tea *(M. piperita* L.*). Phytotherapy Research, 20*, 619-633.

Mimica, D.N., Bozin, B., Soković, M., Mihajlović, B., & Matavulj, M. (2003). Antimicrobial and antioxidant activities of three *Mentha* species essential oils. *Planta Medica, 69*, 413–419.

Nedkov, N., Kanev, K., Kovacheva, N., Stanev, S., Djurmanski, A., Seikova, K., Lambev, K., & Dobreva, A. (2005). *Handbook of medical and essential plants*, Helikon, Kazanlik.

Raqiya, M.S.M., & Hossain, M. (2017). Evaluation of antioxidant and cytotoxic activities of different extracts of folk medicinal plant *Hapllophyllum tuberculatum*. *Egyptian Journal of Basic and Applied Sciences, 4*(2), 101–106.

Stanev, S., & Jelyazkov, V. (2004). Peppermint oil for irritable bowel syndrome. *Acta Horticulturae, 629*, 149-152.

Sivropoulou, A., Kokkini, S., Lanaras, T., & Arsenakis, T. (1995). Antimicrobial activity of mint essential oil. *Journal of Agricultural and Food Chemistry, 43*(9), 2384–2388

Spirling, L.I., & Daniels, I.R. (2001). Botanical perspectives on health peppermint: more than just an after-dinner mint. *Journal of the Royal Society of Health, 121*, 62-63.

Sun, Z., Wang, H., Wang, J., Zhou, L., & Yang, P. (2014). Chemical composition and anti- inflammatory, cytotoxic and antioxidant activities of essential oil from leaves of *Mentha piperita* Grown in China. *PLoS ONE*, *9*(12), 114767.

Stepan, M., Vardan, M., Juletta, A., Anahit, T., Mahsa, D., & Bella, S. (2016). Productivity, biochemical indices and antioxidant activity of peppermint (*Mentha piperita* l.) and basil (*Ocimum basilicum* L.) in conditions of hydroponics. *Journal of Aquaculture Research & Development, 7*(6), 1-4.

Sujana, T.M., Sridhar, P., Josthna, C.V., & Naidu, V. (2013). Antibacterial activity and phytochemical analysis of *Mentha piperita* L. (Peppermint)-An important multipurpose medicinal plant. *American Journal of Plant Sciences, 4*, 77-83.

Tabari, M.A., Youssefi, A.Z., Ghasemi, F., Tabari, R.G., Esmaili, G.H., & Behzadi, M.Y. (2012). In vitro antimicrobial activity of peppermint oil and crude extracts. *Mid-East J. Sci. Res., 11*(4), 536-540.

Tahiya, H.A., Amira, H.S.M., Hossain, M.A., Weli, A.M., & Al-Riyam,i Q. (2014). Comparative study of phytochemical screening, antioxidant and antimicrobial capacities of fresh and dry leaves crude plant extracts of *Datura metel* L. *Journal of King Saud University Science, 26*, 237-243.

Zainab, S.S.H., & Hossain, M.A. (2016). Biological activities of different leaves crude extracts of neem used locally in Ayurvedic medicine. *Pacific Science Review A: Natural Science and Engineering, 18*, 128-131.

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Research Article

Mineral contents, antimicrobial profile, acute and chronic toxicity of the aqueous extract of Moroccan *Thymus vulgaris* in rodents

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Abstract: Moroccan flora is rich in plants used in traditional medicine, but the further scientific investigation is necessary. The aim of the research was to evaluate the nutritional content and antimicrobial activity of Moroccan *Thymus vulgaris*, as well as its possible acute and chronic toxicological effects on rodents. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used to determine the mineral content. The antimicrobial activity was determined using a well-diffusion test, a minimum inhibitory concentration (MIC), and a minimum bactericidal/fungicidal concentration (MBC/MFC) assay. Acute and chronic toxicity studies were conducted *in vivo* on mice and rats, respectively. Following that, haematological, serum-biochemistry, and histological investigations were performed. Moroccan Thyme was shown to be a source of numerous minerals which are necessary for health promotion. All antimicrobial testing, disc diffusion, MIC, and MBC tests revealed that thyme had potent antibacterial activity against all microorganisms tested. *Staphylococcus aureus* was the most susceptible bacterium, followed by *Salmonella enterica* and *Escherichia coli*. Additionally, thyme exhibited great antifungal efficacy against *Candida albicans*. The acute toxicity results indicated that the aqueous extract of *T. vulgaris* is almost non-toxic when taken orally. According to the chronic toxicity study, the extract is generally safe when taken orally over an extended period of time. The biochemical and haematological characteristics of the serum and blood were within acceptable limits, and histological examination revealed no abnormalities. In conclusion, the findings of this investigation, confirm the antimicrobial efficacy of the aqueous extract of Moroccan *T. vulgaris* and its safety for experimental animals.

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1. INTRODUCTION

Since ancient times, natural products have been the main source of the most active ingredients in drugs and remedies (Mrabti *et al.*, 2018, Bouyahya *et al.*, 2020a; Bouyahya *et al*., 2021a; Bouyahya *et al.*, 2021b; Bouyahya *et al.,* 2021c). Despite the dominance of synthetic chemical drugs in the current modern era, natural products still receive great attention from the scientific community and the population. About 80% of the World's inhabitants rely on these products for primary healthcare, and almost half of the new medications approved since 1994 are based on natural products (Harvey, 2008). Recently, a new concept has emerged and is widely spread all over the globe, which is "functional food" or nutraceuticals. These are foods rich in nutrients and associated with a number of powerful health benefits, such as berries, some vegetables, green tea, fruits, nuts, and many more (Gupta & Mishra, 2020).

Antimicrobial resistance, according to the World Health Organization, is a worldwide public health concern that must be addressed with the greatest seriousness (Mendelson & Matsoso, 2015). The issue is exacerbated by the rise of emerging infectious diseases, with 335 new infectious diseases identified between 1940 and 2004 (Jones *et al*., 2008). Antibiotic abuse hastened the emergence of antibiotic-resistant bacteria and antibiotic resistant genes, significantly lowering their therapeutic value against human and animal infections (Bouyahya, *et al.*, 2017c; Qiao *et al.*, 2018). So, there is a real need for new antimicrobial drugs, and medicinal plants could be a good source for novel antimicrobial agents (Abdallah, 2011; Bouyahya *et al.*, 2017b; Bouyahya *et al.*, 2019). Secondary metabolites (phytochemical compounds) are produced by plants that are not necessary for regular growth or development but are vital for reproduction and defense mechanisms against herbivors, fungi, bacteria and viruses, among others. These substances include terpenoids, flavonoids, phenolic acids, and other groups and have a high potential for acting as medicines (Anand *et al.*, 2019; Bouyahya, *et al.*, 2020a; Chamkhi *et al.*, 2021, 2022; Salehi *et al.*, 2021; Sharifi-Rad *et al.*, 2021). Extensive studies have shown that numerous medicinal plants possess extraordinary antimicrobial, antioxidant, anticancer, and anti-inflammatory properties (Noumi *et al.*, 2020; Reddy *et al.*, 2020; Hajlaoui *et al.*, 2021; Khalfaoui *et al.*, 2021; Al Kaabi *et al.*, 2022), and large drug companies should investigate the prospect of using these plants in the development of novel antimicrobials (Cowan, 1999). Scientific research has proved the effectiveness of certain plants' extracts against a variety of microbes and their activities were competitive to antibiotics, including curry leaf plant (*Murraya koenigii*, Rutaceae) (Joshi *et al.*, 2018; Abuga *et al.*, 2020), ginger (*Zingiber officinale*, Zingiberaceae) (Zare-Shehneh *et al.*, 2014; Chakotiya *et al.*, 2017), coriander (*Coriandrum sativum*, Apiaceae) (Matasyoh *et al.*, 2009; Zare-Shehneh *et al.*, 2014), rosemary (*Salvia rosmarinus*, Lamiaceae) (Miraj & Kiani, 2016; Pieracci *et al.*, 2021) and black cumin (*Nigella sativa*, Ranunculaceae) (Salman *et al.*, 2008; Randhawa *et al.*, 2017).

Thymus vulgaris L. known as thyme, belongs to the Lamiaceae family. It is a perennial short-shrub (up to 25 cm high), with tiny oval to oblong leaves (6-12 mm) (Escobar *et al.*, 2020). This herb is a famous functional food, known since ancient civilizations, indigenous to the Mediterranean countries and Northern Africa (Oliviero *et al.*, 2016). It is a popular food in Morocco. Indeed, it has been used as a flavoring ingredient, cooking plant, and herbal medicine for ages (Mustafa *et al.*, 2020). *T. vulgaris* has a long history of usage in traditional medicine to cure a variety of ailments, such as respiratory disorders, toothaches, urinary tract diseases, gastrointestinal disorders, and microbial infections (Abdallah, 2016). In the last decades, various pharmacological investigations carried out on thyme revealed interesting biological activities. Some studies reported that it has antioxidant, anti-inflammatory, antiviral, insecticidal, and antibacterial properties (Prasanth *et al.*, 2014). *T. vulgaris* is a typical aromatic plant. Its essential oils reveale that it contains up to 47 compounds. Thymol, *p*-cymene, and carvacrol are the major constituents, together comprising about 79.2% of the essential oil

constituents (Rota *et al.*, 2008). Morocco is rich in diverse flora and herbs grown in this area are an integral part of Moroccan traditions and culture (Eddouks *et al.*, 2002a; Mrabti *et al.*, 2021). However, there is still a lot of this herbal wealth that needs scientific verification. Besides, the chemical composition of thyme and, subsequently, its biological activities might vary greatly from one region to another, and it is critical to figure out what the true composition of wild plants is in different localities (Guillén & Manzanos, 1998). Therefore, we have proposed in this study to evaluate the mineral contents, Acute and chronic toxicity of the aqueous extract of Moroccan thyme, besides the antimicrobial properties.

2. MATERIAL and METHODS

2.1. Plant Material Collection and Extraction

The aerial parts of *T. vulgaris* were collected from north-eastern Morocco, about 30 km away from Taza (Morocco) in March 2021. The plant material was authenticated by a botanist in the laboratory of botany at the Scientific Institute of Rabat/Morocco, and a voucher number, RAB-1201, was deposited in the herbarium for future reference. Plant material was dried in the shade at room temperature, powdered to achieve a mean particle size, and kept in the dark until future use. The powder was extracted by infusion (for toxicological assessment) and maceration with 80% methanol (for antimicrobial profile). Briefly, 50 g of plant powders were infused in 500 mL of distilled water for a period of 30 min, then filtered and evaporated under vacuum at 50 °C using a rotary evaporator. The recovered extract was frozen and lyophilized to remove all traces of water.

2.2. Minerals Content

The mineral content of *T. vulgaris* was determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES). Tests were carried out on *T. vulgaris* powder using the method we used before (Zaazaa *et al.*, 2021).

2.3. Well-Diffusion Assay

The antibacterial activity of *T. vulgaris* was determined using an agar well-diffusion method, which was modified somewhat from a previously reported assay (Abdallah *et al.*, 2021) with minor modifications. 25 mL of an autoclaved molten nutrient agar (Oxoid Ltd, Ireland) for bacteria or Sabouraud dextrose agar (Thermo Fisher Scientific, USA) for yeast was poured in sterile plates (90 mm diameter) and allowed to solidify. Microorganisms were distributed over the appropriate agar plate (adjusted to 0.5 McFarland). Using a sterilized cork borer. On the inoculated plates, three wells (6 mm in diameter) were bored. 100 µL of thyme methanolic extract was added to these wells, except for one well in each plate that was filled with 100 µL of the reference antibiotic (2.5 mg/mL chloramphenicol for bacteria or 5 mg/mL clotrimazole for yeast). After 24 hours of incubation at 35 ^{o}C, all plates were inspected for inhibition zones, which were measured in millimeters (mm). The test was repeated three times and the mean was calculated.

2.4. Minimum Inhibitory Concentration Assay (MIC)

Microorganisms which showed sensitivity in the well-diffusion assay were subjected to MIC test, using the micro-dilution method (Gulluce *et al.*, 2006) with slight modifications. Briefly, tested microorganisms were sub-cultured for up to 12 hours, and inoculum was taken and adjusted to (0.5 McFarland standard). Thyme extract was diluted in two-fold serial dilution to get a concentration range from 3.9 to 250 mg/mL in nutrient broth for bacteria or Sabouraud dextrosebroth for yeast. The 96-well plates were prepared by loading 95 µL of double-strength nutrient broth and 5 µL of inoculum. The first well was filled with 100 µL of extract (250 mg/mL) and then, from their successive dilutions, 100 µL was put into seven wells in a row. As a negative control, the last well was filled with 195 µL of nutrient broth without extract. The

prepared micro-wells were loaded with 5 µL of the inoculum in the appropriate medium, according to the type of microbe. Plates were covered with sterile plate sealer (Bio-Rad Laboratories Inc. USA) and incubated overnight at 35 °C with frequent gentle shaking. Determination of microbial growth in the 96-well plates containing the formulated media was performed using a microplate reader (Bio-Rad Laboratories Inc. USA) at 595 nm. The test was repeated three times to pinpoint the MIC value accurately.

2.5. Minimum Bactericidal/Fungicidal Concentration Assay

The minimum bactericidal concentration (MBC) for bacteria and minimum fungicidal concentration (MFC) for yeast were determined after the MIC test (Carolina et al., 2013). In brief, 50 µL was pipetted from each MIC tube and spotted over plates containing nutrient agar for bacteria or Sabouraud dextrose agar for yeast, then incubated at 35 °C for 24 hours. After incubation, the plates were checked for microbial growth. MBC was defined as the lowest MIC that showed no observable growth. The MBC/MIC was also determined to be bacteriostatic or bactericidal.

2.6. Experimental Animals

Adult Swiss albino mice and Wistar rats were used for acute treatment and ninety-day toxicity studies, respectively. Animals were obtained from the animal colony of the Department of Pharmacology and Toxicology of the Faculty of Medicine and Pharmacy, Mohamed V University (Rabat-Morocco). Animals were kept in standard animal house conditions including a 12 h light/dark cycle and a room temperature of 20–22 °C. All experimental procedures were performed based on the guidelines of the Ethical Committee for the care and use of laboratory animals.

2.7. Acute Oral Toxicity Study

The acute toxicity of *T. vulgaris* extract was evaluated according to OECD standards. Organization for Economic Cooperation and Development (OECD) 423 (OECD, 2001). A total of 60 mice weighing 25-35 g were randomly divided into six experimental groups of 10 mice each (6 males and 6 females per group). After overnight fasting, an aqueous extract (dissolved in aqueous solution) was administered by oral gavage as a single dose of 500, 1000, 2000 and 5000 mg / kg body weight, respectively. After treatment, the animals were observed for 14 days while they had unrestricted access to water and food.

2.8. Chronic Toxicity Study

The chronic toxicity study was performed according to OECD Test Guidelines 408 for 90 days with certain modifications (El Kabbaoui *et al.*, 2017). A total of 48 male and female Wister rats weighing between 190 and 250 g were randomly divided into four groups (n = 6 males and 6 females/group). After conceiving the animals in 4 groups of 6 rats each, the treated group received daily gastric gavage at doses (500, 700, and 1000 mg/kg) of the aqueous extract tested at the time when the control group received physiological solution (vehicle) for 90 days. During the experimental period, the body weights of all groups were measured once a week. Animals were visually observed for mortality, behavioral changes, physical appearance changes, and signs of illness.

2.9. Hematology and Serum Biochemistry

For the hematological investigation, all animals were fasted overnight but were allowed access to water ad libitum. Rats were then anesthetized and blood samples were taken from the abdominal aorta. Whole blood was collected in EDTA tubes and processed immediately for hematologic analysis. The parameters measured were red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), white blood cell count (WBC),

neutrophils (NEU), eosinophils (EOS), basophils (BASO), lymphocytes (LYM), and monocytes (MONO), platelet count (PLT). The hematological analysis was performed using an automatic hematological analyzer (Sysmex XN-550). Biochemical tests are done by using dry tubes that have been drained of blood. They were centrifuged at 3000 rpm at 4 °C for 10 minutes to get the serum, which was used to figure out the total serum protein (Anand et al., 2019), the total bilirubin (T-BIL), the alkaline phosphatase (Major, 1954), the ALT, and the AST, as well as uric acid (URIC), urea, creatinine, total cholesterol (TC), triglycerides (TG), and glucose (GLU) using an auto-analyzer called the "Cobas c 502."

2.10. Histopathology

Rats were sacrificed by sodium pentobarbital overdose. Liver, kidney, and spleen samples were obtained immediately and fixed in 10% phosphate buffered formalin for further examination. Tissue samples were cut into 2–3 mm thick slices with a surgical blade. The samples were processed using a tissue processor and embedded in paraffin. Paraffin blocks were cut using a microtome cutter and stained with hematoxylin and eosin before observation under light microscopy.

2.11. Statistical Analysis

Data was expressed as the mean ± standard deviation (Mann *et al.*, 2000). Statistical significance between control and treated groups was determined by one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. GraphPad Prism version 6.0 for Windows was used for statistical analysis. Data analyses from male and female groups were done separately, and the differences were considered statistically significant at $p<0.05$.

3. RESULTS

3.1. Mineral Composition

As shown in Table 1, the mineral contents of the edible aerial parts of Moroccan thyme have some important minerals including macro-elements (Ca, P, K, Mg, and Na), the microelements (S, Co, Fe, B, Cu, Zn, Mn, and V). The result of the analyses was established to give nutrient values per mg/kg of dried weight. According to our results, the contents of Calcium (Ca) and Potassium (K) were very high, with a concentration of 13 574.51 mg/kg and 10764.47mg/kg respectively, while elements such as Cobalt (Co) were the least abundant microelements with a concentration of 0.001 mg/kg.

Table 1. Levels of some mineral contents in the aerial parts of *T. vulgaris.*

Mineral	Content in mg/kg dw
Macro-elements	
K	10764.47
Mg	2476.26
Na	509.85
Ca	13574.51
P	1078.73
Micro-elements	
B	26.83
Cu	31.59
S	89.190
Mn	36.23
Fe	419.26
Zn	42.08
Co	0.001
Cr	1.224
V	5.881

3.2. Antimicrobial Activity

The antimicrobial activity of *T. vulgaris* was screened by the agar well-diffusion assay. The methanolic extract of *T. vulgaris* exhibited noticeable antimicrobial activity against all tested microorganisms (Figure 1). The statistical analysis revealed that Thyme has significant antimicrobial activity against all tested microorganisms at (P 0.05, one-way ANOVA), as shown in (Table 2). For antibacterial activity, *Staphylococcus aureus* was found to be the most sensitive (32.5±07 mm), *Salmonella enterica* came next (18.5±0.7 mm), followed by *Escherichia coli* (13.5±0.7 mm). Some bacteria were comparable in sensitivity against the extract to the antibiotic, such as *Staphylococcus aureus* (Figure 1 and Table 2). For antifungal activity, *Candida albicans* exhibited significant sensitivity to the extract (25.5±0.7 mm) and was comparable to the antibiotic used. The MIC and MBC/MFC results are shown in (Table 3). Bacteriostatic or bactericidal activity was assessed by MBC/MIC. When the MBC/MIC or MFC/MIC ratio is less than or equal to 4, the extract is considered bactericidal/fungicidal, whereas if the ratio is higher than 4, the extract is bacteriostatic/fungistatic (Abdallah, 2016). Accordingly, methanol extract of thyme was highly fungicidal to *Candida albicans* (at <3.9 mg/mL), followed by other bacteria, where it was highly bactericidal to *Staphylococcus aureus* (15.6 mg/mL) and *Escherichia coli* (31.2 mg/mL), and to a lesser degree with *Salmonella enterica* (7.8 mg/mL).

Figure 1. The inhibition zone (mm) of *T. vulgaris* against tested microorganisms. (Ext.) represent the methanolic crude extract (500 mg/ml), (Chl.) chloramphenicol (2.5 mg/ml), (Clo.) clotrimazole (5 mg/ml).

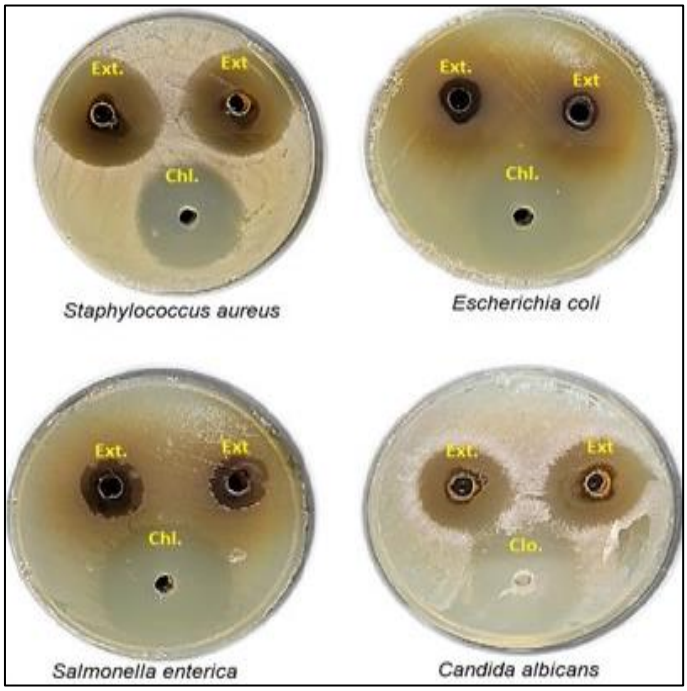


Table 2. Antimicrobial activity of methanol extract of thyme against tested microorganisms

Tested microorganisms	Mean inhibition zone diameter (mm)		
	T. vulgaris (500 mg/mL)	Chloramphenicol (2.5 mg/mL)	Clotrimazole (5 mg/mL)
Staphylococcus aureus	32.5±0.7[a]	32.5±0.7[a]	-
Salmonella enterica	18.5±0.7[b]	27.5±0.7[b]	-
Escherichia coli	13.5±0.7[c]	24.5±0.7[c]	-
Candida albicans	25.5±0.7[b]	-	31.5±2.1[a]

*Mean±Standard deviation, Different letters (a-c) indicate significantly differences (p<0.05).

Table 3. MIC, MBC and MBC/MIC rations of thyme methanol extract against tested microorganisms.

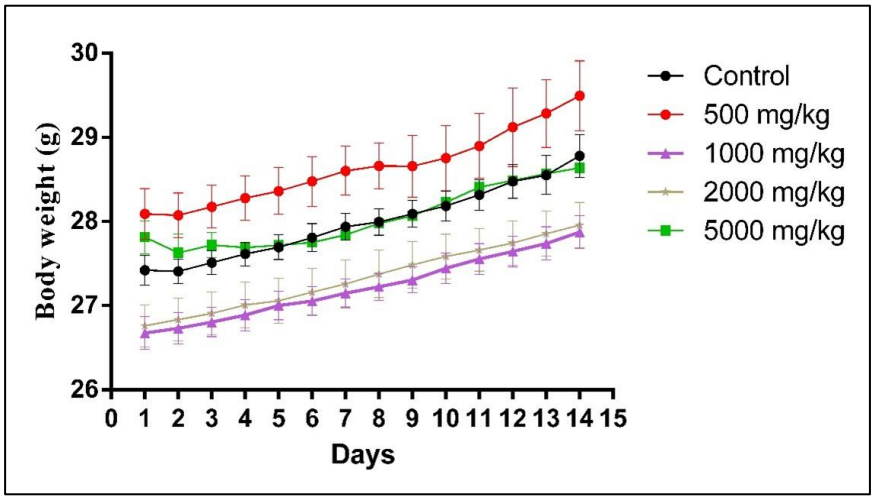
Microorganisms	Methanol extract of thyme (mg/mL)		
	MIC	MBC or MFC*	MBC/MIC
Staphylococcus aureus	15.6	15.6	1.0
Salmonella enterica	7.8	31.2	4.0
Escherichia coli	31.2	31.2	1
Candida albicans	< 3.9	< 3.9	0

*MFC test for the yeast *Candida albicans*, MBC for other tested bacteria.

3.3. Acute Toxicity Study

Regarding acute toxicity evaluation of thyme aqueous extract, administration of different 500 mg/kg,1000 mg/kg, 2000 mg/kg and 5000 mg/kg doses of aqueous extracts of the aerial parts of *T. vulgaris* did not show any signs of toxicity like restlessness, motor activity, breathing, and diarrhea, etc. There was no difference in body weight gained compared to the witness. As it is shown in (Figure 2), in other words, all mice survived for the first 24 h and up to 14 days of cage side follow-up. This implies that, the median lethal dose (LD_{50}) of the extract causing 50% of the deaths of the animals is said to be greater than 5000 mg/kg, suggesting a good safety margin.

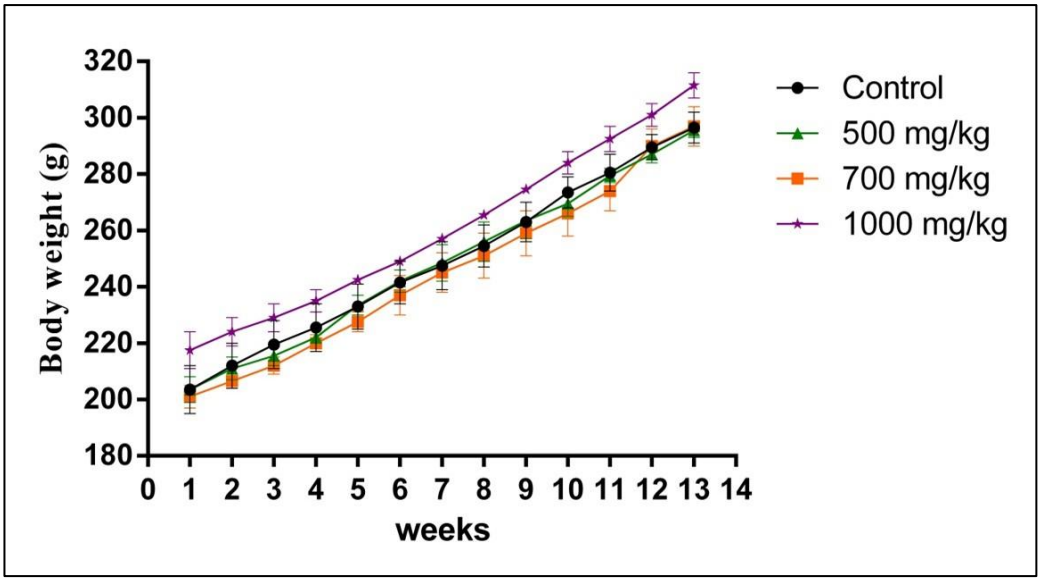
Figure 2. Acute toxicity of *T. vulgaris* aqueous extract.



3.4. Sub-Chronic Toxicity Study

Since body weight is the best indicator of good health and efficient metabolic homeostasis. Throughout the study period, we found no signs of toxicity or deaths of animals at any dose up to the maximum of 1000 mg/kg during the 13 weeks of repeated oral treatment. No statistically significant difference in body weight was noted between the control group and the treated group, either females or males. As it is shown in (Figure 3), which allows us to deduce the absence of signs of toxicity in rats treated with the aqueous extract of the plant studied and the tolerance of this extract in rats.

Figure 3. Chronic toxicity of *T. vulgaris* aqueous extract.



3.5. Hematology Analysis

The hematological parameters of male and female rats after 90 days of treatment with aqueous extract of *T. vulgaris* are shown in (Tables 4 and 5). The effect of sub-chronic oral administration of different doses of 500, 700 and 1000 mg/kg of extract showed no significant difference from the control group. All hematological parameters were within normal limits.

Table 4. Hematological parameters of male's rats after 90 days of treatment with *T. vulgaris* extract.

Parameters	Control	*T. vulgaris* (mg/kg B.W)		
		500	700	1000
WBC (10^3/mm^3)	9.41 ± 0.60	9.24 ± 0.81	9.92 ± 0.63	9.19 ± 0.92
RBC (10^6/mm^3)	7.53 ± 0.29	7.96 ± 0.25	8.19 ± 0.62	8.26 ± 0.45
HCT (%)	38.92 ± 2.02	42.38 ± 4.39	42.56 ± 5.43	39.71 ± 2.29
HGB (g/dL)	13.64 ± 0.69	13.74 ± 0.79	13.16 ± 0.89	14.21 ± 0.54
MCV (µm^3/red cell)	58.02 ± 2.04	57.85 ± 2.22	57.71 ± 3.72	56.37 ± 2.28
MCH (pg/ red cell)	17.07 ± 0.12	17.27 ± 1.03	16.91 ± 0.05	16.83 ± 0.02
MCHC (g/dL)	33.24 ± 1.02	32.82 ± 0.01	33.07 ± 0.09	33.13 ± 0.01
PLT (10^3 cells/mm^3)	892.13 ± 107.11	884.72 ± 103.07	878.03 ± 105.06	863.03 ± 99.01
LYM (%)	73.11 ± 4.06	72.02 ± 4.04	71.57 ± 5.01	71.83 ± 4.21
NEU (%)	24.02 ± 2.09	24.50 ± 0.11	24.22 ± 1.03	24.87 ± 1.05
MONO (%)	3.48 ± 0.02	3.67 ± 0.06	3.35 ± 0.02	3.73 ± 0.04
BASO (%)	0.05 ± 0.01	0.05 ± 0.10	0.06 ± 0.02	0.06 ± 0.04
EOS (%)	1.57 ± 0.01	1.52 ± 0.04	1.49 ± 0.07	1.3 ± 0.03

Values are expressed as mean ± SD, n = 6.

Table 5. Hematological parameters of female rats after 90 days of treatment with *T. vulgaris* extract.

Parameters	Control	*T. vulgaris* (mg/kg B.W)		
		500	700	1000
WBC (10^3/mm^3)	8.46 ± 0.57	9.11 ± 0.02	9.21 ± 0.09	9.24 ± 0.03
RBC (10^6/mm^3)	8.32 ± 0.61	8.35 ± 0.04	8.56 ± 0.07	8.42 ± 0.06
HCT (%)	41.12 ± 0.05	39.15 ± 0.08	39.82 ± 0.02	40.16 ± 0.09
HGB (g/dL)	13.41 ± 0.01	13.37 ± 0.09	13.72 ± 0.08	13.42 ± 0.08
MCV (µm^3/red cell)	54.22 ± 0.06	55.33 ± 0.01	53.22 ± 0.09	53.45 ± 0.03
MCH (pg/ red cell)	16.71 ± 0.03	16.48 ± 0.09	16.57 ± 0.01	16.37 ± 0.05
MCHC (g/dL)	33.12 ± 2.04	32.87 ± 1.05	32.91 ± 0.72	33.08 ± 2.01
PLT (10^3 cells/mm^3)	871.37 ± 1.64	873.13 ± 1.53	901.75 ± 1.01	904.17 ± 0.08
LYM (%)	71.51 ± 0.04	70.63 ± 0.09	70.82 ± 0.07	71.51 ± 0.02
NEU (%)	23.13 ± 1.09	23.22 ± 0.02	23.81 ± 0.08	23.79 ± 1.08
MONO (%)	3.21 ± 0.02	3.14 ± 0.04	3.61 ± 0.05	3.47 ± 0.09
BASO (%)	0.01 ± 0.02	0.01 ± 0.04	0.02 ± 0.01	0.02± 0.03
EOS (%)	1.63 ± 0.08	1.65 ± 0.06	1.62 ± 0.07	1.60 ± 0.09

Values are expressed as mean ± SD, n = 6.

3.6. Clinical Biochemistry Analysis

Tables 6 and 7 summarize the levels or activities of biochemical parameters in male and female rats. All the tested doses of *T. vulgaris* extract did not induce a significant change in the concentration of ALT, AST, total bilirubin, total protein, albumin, creatinine, urea and uric acid. However, the extracts studied at doses of 700 and 1000 mg / kg caused a significant reduction in the level of total cholesterol, triglycerides, in both groups of rats compared to the control groups. Although *Thymus* extract induced various significant decreases (p<0.05 and p<0.01) in the fasting glucose levels of the treated groups of rats, which was labeled at the dose of 1000 mg / kg, (p<0.01).

Table 6. Biochemical parameters of male rats after 90 days of treatment with *T. vulgaris* extract.

Parameters	Control	*T. vulgaris* (mg/kg B.W)		
		500	700	1000
AST (UI/L)	145.72 ± 1.03	145.83 ± 1.04	146.15 ± 1.19	146.33 ± 1.02
ALT (UI/L)	56.32 ± 0.05	52.80 ± 0.03	49.97 ± 0.08	54.45 ± 0.03
ALP (UI/L)	185.07 ± 1.04	181.45 ± 1.03	179.22 ± 1.05	182.31 ± 1.02
Total bilirubin (mg/L)	0.49 ± 0.02	0.48 ± 0.07	0.51 ± 0.04	0.52 ± 0.01
Total proteins (g/L)	68.17 ± 1.01	69.25 ± 0.02	67.23 ± 0.08	67.85 ± 1.06
Total cholesterol (g/L)	0.64 ± 0.06	0.61 ± 0.03	0.52 ± 0.02*	0.51 ± 0.01**
Triglycerides (g/L) HDL	0.70 ± 0.03	0.62 ± 0.02*	0.58 ± 0.02**	0.58 ± 0.02**
Glucose (g/L)	1.09 ± 0.16	0.93 ± 0.03*	0.90 ± 0.05**	0.88 ± 0.05**
Urea (g/L)	0.35 ± 0.03	0.36 ± 0.04	0.36 ± 0.03	0.36 ± 0.05
Creatinine (mg/L)	4.17 ± 0.09	3.81 ± 0.02	4.12 ± 0.09	4.22 ± 0.02
Uric acid (mg/L)	11.25 ± 1.04	11.57 ± 1.33	11.83 ± 0.75	11.97 ± 1.02

Values are expressed as mean ± SD, n = 6. Compared to control group (one-way ANOVA followed by Dunnet's post-hoc test). $^*p<0.05$ compared to control group (one-way ANOVA followed by Dunnet's post-hoc test). $^{**}p<0.01$ compared to control group (one-way ANOVA followed by Dunnet's post-hoc test).

Table 7. Biochemical parameters of female rats after 90 days of treatment with *Thymus* extracts.

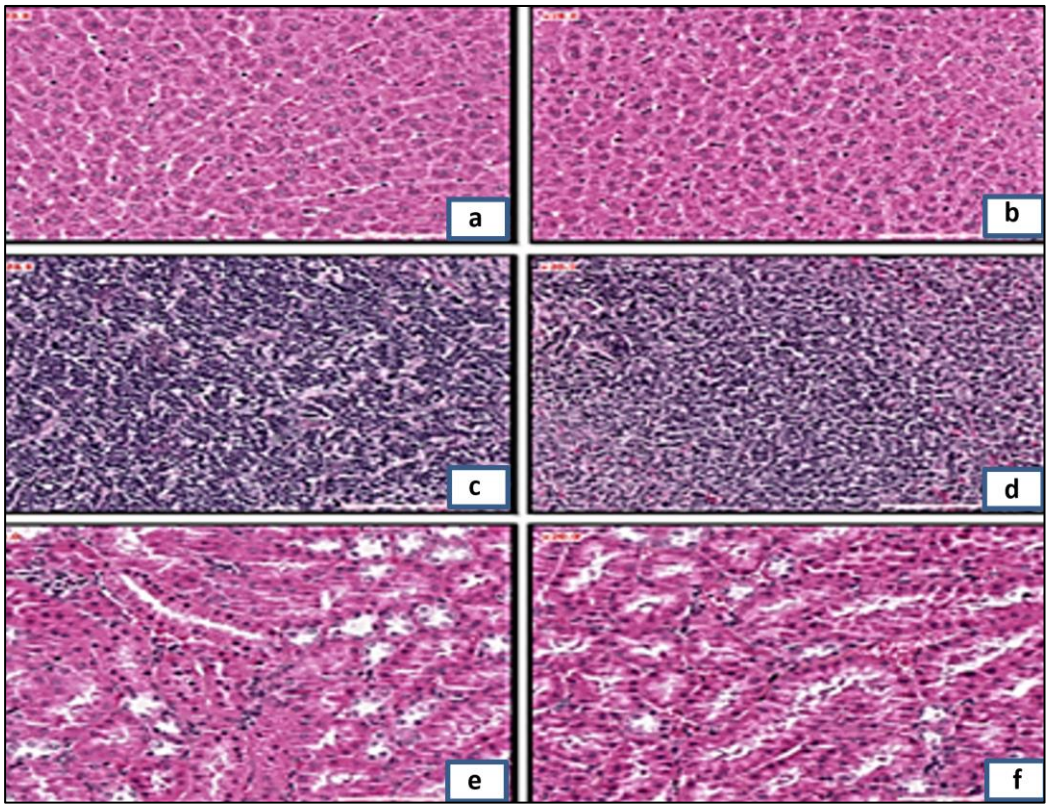
Parameters	Control	*T. vulgaris* (mg/kg B.W)		
		500	700	1000
AST (UI/L)	148.27 ± 1.01	148.83 ± 1.02	147.15 ± 1.03	148.33 ± 1.09
ALT (UI/L)	54.12 ± 0.03	53.80 ± 0.01	54.27 ± 0.02	54.15 ± 0.08
ALP (UI/L)	195.11 ± 0.04	191.18 ± 0.03	194.16± 0.04	192.21 ± 0.03
Total bilirubin (mg/L)	0.47 ± 0.14	0.47 ± 0.25	0.49 ± 0.21	0.48 ± 0.31
Total proteins (g/L)	67.05 ± 0.02	67.75 ± 0.01	68.33 ± 0.03	67.39 ± 0.04
Total cholesterol (g/L)	0.61 ± 0.06	0.53 ± 0.08	0.49 ± 0.04**	0.48 ± 0.04**
Triglycerides (g/L) HDL	0.69 ± 0.01	0.50 ± 0.01	0.47 ± 0.03**	0.43 ± 0.02**
Glucose (g/L)	0.99 ± 0.13	0.84 ± 0.04*	0.80 ± 0.06**	0.80 ± 0.05**
Urea (g/L)	0.36 ± 0.01	0.36 ± 0.02	0.35 ± 0.01	0.35 ± 0.03
Creatinine (mg/L)	3.87 ± 0.05	3.91 ± 0.01	4.01 ± 0.05	4.11 ± 0.03
Uric acid (mg/L)	12.78 ± 0.01	12.98 ± 0.03	13.03 ± 0.05	13.07 ± 0.02

Values are expressed as mean ± SD, n = 6. *$p<0.05$ (one-way ANOVA followed by Dunnet's post-hoc test). **$p<0.01$ (one-way ANOVA followed by Dunnet's post-hoc test).

3.7. Histological Analysis

Microscopic examination of liver, spleen, and kidney tissues was performed on rats that were either untreated (a, c, e) or treated with 1000 mg/kg of *T. vulgaris* aqueous extract (b, d, f, respectively). Demonstrate a normal morphology comparable to that of the control group, indicating that repeated oral administration of the aqueous extract of *T. vulgaris* for 13 weeks by gavage generated no detrimental modifications or morphological abnormalities (Figure 4).

Figure 4. Chronic toxicity (tissue histology). There no difference between treated and control group. On the right, a (1000 mg/kg), b (700 mg/kg) and c (500 mg/kg) represent treated groups. On the left, a (1000 mg/kg), b (700 mg/kg) and c (500 mg/kg) represent no treated groups.



4. DISCUSSION

Calcium is the most abundant mineral in this plant, owing to its role in the formation and strength of bones and teeth. Additionally, it is involved in a broad variety of biological processes, including muscular contraction and nerve impulse transmission (Vannucci *et al.*, 2018). Our results also suggested that Moroccan thyme was rich in macro- and micro-elements, which are necessary for health maintenance through supporting metabolism, energy generation, growth, and healing (Soni *et al.*, 2010). Our findings corroborated previous studies on the mineral content of *T. vulgaris*, as the Turkish variety of this plant also included highest concentrations of Ca, Fe, K, Mg, and S, when compared to thirty-two Turkish medicinal herbs (Özcan, 2004).

Other studies revealed also the richness of *Thymus* species in mineral compounds Indeed, according to study of Ouknin *et al.* (2018), it was revealed that *T. zygis* subsp. gracilis, *T. pallidus*, *T. willdenowii* containing numerous mineral compounds with certain variability depending on plant species and plant parts (Ouknin *et al.*, 2018). Moreover, other species belonging to *Thymus* genus such as *T. capitatus* and *T. broussonetii* containing also different mineral compounds. The variability of these components depends to different factors such as the genetic of specie, plant parts used, and climatic changes (Bouyahya *et al.*, 2020c; Naceiri Mrabti *et al.*, 2021).

Additionally, it was found that species belonging to the genus *Thymus* have a wide variety of chemical constituents; this diversity can be explained by both endogenous (plant varieties, organ tested and vegetative state) and exogenous factors such as soil characteristics, seasons and climatic features (Mseddi *et al.*, 2020).

The findings of this study also revealed that *T. vulgaris* showed good antimicrobial activity against all tested microorganisms to varied degrees using the well diffusion method. The well-diffusion assay is a simple and effective method to determine the possible presence of antimicrobial agents in tested plant extracts (Savaroğlu *et al.*, 2011). Previous studies have been done on thyme grown in various regions worldwide and showed antimicrobial potential of *T. vulgarus* (Al-Bayati, 2008; Jafari *et al.*, 2020).

Indeed, it was reported that thyme essential oils showed various degrees of antibacterial activity, but higher antifungal activity was recorded against *C. albicans*. Tural & Turhan (2017) mentioned that thyme essential oils have remarkable antibacterial effects, against *S. aureus*, *E. coli* and *L. monocytogenes*. Moreover, the current study exhibited that, based on the MBC/MIC values, *T. vulgaris* has potent fungicidal (against *C. albicans*) and bactericidal effects especially against *S. aureus* and *E. coli*. Previous published studies confirmed the thyme essential oils recorded remarkable MIC values, which reflect its effectiveness as a potent antimicrobial against different microorganisms *(*Borugă *et al.*, 2014; Fournomiti *et al.*, 2015; Imelouane *et al.*, 2011). Therefore, the aqueous extract of thyme has an effective antimicrobial properties. There is a robust relationship between the phytochemical structures of thyme and its antimicrobial activity, which could be related to terpenes present in thyme, and this compound is thought to cause rupture of the microbial membrane by a lipophilic mechanism (Imelouane *et al.*, 2011).

T. vulgaris, on the other hand, is regularly consumed by Moroccans with no obvious toxic effects documented (Eddouks *et al.*, 2002b). The scientific assessment of this reputation needs an examination of the plant's long- or medium-term effect on the organism. To the best of our knowledge, no study assessing the safety of the aqueous extract of *T. vulgaris* has been conducted in Morocco. As a result, the oral route was selected for the acute toxicity investigation since it is the most often utilized route in people under normal settings. The results obtained indicated that there were no indicators of toxicity and no death in mice during a 14-

day period with any of the dosages examined, indicating that its usage is safe and without harmful consequences.

Concerning chronic toxicity, the male and female rats administered with the aqueous extract of *T. vulgaris* exhibited a normal body weight evolution, indicating the lack of toxicity. Hematological examination revealed no significant changes in the parameters measured in rats treated with *T. vulgaris* aqueous extract. The aqueous extract also did not influence biochemical parameters at any of the dosages studied. However, this extract resulted in a considerable decrease in total cholesterol and triglycerides.

In agreement with our results, another study found that supplementing drinking water with thyme extract reduces plasma triglycerides and total cholesterol (Abdulkarimi *et al.*, 2011). Thus, this extract resulted in a considerable drop in blood glucose levels throughout therapy, indicating that it is suitable for use in the treatment of diabetes (Tuama, 2016). A similar study conducted in Peru showed no evidence of toxicity in acute and repeated 28-day oral dosage toxicity tests of *T. vulgaris* essential oil in rats (Rojas-Armas *et al.*, 2019).

During the experiment, oral administration of the aqueous extract of *T. vulgaris* caused no detrimental alterations or morphological abnormalities in the tissues examined and this was also in harmoney with previous studies (Benourad *et al.*, 2014). On the contrary, one study revealed that it had a healing impact on liver tissues when rabbits were exposed to methotrexate (MTX)-induced toxicity (Swayeh *et al.*, 2014).

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Declaration of Conflicting Interests and Ethics

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

Authorship contribution statement

Nidal Naceiri Mrabti: Resources, Software, Formal Analysis, and Writing-original draft original draft. **Hanae Naceiri Mrabti**: Resources, Software, Formal Analysis, and Writing-original draft original draft. **Latifa Doudach**: Resources, Software, Formal Analysis, and Writing-original draft original draft. **Zineb Khalil**: Resources, Software, Formal Analysis, and Writing-original draft original draft. **Mohamed Reda Kachmar**: Resources, Software, Formal Analysis, and Writing-original draft original draft. **Mouna Mekkaoui**: Resources, Software, Formal Analysis, and Writing-original draft original draft. **Ryan A. Sheikh**: Investigation, Visualization, and Editing. **Emad M. Abdallah**: Investigation, Visualization, and Editing. **Gokhan Zengin**: Investigation, Visualization, and Editing. **Samiah Hamad Al-Mijalli**: Investigation, Visualization, and Editing. **Abdelhakim Bouyahya**: Methodology, Supervision, and Validation. **Moulay El Abbes Faouzi**: Methodology, Supervision, and Validation. **Menana Elhallaoui**: Methodology, Supervision, and Validation.

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REFERENCES

Abdallah, E.M. (2011). Plants: An alternative source for antimicrobials. *Journal of Applied Pharmaceutical Science*, *1*(6), 16–20.

Abdallah, E.M. (2016). Antibacterial activity of *Hibiscus sabdariffa* L. calyces against hospital isolates of multidrug resistant Acinetobacter baumannii. Journal of Acute Disease, *5*(6), 512–516. https://doi.org/10.1016/j.joad.2016.08.024

Abdallah, E.M., Mujawah, A.A.H., & Al-Mijalli, S.H. (2021). GC-MS and Antibacterial potential of methanolic extract hyphaene Thebaica L. Fruit pulp against antibiotics-resistant pathogens. *Journal of Pure and Applied Microbiology*, *15*(3), 1655–1664. https://doi.org/10.22207/jpam.15.3.62

Abdulkarimi, R., Daneshyar, M., & Aghazadeh, A. (2011). Thyme (*Thymus vulgaris*) extract consumption darkens liver, lowers blood cholesterol, proportional liver and abdominal fat weights
in broiler chickens. *Italian Journal of Animal Science*, *10*(2), e20. https://doi.org/10.4081/ijas.2011.e20

Abuga, I., Sulaiman, S.F., Wahab, R.A., Ooi, K.L., & Rasad, M.S.B.A. (2020). In vitro antibacterial effect of the leaf extract of *Murraya koenigii* on cell membrane destruction against pathogenic bacteria and phenolic compounds identification. *European Journal of Integrative Medicine*, *33*, 101010.

Al Kaabi, S.K.S., Senthilkumar, A., Kizhakkayil, J., Alyafei, M.A.M., Kurup, S.S., Al Dhaheri, A.S., & Jaleel, A. (2022). Anticancer activity of *Moringa peregrina* (Forssk.) Fiori.: A Native plant in traditional herbal medicine of the United Arab Emirates. *Horticulturae*, *8*(1), 37.

Al-Bayati, F.A. (2008). Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts. *Journal of Ethnopharmacology*, *116*(3), 403–406.

Anand, U., Jacobo-Herrera, N., Altemimi, A., & Lakhssassi, N. (2019). A comprehensive review on medicinal plants as antimicrobial therapeutics: Potential avenues of biocompatible drug discovery. *Metabolites*, *9*(11), 258.

Benourad, F., Kahvecioglu, Z., Youcef-Benkada, M., & Colet, J.-M. (2014). Prospective evaluation of potential toxicity of repeated doses of *Thymus vulgaris* L. extracts in rats by means of clinical chemistry, histopathology and NMR-based metabonomic approach. *Drug Testing and Analysis*, *6*(10), 1069–1075.

Borugă, O., Jianu, C., Mişcă, C., Goleţ, I., Gruia, A.T., & Horhat, F.G. (2014). *Thymus vulgaris* essential oil: Chemical composition and antimicrobial activity. *Journal of Medicine and Life*, *7*(Spec Iss 3), 56–60.

Bouyahya, A., Dakka, N., Et-Touys, A., Abrini, J., & Bakri, Y. (2017a). Medicinal plant products targeting quorum sensing for combating bacterial infections. *Asian Pacific Journal of Tropical Medicine*, *10*(8), 729–743. https://doi.org/10.1016/j.apjtm.2017.07.021

Bouyahya, A., Et-Touys, A., Bakri, Y., Talbaui, A., Fellah, H., Abrini, J., & Dakka, N. (2017b). Chemical composition of *Mentha pulegium* and *Rosmarinus officinalis* essential oils and their antileishmanial, antibacterial and antioxidant activities. *Microbial Pathogenesis*, *111*, 41–49.

Bouyahya, A., Dakka, N., Talbaoui, A., Et-Touys, A., El-Boury, H., Abrini, J., & Bakri, Y. (2017c). Correlation between phenological changes, chemical composition and biological activities of the essential oil from Moroccan endemic Oregano (*Origanum compactum* Benth). *Industrial Crops and Products*, *108*, 729-737. https://doi.org/10.1016/j.indcrop.2017.07.033

Bouyahya, A., Abrini, J., Dakka, N., & Bakri, Y. (2019). Essential oils of *Origanum compactum* increase membrane permeability, disturb cell membrane integrity, and suppress quorum-sensing phenotype in bacteria. *Journal of Pharmaceutical Analysis*, *9*(5), 301-311. https://doi.org/10.1016/j.jpha.2019.03.001

Bouyahya, A., El Omari, N., Elmenyiy, N., Guaouguaou, F.-E., Balahbib, A., El-Shazly, M., & Chamkhi, I. (2020a). Ethnomedicinal use, phytochemistry, pharmacology, and toxicology of *Ajuga iva* (L.,) Schreb. *Journal of Ethnopharmacology*, *258*, 112875.

Bouyahya, A., Belmehdi, O., Benjouad, A., El Hassani, R. A., Amzazi, S., Dakka, N., & Bakri, Y. (2020b). Pharmacological properties and mechanism insights of Moroccan anticancer medicinal plants: What are the next steps? *Industrial Crops and Products*, *147*, 112198.

Bouyahya, A., Chamkhi, I., Guaouguaou, F.-E., Benali, T., Balahbib, A., El Omari, N., Taha, D., El-Shazly, M., & El Menyiy, N. (2020c). Ethnomedicinal use, phytochemistry, pharmacology, and food benefits of *Thymus capitatus. Journal of Ethnopharmacology*, *259*, 112925. https://doi.org/10.1016/j.jep.2020.112925

Bouyahya, A., El Menyiy, N., Oumeslakht, L., El Allam, A., Balahbib, A., Rauf, A., Muhammad, N., Kuznetsova, E., Derkho, M., & Thiruvengadam, M. (2021a). Preclinical and clinical antioxidant effects of natural compounds against oxidative stress-induced epigenetic instability in tumor cells. *Antioxidants*, *10*(10), 1553.

Bouyahya, A., El Omari, N., Elmenyiy, N., Guaouguaou, F.-E., Balahbib, A., Belmehdi, O., Salhi, N., Imtara, H., Mrabti, H. N., & El-Shazly, M. (2021b). Moroccan antidiabetic medicinal plants: Ethnobotanical studies, phytochemical bioactive compounds, preclinical investigations, toxicological validations and clinical evidences; challenges, guidance and perspectives for future management of diabetes worldwide. *Trends in Food Science & Technology*, *115*, 147–254.

Bouyahya, A., Guaouguaou, F.-E., El Omari, N., El Menyiy, N., Balahbib, A., El-Shazly, M., & Bakri, Y. (2021c). Anti-inflammatory and analgesic properties of moroccan medicinal plants: Phytochemistry, in vitro and in vivo investigations, mechanism insights, clinical evidences and perspectives. *Journal of Pharmaceutical Analysis*, *12*, 35–37.

Carolina, A., Santana, E.F. De, Marcos, A., Coutinho, F.N., Henrique, R., Castro, A. De, & Nelly, M. (2013). Which approach is more effective in the selection of drugs. *Evidence-Based Complementary and Alternative Medicine*, *2013*, 2005–2010.

Chakotiya, A.S., Tanwar, A., Narula, A., & Sharma, R. K. (2017). *Zingiber officinale*: Its antibacterial activity on Pseudomonas aeruginosa and mode of action evaluated by flow cytometry. *Microbial Pathogenesis*, *107*, 254–260.

Chamkhi, I., Benali, T., Aanniz, T., El Menyiy, N., Guaouguaou, F.-E., El Omari, N., El-Shazly, M., Zengin, G., & Bouyahya, A. (2021). Plant-microbial interaction: The mechanism and the application of microbial elicitor induced secondary metabolites biosynthesis in medicinal plants. *Plant Physiology and Biochemistry*, *167*, 269–295.

Chamkhi, I., Charfi, S., Hachlafi, N.E., Mechchate, H., Guaouguaou, F.-E., El Omari, N., Bakrim, S., Balahbib, A., Zengin, G., & Bouyahya, A. (2022). Genetic diversity, antimicrobial, nutritional, and phytochemical properties of *Chenopodium album:* A comprehensive review. *Food Research International*, *154*, 110979.

Cowan, M.M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, *12*(4), 564–582.

Eddouks, M., Maghrani, M., Lemhadri, A., Ouahidi, M.-L., & Jouad, H. (2002a). Ethnopharmacological survey of medicinal plants used for the treatment of diabetes mellitus, hypertension and cardiac diseases in the south-east region of Morocco (Tafilalet). *Journal of Ethnopharmacology*, *82*(2), 97–103. https://doi.org/10.1016/S0378-8741(02)00164-2

Eddouks, M., Maghrani, M., Lemhadri, A., Ouahidi, M.-L., & Jouad, H. (2002b). Ethnopharmacological survey of medicinal plants used for the treatment of diabetes mellitus, hypertension and cardiac diseases in the south-east region of Morocco (Tafilalet). *Journal of Ethnopharmacology*, *82*(2–3), 97–103.

El Kabbaoui, M., Chda, A., El-Akhal, J., Azdad, O., Mejrhit, N., Aarab, L., Bencheikh, R., & Tazi, A. (2017). Acute and sub-chronic toxicity studies of the aqueous extract from leaves of *Cistus ladaniferus* L. in mice and rats. *Journal of Ethnopharmacology*, *209*(April), 147–156. https://doi.org/10.1016/j.jep.2017.07.029

Escobar, A., Pérez, M., Romanelli, G., & Blustein, G. (2020). Thymol bioactivity: A review focusing on practical applications. *Arabian Journal of Chemistry*, *13*(12), 9243–9269. https://doi.org/10.1016/j.arabjc.2020.11.009

Fournomiti, M., Kimbaris, A., Mantzourani, I., Plessas, S., Theodoridou, I., Papaemmanouil, V., Kapsiotis, I., Panopoulou, M., Stavropoulou, E., Bezirtzoglou, E. E., & Alexopoulos, A. (2015). Antimicrobial activity of essential oils of cultivated oregano (*Origanum vulgare*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) against clinical isolates of *Escherichia coli* , *Klebsiella oxytoca*, and *Klebsiella pneumoniae*. *Microbial Ecology in Health & Disease*, *26*(0). https://doi.org/10.3402/mehd.v26.23289

Guillén, M.D., & Manzanos, M.J. (1998). Study of the composition of the different parts of a Spanish thymus vulgaris L. plant. *Food Chemistry*, *63*(3), 373-383. https://doi.org/10.1016/S0308-8146(98)00013-2

Gulluce, M., Aslan, A., Sokmen, M., Sahin, F., Adiguzel, A., Agar, G., & Sokmen, A. (2006). Screening the antioxidant and antimicrobial properties of the lichens *Parmelia saxatilis*, *Platismatia glauca*, *Ramalina pollinaria*, *Ramalina polymorpha* and *Umbilicaria nylanderiana*. *Phytomedicine*, *13*(7), 515-521. https://doi.org/10.1016/j.phymed.2005.09.008

Gupta, E., & Mishra, P. (2020). Functional Food with Some Health Benefits, So Called Superfood: A Review. *Current Nutrition & Food Science*, *17*(2), 144-166. https://doi.org/10.2174/1573401316999200717171048

Hajlaoui, H., Arraouadi, S., Noumi, E., Aouadi, K., Adnan, M., Khan, M.A., Kadri, A., & Snoussi, M. (2021). Antimicrobial, antioxidant, anti-acetylcholinesterase, antidiabetic, and pharmacokinetic properties of *Carum carvi* L. and *Coriandrum sativum* L. essential oils alone and in combination. *Molecules*, *26*(12), 3625.

Harvey, A.L. (2008). Natural products drug discovery. *Drug Discovery Today*, *13*(19–20), 894–901. https://doi.org/10.1016/j.drudis.2008.07.004

Imelouane, B., Amhamdi, H., Whathelet, J., Ankit, M., Khedid, K., & EL Bachiri, A. (2011). Chemical composition and antimicrobial activity of essential oil of *Thymus vulgaris* from Yemen. *Turkish Journal of Biochemistry*, *36*(4), 342–349.

Jafari, B., Fatemi, S., Pashazadeh, M., Al-Snafi, A.E., & Shariat, A. (2020). Antibacterial effects of *Thymus vulgaris*, *Mentha pulegium*, *Crocus sativus* and *Salvia officinalis* on pathogenic bacteria: A brief review study based on gram-positive and gram-negative bacteria. *Jorjani Biomedicine Journal*, *8*(3), 58–74.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, *451*(7181), 990–993.

Joshi, T., Jain, T., Mahar, R., Singh, S.K., Srivastava, P., Shukla, S.K., Mishra, D.K., Bhatta, R.S., Banerjee, D., & Kanojiya, S. (2018). Pyranocarbazoles from *Murraya koenigii* (L.) Spreng. As antimicrobial agents. *Natural Product Research*, *32*(4), 430–434.

Khalfaoui, A., Noumi, E., Belaabed, S., Aouadi, K., Lamjed, B., Adnan, M., Defant, A., Kadri, A., Snoussi, M., & Khan, M.A. (2021). LC-ESI/MS-Phytochemical Profiling with antioxidant, antibacterial, antifungal, antiviral and in silico pharmacological properties of Algerian *Asphodelus tenuifolius* (Cav.) organic extracts. *Antioxidants*, *10*(4), 628.

Major, R.H. (1954). A history of medicine. In *A history of medicine* (pp. 563–563).

Mann, C.M., Cox, S.D., & Markham, J.L. (2000). The outer membrane of *Pseudomonas aeruginosa* NCTC 6749 contributes to its tolerance to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Letters in Applied Microbiology*, *30*(4), 294–297.

Matasyoh, J.C., Maiyo, Z.C., Ngure, R.M., & Chepkorir, R. (2009). Chemical composition and antimicrobial activity of the essential oil of *Coriandrum sativum. Food Chemistry*, *113*(2), 526–529.

Mendelson, M., & Matsoso, M. P. (2015). The World Health Organization global action plan for antimicrobial resistance. *SAMJ: South African Medical Journal*, *105*(5), 325–325.

Miraj, S., & Kiani, S. (2016). A review study of therapeutic effects of *Salvia officinalis* L. *Der Pharmacia Lettre*, *8*(6), 299-303.

Mrabti, H.N., Bouyahya, A., Ed-Dra, A., Kachmar, M.R., Mrabti, N.N., Benali, T., Shariati, M.A., Ouahbi, A., Doudach, L., & Faouzi, M.E.A. (2021). Polyphenolic profile and biological properties of Arbutus unedo root extracts. *European Journal of Integrative Medicine*, *42*(September 2020), 101266. https://doi.org/10.1016/j.eujim.2020.101266

Mrabti, H.N., Jaradat, N., Fichtali, I., Ouedrhiri, W., Jodeh, S., Ayesh, S., Cherrah, Y., & Faouzi, M.E.A. (2018). Separation, identification, and antidiabetic activity of catechin isolated from *Arbutus unedo* L. Plant roots. *Plants*, *7*(2), 31. https://doi.org/10.3390/plants 7020031

Mseddi, K., Alimi, F., Noumi, E., Veettil, V.N., Deshpande, S., Adnan, M., Hamdi, A., Elkahoui, S., Alghamdi, A., & Kadri, A. (2020). *Thymus musilii* Velen. as a promising source of potent bioactive compounds with its pharmacological properties: In vitro and in silico analysis. *Arabian Journal of Chemistry*, *13*(8), 6782–6801.

Mustafa, S., Hina, S., Mahmood, S., Mueen-ud-din, G., & Alam, M.Q. (2020). Exploring phytochemical potential of nature' s bliss *Thymus vulgaris* L. Mini review. *International Journal of Botany Studies*, *5*(3), 493–496.

Naceiri Mrabti, H., Doudach, L., El Menyiy, N., Bourhia, M., Mohammad Salamatullah, A., Reda Kachmar, M., Belmehdi, O., El Moudden, H., Naceiri Mrabti, N., & Harhar, H. (2021). Phytochemistry and Pharmacology of *Thymus broussonetii* Boiss. *Evidence-Based Complementary and Alternative Medicine*, *2021*, Article ID 6350035, 15 pages

Noumi, E., Snoussi, M., Anouar, E.H., Alreshidi, M., Veettil, V.N., Elkahoui, S., Adnan, M., Patel, M., Kadri, A., & Aouadi, K. (2020). HR-LCMS-based metabolite profiling, antioxidant, and anticancer properties of *Teucrium polium* L. methanolic extract: Computational and in vitro study. *Antioxidants*, *9*(11), 1089.

OECD (2001). Acute Oral Toxicity – Fixed Dose Procedure (chptr). In *Guidelines for the Testing of Chemicals* (pp. 1–14).

Oliviero, M., Romilde, I., Beatrice, M.M., Matteo, V., Giovanna, N., Consuelo, A., Claudio, C., Giorgio, S., Maggi, F., & Massimo, N. (2016). Evaluations of thyme extract effects in human normal bronchial and tracheal epithelial cell lines and in human lung cancer cell line. *Chemico-Biological Interactions*, *256*, 125–133. https://doi.org/10.1016/j.cbi.2016.06.024

Ouknin, M., Romane, A., Arjouni, M.Y., & Majidi, L. (2018). Mineral composition, multivariate analysis of some oligo-elements and heavy metals in some species of genus *Thymus*. *Journal of Materials and Environmental Science*, *9*(3), 980–985.

Özcan, M. (2004). Mineral contents of some plants used as condiments in Turkey. *Food Chemistry*, *84*(3), 437–440.

Pieracci, Y., Ciccarelli, D., Giovanelli, S., Pistelli, L., Flamini, G., Cervelli, C., Mancianti, F., Nardoni, S., Bertelloni, F., & Ebani, V.V. (2021). Antimicrobial activity and composition of five *Rosmarinus* (Now *Salvia* spp. and varieties) essential oils. *Antibiotics*, *10*(9), 1090. https://doi.org/10.3390/antibiotics10091090

Prasanth, R., Kandisa, R.V., Varsha, P., & Satyam, S. (2014). Review on *Thymus vulgaris* traditional ues and pharmacological properties. *Medicinal & Aromatic Plants*, *03*(04), 3–5. https://doi.org/10.4172/2167-0412.1000167

Qiao, M., Ying, G.-G., Singer, A.C., & Zhu, Y.-G. (2018). Review of antibiotic resistance in China and its environment. *Environment International*, *110*, 160–172.

Randhawa, M.A., Alenazy, A.K., Alrowaili, M.G., & Basha, J. (2017). An active principle of *Nigella sativa* L., thymoquinone, showing significant antimicrobial activity against anaerobic bacteria. *Journal of Intercultural Ethnopharmacology*, *6*(1), 97-101. https://doi.org/10.5455/jice.20161018021238

Reddy, M.N., Adnan, M., Alreshidi, M.M., Saeed, M., & Patel, M. (2020). Evaluation of anticancer, antibacterial and antioxidant properties of a medicinally treasured fern *Tectaria coadunata* with its phytoconstituents analysis by HR-LCMS. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, *20*(15), 1845–1856.

Rojas-Armas, J., Arroyo-Acevedo, J., Ortiz-Sánchez, M., Palomino-Pacheco, M., Castro-Luna, A., Ramos-Cevallos, N., Justil-Guerrero, H., Hilario-Vargas, J., & Herrera-Calderón, O. (2019). Acute and repeated 28-day oral dose toxicity studies of *Thymus vulgaris* L. essential oil in rats. *Toxicological Research*, *35*(3), 225–232.

Rota, M.C., Herrera, A., Martínez, R.M., Sotomayor, J.A., & Jordán, M.J. (2008). Antimicrobial activity and chemical composition of Thymus vulgaris, Thymus zygis and Thymus hyemalis essential oils. *Food Control*, *19*(7), 681-687. https://doi.org/10.1016/j.foodcont.2007.07.007

Salehi, B., Quispe, C., Chamkhi, I., El Omari, N., Balahbib, A., Sharifi-Rad, J., Bouyahya, A., Akram, M, Iqbal, M., Docea, A.O., Caruntu, C., Leyva-Gómez, G., Dey, A, Martorell, M., Calina, D., López, V., & Les, F. (2021) Pharmacological Properties of Chalcones: A Review of Preclinical Including Molecular Mechanisms and Clinical Evidence. *Frontiers in Pharmacology*, *11*, 592654. https://doi.org/10.3389/fphar.2020.592654

Salman, M.T., Khan, R.A., & Shukla, I. (2008). Antimicrobial activity of *Nigella sativa* Linn. Seed oilagainst multi-drug resistant bacteria from clinical isolates. *Natural Product Radiance*, *7*(1), 10-14.

Savaroğlu, F., İşçen, C.F., Vatan, A.P., Kabadere, O., & Sİlhan, S. (2011). Determination of antimicrobial and antiproliferative activities of the aquatic moss *Fontinalis antipyretica* Hedw. *Turkish Journal of Biology*, *35*(3), 361–369.

Sharifi-Rad, J., Dey, A., Koirala, N., Shaheen, S., El Omari, N., Salehi, B., Goloshvili, T., Silva, N.C.C., Bouyahya, A., & Vitalini, S. (2021). *Cinnamomum* species: Bridging phytochemistry knowledge, pharmacological properties and toxicological safety for health benefits. *Frontiers in Pharmacology*, *11*(12), 1541. https://doi.org/10.3390/plants11121541

Soni, M.G., Thurmond, T.S., Miller III, E.R., Spriggs, T., Bendich, A., & Omaye, S.T. (2010). Safety of vitamins and minerals: Controversies and perspective. *Toxicological Sciences*, *118*(2), 348–355.

Swayeh, N.H., Abu-Raghif, A.R., Qasim, B.J., & Sahib, H.B. (2014). The protective effects of Thymus Vulgaris aqueous extract against Methotrexate-induced hepatic toxicity in rabbits. *International Journal of Pharmaceutical Sciences Review and Research*, *29*, 187–193.

Tuama, R.J. (2016). Effect of thyme (*Thymus vulgaris* L.) oil on some biochemical parameters of diabetic female rats. *World Journal of Pharmaceutical Research*, *4*(6), 320–325. https://doi.org/10.13140/RG.2.2.24589.49122

Tural, S., & Turhan, S. (2017). antimicrobial and antioxidant properties of thyme (*Thymus vulgaris* L.), Rosemary (*Rosmarinus officinalis* L.) and laurel *(Lauris nobilis* L.) essential oils and their mixtures. *The Journal of Food*, *42*(5), 588-596. https://doi.org/10.15237/gida.gd17030

Vannucci, L., Fossi, C., Quattrini, S., Guasti, L., Pampaloni, B., Gronchi, G., Giusti, F., Romagnoli, C., Cianferotti, L., Marcucci, G., & Brandi, M.L. (2018). Calcium Intake in bone health: A focus on calcium-rich mineral waters. *Nutrients*, *10*(12), 1-12. https://doi.org/10.3390/nu10121930

Zaazaa, L., Naceiri Mrabti, H., Ed-Dra, A., Bendahbia, K., Hami, H., Soulaymani, A., & Ibriz, M. (2021). Determination of Mineral Composition and Phenolic Content and Investigation of Antioxidant, Antidiabetic, and Antibacterial Activities of Crocus sativus L. Aqueous Stigmas Extracts. *Advances in Pharmacological and Pharmaceutical Sciences*, *2021*, Article ID 7533938, 7 pages. https://doi.org/10.1155/2021/7533938

Zare-Shehneh, M., Askarfarashah, M., Ebrahimi, L., Kor, N.M., Zare-Zardini, H., Soltaninejad, H., Hashemian, Z., & Jabinian, F. (2014). Biological activities of a new antimicrobial peptide from *Coriandrum sativum*. *International Journal of Biosciences*, *4*(6), 89–99.

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Review Article

Review on phytochemicals and biological activities of natural sweeteners *Stevia rebaudiana* Bertoni

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Abstract: Diabetes is a chronic metabolic disease that creates high blood sugar level. Therefore, diabetes awareness is necessary to prevent diabetes by reducing sugar intake and using low-calorie alternative sweeteners instead. *Stevia rebaudiana* is a medicinal plant species belonging to the Compositae family. It is a sweet herb that contains diterpene glycosides, which are directly responsible for the sweet taste, but they have no caloric value. Since ancient times, there have been several reports on the use of *S. rebaudiana* as an alternative sweetener and extended research has been conducted on its phytochemicals and biological activities. The plant contains a good number of phytochemicals with significant biological activities, namely polyphenolic derivatives, diterpenes glycosides, alkaloids, glycosides, tannins, chlorophylls, carotenoids, etc. For industrial use, those phytochemicals could be extracted from the selected plant and used for the preparation of nutraceuticals and food additives. *S. rebaudiana* is a natural herb; therefore, it has fewer or minimal adverse effects on human health. The selected plant in various forms is used for the treatment of diabetes, colon cancer, obesity, cavities, and others. However, the literature review shows that the information on this plant and its uses is not systematic. The purpose of the present review is to explore the status of phytochemicals and biological activities of the selected plant for young researchers. Therefore, the updated data will help them to develop new nutraceuticals and food additives that could help in the production of pharmaceuticals to treat different ailments.

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1. INTRODUCTION

Plants have been the primary source of safe medicine to cure various ailments. Traditionally, plants have been used to treat diseases since ancient times due to various biological and pharmacological activities (Zarawska, 1975; Kakkar & Bais, 2014; Rana & Bais, 2014). Since ancient times, herbs have been used as vital primary medicines to treat various ailments by different communities all over the world, including the Sultanate of Oman (Tandon *et al.,* 2004).

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Several evidence-based reports have been published on the clinical trials as well as quality control of herbal medicines (Steven & Ehrlich, 2009). The biological activities of more than 250,000 plant species have been well-discussed (Schoonhoven, 1982). Based on recent estimates, there are approximately 500 000 phytochemicals, but only 30 000 have properly been examined regarding their biological activities (Bemays & Chapman, 1994). Plant phytochemicals have a habitual interest in agriculture and pharmaceutical sectors because of the growth of organic products from plant sources in the United States (Isman, 1994).

The anticancer drug Taxol is manufactured from plants. The increasing aspiration by the communities for alternative medicine has added motivation for scientists to advance research in this field (Klayman, 1985; Phillipson & Wright, 1991; Beutler *et al.,* 1995).

The use of medicinal plants was first reported in Mesopotamia in about 2600 BC.; however, the therapeutic potential of medicinal plants was not emphasized in this record. In Ancient Egypt "Ebers Papyrus" dating from 1550 BC explained more than 800 natural remedies including the use of plants and their extracts, animal parts, and various minerals. The Assyrians, who governed Mesopotamia at the beginning of the first millennium BC, significantly merged antique knowledge about herbal remedies to treat diseases. From about 1100 BC, the Chinese Materia Medica was broadly explained and documented herbalists were fully focusing on cataloguing and documenting the therapeutic effects of plant compounds that they isolated from the plants. During that time, Shennong Bencao Jing (100-200 AD) in China accumulated and added more than 400 plant-based herbal drugs in Materia Medica of Dioscorides (Unschuld, 1988; Tang & Eisenbrand, 1992).

The genus *Stevia* Cav. is considered a sweet herb and it originated in Brazil and Paraguay. At the beginning of the sixteenth century, the people of Europe learned first that the leaves of Stevia are approximately 20-30 times sweeter than sugar. Since then dried leaves have been stored and used in raw form instead of synthetic sugar. The growth rate of the *Stevia* is slow and it does not disturb or increase the blood sugar levels in the living organisms. Several reports showed that *Stevia* extracts do not have any significant neurological or urological side effects (Tanaka *et al.,* 1997). Recently, based on its health benefits, *Stevia* is commercially produced in several countries including the United States, the Republic of China, Portugal, Paraguay, Brazil, Thailand, Poland, the Netherlands, India, and Korea, but Japan is the largest consumer of *Stevia* as an artificial sweetener in the World. Due to the importance of the selected plant, the purpose of this present review is to raise awareness of the status of phytochemicals and biological activities for young researchers.

1.1. Plant Description

S. rebaudiana is a flowering plant that belongs to the genus *Stevia* and Compositae family. It has several common names such as candy leaf, sweet leaf or sugar leaf (Katayama *et al.,* 1976; Goyal *et al.,* 2010; Misra *et al.,* 2011). It is one of the 154 members of the genus of *Stevia*, but only two species are sweet to taste. It is native to the border areas of Brazil and Paraguay. However, some countries gave more effort to produce *Stevia* for commercial purposes. Due to its sweetness, nowadays, it is commercially cultivated in various countries, namely Paraguay, Argentina, Germany, Republic of China, United Kingdom, Japan, Spain, Republic of South Korea, Canada, Australia, Mexico, Belgium, USA, Brazil, Israel, Malaysia, Indonesia, Taiwan, Thailand, Tanzania, and India. Now the leading *Stevia* producing country is China, which commercializes its processed products as artificial sweeteners. Currently, Japan is considered a major importer market for *Stevia*. The plant grows well similar to other vegetable crops and normally it grows well in subtropical countries. Due to its importance, *Stevia* has become cash crop in many countries and its cultivation is closely monitored by Ministry of Agriculture while measuring various parameters such as height, leaf weight, growth, insects as well as the content of sweeteners in the plant. The plantation of *Stevia* is from February to March each year and

the plant is harvested in June or July. It is a flowering plant and the flowers become within 54-100 days (Goyal *et al.,* 2010; Misra *et al.,* 2011). The concentration of sweetening ingredients in this plant is known to increase with the maturity of the plant.

1.2. Morphology

S. rebaudiana is a medicinal plant belonging to the Compositae family. The plant is a small perennial tropical shrub height up to 30 cm (Madan *et al.,* 2010, Figure 1). A mature plant could reach a maximum of 80 cm in height with woody stems. The leaves are oppositely arranged and the surfaces of the leaves are slightly glandular and have two discrete sizes. The leaves are oval in shape and the size of the leaves varies with environmental conditions such as types of soil, irrigation process, sunlight, air quality, etc (Madan *et al.,* 2010). The leaves have a sweet taste that remains in the mouth for at least an hour.

Figure 1. Different parts of *S. rebaudiana*

Plant **Seeds** **Rhizome**

The plant has spirally arranged small white tubular flowers. The shape of the fruit is oval and there is one seed in the seed coat. The seeds are about 3 mm long (Figure 1). The rhizome of this plant itself is slightly branching into roots (Figure 1). The selected plant grows well if the weather conditions are about 80% humidity and the temperature is within the range of 20-29°C (Carneiro, 1930; Robinson, 1930; Brandle *et al.,* 1998; Meireles *et al.,* 2006).

1.3. Taxonomy of the *Stevia* Genus

The genus *Stevia* contains more than 240 species. Among the Gulf countries, only Iran and Iraq have a few endemic species, but they are not sweet. The climate in most parts of Oman is not suitable to grow this plant, as an experiment, the selected plant was planted on Jabal Al Akhdar and the plant is still surviving. Therefore, the mountains in Oman can be used for growing the selected plant species. Among the species, humans used only forteen species of *S. rebaudiana* for artificial sugar (Meireles *et al.,* 2006). The taxonomy and the most common species of *Stevia* are presented in Table 1 and Table 2.

Table 1. Taxonomy and species of *Stevia*

Kingdom	Plantae
Subkingdom	Viridiplantae - green plants
Infrakingdom	Streptophyta - land plants
Superdivision	Embryophyta
Division	Vascular plants
Subdivision	Spermatophytina - seed plants
Class	Magnoliopsida
Superorder	Asteranae
Order	Asterales
Family	Asteraceae (Compositae)
Genus	*Stevia* Cav.

Table 2. Most common species of *Stevia*

- *Stevia anisostemma*
- *Stevia bertholdii*
- *Stevia crenata*
- *Stevia dianthoidea*
- *Stevia enigmatica*
- *Stevia eupatoria*
- *Stevia lemmonii*
- *Stevia micrantha*
- *Stevia ovata*
- *Stevia plummerae*
- *Stevia rebaudiana*
- *Stevia salicifolia*
- *Stevia serrata*
- *Stevia tunguraguensis*
- *Stevia viscida*

1.3. Traditional Use

S. rebaudiana is a medicinal plant and it is considered an alternative sugar instead of synthetic sweeteners. Therefore, the plant is used mainly to stabilize the blood glucose in living organisms. In addition, the leaves are used for the treatment of other diseases, namely diabetes, cardiac diseases, high blood pressure, and weight loss in various alternative traditional systems (Saravanan *et al.,* 2012; Mann *et al.,* 2014). In the beginning, Japan and Korea used this plant extracts for the substitution of sucrose, but their governments in 1991 banned the plant extracts due to some issues. However, in 1991 the US Food and Drug Administration (USFDA) removed the ban, but they have imposed some restrictions on extracts and prepared finished products from the leaves of *S. rebaudiana*. Based on the chemical ingredients, the UK government raised several questions regarding the safety of the use of plant extracts. Due to chemical ingredients, during that time it has generated lots of misunderstandings about the safe use of *Stevia* (Shivanna *et al.,* 2013). After a long discussion, in addition to supporting the use based on the research and comprehensive international response, the USFDA approved the use of the crude extract of *Stevia* leaves as a safe natural and non-nutritive sweetener (NNS) (Schoonhoven, 1982). The leave powders of *Stevia* are used to treat the regulation of the heart rate (Shivanna *et al.,* 2013; Siddique *et al.,* 2014). Currently, *Stevia* is used as an artificial sugar as it does not affect the blood sugar of diabetics' patients. The plant does not have any adverse effects on renal functions like synthetic sweeteners. Not only that, the plant itself and its extracts have significant biological activities like anti-fungal, antioxidant, antihypertensive and anti-

bacterial properties. Therefore, several previous reports supported that the parts of the plant could be used in herbal preparation for the treatment of diseases such as tonics for diabetic patients, daily usage products e.g. mouthwashes and toothpaste (Meireles *et al.,* 2006). As a tea the leaves of *Stevia* give significant relief to diarrhoea (Shivanna *et al.,* 2013).

1.5. *Stevia* Commercial Products

Currently, several commercial products from *Stevia* are available in the global market. Table 3 presents some of the available commercial products. According to the needs, the stevia products can be collected directly from the *Stevia* producing companies as well as from pharmacies. Nowadays, the products can be bought from companies or pharmacies through Internet sources.

Table 3. Different types of **c**ommercial products from *Stevia*

Product name	Companies name	Type of medicine
Stevia powder	Stevia LLC, USA	Crystals
Extract	Life Extension Foundation, USA	Powder
JAJ Stevioside	JAJ Group USA	Powder
Liquid extract of stevia	Baar Product, Inc, USA	Liquid
Stevia Dark Liquid	Stevia NOW	Liquid concentrate
Pure powder extract	Stevia NOW	Powder extract
Stevia crude extract	Stevia NOW	Tablets

2. PLANT PHYTOCHEMICAL

Until now a good number of studies have been conducted on the phytochemicals of *S. rebaudiana*. Most scientists claimed that several groups of phytochemicals are present in the leaves. They are mainly diterpenes glycosides; alkaloids, steroids, tannins, saponins, flavonoids, glycosides, sterol, triterpenes, and anthraquinones (Table 4). Our previous study and several other studies on *Stevia* also showed that the plant leaves contain alkaloids, steroids, tannins, saponins, flavonoids, glycoside, sterol, triterpenes, and anthraquinones including phlobatannins (Hutapea, 1997; Genus Jan, 2002). The plant leaves also contain other vital compounds such as vitamins, folic acid, amino acids, diterpenes glycosides, and derivatives of caffeic acids, nutrients, and several other minor compounds (Soejarto *et al.,* 1982; Barriocanal *et al.,* 2008; Kobus-Moryson and Gramza-Michałowska, 2015; Jan *et al.,* 2021; Lemus-Mondaca *et al.,* 2021). In addition, reports showed that the essential oil of the selected plant contains palmitic, stearic, palmitoleic, oleic, linolenic and linoleic acids (Soejarto *et al.,* 1982). The plant leaves were analyzed by using atomic absorption spectrophotometry (AAS), the plant leaves contain high content of potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), sulphur (S) and sodium (Na). However, metals such as iron (Fe), copper (Cu), cobalt (Co), manganese (Mg), zinc (Zn), selenium (Se) and molybdenum (Mo) are present in smaller amounts. Some research also mentioned that the percentage of essential oil in the leaves is high compared to other plants. The oil of the selected plant contains a high percentage of carbohydrates, ash and protein. The range of foreign matter (2.86%), total ash (2.9% w/w), and moisture content (9.47%) have also been estimated (Soejarto *et al.,* 1982). The leaves are sweeter than the other parts of this plant such as flowers, stems, seeds, and roots. Based on the previous report, the leaves of this plant are 150 times sweeter than synthetic sugar (Soejarto *et al.,* 1982). The leaves contain several diterpene derivatives that are responsible for the sweet taste especially steviol glycosides. It is also reported that the leaves contain a maximum amount of diterpene glycosides derivatives during the flowering time (Soejarto *et al.,* 1982).

Table 4. Group of phytochemicals in the *Stevia*

Groups	Methanol extract	Hexane extract	DCM extract	Ethyl acetate extract	Butanol extract	Water extract
Alkaloids	+	-	-	+	-	-
Steroids	+	+	-	+	-	+
Tannins	+	-	+	+	+	+
Saponins	+	-	-	+	+	+
Flavonoids	+	-	-	+	+	+
Glycosides	+	-	+	+	+	+
Sterols	+	+	-	-	-	-
Triterpenes	-	+	-	-	-	-
Anthraquinones	+	-	+	+	-	-

+ = Present and - =Absent

2.1. Cariogenic and Mutagenic Effects

Previously, Das *et al.* conducted one study to explore the side effects of the prolonged use of stevia products or chemical ingredients: stevioside and rebaudioside-A. However, they didn't find any side effects due to the use of stevia (Das *et al.,* 1992). Regarding the mutagenic effects, several studies have been conducted on stevia by several authors, but the results are contradictory. Therefore, based on the previous report, the FDA stopped listing this herb as a substitute for sugar until the safety issue is settled (FDA, 2019).

2.2. Human Studies

Since the old times, *S. rebaudiana* has been used a natural sweetener instead of synthetic sugar. However, there is a lack of information on a clinical study of the *Stevia* plant and the chemical ingredients in the plant. Glucose tolerance tests were conducted on the effects of administering *Stevia* extracts. Based on the glucose tolerance tests before and after taking the *Stevia* extract, the results showed that the stevia extracts increased glucose tolerance and decreased plasma glucose concentration (Curi *et al.,* 1986). Later, Jeppesen *et al.,* worked on the derivatives of diterpene steviol and stevioside and the results showed that both ingredients can affect directly the pancreas for releasing insulin. His group concluded that the stevia plant could be useful to manage type 2 diabetes (Jeppesen *et al.,* 2000).

2.3. Total Phenols Content

Most of the researchers estimated the total phenol content of *S. rebaudiana* extracts with the modified FCR (Folin-Ciocalteu reagent) method (Amri & Hossain, 2018; Maisa *et al.,* 2021; Hossain *et al.,* 2022). The samples were analysed against the gallic acid standard and measured the absorbance by the UV-visible spectrophotometer method at the wavelength of 760 nm. The literature showed that most of the extracts from the *Stevia* gave a significant amount of total phenol content ranging from 7 to 15 mg/100g in the powder samples. The ingredients belonging to phenol groups are biologically active ingredients and they might be used for the prevention and treatment of diseases. Plant-drive phenolic compounds are widely called antioxidants (Amri & Hossain, 2018). The phenolic ingredients can stop the free radical reactions with other compounds in the human body. As a result, the phenolic compounds can prevent DNA damage and chronic health effects.

2.4. Total Flavonoid Content

The estimation of the total flavonoid content of varied polarities extracts of *S. rebaudiana* was conducted by the modified $AlCl_3$ method against quercetin standard as described by several authors (Al-Jadidi & Hossain, 2016; Al-Oraimi & Hossain, 2016; Al-Saeedi *et al.,* 2016).

Different polarities of plant extracts were analysed and the absorbance was measured by using the UV-visible spectrophotometric method at the wavelength 415 nm. The literature showed that among the six extracts e.g., methanol, hexane, ethyl acetate, chloroform, butanol and water extracts, the ethyl acetate extract gave the highest total phenol content ranging from 3 to 9 mg/100g powder samples (Al-Jadidi & Hossain, 2016). All flavonoid ingredients belong to phenol groups and they are biologically active and they can be used for the prevention and treatment of diseases. Plant-ingredient flavonoids contain compounds that are quite stable and they can also protect the human body from different cell reactions.

3. BIOLOGICAL ACTIVITIES

Since the old times, scientists have been paying attention to the biological activities of the plant extracts and confirmed their *biological activities such as antioxidant, antibacterial, antifungal,* antidiabetics, antihypertensive, cytotoxic, anti-inflammatory activities etc. *S. rebaudiana* gave significant biological activities. The literature search showed that plant extracts with different polarities displayed varied activities due to the chemical ingredients present in the extract. Biological activities play a vital role in the treatment of different diseases. The selected plant species showed varied biological activities; therefore, the selected species is used to treat various diseases including diabetes.

3.1. Antioxidant Activity

In general, the antioxidant activity of plant extracts was determined by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing ability of plasma (FRAP), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) which was described by most of the authors (Al-Habsi & Hossain, 2018; Abdulsattar & Hossain, 2020; Al-Rashdi *et al.,* 2021; Al-Qassabi *et al.,* 2018; Hossain *et al.,* 2019). The same methods have been used to determine the antioxidant activity of various *S. rebaudiana* extracts and showed that the plant extracts with varied polarities gave varied antioxidant activity. The majority of the previous work showed that in the case of most plant species, the polar extracts have more significant activity than non-polar extracts (Al-Jadidi & Hossain, 2016; Saravanan *et al.,* 2012). However, some authors, found the reverse results meaning that non-polar extracts showed higher activity compared to polar extracts. Nevertheless, the total average antioxidant activities are quite high about 89% DPPH inhibition against ascorbic acid either in the case of polar or non-polar extracts (Shivanna *et al.,* 2014). In conclusion, the consumption of food with high antioxidants could reduce the risk of chronic diseases, including cardiovascular disease, colon cancers, diabetes, hypertension, etc. Free radicals from antioxidants are the vital source to prevent or reduce human cell damage through oxidation. The mechanism of preventing human cell damage by antioxidants is still.

3.2. Antimicrobial Activity

Still now, the researchers are using *in-vitro* and *in-vivo* studies to determine the antimicrobial activity of plant crude extracts (Al-Jabri & Hossain, 2018; Weli *et al.,* 2020; Al-Saeghi *et al.,* 2022). For the screening of the plant extracts, the scientists use the *in-vitro* method, however for clinical trials scientists use the *in-vivo* model. Scientists studied the selected *S. rebaudiana* extracts and found the best extracts that could be used to treat all kinds of infectious diseases without adverse effects (Curi *et al.,* 1986; Maisa *et al.,* 2021). The best extract with significant antimicrobial activity was found in hexane extract against Gram (+) bacterial strains within the range of 0-13 mm (Al-Jadidi & Hossain, 2016). However, other various polarities extracts from the leaves of *S. rebaudiana* also gave activity against all other Gram (+and -) with the range of 0-10 mm. The previous studies showed that all varied polarity extracts from this plant species gave moderate activity against the Gram (+ and -) bacterial strains. Based on the antimicrobial activity among the extracts, the hexane extract could be used as an alternative natural safe antibiotic instead of a synthetic antibiotic.

3.3. Anti-diabetic Activity

Diabetes is the deficiency of insulin. Most of the vital organs were affected due to diabetics. Scientists are looking for a natural safe remedy to treat or manage diabetes. *In-vitro* and *in-vivo* antidiabetic activity of the plant extracts was determined by using α-amylase inhibitory and α-glucosidase inhibitory bioassay as described by several authors (Ahmad & Ahmad, 2018; Dhasarathan & Theriappan, 2011). The same therapeutic approaches were used for the screening of the crude extracts of the selected plant species for their antidiabetic activity. The inhibition results obtained from both approaches showed that the stevia extracts significantly reduced postprandial elevation of blood glucose. Previous studies showed that all fresh extracts of the selected plant inhibit the α-amylase with an IC_{50} value of 2.679 µg/ml, which was higher than the standard acarbose (1.736 µg/mL). However, in the case of all commercial and wild samples, the inhibition capacity towards α-amylase deteriorated once the maximum capacity was reached, which was probably due to the saturation of the enzyme. The inhibition could be due to the ingredients that inhibit the α-amylase enzyme.

4. CONCLUSION

S. rebaudiana is a flowering plant that belongs to the genus *Stevia*. The selected plant is used mainly to stabilize the blood glucose in living organisms. In addition, the leaves are used for the treatment of other diseases, namely diabetes, cardiac diseases, control of blood pressure and weight loss in various alternative traditional systems. The focus of this present review is on the phytochemicals, pharmacological and toxicological activities status of the plant species. The plant can be used as a natural antioxidant and antibiotics as supplements and drugs. Therefore, further clinical and pathological studies must be conducted to investigate the unexploited potentials of the *Stevia* plant before using the plant as a drug to treat diseases.

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Declaration of Conflicting Interests and Ethics

The authors declare that in this review there is no conflict of interest. This study complies with research and publishing ethics. The authors are responsible for scientific and legal responsibility for manuscripts published in IJSM.

Authorship Contribution Statement

Amzad Hossain: Conceptualization; Data curation; Data analysis; Wrote a first draft of the review. **Said Al Harthy:** Literature survey; Data collection; Edit data. **Salem Said Jaroof Al-Touby:** Reviewing and Editing. **Yahya Bin Abdullah Alrashdi:** Reviewing and Editing

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REFERENCES

Abdulsattar, A.M., & Hossain, M.A. (2020). Antibacterial and antioxidant potential of *Tetraena simplex* extracts of various polarities. *Toxicology Reports 7*, 925–929.

Ahmad, U., & Ahmad, R.S. (2018). Anti diabetic property of aqueous extract of *Stevia rebaudiana* Bertoni leaves in Streptozotocin-induced diabetes in albino rats. *BMC Complementary and Alternative Medicine*, *18*, 179-185.

Al-Habsi, A.A.S., & Hossain, M.A. (2018). Isolation, structure characterization and prediction of antioxidant activity of two new compounds from the leaves of *Dodonaea viscosa* native to the Sultanate of Oman. *Egyptian Journal of Basic Applied Sciences, 5*, 157–164.

AL-Jabri, N.N., & Hossain, M.A. (2018). Chemical composition and antimicrobial potency of locally grown lemon essential oil against selected bacterial strains. *Journal of King Saud University Sciences, 30*, 14-20.

Al-Jadidi, H.S.K., & Hossain, M.A. (2016). Determination of the total phenols, flavonoids and antimicrobial activity of the crude extracts from locally grown neem stems. *Asian Pacific Journal of Tropical Diseases, 6*(5)*,* 376-379.

AL-Oraimi, A.A., & Hossain, M.A. (2016). In vitro total flavonoids content and antimicrobial capacity of different organic crude extracts of *Dodonaea viscosa*. *Journal of Biologically Active Products from Nature, 6*(2), 166-172.

Al-Qassabi, J.S.A., Weli, A.M., & Hossain, M.A. (2018). Comparison of total phenols content and antioxidant potential of peel extracts of local and imported lemons samples. *Sustainable Chemistry and Pharmacy, 8*, 71–75.

Al-Rashdi, R.S.Y., Hossain. M.A., & Al-Touby, S.S.J. (2021). Antioxidant and antibacterial activities of leaves crude extracts of *Adenium obesum* grown in Oman National Botanical Garden. *Advances in Biomarkar Science and Technology, 3*, 8-14.

Al-Saeedi, A.H., Al-Ghafri, M.T.H., & Hossain, M.A. (2016). Comparative evaluation of total phenols, flavonoids content and antioxidant potential of leaves and fruit extracts of Omani *Ziziphus jujuba* L. *Pacific Science. Review A: Natural Science and Engineering, 18*, 78-83.

Al-Saeghi, S.S., Hossain, M.M., & Al-Touby, S.S.J**.** (2022). Characterization of antioxidant and antibacterial compounds from the aerial parts of *Haplophyllum tuberculatum. Journal Bioresources and Bioproducts, 7*, 52-62.

Amri, F.S., & Hossain, M.A. (2018). Comparison of total phenols, flavonoids and antioxidant potential of local and imported ripe bananas. *Egyptian Journal of Basic Applied Sciences*, *5*, 245-251.

Barriocanal, L., Palacios, M., Benitez, G., Benitez, S., Jimenez, J., & Jimenez, N. (2008). Apparent lack of pharmacological effect of steviol glycosides used as sweeteners in humans, a pilot study of repeated exposures in some normotensive and hypotensive individuals and in type 1and type 2 diabetics. *Regulatory Toxicology and Pharmacology*, *51*, 37–41.

Bemays, E.A., & Chapman, R.F. (1994). *Host plant selection by phytophagous insects*. Chapman and Hall, NY. USA.

Beutler, J.A., Caredelline, J.H., McMahon, J.B., Shoemaker, R.H., & Boyd, M.R. (1995). *Phytochemistry of medicinal plants*. ed. Amason J. T., Mata R., Romeo J. T. Plenum Press, NY, USA.

Brandle, J.E., Starratt, A.N., & Gijzen, M. (1998). *Stevia rebaudiana*: Its agricultural, biological, and chemical properties. *Canadian Journal of Plant Science, 78*, 527–536.

Carneiro, J.W.P. (1930). *Stevia rebaudiana* (Bert.) Bertoni: produção de sementes. UEM: Maringá. 61.

Curi, R., Alvarez, M., & Bazotte, R.B. (1986). Effect of Stevia rebaudiana on glucose tolerance in normal adult humans. *Brazilian Journal of Medical and Biological Research, 19*(6),771–774.

Das, S., Das, A.K., & Murphy, R.A. (1992). Evaluation of the cariogenic potential of the intense natural sweeteners stevioside and rebaudioside A. *Caries Research, 26*(5), 363–366.

Dhasarathan, P., & Theriappan, P. (2011). Evaluation of anti-diabetic activity of *Strychonous potatorum* in alloxan induced diabetic rats. *Journal of Medical Science, 2*(2)*,* 670–674.

Gonsumer. (2019). *National Technical Information Services*. 5285 Port Royal Road, Springfield, VA 22161, pp. 152–157.

Genus Jan, M.C. (2002). Safety evaluation of stevia and Stevioside. *Journal of Natural Product Chemistry*, *27*(8), 299–319.

Goyal, S.K., Samsher, N., & Goyal, R.K. (2010). Stevia (*Stevia rebaudiana*) a bio-sweetener: a review. *International Journal of Food Science and. Nutrition, 61*(1)*,* 1–10.

Hossain, M.A., Alrashdi, Y.B.A., & Al-Touby, S.S.J. (2022). A review on essential oil analyses and biological activities of the traditionally used medicinal plant *Thymus vulgaris* L. *International Journal of Secondary Metabolite*, *9*(1)*,* 103–111.

Hossain, M.A., Weli, A.M., & Ahmed, S.H.I. (2019). Comparison of total phenols, flavonoids and antioxidant activity of various crude extracts of *Hyoscyamus gallagheri* traditionally used for the treatment of epilepsy. *Clinical Phytoscience, 5*, 20-29.

Hutapea, A.M. (1997). Digestion of stevioside (a natural sweetener) by various digestive enzymes. *Journal of Clinical Biochemistry and Nutrition*, *23*(3)*,* 177–186.

Isman, M.B. (1994). Botanical insecticides. *Pesticides Outline, 5*, 26-30.

Jan, S.A., Habib, N., & Shinwari, Z.K. (2020). The anti-diabetic activities of natural sweetener plant Stevia: an updated review. *SN Applied Science, 3*, 517-520.

Jeppesen, P.B., Gregersen, S., & Poulsen, C.R. (2000). Stevioside acts directly on pancreatic beta cells to secrete insulin: Actions independent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive K+-channel activity. *Metabolism*, *49(2),* 208–214.

Kakkar, S., & Bais, S. (2014). A review on protocatechuic acid and its pharmacological potential. *ISRN Pharmacy*, *9,* 2014-2020.

Katayama, O., Sumida, T., Hayashi, H., & Mitsuhashi, H. (1976). *The practical application of Stevia and research and development data*, I.S.U. Company, Japan, p.747

Klayman, D. (1985). Qinghaosu (Artemisinin) an antimalarial from China. *Science, 228,* 1048-1055.

Kobus-Moryson, M., & Gramza-Michałowska, A. (2015). Directions on the use of stevia leaves (*Stevia rebauidana*) as an additive in food products. *Acta Scientiarum Polonorum Technologia Alimentaria, 14*(1), 5–13.

Lemus-Mondaca, R., Vega-Galvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry, 132*, 1121–1132.

Madan, S., Ahmad, S., Singh, G.N., Kohli, K., Kumar, Y., Singh, R., & Garg, M. (2010). *Stevia rebaudiana* (Bert.) Bertoni – A review. *Indian Journal of Natural Products Resources, 1*, 267-86.

Maisa, S., Al-Touby, S.S.J., & Hossain, M.A. (2021). Total phenols content and antioxidant activity of different polarity crude extracts of *Dodonaea viscosa. Indian Drugs*. *58* (08), 79-83.

Mann, T.S., Agnihotri, V.K., Kumar, D., Pal, P.K., Koundal, R., Kumar, A., & Padwad, Y.S. (2014). In vitro cytotoxic activity guided essential oil composition of flowering twigs of *Stevia rebaudiana*. *Natural Product Communications, 9*(5)*,* 715–718

Meireles, M.A.A., Wang, G., Hao, Z., Shima, K., & Silva, J.A.T. (2006). *Stevia (Stevia rebaudiana Bertoni): futuristic view of the sweeter side of life. In: Jaime A. Teixeira da Silva ed. Floriculture, ornamental and plant biotechnology: Advances and Topical Issues* IV, Edition: 1, Global Science Books 416- 425.

Misra, H., Soni, M., Silawat, N., Mehta, D., Mehta, B.K., & Jain, D.C. (2011). Antidiabetic activity of medium-polar extract from the leaves of *Stevia rebaudiana* Bert. (Bertoni) on alloxan-induced diabetic rats. *Journal of Pharmacy and Bioallied Sciences, 3*(2)*,* 242–8.

Phillipson, J.D., & Wright, C.W. (1991). Can ethnopharmacology contribute to the development of antimalarial agents. *Journal of Ethnopharmacology*, *32*, 155-165.

Rana, N., & Bais, S. (2014). *Neuroprotective effect of J. communis in Parkinson disease induced animal models*, Pharmacology Department, Punjab Technical University, Punjab, India.

Robinson, B.L. (1930). *Contribution from Gray Herbarium of Harvard University*, The Gray Herbarium of Harvard University, Cambridge.

Saravanan, R., Vengatash B.K., & Ramachandran, V. (2012). Effect of Rebaudioside A, a diterpenoid on glucose homeostasis in STZ-induced diabetic rats. *Journal of Physiology and Biochemistry, 68*(3)*,* 421–431.

Schoonhoven, L.M. (1982). Biological aspects of antifeedants. *Entomolgia Experimentalis et Applcata, 31*, 57-69.

Shivanna, N., Naika, M., Khanum, F., & Kaul, V.K. (2013). Antioxidant, anti-diabetic and renal protective properties of *Stevia rebaudiana*. *Journal of Diabetes Complications, 27*(2), 103–113.

Siddique, A.B., Rahman, S.M.M., Hossain, M.A., & Rashid, M.A. (2014). Phytochemical screening and comparative antimicrobial potential of different extracts of *Stevia rebaudiana* Bertoni leaves. *Asian Pacific Journal of Tropical Diseases, 4*(4)*,* 275–280.

Soejarto, D.D, Kinghorn, A.D., & Farnsworth, N.R. (1982). Potencial sweetening agentsof plant origin. III. Organoleptic evaluation of Stevia leaf herbarium samples for sweetness. *Journal of Natural Products, 45*, 590–599.

Steven, D., & Ehrlich, N.M.D. (2009). *Solutions acupuncture, a private practice specializing in complementary and alternative medicine.* Healthcare Network, Phoenix, Ariz, USA.

Tandon, V., Kapoor, K., & & Gupta, B.M. (2004). Herbal drug research in India: a trend analysis using IJP as a marker. *Indian Journal of Pharmacy, 36* (2)*,* 99– 100.

Tanaka, T., Zhang, H., Jiang, Z.H., & Kouno, I. (1997). Relationship between hydrophobicity and structure of hydrolysable tannins, and association of tannins with crude drug constituents in aqueous solution. *Chemical and Pharmaceutical Bullutin, 45*, 1891-1897.

Tang, W., & Eisenbrand, G. (1992). *Chinese drugs of plant origin. chemistry, pharmacology, and use in traditional and modern medicine*. Springer-Verlag. Berlin, 1.

Unschuld, P.U. (1988). *Culture and pharmaceutics: some epistemological observations on pharmacological systems in ancient Europe and Medieval China. In: The context of medicines in developing countries. Studies in pharmaceutical anthropology.* Kluwer Academic Publishers. Dordrecht, pp. 188.

Weli, A.M., Al-Saadia, H.S., Al-Fudhaili, R.S., Hossain, M.A., Putit, Z.B., & Jasim, M.K. (2020). Cytotoxic and antimicrobial potential of different leaves extracts of *R. fruticosus* used traditionally to treat diabetes. *Toxicology Reports, 7*, 183–187.

Zarawska, E.L. (1997). Biflavonoids in *Juniperus species* (Cupressaceae). *Polish Journal of Pharmacology and Pharmacy*, *27*(1), 81–87.

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Research Article

Chemical profiling of *Oxalis* species growing wild in Egypt using HRLC/MS Spectrometry

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Abstract: Three medicinally promising *Oxalis* species, namely *O. pes-caprae, O. corymbosa* & *O. latifolia* were collected from Egyptian flora and their methanolic extracts were subjected to LC-QTOF-MS analysis to annotate their chemical profiles. Subsequently, 50 compounds belonging to various chemical classes were identified and characterized, of which 34 compounds were first reported from *Oxalis* L.. Moreover, five flavone compounds were separated and identified from *O. pes-caprae*; their structures were elucidated using acid hydrolysis, UV/vis, ^{1}H-NMR, and HR-ESI-MS. The chemotaxonomic relationship of the studied species was evaluated and the extracted data were statistically analyzed and classified *Oxalis* sp. into two distinct clusters. Each cluster was characterized by special chemical features that helped in distinguishing between them.

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1. INTRODUCTION

Oxalis L. is a genus of family Oxalidaceae R.Br. representing nearly 500 accepted species being worldwide distributed, with special richness in the area from South America to the southern region of North America and at the South-Western Cape region of South Africa (Oberlander *et al*., 2002; The plant list, 2013). The genus is represented in Egypt by five species and one variety (Täckholm, 1974; El-Khanagry, 2005).

The pharmacological and therapeutic potential of some *Oxalis* plants was evaluated by identifying their biologically active chemical constituents, such as *O. pes-caprae* L. which has antioxidant and neuroprotective activities (Gaspar *et al*., 2018). While some other species have been reported for their antioxidant, anti-diabetic, anti-cancer, antibacterial, and enzyme-inhibiting activities (Prasad Pandey *et al*., 2020; Sarfraz *et al*., 2022).

O. corymbosa DC. and *O. latifolia* Kunth had not been subjected to any chemical study prior to work on the nonpolar fraction by Draz *et al*. (2021). Limited studies have been reported about the other *Oxalis* species. Some of those works concerned with their nonpolar chemical profiles

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(Draz *et al.*, 2021) while others provided their chemical profiles using polar fractions (Güçlütürk *et al*., 2012; Gaspar *et al*., 2018; Prasad Pandey *et al*., 2020). In addition, the separation, and identification of flavonoids and phenolic acids from *O. pes-caprae* were carried out by Della Greca *et al*. (2009) and Güçlütürk *et al*. (2012).

Lourteig (2000) proposed a system for classifying *Oxalis* L. based on its morphological characters and concluded *O. corymbosa* and *O. latifolia* under section Ionoxalis, while *O. pes-caprae* was placed under section Cernuae. There weren't any classifying systems to concern with the chemical characters of this genus before, so the present study aims to evaluate the chemotaxonomic relationship between the studied species.

2. MATERIAL and METHODS

2.1. Plant Materials

Three *Oxalis* plants were collected from different localities in Egypt by Amal A. Draz and were identified and deposited in the Herbaria of the Faculty of Science, Cairo University (CAI) and National Research Centre (CAIRC) by Dr. Eman M. Shamso. *O. corymbosa* DC. (voucher No. A303) was collected from the National Research Centre gardens, Dokki, Giza in March, 2019, while *O. pes-caprae* L. (voucher No. A304) was collected from Bramly's grotto, Burg El-Arab in February, 2019 as well as *O. latifolia* Kunth (voucher No. A305) was collected from Faculty of Science gardens, Cairo University, Giza in April, 2019.

2.2. LC/QTOF/MS Analysis

The LC-QTOF-MS analysis was performed using plant materials that had been cleaned, dried, and ground into a fine powder. Fifty grams of each plant were soaked in 70% MeOH three times, 2 days/each time; the extracts were filtered and evaporated under reduced pressure and temperature till full dryness using Rota-vapour apparatus. Next, 30 mg of each prepared crude extract was analyzed using the -ve mode (Triple TOF 5600+ Sciex) in accordance with the method of Eissa *et al*. (2020). The profiling and annotation of their chemical constituents were described (Table 1).

2.3. Isolation of Pure Compounds

Air-dried and powdered 1.6 kg of *O. pes-caprae* was extracted using 70% MeOH, filtered, and then dried using Rota-vapour apparatus. The crude extract was then suspended in distilled water (500 ml) and fractionated with ethyl acetate (65 g) and n-butanol (98 g). For further separation, each fraction was subjected to column and paper chromatographyaccording to the method of Hussein *et al*. (2018). The identification of the separated compounds was achieved through: NMR experiments that were carried out by using Jeol EX-500 spectrometer: 500 MHz (^{1}H NMR); UV absorption spectra were recorded on a Shimadzu model-2401CP spectrophotometer, and HR-ESI-MS on a Triple TOF 5600$^+$Sciex. Authentic samples were obtained from the department of phytochemistry and plant systematics, NRC. Five compounds were separated, two of which were identified via ^{1}H-NMR, UV/vis, and HR-ESI-MS (compounds 29 & 37), while the other three compounds were identified through co-chromatography with authentic standards (compounds 40, 38 & 33) (Table 1).

2.4. Statistical Analysis

Multivariate statistical analysis of 50 character states was performed using Minitab 21 software for detecting the affinities among the variable chemical profiles of the investigated plants and a dendrogram was plotted.

3. RESULTS and DICUSSION

3.1. LC/QTOF/MS Analysis

The LC/QTOF/MS analysis revealed the identification and characterization of 50 compounds belonging to different chemical classes from the methanolic extracts of three *Oxalis* L. plants (*O. corymbosa*, *O. latifolia* & *O. pes-caprae*) (Table 1; Figure 1).

3.1.1. *Phenolic acid derivatives*

They were recorded in different *Oxalis* species. Compounds **(1), (2), (3),** and **(4)** were identified in all of the studied taxa, whereas the rest of compounds showed a selective occurrence in certain species and were absent in others.

Compound **(5)** was only detected in the extract of *O. pes-caprae* at *m/z* 153.0188 and R_t 4.94 min It produced a major daughter fragment ion at *m/z* 109 due to loss of CO_2, that supported its identification as 3,4-Dihydroxybenzoic acid (Ruan *et al*., 2019).

Compound **(6)** was detected at R_t 4.96 min with *m/z* 359.0782 in all of the studied taxa except for *O. latifolia.* It gave fragmentation pattern of *m/z* 197, 179, 161 that was in compliance with that of rosmarinic acid (Wang *et al*., 2021).

Compound **(7)** was characteristic of *O. latifolia*. This compound appeared at *m/z* 137.0247 (at R_t 6.97 min.) and produced a daughter fragment ion at *m/z* 93, suggesting its identification as "salicylic acid" (Lin *et al*., 2020).

Although compound **(8)** was only absent from *O. Latifolia,* it was reported at *m/z* 167.0356 (at R_t 7.14 min.) and produced a fragment ion at *m/z* 152 due to the loss of CH_3 group in the remaining studied taxa. Thus, it was suggested to be "5-Methoxysalicylic acid" (Massbank Record: PS104208).

3.1.2. *Carboxylic acids*

A wide variation of carboxylic acids have been detected in the studied taxa, of which the compounds **(9)**, **(10), (12)**, **(13), (14),** and **(15)** were detected in all of the studied taxa, while the remaining compounds showed a selective occurrence among the studied taxa that developed a level of variation.

Compound **(11)** was identified as succinic acid as it appeared at *m/z* 117.0176 (R_t 1.19) and produced a series of fragments at *m/z* 99 $[M\text{-}H\text{-}H_2O]^-$ and *m/z* 73 $[M\text{-}H\text{-}CO_2]^-$ (Eissa *et al*., 2020). This compound was detected in all of the investigated extracts except *O. latifolia*.

Compound **(16)** at R_t 1.25 min and *m/z* 195.0516 was identified as gluconic acid as it produced a fragmentation pattern of *m/z* 129, 177 and 159 (Lucas, 2021).

Compound **(17)** was exclusively detected in extracts of *O. corymbosa* and *O. latifolia*. Its fragment ion peak at R_t 1.26 min and *m/z* 160.0615 produced the fragment ions *m/z* 142 (due to loss of H_2O molecule) and *m/z* 116 (due to loss of CO_2 molecule) on the MS/MS fragmentation. This fragmentation spectrum was mostly compatible with that of "1H-indole-3-carboxylic acid" (Gamir *et al*., 2012).

Compound **(18)** at R_t 1.27 min and *m/z* 105.0199 was absent only in *O. pes-caprae*. The fragmentation pattern of this compound was mostly compatible with that of glyceric acid, as it produced fragment ions at *m/z* 75, 73, 59, 57, 47 (Lucas, 2021).

Compound **(19)** that appeared at R_t 1.28 min and *m/z* 147.0288 was identified as citramalate as it showed the fragmentation pattern of *m/z* 129, 103, 87, 85 (Wu *et al*., 2010). This compound was only present in *O. latifolia*.

Compound **(20)** was only detected in *O. corymbosa* extract at *m/z* 149.0451 (R_t 1.29 min.), and on MS/MS fragmentation, it produced a major fragment ion at *m/z* 73 in a fragmentation pattern being characteristic of tartaric acid (Brent *et al*., 2014).

Compound **(21)** appeared at R_t 1.38 min and *m/z* 145.0613 was only recorded in the extracts of section Ionoxalis representatives (*O. corymbosa* and *O. latifolia*). It gave fragment ions at *m/z* 127, 101 in a MS/MS fragmentation pattern that was characteristic of 2-Methylglutaric acid (Lucas, 2021).

Compounds **(22), (23), and (25)** were detected only in *O. pes-caprae*. Those compounds had molecular ion peaks at *m/z* 175.0616 (at R_t 1.46 min), 138.0556 (at R_t 1.51 min), and 147.0459 (at Rt 4.83 min), respectively. Compound **(22)** gave a fragmentation pattern of *m/z* 157 (due to the loss of an H_2O molecule), 131 (due to the loss of a CO_2 molecule), 129, 115, 113 and 85 being characteristic of 2-Isopropylmalic acid (Ricciutelli *et al*., 2019). Compound **(23)** gave a fragment ion at *m/z* 94, so it was characterized as 6-Hydroxynicotinic acid (Llorach *et al*., 2009)**.** Compound **(25)** gave fragment ions at *m/z* 119 and 103 (due to the loss of a CO_2 molecule) that confirmed its identification as cinnamic acid (Zhang *et al*., 2010).

Compound **(24)** appeared at R_t 2.15 min and *m/z* 353.0878 was characterized as chlorogenic acid. Its spectrum showed fragment ions at *m/z* 191, 179, 173 (Zhang *et al*., 2010). This compound was recorded in the extracts of all studied taxa except *O. corymbosa*.

3.1.3. *Flavonoids*

Seventeen flavonoid compounds were identified from the plants under investigation, of which compounds **(29** & **37)** were separated from *O. pes-caprae* and identified using UV/vis and ^{1}H-NMR techniques, and for further accuracy they were identified along with the compounds **(38**, **33** & **40)** from the same species via co-chromatography with authentic standard samples (Figure 2).

Compounds (**26)** (R_t 4.36 min., *m/z* 449.1074) and **(36)** (R_t 7.95 min., *m/z* 433.10678) were identified as eriodictyol-7-*O*-glucoside and naringenin-7-*O*-glucoside, respectively, as confirmed by comparing their fragmentation patterns (*m/z* 287, 269) and (271, 151), respectively with the literature (Krasteva & Nikolov, 2008; Kang *et al*., 2016). Those two compounds were detected in all of the studied taxa except for *O. latifolia.*

Compound **(27)** has molecular ion peak at *m/z* 289.0729 that appeared at R_t 4.93 min and gave fragment ions at *m/z* 245, 205, 179, 149, 123, and 109 being characteristic of "Catechin" (Kang *et al*., 2016). Catechin was detected only in *O. corymbosa.*

Compounds **(28)**, **(29),** and **(37)** are luteolin-*C*-glycoside derivatives that appeared at R_t 6, 6.42 & 8.11 with molecular ion peaks at *m/z* 447.0924, 461.1070 & 447.0887, respectively. They were detected in all of the studied taxa except compound **(29)** which was absent from *O. corymbosa*. Compounds **(28)** & **(37)** have the same fragment ions being *m/z* 429 $[M\text{-}H\text{-}H_2O]^-$, 357 $[M\text{-}H\text{-}90]^-$, and 327 $[M\text{-}H\text{-}120]^-$. The intensities of the fragment ions of the two compounds were different, being 327 (100%), 357 (55%) for compound **(28)** and 327 (100%), 357 (19%) for compound **(37)** that suggested their identification as luteolin-6-*C*-glucoside and luteolin-8-*C*-glucoside, respectively (Hassan *et al*., 2019) with further confirmation of compound **(37)** identification through UV/vis and ^{1}H-NMR (Almahy & Fouda, 2013). Compound **(29)** produced daughter fragment ions at *m/z* 446, 313, 298 that suggested its identification as luteolin-7-OCH_3-6-*C*-glucoside "swertiajaponin" (Wang *et al*., 2008), an identification that was further confirmed using UV/vis and ^{1}HNMR (Kumarasamy *et al*., 2004).

Compound **(31)** was only detected in *O. latifolia* at R_t 6.78 min and *m/z* 463.0924 with a major fragment ion at *m/z* 301, suggesting its identification as "quercetin 3-hexoside" (Marzouk *et al*., 2018).

Compound **(38)** at R_t 9.52 min was identified as luteolin. It gave molecular ion peak at *m/z* 285.0387 with production of fragments 241, 217, 151, and 133 that corresponds to the luteolin aglycon (Marzouk *et al*., 2018), an identification that was more confirmed via co-chromatography with an authentic standard. While compound **(33)** was identified as luteolin-7-*O*-glucoside that appeared at R_t 7.21 and gave a molecular ion peak at *m/z* 447.0934 with producing the characteristic fragment ion of luteolin aglycon at *m/z* 285 that corresponds to the loss of the glucoside moiety $[M-H-162]^-$ in addition to producing *m/z* 179, 151 (Marzouk *et al*., 2018), an identification that was more confirmed via co-chromatography with an authentic standard.

Compound **(34)** was detected only in *O. pes-caprae* and *O. corymbosa* at R_t 7.64 min with molecular ion peak *m/z* 447.0917 and gave fragment ions at *m/z* 285, 133, so it was identified as maritimetin-6-*O*-hexoside (Cao-Ngoc *et al*., 2020).

Compound **(40)** at R_t 10.45 and *m/z* 269.0469 was identified as being apigenin by comparing its fragmentation product ions (225, 151) with the literature and its co-chromatography with an authentic standard (Brito *et al*., 2014). Apigenin was detected only in *O. pes-caprae* and *O. latifolia*. The fragmentation pattern of compound **(32)** at R_t 6.87 min and *m/z* 577.1547 showed a daughter fragment ion at *m/z* 269 that was characteristic of the apigenin aglycon (due to losing neohesperidoside moiety) and another fragment ion at *m/z* 413 that indicated the loss of a rhaminose moiety. This fragmentation pattern supported its identification as apigenin 7-*O*-neohesperidoside (Brito *et al*., 2014). In the same manner, compound **(35)** at R_t 7.84 min and *m/z* 431.1192, it was found that it produced a fragment ion at *m/z* 269 which is characteristic of the apigenin aglycon after losing the glucose moiety from the original compound and its isotopic fragment also appeared at *m/z* 268 in addition to another fragment ion at *m/z* 311 and this supported its identification as apigenin-7-*O*-glucoside (Marzouk *et al*., 2018).

Compound **(39)** was identified as naringenin (at R_t 10.13 min and *m/z* 271.0635) as it gave fragment ions at *m/z* 151, 125, 107 (Kang *et al*., 2016).

Compound **(41)** was recorded only in the extract of *O. pes-caprae* (section: Cernuae) at R_t 12.17 min and *m/z* 267.0652. It gave fragment ions at *m/z* 252, 224 that were characteristic of formononetin (Zhao *et al*., 2020).

Compound **(42)** appeared at R_t 14.67 min with a molecular ion peak at *m/z* 283.0672 and fragment ions at *m/z* 268, 240, 211, a fragmentation pattern being characteristic of acacetin (Ben Salah *et al*., 2019). This compound was present only in *O. corymbosa* extract.

One coumarin was identified from the taxa under study being compound **(43)** that was detected only in *O. pes-caprae* at R_t 8.33 min and *m/z* 177.0925. It produced fragment ions at *m/z* 149, 133 that suggested its identification as esculetin (6,7-Dihydroxycoumarin) (Ruan *et al*., 2019).

Compound **(45)** was characterized as a stilbene. It was recorded only in the extract of *O. corymbosa*. Its molecular ion peaks appeared at *m/z* 227.0718 and R_t 9.1 min with producing daughter fragment ions at *m/z* 185, 183, 159, 157, and 143 that was characteristic of resveratrol (Guerrero *et al*., 2020).

Compound **(50)** belonging to the class of sugar alcohols was detected at R_t 1.39 min and *m/z* 181.0717. It gave fragmentation pattern of *m/z* 163, 119, 89, and therefore it was identified as mannitol (Gervasoni *et al*., 2016). This compound was detected in *O. pes-caprae* and *O. corymbosa*.

Figure 1. LC-QTOF/MS analysis of methanolic extracts of *Oxalis* sp.

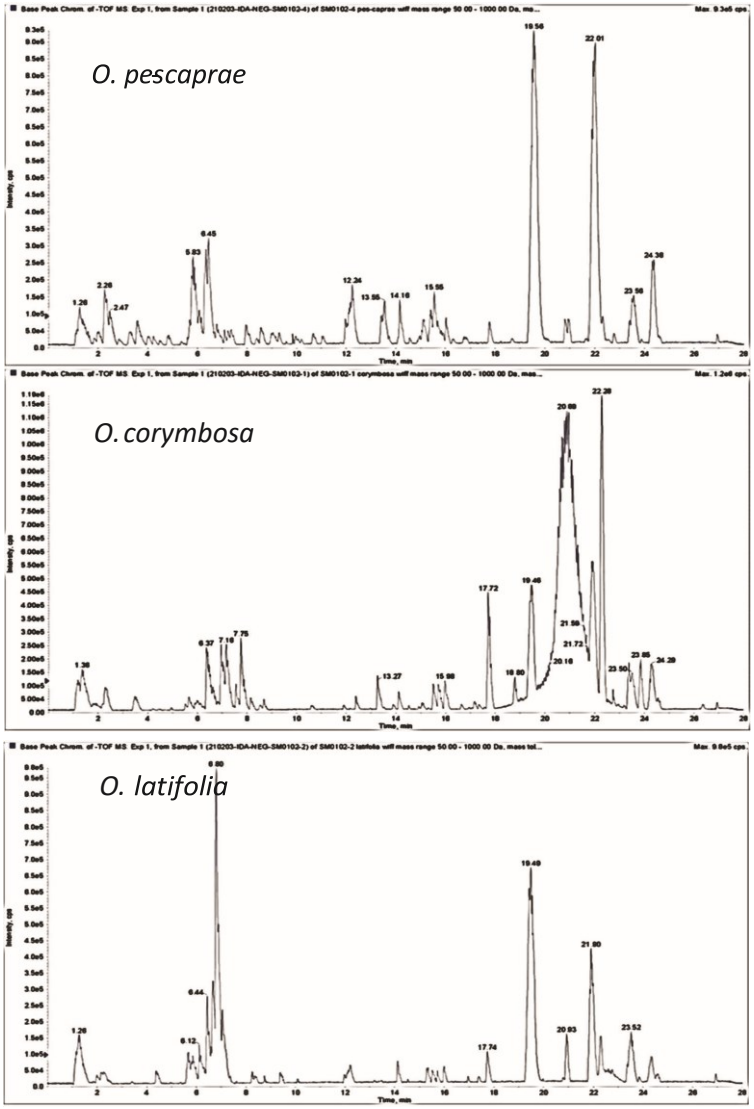
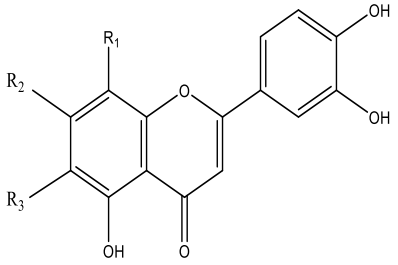
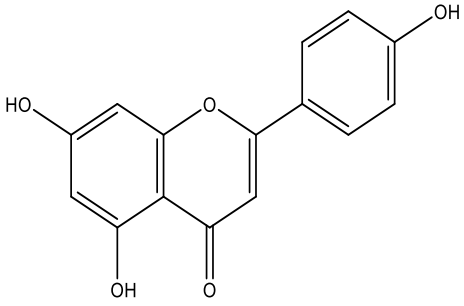


Figure 2. Chemical structures of isolated compounds from *Oxalis pes-caprae*.



Compound	R_1	R_2	R_3
29:	H	OMe	Glc
37:	Glc	OH	H
38:	H	OH	H
33:	H	Glc	H



Compound (40)

Table 1. Different chemical classes detected in the studied *Oxalis* taxa by using LC-QTOF-MS.

NO.	R_t (Min.)	M.Wt	$[M-H]^-$	Compound name	MS/MS spectrum	Section			Reference
						Cernuae	Ionoxalis		
						Oxalis pes-caprae	*Oxalis corymbosa*	*Oxalis Latifolia*	
phenolic acid derivatives									
1	1.98	180	179.0341	Caffeic acid #	135, 161	+	+	+	Marzouk *et al.*, 2018
2	2	164	163.03873	*P*-Coumaric acid #	93, 119	+	+	+	Zhong *et al.*, 2020
3	2.25	194	193.04855	ferulic acid #	178, 149	+	+	+	Zhong *et al.*, 2020
4	3.31	138	137.0238	*P*-Hydroxybenzoic acid #	93	+	+	+	Zhong *et al.*, 2020
5	4.94	154	153.0188	3,4-Dihydroxybenzoic acid	109	+	-	-	Ruan *et al.*, 2019
6	4.96	360	359.07828	Rosmarinic acid	161, 179, 197	+	+	-	Wang *et al.*, 2021
7	6.97	138	137.02477	Salicylic acid	93	-	-	+	Lin *et al.* 2020
8	7.14	168	167.0356	5-Methoxysalicylic acid	152	+	+	-	(Massbank Record: PS104208)
Carboxylic acids									
9	1.16	134	133.01486	Malic acid #	70, 71, 115	+	+	+	Brent *et al.*, 2014
10	1.17	210	208.9362	Galactaric acid	71, 85, 191	+	+	+	Lucas, 2021
11	1.19	118	117.01762	Succinic acid	73, 99	+	+	-	Eissa *et al.*, 2020
12	1.21	116	115.00307	Maleic acid	71	+	+	+	Brent *et al.*, 2014
13	1.22	174	173.00958	Cis-Aconitate	85, 111, 129	+	+	+	Lucas, 2021
14	1.23	192	190.97238	Citric acid #	111, 129	+	+	+	Marzouk *et al.*, 2018
15	1.24	192	191.05583	Quinic acid	85, 111, 127, 173	+	+	+	Marzouk *et al.*, 2018
16	1.25	196	195.05165	Gluconic acid	129, 159, 177	+	+	-	Lucas, 2021
17	1.265	161	160.06158	1H-indole-3-carboxylic acid	142, 116	-	+	+	Gamir *et al.*, 2012
18	1.27	106	105.01991	Glyceric acid	73, 75, 57, 59, 47	-	+	+	Lucas, 2021
19	1.28	148	147.02885	Citramalate	85, 87, 129, 103	-	-	+	Wu *et al.*, 2010
20	1.29	150	149.04518	Tartaric acid #	73	-	+	-	Brent *et al.*, 2014
21	1.38	146	145.06139	2-Methylglutaric acid	101, 127	-	+	+	Lucas, 2021

Table 1. *Continues.*

22	1.46	176	175.06165	2-Isopropylmalic acid	157, 129, 131, 115, 113, 85	+	-	-	Ricciutelli *et al.*, 2019
23	1.51	139	138.0556	6-Hydroxynicotinic acid	94	+	-	-	Llorach *et al.*, 2009
24	2.15	354	353.0878	Chlorogenic acid [#]	173, 179, 191	+	-	+	Zhang *et al.*, 2010
25	4.83	148	147.04594	Cinnamic acid	103, 119	+	-	-	Zhang *et al.*, 2010
					Flavonoids				
26	4.36	500	449.10742	Eriodictyol-7-*O*-glucoside	269, 287	+	+	-	Krasteva & Nikolov, 2008
27	4.93	290	289.07294	Catechin	109, 123, 149, 179, 205, 245	-	+	-	Kang *et al.*, 2016
28	6.00	448	447.09247	Luteolin-6-*C*-glucoside [#]	285, 297, 327, 357, 429	+	+	+	Hassan *et al.*, 2019
29	6.42	462	461.10702	Luteolin-7-OCH_3-6-*C*-glucoside [$]	298, 313, 415, 446	+	-	+	Wang *et al.*, 2008; Kumarasamy *et al.*, 2004
30	6.45	578	577.2334	Vitexin-2"-O-rhamnoside	269, 293, 311, 341, 413	+	+	+	Hassan *et al.*, 2019
31	6.78	464	463.09241	Quercetin 3-hexoside	301	-	-	+	Marzouk *et al.*, 2018
32	6.87	578	577.15472	Apigenin 7-*O*-neohesperidoside	413, 269	+	+	+	Brito *et al.*, 2014
33	7.21	448	447.09341	Luteolin-7-*O*-glucoside [*#]	285, 179, 151	+	+	+	Marzouk *et al.*, 2018
34	7.64	448	447.09177	Maritimetin-6-*O*-hexoside	285, 133	+	+	-	Cao-Ngoc *et al.*, 2020
35	7.84	432	431.11929	Apigenin-7-*O*-glucoside [#]	268, 269, 311	+	+	+	Marzouk *et al.*, 2018
36	7.95	434	433.10678	Naringenin-7-*O*-glucoside	271, 151	+	+	-	Kang *et al.*, 2016
37	8.11	448	447.08871	Luteolin-8-*C*-glucoside [#$]	327, 357, 429	+	+	+	Hassan *et al.*, 2019; Almahy & Fouda, 2013
38	9.52	286	285.03873	Luteolin [*#]	133, 217, 241, 151	+	+	+	Marzouk *et al.*, 2018
39	10.13	272	271.06351	Naringenin	107, 125, 151	-	+	+	Kang *et al.*, 2016
40	10.45	270	269.04691	Apigenin [*#]	151, 225	+	-	+	Brito *et al.*, 2014
41	12.17	268	267.06525	Formononetin	224, 252	+	-	-	Zhao *et al.*, 2020

Table 1. *Continues.*

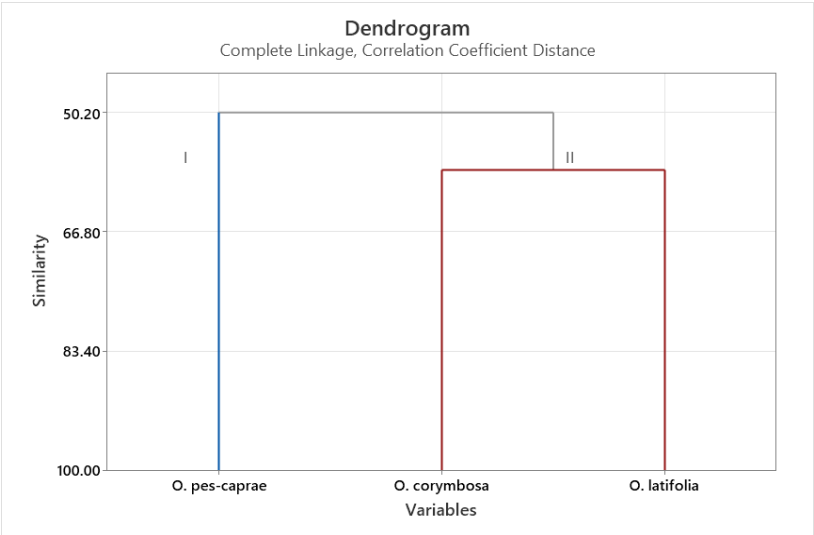
42	14.67	284	283.06726	Acacetin [#]	268, 240, 211	-	+	-	Ben Salah *et al*., 2019
Coumarins									
43	8.33	178	177.0925	Esculetin	133, 149	+	-	-	Ruan *et al*., 2019
Stilbenes									
44	4.81	406	405.17596	Astringin	225, 243	+	+	+	Guerrero *et al*., 2020
45	9.10	228	227.07181	Resveratrol	143, 157, 159, 183, 185	-	+	-	Guerrero *et al*., 2020
Terpenes									
46	1.26	136	135.02867	Gamma-terpinene	89, 117	+	+	+	(MassBank Record: PT208863).
Sugars									
47	1.21	260	259.02151	Mannose 1-phosphate	79	+	+	+	Cocuron & Alonso 2014
48	1.41	180	179.0554	Hexoside	89, 161	+	+	+	Jin *et al*. 2018
49	1.47	342	341.10941	Sucrose	89, 119, 161, 179	+	+	+	Jin *et al*. 2018
Sugar alcohols									
50	1.39	182	181.07173	Mannitol	89, 119, 163	+	+	-	Gervasoni *et al*. 2016

Notes: [#]Compounds that were previously identified from genus *Oxalis* L.
[$] Compounds that were separated and identified in the present study from *O. pes-caprae* using [1]H-NMR and UV/vis.
[*]Compounds that were identified in the present study from *O. pes-caprae* via co-chromatography.
+ = present, - = absent

3.2. Statistical Analysis

The statistical analysis was presented in a dendrogram that showed two clusters (I & II) at similarity level 50.2%. Cluster I ended with *O. pes-caprae* while cluster II included both *O. corymbosa* and *O. latifolia* at 58.19% similarity level, results being in accordance with Lourteig's (2000) findings about the sectional affinity of those species (Figure 3).

Figure 3. Dendrogram for the numerical analysis of the extracted chemical data.



3.3. Chemosystematic Evaluation

According to Lourteig (2000), the investigated members of *Oxalis* in this study could be distributed under two sections *viz*. section Cernuae Knuth includes *O. pes-caprae* and section Ionoxalis (small) Knuth comprises *O. corymbosa* and *O. latifolia*.

On comparing the chemical profiles of the studied taxa, significant differences among them were recorded, where *O. pes-caprae* can be recognized by the presence of 3, 4-Dihydroxybenzoic acid, esculetin, formononetin, cinnamic acid, 6-Hydroxynicotinic acid and 2-Isopropylmalic acid in its extract and lacking of naringenin, glyceric acid, 1H-indole-3-carboxylic acid and 2-Methylglutaric acid compounds.

O. corymbosa was characterized by presence of catechin, tartaric acid, acacetin and resveratrol, in addition to the absence of chlorogenic acid, apigenin and luteolin-7-OCH_3-6-*C*-glucoside in its extract.

O. latifolia can be distinguished from the other species by lacking of maritimetin-6-*O*-hexoside, 5-Methoxysalicylic acid, succinic acid, gluconic acid, eriodictyol-7-*O*-glucoside, mannitol, naringenin-7-*O*-glucoside, and rosmarinic acid in its extract, as well as the presence of salicylic acid, citramalate, and quercetin 3-hexoside. Among the studied plants, *O. latifolia* is the only species being characterized with its ability of forming flavonol compounds (quercetin 3-hexoside), these compounds were previously reported in *O. corniculata* L. (Prasad Pandey *et al*., 2020).

The present study also helps in understanding the chemotaxonomic relationship between the studied *Oxalis* species at the sectional level. Certain identified compounds confirmed the sectional demarcation of *Oxalis* as suggested by Lourteig (2000). Section Cernuae represented by *O. pes-caprae* could be distinguished from section Ionoxalis by the presence of 3,4-dihydroxybenzoic acid, 2-isopropylmalic acid, 6-hydroxynicotinic acid, cinnamic acid, formononetin, and esculetin, and lacking of glyceric acid, 2-methylglutaric acid, 1H-indole-3-carboxylic acid and naringenin. Coumarin and isoflavone compounds (esculetin and formononetin) were recorded in *O. pes-caprae* for the first time, and they have never been detected in any other *Oxalis* species, so it may be a valuable compound in distinguishing between *Oxalis* sections.

Our chemical and statistical findings were in consistence with Lourteig (2000) findings that supported her taxonomic treatment concerning with the sectional affinities of the species under investigation.

As new chemical classes were detected in the present work from *Oxalis* plants (flavanones, stilbenes and catechin) and to have a complete overview concerning the chemotaxonomic evaluation of *Oxalis* sections and their species, more investigations are needed for the rest of the worldwide distributed *Oxalis* species and in particular for section Cernuae.

4.CONCLUSION

This is the first chemical report to shed light on the polar constituents of Egyptian *Oxalis* that was an informative achievement with respect to both *O. corymbosa* and *O. latifolia* that had not been previously subjected to any chemical separation and identification processes. The obtained results suggested significant chemical variations among the three *Oxalis* sp. that could be a diagnostic character in their identification. All the chemical and statistical data supported the grouping of the studied taxa within two sections under genus *Oxalis* L. being Ionoxalis and Cernuae.

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Amal A. Draz: Design, analysis and interpretation. **Salwa A. Kawashty**: Supervision, data collection and processing. **Sameh R. Hussein:** Conception, supervision, critical review. **Eman M. Shamso**: Supervision, writing. **Hasnaa A. Hosni:**Supervision.

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REFERENCES

Almahy, H.A., & Fouda, H.A.R. (2013) Isolation of luteolin 8-*C*-*β*-D-glucopyranoside from the roots of *Salvadora persica* (Rutaceae). *Journal of Current Chemical and Pharmaceutical Sciences*, *3*(1), 49-53.

Ben Salah, H., Smaoui, S., Abdennabi, R., & Allouche, N. (2019). LC-ESI-MS/MS phenolic profile of *Volutaria lippii* (L.) Cass. extracts and evaluation of their in vitro antioxidant, Antiacetylcholinesterase, antidiabetic, and antibacterial activities. *Evidence-Based Complementary and Alternative Medicine*, *2019*. https://doi.org/10.1155/2019/9814537

Brent, L.C., Reiner, J.L., Dickerson, R.R., & Sander, L.C. (2014). Method for characterization of low molecular weight organic acids in atmospheric aerosols using ion chromatography mass spectrometry. *Journal of Analytical Chemistry, 86*(15), 7328-7336. https://doi.org/10.1021/ac403937e

Brito, A., Ramirez, J.E., Areche, C., Sepúlveda, B., & Simirgiotis, M.J. (2014). HPLC-UV-MS profiles of phenolic compounds and antioxidant activity of fruits from three *citrus* species consumed in Northern Chile. *Molecules*, *19*(11), 17400-17421. https://doi.org/10.3390/molecules191117400

Cao-Ngoc, P., Leclercq, L., Rossi, J.C., Hertzog, J., Tixier, A.S., Chemat, F., Nasreddine, R., Al HamouiDitBanni, G., Nehmé, R., Schmitt-Kopplin, P., & Cottet, H. (2020). Water-based extraction of bioactive principles from black currant leaves and *chrysanthellum americanum*: A comparative study. *Foods*, *9*(10), 1478. https://doi.org/10.3390/foods9101478

Cocuron, J.C., & Alonso, A.P. (2014). *Liquid chromatography tandem mass spectrometry for measuring ¹³C-labeling in intermediates of the glycolysis and pentose phosphate pathway*. Plant Metabolic Flux Analysis, Springer. https://doi.org/10.1007/978-1-62703-688-7_9. PMID: 24222414.

Dellagreca, M., Previtera, L., Purcaro, R., & Zarrelli, A. (2009). Phytotoxic Aromatic Constituents of *Oxalis pes-caprae*. *Chemistry & Biodiversity*, *6(1)*, 459-465. https://doi.org/10.1002/cbdv.200800179

Draz, A.A., Shamso, E.M., Hosni, H.A., Kawashty, S.A., & Hussein, S.R. (2021). Comparative study of *Oxalis* L. species growing wild in Egypt: GC-MS analysis and chemosystematic significance. *Egyptian Journal of Chemistry*, *64*(9), 5251-5256. https://doi.org/10.21608/EJCHEM.2021.72328.3592

Eissa, M.A., Hashim, Y.Z., El-Kersh, D.M., Abd-Azziz, S.S., Salleh, H.M., Isa, M.L.M., & Abd Warif, N.M. (2020). Metabolite Profiling of *Aquilaria malaccensis* leaf extract using Liquid Chromatography-Q-TOF-Mass spectrometry and investigation of its potential antilipoxygenase activity in-vitro. *Processes*, *8*(2), 202. https://doi.org/10.3390/pr8020202

El-Khanagry, S.S.G. (2005). New records of dicotyledonous taxa to the flora of Egypt. *Bulletin of Faculty of Agriculture, Cairo University, 56*(1), 89-105.

Gamir, J., Pastor, V., Cerezo, M., & Flors, V. (2012). Identification of indole-3- carboxylic acid as mediator of priming against cucumerina. *Plant Physiology and Biochemistry*, *61*, 169-179. https://doi.org/10.1016/j.plaphy.2012.10.004

Gaspar, M.C., Fonseca, D.A., Antunes, M.J., Frigerio, C., Gomes, N.G., Vieira, M., Santos, A.E., Cruz, M.T., Cotrim, M.D., & Campos, M.G. (2018). Polyphenolic characterisation and bioactivity of an *Oxalis pes*-caprae L. leaf extract. *Natural Product Research*, *32*, 732-738. https://doi.org/10.1080/14786419.2017.1335728

Gervasoni, J., Schiattarella, A., Giorgio, V., Primiano, A., Russo, C., Tesori, V., Scaldaferri, F., Urbani, A., Zuppi, C., & Persichilli, S. (2016). Validation of an LC-MS/MS method for urinary lactulose and mannitol quantification: Results in patients with irritable bowel syndrome. *Disease Markers*, *2016*, ID 5340386, 6. https://doi.org/10.1155/2016/5340386

Güçlütürk, I., Detsi, A., Weiss, E.K., Ioannou, E., Roussis, V., & Kefalas, P. (2012). Evaluation of anti-oxidant activity and identification of major polyphenolics of the invasive weed *Oxalispes*-caprae. *Phytochemical Analysis, 23*(6), 642-646. https://doi.org/10.1002/pca.2367

Guerrero, R.F., Valls-Fonayet, J., Richard, T., & Cantos-Villar, E. (2020). A rapid quantification of stilbene content in wine by ultra-high pressure liquid chromatography–Mass spectrometry. *Food Control*, 108, 106821. https://doi.org/10.1016/j.foodcont.2019.106821

Hassan, W.H., Abdelaziz, S., & Al Yousef, H.M. (2019). Chemical composition and biological activities of the aqueous fraction of *Parkinsonea aculeata* L. growing in Saudi Arabia. *Arabian Journal of Chemistry, 12*(3), 377-387. https://doi.org/10.1016/j.arabjc.2018.08.003

Hussein, S.R., Latif, R.R.A., Marzouk, M.M., Elkhateeb, A., Mohammed, R.S., Soliman, A.A., & Abdel-Hameed, E.S.S. (2018). Spectrometric analysis, phenolics isolation and cytotoxic activity of *Stipagrostis plumosa* (Family Poaceae). *Chemical Papers-Chemicke Zvesti*, *72*(1), 29-37. https://doi.org/10.1007/s11696-017-0254-0

Jin, J., Lao, J., Zhou, R., He, W., Qin, Y., Zhong, C., Xie, J., Liu, H., Wan, D., Zhang, S., & Qin, Y. (2018). Simultaneous identification and dynamic analysis of saccharides during steam processing of rhizomes of *Polygonatum cyrtonema* by HPLC–QTOF–MS/MS. *Molecules*, *23*(11), 2855. https://doi.org/10.3390/molecules23112855

Kang, J., Price, W.E., Ashton, J., Tapsell, L.C., & Johnson, S. (2016). Identification and characterization of phenolic compounds in hydromethanolic extracts of *sorghum* whole grains by LC-ESI-MS^n. *Food Chemistry*, *211*, 215-226. https://doi.org/10.1016/j.foodchem.2016.05.052

Krasteva, I., & Nikolov, S. (2008). Flavonoids in *Astragalus corniculatus*. *Quím Nova*, *31*, 59-60. https://doi.org/10.1590/S0100-40422008000100012

Kumarasamy, Y., Byres, M., Cox, P.J., Delazar, A., Jaspars, M., Nahar, L., Shoeb, M., & Sarker, S.D. (2004). Isolation, structure elucidation, and biological activity of flavone 6-*C*-glycosides from *Alliaria petiolata*. *Chemistry of Natural Compounds*, *40*(2), 122-128.

Lin, Y.T., Mao, Y.W., Imtiyaz, Z., Chiou, W.F., & Lee, M.H. (2020). Comprehensive LC-MS/MS-based phytochemical perspectives and osteogenic effects of *Uraria crinita*. *Food Function*, *11*(6), 5420-5431. https://doi.org/10.1039/D0FO00782J

Llorach, R., Urpi-Sarda, M., Jauregui, O., Monagas, M., & Andres-Lacueva, C. (2009). An LC-MS-based metabolomics approach for exploring urinary metabolome modifications after

cocoa consumption. *Journal of Proteome Research*, *8*(11), 5060-5068. https://doi.org/10.1021/pr900470a

Lourteig, A. (2000). *Oxalis* L., subgenera Monoxalis (Small) Lourt., *Oxalis* y Trifidus Lourt. *Bradea*, *7*(2), 201–629.

Lucas, A.R. (2021). Viruses as therapeutics: Methods and protocols. Springer.

Marzouk, M.M., Hussein, S.R., Elkhateeb, A., El-shabrawy, M., Abdel-Hameed, E.S.S., & Kawashty, S.A. (2018). Comparative study of *Mentha* species growing wild in Egypt: LC-ESI-MS analysis and chemosystematic significance. *Journal of Applied Pharmaceutical Science*, *8*, 116-122. https://doi.org/10.7324/JAPS.2018.8816

MassBank Europe, Mass Spectral DataBase. Available online: https://massbank.eu/MassBank/ (accessed on 19thJanuary, 2022).

Minitab 21, Statistical & Data Analysis Software. Available online: https://www.minitab.com/en-us/support/downloads/ (accessed on 17thFebruary, 2022).

Oberlander, K.C., Dreyer, L.L., & Elser, K.J. (2002). Biogeographv of *Oxalis* (Oxalidaceae) in South Africa: A preliminary study. *Bothalia*. *African Biodiversity & Conservation*, *32*(1), 97-100. https://doi.org/10.4102/abc.v32i1.472

Prasad Pandey, B., Prakash Pradhan, S., & Adhikari, K. (2020). LC-ESI-QTOF-MS for the profiling of the metabolites and in vitro enzymes inhibition activity of *Bryophyllum pinnatum* and *Oxalis corniculata* collected from Ramechhap District of Nepal. *Chemistry & Biodiversity*, *17*, e2000155. https://doi.org/10.1002/cbdv.202000155

Ricciutelli, M., Moretti, S., Galarini, R., Sagratini, G., Mari, M., Lucarini, S., Vittori, S., & Caprioli, G. (2019). Identification and quantification of new isomers of isopropyl-malic acid in wine by LC-IT and LC-Q-Orbitrap. *Food Chemistry*, *294*, 390-396. https://doi.org/10.1016/j.foodchem.2019.05.068

Ruan, J., Yan, J., Zheng, D., Sun, F., Wang, J., Han, L., Zhang, Y., & Wang, T. (2019). Comprehensive chemical profiling in the ethanol extract of *Plucheaindica* aerial parts by liquid chromatography/mass spectrometry analysis of its silica gel column chromatography fractions. *Molecules*, *24*(15), 2784. https://doi.org/10.3390/molecules24152784

Sarfraz, I., Rasul, A., Hussain, G., Shah, M. A., Nageen, B., Jabeen, F., Selamoğlu, Z., Uçak, İ., Asrar, M., & Adem, S. (2022). A review on phyto-pharmacology of *Oxalis corniculata*. Comb. *Combinatorial Chemistry & High Throughput Screening*, *25*(7), 1181-1186. https://doi.org/10.2174/1386207324666210813121431

Täckholm, V. (1974). Students' Flora of Egypt (2nd ed.). Cairo University, Cairo.

The plant list (2013). Version1.1.. Available online: http://www.theplantlist.org/ (accessed 1st January, 2021).

Wang, X., Qian, Y., Li, X., Jia, X., Yan, Z., Han, M., Qiao, M., Ma, X., Chu, Y., Zhou, S., & Yang, W. (2021). Rapid determination of rosmarinic acid and its two bioactive metabolites in the plasma of rats by LC–MS/MS and application to a pharmacokinetics study. *Biomedical Chromatography*, *35*(2), e4984. https://doi.org/10.1002/bmc.4984

Wang, Z., Ma, C., Tang, S., Xiao, H., Kakiuchi, N., Kida, H., & Hattori, M. (2008). Qualitative and quantitative analysis of *Swertia* herbs by high performance liquid chromatography-diode array detector-mass spectrometry (HPLC-DAD-MS). *Chemical and Pharmaceutical Bulletin*, *56*(4), 485-490. https://doi.org/10.1248/cpb.56.485

Wu, B., Zhang, B., Feng, X., Rubens, J.R., Huang, R., Hicks, L.M., Pakrasi, H.B., & Tang, Y.J. (2010). Alternative isoleucine synthesis pathway in cyanobacterial species. *Microbiology*, *156*(2), 596-602. https://doi.org/10.1099/mic.0.031799-0

Zhang, J., Chen, M., Ju, W., Liu, S., Xu, M., Chu, J., & Wu, T. (2010). Liquid chromatography/Tandem mass spectrometry assay for the simultaneous determination of chlorogenic acid and cinnamic acid in plasma and its application to a pharmacokinetic study.

Journal of Pharmaceutical and Biomedical Analysis, *51*(3), 685-690.-https://doi.org/10.1016/j.jpba.2009.09.039

Zhao, X., Zhang, S., Liu, D., Yang, M., & Wei, J. (2020). Analysis of flavonoids in *dalbergia odorifera* by Ultra-Performance Liquid Chromatography with Tandem mass spectrometry. *Molecules*, *25*(2), 389. ; https://doi.org/10.3390/molecules25020389

Zhong, B., Robinson, N.A., Warner, R.D., Barrow, C.J., Dunshea, F.R., & Suleria, H.A. (2020). LC-ESI-QTOF-MS/MS characterization of seaweed phenolics and their antioxidant potential. *Marine Drugs*, *18*(6), 331. https://doi.org/10.3390/md18060331

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Review Article

Medicinal uses, chemical constituents and biological activities of *Rumex abyssinicus*: A Comprehensive review

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Abstract: *Rumex abyssinicus* is a valuable medicinal plant species that is native to tropical Africa. Traditionally, *R. abyssinicus* is used to treat different disease such as, liver diseases, hepatitis, malaria, scabies, blood pressure, jaundice, wound and pneumonia. The purpose of the current study was to review the literature on the ethnomedicinal uses, chemical constitutes and biological activities of *R. abyssinicus* in an attempt to create information for future studies aimed towards exploring the therapeutic ability of the species. A scientific search engines, namely Google Scholar, PubMed, Scopus, Science Direct and Web of knowledge for the search terms: *Rumex abyssinicus*, ethnomedicinal studies, phytochemical investigations, and pharmacological activities were undertaken. The search strategy included all articles with descriptors that were available until December 30, 2021. Only published works in English have been used on this study. The data was collected using textual descriptions of the studies, tabulation, grouping, and figures. The principal phytochemicals of *R. abyssinicus* are anthraquinones, flavonoids, terepenoids and phenolic compounds. The *in vitro* and *in vivo* studies on the crude extracts and compounds of *R. abyssinicus* showed antibacterial, antioxidant, anticancer, anti-inflammatory, antifungal, wound healing, antialzeimer's and hepatoprotective activities of it. *R. abyssinicus* afforded drug leads such as helminthosporin (**4**) with anti-alzheimer and physicon (**3**) with antifungal and antioxidant activity. *R. abyssinicus* have traditionally been used to cure a variety of diseases. Pharmacological actions of phytochemicals were shown to be promising. Despite this, further studies on crude extracts and promising compounds are needed to find new drug candidates.

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1. INTRODUCTION

Rumex abyssinicus is a flowering plant (genus: Rumex, family: *Polygonaceae*) native to tropical Africa. Often used in traditional African medicine for the treatment of hepatitis, asthma, stomachache, anthrax, hypertension, pneumonia, cancer and Diabetes Mellitus (Gorsi & Miraj, 2002; Mishra *et al*., 2018). It is a perennial plant with a thick, fleshy rhizome that develops up to three meters tall. The plant grows in a variety of soils, from mild loam to clay loam, but

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favors sufficiently heavy soils that retain moisture. It can grow in any soil pH condition, including acid, neutral, and alkaline soils, and in semi-shade or no-shade areas. The plant's leaves is usually sagittate, and the inflorescence and lots of branches. The plant is propagated by means of seeds (Awas, 2007). It is medicinal and meals additive plant. The shoots and leaves are safe to eat, and the tuber can be used as a tea to help with meal insecurity (Duguma, 2020; Hassen, 2021). The rhizomes have been used to refine butter, giving it a deep yellow color, while the roots are utilized for therapy (Mekonnen *et al*., 2010). In Ethiopia, *R. abyssinicus* (makmeko in Amharic) is widely distributed species with wide spectrum of ethnomedicinal uses such as hepatitis, hypertension, tuberculosis, jaundice and high blood pressure (Tsegay *et al*., 2019; Tuasha *et al*., 2018). Given the species' established ethnomedicinal use, *R. abyssinicus* has the potential to promote a vital task in community primary healthcare across its natural distribution. As a result, the goal of this review was to get an in-depth assessment of existing data and literature on *R. abyssinicus'* medicinal benefits, chemical constituents, and biological activities in order to compile a database of records that could be used in future studies aimed at determining the species' therapeutic potential.

Figure 1. Leave and steam parts of *R. abyssinicus* (Workneh, 2017).

2. METHODOLOGY OF THE REVIEW

A scientific search engines, namely Google Scholar, PubMed, Scopus, Science Direct and Web of knowledge for the search terms: *Rumex abyssinicus* and synonyms of the species, ethnomedicinal studies, phytochemical investigations, and pharmacological activities were undertaken. The search strategy included all articles with descriptors that were available until December 30, 2021. Only published works in English have been used on this study. The data was collected using textual descriptions of the studies, tabulation, grouping, and figures. The worldwide plant name index (https://www.ipni.org) and the Kew Botanical Garden plant name database (https://www.kew.org) were used to check species names and its synonyms.

3. ETHNOMEDICINAL USES

Throughout the species' distributional area, the leaves, fruits, roots, rhizomes, and stem are said to have numerous traditional medicinal characteristics and treat a variety of human and animal ailments (Table 1). In Ethiopia the root decoction of *R. abyssinicus* macerated with water and taken orally as remedy of liver diseases (Gabriel & Guji, 2014). In Uganda, chewing *R. abyssinicus* leaves is used to treat gynecological morbidity and allergies (Gumisiriza *et al*., 2021; Kamatenesi-Mugisha *et al*., 2007). The majority of the *R. abyssinicus* remedies are used as mono therapies indicated in (Table 1). However, multi therapies/ a mixture of dry leaf of *R. abyssinicus* with leaf of *Zehneria scabra* is pounded, powdered, mixed with milk and boiled, then drunk when it is cooled for malaria remedy (Omara, 2020).

Table 1. Ethnomedicinal uses and preparation methods of different parts of *R. abyssinicus*

Disease Treated	Parts Used	Method of Preparation	Country Practiced	Ref.
Hepatitis	Root, Aerial parts	The roots are crushed, powdered, and mixed with bat meat that has been dried and powdered, then eaten once or twice. The fluid is ingested after chewing the roots.	Ethiopia	(Limenih *et al*., 2015; Novotna *et al*., 2020; Suleman & Alemu, 2012; Tegen *et al*., 2021)
Liver disease,	Root	Orally, the root is mashed, macerated with water, and consumed or Powdered and drunk 1 tea cup with coffee/tea	Ethiopia	(Etana, 2010; Gabriel & Guji, 2014)
Gonorrhea	Root	Pounding the fresh roots, boiling, adding butter and drinking one water glass daily until cured.	Ethiopia	(Eshete *et al*., 2016)
TB	Root	steeping the fresh/dried root in boiled water and drunk a cup of the resulting decoction	Ethiopia	(Gidey *et al*., 2015)
Bone Tuberculosis	Root	The paste is softened with cow butter	Ethiopia	(Moravec *et al*., 2014)
Scabies	Leaves	Fresh leaves are pulverized and blended and applied to the injured area	Ethiopia	(Megersa, 2010)
Malaria	Leaves	The dry leaves of *R. abyssinicus* and *Z. scabra* are powdered, combined with milk, and boiled, then cooled or consumed orally as a decoction	Ethiopia, Kenya	(Eskedar, 2011; Omara, 2020)
Gynecological morbidity	Leaves/ Steams	Chewing, Squeezing and boiling	Uganda	(Kamatenesi-Mugisha *et al*., 2007)
Allergy	Leaves	Chewing	Uganda	(Gumisiriza *et al*., 2021)
Heamaturia	Roots	Pounding	Ethiopia	(Dinbiso *et al*., 2020)
Eye infections	Leaves	Eye drops/ boiled	Kenya	(Odongo *et al*., 2018)
Hypertension	Leaves	The dried leaves is boiled in water and the liquid is consumed	Ethiopia	(Tsegay *et al*., 2019)
Ascariasis	Root	Pulverized the root, mixed it with a small amount of water, and drank the solution	Ethiopia	(Dalle, 2019; Getu, 2017; Khan *et al*., 2018)
Fungal and bacterial infection	Leaves/ Steams	Chewing, pounding and Smearing	Uganda	(Kamatenesi-Mugisha *et al*., 2008)
Breast cancer	Rhizome	Concoction taken oral	Ethiopia	(Bussa & Belayneh, 2019)
Amoeba	Root	Fresh root crush, mix with tea/milk and drink	Ethiopia	(Regassa, 2013; Sina & Degu, 2015)
Gonorrhea	Root	Infusion/ decoction	Ethiopia, Rwanda	(Silva *et al*., 2020; Teka *et al*., 2020)
Liver complaint, Kidney problem	Root	Infusion/ decoction	Ethiopia	(Teka *et al*., 2020)
Syphilis	Leaves	pound and mixed with water and drunk, 150mL 3x daily	Uganda	(Gumisiriza *et al*., 2019)

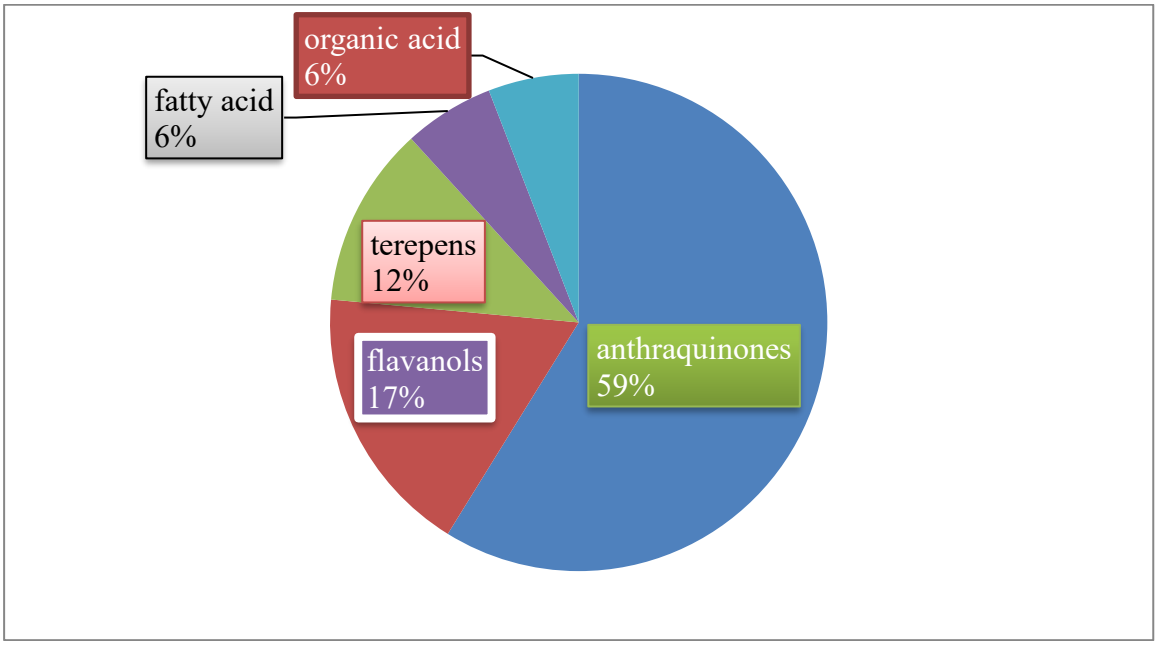
Table 1. Continued

Jaundice	Root	Fresh roots are pulverized, boiled, and given orally in a cup	Ethiopia	(Tuasha *et al*., 2018)
Pneumonia	Stem	Crushed/ chewed	Kenya	(Kamau *et al*., 2016)
Diabetes Mellitus	Aerial Parts	The aerial parts are decocted after being crushed	Ethiopia	(Suleman & Alemu, 2012)
Skin infection	Leaves	Pulverized and mashed leaves are applied to the affected area after being soaked in water	Ethiopia	(Birhanu *et al*., 2015)
Anthrax	Root	For five days, dry roots are crushed, boiled in water, and drunk with honey	Ethiopia	(Birhan *et al*., 2017)
Expel delayed embryo in the uterus	Root	After powdering the dry root and mixing it with water, drink it	Ethiopia	(Chekole, 2017)
Headache	Leave/Root	Adding to tea	Ethiopia	(Teklay *et al*., 2013)
Goiter	Root	Dry roots chewed and swallowed	Ethiopia	(Sina & Degu, 2015)
Snake bite	Root	Pulverize the root and mix it with honey before chewing and swallowing	Ethiopia	(Assefa *et al*., 2019)
Scabies	Whole plant	Pounded whole plant, mixed with water, and used to wash the animal	Ethiopia	(Kefalew *et al*., 2015)
Blackleg	Root	Dry root pound and drunk to the cattle with ATELLA (By product of local alcohol, Tella)	Ethiopia	(Kefalew *et al*., 2015)
Tooth diseases	Root	Chewing / hold with teeth	Ethiopia	(Gebru *et al*., 2021)
Vomiting	Root	Drink as herbal tea	Ethiopia	(Gebru *et al*., 2021)
Diuretic, carminative	Leaves	-	Pakistan	(Muhammad *et al*., 2021)
Gastrointestinal disorders	Root	-	Ethiopia	(Guadie *et al*., 2020)

4. CHEMICAL CONSTITUENT

Flavonoids, phenols, anthraquinones, saponins, cardiac glycosides, tannins, steroids, and quinones were detected in the qualitative phytochemical screening of *R. abyssinicus*, which could be linked to the plant's therapeutic effectiveness (Awoke & Gedamu, 2020; Kebede *et al*., 2021; Workineh, 2021; Workneh, 2017). Previous investigation into the chemical constituents of *R. abyssinicus* have led to the isolation of antraquinones (**1-10**), flavanols (**11-13**), terepens (**14-15**), fatty acid (**16**) and organic acid (**17**) (Figure 1). The chemical structures of these compounds are shown in Figures 2 and summarized in (Table 2).

Figure 1. Structure classes from *R. abyssinicus*.



4.1. Anthraquinones

Anthraquinones are a significant class of compounds with numerous applications. Anthraquinone-containing plants, such as rhubarb and aloe, have been known and utilized in folk medicine for over 4000 years (Monks *et al*., 1992). Biologically active anthraquinone derivatives have also been identified in bacteria, fungi and insects (Malik & Müller, 2016). Both natural and synthetic anthraquinones are widely used in textile dying, paints, imaging devices, foods, cosmetics, and pharmaceuticals (Malik *et al*., 2015). So far, 10 anthraquinones (**1-10**) have been isolated and identified from various parts of this plant, including the rhizome, root tuber, and whole plant (Augustin *et al*., 2020; Fassil *et al*., 1985; Kengne *et al*., 2021; Munavu *et al*., 1984; Shifa *et al*., 2021; Tala *et al*., 2018). Out of ten, six anthraquinones have been investigated its pharmacological activities (Augustin *et al*., 2020; Kengne *et al*., 2021).

4.2. Flavanols

Flavanols are distinguished sub-group of flavonoids by the absence of a double bond between C-2 and C-3 and the absence of a carbonyl group on the C ring (C-4), as well as the presence of hydroxyl group(s) on C-3 or C-4. Flavanols have previously been found in cereals, legumes, fruits, vegetables, forages, hops, beers, red wine, tea, cocoa, grapes, and apples, among other foods (Manach *et al*., 2004). Three flavanols (**11-13**) have been isolated and identified from this plant's root parts so far (Tala *et al*., 2018).

4.3. Terpenes

Terpenes have the chemical formula $(C_5H_8)_n$, which is defined by the unit isoprene. From the whole part and root of *R. abyssinicus* two compounds (**14** and **15**) were isolated (Fufa *et al*.,

2016; Kengne *et al*., 2021). The compounds' pharmacological activities have not been investigated.

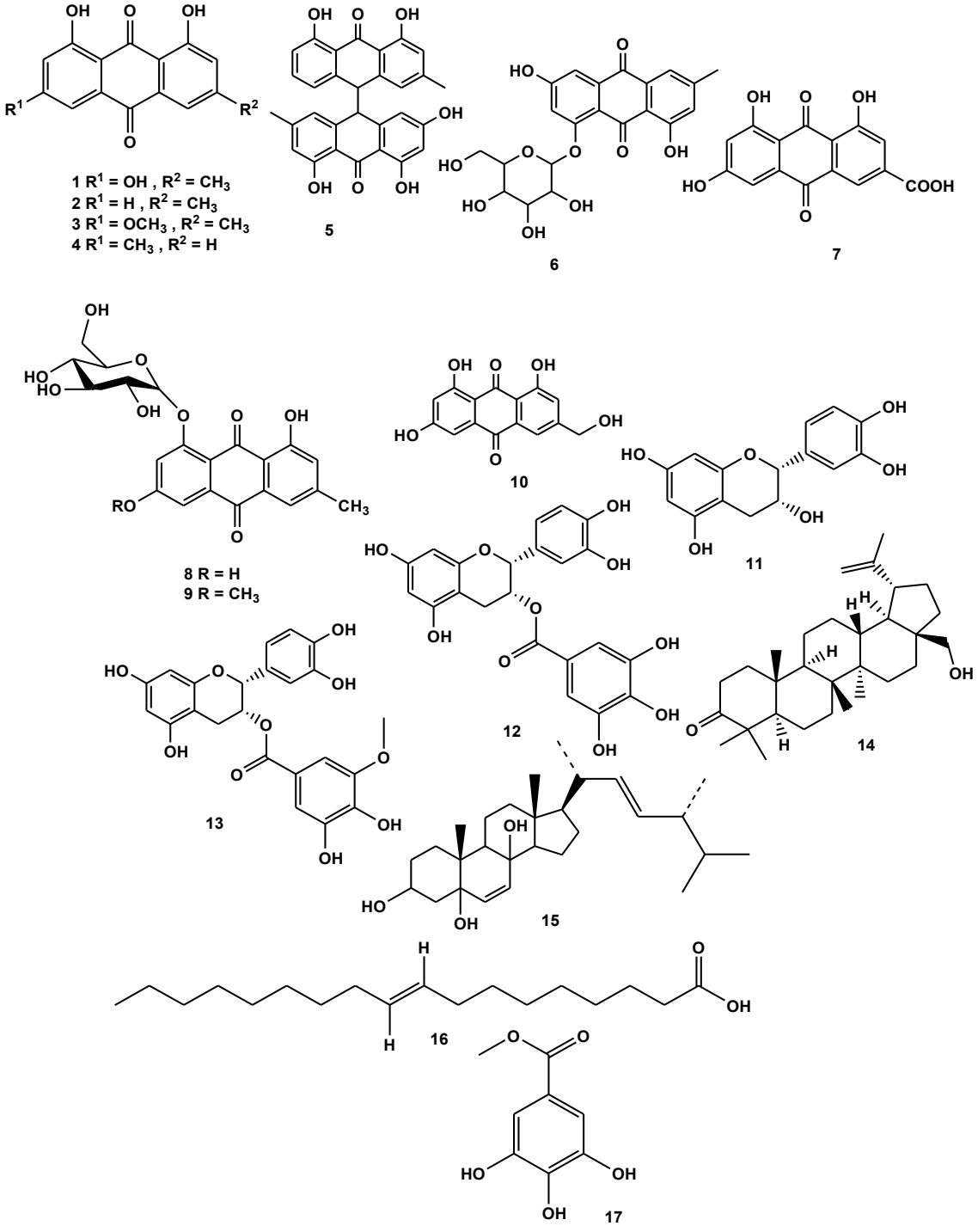
4.4. Organic and Fatty cids

Organic and fatty acid are another important component of *R. abyssinicus*. The compound (**16** and **17**) was isolated in the roots of the plant (Fufa *et al*., 2016; Tala *et al*., 2018). The compounds' pharmacological activities have not been investigated.

Table 2. Compounds isolated and identified from *R. abyssinicus*

Compound	Plant part (s)	Ref
Anthraquinones		
Emodin (**1**)	Rhizome, root, tuber, root bark, whole plant	(Augustin *et al*., 2020; Awoke & Gedamu, 2020; Fassil *et al*., 1985; Kengne *et al*., 2021; Munavu *et al*., 1984; Shifa *et al*., 2021; Tala *et al*., 2018)
Chrysophanol (**2**)	Rhizome, root, tuber, whole plant	(Augustin *et al*., 2020; Fassil *et al*., 1985; Kengne *et al*., 2021; Munavu *et al*., 1984; Shifa *et al*., 2021; Tala *et al*., 2018)
Physicon (**3**)	Rhizome, root, tuber, whole plant	(Augustin *et al*., 2020; Fassil *et al*., 1985; Kengne *et al*., 2021; Munavu *et al*., 1984; Shifa *et al*., 2021; Tala *et al*., 2018)
Helminthosporin (**4**)	Rhizome	(Augustin *et al*., 2020)
Palmadin C (emodin-chrsophanol bianthrone) (**5**)	Tuber	(Fassil *et al*., 1985)
Emodin-8-β-D-glucoside (**6**)	Tuber	(Fassil *et al*., 1985)
Emodic acid (**7**)	Root	(Tala *et al*., 2018)
Emodin-8-O-β-D-glucopyranoside (**8**)	Root, whole plant	(Kengne *et al*., 2021; Tala *et al*., 2018)
Physicon- 8-O-β-D- glucopyranoside (**9**)	Root, whole plant	(Kengne *et al*., 2021; Tala *et al*., 2018)
6-hydroxyemodin (**10**)	Whole plant	(Kengne *et al*., 2021)
Flavanol		
Epicatechin (**11**)	Root	(Tala *et al*., 2018)
Epicatechin-3-O-gallate (**12**)	Root	(Tala *et al*., 2018)
Epicatechin-3-O-(4″methyl) gallate (**13**)	Root	(Tala *et al*., 2018)
Terpenes		
Betulone (**14**)	Root	(Fufa *et al*., 2016)
Ergosta-6,22- diene-3,5,8-triol (**15**)	Whole plant	(Kengne *et al*., 2021)
Fatty Acid		
Oleic acid (**16**)	Root	(Fufa *et al*., 2016)
Organic acid		
Methylgallate (**17**)	Root	(Tala *et al*., 2018)

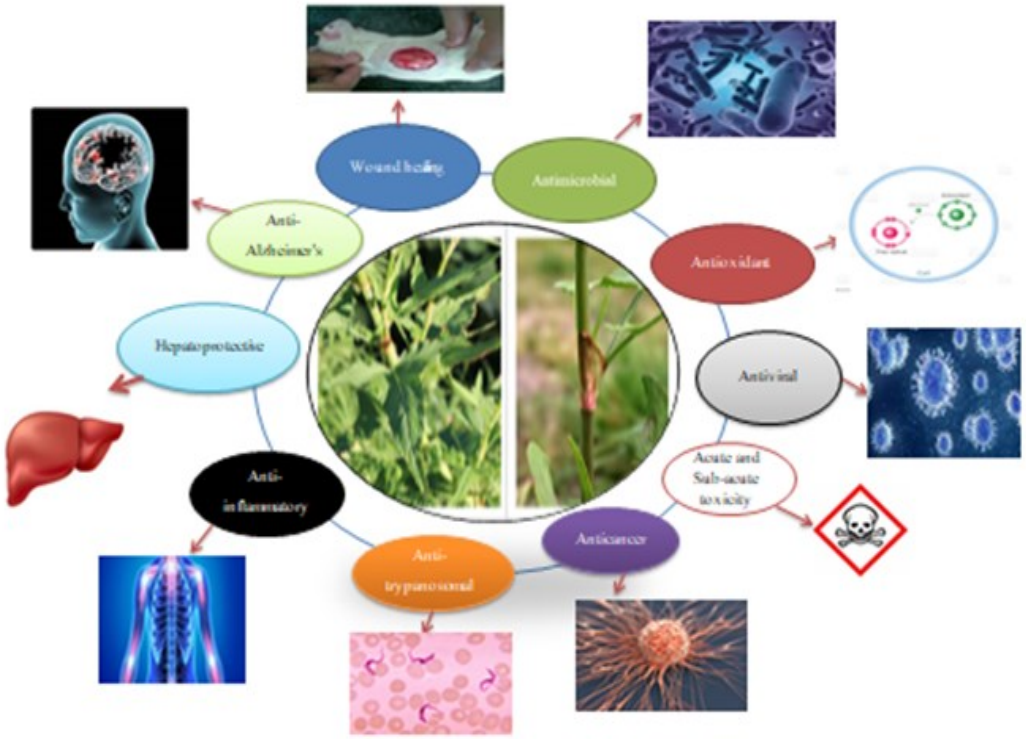
Figure 2. Compound chemical structures isolated from different parts of *R. abyssinicus*



5. BIOLOGICAL ACTIVITY

Healthcare techniques that are both modern and traditional usually coexist and complement one another. In the search for new medications, ethnomedicinal approaches are becoming commonly adopted (Gurib-Fakim, 2006). Recent interest in examining plant constituents for their pharmacological activity and screening for useful and safe phytochemicals has renewed (Nigussie *et al*., 2021). Various *in vitro* and *in vivo* pharmacological activities of *R. abyssinicus* such as antibacterial, antifungal, anti-Alzheimer's, antioxidant, anti-inflammatory, antiviral, anticancer, hepatoprotective and wound healing activity are showed in Figure 3 and mentioned below.

Figure 3. Graphical representation of pharmacological activities of *R. abyssinicus*



5.1. Antibacterial activity

Shifa *et al*. (2021) used the agar disc diffusion method using gentamicin as the standard drug to examined acetone extract of *R. abyssinicus* roots against *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, and *E. coli*. The extract revealed zone of growth inhibition against *S. aureus* (21 mm) and *P. aeruginosa* (22.5 mm) with the positive standard gentamicin has the range (34.5 to 32 mm) respectively. Additionally, chysophanol (**2**) and emodin (**1**) exhibited comparable zones of growth inhibition to the positive standard gentamicin (26 mm) against *S. aureus* (23 mm) and *K. pneumoniae* (22.5 mm). Mekonnen and Desta (2021) used the agar well diffusion method with amoxicillin as a positive control to study ethanol extract of *R. abyssinicus* rhizomes against *S. aureus*, *Methicillin-resistant S. aureus*, *S. pneumonia*, *E. coli*, and *S. flexneri*. The rhizome extract revealed inhibition zones (in mm) of 20.33, 21.67, 19.17, 18.17, and 21.67, respectively compared to amoxicillin with zone of growth inhibition in the range (10 to 14mm).

Using the Kirby-Bauer agar disc diffusion method, Getie *et al*. (2003) evaluated methanol extract of *R. abyssinicus* roots against *S. aureus*, *S. pyogenes*, *C. diphtheria*, *P. aeruginosa*, and *E. coli*. The extract exhibited poor antibacterial activity against *S. aureus*, *S. pyogenes*, and *C. diphtheria*, with inhibition zones of 8 mm, 8 mm, and 12 mm, respectively. Kengne *et al*. (2021) used a broth micro dilution method with ciprofloxacin as a positive control to examine a methanol extract of the whole part of *R. abyssinicus* and an EtOAc fraction against *S. aureus*, *methicillin sensitive S. aureus*, *methicillin resistant S. aureus*, *P. aeruginosa*, and *S. flexneri*. The methanol extract shows antibacterial activities against *P. aeruginosa*, *S. flexneri*, *S. aureus*, *methicillin sensitive S. aureus* and *methicillin resistant S. Aureus* with MIC values 64 µg/mL, 128 µg/mL, 64 µg/mL, 64 µg/mL and 64 µg/mL while the maximum activity was reported against *S. flexneri* and *S. aureus* with an EtOAc fraction MIC of 32 µg/mL. Additionally, at a MIC of 8 µg/mL, Physicon (**3**) inhibited the growth of *P. aeruginosa*, *S. flexneri*, and *S. aureus*, while Emodin (**1**) and a mixture of Emodin-8-O—D-glucopyranoside (**8**) and Physcion-8-O-D-glucopyranoside (**9**) inhibited the growth of bacteria at 16 µg/mL, 8 µg/mL respectively.

Using the agar disc diffusion method and chloromophynacol as a reference drug, Awoke and Gedamu (2020) investigated methanol and ethyl acetate extracts of *R. abyssinicus* roots against *S. aureus*, *L. Monocytogenes*, *K. pneumonia*, and *E. coli*. The extract revealed zone of growth inhibition against *S. aureus* (13.65 mm), *L. Monocytogenes* (13.16 mm), *K. pneumonia* (16.53mm) and *E. coli* (10mm) with the chloromofinacol positive control at the range (8.09 to 9.45 mm). Similarly, the ethyl acetate extract revealed zone of growth inhibition against *S. aureus* (16.6mm) and *L. Monocytogenes* (10.8 mm), *K. pneumonia* (13.43 mm) and *E.coli* (12.91 mm) with the positive standard chloromophynacol has the range (8.09 to 9.45 mm).

5.2. Antioxidant Activity

Using radical scavenging activities with ascorbic acid as the positive control, Adamu *et al*. (2020) investigated the antioxidant activities of the crude methanol extracts and solvent fractions of *R. abyssinicus* rhizomes. The crude extract had an IC_{50} value of 13.1 µg/mL, which is lower than the positive control's IC_{50} value of 4.9 µg/mL, indicating that it has weaker antioxidant activity. Similarly, the IC_{50} values for petroleum ether, chloroform, ethyl acetate, butanol, and aqueous fractions were 34.4, 8.0, 9.9, 6.1, and 24.1 µg/mL, respectively. Using DPPH radical scavenging assays, Kengne *et al*. (2021) investigated the antioxidant properties of methanol extracts of *R. abyssinicus* whole plant. The extract had an EC_{50} value of 62 µg/mL, which is lower than the standard drug's EC_{50} value of 1.81 µg/mL, indicating weaker antioxidant activity. The reported compound physicon (**3**) showed a significant scavenging ability against the DPPH radical, with an EC_{50} value of 3.08 µg/mL, which was close to the reference drug's EC_{50} value of 1.81 µg/mL.

5.3. Antiviral Activity

To assess antiviral activities of methanol extracts of *R. abyssinicus* root against Coxsackie and influenza A viruses, Getie *et al*. (2003) used virus-specific phosphorothioate oligodeoxynucleotides technique with guanidine hydrochloride for CVB3 and Acute Macular Neuroretinopathy for influenza A virus as a positive control. The methanol extract demonstrated actions with IC_{50} and IC_{100} values ranging from 7.8 mg/mL to 125 mg/mL in a dose-dependent manner, and their influence altered to be similar to virustatic positive control drugs (at IC_{100}).

5.4. Anticancer Activity

Using quantitative colorimetric assay via the oxidation-reduction indicator resazurin method, Worku *et al*. (2013) evaluated the in vitro anticancer activities of methanol extracts of *R. abyssinicus* rhizomes. With an IC_{50} value of 3 µg/mL, the extract indicated cell proliferation in human prostate cancer cells (LNCaP), Leukemia (THP-1) and human astrocytoma cells (1321N1). Girma *et al*. (2015) used dimethylhydrazine (DMH) induced colon carcinogenesis rats to investigate the in vivo colon cancer chemopreventive activity of methanol extracts of *R. abyssinicus* rhizomes, administering 250 mg/kg and 500 mg/kg body weight of extract orally to different groups of rats. The findings revealed that extract doses of 250 mg/kg and 500 mg/kg body weight significantly reduced the incidence of aberrant crypts (ACs) and aberrant crypt foci (ACF).

5.5. Anti-trypanosomal Activities

Using a quantitative colorimetric assay with the oxidation-reduction indicator resazurin, Worku *et al*. (2013) examined the antitryposomal activity of methanol extracts of *R. abyssinicus* rhizomes *in vitro*. IC_{50} values for the extract against *Trypanosoma brucei* cells ranged from 33 to 333µg/mL.

5.6. Anti-inflammatory Activity

Using the cyclooxygenase (COX) inhibition assay with indomethacin as a positive control, Getie *et al*. (2003) investigated the anti-inflammatory effect of methanol crude root extract of *R. abyssinicus*. PGE2 production was demonstrated in the extract, with IC_{50} values ranging from 0.05 to 10µg/mL. Mulisa *et al*. (2015) used the carrageenan-induced rat paw edema assay in adult Swiss albino mice to examine in vivo anti-inflammatory activities of methanol extracts of *R. abyssinicus* rhizomes, with 250, 500, and 750 mg/Kg body weight of the extract administered orally to different groups of mice, with indomethacin (10 mg/kg) as the standard drug. After 2 hours of carrageenan injection, the two higher dosages of the extract (500 and 750 mg/kg) as well as the standard drug significantly (p<0.001) inhibited edema, whereas the lower dose (250 mg/kg) of the extract showed anti-inflammatory effect (p<0.05) after 3 hours of edema induction.

5.7. Antifungal Activity

Using the agar disc diffusion method and ketoconazole as a positive control, Kebede *et al*. (2021) nvestigated the antifungal activity of methanol extracts of *R. abyssinicus* leaves against *C. albicans* and *T. mentagrophytes*. The methanol extract (30 µg/mL) inhibited activities with a zone of inhibition ranging from 22 to 26 mm, whereas ketoconazole, the positive control, inhibited activities with a zone of inhibition ranging from 26 to 28 mm. The MIC values for the pathogens that were tested ranged from 32 to 64 µg/mL. With positive control Fluazinan 500F, Tala *et al*. (2018) identified anthraquinones and flavanols compounds from the root of *R. abyssinicus* extract displayed plant pathogens motility inhibitory and lytic properties against Phytophthora capsici zoospores. With a MIC of 15µg/mL, the isolated compound physcion (**3**) displayed the strongest motility inhibitory effect, as well as lytic activity against zoospores at 500µg/mL. Using the broth microdilution method and fluconazole as a positive control, Kengne *et al*. (2021) examined antifungal activity of methanol extracts of whole parts of *R. abyssinicus* and EtOAc fraction against *C. neoformans* and *C. albicans*. At a MIC of 32 µg/mL, the methanol extract and EtOAc fraction showed activity against C.neoformans, whereas the isolated compounds phsicon (**3**) and emodin (**1**) showed activity against *C. neoformans* and *C. albicans* at a MIC of 8 µg/mL.

5.8. Hepatoprotective Activity

Adamu *et al*. (2020) investigated the hepatoprotective properties of methanol crude extracts of R. abyssinicus rhizomes in female Swiss albino mice using the toxic carbon tetrachloride (CCl_4) technique and silymarin as the standard drug that induces liver damage. The extract of *R. abyssinicus* at a dose of 500 mg/Kg reduced elevated levels of serum alanine aminotransferase (ALT, 51.3%) and alkaline phosphatase (ALP, 63.8%) better than the standard drug silymarin 100 mg/Kg (69%) and significantly reduced the deleterious histopathological changes in the liver following carbon tetrachloride intoxication.

5.9. Anti-Alzheimer's Activity

Augustin *et al*. (2020) used the Ellman assay with donepezil hydrochloride as a positive control to investigate the anti-alzheimer effects of ethyl acetate rhizome extract of *R. abyssinicus*. The extract showed dual cholinesterase inhibitor action against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), with IC_{50} values of 2.7 and 11.4 µg/mL, respectively, which is lower than the positive control donepezil HCl, which had IC_{50} values of 0.049 and 5.52 µg/mL. Helminthosporin (**4**), Emodin (**1**), Chrysophanol (**2**), and Phsicon (**3**), all isolated anthraquinone compounds, demonstrated substantial inhibition of acetylcholinesterase (AChE) with IC_{50} values of 2.63, 15.21, 33.7, and 12.16 µg/mL, respectively. Helminthosporin (**4**) also demonstrated considerable inhibition of butyrylcholinesterase (BChE) with an IC_{50} value of

2.99 µg/mL, which is higher activity than donepezil HCl, the positive control, which had an IC_{50} value of 5.52 µg/mL.

5.10. Wound Healing Activity

Mulisa *et al*. (2015) used excision and incision models in adult Swiss albino mice to investigate the wound healing capabilities of methanol rhizomes extracts of *R. abyssinicus*, with nitrofurazone ointment as a control. In both excision and incision models, treatment with 5% and 10% (w/w) methanol extract ointment exhibited significant wound recovery activities, with higher activity when compared to nitrofurazone ointment.

5.11. Acute and Sub-acute Toxicity Activity

Adamu *et al*. (2020) evaluated *in vivo* acute toxicity of methanol rhizome extracts of *R. abyssincus* using female Swiss mice. A total of five starved mice were given a test sample 2000 mg/Kg body weight orally and the numbers of deaths occurring in 24 h were noted. The extract's LD_{50} in mice was greater than 2000 mg/kg, indicating that it was non-toxic. Alelign *et al*. (2020) evaluated *in vivo* acute and sub-acute toxicity of methanol rhizome extract of *R. abyssinicus* using female Swiss mice also showed that LD_{50} in female Swiss mice were higher than 2 µg/kg, suggesting that the extract was of non-toxic. Similarly, Enyew *et al*. (2020) examined *in vivo* acute and sub-acute toxicity of methanol rhizome extracts of *R. abyssinicus*, LD_{50} in Swiss Albino mice greater than 2 µg/kg.

6. EXTENDED APPLICATION

6.1. Industrial Application in The Leather Industry

Raw hides and skins are preserved before being turned into leather to protect the skin protein from microbial attack. The most extensively used preservation method in the world is the salting process, which employs around 50% w/w common salt. This typical system generates an excessive amount of saline wastewater, which adds to major pollution in terms of total dissolved solids and chlorides, and is discharged into the effluent throughout the manufacturing process. The treatment plants operational and maintenance costs increase as the soaking produces more salinity. Furthermore, ground water pollution around tanning industries is pressuring tanners to minimize or eliminate the use of salts in the leather manufacturing process (Vankar & Dwivedi, 2009).

Mohammed *et al*. (2016) investigated a less-salt preservation system based on *R. abyssinicus* root powder as a cleaner alternative to conventional salt-based preservation approach, with a positive control of 50 % (w/w) sodium chloride (NaCl). The results revealed that curing the raw goatskins with 10% (w/w) *R. abyssinicus* root powder and 15% (w/w) common salt is successful in preserving them. The pollution-reduction generation also shows a % reduction in total dissolved solids and a 70 % reduction in chloride. Aqueous root extracts of *R. abyssinicus* were employed as a cleaner way for dying in product production in another investigation. The 10% (w/w) aqueous root powder of *R. abyssinicus* demonstrated good fastness and organoleptic features, according to the results (Mohammed *et al*., 2018).

7. CONCLUSION

The ethnomedicinal uses, chemical constituents, and biological activity of *R. abyssinicus* are summarized in this review. Anthraquinones, flavonoids, terepenoids, phenolic compound and fatty acid had been confirmed to be the principle active constituents of *R. abyssinicus*. Biological activity has additionally targeted on antibacterial, antioxidant, anticancer, anti-inflammatory, antifungal, wound healing and antialzeimer's from different extracts and reported compounds of *R. abyssinicus*. The various uses of *R. abyssinicus* amongst different ethnic groups and the scientific evidence of its chemical constituents and biological activities imply healing capability of the species. However, there may be need for clinical studies of crude

extracts and compounds reported from the species using *in vivo* models. Therefore, further research of *R. abyssinicus* need to attention on complete phytochemical analyses of the species and to more accurately outline its biological activities. Additionally, new biological evaluations are still needed to scientifically validate some of its ethnobiological applications in order that the species can be used as a future useful resource for disease treatment and management. *R. abyssinicus* is also now no longer completely evaluated regarding its safety as herbal medicine. Further research want to assess the toxicity of the species for further uses of the plant extracts, fractions and isolated compounds in pharmaceutical industries.

Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Gashaw Nigussie came up with the idea, acquired information, wrote and edited the original paper. **Tiruwork Fanta** and **Mekdes Alemu**, proofread and modified the final version. The contents of this manuscript were read by all of the authors, and they all agreed to bear responsibility for it.

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REFERENCES

Adamu, B.A., Emiru, Y.K., Sintayehu, B., Araya, E.M., Periasamy, G., & Hiben, M.G. (2020). In vivo Hepatoprotective and in vitro Radical Scavenging Activities of Extracts of *Rumex abyssinicus* Jacq. Rhizome. *Journal of Experimental Pharmacology*, *12*, 221. https://doi.org/10.2147/JEP.S258566

Alelign, T., Chalchisa, D., Fekadu, N., Solomon, D., Sisay, T., Debella, A., & Petros, B. (2020). Evaluation of acute and sub-acute toxicity of selected traditional antiurolithiatic medicinal plant extracts in Wistar albino rats. *Toxicology Reports*, *7*, 1356-1365. https://doi.org/10.1016/j.toxrep.2020.10.001

Assefa, T., Nigussie, N., Mullualem, D., Sinshaw, G., & Adimasu, Y. (2019). The Role of Medicinal Plants in Traditional Medicine in Adwa District, Tigray, Northern Ethiopia. *Asian Plant Research Journal*, 1-11.

Augustin, N., Nuthakki, V.K., Abdullaha, M., Hassan, Q.P., Gandhi, S.G., & Bharate, S.B. (2020). Discovery of helminthosporin, an anthraquinone isolated from *Rumex abyssinicus* Jacq as a dual cholinesterase inhibitor. *ACS omega*, *5*(3), 1616-1624. https://doi.org/10.1021/acsomega.9b03 693

Awas, T. (2007). *Plant diversity in Western Ethiopia: ecology, ethnobotany and conservation* [Dictoral dessertation, University of Oslo]. University of Oslo Libraries. http://urn.nb.no/URN:NBN:no-15871

Awoke, S., & Gedamu, C. (2020). Chemical Studies and Antibacterial Activity of the Root of *Rumex abyssinicus*. *Earthline Journal of Chemical Sciences*, *4*(2), 155-173.

Birhan, Y., Kitaw, S., Alemayehu, Y., & Mengesha, N. (2017). Ethnobotanical study of medicinal plants used to treat human diseases in Enarj Enawga district, East Gojjam zone, Amhara region, Ethiopia. *SM Journal of Medicinal Plant Studies*, *1*(1), 1-9.

Birhanu, T., Abera, D., Ejeta, E., & Nekemte, E. (2015). Ethnobotanical study of medicinal plants in selected Horro Gudurru Woredas, Western Ethiopia. *Journal of Biology, Agriculture and Healthcare*, *5*(1), 83-93.

Bussa, N., & Belayneh, A. (2019). Traditional medicinal plants used to treat cancer, tumors and inflammatory ailments in Harari Region, Eastern Ethiopia. *South African Journal of Botany*, *122*, 360-368. https://doi.org/10.1016/j.sajb.2019.03.025

Chekole, G. (2017). Ethnobotanical study of medicinal plants used against human ailments in Gubalafto District, Northern Ethiopia. *Journal of Ethnobiology and Ethnomedicine*, *13*(1), 55. https://doi.org/10.1186/s13002-017-0182-7

Dalle, M.G.G. (2019). Ethnobotanical Study of Medicinal Plants in Nagelle Arsi District, West Arsi Zone of Oromia, Ethiopia. *Journal of Natural Sciences Research*, *9*(13).

Dinbiso, T.D., Tolosa, T.T., & Begna, F.D. (2020). *Ethnoveterinary Practices of Medicinal Plants and Non-plant Remedies Used in Animal Health Management in Dawuro Zone, Southern Ethiopia*. Resarch Square. https://doi.org/10.21203/rs.3.rs-94568/v2

Duguma, H.T. (2020). Wild edible plant nutritional contribution and consumer perception in Ethiopia. *International Journal of Food Science*, *2020*.

Enyew, E., Muche, A., Endale, A., & Goshu, B. (2020). Evaluation of the Acute and Sub-Acute Toxic Effects of 80% Methanol Rhizome Extracts of *Rumex abyssinicus* jacq. (Plygonaceae) on Histopathology of Liver, Kidney and Some Blood Parameters. *EC Pharmacology and Toxicology*, *9*(2), 01-12.

Eshete, M.A., Kelbessa, E., & Dalle, G. (2016). Ethnobotanical study of medicinal plants in Guji agro-pastoralists, Blue Hora District of Borana Zone, Oromia region, Ethiopia. *Journal of Medicinal PlantsSstudies*, *4*(2), 170-184.

Eskedar, A. (2011). *Ethnobotanical study on medicinal plants used by local communities in Debark Wereda, north Gondar zone, Amhara regional state, Ethiopia* [MSc thesis, Addis Ababa University]. Addis Ababa University Libraries. http://etd.aau.edu.et/handle/123456789/4244

Etana, B. (2010). Ethnobotanical study of traditional medicinal plants of Goma Wereda, Jimma Zone of Oromia Region, Ethiopia [MSc Thesis, Addis Ababa University]. Addis Ababa University Libraries. http://etd.aau.edu.et/handle/123456789/11339

Fassil, Y., Bezabeh, A., Abegaz, B., Botta, B., Monache, G.D., & Monache, F.D. (1985). Anthracene derivatives from *Rumex abyssinicus*. *Journal of Natural Products*, *48*(1), 148-148.

Fufa, F., Padmanabhan, R., & Gurmessa, G. (2016). Phytochemical investigation and in vitro antibacterial evaluation on root extracts of *Rumex abyssinicus*. *Natural Products Chemistry & Research*, *4*(6), 1-14.

Gabriel, T., & Guji, T. (2014). Ethnopharmacological survey of medicinal plants in Agaro district, Jimma zone, South West Ethiopia. *International Journal of Pharmaceutical Sciences and Research*, *5*(8), 3551. https://doi.org/10.13040/IJPSR.0975-8232.5(8).3551-59

Gebru, M.G., Lulekal, E., Bekele, T., & Demissew, S. (2021). Use and management practices of medicinal plants in and around mixed woodland vegetation, Tigray Regional State, Northern Ethiopia. *Ethnobotany Research and Applications*, *21*, 1-26.

Getie, M., Gebre-Mariam, T., Rietz, R., Höhne, C., Huschka, C., Schmidtke, M., Abate, A., & Neubert, R. (2003). Evaluation of the anti-microbial and anti-inflammatory activities of the medicinal plants *Dodonaea viscosa*, *Rumex nervosus* and *Rumex abyssinicus*. *Fitoterapia*, *74*(1-2), 139-143.

Getu, A. (2017). *Plant diversity and ethnobotany of medicinal and wild edible plants in Amaro district of Southern nations, nationalities and peoples region and Gelana district of Oromia region, Southern Ethiopia* [Msc Thesis, Addis Ababa University]. Addis Ababa univesristy Libraries. http://etd.aau.edu.et/handle/123456789/9230

Gidey, M., Beyene, T., Signorini, M.A., Bruschi, P., & Yirga, G. (2015). Traditional medicinal plants used by Kunama ethnic group in Northern Ethiopia. *Journal of Medicinal Plants Research*, *9*(15), 494-509. https://doi.org/10.5897/JMPR2014.5681

Girma, B., Yimer, G., & Makonnen, E. (2015). Effect of *Rumex Abyssinicus* on preneoplastic lesions in dimethylhydrazine induced colon carcinogenesis in rats. *BMC Complementary and Alternative Medicine*, *15*(1), 1-7. https://doi.org/10.1186/s12906-015-0883-1

Gorsi, M.S., & Miraj, S. (2002). Ethenomedicinal survey of plants of Khanabad village and its allied areas, District Gilgit. *Asian Journal of Plant Sciences*. https://doi.org/10.3923/ajps.2002.604.615

Guadie, A., Dakone, D., Unbushe, D., Wang, A., & Xia, S. (2020). Antibacterial activity of selected medicinal plants used by traditional healers in Genta Meyche (Southern Ethiopia) for the treatment of gastrointestinal disorders. *Journal of Herbal Medicine*, *22*, 100338. https://doi.org/10.1016/j.hermed.2020.100338

Gumisiriza, H., Birungi, G., Olet, E. A., & Sesaazi, C. D. (2019). Medicinal plant species used by local communities around queen elizabeth national park, maramagambo central forest reserve and ihimbo central forest reserve, south western Uganda. *Journal of Ethnopharmacology*, *239*, 111926. https://doi.org/10.1016/j.jep.2019.111926

Gumisiriza, H., Sesaazi, C.D., Olet, E.A., Kembabazi, O., & Birungi, G. (2021). Medicinal plants used to treat" African" diseases by the local communities of Bwambara sub-county in Rukungiri District, Western Uganda. *Journal of Ethnopharmacology*, *268*, 113578. https://doi.org/10.1016/j.jep.2020.113578

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*, *27*(1), 1-93. https://doi.org/10.1016/j.mam.2005.07.008

Hassen, A. (2021). Diversity and potential contribution of wild edible plants to sustainable food security in North Wollo, Ethiopia. *Biodiversitas Journal of Biological Diversity*, *22*(6).

Kamatenesi-Mugisha, M., Oryem-Origa, H., Odyek, O., & Makawiti, D.W. (2008). Medicinal plants used in the treatment of fungal and bacterial infections in and around Queen Elizabeth Biosphere Reserve, western Uganda. *African Journal of Ecology*, *46*, 90-97. https://doi.org/10.1111/j.1365-2028.2008.00935.x

Kamatenesi-Mugisha, M., Oryem-Origa, H., & Olwa-Odyek. (2007). Medicinal plants used in some gynaecological morbidity ailments in western Uganda. *African Journal of Ecology*, *45*, 34-40. https://doi.org/10.1111/j.1365-2028.2007.00735.x

Kamau, L.N., Mbaabu, P.M., Mbaria, J.M., Gathumbi, P.K., & Kiama, S.G. (2016). Ethnobotanical survey and threats to medicinal plants traditionally used for the management of human diseases in Nyeri County, Kenya. *CELLMED*, *6*(3), 21.21-21.15.

Kebede, T., Gadisa, E., & Tufa, A. (2021). Antimicrobial activities evaluation and phytochemical screening of some selected medicinal plants: A possible alternative in the treatment of multidrug-resistant microbes. *PloS One*, *16*(3), e0249253. https://doi.org/10.1371/journal.pone.0249253

Kefalew, A., Asfaw, Z., & Kelbessa, E. (2015). Ethnobotany of medicinal plants in Ada'a District, East Shewa Zone of Oromia regional state, Ethiopia. *Journal of Ethnobiology and Ethnomedicine*, *11*(1), 1-28. https://doi.org/10.1186/s13002-015-0014-6

Kengne, I.C., Feugap, L.D.T., Njouendou, A.J., Ngnokam, C.D.J., Djamalladine, M.D., Ngnokam, D., & Voutquenne-Nazabadioko, L. (2021). Antibacterial, antifungal and antioxidant activities of whole plant chemical constituents of *Rumex abyssinicus*. *BMC Complementary Medicine and Therapies*, *21*(1), 1-14. https://doi.org/10.1186/s12906-021-03325-y

Khan, M. A., Agize, M., Shonga, A., & Tora, A. (2018). *The utilization and conservation of plants of medicinal value by local traditional medicinal practitioners and the associated indigenous knowledge in Dawuro Zone of Ethiopia: Northeast Africa—an ethnobotanical*

approach. In Plant and Human Health (Vol. *1*, pp. 267-321). Springer. https://doi.org/10.1007/978-3-319-93997-1_7

Limenih, Y., Umer, S., & Wolde-Mariam, M. (2015). Ethnobotanical study on traditional medicinal plants in Dega Damot woreda, Amhara Region, North Ethiopia. *International Journal of Research in Pharmacy and Chemistry*, *5*(2), 258-273.

Malik, E.M., Baqi, Y., & Müller, C.E. (2015). Syntheses of 2-substituted 1-amino-4-bromoanthraquinones (bromaminic acid analogues)–precursors for dyes and drugs. *Beilstein Journal of organic chemistry*, *11*(1), 2326-2333. https://doi.org/10.3762/bjoc.11.253

Malik, E.M., & Müller, C.E. (2016). Anthraquinones as pharmacological tools and drugs. *Medicinal research reviews*, *36*(4), 705-748. https://doi.org/10.1002/med.21391

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, *79*(5), 727-747. https://doi.org/10.1093/ajcn/79.5.727

Megersa, M. (2010). *Ethnobotanical study of medicinal plants in Wayu Tuka Wereda, East Wollega zone of Oromia region, Ethiopia* [Msc Thesis, Addis Ababa University]. Addis Ababa University Libraries. http://213.55.95.56/bitstream/handle/123456789/6984

Mekonnen, A., & Desta, W. (2021). Comparative study of the antioxidant and antibacterial activities of Rumex abyssinicus with commercially available Zingiber officinale and Curcuma longa in Bahir Dar city, Ethiopia. *Chemical and Biological Technologies in Agriculture*, *8*(1), 1-11. https://doi.org/10.1186/s40538-020-00198-0

Mekonnen, T., Urga, K., & Engidawork, E. (2010). Evaluation of the diuretic and analgesic activities of the rhizomes of Rumex abyssinicus Jacq in mice. *Journal of Ethnopharmacology*, *127*(2), 433-439. https://doi.org/10.1016/j.jep.2009.10.020

Mishra, A. P., Sharifi-Rad, M., Shariati, M. A., Mabkhot, Y. N., Al-Showiman, S. S., Rauf, A., Salehi, B., Župunski, M., Sharifi-Rad, M., & Gusain, P. (2018). Bioactive compounds and health benefits of edible Rumex species-A review. *Cellular and Molecular Biology*, *64*(8), 27-34. http://dx.doi.org/10.14715/cmb/2018.64.8.5

Mohammed, S.A., Madhan, B., Demissie, B.A., Velappan, B., & Selvi, A.T. (2016). *Rumex abyssinicus* (mekmeko) Ethiopian plant material for preservation of goat skins: approach for cleaner leather manufacture. *Journal of Cleaner Production*, *133*, 1043-1052. https://doi.org/10.1016/j.jclepro.2016.06.043

Mohammed, S.A., Panda, R.C., Madhan, B., & Demessie, B.A. (2018). *Rumex abyssinicus* (mekmeko) extract as cleaner approach for dyeing in product manufacture: Optimization and modeling studies. *Asia-Pacific Journal of Chemical Engineering*, *13*(2), e2165. https://doi.org/10.1002/apj.2165

Monks, T.J., Hanzlik, R.P., Cohen, G.M., Ross, D., & Graham, D.G. (1992). Quinone chemistry and toxicity. *Toxicology and Applied Pharmacology*, *112*(1), 2-16. https://doi.org/10.1016/0041-008X(92)90273-U

Moravec, I., Fernandez, E., Vlkova, M., & Milella, L. (2014). Ethnobotany of medicinal plants of northern Ethiopia. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas*, *13*(2), 126-134.

Muhammad, M., Badshah, L., Shah, A.A., Shah, M.A., Abdullah, A., Bussmann, R.W., & Basit, A. (2021). Ethnobotanical profile of some useful plants and fungi of district Dir Upper, Tehsil Darora, Khyber Pakhtunkhwa, Pakistan. *Ethnobotany Research and Applications*, *21*, 1-15.

Mulisa, E., Asres, K., & Engidawork, E. (2015). Evaluation of wound healing and anti-inflammatory activity of the rhizomes of *Rumex abyssinicus* J.(*Polygonaceae*) in mice. *BMC Complementary and Alternative Medicine*, *15*(1), 1-10. https://doi.org/10.1186/s12906-015-0878-y

Munavu, R.M., Mudamba, L., & Ogur, J. (1984). Isolation and characterization of the major anthraquinone pigments from Rumex abysinica. *Planta medica*, *50*(01), 111-111.

Nigussie, G., Ibrahim, F., & Neway, S. (2021). A Phytopharmacological Review on a Medicinal Plant: *Cordia africana* Lam. *Journal of Tropical Pharmacy and Chemistry*, *5*(3), 254-263. https://doi.org/10.25026/jtpc.v5i3.267

Novotna, B., Polesny, Z., Pinto-Basto, M.F., Van Damme, P., Pudil, P., Mazancova, J., & Duarte, M.C. (2020). Medicinal plants used by 'root doctors', local traditional healers in Bié province, Angola. *Journal of Ethnopharmacology*, *260*, 112662. https://doi.org/10.1016/j.jep.2020.112662

Odongo, E., Mungai, N., Mutai, P., Karumi, E., Mwangi, J., & Omale, J. (2018). Ethnobotanical survey of the medicinal plants used in Kakamega County, Western Kenya. *Applied Medical Research*, *4*(1), 22.

Omara, T. (2020). Antimalarial plants used across Kenyan communities. *Evidence-Based Complementary and Alternative Medicine*, *2020*. https://doi.org/10.1155/2020/4538602

Regassa, R. (2013). Assessment of indigenous knowledge of medicinal plant practice and mode of service delivery in Hawassa city, southern Ethiopia. *Journal of Medicinal Plants Research*, *7*(9), 517-535. https://doi.org/10.5897/JMPR12.1126

Shifa, M., Abdissa, D., & Asere, T.G. (2021). Chemical constituents of *Rumex abyssinicus* roots and evaluation of its antimicrobial activities. *Journal of the Turkish Chemical Society Section A: Chemistry*, *8*(1), 21-46. https://doi.org/10.18596/jotcsa.797560

Silva, O., Caldeira, G., & Serrano, R. (2020). A review of the role of medicinal plants on Neisseria gonorrhoeae infection. *European Journal of Integrative Medicine*, 101211. https://doi.org/10.1016/j.eujim.2020.101211

Sina, B., & Degu, H.D. (2015). Knowledge and use of Wild Edible Plants in the Hula district of the Sidama Zone. *International Journal of Bio-resource and Stress Management*, *6*(3), 352-365. https://doi.org/10.5958/0976-4038.2015.00052.4

Suleman, S., & Alemu, T. (2012). A survey on utilization of ethnomedicinal plants in Nekemte town, East Wellega (Oromia), Ethiopia. *Journal of herbs, spices & medicinal plants*, *18*(1), 34-57. https://doi.org/10.1080/10496475.2011.645188

Tala, M., Ansary, M., Talontsi, F., Kowa, T., Islam, M.T., & Tane, P. (2018). Anthraquinones and flavanols isolated from the vegetable herb *Rumex abyssinicus* inhibit motility of Phytophthora capsici zoospores. *South African Journal of Botany*, *115*, 1-4. https://doi.org/10.1016 /j.sajb.2017.11.015

Tegen, D., Dessie, K., & Damtie, D. (2021). Candidate Anti-COVID-19 Medicinal Plants from Ethiopia: A Review of Plants Traditionally Used to Treat Viral Diseases. *Evidence-Based Complementary and Alternative Medicine*, *2021*. https://doi.org/10.1155/2021/6622410

Teka, A., Asfaw, Z., Demissew, S., & Van Damme, P. (2020). Traditional uses of medicinal plants practiced by the indigenous communities in Gurage Zone, south central Ethiopia. *Ethnobotany Research and Applications*, *19*, 1-31.

Teklay, A., Abera, B., & Giday, M. (2013). An ethnobotanical study of medicinal plants used in Kilte Awulaelo District, Tigray Region of Ethiopia. *Journal of ethnobiology and ethnomedicine*, *9*(1), 65. https://doi.org/10.1186/1746-4269-9-65

Tsegay, B., Mazengia, E., & Beyene, T. (2019). Diversity of medicinal plants used to treat human ailments in rural Bahir Dar, Ethiopia. *Asian Journal of Forestry*, *3*(2), 75-82.

Tuasha, N., Petros, B., & Asfaw, Z. (2018). Medicinal plants used by traditional healers to treat malignancies and other human ailments in Dalle District, Sidama Zone, Ethiopia. *Journal of ethnobiology and ethnomedicine*, *14*(1), 15. https://doi.org/10.1186/s13002-018-0213-z

Vankar, P.S., & Dwivedi, A.K. (2009). Raw skin preservation through sodium salts—a comparative analysis. *Desalination*, *249*(1), 158-162.

Workineh, Y.T. (2021). *Phytochemical Analysis and Determination of Antioxidant and Antibacterial Activities of Leaf extracts of Rumex nervosus (Embacho)* [Msc Thesis, Addis Ababa University]. Addis Ababa University.

Workneh, D. (2017). *Effect of Iron, Copper and Zinc metal ions on the antibacterial activity of crude extract of Aloe pulcherrima And Rumex abyssinicus leaves* [Msc Thesis, Jimma University]. Jimma University.

Worku, N., Mossie, A., Stich, A., Daugschies, A., Trettner, S., Hemdan, N.Y., & Birkenmeier, G. (2013). Evaluation of the in vitro efficacy of *Artemisia annua*, *Rumex abyssinicus*, and *Catha edulis* Forsk extracts in cancer and Trypanosoma brucei cells. *International Scholarly Research Notices*, *2013*. https://doi.org/10.1155/2013/910308

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Research Article

Influence of drying method and infusion time on purple basil leaves tea

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Abstract: In this study, purple basil leaves were dried in 3 different methods (oven, microwave, and shade drying) and then infused 5 different times in hot water at 90 °C. Analyses of total phenolic content, antioxidant activity, total monomeric anthocyanin content, and color parameters were performed on tea samples. When the analysis results were evaluated, the best results were determined in the tea samples prepared from the leaves dried in the oven and in the shade, at brewing times of 10 and 15 minutes. 45 minutes was the best infusion time for the tea sample prepared from microwave dried leaves. When the three different drying methods were compared, the shade drying method gave better results than the other drying methods. According to the highest values obtained from the analysis results, the effect of the functional compounds in the purple basil leaves on the tea was calculated. It was determined that the highest percentage of phenolic compounds in tea was found in the samples prepared by the shade drying method.

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1. INTRODUCTION

Herbal teas are popular beverages preferred in the daily diet in recent years because they are sugar-free, calorie-free and offer different tastes. For a healthy adult person, daily average consumption of tea and herbal teas is recommended up to 2 liters (Schulzki *et al*., 2017). Hundreds of pure or blended herbal teas sold in health food stores contain active phytochemicals with various biological properties. These phytochemicals help prevent or treat many diseases such as allergic reactions, insomnia, headaches, anxiety, intestinal disorders, depression, and high blood pressure. In addition, studies have reported that herbal teas have anticarcinogenic, antioxidant (Jin *et al*., 2016; Schulzki *et al*., 2017), and antimicrobial (Chan *et al*., 2010; Oh *et al*., 2013) activities. Herbal preparations consumed in the form of tea are usually infused from the leaves, flowers, seeds, fruits, stems, and roots of the plant (Oh *et al*., 2013; Jin *et al*., 2016; Schulzki *et al*., 2017). Basil (*Ocimum basilicum* L.), one of the important industrial, pharmaceutical and aromatic plant species of the Lamiaceae family, has an important place in various food applications, essential oil production, ornamental, pharmaceutical, and cosmetic industries (Ekren *et al*., 2012; Fratianni *et al*., 2017). Components of sweet basil include various phenolic acids (such as cinnamic, sinapic, caffeic, rosmarinic, caffeic, and ferulic acid) and flavonoids (such as apigenin and catechin). These compounds act as free

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radical scavengers and metal chelators by showing strong antioxidant effects against free radicals known to be harmful to humans (Flanigan and Niemeyer, 2014; McCance *et al*., 2016; Fratianni *et al*., 2017). Basil leaves and basil tea are beneficial in medical treatments for digestion, cough, headache, worms, diarrhea, and stomachache (Ekren *et al*., 2012; Ahmad *et al*., 2016).

Purple basil varieties have rich anthocyanin content. Anthocyanins belong to one of the largest classes of secondary metabolites known as flavonoids and dissolve in water to form red-blue color pigments (Ahmad *et al*., 2016). Anthocyanins offer numerous benefits in relation to antioxidant properties and radical-scavenging abilities for human health, as well as having different functions for plants (Flanigan and Niemeyer, 2014; McCance *et al*., 2016). The shelf life of fresh purple basil is short due to the high water content (Fratianni *et al*., 2017). Therefore, it is more appropriate to use fresh purple basil leaves as tea in the food industry by drying them. The aim of this study is to determine the effects of various infusion times of purple basil (*O. basilicum* L.) leaf tea, dried by different methods, on total phenolic, total monomeric anthocyanin, antioxidant activity, and color parameters.

2. MATERIAL and METHODS

2.1. Drying of Purple Basil Leaves and Brewing

Purple basil plant was supplied from the local market in Balikesir, Turkey. The purple basil sprigs were washed under running water and leaves were separated from their stems by hand. The leaves were spread on a qualitative filter paper (Whatman) to remove the wash water. The leaves were dried by oven, microwave, and shade drying methods. Microwave drying was carried out in a microwave oven (Arçelik, MD 564) at 90 W for 2 min. For oven drying, the leaves were spread on a tray and dried in an oven drier (Nuve, Turkey) set at 80 ^{o}C. For shade drying, the leaves were spread on qualitative filter paper and kept away from direct sunlight and dried at room temperature. Drying in all methods was completed when the products reached a dry matter of 95±1%. The boiled distilled water was cooled to 90°C. It was then added to the dried purple basil leaves (1.0 g) and infused for 5, 10, 15, 30 and 45 minutes. Prepared teas were stored at -20 °C until analysis. At the end of each infusion time, leaves were removed from the infusions by using a sieve and immediately cooled to room temperature.

2.2. Extraction Procedure for Antioxidants and Polyphenolic Compounds

A 0.1 g of ground dried purple basil weighed into a tube and 15 mL of acidified methanol was added. The mixture was shaken at 35°C for 60 minutes in a shaking water bath, centrifuged at 7000 rpm for 10 minutes and the supernatant was collected in a jar and the residue was re-extracted with fresh methanol and according to the same procedure and the supernatants were combined. Total phenolic compounds, monomeric anthocyanin, and antioxidant activity were analyzed in the prepared extracts.

2.3. Determination of Total Phenolic Compounds

Total phenolic substance amounts were determined according to the method developed by Singleton and Rossi (1965). The solution mixture prepared for this method consists of 0.5 mL of methanol extract, 2.5 mL of Folin-Ciocaleu solution (0.2 N), and 2.0 mL of Na_2CO_3 (75 g/L). After the samples were treated with the solution mixture, absorbance was read at 765 nm after 120 min in the spectro-photometer (U-1800, Hitachi, Japan). The results obtained were given as gallic acid (GAE)/kg dry weight (DW).

2.4. Determination of Antioxidant Activity

Antioxidant capacities of extract and tea samples Brand-Williams *et al*. (1995) developed by the DPPH (2,2-Diphenyl-1-picrylhydrazil) method. In this method, 0.1 mL of the test materials

were taken and 3.9 mL of methanolic DPPH ($6x10^{-5}$ M) solution was added to it to complete the total volume to 4 mL. Afterward, the obtained mixtures were incubated at 25 °C for 30 min in the dark, and their absorbance was determined at 515 nm in a spectrophotometer. The data obtained are given in mmol Trolox Equivalent/kg.

2.5. Total Monomeric Anthocyanin Content

TMAC was determined according to the pH differential analysis reported by Lee *et al*. (2008). According to the method, 1 mL sample taken from the extracts was diluted separately in 4 mL KCl (pH 1.0, 0.025 M) and $C_2H_3NaO_2$ buffers (pH 4.5, 0.4 M). Approximately 30 minutes after dilution, absorbance was obtained at two different wavelengths (515 and 700 nm), and absorbance differences were used to calculate the anthocyanin content.

2.6. Color Parameters

L* (lightness), a* (position between red and green), b* (position between yellow and blue), C* (chroma), and h (hue) color values of tea samples were measured by using a Minolta colorimeter (CM-5, Minolta, Japan).

2.7. Statistical Analysis

Study results were expressed as mean ± standard deviation. Two-way analysis of variance (ANOVA) at a 95% confidence level was used to determine a statistically significant difference between drying methods and infusion times. Statistical analyzes were calculated using the MINITAB (Minitab Inc. USA) software program.

3. RESULTS and DISCUSSION

3.1. Changes in Total Phenolic Content (TPC)

The TPC of dried purple basil samples is shown in Table 1. Oven, shade, and microwave dried basil contained 22.38, 45.16 and 34.81 mg/g DW phenolic compounds, respectively. Flanigan and Niemeyer (2014) found mean TPC levels in purple basil cultivars in the range of 13.1 mg GAE/g DW for Johnny's Purple Ruffles to 26.9 mg GAE/g DW for Purple Delight basil. Another study reported the mean TPC levels of three different varieties of purple basil harvested at different maturity stages in the range of 3.30-20.08 mg/g DW (McCance *et al*., 2016).

The TPC results we obtained in the current study are slightly higher than the concentrations obtained by Flanigan and Niemeyer (2014) and McCance *et al*. (2016). Total phenolic compounds, monomeric anthocyanin contents, and antioxidant activities of tea samples are shown in Table 2. Among all purple basil teas, total phenolic content varied between 158.89 and 325.20 mg/L. The lowest TPC value was determined in the oven dried samples and the highest value was in the shade dried samples. When the brewing times are compared; the total phenolic content was found as 174.65 mg/L in 5 minutes infusion time in samples prepared by oven drying method. The TPC of the tea prepared with microwave dried samples in 45 minutes of brewing time was 308.35 mg/L. The highest TPC was calculated as 325.20 mg/L in the samples prepared with shade dried basil infused for 15 minutes. While there was a difference between the phenolic substance content of the purple basil samples dried by different methods, the purple basil teas brewed for different periods did not make a statistically ($p>0.05$) significant difference in terms of phenolic substance.

Table 1. TPC, TMAC and antioxidant activities of dried purple basil leaves

	Dried Sample		
	Oven	Microwave	Shade
TPC*	22.38±0.65	34.81±0.07	45.16±1.03
TMAC**	1.74±0.04	4.45±0.17	10.91±0.12
AA***	11.28±0.10	10.86±7.84	14.35±4.23

*mg GAE/g DW, **mg/ g DW, *** mmol TE/100 g DW

The highest transition rates were calculated as 78.05, 88.60 and 72.02% for oven dried basil infused for 5 min, microwave dried basil infused for 45 min, and shade dried basil infused for 15 min, respectively. As general, the highest phenolic transfer was seen in the tea sample prepared by the microwave drying method.

It is possible to find many studies in which different herbs are prepared and analyzed in tea form. However, in the literature studies, there is no study on purple basil leaf tea and different infusion times. Hajiaghaalipour *et al*. (2016) stated that the TPC of white, green, and black tea infusions was significantly affected by the steeping time and temperature. In all of the samples, the highest TPC for white tea was determined in long-term hot water infusion, in long-term cold water infusion for green tea, and in short-term hot water infusion for black tea. The lowest TPC was determined in a short-term cold water infusion. Atoui *et al*. (2005) observed that the amounts of total phenolics ranged from 88.1 to 1216 mg GAE/cup in Greek mountain tea and Chinese green tea, respectively. In another study, the values of total phenolic content varied widely in the leafy herbal tea extracts, ranged from 10.98 to 144.52 mg GAE/g herb tea (Oh *et al*., 2013). It was stated that *Centella asiatica* herbal teas should be prepared in 100 ºC water for 10 minutes to obtain the maximum TPC. The TPC values of unfermented, partially fermented (120 min) and fermented (24 h) *C. asiatica* herbal teas were 7.3, 6.8, and 5.3 mg GAE/ g, respectively (Ariffin *et al*., 2011). The highest TPC value was detected in *C. asiatica* tea infused at 100 °C for 5 minutes (45.99 mg/L GAE) and at 80 °C for 10 minutes (45.53 mg/L GAE) (Siah *et al*., 2011).

3.2. Changes in TMAC Color Parameters

As seen in Table 1, the total monomeric anthocyanin contents of purple basil samples were 1.74 mg/g DW for the oven drying method to 10.91 mg/g DW for the shade drying method. The result obtained in purple basil sample dried in microwave oven was found as 4.45 mg/g DW. The average anthocyanin concentrations of basil cultivars harvested at varying maturities are presented in a study done by McCance *et al*. (2016). Accoring to their results, anthocyanin concentrations ranged from 2.07 mg/g DW for Sweet Petra Dark Red to 9.72 mg/g DW for Purple Ruffles. These values are lower than the results of Flanigan and Niemeyer (2014), who observed anthocyanin contents of Rubin and Purple Ruffles (Richter's Herbs) cultivars as 7.55 and 16.6 mg/g DW, respectively.

Table 2 shows the monomeric anthocyanin contents of purple basil teas. TMAC varied from 4.86 to 27.99 mg/L. The lowest value was found in the sample prepared from oven dried leaves, whereas the highest value was in the sample prepared from shade dried sample. In the samples prepared by oven drying method, at the infusion time of 15 min. total monomeric anthocyanin content was found as 5.29 mg/L. TMAC value of tea prepared by microwave dried samples at the infusion time of 45 minutes was 19.69 mg/L. In the samples prepared by shade dried basil at the infusion time of 10 minutes, TMAC has been detected as 27.99 mg/L. According to our results, the TMAC of purple basil tea was affected by different drying methods. Monomeric anthocyanin in the oven drying method was found to range from 4.86 to 5.29, but this value is

higher in the shade drying method (19.65-27.99 mg/L). The effect of different brewing times on the TMAC was not found to be statistically ($p>0.05$) significant.

The highest anthocyanin transition rates were calculated as 30.40, 44.24 and 25.65% for oven dried basil infused for 15 min, microwave dried basil infused for 45 min, and shade dried basil infused for 10 min, respectively. As in the case of total phenolic compounds, the best drying method was microwave drying in terms of anthocyanins transfer.

Vinokur *et al*. (2006) obviously reported that the total anthocyanin content in rose teas was correlated with the petal color, and higher anthocyanin values obtained in the samples prepared from the red-flowered cultivars. Zijuan (*Camellia sinensis* var. *assamica*) with purple leaves and rich anthocyanin content is widely consumed in China. Anthocyanin levels in infusions made from various Zijuan tea products have been reported to be in the range of 115.4-198.2 µg/mL (Lv *et al*., 2015). In the study, the total monomeric anthocyanin content was determined in selected Kenyan tea cultivars processed into black and green tea products. Green tea (2.25-108.26 mg/L) had significantly higher total monomeric anthocyanins than black tea (0.82-45.48 mg/L) (Kerio *et al*., 2012).

3.3. Changes in Antioxidant Activity

Table 1 shows the DPPH assay for the free radical scavenging activity of methanolic extracts of the dried purple basil samples. The antioxidant activity results of purple basil samples were calculated as 11.28, 10.86, and 14.35 mmol Trolox equivalent/100 g DW for oven dried, microwave dried, and shade dried samples, respectively. Drying methods had a significant effect antioxidant activity of purple basil leaves. The decrease in antioxidant activity could arise from the decrement in phenolic content of the samples with the effect of the drying.

Flanigan and Niemeyer (2014) reported the antioxidant capacities of purple basil cultivars ranging from 24.6 mmol/100 g DW for Johnny's Purple Ruffles to 34.8 mmol/100 g DW for Purple Delight basil. The authors attributed the free radical scavenging activity of the teas to phenolic components (Bhebhe *et al*., 2015; Abdullah and Mazlan, 2020). Higher phenolic content and composition of major phenolic compounds are associated with higher antioxidant activity. According to our analysis results; the highest antioxidant activity value was found in shade dried sample and followed by microwave dried sample. Total phenolic content and antioxidant activity results were in parallel. Our results indicate that the total monomeric anthocyanin content also plays an important role in the measured antioxidant capacities of purple basil leaves tea.

Antioxidant activity values of tea samples are shown in Table 2. A wide range of variations exists in the antioxidant capacity of all purple basil teas. The antioxidant capacity ranged from 0.229 to 0.855 mmol Trolox equivalent/L.

Usually, the lowest DPPH value was found in the sample dried in oven, whereas the highest value was in the shade dried leaves. In the samples prepared by oven drying method, the highest value was found with 0.250 mmol Trolox equivalent/L at the infusion time of 15 min. The DPPH value of the tea prepared with microwave dried samples in 45 min. infusion time gave the highest result with 0.758 mmol TE/L. In the samples prepared with basil dried in the shade, the highest DPPH value was determined as 0.855 mmol TE/L at the infusion time of 10 minutes. While the effect of purple basil samples dried with different methods in antioxidant activity was statistically significant ($p<0.05$), differences in brewing times did not make a significant difference on antioxidant activity ($p>0.05$).

The effect of infusion time and temperature on Turkish black tea is examined by Kelebek (2016). Antioxidant capacity, depending on brewing (infusion) time and temperature, showed statistically significant results ($p<0.05$). It was determined that ABTS and DPPH levels reached the highest values numerically in teas with a 10-minute brewing time. The IC_{50} of the DPPH

radical scavenging assay of white, green, and black teas steeped at different temperatures and times was investigated. This value was found between 84.03-101.5 µg/mL in white teas, 79.4-98.33 µg/mL in green teas and 91.55-105.6 µg/mL in black teas (Hajiaghaalipour *et al*., 2016). It has been reported that at least 15 minutes of steeping period is required for green tea samples to exhibit the highest antioxidant activity (Abdullah and Mazlan, 2020). In another study, the antioxidant activities of teas with different infusion times were optimized according to the extraction procedure. No statistically significant difference was detected in the samples extracted with green tea in 5-30 min periods ($p>0.05$). In rose tea samples, the highest radical scavenging activity was determined during the 20 min brewing period.

These brewing times were used in the brewing methods of both green tea and rose leaf tea samples in later studies (Vinokur *et al*., 2006). Comparing tropical and temperate herbal teas with *C. sinensis* teas, Chan *et al*. (2010) found that lemon myrtle, guava, oregano, and black teas gave similar results. In a study comparing green tea with different herbal teas, it was reported that most of the herbal teas showed lower antioxidant activity than green tea (Jin *et al*., 2016).

The antioxidant capacity, measured by the DPPH method ranged from 0 to 273.43 mmol TE/ 100 g DW in herbal teas and between 140.13 and 221.21 mmol TE/ 100 g DW in green teas. According to results of Siah *et al*. (2011), the highest antioxidant activity was (52.8%) in *C. asiatica* tea infused for 5 minutes at 100 °C, and the lowest value was (32%) in tea infused at 60 °C for 3 minutes.

The total antioxidant capacity values of sweet basil (*O. basilicum*) herbal tea as dried obtained from the Turkish market, measured by CUPRAC and ABTS assays, were determined as 1.18 mmol TE/g and 0.77 mmol TE/g, respectively (Apak *et al*., 2006).

Table 2. TPC, TMAC and DPPH values of tea samples.

Drying method	Infusion time (min)	TPC*	TMAC*	DPPH**
Oven	5	174.65±13.04	5.23±0.43	0.293±0.017
	10	170.85±0.76	5.22±0.04	0.277±0.013
	15	171.83±2.61	5.29±0.04	0.250±0.017
	30	158.89±15.33	5.05±0.02	0.229±0.014
	45	163.78±2.39	4.86±0.10	0.270±0.003
Microwave	5	297.91±6.52	19.58±0.55	0.732±0.039
	10	277.80±15.76	17.36±0.22	0.702±0.042
	15	285.09±9.35	17.78±0.51	0.734±0.070
	30	306.50±0.77	19.24±0.39	0.748±0.017
	45	308.35±0.87	19.69±0.13	0.758±0.011
Shade	5	312.91±5.22	26.54±1.06	0.843±0.003
	10	307.15±28.80	27.99±3.07	0.855±0.040
	15	325.20±4.67	26.60±0.74	0.814±0.029
	30	324.87±14.13	19.65±2.20	0.774±0.061
	45	324.76±27.72	22.26±3.82	0.793±0.155

*(mg/L), ** mmol trolox equivalent/L

3.4. Changes in Color Parameters

L*, a*, b*, h, and C* values of purple basil teas dried by different methods are summarized in Table 3. Among all the purple basil leaves teas, a* value ranged from 7.11 to 15.72, with the lowest values coming from the oven drying method, whereas the highest value was from the shade drying method. While brewing time was not statistically ($p>0.05$) significant on the a* value of purple basil tea, drying method had significant effect on this color parameter. Among all the purple basil leaves teas, the L* value ranged from 62.84 to 77.67, the b* value ranged

from 17.42 to 32.02, the C* value ranged from 22.77 to 32.83, and the h value ranged from 49.85 to 77.70. According to these data, while the effect of different drying methods on the L* and h values of purple basil tea was statistically (p<0.05) significant, the brewing time did not have a significant effect on this color parameter. The effect of purple basil samples dried by different methods and purple basil tea brewed for different times on the b* and C* values were found to be statistically (p<0.05) significant.

Consumer preferences are influenced by the color properties of herbal teas. Color differences were observed between different experiments in relation to time and temperature (Kelebek, 2016). In a previous study (Jin *et al*., 2016), among all the herbal teas, lightness (L*) has been reported between 46.38-99.98. The a*, b*, C*, and h values of herbal teas have ranged between -7.68- 44.85, 0.35-24, 0.35-86.26, and 17.65-109.78, respectively. Kelebek (2016) found that the L*, a*, and b* values are significantly correlated with infusion time and temperature. The L* value is negatively correlated with infusion time and temperature and the tea infusions become darker with a long infusion time. Moreover, the values of a* and b* of the tea are positively correlated with infusion time and temperature. No proportional relationship was found between L*, b*, C*, and h values with the drying methods and infusion time (min). The a* values of the prepared tea samples were significantly affected by the drying methods and infusion times. Redness value (a*) is increased in proportion to the amount of anthocyanin contained in the prepared purple basil leaves tea. a* value results are one of the color parameters that are very important for this study. The purple basil tea prepared by the shade drying method gave the highest a* value results with respect to the other drying methods. The highest result was 15 minutes of infusion time (15.72). The lowest color values were obtained from samples prepared by the oven drying method. The results were found to be parallel to the total monomeric anthocyanin content.

Table 3. L*, a*, b*, C* and h color values of tea samples.

Drying methods	Infusion time (min)	L*	a*	b*	C*	h
Oven drying	5	75.94±1.13	7.11±2.00	32.02±2.32	32.83±2.69	77.70±2.55
	10	76.19±0.02	7.61±0.12	31.10±0.30	32.00±0.30	76.25±0.09
	15	77.67±0.43	7.78±0.17	31.41±0.41	32.36±0.44	76.09±0.13
	30	76.13±3.45	7.76±0.05	29.65±2.25	30.43±2.40	77.13±0.72
	45	75.72±0.08	7.56±0.06	31.49±0.16	32.39±0.14	76.50±0.16
Microwave drying	5	71.38±0.93	13.69±0.34	27.06±0.59	30.32±0.68	63.18±0.07
	10	73.63±0.67	11.87±0.21	24.83±0.07	27.52±0.03	64.44±0.45
	15	71.63±0.66	12.54±0.17	26.76±0.40	29.55±0.43	64.89±0.03
	30	68.80±0.14	13.88±0.24	28.58±0.28	31.77±0.36	64.10±0.16
	45	68.73±0.40	14.55±0.15	29.27±0.29	32.69±0.33	63.57±0.02
Shade drying	5	65.07±0.21	14.66±0.42	17.42±0.39	22.77±0.03	49.93±1.43
	10	63.46±0.52	15.52±1.29	18.54±0.31	24.21±0.59	50.15±2.80
	15	65.29±0.28	15.72±0.05	18.63±0.24	24.37±0.21	49.85±0.29
	30	62.84±0.61	15.21±0.27	24.35±0.78	28.72±0.53	57.99±1.27
	45	64.71±2.46	15.12±1.69	22.59±0.23	27.21±1.13	56.33±2.68

4. CONCLUSION

This study provides a foundation for the TPC and TMAC content, antioxidant activity and color parameters of a large group of purple basil leaves teas. The highest results in terms of TPC, TMAC, DPPH, and a* values were obtained with 5, 10, and 15 min infusion time in tea samples prepared with purple basil dried in an oven and shade. If the microwave drying method is preferred, an infusion time of 45 min is recommended. The results showed that purple basil leaf tea can be an economical dietary source as well as a healthy, natural antioxidant. These study

data may be of benefit to researchers and people adopting traditional complementary therapies. According to the results obtained in this study, the shade drying method values of purple basil leaves tea are generally better than oven and microwave drying methods. In order to shorten the drying time between these methods, drying in the microwave could be a good alternative to the shade drying method.

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Ayca Gulhan: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing-original draft. **Hacer Coklar:** Methodology, Supervision, and Validation. **Mehmet Akbulut:** Supervision, Writing-original draft.

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REFERENCES

Abdullah, S.S.S., & Mazlan, A.N. (2020). Quantification of polyphenols and antioxidant activity in several herbal and green tea products in Malaysia. *Materials Today: Proceedings, 31*, 106-113. https://doi.org/10.1016/j.matpr.2020.12.1083

Ahmad, M., Khan, M.P.Z., Mukhtar, A., Zafar, M., Sultana, S.&Jahan, S. (2016). Ethnopharmacological survey on medicinal plants used in herbal drinks among the traditional communities of Pakistan. *Journal of Ethnopharmacology, 184*, 154-186. https://doi.org/10.1016/j.jep.2016.02.039

Apak, R., Güçlü, K., Özyürek, M., Karademir, S.E., & Erçağ, E. (2006). The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *International Journal of Food Science and Nutrition*, *57* (5/6), 292-304. https://doi.org/10.1080/09637480600798132

Ariffin, F., Chew, S.H., Bhupinder, K., Karim, A.A., & Huda, N. (2011). Antioxidant capacity and phenolic com-position of fermented *Centella asiatica* herbal teas. *Journal of the Science of Food and Agriculture*, *91*, 2731-2739. https://doi.org/10.1002/jsfa.4454

Atoui, A.K., Mansouri, A., Boskou, G., & Kefalas, P. (2005). Tea and herbal infusions: Their antioxidant activity and phenolic profile. *Food Chemistry*, *89*, 27-36. https://doi.org/10.1016/j.foodchem.2004.01.075

Bhebhe, M., Chipurura, B. & Muchuweti, M., (2015). Determination and comparison of phenolic compound content and antioxidant activity of selected local Zimbabwean herbal teas with exotic *Aspalathus linearis. South African Journal of Botany*, *100*, 213-218. https://doi.org/10.1016/j.sajb.2015.06.006

Brand-Williams, W., Cuvelier, M.E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology*, *28*, 25-30. https://doi.org/10.1016/S0023-6438(95)80008-5

Chan, E.W.C., Lim, Y.Y., Chong, K.L., Tan, J.B.L., & Wong, S.K. (2010). Antioxidant properties of tropical and temperate herbal teas. *Journal of Food Composition and Analysis*, *23*, 185-189. https://doi.org/10.1016/j.jfca.2009.10.002

Ekren, S., Sönmez, Ç., Özçakal, E., Kukul Kurttaş, Y.S., Bayram, E., & Gürgülü, H. (2012). The effect of different irrigation water levels on yield and quality characteristics of purple basil (*Ocimum basilicum L.*). *Agricultural water management, 109*, 155-161. https://doi.org/10.1016/j.agwat.2012.03.004

Flanigan, P.M., &Niemeyer, E.D. (2014), Effect of cultivar on phenolic levels, anthocyanin composition, and antioxidant properties in purple basil (*Ocimum basilicum L.*). *Food Chemistry, 164*, 518-526. https://doi.org/10.1016/j.foodchem.2014.05.061

Fratianni, F., Cefola, M., Pace, B., Cozzolino, R, Giulio, B.D., Cozzolino, A., d'Acierno, A., Coppola, R., Logrieco, A. F., & Nazzaro, F. (2017). Changes in visual quality, physiological and biochemical parameters assessed during the postharvest storage at chilling or non-chilling temperatures of three sweet basil (*Ocimum basilicum L.*) cultivars. *Food Chemistry, 229*, 752-760. https://doi.org/10.1016/j.foodchem.2017.02.137

Hajiaghaalipour, F., Sanusi, J., & Kanthimathi, M.S. (2016). Temperature and Time of Steeping affect the antioxidant properties of white, green, and black tea infusions. *Journal of Food Science, 81*(1), 246-254. https://doi.org/10.1111/1750-3841.13149

Jin, L., Li, X., Tian, D., Fang, X., Yu, Y., Zhu, H., Ge, Y., Ma, G., Wang, W., Xiao, W., & Li, M. (2016). Antioxidant properties and color parameters of herbal teas in China. *Journal Industrial Crops and Products, 87*, 198-209. https://doi.org/10.1016/j.indcrop.2016.04.044

Kelebek, H. (2016). LC-DAD–ESI-MS/MS characterization of phenolic constituents in Turkish black tea: Effect of infusion time and temperature. *Food Chemistry,* 204, 227-238. https://doi.org/10.1016/j.foodchem.2016.02.132

Kerio, L.C., Wachira, F.N., Wanyoko, J.K., & Rotich, M.K. (2012). Characterization of anthocyanins in Kenyan teas: Extraction and identification. *Food Chemistry, 131*, 31-38. https://doi.org/10.1016/j.foodchem.2011.08.005

Lee, J., Rennaker, C., & Wrolstad, R.E. (2008). Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods. *Food Chemistry*, *110*(3), 782-786. https://doi.org/10.1016/j.foodchem.2008.03.010

Lv, H., Dai, W., Tan, J., Guo, L., Zhu, Y., & Lin, Z. (2015). Identification of the anthocyanins from the purple leaf coloured tea cultivar Zijuan (*Camellia sinensis* var. assamica) and characterization of their antioxidant activities. *Journal of Functional Foods*, *17*,449-458. https://doi.org/10.1016/j.jff.2015.05.043

McCance, K.R., Flanigan, P.M., Quick, M.M., & Niemeyer, E.D. (2016). Influence of plant maturity on anthocy-anin concentrations, phenolic composition, and antioxidant properties of 3 purple basil (*Ocimum basilicum L.*) cultivars. *Journal of Food Composition and Analysis, 53*, 30-39. https://doi.org/10.1016/j.jfca.2016.08.009

Oh, J., Jo, H., Cho, A.R., Kim, S., & Han, J. (2013). Antioxidant and antimicrobial activities of various leafy herbal teas. *Food Control*, *31*, 403-409. https://doi.org/10.1016/j.foodcont.2012.10.021

Šavikin, K., Zdunić, G., Janković, T., Gođevac, D., Stanojković, T., & Pljevljakušić, D. (2014). Berry fruit teas: Phenolic composition and cytotoxic activity. *Food Research International, 62*, 677-683. https://doi.org/10.1016/j.foodres.2014.04.017

Schulzki, G., Nüblein, B., & Sievers, H. (2017). Transition rates of selected metals determined in various types of teas (*Camellia sinensis* L. Kuntze) and herbal/fruit infusions. *Food Chemistry*, *215*, 22-30. https://doi.org/10.1016/j.foodchem.2016.07.093

Siah, W.M., Azman, M.A., Jeeven, K., Noor Hayazan, M.D., & Mohd Tahir, S. (2011). Effect of infusion conditi-ons on total phenolic content and antioxidant activity in *Centella asiatica*

tea. *Journal of Tropical Agriculture and Food Science, 39*(2), 149-156. https://doi=10.1.1.1082.7283

Singleton, V.L., & Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, *16*(3), 144-158.

Vinokur, Y., Rodov, V., Reznick, N., Goldman, G., Horev, B., Umiel, N., & Friedman. H. (2006). Rose petal tea as an antioxidant-rich beverage: cultivar effects. *Journal of Food Science*, *71*(1), 42-47. https://doi.org/10.1111/j.1365-2621.2006.tb12404.x

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Research Article

Pharmacokinetics, drug-likeness, antibacterial and antioxidant activity of secondary metabolites from the roots extracts of *Crinum abyssinicum* and *Calotropis procera* and *in silico* molecular docking study

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Abstract: *Crinum abyssinicum* and *Calotropis procera* were traditionally used for the treatment of different diseases such as hypertension, diabetes, hepatitis B, skin infection, anticancer, asthma, fever, and diarrhea. The structures of the compounds were characterized by ^{1}H NMR, ^{13}C NMR, and DEPT-135 spectra. Compounds **1-3** were reported herein for the first time from the species of *C. abyssinicum*. The DCM/MeOH (1:1) and MeOH roots extracts of *C. abyssinicum* showed significant inhibitory activity against *S. aureus* and *P. aeruginosa* with a mean inhibition zone of 16.67 ± 1.20 and 16.33 ± 0.33 mm, respectively. Compounds **4** and **5** showed promising activity against *E. coli* with a mean inhibition zone of 17.7 ± 0.8 and 17.7 ± 1.2 mm, respectively. The results of DPPH activity showed the DCM: MeOH (1:1) and MeOH roots extracts of *C. abyssinicum* inhibited the DPPH radical by 52.86 ± 0.24 % and 45.6 ± 0.11 %, respectively, whereas compound **5** displayed 85.7 % of inhibition. The drug-likeness analysis showed that compounds **2-4** satisfy Lipinski's rule of five with zero violations. Compounds **2**, and **6** showed binding affinities of −6.0, and −6.7 kcal/mol against *E. coli* DNA gyrase B, respectively, while **3** and **5** showed −5.0 and −5.0 kcal/mol, respectively against human peroxiredoxin 5. Therefore, the *in vitro* antibacterial, radical scavenging activity along with the molecular docking analysis suggest the potential use of the extracts of *C. abyssinicum* and compounds **2, 5, 6,** and **3, 5** can be considered as promising antibacterial agents and free radical scavengers, respectively.

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1. INTRODUCTION

The genus *Crinum* is one of the medicinal plants that belong to the family Amaryllidaceae. Globally, around 180 Crinum species were described and widely distributed in Africa, America, southern Asia, and Australia (Lawal & Dangoggo, 2014). Out of these, four species are found in Ethiopia *i.e.*, *Crinum abyssinicum, Crinum bambusetum, Crinum macowanii*, and *Crinum ornatum* (Nordal & Sebsebe, 2010). *Crinum abyssinicum* and *Crinum bambusetum* are endemic species of Ethiopia (Nordal & Sebsebe, 2010). *Crinum abyssinicum* is a species of bulbous

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plant that belongs to the family of the Amaryllidaceae (Figure 1). In Ethiopia, it is commonly known as shinkurta/bokolo Werabessa/ Murquffaa/ Chopi in Afan Oromo, Yejib Shinkurt in Amharic, Galadiweese in Sidamigna (Abebe *et al*., 2003). In Ethiopia, *Crinum* species have been reported to be used in various health care systems for the treatment of a variety of diseases such as hypertension and diabetes (Regassa, 2013), animals' internal parasites (Tamiru *et al*., 2013), skin infection (Yineger *et al*., 2008), rheumatoid arthritis (Kloos *et al*., 2014), snake bite (Mekuanent *et al*., 2016), malaria (Asnakech *et al*., 2019) and antitumor (Teklehaymanot & Giday, 2007). Traditionally the dried roots of *C. abyssinicum* mixed with water or butter is applied topically or oral.

Figure 1. (a) Aerial part and (b) bulb of *C. abyssinicum* (Illubabor, Oromia, Picture taken by Melaku Tegegn on November 20, 2021).

(a)

(b)

Crinum species have been subjected to extensive chemical and biological investigations due to their richness in pharmacologically active principles (Wildman, 1960). A large number of alkaloids and non-alkaloid compounds have been reported from different *Crinum* species (Refaat *et al*., 2012; 2013). Amaryllidaceae alkaloids, augustamine (Ali *et al*., 1983; Ramadan, 1986; Machocho *et al*., 2004), *β*-carboline, phenanthridine (Razafimbelo *et al*., 1996), sceletium (Döpke *et al*., 1981), ismine (Ghosal, 1981; Highet & Ismine, 1961) and clivimine type alkaloids were reported from the genus. Other secondary metabolites including flavonoids, chromones, coumarins, terpenoids, steroids, phenolics, simple glycosides, and long-chain hydrocarbons were also reported (Refaat *et al*., 2013). In Ethiopia, the bulb extract, 6-hydroxycrinamine, and lycorine possessed significant antiproliferative activity, lycorine being the most active exhibiting GI_{50} values of 2.8 μg/mL and 3.4 μg/mL against A2780 and MV4-11 cells, respectively (Besufekad *et al*., 2020). To the best of our knowledge, there is no scientific report in the literature concerning the antioxidant and antimicrobial effects of the plant.

Calotropis procera is a soft-wooded, evergreen, perennial shrub in the family Asclepiadaceae. They are commonly known as milkweeds because of the latex they produce. It includes 320 genera and 2,000 species. It is widely distributed in Asia, Africa, and America (Ramos *et al*., 2006). In Ethiopia, this plant species is known by local names of Tobiaw (Amharic), Qimbo (Afan Oromo), Ghinde'a (Tigregna), Galaqto (Afar) (Harini and Nithyalakshmi, 2017). Traditionally, *Calotropis* is used alone or with other medicines to treat common diseases such as fever, rheumatism, cold, eczema, diarrhea, and treatment of boils (Pathyusha, 2012; Abhishek *et al*., 2010). In traditional folk medicine, the plant has been employed as an antifungal and antipyretic agent (Shrivastava *et al*., 2013). In Ethiopia, the latex of *C. procera* (Asclepiadaceae) (Figure 2) is among the herbal drugs used for the treatment of blackleg to treat cattle by "Zay" people (Giday & Teklehaymanot, 2013).

Figure 2. Aerial part of *C. procera* (Adama, Oromia, Picture by Getachew Tegegn on August 16, 2020).

Previous pharmacological reports disclosed that *C. procera* exhibit wide spectrum of pharmacological activity including antimicrobial, anthelmintic, anti-inflammatory, analgesic and antipyretic, anticancer, antidiabetic, antifungal, antioxidant, larvicidal activity, anticonvulsant, anti-ulcer effects, and wound healing (Hassan *et al*., 2006; Al-Snafi, 2015). In Ethiopia, pharmacological studies of ethanolic extract from the leaves and latex *C. procera* demonstrated antimicrobial activity against *S. aureus, E. coli, Bacillus cereus, Proteus mirabilis, Proteus vulgaris, Klebsiella pneumoniae, Shigella dysenteries* and *Pseudomonas aeruginosa* (Chavan, 2016). Phytochemical studies indicated that root extracts of *C. procera* contained alkaloids, flavonoids, glycosides, saponins, and terpenes (Schimmer and Mauthner, 1996). Inspired by these reports, in the present work, chemical constituents of roots extract of *C. abyssinicum* and *C. procera*, evaluation of the antibacterial and radical scavenging activities of extracts and isolated compounds along with *in silico* molecular docking, ADMET, and Toxicity analysis were presented.

2. MATERIAL and METHODS

2.1. General Procedure

The compound purity was determined by analytical TLC. Analytical TLC was run on a 0.25 mm thick layer of silica gel GF254 (Merck) on aluminum plate. Spots were detected by observation under UV light (254 and 365 nm). The vanillin spraying agents were used as detecting reagent. Column chromatography was performed using silica gel (60-200 mesh) Merck. Samples were applied on a column by adsorbing on silica gel. The solvent was removed using rotavapor under vacuum at 40^{o}C. NMR spectra were recorded using Bruker Avance 400 MHz spectrometer.

2.2. Plant Materials Collection and Identification

The roots of *C. procera* and *C. abyssinicum* were collected from Adama and Illubabor zones of the Oromia Region, Ethiopia on August 16, 2020, and November 20, 2021, respectively. Identification and authentication of the plant's specimen were done by botanist Mr. Melaku Wondaferash at the National Herbarium, Addis Ababa University, and voucher specimens were deposited (GT-002/2020 for *C. procera* and GT-003/2021 for *C. abyssinicum*). The plants were chopped into small pieces, air dried under shade at room temperature, and pulverized using a Willy electrical mill.

2.3. Extraction and Isolation

The powdered roots of *C. abyssinicum* (800 g) and *C. procera* (550 g) were extracted with CH_2Cl_2/MeOH (1:1) 3L three times for 48h in each case by shaking using a mechanical shaker

at room temperature. The filtrates were concentrated *in vacuo* on a rotary evaporator at 40 ^{o}C to obtain 16 g (2%) reddish solid and 15.2 g (2.76 %) yellowish solid crude extracts, respectively. The mark left in each case was further extracted with methanol (100%). The filtrate was concentrated using a vacuum rotary evaporator to yield 3 g (0.375 %) reddish and 10.2 g (1.85 %) yellowish crude extracts, respectively.

The dichloromethane/methanol (1:1) roots extract (14 g) of *C. abyssinicum* was adsorbed to an equal amount of silica gel and subjected to silica gel column chromatography (200 g). Elution was done with an increasing gradient of ethyl acetate in *n*-hexane followed by methanol in dichloromethane. A total of 111 fractions were collected (each 50 mL). Concentrated fractions were subjected to thin-layer chromatography to monitor the composition profile and fractions that showed similar R_f values and the same characteristic color on TLC were combined and further purified. Combined fractions were dried in glass vials and yields were determined. Fraction 14 (1% ethyl acetate in *n*-hexane) afforded compound **1** (100 mg). Fractions 27-29 (4.5 % ethyl acetate in *n*-hexane as eluent) were combined and purified by silica gel column chromatography (eluent, gradient of ethyl acetate in *n*-hexane) to give compound **2** (90 mg). Fractions 57-59 (0.5 % methanol in dichloromethane) were combined to give compound **3** (40 mg).

The crude dichloromethane/methanol (1:1) extract (10 g) of *C. procera* was adsorbed on an equal mass of silica and subjected to silica gel column chromatography (200 g). Elution was conducted with an increasing gradient of ethyl acetate in *n*-hexane followed by an increasing gradient of methanol in dichloromethane. A total of 50 fractions were collected (each 50 mL). Fractions 22-30 showed three spots on TLC (*n*-hexane/EtOAc 4/1, 800 mg) and were further purified using silica gel column chromatography with *n*-hexane to afford 54 subfractions. Subfractions 7-11 showed a single spot (*n*-hexane: EtOAc,4:1) to give compound **4** (70 mg). Subfractions 20-31 showed a single spot (*n*-hexane:EtOAc, 7:3) to give compound **5** (60 mg). Fractions 31-34 gave single spot-on TLC (mobile phase *n*-hexane/EtOAc 7:3 as eluent) to give compound **6** (20 mg).

2.4. Antibacterial Activity

All samples were evaluated for their antibacterial activity against two-Gram positive bacteria (*Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615) and two Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922) using the disc diffusion method. The microbial cultures were grown overnight at 37°C in nutrient broth, distilled water was used to adjust to 0.5 McFarland standard, and lawn inoculated onto Mueller Hinton agar (MHA) plates.100 µg were dissolved in each sample in 0.1 mL DMSO and adjusted to a concentration of 250 and 125 mg/mL. 6 mm diameter of sterile filter paper discs were soaked in 1 mL DMSO solution of the compounds at 250 and 500 µg/mL concentrations. Then, the saturated paper discs were placed on the center of every MHA plate. The inhibition zones were measured and compared with those produced by the reference antibiotics, Ciprofloxacin (each at 10 µg/disc). The resulting diameters of zones of inhibition produced by the plant extracts and standard antibiotics were measured using a ruler and reported in millimeters. The mean of zones of inhibition was calculated for each extract and the standard antibiotics. The results are expressed as M ± SEM of triplicate (Table 6) (Valgas *et al*., 2007; Singh and Jain, 2011). The standard drug used as a positive control was Ciprofloxacin, and DMSO was used as a negative control.

2.5. Antioxidant Activity Assay

The antioxidant activities of the plant extract and their constituents were studied using DPPH methods. The DPPH radical scavenging activities of the extracts and isolated compounds were evaluated using DPPH assay following procedure (Rivero-Perez *et al*., 2007). Serial dilutions

were carried out with the stock solutions 0.5 mg mL^{-1} of the plant extract and its constituents to obtain concentrations of 50, 25, 12.5, and 6.25 µg mL^{-1}. The solutions were prepared using methanol as solvent. 4 mL from each four diluted concentrations of the samples were mixed with 1 mL of 2,2-diphenyl-1-picryl hydrazyl (DPPH) solution that was prepared by dissolving 4 mg of DPPH in 100 mL of MeOH. The resulting solution was placed in an oven at 37°C for 30 min and subjected to a UV-Vis spectrophotometer to record absorbance at 517 nm. The percentage of DPPH inhibition is calculated according to the following formula (Proestos *et al*., 2013).

$$(\%)\ \text{inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Where A control was the absorbance of the DPPH solution and A sample was the absorbance of a tested sample. Samples were analyzed in triplicate. Ascorbic acid was used as a positive control.

2.6. *In silico* Pharmacokinetics (ADME), Drug-likeness and Toxicity Prediction

Computational technology has reduced experimental drug trials and improved the success rate; hence, it's become an important tool in drug candidate identification. The screening of bioavailability and pharmacokinetic properties like absorption, distribution, metabolism, and excretion parameters are evaluated to determine their activity within the human body. They evaluated using the Swiss ADME online web tool. The structures of isolated compounds were converted to their canonical simplified molecular-input line-entry system (SMILE), and the SMILES of all selected ligands were used as input data and submitted to SwissADME and PreADMET tool to estimate *in-silico* pharmacokinetic parameters such as the number of hydrogen donors, hydrogen acceptors, and rotatable bonds, and total polar surface area of a compound. The computer program further gave a compiled result on the lipophilicity and hydrophilicity of those molecules by integrating results obtained from various Log P and S prediction programs called ILOGP, XLOGP3, WLOGP, ESOL, and SILICOS-IT. Log P, a measure of the lipophilicity of the molecule is the logarithm of the ratio of the concentration of drug substance between two solvents in an unionized form. The drug-likeness of the isolated compounds was predicted by adopting Lipinski's Rule of 5. It denotes that the drugs and/or candidates should obey the rule of 5 parameters like hydrogen-bond donors (HBDs) < 5, hydrogen-bond acceptors (HBAs) < 10, a molecular mass < 500 Da, log P not > 5, and total polar surface area (TPSA) shouldn't be > 140 Å (Lipinski *et al*., 1997). The rule was developed to set drug-likeness ground rules for new molecular entities (NMEs) (Lipinski, 2000). The Rule of 5 predicts molecules with more than 5 H-bond donors, 10 H-bond acceptors, molecular weight of more than 500 Da, and also the calculated Log P (Log P) greater than 5 likely had poor absorption or permeation of the molecular entities. Hence, molecules will unlikely to become orally bioavailable as a drug if their properties fall outside these boundaries (Tareq & Khan, 2010). Lipinski's rule suggests an upper limit of 5 for druggable compounds. For the chemical substance, the lower the log P values the stronger the lipophilicity.

The absorption of drugs depends on water solubility, P-glycoprotein substrate (P-gp substrate), skin permeability (log Kp) levels, Gastro-Intestinal absorption (GSI), and membrane permeability. The drug distribution depends on the blood-brain barrier (BBB). Excretion depends upon the total clearance, and renal OCT2 substrate (Mahanthesh *et al*., 2020; Han *et al*., 2019). The volume of distribution and metabolism was evaluated with the help of CYP models, namely CYP1A2 inhibitor, CYP2C19 inhibitor, CYP2C9 inhibitor, and CYP3A4 inhibitor. The toxicity profile of the isolated compounds was predicted. ProTox-II server were accustomed to determine the toxicological endpoints (Hepatotoxicity, Carcinogenicity, Immunotoxicity, Mutagenicity), toxicity class and also the level of toxicity (LD50, mg/Kg) of

the isolated compounds (Banerjee *et al*., 2018; Drwal *et al*., 2014). The results were compared with ciprofloxacin used as a standard clinical drug (Table 9).

2.7. Molecular Docking Study

AutoDock Vina version 4.2 with the standard protocol was established to dock the proteins (PDB ID: 6F86, PDB ID: 1HD2 and PDB ID: 3T07) and also isolated compounds **2, 3, 4, 5** and **6** into the active site of proteins (Seeliger and Groot; Trott and Olson, 2010; Tapera *et al.*, 2022). The 2D structures of isolated compounds (**2, 3, 4, 5, 6**) were drawn using the ChemOffice tool (ChemDraw 16.0). ChemBio3D was used to minimize the energy of each molecule. The energy-minimized ligand molecules were then used as input for AutoDock Vina, so as to hold out the docking simulation (Trott and Olson, 2010). The crystal structures of receptor molecules *E. Coli* DNA gyrase B (PDB ID: 6F86), Pyruvate Kinase (PDB ID: 3T07), and human peroxiredoxin 5 (PDB ID: 1HD2) were downloaded from the protein data bank. All the ligands were individually docked into the target based on ligand-protein interactions. As per standard protocol protein preparation was done (Narramore *et al*., 2019), by removing the chosen water molecules, cofactors, and previously attached ligands. The protein was prepared by adding polar hydrogens using auto preparation of target protein file AutoDock 4.2.6 (MGL tools 1.5.6). To set the grid box for docking simulations the graphical user interface program AutoDock 4.2.6 was used. We tried several different docking pockets and poses, and at last, the grid was generated as per the best results achieved. The docking algorithm provided with AutoDock Vina v.1.2.0 was used to search for the best docked conformation between ligand and protein (Poustforoosh *et al.*, 2022). A maximum of nine conformers were considered for each ligand during the docking process. For analyzing the interactions between the target receptor and ligands by discovery studio visualizer and PyMOL, conformations with the most favorable (least) free binding energy were selected (Poustforoosh *et al.*, 2021). The ligands are represented in several colors, H-bonds and also the interacting residues are represented in stick model representation.

2.8. Statistical data analysis

Antibacterial and antioxidant data obtained by triplicate measurements were reported as mean ± standard error of the mean (SEM). GraphPad Prism version 8.0.2 for Windows was established to perform the analysis. Groups were analyzed for significant differences using a linear model of variance analysis (ANOVA) test for comparisons was performed.

3. RESULTS and DISCUSSION

3.1. Characterization of Isolated Compounds

The dichloromethane/methanol (1:1) extract of the roots of *C. abyssinicum* and *C. procera* was subjected to silica gel column chromatographic fractionation which afforded six compounds (**1-6**) of which compounds **1-3** were reported herein for the first time from *C. abyssinicum* whereas compound **2** was reported herein for the first time from the plant source. The structures of the isolated compounds were characterized by ^{1}H NMR, ^{13}C NMR, and DEPT-135 as discussed here below.

Compound **1** was obtained as a yellow oil with R_f value of 0.40 (*n*-hexane/EtOAc (9:1), as eluent. Its ^{1}H NMR spectrum (400 MHz, $CDCl_3$) showed signals at δ 2.3 (t, 2H, H-2), δ 5.4 (brs, 1H, H-7) and δ 0.9 (t, 3H, H-18) attributed to methylene protons attached to a carboxyl group, olefinic proton, and terminal methyl protons, respectively. The ^{13}C NMR (100 MHz, $CDCl_3$) spectral data with the aid of DEPT-135 revealed a total of 18 carbon signals of which two olefinic methine signals at δ 130.0, and 127.9, and fourteen methylene signals at δ 34.0, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.3, 29.2, 29.1, 27.2, 25.6, 24.7 and 22.7 are clearly evident. The most downfield and upfield signals appearing at δ 179.9 and 14.1 attributed to the carboxyl

group and terminal methyl, respectively. The above spectral data are in good agreement with data reported in the literature for (*E*)-Octadec-7-enoic acid (Ibrahim *et al*., 2012).

Compound **2** (40 mg) was isolated as a yellow oil from the DCM/MeOH (1:1) with R_f value of 0.6 (*n*-hexane:EtOAc (5.5/4.5), as eluent). Its ^{1}H NMR spectral data (400 MHz, $CDCl_3$) showed a signal due to terminal methyl protons at δ 0.9 (3H, t, *J*= 6.7 Hz). The spectrum also displayed methylene signals at δ 2.3 (2H, t) and 1.6 (2H, brs) of which the former suggests methylene is connected to a carbonyl group. The proton signal at δ 3.7 (1H, *m*, H-2′) accounted for the presence of oxygenated methine proton connected to oxygen whereas as signals at δ 4.1 (dd, *J* = 12.0, 4.5 Hz, 2H, H-1′), 3.9 (m, 1H, H-3′a) and 3.7 (m, 1H, H-3′b) attributed to oxygenated methylene protons. The ^{13}C NMR spectrum with the aid of DEPT-135 spectrum showed a total of 20 well resolved carbon signals of which four olefinic carbons signals at δ 130.2, 130.0, 128.1 and 127.9, methylene carbon signals at δ 34.2, 31.9, 31.5, 29.7, 29.5, 29.3, 29.1, 27.2, 25.6, 24.9 and 22.7, oxygenated methylene signals at δ 65.1 and 63.3, and sp^3 oxygenated methine at δ 70.3 are clearly observed. Most downfield signals appearing at δ 174.4 are attributed to the ester carbonyl whereas the upfield signal at δ 14.1 suggests a terminal methyl group. The above spectral data is in good agreement with data reported for penicilloitins B, previously reported from marine endophytic *Penicillium* species, where the hydroxyl group at C-13 is dehydrated to double bond in case of compound **2** (Table 1) (Mourshid *et al*., 2016).

Table 1. Comparison of the spectral data of compound 2 and penicilloitins B ($CDCl_3$, δ in ppm).

Position	Compound **2**			Mourshid *et al*., 2016
	^{1}H NMR	^{13}C NMR	Multiplicity	^{13}C NMR
1		174.4	Carbonyl	174.3
2	2.3 (d, *J* = 7.2 Hz, 2H)	34.2	CH_2	34.1
3	1.6 (brs, 2H, H-3,4)	24.9	CH_2	24.8
4		25.6	CH_2	25.7
5	1.3 (d, *J* = 17.1 Hz, H-5,6,7,8)	29.3	CH_2	29.3
6		29.7	CH_2	29.7
7		29.5	CH_2	29.5
8		29.1	CH_2	29.0
9	2.0 (brq, *J* = 13.3, 5.0 Hz, 2H)	27.2	CH_2	27.3
10	5.4 (d, *J* = 11.6 Hz, 1H)	130.2	= CH	133.4
11		127.9	= CH	125.2
12		31.5	CH_2	35.3
13		128.1	= CH	71.5
14		130.0	= CH	36.8
15		31.9	CH_2	31.8
16		22.7	CH_2	22.0
17	0.9 (t, *J* = 5.0 Hz, 3H)	14.1	CH_3	14.0
1’	4.2 (d, *J* = 17.5 Hz, 1H), 4.1 (t, *J* = 6.1 Hz, 1H)	65.1	CH-O	65.3
2’	3.7 (m, 1H)	70.3	CH_2O-	70.2
3’	3.9 (d, *J* = 22.3 Hz, 1H), 3.86 – 3.75 (m, 1H)	63.3	CH_2O-	63.3

Compound **3** was obtained as a reddish crystal with R_f value of 0.56 (DCM: MeOH, 9.5:0.5) as eluent. The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) showed the presence of an olefinic proton signal at δ 5.3 (brs, 1H, H-6, 7)**.** The compound exhibited the doublet signals at δ 4.1 (1H, d, *J* = 8.0 Hz, H-1′a), and 3.8 (1H, d, *J* = 7.8 Hz, H-1′b) due to oxygenated methylene protons. The

signal at δ 1.3 (brs, 6H) is the characteristic signal for many methylene protons in the compound which was supported by the appearance of an intense carbon signal at δ 29.7 in the ^{13}C NMR spectrum. The broad singlet signal observed at δ 2.3 is ascribed to methylene protons attached to a carbonyl group. An upfield proton signal at δ 0.9 (brs, 6H) is evident for the presence of two terminal methyl protons in the compound. The ^{13}C-NMR (400 MHz, $CHCl_3$) spectrum with the aid of DEPT-135 revealed 20 carbon signals of which fourteen methylene carbons at δ 34.4, 34.3, 31.9, 31.5, 29.7, 29.5, 29.4, 29.3, 28.6, 27.2, 25.9, 25.0, 24.8 and 22.7. The downfield signals at δ 64.4 correspond to an oxygenated methylene carbon and the most upfield carbon signal at δ 14.2, and 14.1accounts for the presence of two terminal methyl protons. The presence of two olefinic sp^2 signals at δ 129.9 and 129.8 suggest the presence of one double bond. The presence of ester carbonyl carbon was evident at δ 174.1. On the basis of the above spectral data, the structure of compound **3** was found to be identical to data reported for Ethyl (E)-octadec-8-enoate (Barange *et al*., 2020) reported herein for the first time from the genus *Crinum.*

Compound **4** was obtained as a yellowish solid with R_f value of 0.51 (*n*-hexane/Ethyl acetate 4/1) as eluent. Its ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) showed the presence of an olefinic proton signal at δ 5.4 (m, 1H, H-4) and 5.1 (m, 1H, H-5)**.** Oxygenated methylene protons appeared at δ 4.3-4.1 (m, 1H) and 3.8-3.5 (m, 1H). An upfield proton signal at δ 0.9 (m, 3H) is evident for the presence of methyl group in the compound.

The ^{13}C-NMR spectrum with aid of DEPT-135 showed a total of well resolved 14 carbon signals including seven methylene signals at δ 33.8, 31.9, 30.4, 29.7, 29.4, 28.9, 23.7, and 22.7, methine carbon at δ 38.7, oxygenated methylene carbons at δ 63.1 and 68.2 and terminal methyl at δ 14.1, and two sp^2 methines at δ 130.9 and 128.8. The above spectral data is comparable with literature reported data for (4*Z*)-dodec-4-en-1-ol (D´yakonov *et al*., 2020) and the only difference is additional hydroxyethyl at C-2 in the case of compound **4** Table 2.

Table 2. ^{13}C-NMR spectral data of compound **4** and the reported ^{13}C-NMR data for (4*Z*)-dodec-4-en-1-ol (D´yakonov *et al*., 2020).

Position	NMR data of compound **4**			D´yakonov *et al*., 2020	
	^{1}H-NMR	^{13}C-NMR	Multiplicity	^{13}C-NMR	Multiplicity
1		63.1	CH_2O-	61.9	CH_2O-
2		38.7	CH	32.5	CH_2
3		33.8	CH_2	23.5	CH_2
4	5.38(m,1H)	128.8	= CH	128.8	= CH
5	5.1(m, 1H)	130.0	= CH	130.4	= CH
6		23.7	CH_2	27.1	CH_2
7		31.9	CH_2	31.8	CH_2
8		28.9	CH_2	29.2	CH_2
9		29.4	CH_2	29.2	CH_2
10		29.7	CH_2	29.7	CH_2
11		22.7	CH_2	22.6	CH_2
12	0.9 (m, 3H)	14.1	CH_3	13.9	CH_3
1'		30.4	CH_2	-	-
2'		68.2	CH_2O-	-	-

Compound **5** was obtained as a white solid with an R_f value of 0.46 (*n-hexane*/EtOAc (3.5:1.5) as eluent. Its ^{1}H-NMR spectrum (400 MHz, $CDCl_3$, Table 3) showed three olefinic protons at δ 5.8 (dd, 1H), 5.4 (m, 1H), 5.2 (dd, *J* = 15.1, 8.6 Hz, 1H). The signal at δ 4.4 (1H, s, H-3) corresponds to oxygenated methylene proton. The appearance of the singlets at δ 1.4, 0.8, and 0.8 confirm the presence of three methyl groups attached to quaternary carbons. The presence of the peak at δ 2.4 revealed that the methyl proton is attached to the carbonyl group.

The spectrum also revealed the presence of methyl proton signals at δ 1.0 (dd, *J* = 11.5, 7.4 Hz, 3H), 0.9 (dd, *J* = 7.1, 2.7 Hz, 3H), 0.9 (dd, *J* = 13.5, 7.4, 1.9 Hz, 9H) and 0.8 (d, *J* = 7.1 Hz, 3H).

The ^{13}C NMR ($CDCl_3$ 400 MHz, Table 3) spectrum in combination with DEPT-135 displayed 31 carbon signals corresponding to seven methyl groups at δ 20.2, 19.8, 19.5, 19.0, 14.1, 12.2, and 12.0, nine methylene groups at δ 39.6, 38.6, 37.1, 34.2, 29.7, 28.2, 24.1, 22.7 and 21.0. Eight sp^3 methine carbons were observed at δ 56.0, 55.9, 53.6, 51.2, 45.8, 40.5, 36.,1 and 31.9. The peaks observed at δ 38.0 and 42.5 in the ^{13}C NMR spectrum were absent in DEPT-135 spectrum suggesting the presence of two sp^3 quaternary carbon atoms that belong to C-10 and C-13, respectively. The spectrum also showed sp^3 oxygenated methine at δ 73.1 (C-3) and ester carbonyl at δ 168.8 along with olefinic methines at δ 126.2 (C-7), 138.1 (C-23), and 129.4 (C-22). The above spectral data of compound **5** is comparable with data reported for the spinasterol skeleton and the only difference is the presence of extra acyl moiety at C-3, in the case of compound **5**.

Table 3. ^{1}H NMR and ^{13}C NMR data of compound **5** and literature ^{13}C-NMR data of spinasterol.

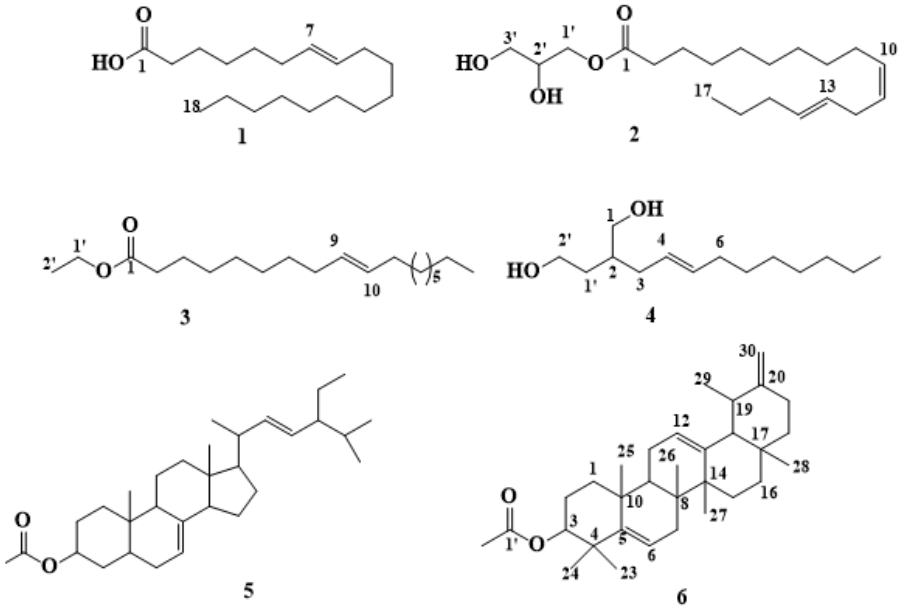
Position	Compound **5**			Meneses-Sagrero, *et al*., 2017)	Yang *et al*., 2017
	^{1}H-NMR	^{13}C-NMR	Multiplicity	^{13}C-NMR	^{13}C-NMR
1		37.1	CH_2	37.2	37.2
2		31.9	CH_2	31.5	31.5
3		73.2	CH-O	71.1	71.1
4		38.6	CH_2	38.0	38.0
5		45.8	CH	40.3	40.8
6		29.7	CH_2	29.7	29.7
7	5.8 (t, 1H)	126.2	= CH	117.5	117.5
8		-	CH	139.6	139.6
9		53.6	CH	49.5	49.5
10	-	38.0	C	34.2	34.2
11		21.0	CH_2	21.6	21.6
12		39.6	CH_2	39.5	39.5
13	-	42.5	C	43.3	43.3
14		55.9	CH	55.1	55.1
15		23.1	CH_2	23.1	23.0
16		28.2	CH_2	28.5	28.5
17		56.0	CH	55.9	55.9
18		12.2	CH_3	12.0	12.2
19		12.2	CH_3	13.0	13.0
20		40.5	CH	40.8	40.3
21		19.5	CH_3	21.4	19.0
22	5.4 (1H, m)	138.1	CH	138.2	138.2
23	5.2 (1H, m)	129.5	CH	129.5	129.5
24		51.2	CH	51.3	51.3
25		31.9	CH	31.9	31.9
26		21.2	CH_3	21.1	21.4
27		21.1	CH_3	18.99	21.0
28		25.4	CH_2	25.4	25.4
29		12.0	CH_3	12.0	12.1
30	-	168.8	Ester	-	-
31		21.0	CH_3	-	-

Compound **6** was isolated as a colorless powder with an R_f value of 0.6 (*n*-hexane: EtOAc, 1:1) as eluent from DCM:MeOH (1:1) extract of the roots of *C. procera*. Its ^{1}H NMR spectrum showed two downfield protons at δ 5.4 (m, 1H, H-6) and 5.1 (t, *J* = 3.5 Hz, 1H, H-12) allocated to H-6 and H-12 and pair of doublets at δ 4.6 and 4.5 (*J* = 4.6, 2.2 Hz*)* associated with the C-30 exocyclic methylene group. A doublet proton at δ 2.2 was attributed to methine proton H-18 proton. The ^{1}H NMR spectrum also showed signals for ten methyl groups, of these, six of them were positioned at quaternary carbons corresponding to the singlet signals. The ^{13}C NMR spectrum showed carbon signals corresponding to those of the ursane-type triterpenes (Ali et al., 1998, 2000). Olefinic carbons appeared at δ 145.2 (C-5), 121.6 (C-6), 124.3 (C-12), and 139.6 (C-13), of which the former is sp^2 quaternary carbon, whereas signals of exocyclic methylene appeared at δ 154.6 (C-20) and 107.2 (C-30). The signals at δ 80.9 is attributed to sp^3 oxygenated methine at C-3. The NMR spectral data of compound **6** is in good agreement with data reported for calotroproceryl acetate A, previously reported from the same species (Table 4) (Ibrahim *et al*., 2012).

Table 4. Comparison of the ^{13}C-NMR spectral data of compound **6** and calotroproceryl acetate A ($CDCl_3$, δ in ppm).

Position	NMR data of compound 7		Ibrahim *et al*., 2012
	^{1}H-NMR	^{13}C-NMR	^{13}C-NMR
1		38.4	38.5
2		28.1	28.1
3	3.7 (1H, brs)	80.9	81.0
4		36.8	37.8
5		145.2	145.2
6	5.4 (t, *J* = 14.5 Hz, 1H)	121.6	121.6
7		32.87	33.3
8		40.0	40.0
9		48.6	47.6
10		37.7	37.7
11		23.4	23.6
12	5.14 (t, *J* = 3.5 Hz, 1H)	124.3	124.3
13		139.6	139.6
14		40.9	42.1
15		26.6	26.9
16		23.6	23.7
17		34.5	34.7
18		50.4	48.6
19		39.7	41.5
20		154.6	154.7
21		32.9	32.9
22		38.9	39.7
23		28.8	29.1
24		15.9	15.8
25		16.8	16.8
26		16.9	16.9
27		25.8	26.6
28		17.5	17.5
29		19.5	18.3
30	4.62 (dd, *J* = 4.6, 2.2 Hz, 2H)	107.2	107.1
1′	-	171.0	171.1
2′		21.3	21.3

Figure 3. Compounds isolated from the roots of *C. abyssinicum* and *C. procera*.



3.2. Antibacterial Activity of The Extracts and Isolated Compounds

The DCM/MeOH (1:1) root extracts of *C. abyssinicum* (Table 5, Figure 4) showed antibacterial activity against Gram-negative bacteria *P. aeruginosa* with an inhibition zone of 12.67 ± 1.76 at a concentration of 250 µg/mL and Gram-positive bacteria *S. aureus, S. pyogenes* with 16.67 ± 1.20 and 12.67 ± 1.20 mm diameter zone of inhibition at 250 µg/mL, respectively. However, unlike the above tested bacterial strains, the activity shown against *S. aureus* at 250 µg/mL was promising compared to ciprofloxacin used as a standard antibiotic. Whereas the DCM/MeOH (1:1) extract showed no activity against *E. coli*. The MeOH extracts of the root of *C. abyssinicum* (Table 5) showed activity only against Gram-negative bacteria *P. aeruginosa*, *E. coli* with a mean inhibition zone of 16.33 ± 0.33 and 10.33 ± 0.33 mm diameter at 250 µg/mL, respectively. However, the activity demonstrated against *P. aeruginosa* compared to ciprofloxacin was pronounceable.

The DCM/MeOH (1:1) extract of *C. abyssinicum* displayed better activity than the MeOH extract against *S. aureus* and *S. pyogenes*. In contrast, MeOH extract inhibited better activity against *E. coli* than DCM/MeOH (1:1) extract. Accordingly, the standard drug ciprofloxacin revealed higher antibacterial activity in comparison with the plant crude extracts. The negative control DMSO did not show any inhibition effect against the tested bacterial species. The activity showed by DCM/MeOH extract of roots of *C. procera* (Table 5, Figure 4) exhibited relatively better activity against Gram-negative bacteria with mean inhibition zone 11.67 ± 0.33 and 11.33 ± 0.67 in mm observed at 250 µg/ mL for *P. aeruginosa* and *E. coli*., respectively. Whereas *S. pyogenes* was found to inhibit moderate activity with mean inhibition zones of 10.33 ± 0.33 and 10.67 ± 0.67 mm at 125 and 250 *µ*g/mL, respectively. The MeOH extract (Table 5) showed no activity against all bacterial species used in the study. The DCM/MeOH (1:1) extract displayed better activity than the MeOH extract against all the tested bacterial species. Besides, the inhibition zone of all the extracts in the present work, the activity increased with increasing concentration in a dose-dependent manner. The MeOH root extract of *C. abyssinicum* had strong activity against *P. aeruginosa* and *E. coli* comparable to the root of *C. procera*. Similarly, its CH_2Cl_2/MeOH extract was indicated to be active against *S. aureus* with inhibition diameters 16.67 ± 1.20 and 15.67 ± 0.33 mm at 250 µg/ml and 125 µg/ml, respectively.

Compounds **1**, **2**, **4,** and **5** showed promising activity against *E. coli* with a mean inhibition zone of 13.67 ± 0.67, 14.67 ± 0.33, and 17.7 ± 0.8 mm at 250 µg/mL, respectively, compared to ciprofloxacin (31.3±0.3 mm at 250 µg/mL). Compound **2** showed moderate activity against

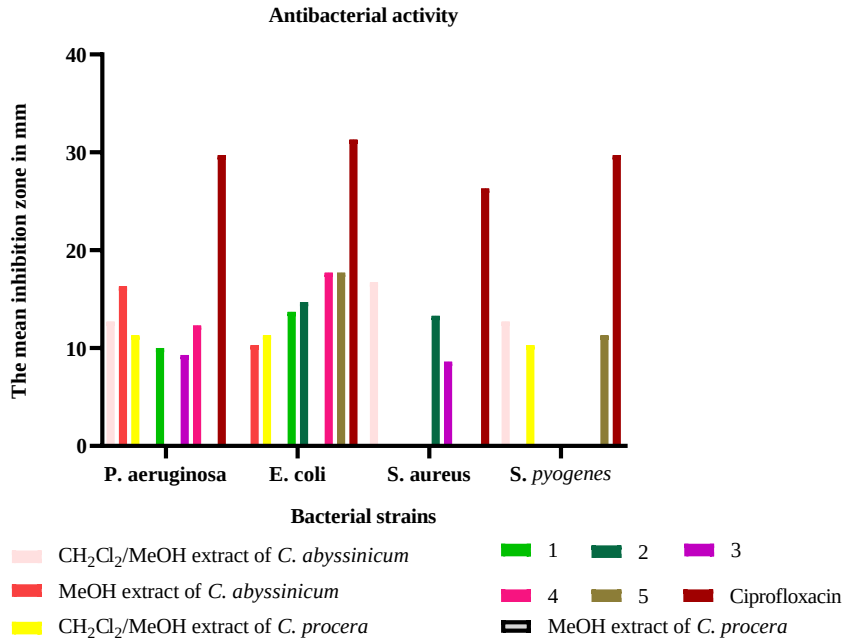
S. aureus (13.33 ± 0.67 mm at 250 µg/mL) compared to ciprofloxacin (26.3 ± 0.3 mm at 250 µg/mL). Compounds **1** and **4** exhibited moderate activity against *P. aeruginosa* with mean inhibition zone 10 ± 1.53 mm and 12.3 ± 1.5 mm at 250 µg/mL, respectively. The negative control DMSO did not show any inhibition effect against the tested bacterial species.

Table 5. Inhibition zone (in mm) of the DCM/MeOH (1:1), methanol root extracts and isolated compounds of C. abyssinicum and Calotropis *Procera* against selected bacterial species.

Samples	Conc. µg/mL	Inhibition zone in diameter (mm)			
		P. aeruginosa	*E. coli*	*S. aureus*	*S. pyogenes*
CH_2Cl_2/MeOH extract of *C. abyssinicum*	250 µg/mL	12.7 ± 1.8	NA	16.7 ± 1.2	12.7 ± 1.2
	125 µg/mL	11.3 ± 1.4	NA	15.7 ± 0.3	10.3 ± 0.3
MeOH extract of *C. abyssinicum*	250 µg/mL	16.3± 0.3	10.3 ± 0.3	NA	NA
	125 µg/mL	15.7 ± 0.3	9.7 ± 0.3	NA	NA
CH_2Cl_2/MeOH extract of *C. procera*	250 µg/mL	11.3 ± 0.2	11.3 ± 0.5	NA	10.3 ± 0.3
	125 µg/mL	10.7 ± 0.3	10.3 ± 0.7	NA	10.7 ± 0.7
MeOH extract of *C. procera*	250 µg/mL	NA	NA	NA	NA
	125 µg/mL	NA	NA	NA	NA
Compound **1**	250 µg/mL	10 ± 1.5	13.7 ± 0.7	NA	NA
	125 µg/mL	9.7 ± 1.2	12 ± 1.5	NA	NA
Compound **2**	250 µg/mL	NA	14.7 ± 0.3	13.3 ± 0.7	NA
	125 µg/mL	NA	13 ± 0.6	10.7 ± 0.7	NA
Compound **3**	250 µg/mL	9.3 ± 0.3	NA	8.6± 1.53	NA
	125 µg/mL	7.7 ± 0.3	NA	7.3 ± 0.3	NA
Compound **4**	250 µg/mL	12.3 ± 1.5	17.7 ± 0.8	NA	NA
	125 µg/mL	11.7 ± 1.2	16 ± 0.6	NA	NA
Compound **5**	250 µg/mL	NA	17.7 ± 1.2	NA	11.3 ± 1.2
	125 µg/mL	NA	15.7 ± 0.9	NA	9.3 ± 1.2
Ciprofloxacin	250 µg/mL	29.7 ± 0.3	31.3 ± 0.3	26.3 ± 0.3	29.7 ± 0.3
	125 µg/mL	29.3 ± 0.3	29.7 ± 0.3	26.0 ± 0.3	29.3 ± 0.3
DMSO	250 µg/mL	NA	NA	NA	NA
	125 µg/mL	NA	NA	NA	NA

NA: No activity; DMSO = Dimethyl sulfoxide; Ciprofloxacin and DMSO were used as the positive and negative controls, respectively.

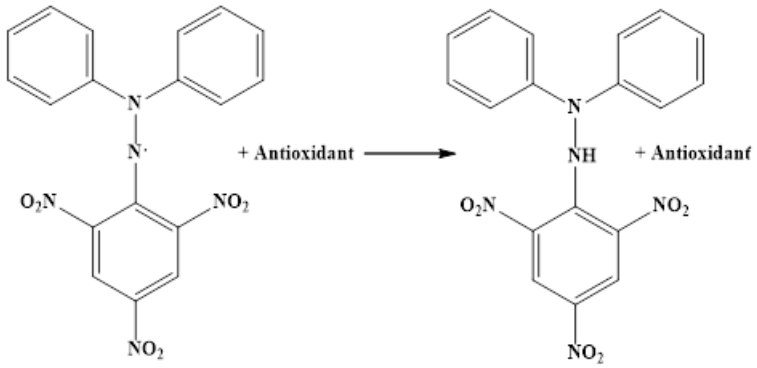
Figure 4. The inhibition zone of the extracts and isolated compounds in mm (mean ± SD) at 250 μg/mL.



3.3. Radical Scavenging Assay

DPPH is widely used to test the ability of compounds to act as free radical scavengers and to evaluate the antioxidant activity of compounds. It is a stable free radical, which is because of the delocalized electron. The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 5l7 nm (Hangun-Balkir and McKenney, 2012). The decrease in absorbance at 517 nm (Gulcin *et al*., 2010) in addition to the change in color of the DPPH from purple to yellow indicates the antioxidant activity of the samples. Furthermore, the color turns from purple to yellow as soon as the odd electron of the DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H (Luqman *et al*., 2012) (Figure 5).

Figure 5. The structure of DPPH radical and its product (DPPH-H).



DPPH radical (Purple) DPPH-H (Yellow)

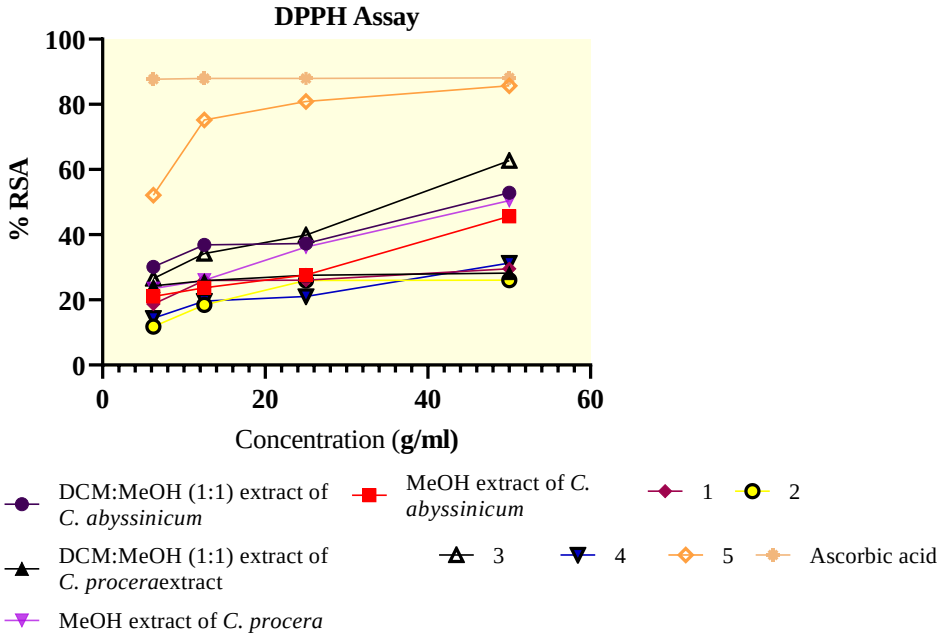
In this study, the DPPH radical scavenging activities of the *C. abyssinicum* extract and isolated compounds were examined by comparison with ascorbic acid which is used as a positive control, and the result is depicted in (Table 6, Figure 6). The results showed that the DCM:MeOH (1:1) and MeOH root extracts of *C. abyssinicum* inhibited the DPPH radical by 52.86 and 45.6 at 50 μg/mL, respectively. The result obtained was found to be moderate as compared to ascorbic acid which is used as a positive control with percent inhibition of radical by 88.10 % at the same concentration. The isolated compounds (**1-5)** from the DCM: MeOH (1:1) root extracts of *C. abyssinicum* and *C. procera* inhibited the DPPH radical by 29.53, 26.07, 62.74, 31.31, and 85.7 at 50 μg/mL, respectively. The IC_{50} values of the isolated compounds (Table 6) are calculated. The lower the IC_{50}, the higher the antioxidant activity of substances. The IC_{50} values of isolated compounds vary from 0.3 µg/mL to 20.04 µg/mL. The isolated compounds displayed lower IC_{50} values for compound **5** (85.7 %, IC_{50} value 0.30 μg/mL) which showed promising antioxidant potential as compared to ascorbic acid (88.10 %, IC_{50} value 0.14 μg/mL) used as a standard antioxidant. The activities shown by isolated compounds showed moderate antioxidant activity.

Table 6. Percent radical scavenging activity of the plants extract and isolated compounds

Tested samples	Concentration (μg/mL)				
	6.25	12.5	25	50	IC_{50}
DCM:MeOH (1:1) extract of *C. abyssinicum*	30.12 ± 0.1	36.91 ± 0.2	37.26 ± 0.1	52.86 ± 0.2	4.1
MeOH extract of *C. abyssinicum*	21.07 ± 0.4	23.69 ± 0.1	27.62 ± 0.2	45.6 ± 0.1	5.2
DCM:MeOH (1:1) extract of *C. procera*	24.29 ± 0.2	25.84 ± 0.4	27.5 ± 0.1	28.21 ± 0.1	20.0
MeOH extract of *C. Procera*	23.45 ± 0.1	26.08 ± 0.3	36.19 ± 0.2	50.48 ± 0.2	4.3
Compound **1**	18.69 ± 0.4	25.95 ± 0.2	26.07 ± 0.1	29.53 ± 0.5	10.1
Compound **2**	1.79 ± 0.1	18.45 ± 0.3	25.95 ± 0.4	26.07 ± 0.2	8.4
Compound **3**	26.55 ± 0.6	34.17 ± 0.13	39.88 ± 0.1	62.74 ± 0.12	3.3
Compound **4**	14.41 ± 0.11	19.64 ± 0.12	21.07 ± 0.11	31.31 ± 0.12	7.9
Compound **5**	52.1 ± 0.01	75.2 ± 0.04	80.9 ± 0.02	85.7 ± 0.03	0.3
Ascorbic acid	81.74 ± 0.12	85.78 ± 0.12	87.98 ± 0.12	88.10 ± 0.05	0.14

Samples were reported as Mean± SEM; Ascorbic acid was used as positive control

Figure 6. Percentage radical scavenging activities of DPPH radical by the tested samples.



3.4. *In silico* Pharmacokinetics (ADME), Drug-Likeness and Toxicity Studies Analysis

Physicochemical properties, drug-likeness, boiled-egg model, and pharmacokinetic properties (ADME) of compounds **1-6** were determined using the SwissADME Web tool (Daina *et al*., 2017). The percent absorption (%Abs) of the was calculated using the formula %Abs=109 – 0.345 TPSA (Remko, 2009). The toxicity profile of the compounds was predicted using the ProTox-II Web tool (Banerjee *et al*., 2018). Drug-likeness is a prediction that screens whether a particular organic molecule has properties consistent with being an orally active drug (Lipinski *et al*., 1997). The drug-likeness of the isolated compounds was characterized according to "Lipinski's rule." According to Lipinski rule, the potential molecules should have the following physicochemical properties (Lipinski, 2001), such as (i) hydrogen bond donors

(HBDs) less than 5, (ii) hydrogen bond acceptors (HBAs) less than 10, (iii) a molecular mass less than 500 Da, (iv) log *P* not greater than 5, and (*v*) total polar surface area (TPSA) which should not be greater than 140 Å. In the present investigation, compounds **2, 3,** and **4** obeyed Lipinski's rule of five and are likely to be orally active. Low molecular weight (MW) signifies that the molecules are light and can easily pass through the cell membrane. Low molecular weight (MW 500) chemicals are favored for oral absorption, (Lipinski, 2001) whereas compounds with MW > 500 Da are absorbed via an alternate route, generally the membrane (Refsgaard *et al*., 2005). The present study revealed that all of the molecular weight of the studied (Table 7) compounds were less than 500 Da. An abnormal increase in values may result in a considerably lower absorption rate or poor permeation (Lipinski, C.A., 2004). The TPSA value of all the studied compounds was noticed in the range of 26.3–66.76 $Å^2$. This indicates that the results are less than 140 $Å^2$, indicating that intestinal absorption is good and if limits are beyond the score, then the drug does not possess passive cellular permeability (Turner *et al*., 2004). The optimal good bioavailability range of lipophilicity (log P) is between 0 and 3, which refers to the state of a good balance between solubility and permeability. The distribution coefficients (logP) of the isolated compounds (Table 7) were in the range of 3.36 to 5.29. The log(P) of all isolated compounds was found to be greater than three as predicted in (Table 7) which might be due to the small number of hydroxyl groups present in all compounds. Compounds that meet these criteria have been shown to have better pharmacokinetics and bioavailability characteristics. The % Abs analysis of the isolated compounds and control (Table 7) showed that all the isolated compounds have the highest percent absorption than the control.

Table 7. Drug-likeness predictions of compounds **1-6**, computed by SwissADME.

Formula	MW	NRBs	NHBAs	NHBDs	MR	TPSA	%Absn	iLOGP	Lipinski rule of five
$C_{18}H_{34}O_2$	282.46	15	2	1	89.94	37.3	96.13	4.22	1
$C_{20}H_{36}O_4$	340.5	17	4	2	100.91	66.76	85.97	4.66	0
$C_{16}H_{30}O_2$	310..51	17	2	0	99.06	26.3	99.93	5.03	1
$C_{14}H_{28}O_2$	228.37	11	2	2	71.26	40.46	95.04	3.36	0
$C_{31}H_{50}O_2$	454.73	7	2	0	142.49	26.3	99.93	5.29	1
$C_{32}H_{48}O_2$	464.72	2	2	0	143.93	26.3	99.93	4.79	1
Ciprofloxacin	331.34	3	5	2	95.25	74.57	83.27	2.24	0

NRB = number of rotatable bonds, NHD = number of hydrogen donors, NHA = number of hydrogen acceptors, and TPSA = total polar surface area.

The ability of molecules to penetrate the outer layer of the skin is described by skin permeability (Kp). The developed compounds' log Kp values (Table 8) were all determined to be within the permissible range of - 8.0 to - 1.0 (Gaur, 2015). The SwissADME prediction parameters have shown that the GIA exhibits good oral absorption except for compounds **5** and **6**. The results of the BBB permeability test performed on studied compounds (Table 8) demonstrated that compounds **1, 5,** and **6** lack BBB permeability. The P-gp affects the absorption, distribution, and clearance of a variety of substances. As a result, identifying permeability glycoprotein substrates is critical for identifying prospective medicines and optimizing them. The results show that except for compound **2,** and clinical drugs, no compounds were substrates of permeability glycoprotein (P-gp).

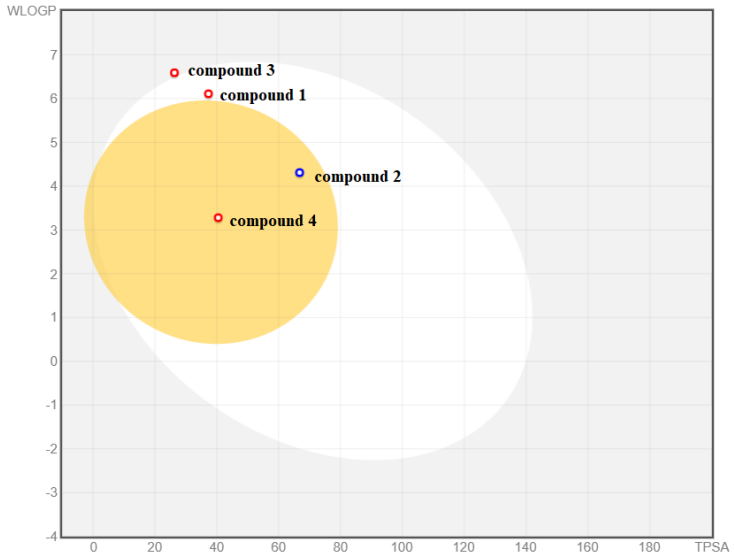
Table 8. ADME Predictions of studied compounds (**1-6**), computed by SwissADME and PreADMET.

Formula	log Kp (cm/s)	GI abn	BBB prn	Pgp substrate	CYP1A2 In	CYP2C19 In	CYP2C9 In	CYP2D6 In	CYP3A4 In
$C_{18}H_{34}O_2$	-2.6	High	No	No	Yes	No	Yes	No	No
$C_{20}H_{36}O_4$	-4.65	High	Yes	Yes	Yes	Yes	Yes	Yes	No
$C_{16}H_{30}O_2$	-3.69	High	Yes	No	Yes	No	No	No	No
$C_{14}H_{28}O_2$	-4.89	High	Yes	No	Yes	No	No	Yes	No
$C_{31}H_{50}O_2$	-2.46	Low	No	No	No	No	Yes	No	No
$C_{32}H_{48}O_2$	-3.45	Low	No	No	No	No	No	No	No
Ciprofloxacin	-9.09	High	No	Yes	No	No	No	No	No

logKp: Skin permeation, GI: Gastro-intestinal; BBB: Blood brain barrier; P-gp: Permeability glycoprotein; CYP: Cytochrome-P, In: Inhibitor, abn: absorption, prn: permission

Inhibitory drug metabolism fails when CYP enzymes are inhibited. Knowledge about the interaction of molecules with cytochromes (CYP) P450 enzymes is essential for the liver's drug metabolism. The results show that compound **1** inhibited cytochromes CYP1A2 and CYP2C9. Compounds **1**, **2**, and **5** act as an inhibitor for CYP2C19. All the screened compounds did not act as an inhibitor for CYP3A4, whereas compounds **2, 3,** and **4** inhibited CYP1A2. However, compound **6** did not act as any cytochrome inhibitor.

Figure 7. BOILED-Egg model for predicting gastrointestinal absorption and brain access.



The BOILED-Egg model allowed for the correlation between the prediction of gastrointestinal (GI) absorption and blood-brain barrier (BBB) penetration with the alignment between lipophilicity (WlogP) and polarity (TPSA) properties. The white region was for a high probability of passive absorption by the gastrointestinal tract, and the yellow region (yolk) was for a high probability of brain penetration. Yolk and white areas were not mutually exclusive. In addition, the points were colored in blue if predicted was actively effluxed by P-gp (PGP+) and in red if predicted was as a non-substrate of P-gp (PGP−).

The BOILED-Egg (Figure 7) prediction model showed that compound **1** was predicted as passively absorbed but not accessing the brain (in the white) and PGP– (red dot), Whereas, compound **2** was predicted as brain-penetrant (in the yolk) and actively effluxed (blue dot). Compound **3** was predicted as not absorbed and not brain penetrant (outside the Egg). Compound **4** was predicted as brain-penetrant (in the yolk) and not subject to active efflux (red dot). Compounds **5** and **6** were predicted as not absorbed and not BBB permeant because outside of the range of the plot.

The organ toxicity (hepatotoxicity) and toxicological endpoints (carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity) of the isolated compounds and their toxicity class and LD_{50} were predicted using Pro Tox II server (Table 9). Toxicological endpoints prediction analysis indicated median lethal dose (LD50) values ranging from 48–39800 mg/Kg. Compounds **3** and **6** were predicted to be carcinogenic. Compounds **5**, and **6** were predicted to be immunotoxic. However, all the compounds including clinical drugs have shown non-hepatotoxic. Whereas compound **5** was predicted to be cytotoxic.

Table 9. Toxicity prediction of compounds **1-6**, computed by ProTox-II property explorer.

Formula	Hep	Carc	Immu	Muta	Cyto	LD50 (mg/kg)	Toxicity class
$C_{18}H_{34}O_2$	inactive	Inactive	Inactive	Inactive	inactive	48	2
$C_{20}H_{36}O_4$	inactive	Inactive	Inactive	Inactive	inactive	39800	6
$C_{16}H_{30}O_2$	inactive	Active	inactive	Inactive	inactive	5000	5
$C_{14}H_{28}O_2$	inactive	Inactive	inactive	Inactive	inactive	4300	5
$C_{31}H_{50}O_2$	inactive	inactive	active	Inactive	active	5000	5
$C_{32}H_{48}O_2$	inactive	Active	active	Inactive	inactive	3000	5
Ciprofloxacin	inactive	Inactive	inactive	Active	inactive	2000	4

Hep: Hepatotoxicity, Carc: Carcinogenicity, Immu: Immunotoxicity, Muta: Mutagenicity, Cyto: Cytotoxicity.

3.5. Molecular Docking Studies

For additional support of the *in vitro* antibacterial and antioxidant activities of the compounds, molecular docking studies of the isolated compounds (**2, 3, 4, 5,** and **6**) with the binding sites of *E. coli* DNA gyrase B (PDB ID: 6F86), Pyruvate Kinase (PDB ID: 3T07) and human peroxiredoxin 5 (PDB ID: 1HD2) were performed in order to predict the protein-ligand interactions.

In the present study, the molecular docking analysis of the isolated compounds **2, 4** and **6** was carried out to investigate their binding pattern with bacterial gyrase and the results were compared with the standard antibacterial drug ciprofloxacin (Durcik *et al.*, 2020). The result showed a binding pocket of DNA gyrase B of isolated compounds (6, **4, 2**) were found to have minimum binding energy ranging from –5.5 to –6.7 kcal/mol, respectively (Table 10). The binding affinity, H-bond, and residual interaction of compounds **2, 4,** and **6** along with ciprofloxacin are presented in Table 10. Compounds **2, 4,** and **6** showed hydrogen bond interaction with active site amino acid residue Val-120, Asn-40, and Asn-46, respectively. Compared to ciprofloxacin, compounds **2, 4,** and **6** showed significant interaction within the active site of the protein with the key amino acids Asp-73, Asn-46, Arg-76, Glu-50, Gly-77, Pro-79, Ile-78, Ile-94, Ile-78, and Ala-47. Furthermore, compounds **2, 4,** and **6** have similar residual amino acid Val-43, Val-71, Val- 167, Ile-94, Pro-79, and Ile-78 interactions and comparable binding affinities. The binding interactions of compounds **2, 4, 6,** and ciprofloxacin against *E. coli* DNA gyrase B were shown in Figure 8. The *in-silico* results are in good agreement with *in vitro* results. Hydrogen bonds between compounds and amino acids are shown as green dashed lines, hydrophobic interaction was shown as pink lines.

The molecular docking analysis of compounds **3, 5,** and **6** was carried out to investigate their binding pattern with human peroxiredoxin 5 (PDB ID: 1HD2) and compared with the Ascorbic Acid (Bernard *et al.*, 2001). The isolated compounds (**3, 5,** and **6**) were found to have minimum binding energy ranging from -2.7 to -6.5 kcal/mol (Table 11). Results obtained from the molecular docking study demonstrated that compounds **3** (-5.0 kcal/mol) and **5** (-6.5 kcal/mol) displayed higher binding affinity values compared to ascorbic acid (- 4.5 kcal/mol), while the lowest binding score was showed by compound **6** (–2.7 kcal/mol). This agrees with the

experimental findings that compounds **3** and **5** have potentially good antioxidant activity. Compared to ascorbic Acid, the isolated compounds **3** and **5** showed similar residual interactions with amino acid residues Pro-40, Thr-44, Pro-45, Arg-127, Gly-148, and Leu-149 and H-bonding interaction with Gly-46, Cys-47, and Thr-147. Compounds **3** (Arg-127, Thr-44) and compound **5** (Arg-124, Asn-76) exhibited additional hydrogen bonding interaction with amino acid residue. The binding affinity, H-bond, and residual interaction of compounds **3, 5,** and **6** are summarized in Table 11, and the binding interactions of **3, 5,** and **6** against human peroxiredoxin 5 are depicted in Figure 9.

Figure 8. The binding interactions of compounds 2, 4 and 6 against E. coli DNA gyrase B (PDB ID:6F86).

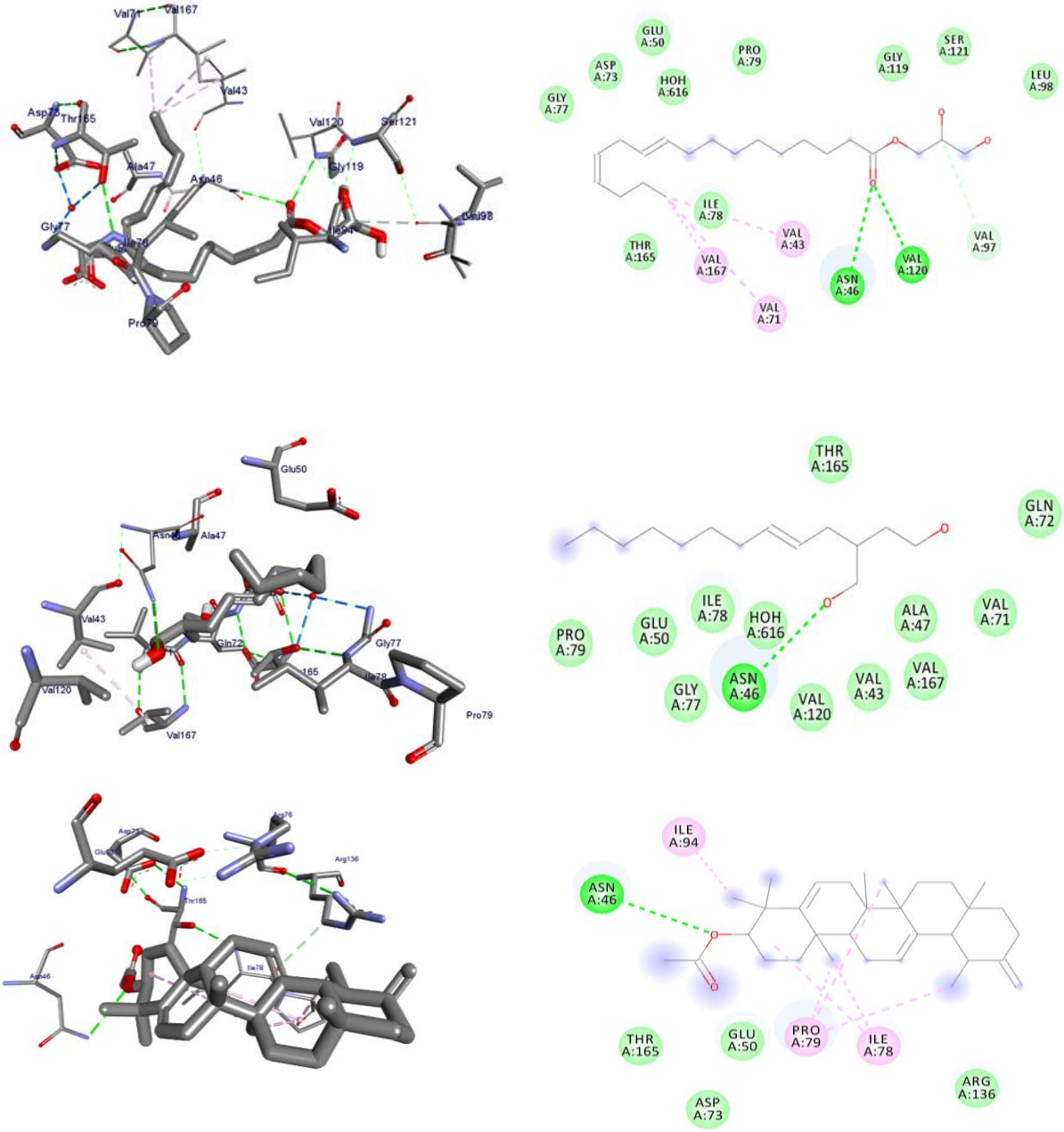


Table 10. Molecular docking scores and residual amino acid interactions of isolated compounds against E. coli DNA gyrase B (PDB ID: 6F86).

Ligands	Affinity (kcal/mol)	H-bond	Residual amino acid interactions	
			Hydrophobic/Pi-Cation	Van dar Waals
2	-6.0	Val-120, Asn-40	Val-43, Val-71, Val-167	Gly-77, Asp-73, Glu-50, Pro-79, Gly-119, Ser-121, Leu-98, Val-97, Thr-165
4	- 5.5	Asn-46	--	Val-43, glu-50, Pro-79, Gly-77, Ile-78, Thr-165, Val-120, Val-167, Val-71, Gn-72
6	-6.7	Asn-46	Ile-94, Pro-79, Ile-78	Glu-50, Asp-73, Thr-165, Arg-136
Ciprofloxacin	–7.2	Asp-73, Asn-46, Arg-76	Glu-50, Gly-77, Pro-79, Ile-78, Ile-94, Ile-78	Ala-47

Figure 9. The binding interactions of compounds **3, 5,** and **6** against human peroxiredoxin 5 (PDB ID: 1HD2).

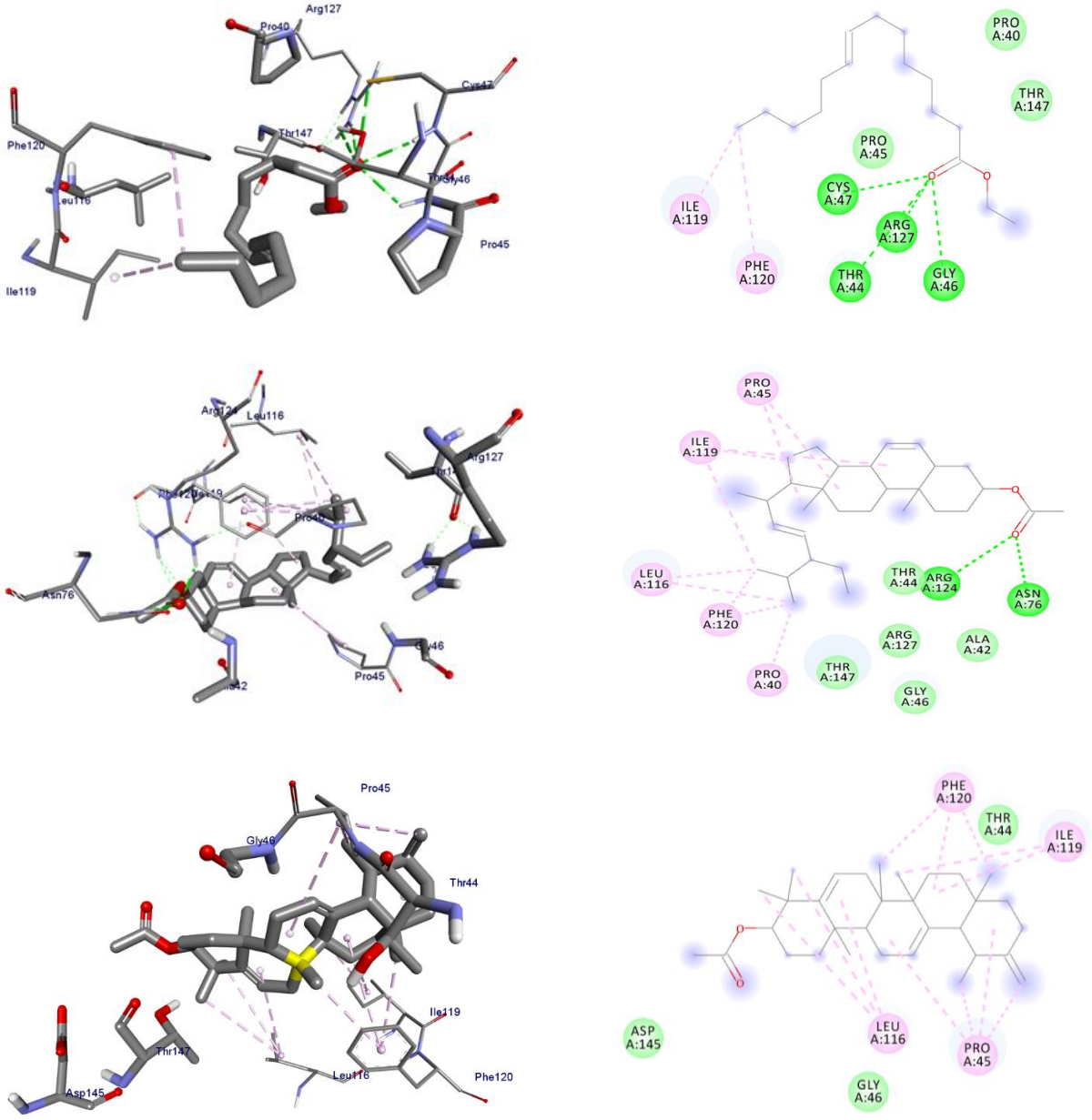


Table 11. Molecular docking scores and residual amino acid interactions of isolated compounds against human peroxiredoxin 5 (PDB ID: 1HD2).

Ligands	Affinity (kcal/mol)	H-bond	Residual interactions	
			Hydrophobic/Pi-Cation	Van dar Waals
3	-5.0	Cys-47, Arg-127, Gly-46, Thr-44	Ile-119, Phe-120	Ile-361, thr-353, Ser-354, Asn-465, Thr-464
5	-6.5	Arg-124, Asn-76	Pro-40, Pro-45, Ile-119, Phe-120, Leu-116	Ile-361, thr-353, Ser-354, Asn-465, Thr-464
6	-2.7	--	Ile-119, Phe-120, Leu-116, Pro-45	Thr-44, Asp-145, Gly-46
Ciprofloxacin	-4.5	Gly-46, Cys-47, Thr-147	---	Pro-40, Thr-44, Pro-45, Arg-127, Gly-148, Leu-149

In this study, molecular docking interactions of the isolated compound 4 and 6 against Pyruvate Kinase was studied and compared with ciprofloxacin used as an antibacterial drug (El Sayed *et al.*, 2020). The isolated compounds 4 and 6 were found to have a binding affinity of 4.5 and 5.3 kcal/mol, respectively (Table 12). Compared to ciprofloxacin, compound 4 and 6 showed similar residual interactions with amino acid residues Ile-361, Ala-358, Ser-354, Thr-353, and Thr-348 and H-bonding interaction with Ser-362 while compound 6 (Asn-465) exhibited additional hydrogen bonding interaction with amino acid residue. Binding interactions of the isolated compounds 4, 6, and ciprofloxacin against Pyruvate Kinase were shown in Figure 10.

Figure 10. The binding interactions of compounds 4 and 6 against Pyruvate Kinase (PDB ID: 3T07).

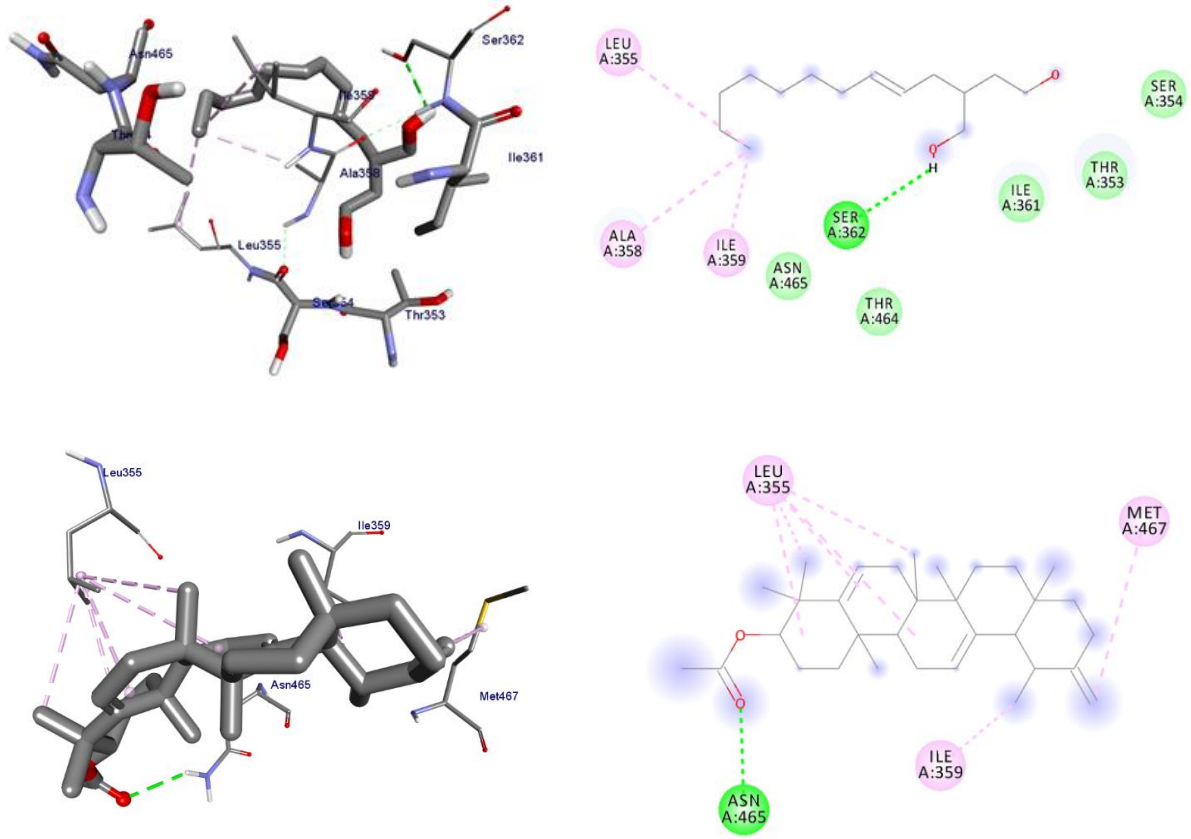


Table 12. Molecular docking scores and residual amino acid interactions of isolated compounds against Pyruvate Kinase (PDB ID: 3T07).

Ligands	Affinity (kcal/mol)	H-bond	Residual interactions	
			Hydrophobic/Pi-Cation	Van dar Waals
4	-4. 5	Ser-362	Ala-358, Ile-359, Leu-355	Ile-361, thr-353, Ser-354, Asn-465, Thr-464
6	-5.3	Asn-465	Ile-359, Leu-355	---
Ciprofloxacin	-5.6	Ser-362	Ile-361, Ala-358	Ser-354, Thr-353, Thr-348

4. CONCLUSION

The present study identified six compounds from dichloromethane/methanol (1:1) root extracts of *Crinum abyssinicum* and *Calotropis procera* of which compounds **1**-**3** were reported for the first time from *C. abyssinicum*. Compound **2**, a derivative of Penicilloitins B, was reported herein for the first time from a plant source, previously reported from culture broth of a marine endophytic *Penicillium* species. The DCM/MeOH (1:1) and MeOH root extracts of *C. abyssinicum* showed significant inhibitory activity against *S. aureus* and *P. aeruginosa* with a mean inhibition zone of 16.67 ± 1.20 mm and 16.33 ± 0.33 mm at 250 µg/mL, respectively, compared to ciprofloxacin (29.7 ± 0.3 mm and 26.3 ± 0.3 mm, respectively, at 250 µg/mL). Compounds **4** and **5** showed 17.7 ± 0.8mm and 17.7 ±1.2 mm mean inhibition zone against *E. coli*, respectively, at 250 µg/mL compared to ciprofloxacin (31.3±0.3 mm at 250 µg/mL). Compound **2** showed moderate activity against *S. aureus* (13.33 ± 0.67 mm at 250 µg/mL) compared to ciprofloxacin (26.3 ± 0.3 mm at 250 µg/mL). The results of DPPH activity showed that the DCM: MeOH (1:1) and MeOH root extracts of *C. abyssinicum* inhibited the DPPH radical by 52.86 ± 0.24 % and 45.6 ± 0.11 % at 50 µg/mL, respectively, whereas compounds (**1**-**5**) displayed 29.53, 26.07, 62.74, 31.31 and 85.7 % of inhibition, respectively, at 50 µg/mL compared to ascorbic acid (88.10 ± 0.0 % at 50 µg/mL). The drug-likeness analysis showed that compounds **2** and **4** satisfy Lipinski's rule of five with zero violations. The LD_{50} and toxicity class values obtained by *insilico* toxicity profile analysis of the compounds suggested that none of the compounds has hepatotoxicity and mutagenicity. The docking binding affinity and *in vitro* assay results suggest that compounds **2** and **6** can be considered as a potential antibacterial against *S. aureus*. Compounds **3** and **5** can be considered promising free radical scavengers. Therefore, the *in vitro* antibacterial, radical scavenging activity along with the molecular docking analysis suggest the potential use of the extracts of *C. abyssinicum* and compounds **2, 4,** and **6** as promising antibacterial agents whereas compounds **3** and **5** can be considered as promising free radical scavenger after further works which corroborate the traditional uses of the roots of the plants.

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Getachew Tegegn: Conducted experimental work and wrote the original draft. **Milkyas Endale** and **Yadessa Melaku:** Supervised the experimental work and edited the manuscript. Rajalakshmanan Eswaramoorthy: conducted computational analysis and edited the manuscript.

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REFERENCES

Abebe, D., Debella, A.,Urga, K. (2003). *Medicinal Plants and Other Useful Plants of Ethiopia: Illustrated Checklist*. Addis Ababa. Ethiopian Health and Nutrition Research Institute.

Abhishek, D., Mohit, C., Ashish, G., Ameeta, A. (2010). Medicinal utility of *Calotropis Procera* (Ait.) R. Br. as used by natives of village Sanwer of Indore District, Madhya Pradesh. *IJPLS*, *1*(3), 188-190

Adesanya, S.A., Olugbade, T.A., Odebiyil, O.O., Aladesanmi, J.A. (1992). Antibacterial Alkaloids in Crinum jagus, *International Journal of Pharmacognosy*, *4*, 303-307.

Ali, A.A., Hambloch, H., Frahm, A.W. (1983). Relative configuration of the alkaloid augustamine. *Phytochemistry*, *22*(1), 283–287.

Ali, M., Gupta, J., Neguerulea, M.V., Perez-Alonso, M.J. (1998). New ursan-type triterpenic esters from the roots of Calotropis gigantea. *Pharmazie*, *53*, 718– 721.

Ali, M., Ravinder, E., Ramachandram, R. (2000). New ursane-type triterpenic esters from the stem bark of Thevetia peruviana. *Pharmazie*, *55*, 385–389.

Al-Snafi, A.E. (2015). The Constituents and Pharmacological Properties of Calotropis Procera. An Overview. *International Journal of Pharma Research & Review*, *5*, 259-275

Asnakech, S., Awas, T., Gure, A. (2019). The Qualitative and Quantitative Phytochemical Investigation of Crinum Species in Ethiopia. *International Journal of Photochemistry and Photobiology*, *3*(1), 1-9.

Banerjee, P., Eckert, A.O., Schrey, A.K., Preissner, R. (2018). ProTox-II: a webserver for the prediction of toxicity of chemicals. *Nucleic Acids Research*, *46*(1), 257-263.

Barange, S.H., Raut, S.U., Bhansali, K.J., Balinge, K.R., Patle, D.S., Pundlik Rambhau Bhagat, P.R. (2020). Biodiesel production via esterification of oleic acid catalyzed by Brønsted acid-functionalized porphyrin grafted with benzimidazolium-based ionic liquid as an efficient photocatalyst. *Biomass Conversion and Biorefinery*, (2021). https://doi.org/10.1007/s13399-020-01242-7

Bekele, B., Adane, L.,Tariku, Y., Hailu, A.(2013). Evaluation of Antileishmanial Activities of Triglycerides Isolated from Roots of *Moringa stenopetala*. *Medicinal Chemistry Research*, *22*(10), 4592-99.

Bernard, A., Clippe, A., Declercq, J. P., Evrard, C., Knoops, B., & Stricht, D.V. (2001). Crystal Structure of Human Peroxiredoxin 5, a Novel Type of Mammalian Peroxiredoxin at 1.5 A Resolution. *Journal of Molecular Biology, 311(4)*, 751-759. https://doi.org/10.1006/JMBI.2001.4853

Besufekad, A., Tadesse, S., Hymete, A., Bisrat, D. (2020). Antiproliferative Effects of Alkaloids from the Bulbs of *Crinum abyscinicum* Hochst. ExA. Rich. *Evidence-Based Complementary and Alternative Medicine*, 1-8. https://doi.org/10.1155/2020/2529730

Chavan, P.A. (2016). Evaluation of Antimicrobial activity of Various Medicinal Plants Extracts of Latur Zone against Pathogens. *International Journal of Life Sciences Scientific Research*, *2*(5), 612-618.

Daina, A., Michielin, O., Zoete, V. (2017). SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. *Scientific Reports*, *7*, 42717.

Döpke, W., Sewerin, E., Trimino, Z. Juliérrez, C.Z. (1981). Isolation, structure and stereochemistry of a new alkaloid from *Crinum oliganthum*. *Z Chem*, *21*(10), 358.

Drwal, M.N., Banerjee, P., Dunkel, M., Wettig, M.R., Preissner, R. (2014). ProTox: a web server for the in-silico prediction of rodent oral toxicity. *Nucleic Acids Research, 42*(1), 53–58. https://doi.org/10.1093/nar/gku401

Durcik, M., Skok, Z., Ilas, J., Zidar, N., Zega, A., Szili, P. E., Draskovits, G., Révész, T., Kikelj, D., Nyerges, A., Pál, C., Maši˘c, L. P., & Tomaši˘c, T. (2020). Hybrid inhibitors of DNA gyrase A and B design: Synthesis and evaluation. *Pharmaceutics*, *13*(1), 6. https://doi.org/10.3390/pharmaceutics13010006

D´yakonov, V.A., Makarov, A.A., Andreev, E.N., Makarova, E.Kh., Dzhemileva, L.U., Khalilov, L.M., Dzhemilev, U.M. (2020). Catalytic cycloalumination of 1,2-dienes in the total synthesis of natural grenadamide and lyngbyoic acid. *Russian Chemical Bulletin, International Edition, 69*(2), 0386-0389.

El Sayed, M.T., Sarhan, A.E., Ahmed, E., Khattab, R.R., Elnaggar, M., El-Messery, S.M., Shaldam, M.A., & Hassan, G.S. (2020). Novel pyruvate kinase (PK) inhibitors: New target to overcome bacterial resistance. *ChemistrySelect*, *5*(11), 3445-3453. https://doi.org/10.1002/slct.202000043

Fennell, C.W., Van Staden, J. (2001). *Crinum* species in traditional and modern medicine. *Journal of Ethnopharmacology*, 78(1), 15–26.Gaur, R., Thakur, J.P., Yadav, D.K, Kapkoti, D.S., Verma, R.K., Gupta, N., Khan, F., Saikia D., Bhakuni, R.S. (2015). Synthesis, antitubercular activity, and molecular modeling studies of analogues of isoliquiritigenin and liquiritigenin, bioactive components from Glycyrrhiza glabra. *Medicinal Chemistry Research., 24*(9), 3494– 3503. https://doi.org/10.1007/s00044-015-1401-1

Ghosal, S. (1981). *Special Lecture, Sixth Indo-Soviet symposium on Chemistry of Natural Products*. NCL, Pune, India, 71–72.

Giday, M., Teklehaymanot, T. (2013). Ethnobotanical study of plants used in management of livestock health problems by Afar people of Ada'ar District, Afar Regional State, Ethiopia, *Journal of Ethnobiology and Ethnomedicine, 9(*8), 1-10. https://doi.org/10.1186/1746-4269-9-8

Gulcin, I., Huyut, Z., Elmastas, M., Aboul-Enein, H.Y. (2010) Radical scavenging and antioxidant activity of tannic acid. *Arabian Journal of Chemistry*, *3*, 43–53.

Han, Y., Zhang, J., Hu, C.Q., Zhang, X., Ma, B., Zhang, P. (2019). In silico ADME and toxicity prediction of ceftazidime and its impurities. *Frontiers in Pharmacology*, *10*, https://doi.org/10.3389/fphar.2019.00434

Hangun-Balkir, Y., McKenney, M. (2012). "Determination of Antioxidant Activities of Berries and Resveratrol." *Green Chemistry Letters and Reviews, 5*(2), 147–53.

Harini, K. and Nithyalakshmi, V. (2017). Phytochemical Analysis and Antioxidant Potential of *Cucumis Melo* Seeds.*International Journal of Life Sciences Scientific Research, 3*(1), 863-867.

Hassan, S.W., Bilbis, F.L., Ladan, M.J., Umar, R.A., Dangoggo, S.M., Saidu, Y., Abubakar, M.K., Faruk, U.Z. (2006), Evaluation of antifungal acivity and phytochemical analysis of leaves, roots and stem barks extracts of Calotropis procera (asclepiadaceae), *Pakistan Journal of Biological Sciences*, *9*(14), 2624-2629.

Highet, RJ.,. (1961). Ismine. *Journal of Organic Chemistry*, *26*(11), 4767–4768. https://doi.org/10.1021/jo01069a564

Hussein, K. Eswaramoorthy, R., Melaku, Y., Endale, M. (2021). Antibacterial andAntioxidant Activity of Isoflavans from the Roots of *Rhynchosiaferruginea*and *in Silico* Study on DNA Gyrase and Human Peroxiredoxin. *International Journal of Secondary Metabolite, 8*(4), 321-336.

Ibrahim, S.R.M, Mohamed, G.A, Shaala, L.A., Laetitia Moreno Y. Banuls, L.M.Y., Goietsenoven, G.V, Kiss, R., Youssef, D.T.A. (2012). New ursane-type triterpenes from the root bark of Calotropis procera. *Phytochemistry Letters*, *5*, 490–495.

Jäger, A.K. et al. (2004). Acetylcholinesterase inhibition of *Crinum* sp. *South African Journal of Botany*, *70*(2), 323–325.

Kloos, H., Menberu, T., Tadele, A., Chanie, T., Debebe, Y., Abebe, A., Zealiyas, K., Tadele,

Lawal, A., Dangoggo, S. (2014). Phytochemical, Proximate and Toxicity Studies of Aqueous Extract of *Crinum ornatum* (Toad's Onion). *Chemical Search Journal*, *5*, 45-50.

Kloos, H., Menberu, T., Tadele, A., Tadele, A., Chanie, T., Debebe, Y., Abebe, A., Zealiyas, K., Tadele, G., Mohammed, M., & Debella, A. (2016). Traditional medicines sold by vendors in Merkato, Addis Ababa: Aspects of their utilization, trade, and changes between 1973 and 2014. *The Ethiopian Journal of Health Development*, *28*(2).

Lipinski, C.A., Franco, L., Dominy, B.W., Feeney, P.J. (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviewers*, *23*, 3-25, https://doi.org/10.1016/S0169409X(96)00423-1

Lipinski, C.A.; Franco, L., Dominy, B., Feeney, W., Experimental P.J. (1997). Computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviewers*, *23*, 3-25. https://doi.org/10.1016/S0169-409X(96)00423-1

Lipinski, C.A. (2000). Drug-like properties and the causes of poor solubility and poor permeability. *Journal of Pharmacological and Toxicological Methods*, *44*(1), 235–249. https://doi.org/10.1016/s1056-8719(00)00107-6

Lipinski, C.A. (2004) Lead- and drug-like compounds: the rule-of-five revolution. *Drug Discovery Today Technologies*, *1*(4), 337–341. https://doi.org/10.101 6/j.ddtec.2004.11.007

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J. (2001) "Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings1," *Advanced Drug Delivery Reviews*, *46*(1-3), 3–26.

Luqman, S., Srivastava, S., Kumar, R., Maurya, A.K., Chanda, D. (2012). Experimental Assessment of *Moringa oleifera* Leaf and Fruit for Its Antistress, Antioxidant, and Scavenging Potential Using *In vitro* and *In vivo* Assays.*Evidence-Based Complementary and Alternative Medicine*, 1–12.

Machocho, A.K., Bastida, J., Codina, C., Viladomat, F., Brun R and Chahabra, S.C. (2004). Augustamine type alkaloids from *Crinum kirkii*. *Phytochemistry*, *65*(23), 3143–3149.

Mahanthesh, M.T., Ranjith, D., Yaligar, R., Jyothi, R., Narappa, G., Ravi, M.V. (2020). Swiss ADME prediction of phytochemicals present in Butea monosperma (Lam.) *Tau. Journal of Pharmacognosy and Phytochemistry.*, *9*(3), 1799-1809.

Mailafiya, M.M., Pateh, U., Hassan, H.S.,2, Sule, M.I., Yusuf, A.J., Bila, A.H. (2020). Isolation and Characterization of Stigmasterol Glycoside from the root bark of Leptadeniahastata. *FUW Trends in Science and Technology Journal*, *5*(2), 394–398.

Mekuanent, T., Zebene, A., Solomon, Z. (2016). Ethnobotanical Study of Medicinal Plants in Chilga District, Northwestern Ethiopia. *Journal of Natural Remedies*, *15*(2), 88–11.

Meneses-Sagrero, S.E., Navarro-Navarro, M., Ruiz-Bustos, E., Del-Toro-Sánchez, C.L., Jiménez-Estrada, M., Robles-Zepeda, R.E. (2017). Antiproliferative activity of spinasterol isolated of Stegnospermahalimifolium (Benth, 1844*). Saudi Pharmaceutical Journal*, *25*, 1137–1143.

Mourshid,S.A., Badr, M.J., Risinger, A.L., Susan, Mooberry, L.S., Youssef, T.A. (2016). Penicilloitins A and B, new antimicrobial fatty acid esters from a marine endophytic Penicillium species. *Zeitschrift fur Naturforschung C.*, *71*(11-12), 387–392. https://doi.org/10.1515/znc-2015-0242

Narramore, S., Stevenson, C. E. M., Maxwell, A., Lawson, D.M., Fishwick, C.W.G. (2019). New insights into the binding mode of pyridine-3-carboxamide inhibitors of *E. coli* DNA gyrase. *Bioorganic and Medicinal Chemistry*, *27*(16), 3546–3550.

Demissew, S. & Nordal, I. (2010). *Aloes and other Lilies of Ethiopia and Eritrea*. Ed, 2: 1-351. Shama Books, Addis Ababa, Ethiopia.

Pathyusha, R.J.B. (2012). Potential of local anesthetic activity of Calotropis procera latex with epinephrine and pH in guinea pig. http//www.pharmatutor.org/articles/Pharmatutor-art-1043.259-275.procera - an overview, *International Journal of Pharmacy Review and Research*, 5(3).

Poustforoosh, A., Faramarz, S., Nematollahi, M. H., Hashemipour, H., Negahdaripou, M., & Pardakhty, A. (2022) In silico SELEX screening and statistical analysis of newly designed 5mer peptide-aptamers as Bcl-xl inhibitors using the Taguchi method. *Comput Biol Med 146*, 105632. https://doi.org/10.1016/J

Poustforoosh, A., Hashemipour, H., Tüzün, B., Pardakhty, A., Mehraban, M., Nematollahi, M. H. (2021) Evaluation of potential anti-RNA-dependent RNA polymerase (RdRP) drugs against the newly emerged model of COVID-19 RdRP using computational methods. *Biophys Chem., 272*, 106564. https://doi.org/10.1016/j.bpc.2021.106564

Proestos, C.H., Lytoudi, K., Mavromelanidou, O., Zoumpoulakis, P., Sinanoglou, V. (2013). Antioxidant Capacity of Selected Plant Extracts and Their Essential Oils. *Antioxidants*, *2*(6), 11–22.

Ramadan, M.A. (1986). Phytochemical investigation of the minor alkaloids and phenolic compounds of *Crinum bulbispermum*Milne. and *Crinum augustum*Rox. cultivated in Egypt. *[Thesis for Ph.D.]. Assiut University*.

Ramos, M.V. et al. (2006). Latex constituents from Calotropis procera (R. Br.) display toxicity upon egg hatching and larvae of Aedes aegypti (Linn.). *Memorias Instituto Oswaldo Cruz, Rio de Janeiro, 101*(5), 503-510.

Ramsewak, S., Nair, G., Murugsan, S. (2001). Insecticidal fatty acids and triglycerides from Dirca palustris. *Journal of Agricultural and Food Chemistry*, *49*, 5852–5856.

Razafimbelo, J., Andriantsiferana, M., Baudouin, G., Tillequin, F. (1996). Alkaloids from *Crinum firmifolium var. hygrophilum*. *Phytochemistry*, *41*(1), 323–326.

Refaat, J., Mohamed S. Kamel, Mahmoud A., Ramadan and Ahmed A. (2012). Crinum; an endless source of bioactive principles: a review, Part II. Crinum Alkaloids: Crinine-Type Alkaloids, I*nternational journal of pharmaceutical sciences and research*, *3*(9), 3091-3100.

Refaat, J., Kamel, M.S., Ramadan, M.A., & Ali, A.A. (2013). C*rinum*; an endless source of bioactive principles: a review. Part Iv: non-alkaloidal constituents, *International Journal of Pharmaceutical Sciences and Research, 4*(3), 941-948.

Refsgaard, H.H., Jensen, B.F., Brockhoff, P.B., Padkjaer, S.B., Guldbrandt, M., Christensen M.S. (2005). In silico prediction of membrane permeability from calculated molecular parameters. *Journal of Medicinal Chemistry*, *48*(3), 805-811. https://doi.org/10.1021/jm049661n

Regassa, R. (2013). Assessment of indigenous knowledge of medicinal plant practiceand mode of service delivery in Hawassa city, southern Ethiopia. *Journal of Medicinal Plant Research*, *7*, 517-535.

Remko, M. (2009). Theoretical study of molecular structure, pKa, lipophilicity, solubility, absorption, and polar surface area of some hypoglycemic agents. *Journal of Molecular Structure, THEOCHEM, 897*(1-3), 73-82.

Ridhay, A., Noor, A., Soekamto, N.H., Harlim, J., Altena, I.V. (2012). A Stigmasterol Glycoside from the root wood of Melochiaumbellata (Houtt) Stapf var. degrabrata. *Indonesian Journal of Chemistry*, *12*(1), 100–103.

Rivero-Perez, M.D., Muniz, P., Sanjase, M.L. (2007). Antioxidant profile of red coines evaluated by total antioxidant capacity, Scavenger activity, and biomarkers of oxidative stress methodologies. *Journal of Agricultural and Food Chemistry, 55*, 5476–5483.

Schimmer, O., Mauthner, H. (1996), Polymetoxylated xanthones from the herb ofCentaurium erythraea with strong antimutagenic properties in Salmonella typhimurium. *Planta Medica, 62*(06), 561-564.

Seeliger, D., De Groot, B.L. (2010). Ligand docking and binding site analysis with PyMOL and Autodock/Vina. *Journal of Computer Aided Molecular Design*, *24*, 417–422.

Shrivastava, A., Singh, S., Singh, S. (2013). Phytochemical investigation of different plant parts of calotropis procera. *International Journal of Scientific and Research Publications*, 3(8), 1-4.

Singh, R.P., Jain, D.A. (2011). Anticandidal potential of *Crinum asiaticum* leaves extract against selected oral and vaginal *Candida* pathogens. *Asian Journal of Biochemical and Pharmaceutical Research*, *1*, 283–91.

Tamiru, F., Terfa, W., Kebede, E., Dabessa, G., Roy, R.K., Sorsa, M. (2013). Ethno-knowledge of plants used in veterinary practices in Dabo Hana District, West Ethiopia.*Journal of Medicinal Plant Research, 7*(40), 2960- 2971.

Tareq, M., Khan, H. (2010). Predictions of the ADMET properties of candidate drug molecules utilizing different QSAR/QSPR modelling approaches, *Current Drug Metabolism, 11*(4), 285–295. https://doi.org/10.2174/138920010791514306

Tapera, M., Kekeçmuhammed, H., Tüzün, B., Sarıpınar, B., Koçyiğit, U. M., Yıldırım, B., Doğa, M, & Zorlue, Y. (2022). Synthesis, carbonic anhydrase inhibitory activity, anticancer activity and molecular docking studies of new imidazolyl hydrazone derivatives. *Journal of molecular structure, 1269*(133816). https://doi.org/10.1016/j.molstruc.2022.133816

Teklehaymanot, T., Giday, M., (2007; 2013). Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. *Journal of Ethnobiology and. Ethnomedicine, 3,* 3-12.

Trott. O., Olson, A.J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, *31*(2),455–461.

Turner, J.V., Maddalena, D.J., Agatonovic-Kustrin, S. (2004). Bioavailability prediction based on molecular structure for a diverse series of drugs. *Pharmaceutical Research, 21*(1), 68-82. https://doi.org/10.1023/b:pham

Valgas, C., de Souza, S.M., Smània, E.F.A., Smânia, J. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, *38*, 369–80.

Wildman, W.C. (1960). *The Alkaloids: Chemistry and Physiology*. Edited by R.H.F. Manske, Academic Press.

Yang, X., Zhou, J., Wang, T., Zhao, L., Ye, G., Shi, F., Li, Y., Tang, H., Dong, Q., Zhou, X., Xu, M., Rong, Q., Chen, H., Yang, X., Cai, Y. (2017). A Novel Method for Synthesis of α-Spinasterol and its Antibacterial Activities in Combination with Ceftiofur. *Fitoterapia*, *119*, 12-19. https://doi.org/10.1016/j.fitote.2017.03.011

Yineger, H., Kelbessa, E., Bekele, T., Molla, E. (2008). Plants used in traditional management of human ailments at Bale Mountains National Park, Southeastern Ethiopia. *Journal of Medicinal Plants Research*, *2*(6), 132-153.

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Research Article

Antioxidant and antimicrobial activities of methanol extracts from *Adonis paryadrica* (Asteraceae) – a critically endangered endemic species growing in the Turkish flora

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Abstract: This study was conducted to determine the antioxidant and antimicrobial activities of methanol extract obtained from flower, leaf and root sections of endemic *Adonis paryadrica* (Boiss.) Kandemir & Aytaç stat. nova. naturally growing in the Turkish flora. The most efficient total phenolic compounds and flavonoid contents were obtained from leaf extract at 21.24 mg GAEs (gallic acid equivalent)/g dw and 54.97 mg REs (rutin equivalent)/g dw, respectively. Among the three different sections of this plant, leaf extracts showed the highest Cupric Reducing Antioxidant Power (CUPRAC) effect with 80.28 µmol TEs (trolox equivalent)/g dw. From the three different sections, the methanol extract of the leaf parts demonstrated strong antibacterial activity against *Bacillus subtilis* with a 16.1 mm zone diameter. These valuable and current findings from these precious plants, which constitute natural resources in terms of biodiversity, contribute innovative information to the literature on endemic plant species.

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1. INTRODUCTION

Plants have been preferred as one of the main sources of natural remedies for centuries, and still today, the new compounds of these valuable organisms continue to be documented as natural sources (Egamberdieva and Tiezzi, 2019). Secondary metabolites are the basis of these natural resources. Ranunculaceae family with a wide distribution in the world contributes to these natural resources pool with its secondary compounds such as alkaloids, glycosides derivatives, saponins, and steroids, including bufadienolides and cardenolides (Hao *et al*., 2017; Kuroda *et al*., 2018). The genus of Adonis L., belonging to the Ranunculaceae family, is represented by approximately 40 species in the world. This genus has 9 species and one subspecies in Turkey. Among these 10 taxa, only *Adonis paryadrica* (Boiss.) Kandemir & Aytaç stat. nova. is a rare endemic species for Turkish flora. This species has been collected in 1858 by Tchihatcheff Pierre from Giresun, Turkey for the first time. After a long break, this species was investigated within the scope of biodiversity study by the Ministry of Agriculture and Forestry of the Republic of Turkey in 2018. Within the scope of the project, the presence

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of the species was confirmed in six locations of Şebinkarahisar-Alucra districts in Giresun and one location in Erzincan Munzur Mountains. The distance between locations with semi-acidic soil characteristics is approximately 20 km from the bird's eye view. The taxon is very insufficient in terms of the number of individuals in all of the determined locations and is stuck in a narrow area. Many limiting factors such as grazing pressure, erosion, road construction activities and insect attacks have encountered in some areas where the species lives (Ministry of Agriculture and Forestry, 2018). For all these reasons, it has been reported that the species is endangered and should be evaluated in the Critically Endangered (CR)" (criteria B2 a b (i, iii) of IUCN 2010) category (IUCN, 2010; Kandemir *et al*., 2019).

There are a limited number of studies on phytochemical components of this genus in the literature (Mohadjerani *et al*., 2014; Kuroda *et al*., 2018; Ucuncu *et al*., 2020). Although a study has been reported on the phytochemical and biological activity of ethanol extracts of *A. paryadrica*, however, there is no comprehensive study in the literature on the biological activities of extracts obtained with other solvents up to the best of our knowledge. The aim of this study was to determine the (i) antioxidant activity, (ii) total phenolic compounds, (iii) total flavonoid contents and (v) antimicrobial activity of the methanol extracts of this valuable rare natural resource as well as the ongoing in situ and ex situ conservation studies. All of these obtained results will shed light on the evaluation of bioactive phytochemical constituents of *A. paryadrica* as medically.

2. MATERIAL and METHODS

2.1. Electrochemical Method

All electrochemical measurements were obtained using an electrochemical analyzer i.e. Vertex®One (Ivium) device which includes electrode cell stand. This electrode cell stand consists of a reference (Ag/AgCl; BASi, MF-2052), a counter (platinum wire; BASi, MW-1032) and a working electrodes (glassy carbon electrode (GCE); BASi MF-2012). In order to make the indicator electrode clean, smooth, polishing the surface of GCE with aluminum silica was applied before each measurement. The electrochemical method as square wave stripping voltammetry (SWSV) was used to determine the amount of antioxidants found in the root, stem and leaves of the *A. paryadrica* on GCE. The operating conditions of SWSV were selected as pulse amplitude of 60 mV, frequency of 100 Hz, step potential of 5 mV, accumulation time of 30 s and accumulation potential of 0 mV. For the supporting electrolyte solution, the Britton-Robinson buffer solution at pH 6.0 was used to collect all SWSV data. pH of solutions was adjusted with a Mettler Toledo brand pH meter with an accuracy of ± 0.05. No pre-purification was applied to the samples. The samples of *A. paryadrica* were prepared for spectrophotometric analysis and used directly.

2.2. Reagents

The analytical standard of rutin was purchased from Aldrich-Sigma. Stock solution for rutin was prepared daily at a concentration of 500 mg/L. Britton Robinson (BR) buffer solution preferred as support electrolyte was prepared with 0.4 M of acetic acid, ortho-phosphoric and boric acid. To adjust the BR buffer solution to pH 6.0, 2.0 M NaOH or 2.0 M HCl solutions were used. Distilled water was used in the whole experimental process.

2.3. Spectrophotometric Methods

The antioxidant activities of the methanolic extracts from plants were expressed as mg trolox equivalent (TEs)/g extract. Details of the spectrophotometric methods can be found in supplementary file (Zengin *et al*., 2015a; Zengin *et al*., 2015b; Apak *et al*., 2006; Kocak *et al*., 2010).

2.4. Preparation of the extracts of *Adonis paryadrica*

Air-dried samples of the aerial parts (2 g) of plants were extracted with 50 ml of methanol for 30 min in a sonication bath at 30 °C. The extracts were filtered and then concentrated under reduced pressure. All samples were stored at –20 °C before using for experiments.

2.5. Activity Test

2.5.1. *Microbial strains*

MeOH extracts of flower, leaf and root parts of the *A. paryadrica* were individually tested against six Gram-positive, seven Gram-negative bacteria and a fungus. Detailed information about the strains of bacteria and fungus were given in the supplementary file.

2.5.2. *Disc diffusion assay*

The assay was performed by following the protocols of the Clinical and Laboratory Standards Institute (2014) and The European Committee on Antimicrobial Susceptibility Testing (2013). Details of the disc diffusion analyses were also specified in the supplementary file.

2.5.3. *Microdilution assay*

Minimum Inhibitory Concentration (MIC) was tested for four Gram-positive and two Gram-negative bacteria. MeOH extracts of flower part of the *A. paryadrica* were individually tested against only three Gram-positive bacteria. For this purpose, the flower part of this species was individually tested against four Gram-positive bacteria and two Gram-negative bacteria. Detailed information about the MIC values were given in the supplementary file.

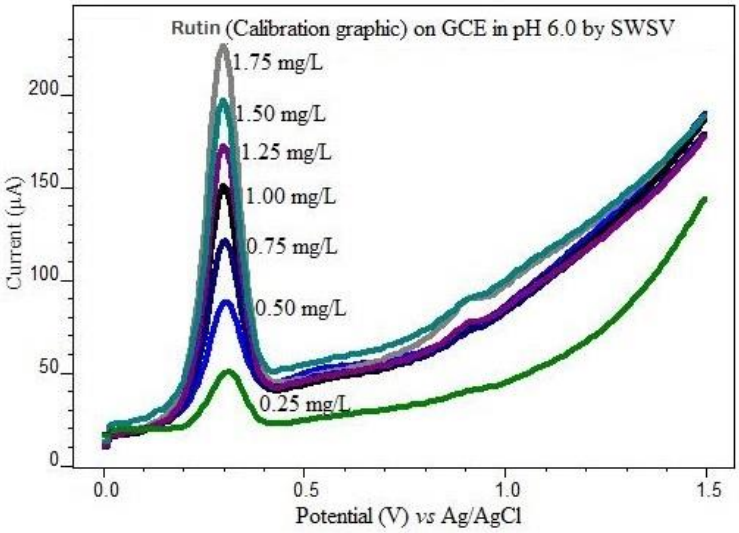
2.6. Statistical Analysis

All spectrophotometric and electrochemical tests were performed in triplicate and the results were expressed as mean with standard deviation (mean ± SD). The electrochemical analyses of antioxidant capacity of *A. paryadrica* samples were carried out by calibration method performed under the optimum condition for analytical standard i.e. rutin. Statistical significance between data was determined by using Tukey's honestly significant difference post hoc test with $\alpha = 0.05$ and ANOVA (one-way analysis of variance) test. Statistical calculations were carried out by using SPSS v. 22.0 software.

3. RESULTS and DISCUSSION

Square wave stripping voltammetry (SWSV) is generally one of the most preferred electrochemical methods for sensitive, selective, cheap and fast analysis of substances that are electroactive types such as phenolic compounds, flavonoids, vitamins, drugs and pesticides (Demir and İnam, 2014; Demir, 2019; Yıldırım *et al*., 2020; Demir and Silah, 2020; Inam *et al*., 2020; Demir *et al*., 2021). Therefore, firstly, SWSV experimental conditions were selected for the rutin, which is one of the standard antioxidants. The operating conditions of SWSV were preferred as pulse amplitude of 60 mV, frequency of 100 Hz, step potential of 5 mV, accumulation time of 30 s, accumulation potential of 0 mV and BR buffer solution at pH 6.0 on GCE. Two oxidation peaks were obtained at 320 mV and 910 mV for rutin by SWSV on GCE in pH 6.0 BR buffer solutions (Figure 1).

Figure 1. Calibration graphic of rutin by SWSV on GCE.

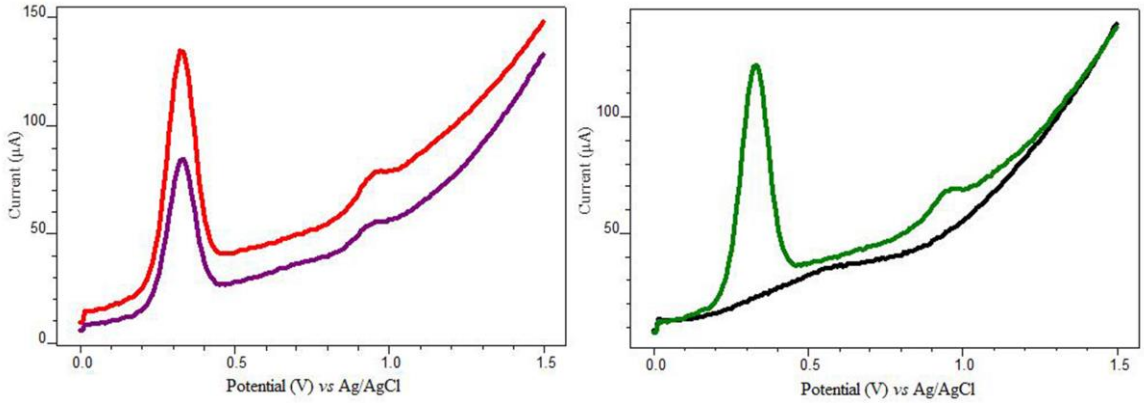


Then, under these conditions, based on the anodic peak at approximately 350 mV, calibration was created with standard addition between 0.25 mg/L and 1.75 mg/L by SWSV method. Linear calibration equation for the rutin agent was obtained by plotting peak signal on SWS voltammograms versus concentrations. The obtained equation on the linear calibration graphics for the rutin is as follows:

$$I_p\ (\mu A) = 105.15\ C\ (mg/L) + 8.247\ R2 = 0.9961 \text{ for the rutin}$$

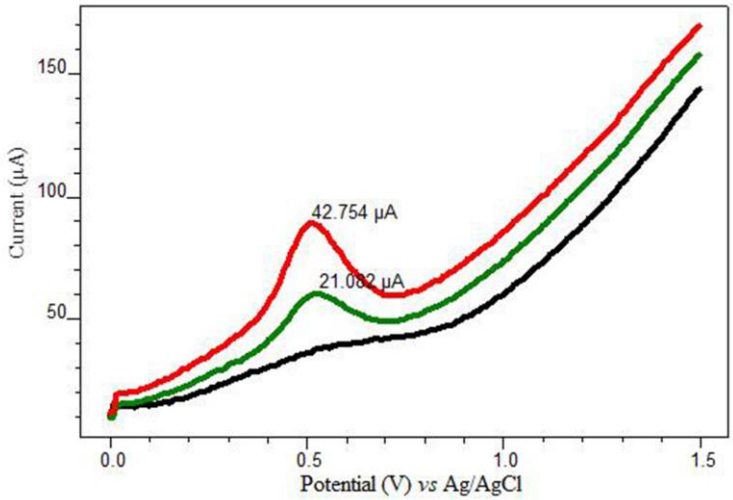
Under the conditions set for standard antioxidant substance, SWS voltammograms of real samples such as for the stem and leaves of the *A. paryadrica* were obtained on GCE in pH 6.0 BR buffer solutions (Figure 2a and 2b).

Figure 2. SWS voltammograms for the a) Flower b) Leaf sample on GCE in pH 6.0 BR buffer solutions.



Two oxidation peaks were obtained at 350 mV and 675 mV of real samples on the glassy carbon electrode (GCE) under SWSV test conditions. The peak potential values obtained for these two samples are almost identical to the peaks exhibited by rutin. Therefore, the amount of antioxidants contained in these two samples can be easily calculated in terms of rutin equivalent. Here, the first anodic peak which is high intensity peak and well-defined at approximately 350 mV was referenced. As a result of three replicate measurements for the 0.05 ml samples, average peak currents for flower samples were found to be 62.29 ± 2.78 µA, while this value was calculated as 95.88 ± 3.56 µA for leaf samples. The total antioxidant capacity (TAC) for the 1 g extract of flower and leaf samples as rutin equivalent were found as 257.0 mg/L and 416.5 mg/L, respectively. The peak obtained for root samples differs from the peak potential values obtained for flower and leaf (Figure 3).

Figure 3. SWS voltammograms for the root sample on GCE in pH 6.0 BR buffer solutions.



The main reason of this difference is dominance of phenolic compound in the root samples that is different from the rutin. However, since the peak potential of the phenolic compounds is between 0.3 V and 0.5 V, it is possible to give the total amount of antioxidants in the root sample as rutin equivalent. According to the results of three replicates for the 0.05 ml extract sample, the peak flow value was found to be 21.425 ± 0.672 µA. The TAC in root sample was calculated as rutin equivalent of 88.29 ± 2.77 mg/L (Table 1).

Table 1. Total antioxidant amounts in equivalent rutin in plant samples by SWSV.

Method	Samples	Total antioxidant capacity Equivalent rutin
SWSV	Flower	257.0 ± 11.45^{b} mg/l
	Root	88.29 ± 2.77^{c} mg/l
	Leaf	416.5 ± 14.67^{a} mg/l

Antioxidant quantification of the endemic plant *Draba cemileae* (Karaer) in leaf, root and stem samples were carried out with the electrochemical method by Cuce *et al*. (2021). The electrochemical measurements are compatible with the data obtained by spectrophotometric-based methods such as traditional Cupric Reducing Antioxidant Power (CUPRAC) reducing, Ferric Reducing Antioxidant Power (FRAP) reducing, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferrous ion chelating (Cuce *et al*., 2021). Moreover, electrochemical methods have been successfully performed to determine the total antioxidants in many products such as fruit juices, coffee samples and many different foodstuffs (Yıldırım *et al*., 2020; Demir *et al*., 2021; Öztürk *et al*., 2021). Therefore, the antioxidant capacities of *A. paryadrica* were investigated in detail by electroanalytical methods, which is a fast, inexpensive, simple, accurate and reliable new antioxidant method.

3.1. Spectrophotometric Methods

3.1.1. *Chemical composition*

The yields of the MeOH extracts obtained from the flower, leaves and roots of *A. paryadrica* were shown in Table 2. The highest yield percentage was obtained from the flower extract with 18.76% followed by the root (16.59%) and leaf (13.80) extracts, respectively.

The spectrophotometric analysis data were also given in Table 2. Leaf parts were found to be the richest in terms of total phenolic substances (21.24 mg GAEs/g) and total flavonoid content (54.97 mg REs/g). The root parts were found poorest in terms of both phenolics (18.59

mg GAEs/g), and flavonoids (0.54 mg REs/g) than other parts . Statistical analyzes showed that the phenolic profiles of flower and root extracts were not significantly different from each other ($p < 0.05$). In addition, when the flavonoid profiles were compared statistically, it was determined that the flower, leaf and root extracts were significantly different from each other ($p < 0.05$).

Table 2. Extraction yield, total phenolic and flavonoid contents of the methanol extracts from *A. paryadrica.*

Samples	Yield (%)	Total phenolics (mg GAEs/g extract)	Total flavonoids (mg REs/g extract)
Flower	18.76	19.17 ± 0.23^{b}	32.42 ± 0.01^{b}
Root	16.59	18.59 ± 0.37^{b}	0.54 ± 0.02^{c}
Leaf	13.80	21.24 ± 0.14^{a}	54.97 ± 0.51^{a}

Within each column, means sharing the different superscripts show comparison between the extracts by Tukey's test at $p < 0.05$. GAEs and REs, gallic acid and rutin equivalents, respectively

Evaluation of the biological activities of valuable natural metabolites of plants is one of the most important research areas today and there are many studies carried out in this field (Cüce *et al*., 2017, 2019; Cüce and Basançelebi, 2021; Sarikurkcu *et al*., 2021). The different techniques and methods used in these studies or the different extraction techniques and solvents for the same species can be shown among the reasons for the results obtained from the studies (Jahanban-Esfahlan *et al*., 2019; Ucuncu *et al*., 2020; Sarikurkcu *et al*., 2020). Due to this situation, the researchers preferred ethanol extracts for *A. paryadrica* in previous studies and reported that they obtained similar results in terms of total phenolic contents with the data in our study (Ucuncu *et al*., 2020). The strength of this study is that the percentage yield and total flavonoid content values were not given in the previous study on *A. paryadrica.*

3.1.2. *Antioxidant activity*

The data regarding the antioxidant activity potentials of the extracts are summarized in Table 3. The extracts differed in terms of radical scavenging capacities. DPPH radical scavenging capacity order of extracts were leaf>flower>root and ABTS radical scavenging capacity order of extracts were root>leaf>flower (Table 3). Based on these data, the highest DPPH radical scavenging effect was found to be 38.26 mg/ml in leaf extracts, while the most effective ABTS radical scavenging effect in root extracts was calculated as 44.78 mg/ml. The DPPH radical scavenging capacity of root extracts (12.58 mg/ml) created a statistically significant negative difference from others. ABTS radical scavenging capacities of the leaf and flower extract were determined to be 39.71 and 33.70 mg/ml, respectively, which is an indicator of the statistical difference between them ($p < 0.05$).

Similar scenarios were experienced in terms of the CUPRAC and FRAP reducing powers of the extracts. While the leaf extract gave the most effective CUPRAC reducing power activity with 80.28 mg/ml, the root extracts had 45.05 mg/ml FRAP reducing power. In terms of CUPRAC reducing power activity, leaf extracts were followed by flower (66.54 mg/ml) and root (49.59 mg/ml) extracts, respectively. On the other hand, in terms of FRAP reducing power activity, root extracts were followed by leaf with 42.11 mg/ml and flower (34.17 mg/ml) extracts, respectively (Table 3).

Table 3. Radical scavenging activities of methanolic extracts from *A. paryadrica*.

Samples	DPPH radical (TEs/g extract)	ABTS cation radical (TEs/g extract)	CUPRAC reducing (TEs/g extract)	FRAP reducing (TEs/g extract)
Flower	30.94 ± 0.11^{b}	33.70 ± 0.20^{c}	66.54 ± 0.25^{b}	34.17 ± 0.53^{c}
Root	12.58 ± 0.19^{c}	44.78 ± 0.01^{a}	49.59 ± 0.41^{c}	45.05 ± 0.01^{a}
Leaf	38.26 ± 0.03^{a}	39.71 ± 0.14^{b}	80.28 ± 0.25^{a}	42.11 ± 0.24^{b}

Within each column, means sharing the different superscripts show comparison between the extracts by Tukey's test at $p < 0.05$. TEs, trolox equivalents

Antioxidant studies of species belonging to the genus Adonis are limited in the literature (Mohadjerani *et al*., 2014; Ucuncu *et al*., 2020; Guo *et al*., 2022). In antioxidant studies of *A. paryadrica* with only ethanol extracts, researchers used different parts of the plant and obtained effective results on the gallic acid equivalent (Ucuncu *et al*., 2020). The data obtained from this study also showed that the difference in the extract had an effect on the antioxidant properties of the Trolox equivalent.

3.2. Antimicrobial Activity

According to the results of the agar disc diffusion test, some bacteria were inhibited by the flower and leaf extracts. The root extract of the plant and negative control (20% DMSO) did not show any antimicrobial activity against bacteria. The inhibition zones against the tested bacteria ranged from 8.21 to 16.1 mm. The highest inhibition zone was obtained from the leaf extract against *B. subtilis* with 16.1 mm (MIC = 62.5 µg/mL). Among the bacteria whose inhibition value was obtained, E. faecalis had the lowest value with a zone diameter of 8.2 mm (MIC = 125 µg/mL). Flower extract was effective as antibacterial against S. aureus and *B. subtilis* and S. faecalis with 9.2 mm, 15.2 mm and 10.2 mm inhibition zone, respectively. MIC values for the mentioned these bacterial species were calculated as 125, 31.25 and 62.5 125 µg/mL, respectively. None of the flower leaf and root extracts showed any inhibition effect on *C. albicans*. Methanolic extracts of *A. paryadrica* demonstrate antimicrobial activity against two Gram-negative and four Gram-positive bacteria according to the disc diffusion assay (Table 4).

Diameter of inhibition zone including disc diameter of 6 mm by the agar dics diffusion method at a concentration of 10 µL of extract/disc. Ofloxacin (10 µg/disc) (OFX), netilmicin (30 µg/disc) (NET30), sulbactam (30 µg) + cefoperazone (75 µg); (105 µg per disc) (SCF) were used as reference antibiotics. Dimethyl sulfoxide (DMSO) (20%) was used as negative control (N.C.). MIC (minimal inhibition concentration) was calculated as µg/ml. The values are the average ± standard deviation of three determinations ($p < 0.05$). - = not detected. Values with different letter(s) in the same line(s) were significantly different ($p < 0.05$)

Due to the fact that microbial contamination is one of the most threatening elements of today, research is focused on this subject. Researchers carry out antimicrobial studies on each pathogenic microorganism against the risks of future epidemics, and herbal phytochemicals come first as a source (Tepe *et al*., 2005; Rios and Recio, 2005; Cüce and Basançelebi, 2021). In the study conducted on the antibacterial effects of ethanol extracts of *A. paryadrica*, the researchers obtained the highest zone diameter of 14 mm from the flower extract on *Yersina pseudotuberculosis* (Ucuncu *et al*., 2020). These researchers generally reported that flower extracts showed more effective antibacterial activity on different bacterial species.

On the other hand, the calculation of the highest inhibition zone on *B. subtilis* with 16.1 mm reveals the difference in the present study. According to these results, the leaf extract of *A. paryadrica* can be said more effective than other parts in terms of the antibacterial activity.

Table 4. Zones of growth inhibition (mm) showing antimicrobial activity of *A. paryadrica*

Bacteria	Disc Diffusion (mm)			MIC (µg/mL)			N.C.	Standard Antibiotic Discs		
	Flower	Leaf	Root	Flower	Leaf	Root		OFX	NET30	SCF30
Gram Negative Bacteria										
K. pneumoniae	-	8.5 ± 0.2^{d}	-	-	125	-	-	16.7 ± 0.6^{c}	18.1 ± 0.7^{b}	21.6 ± 0.8^{a}
E. coli	-	-	-	-	-	-	-	26.6 ± 1.1^{b}	16.6 ± 0.8^{c}	29.0 ± 0.5^{a}
S. marcescens	-	-	-	-	-	-	-	26.6 ± 0.7^{b}	20.0 ± 0.6^{c}	28.4 ± 0.9^{a}
S. typhimurium	-	-	-	-	-	-	-	26.3 ± 0.2^{b}	19.1 ± 1.5^{c}	28.2 ± 0.0^{a}
P. aeruginosa	-	-	-	-	-	-	-	27.8 ± 0.1^{a}	20.6 ± 0.3^{b}	27.4 ± 0.5^{a}
P. vulgaris	-	9.3 ± 0.3^{c}	-	-	31.25	-	-	29.1 ± 0.6^{a}	16.6 ± 0.8^{b}	29.2 ± 0.9^{a}
S. enterica	-	-	-	-	-	-	-	28.7 ± 0.5^{a}	27.4 ± 0.2^{a}	19.5 ± 0.7^{b}
Gram Positive Bacteria										
S. aureus	9.2 ± 0.2^{d}	9.1 ± 0.2^{d}	-	125	125	-	-	29.1 ± 1.0^{a}	20.6 ± 0.5^{c}	25.1 ± 0.6^{b}
B. subtilis	15.2 ± 0.3^{d}	16.11 ± 0.3^{c}	-	31.25	62.5	-	-	30.4 ± 1.4^{a}	29.3 ± 1.5^{a}	28.5 ± 1.5^{b}
S. epidermidis	-	-	-	-	-	-	-	27.5 ± 1.5^{b}	26.2 ± 1.1^{b}	31.7 ± 0.0^{a}
B. thrungiensis	-	-	-	-	-	-	-	25.4 ± 0.5^{b}	23.6 ± 1.0^{c}	28.6 ± 0.8^{a}
E. faecalis	-	8.21 ± 0.1^{d}	-	-	125	-	-	19.3 ± 0.5^{b}	21.2 ± 1.3^{a}	14.3 ± 0.7^{c}
S. faecalis	10.2 ± 0.2^{d}	11.03 ± 0.3^{c}	-	62.5	62.5	-	-	19.9 ± 0.5^{a}	16.6 ± 0.0^{b}	19.9 ± 0.7^{a}
Fungi										
C. albicans	-	-	-	-	-	-	-	20.5 ± 0.5^{b}	19.8 ± 0.6^{c}	18.7 ± 0.0^{d}

4. CONCLUSION

In this study, innovative and traditional antioxidant detection methods were applied to flower, leaf and root methanol extracts of *A. paryadrica*, an important endemic plant, and effective results were obtained. In terms of total antioxidant capacity, electrochemical and spectrophotometric antioxidant analysis methods have generally shown that the leaf parts of this plant are much more effective. Leaf extracts were found to be more effective than the other parts in terms of antimicrobial analyzes on Gram-negative and Gram-positive bacterial species and a fungus. This study is important in terms of revealing the pharmaceutical properties of different extracts obtained from medicinal and aromatic plants grown in our country and have medicinal value. It is envisaged that these studies will constitute a model for similar studies to be carried out in the near future.

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Declaration of Conflicting Interests and Ethics

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

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REFERENCES

Apak, R., Güçlü, K., Özyürek, M., Esin Karademir, S., & Erçağ, E. (2006). The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *International Journal of Food Sciences and Nutrition, 57*(5-6), 292-304. https://doi.org/10.1080/09637480600798132

Clinical and Laboratory Standards Institute (CLSI) (2020). Performance standards for antimicrobial susceptibility testing; twenty-third informational supplements. CLSI document M100-S23, Wayne, PA/USA.

Cuce, M., Demir, E., Tepe, A.S., & Sarikurkcu, C. (2021). *Draba cemileae* (Karaer): Phytochemical composition, antioxidant and enzyme inhibitory activity. *South African Journal of Botany*, *145*, 170-176. https://doi.org/10.1016/j.sajb.2021.07.028

Cüce, M., & Basançelebi, O. (2021). Comparison of volatile constituents, antioxidant and antimicrobial activities of *Thymus leucotrichus* (Lamiaceae) stem and leaves essential oils from both natural resources and *in vitro* derived shoots. *Journal of Essential Oil-Bearing Plants*, *24*(5), 1097-1112. https://doi.org/10.1080/0972060X.2021.2003256

Cüce, M., Bekircan, T., Laghari, A.H., Sökmen, M., Sökmen, A., Uçar, E.Ö., & Kılıç, A.O. (2017). Antioxidant phenolic constituents, antimicrobial and cytotoxic properties of Stachys annua L. from both natural resources and micropropagated plantlets. *Indian Journal of Traditional Knowledge, 16*(3), 407-416.

Cüce, M., Bekircan, T., Laghari, A.H., Sökmen, M., Sökmen, A., Uçar, E.Ö., & Kılıç, A.O. (2019). Phenolic profiles, antimicrobial and cytotoxic properties of both micropropagated and naturally growing plantlets of *Calamintha sylvatica* subsp. *sylvatica*. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, *47*(4), 1145-1152. https://doi.org/10.15835/nbha47411474

Demir, E. (2019). Sensitive and selective pathway of total antioxidant capacity in commercially lemon, watermelon and mango-pineapple cold teas by square wave adsorptive stripping voltammetry. *Gazi University Journal of Science*, *32*(4), 1123-1136. https://doi.org/10.35378/gujs.490523

Demir, E., Göktug, Ö., İnam, R., & Doyduk, D. (2021). Development and characterization of iron (III) phthalocyanine modified carbon nanotube paste electrodes and application for determination of fluometuron herbicide as an electrochemical sensor. *Journal of Electroanalytical Chemistry*, *895*, 115389. https://doi.org/10.1016/j.jelechem.2021.115389

Demir, E., & İnam, R., 2014. Electrochemical behaviour and determination of rimsulfuron herbicide by square wave voltammetry. *International Journal of Environmental Analytical Chemistry*, *94*(13), 1330-1341. https://doi.org/10.1080/03067319.2014.940340

Demir, E., & Silah, H. (2020). Development of a new analytical method for determination of veterinary drug oxyclozanide by electrochemical sensor and its application to pharmaceutical formulation. *Chemosensors*, *8*(2), 25. https://doi.org/10.3390/chemosensors8020025

Demir, E., Silah, H., & Aydogdu, N. (2021). *Electrochemical applications for the antioxidant sensing in food samples such as citrus and its derivatives, soft drinks, Supplementary Food and Nutrients. In Citrus.* IntechOpen.

Egamberdieva, D., & Tiezzi, A. (2019). *Medically Important Plant Biomes: Source of Secondary Metabolites*. Springer. https://doi.org/10.5772/intechopen.96873

European Committee on Antimicrobial Susceptibility Testing. (2016). EUCAST disk diffusion test manual, version 4.0. Retrieved 2016 November 12 from http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/, Accessed 30 September 2021.

Guo, X., Chen, M., He, X., Zhao, Y., Yu, J., Zhu, J., Li, L., Xia, G., & Zang, H. (2022). Phytochemical profiling and antioxidant, enzyme-inhibitory, and toxic activities of extracts from *Adonis ramose* Franch. *Natural Product Research*, 1-5. https://doi.org/10.1080/14786419.2022.2027937

Hao, D.C., He, C.N., Shen, J., & Xiao, P.G. (2017). Anticancer chemodiversity of Ranunculaceae medicinal plants: molecular mechanisms and functions. *Current Genomics*, *18*, 39-59. https://doi.org/10.2174/1389202917666160803151752

Inam, O., Demir, E., & Uslu, B. (2020). Voltammetric pathways for the analysis of ophthalmic drugs. *Current Pharmaceutical Analysis*, 16(4), 367-391.

IUCN. (2010). The IUCN red list of threatened species. Version 2010.4 [accessed 15. 03. 2019].

Jahanban-Esfahlan, A., Ostadrahimi, A., Tabibiazar, M., & Amarowicz, R. (2019). A comparative review on the extraction, antioxidant content and antioxidant potential of different parts of walnut (*Juglans regia* L.) fruit and tree. *Molecules*, *24*(11), 2133. https://doi.org/10.3390/molecules24112133

Kandemir, A., Kaptaner Igci, B., Aytac, Z., & Fisne, A. (2019). Contributions to the systematics of the genus *Adonis* L. (Ranunculaceae) in Turkey. *Gazi University Journal of Science*, *32*(4), 1105-1111. https://doi.org/10.35378/gujs.488934

Kocak, M.S., Sarikurkcu, C., Cengiz, M., Kocak, S., Uren, M.C., & Tepe, B. (2016). *Salvia cadmica*: Phenolic composition and biological activity. *Industrial Crops and Products*, *85*, 204-212. https://doi.org/10.1016/j.indcrop.2016.03.015

Kuroda, M., Kubo, S., Masatani, D., Matsuo, Y., Sakagami, H., & Mimaki, Y. (2018). Aestivalosides A-L, twelve pregnane glycosides from the seeds of *Adonis aestivalis*. *Phytochemistry*, *150*, 75-84. https://doi.org/10.1016/j.phytochem.2018.03.001

Mohadjerani, M., Tavakoli, R., & Hosseinzadeh, R. (2014). Fatty acid composition, antioxidant and antibacterial activities of *Adonis wolgensis* L. extract. *Avicenna Journal of Phytomedicine*, *4*(1), 24-30.

Öztürk, M., Demir, E., & Özdal, T. (2021). Voltammetric and spectrophotometric pathways for the determination of total antioxidant capacity in commercial turnip juice. *Journal of the Turkish Chemical Society Section A: Chemistry*, *8*(1), 163-172. https://doi.org/10.18596/jotcsa.752982

Rios, J.L., & Recio, M.C. (2005). Medicinal plants and antimicrobial activity. *J. Ethnopharmacol*., *100*(1-2), 80-84. https://doi.org/10.1016/j.jep.2005.04.025

Sarikurkcu, C., Ceylan, O., Benabdallah, A., & Tepe, B. (2021). *Stachys germanica* subsp. *heldreichii* (Boiss.) Hayek: Phytochemical analysis, antioxidant and enzyme inhibitory activities. *South African Journal of Botany*, *143*, 291-300. https://doi.org/10.1016/j.sajb.2020.11.009

Sarikurkcu, C., Sahinler, S.S., & Tepe, B. (2020). *Onosma aucheriana*, *O. frutescens*, and *O. sericea:* Phytochemical profiling and biological activity. *Industrial Crops and Products*, *154*, 112633. https://doi.org/10.1016/j.indcrop.2020.112633

T. C. Ministry of Agriculture and Forestry. (2018). *Yellow mountain rose (Adonis cyllenea var. paryadrica) type of action plan*. General Directorate of Nature Conservation and National Parks, November 2018.

Tepe, B., Daferera, D., Sokmen, A., Sokmen, M., & Polissiou, M. (2005). Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (Lamiaceae). *Food Chemistry*, *90*(3), 333-340. https://doi.org/10.1016/j.foodchem.2003.09.013

Ucuncu, O., Baltaci, C., Akar, Z., Duzgun, A.O., Cuce, M., & Kandemir, A. (2020). Biological activities and phytochemical screening of ethanol extracts from *Adonis paryadrica* (Ranunculaceae). *Farmacia*, *68*(6), 1062-1068. https://doi.org/10.31925/farmacia.2020.6.13

Yıldırım, S., Demir, E., & Gök. İ. (2020). Determination of total antioxidant capacities in Turkish and filter coffee samples by electrochemical methods. *Bilecik Şeyh Edebali Üniversitesi Fen Bilimleri Dergisi*, *7*(1), 382-393. https://doi.org/10.35193/bseufbd.736123

Zengin, G., Sarikurkcu, C., Gunes, E., Uysal, A., Ceylan, R., Uysal, S., Gungor, H., & Aktumsek, A. (2015a). Two *Ganoderma* species: profiling of phenolic compounds by HPLC–DAD, antioxidant, antimicrobial and inhibitory activities on key enzymes linked to diabetes mellitus, Alzheimer's disease and skin disorders. *Food & Function Journal*, *6*(8), 2794-2802. https://doi.org/10.1039/C5FO00665A.

Zengin, G., Sarikurkcu, C., Uyar, P., Aktumsek, A., Uysal, S., Kocak, M.S., & Ceylan, R. (2015b). *Crepis foetida* L. subsp. *rhoeadifolia* (Bieb.) Celak. as a source of multifunctional agents: Cytotoxic and phytochemical evaluation. *Journal of Functional Foods*, *17*, 698-708. https://doi.org/10.1016/j.jff.2015.06.041

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Research Article

Determination of heavy metal concentrations and soil samples of *Betula pendula* and *Populus tremula* in Nemrut Crater Lake

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Abstract: The concentrations of Cr, Mn, Fe, Ni, Cu, Zn, Cd, Pb and As heavy metals and the heavy metal accumulation levels of these plants were determined in *Betula pendula* and *Populus tremula* trees, which are the characteristic trees of Nemrut Crater Lake, the second largest crater lake in the world, and the soil samples surrounding them. Heavy metals are considered to be one of the most dangerous and priority pollutants due to their high persistence and toxicity to plant and animal life in the environment. For this reason, the results obtained will contribute to the studies carried out to determine the uptake of heavy metal pollutants in the environment and the self-healing effort of the polluted environments by applying the phytoremediation method.

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1. INTRODUCTION

Pollution can be examined in three groups as physical, biological and chemical pollution according to the types of pollutants. The pollution that arises as a result of the mixture of chemical substances consciously or unconsciously thrown into the nature and industrial wastes is chemical pollution. Examples of pollutants that cause chemical pollution are dyes, detergents, pesticides and petroleum products. Heavy metal pollution, which is accepted as a chemical pollution today, is in the first place among other chemical pollutants because they can arise from various sources, are resistant to environmental conditions, and can easily enter the food chain and accumulate in living things in increasing densities (Uzunoğlu, 1999). Heavy metals in these pollutants pose great danger. Exposure to heavy metals and environmental pollution is seen as an increasing problem around the world (Seaward & Richardson, 1989).

Since the beginning of the industrial revolution, heavy metal pollution has become an increasing problem for the environment. Heavy metals are one of the most important factors that cause pollution in wetlands. These metals are one of the main factors that play an important role in the deterioration of the ecological balance, and the development of living things and cause environmental pollution. Metals such as copper (Cu), zinc (Zn), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and cobalt (Co) are micronutrients that play an important

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role in the growth and development of animals and plants. Furthermore, some heavy metals such as arsenic (As), mercury (Hg), cadmium (Cd) and lead (Pb) are elements that are not important for the development of living things (Niess, 1999).

Despite the increase in environmental pollution, the first remediation studies were carried out on a physical and chemical basis. The efficiency of these remediation methods, which are widely used in areas exposed to environmental pollutants, is limited due to the expensiveness, high energy expenditure and side-waste generation (Hamutoğlu *et al*., 2012). Phytoremediation, which means cleaning or restoration of polluted areas with plants, carries a hope against environmental pollution predominantly caused by nano-industry. It is green biotechnology that minimizes, neutralizes or completely cleans and metabolizes contamination factors that cause toxicity with the usage of plants and rhizospheric microorganisms in terrestrial and wetland areas exposed to pollution for various reasons (Prasad, 2003; Prasad & Fertias, 2003; Park *et al*., 2011; Singh &Prasad, 2011; Zhai, 2013; Bayçu *et al*., 2014).

Heavy metals are naturally found in the environment at trace level, in the inanimate realm, in rocks, soil and water, and in plants and animals in the living world. Although natural physical and chemical processes such as erosion of rocks and volcanic activities contribute to heavy metal enrichment in water bodies, human-induced activities have the most important share in the increase in the system (Akbıyık, 2012). Heavy metals are released into the environment in significant quantities as a result of industrial activities such as mining, energy and fuel production, and excessive usage of pesticides and fertilizers (Halim *et al*., 2003; Samarghandi *et al*., 2007). The metal concentration in the soil typically ranges from 1 to 100,000 mg kg^{-1}. High levels of heavy metals cause deterioration of soil quality, decrease in crop yield and quality (Long *et al*., 2002) and therefore cause significant hazards to humans and other organisms (Blaylock & Huang, 2000).

These elements, which are one of the most important inorganic impurities that mix with soil and water and accumulate in these environments, and are defined as pure substances consisting of the same kind of atoms, can cause many environmental and human health problems such as microbial activity, soil fertility, biological diversity and even poisoning in living things through the food chain (Vanlı and Yazgan, 2008).

Studies are carried out to determine the toxic heavy metal contents, biomonitor and hyperaccumulator properties of plants, and as a result of these studies, the evaluation of the usage of plants within the scope of phytoremediation in terms of human and environmental health gains importance with each passing day. Therefore, the aim of this study is to contribute to the studies carried out to determine the uptake of heavy metal pollutants in the environment and the self-healing effort of the polluted environments by determining the concentrations of Mn, Fe, Cu, Zn, Pb and As heavy metals in *Betula pendula* and *Populus tremula* trees, which are the characteristic trees of Nemrut Crater Lake, and in the soil samples surrounding them, by applying phytoremediation method. Moreover, it is predicted that the plant sap obtained from *Betula sp.* species, which is an important food and medicine plant throughout history, will come to the fore as a functional food and food additive for a healthy life in the coming period (GKGM, 2016). Therefore, determining the heavy metal concentration in different organs of Betula pendula will help to know the effects on human health of these species, which are thought to be used as food and food additives.

2. MATERIAL and METHODS

2.1. Study area

The Nemrut Caldera is one of the most characteristic and original landforms of Turkey in terms of volcanic activities, which was formed as a result of the explosion of Mount Nemrut on the highest peak of the Van and Bitlis Plains and shows the structural features of a great caldera

collapse. Nemrut Crater ranks first in Turkey, fourth in Europe and sixteenth in the world. Nemrut Crater Lake, with a diameter of about 6 km, is the second in the world after Mount Mazama Crater Lake in Oregon, USA. Nemrut Caldera Natural Monument was taken under protection with many protection status until today due to its richness and virginity in terms of its formation and, accordingly, its geological, geomorphological and hydrogeological features, and in terms of floristic and visual landscape values. These are the First Degree Natural Site, Natural Monument, Tourism Area and Wetland-RAMSAR Area having International Importance (Figure 1).

Figure 1. General view photos from the research area

2.2. Plant Materials

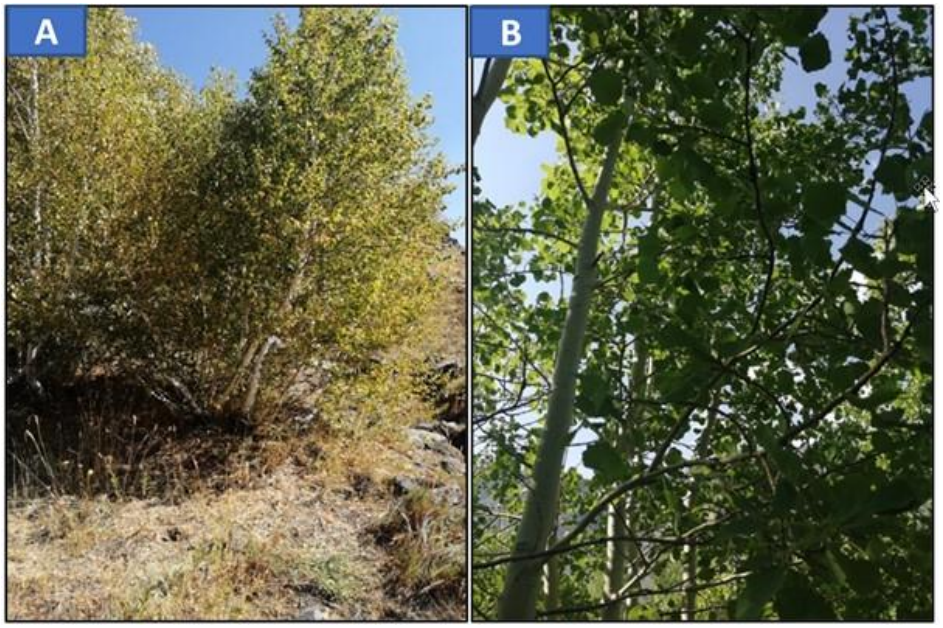
2.2.1. *Betula pendula* Roth

B. pendula is a member of the Betulaceae family. It is a deciduous tree with a smooth stem that grows naturally in Turkey (Figure 2a). It can be up to 15-30 m in length in the environments where it grows naturally. Their stems have a snow-white thin bark. The shoots are thin and slender, hanging down, bare but with abundant resin glands (Anşin and Özkan, 1993). Male flower seeds are cylindrical, 8-10 cm long, in terminal (tip) or lateral catkins (chatons). Male flowers that form in autumn are in bundles 2-4 cm long. The flowers, which are covered with scales, spend the winter months in this way and bloom in March-May (Korkut *et al*., 2010).

2.2.2. *Populus tremula* L. subsp. *tremula*

P. tremula is a member of the Salicaceae family. Although their homeland is unknown, they show natural distribution in many areas from Western Europe, North Africa to the interior of Asia, Siberia and Japan. This tree species is a Class I forest tree that can grow up to 25 m in height, has a cylindrical trunk, dense branches, and a broad conical top (Yaltırık, 1993). The shells are greenish-gray, shiny and flat. Shoots and buds are red chestnut color. It is also shiny as if it was polished. The buds are terminal and spirally arranged on the shoots. The flower-bearing buds are large, plump and blunt-tipped, while the leaf buds are pointed, smaller, light and sticky. The leaves on the long shoots (especially on the log shoots) are egg-shaped with a pointed tip, and the bottom sides are heart-shaped. The leaf margins are irregularly double-row toothed, and the undersides are soft hairy. The leaves on the short shoots are 3-7 x 3-7 cm in size, almost equal in length to the width, circular, blunt-tipped, and the bottom side is in the form of a light heart. The upper surface of the leaves is dark green, the lower surface is grayish green and bare, the edges of the leaf blade are toothed. The petiole is long (6-8 cm), and since it is pressed from the sides, it causes the leaf blade to sway even in a light wind. The petiole scar is in the form of a circle slice and there are three vascular bundle scars on it (Kayacık, 1981; Yaltırık, 1993). Since the petioles of this tree are printed on the sides, the leaves sway even in the slightest wind. That's why the tree is called aspen (Figure 2b).

Figure 2. General view of the studied plant species. A) *Betula pendula*, B) *Populus tremula*



2.3. Heavy Metal in Sediment and Plant Analysis

Some vegetative organs (bark, root, leaf) of *B. pendula* and *P. tremula* trees, which form the climax vegetation of the Nemrut Caldera chosen as the study area, were taken under suitable seasonal conditions. While taking plant samples, soil samples surrounding these plants were taken appropriately. The purpose of obtaining the appropriate vegetative organs such as bark, leaves and roots of these trees, which are the subject of the study, was to determine separately the heavy metal contents accumulated in the underground and above-ground organs of the plant and to make comparisons between these organs. After this process, these plant parts were washed to avoid contamination with tap water, and then passed through the ELGA PURELAB-Q DV25 brand pure water device in the Environmental Engineering Laboratory, and these parts were laid on separate filter papers to be dried. After drying, the plant samples, which were wrapped in filter papers in the MST55 incubator in the same laboratory, were kept for 24 hours at 50 ^{0}C - 80 ^{0}C, and at the end of 24 hours, these samples were placed in separate sealed bags and put into cabinets. Furthermore, the preliminary processes of soil samples taken from 2 different areas where these trees are concentrated were carried out in our Environmental Engineering Laboratory. Soil samples were laid on filter papers and aerated for two weeks. The samples were first subjected to the combustion process in the microwave device, 0.5 g sample was taken and 6 mL 65% HNO_3 and 2 mL 30% H_2O_2 were added. The burned samples were diluted 50 times with 2% HNO_3 (200 µM sample + 9800 µM 2% HNO_3) for reading in the ICP*MS device. In the reading made in the ICP*MS device, the peroxide and nitric acid used in the dilution in the microwave were also subjected to the burning process as a blank and read as a special blank and this value was deducted from the results in the ICP reading. 50-times dilutions were calculated in the ICP device and the results were transferred to an excel file.

3. RESULTS

The concentrations of *B. pendula* and *P. tremula* trees, which are the characteristic trees of Nemrut Crater Lake, the second largest crater lake in the world and of Cr, Mn, Fe, Ni, Cu, Zn, Cd, Pb and As heavy metals in the soil samples surrounding them and the heavy metal accumulation levels of these plants were determined (Table 1, Table 2). Although the limit values of heavy metal amounts (Table 3, Table 4) for plants in polluted and uncontaminated soils in the studies in the international literature differ, as a result of our analysis, the heavy

metal concentrations in the parts of both plants were found well below these values. One of the most important reasons for this is that the natural environment in which these two plants, which are the subject of our study, are located, is free from many pollutants. While it was determined that the amount of Fe (34.79 mg kg^{-1}) measured only in the root part of the *B. pendula* was above the Fe limit value accepted by FAO/WHO in plants, it was found to be normal for other reference values in the literature. When the results obtained from soil samples (Table 3) were compared with the limit values for heavy metals in the soil pollution control regulation and Table 4, it was seen that all heavy metals except Fe are within the limit values. It was determined that the Fe value in the soil taken from both stations (T1, T2) was well above the limit values.

Table 1. Heavy metal values (mg/kg) in the organs of investigated species

Betula pendula			*Populus tremula*			Heavy metals
Leaf	Bark	Root	Leaf	Bark	Root	
0.05111	0.01783	0.6312	0.03946	0.00917	0.3793	Cr
39.170	3.487	21.51	3.631	1.433	5.05	Mn
11.320	1.196	34.790	8.269	1.142	42.62	Fe
0.05962	0.02082	0.2085	0.04614	0.06233	0.5073	Ni
0.2464	0.148	0.1256	0.2176	0.1049	1.673	Cu
8.505	4.245	1.380	4.124	4.679	1.898	Zn
0.00788	0.00483	0.02146	0.05763	0.04721	0.1754	Cd
0.0141	0.00124	0.03382	0.00957	0.00127	0.0456	Pb
0.00586	0.00126	0.01474	0.00814	0.00136	0.0333	As

Table 2. Soil sample values (mg/kg) were taken from the study area.

Soil samples	Cr	Mn	Fe	Ni	Cu	Zn	Cd	Pb	As
Soil1 (T1)	0.3046	12.41	414	0.168	0.9308	3.149	1.101	0.4961	0.270
Soil2 (T2)	0.3667	16.670	576.9	0.221	0.1847	3.856	2.991	0.7054	0.391

Table 3. Reference values for normal and toxic concentrations of heavy metals in plants according to FAO/WHO & Allen (1989).

Heavy Metals	Necessary upper value for soil (mg/kg) (FAO/WHO, 1996).	Allowable value for plants (mg/kg) (FAO/WHO, 1996).	Allowable value for plants (mg/kg) Allen (1989).
Cd	0.8	0.05	0.01-0.3
Zn	50	50	10-100
Cu	36	5	25-40
Cr	100	0.5	0.05 – 0.5
Pb	85	2	2-20
Mn	70	5	50-500
Ni	35	5	5
Fe	4.5	30	40-500
Ar	20	0.2	*

Table 4. Reference values for normal and toxic concentrations of heavy metals in plants (Misra &Mani, 1991; Kastori *et al*., 1997; Schulze *et al*., 2005; Kabata-Pendias, 2007).

Heavy metals	Normal Concentration (mg / kg)	Toxic Concentration (mg / kg)
Pb	5.0-10.0	30.0-300.0
Cd	0.05-0.2	5.0-30.0
Cu	5.0-30.0	20.0-100.0
Cr	0.1-0.5	5.0-30.0
Ni	0.1-5.0	10.0-100.0
Mn	15-100	400
Fe	50-250	>500
As	10-60	<2

4. DISCUSSION and CONCLUSION

Bioconcentration factor (BCF), this value is used to evaluate the metal accumulation in plant regions or the transfer of metals from the soil to the plant root (Chen *et al*., 2004) and to calculate this value, the plant root metal concentration (mg/kg) is divided by the soil metal concentration (mg/kg). The translocation factor (TF), which is important, indicates the degree of phytoremediation of the species (Zhao & Duo, 2015) and is used to evaluate metals transferred from soil-fed plant roots to the plant stem. Additionally, this value represents the plant's ability to absorb and transfer metals from the sediment and then store it in the above-ground parts (Wei *et al.*, 2002). Transport factor (TF) is an indicator of the movement of heavy metals from the root to the upper organs and is calculated by dividing the heavy metal concentration in the stem of the plant to that in the root (Padmavathiamma & Li, 2007). If TF> 1, the plant transferred the metal from the root to the stem. If BCF>1, it is called a plant accumulator (Baker, 1989). The TF and BCF values we obtained as a result of our study are shown in Table 5 and Table 6.

Table 5. TF and BCF values of *Betula pendula*.

Heavy Metals	Translocation Factor (TF)	Bioconcentration Factor (BCF)	
		Aerial parts	Root
Cr	0.1092	0.06894	0.6312
Mn	19.776	42.657	21.51
Fe	0.3597	12.516	34.79
Ni	0.3858	0.08044	0.2085
Cu	3.1401	0.3944	0.1256
Zn	9.2391	12.75	1.38
Cd	5.9226	0.01271	0.02146
Pb	0.4535	0.01534	0.03382
As	0.4830	0.00712	0.01474

It can be said that the TF values of Mn, Cu, Zn and Cd metals in *Betula pendula* plant were greater than 1, especially these metals accumulate excessively in the aboveground organs and the plant showed hyperaccumulator properties in terms of these metals. When we looked at the BCF values, we can think that this plant has accumulator properties since BCF>1 for Mn and Zn metals.

Table 6. TF and BCF values of *Populus tremula*.

Heavy Metals	Translocation Factor (TF)	Bioconcentration Factor (BCF)	
		Aerial parts	Root
Cr	1.2820	0.04863	0.3793
Mn	1.0027	5.064	5.05
Fe	0.2208	9.411	42.62
Ni	0.2138	0.10847	0.5073
Cu	0.1927	0.3225	1.673
Zn	4.6380	8.803	1.898
Cd	0.5977	0.10484	0.1754
Pb	0.2379	0.01085	0.0456
As	0.2852	0.0095	0.0333

When the TF and BCF values of the *Populus tremula* were examined, while TF>1 in terms of Cr, Mn and Zn metals, the only metal with BCF>1 was Zn. Therefore, we can say that this plant has an accumulator feature in terms of Cr, Mn and Zn metals.

Consequently, it was seen that the heavy metal concentrations obtained from the soil samples in this study did not exceed the allowable heavy metal accumulation limit values in the soil in Turkey and in the world and were at an acceptable level. When the data of the plant samples were examined, it was determined that there was heavy metal accumulation, but we can say that it was within the heavy metal limit values accepted for the plants obtained as a result of many studies in the literature with the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO). Only the amount of Fe (34.79 mg kg^{-1}) measured in the root part of the *Betula pendula* plant was determined to be above the Fe limit value accepted by FAO/WHO in plants. It is known that plants with hyperaccumulators that accumulate in the above-ground organs can be used successfully in phytoextraction, and those that accumulate in their roots can be used successfully in phytostabilization processes and can naturally clean the metal pollution in the environment. Due to the limited number of similar studies with *Betula pendula* and *Populus tremula* species, and for the first time, the amount of heavy metal concentrations they can accumulate in their bodies were shown in detail in this study, and these findings will make an important contribution to the literature in phytoremediation applications.

Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

Sukru Hayta: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing -original draft. **Elif Fırat**: Methodology, Supervision, and Validation.

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REFERENCES

Akbıyık, F. (2012). *An investigation on the accumulations of macro and microelements on biotic and abiotic components of feline stream* [Unpublished Master Thesis]. Anadolu University.

Allen, S.E. (1989). *Chemical Analysis of Ecological Material*. 2nd edition. Blackwell Scientific Publications, Oxford, 368 pp.

Baker, A.J.M., & Brooks, R.R. (1989). Terrestrial higher plants which hyperaccumulate metallic elements - a review of their distribution, ecology and phytochemistry. *Biorecovery, 1,* 81-126.

Baycu, G., Tolunay, D., Ozden, H., Csatari, I., Karadag, S., Agba, T., & Rognes, S.E. (2014). An abandoned copper mining site in Cyprus and assessment of metal concentrations in plants and soil. *International Journal of Phytoremediation, 17*(7), 622–631.

Blaylock, M.J., & Huang, J.W. (2000). *Phytoextraction of metals*. In: Raskin, I. ve Ensley, B.D. (eds.), Phytoremediation of Toxic Metals: Using plants to clean-up the environment. Wiley, New York, 53-70.

Chen, Y., Shen, Z., & Li, X. (2004). The use of Vetiver Grass (*Vetiveria zizanioides*) in the phytoremediation of soils contaminated with heavy metals. *Applied Geochemistry, 19,* 1553–1565.

FAO/WHO (1996). *Biotechnology and food safety.* Report of a Joint FAO/WHO Consultation. FAO Food and Nutrition Paper No. 61. Rome, FAO.

GKGM (2016). *Betula* sp. özsuyunun (usaresi) gıdalarda kullanımının güvenilirliğinin değerlendirilmesi hakkında bilimsel görüş. Gıda ve Kontrol Genel Müdürlüğü Risk Değerlendirme Daire Başkanlığı (Bilimsel Rapor).(in Turkish). [*Betula* sp. Scientific opinion on the evaluation of the safety of the use of the juice (usaresi) in food.General Directorate of Food and Control Risk Assesment Department]

Halim, M., Conte, P., & Piccolo, A. (2003). Potential availability of heavy metals to phytoextraction from contaminated soils by exogenous humic substances. *Chemosphere*, *52*, 265.

Hamutoğlu, R., Dinçsoy, A.B., Cansaran-Duman, D., & Aras, S. (2012). Biyosorpsiyon, adsorpsiyon ve fitoremediasyon yöntemleri ve uygulamaları. *Türk Hijyen ve Deneysel Biyoloji Dergisi, 69(4),* 235-53. (in Turkish). [Biosorption, adsorption, phytoremediation methods and applications. [Turkish Bulletin of Hygiene and experimental Biology].

Kabata-Pendias, A., & Mukherjee, A. (2007). *Trace elements from soil to human*. Springer-verlag, Berlin, Heidelberg.

Kayacık, H. (1981). *Orman ve park ağaçları özel sistematiği*. II. Cilt Angiospermae (Kapalı Tohumlular), İstanbul Üniversitesi Yayın No: 2766, İstanbul, s 52-53, 1981. (in Turkish). [*Special systematic of forest and park trees.* Volume II Angiospermae. Istanbul University Pulication No: 2766, Istanbul, 52-53, 1981.]

Kastori, R., Petrović, N., & Arsenijević-Maksimović, I. (1997). *Heavy metals and plants*. In: R kastori (ed.), Heavy Metals in the environment. Institute of Field and vegetable Crops, novi Sad. 196-257.

Korkut, A.B., Şişman E, & Özyavuz, E. (2010). *Peyzaj Mimarlığı* [Landscape architecture]. Ada Ofset Matbaacılık. ISBN: 978-605-88381-0-9. İstanbul.

Long, X.X., Yang, X.E., & Ni, W.Z. (2002). Current status and perspective on phytoremediation of heavy metal polluted soils. *Journal of Applied Ecology, 13,* 757-762.

Misra, S.G., & Mani, D. (1991). *Soil pollution*. Ashish Publishing House, Punjabi Bagh, New Delhi, India.

Niess, D.H. (1999). Microbial heavy-metal resistance. *Applied Microbiol. Biotech., 51,* 730-750.

Padmavathiamma, P.K., & Li, L.Y. (2007). Phytoremediation technology: hyper-accumulation metals in plants. *Water, Air, and Soil Pollution, 184*(1-4), 105-126.

Park, S., Kim, K.S., Kim, J. T., Kang, D., & Sung, K. (2011). Effects of humic acid on phytodegradation of petroleum hydrocarbons in soil simultaneously contaminated with heavy metals. *Journal of Environmental Sciences, 23*(12), 2034-2041.

Prasad, M. N. V., & Freitas, H. M. D. O. (2003). Metal hyperaccumulation in plants biodiversity prospecting for phytoremediation technology. *Electronic Journal of Biotechnology, 6*(3), 275–321.

Prasad, M.N.V. (2003). Phytoremediation of metal-polluted ecosystems: Hype for commercialization. *Russian Journal of Plant Physiology, 50*(5), 686–700.

Samarghandi, M.R., Nouri, J., Mesdaghinia, A.R., Mahvi, A.H., Nasseri, S., & Vaezi, F. (2007). Efficiency removal of phenol, lead and cadmium by means of UV/TiO2/H2O2 Processes. *International Journal of Environmental Science and Technology, 4*, 19-25.

Schulze, E.D., Beck, E., Müller-Hohenstein, K., Lawlor, D., Lawlor, K., & Lawlor, G. (2005). *Plant Ecology*. Springerverlag, Berlin, Heidelberg.

Seaward, M.R.D., & Richardson, D.H.S. (1989). Atmospheric sources of metal pollution an effects on vegatation, In: SHAW A.J. (ed.) Heavy metal tolerance in plants. pp. 75-92.

Singh, A., & Prasad, S.M. (2011). Reduction of heavy metal load in food chain: technology assessment, *Reviews in Environmental Science and Bio/Technology, 10*(3), 199–214.

Uzunoğlu, O. (1999). *Determining some heavy metal concentrations in water and sediments samples taken from Gediz river* [Unpublished Master thesis]. Celal Bayar University.

Vanlı, Ö., & Yazgan, M. (2008). Phytormediation technique in cleaning soils contaminated with heavy metals. http://www.tarimsal.com. (Accesss date: 05.04.2018).

Wei, C.Y., Chen, T.B., & Huang, Z.C. (2002). Cretan bake (*Pteris cretica* L): an arsenic accumulating plant. *Acta Ecology Sinica, 22*, 777-782.

Yaltırık, F. (1993). Dendroloji Ders Kitabı II Angiospermae (Kapalı Tohumlular) [Dendrology Textbook II Angiospermae]., Bölüm I, 2. Baskı, İstanbul, s 46-48, 1993.(in Turkish).

Zhai, G. (2013). Phytoremediation: Green Weapon to Fight Pollution. *Journal of Bioremediation and Biodegradation, 7*, 56-65.

Zhao, S., & Duo, L. (2015). Bioaccumulation of cadmium, copper, zinc, and nickel by weed species from municipal solid waste compost. *Polish Journal of Environmental Studies, 24*(1), 413-417.

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Research Article

Medical physiological perspective to biochemical assays and GC-MS results of corn tassel

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Abstract: Corn tassel (Zea may L.) is rich in phenolic compounds including flavonoids and anthocyanins. The aim of this study is to consider the results of the contents of phenolics (TPC), flavonoids (TFC), flavonol (TF), anthocyanins (TAC), alfa-amylase inhibitory activity, and antioxidant activity including FRAP and metal chelating capabilities (MCC) as potential Antiviral and anti-Rheumatoid arthritis. Significantly high levels of antioxidant capacity, total flavonol and alfa-amylase inhibition were found in ethanolic extracts of corn tassels. It was found that their concentrations are TPC= 40 mg GA/g, TFC= 13 mg QE/g, TF= 45 mg R/g, and TAC= 8 mg cyanidin-3-glucoside/g based on dry extract. Additionally, the extracts showed relatively higher antioxidant activities due to metal chelating capabilities (MCC) were found to be 217 mg Fe^{2+}/g dry extract. From the GC-MS analysis, corn tassel was found to be good source of arctigenin that has antiviral and anti- rheumatic properties. Further, the extracts of corn tassels showed significantly higher α-amylase inhibitory activity up to 90 %. Thus, it was concluded that extracts of corn tassels may be considered as pharmacological potential in rheumatoid and antiviral treatment.

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Bioactive compounds,
Antioxidant activity.

1. INTRODUCTION

Plants as producer of bioactive components are commonly ingested in nutrition and have been utilized as traditional treatments for generations, where they are still used in the form of classical extracts such as infusion, decoction, or maceration in aqueous media (Abraham *et al*., 2021). Among bioactive components, phenolic compounds in medicinal plants and foods are known to dramatically decrease the adverse effects of reactive species on physiological functions of humans. This is very important because oxidative stress and reactive oxygen species (ROS) are considered to be harmful for human as they damage the cells and results in various metabolic disorders.

Corn (Zea may L.) tassel is rich in phenolic compounds including flavonoids and anthocyanins. Some studies reported that corn tassel extract has antioxidant capacity (Mohsen & Ammar, 2009; Wang *et al*., 2014) and high ability to reduce the proliferation of gastric cancer cells (Duangpapeng *et al*., 2018).

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Corn tassels can be included as therapeutic agents in the treatment of diseases Thus, there is need to studies on the composition of this plant seeds.

In this study, biochemical assays including total phenolic content (TPC), total flavonoid content (TFC), total flavonol content (TF), total anthocyanin content (TAC), antioxidant assays such as ferric reducing antioxidant power (FRAP) and metal chelating capacity (MCC) were determined in corn tassels because these parameters project the extent of bioactive components of the samples.

Further, α -amylase inhibition assay (AAI) and GC-MS analysis were also examined. Finally, the results obtained were evaluated taking into account their health correlations by medical physiological approach.

2. MATERIAL and METHODS

2.1. Chemicals

In the current study, all chemicals and solvents were of analytical grade. The chemicals including Folin-ciocalteu's phenol reagent, sodium hydroxide, trichloroacetic acid, ferric chloride trihydrate, potassium hydroxide, formic acid, aluminum chloride, sodium acetate trihydrate, potassium ferricyanide, iodine, phenanthroline monohydrate, ferrous sulfate heptahydrate, dipotassium hydrogen phosphate, and sodium carbonate were obtained from Merck (Germany). Methanol, ethanol, hexane, and sodium dihydrogen phosphate were obtained from Carlo Erba (Spain). Quercetin and ethyl acetate were purchased from Sigma-Aldrich (Germany) and gallic acid anhydrous was taken from ISOLAB GmbH. Rutin trihydrate was obtained from DR Ehrenstorfer™.

2.2. Preparation of Plant Material

Corn tassels were collected from Gaziantep city in Türkiye and washed by tap and distilled water, respectively. After drying at 70 ^{o}C for two days, the samples were ground into a fine powder using grinding mill. Powdered seed samples were extracted firstly by using hexane to defatted for 4 hours at room temperature. After evaporation, the hexane was removed using vacuum-rotary evaporator, the residue was extracted using the mixture of ethanol/water:70/30 (70 mL for 10 g dried sample) at room temperature under reflax for 24 hrs. After filtration (through Whatman filter paper-white), new extract at the same volume was added, and the process was repeated two times more. The filtrates of three days were combined and concentrated under reduced pressure at 40°C by using rotary evaporator with vacuum. The residue was dried at room temperature following oven with vacuum. From the dried sample, 0.03 g was dissolved in 10 mL of methanol and used for both biochemical assays and individual compounds.

2.3. Biochemical Assays

2.3.1. *Total phenolic content* (TPC)

The Folin–Ciocalteu technique was used to determine the total phenolic content of the extract with a minor modification (Abraham *et al*., 2021). Briefly, 1.5 mL of distilled water was added to 0.1 mL of crude extract (0.03 g/10 mL of methanol). After adding 0.1 mL of Folin–Ciocalteu reagent and waiting for 5 min, 1.5 mL of 10 % (w/v) sodium carbonate was added. The mixture was left in the dark for 60 minutes. Absorbance of the samples were measured using UV-VIS spectrophotometer (Thermo Scientific GENESYS 10S UV-VIS spectrophotometer, USA) at 765 nm. The total phenolic content of the extract was converted to gallic acid equivalents (milligrams of gallic acid per gram of dried extract=mg GA/g)) using a regression equation derived from the gallic acid standard calibration curve.

2.3.2. *Total flavonoid content* (TFC)

To determine the total flavonoid content of crude extract, the aluminum chloride colorimetric method was used with minor modification (Do *et al*., 2014). In brief, 0.2 mL of crude extract (0.03 g/10 mL of methanol) was added to 1 mL of 5 % $AlCl_3$ solution, and then 0.1 mL of 1.0 M CH_3COOK solution was added. After adding 2.7 mL of methanol, the mixture was allowed to stand for 60 minutes. The absorbance was measured at 420 nm using UV-VIS spectrophotometer. The quercetin was used as standard reagent. The total flavonoid content was determined using the quercetin calibration curve and represented in milligrams of quercetin equivalents per gram of dry weight (mg Q/g extract).

2.3.3. *Total flavonol content* (TF)

The total flavonol content was determined using a colorimetric technique with aluminum chloride (Iqbal *et al*., 2015). Briefly, 0.5 mL of aluminum chloride (2 %) was added to 0.5 mL extracts (0.03 g/10 mL of methanol) in a test tube. After adding 6.0 mL of sodium acetate (5%), the mixture was vortexed and then was incubated at room temperature in the dark for 2.5 hrs. Absorbance of the solutions was measured at 440 nm. Rutin was used to obtain standard calibration curve. The results were represented as mg rutin (R) equivalent/g of crude extract.

2.3.4. *Total Anthocyanin content* (TAC)

Anthocyanin concentrations were measured using the pH differential method, which was carried out with the use of a UV-vis. spectrophotometer (Mihailović *et al*., 2016). Separately, the tassels extracts were diluted in 0.025 M potassium chloride and 0.40 M sodium acetate solutions and then their acidities were adjusted to pH 1.0 and 4.5 with HCl, respectively. After that, absorbance of the solutions was measured at 520 and 700 nm, respectively. The following formula was used to compute the absorbance (A):

$$A = (A_{520} - A_{700})_{pH=1.0} - (A_{520} - A_{700})_{pH=4.5}$$

The concentration of monomeric anthocyanin in the original sample was calculated as follows Eq. 1:

$$[\text{monomeric anthocyanins}]\ (mg/L) = (A \times MW \times DF \times 1000)/(\varepsilon \times l) \quad (1)$$

The monomeric and total anthocyanin contents were calculated using cyanidin-3-glucoside equivalents (mg cyanidin-3-glucoside/kg dry extract).

The total anthocyanin concentration was calculated using Eq. (2):

$$[\text{total anthocyanins}]\ (mg/L) = (A' \times MW \times DF \times 1000)/(\varepsilon \times l) \quad (2)$$

Where: $A' = (A_{520} - A_{700})_{pH=1.0}$; MW = molecular weight (449.2 g/mol cyanidin-3-glucoside); DF = dilution factor; ε = molar extinction coefficient (26900 L $mol^{-1}cm^{-1}$ cyanidin-3-glucoside), l = path length (1.0 cm).

2.4. Antioxidant Assays

2.4.1. *Ferric reducing antioxidant power* (FRAP)

Based on its antioxidant principles, the reducing power (FRAP) of extracts of corn tassels was measured to create a colorful complex with potassium ferricyanide (Işil Berker *et al*., 2010). Briefly, 0.1 mL sample (0.03 g/10 mL) was added to test tubes containing 2.5 mL of 1% $K_3Fe(CN)_6$ and 2.5 mL of 0.2 M phosphate buffer (pH: 6.6). After incubation for 30 minutes at 50 °C, 2.5 mL of 10 % TCA was added to the tube. The samples were then centrifuged at 3200 rpm for 10 minutes. The 2.5 mL of supernatant was pipetted into a second tube containing 2.5 mL water and 0.5 mL of freshly prepared $FeCl_3$ of 0.1% was added. Absorbance was

measured at 700 nm. The results are expressed as milligrams of Quercetin equivalents per gram of dry mass (mg Q/g dry extract).

2.4.2 *Metal chelating capacity* (MCC)

Because the antioxidants are oxidized when exposed to $FeCl_3$, the amount of $FeCl_3$ utilized should be excessive to ensure that all antioxidants are oxidized. In this approach, the complexing reagent is 1-10-phenanthroline, which produces a complex compound with Fe (II) was used in the MCC determination. The reduction of Fe (III) by antioxidants results in Fe(II). As a result, the amount of Fe-phenanthroline generated will be equal to the antioxidant content of the sample being evaluated (Yefrida *et al*., 2018). So, a 1,10-phenanthroline technique was used to determine metal chelating capacity. A 1.5 mL of distilled water was added to 0.1 mL of the sample solution (0.03 g/10 mL) in the test tube. 1.0 mL of 0.2 % $FeCl_3$, 1.0 mL of 0.2% phenanthroline and 1.4 mL water were added to the mixture. After vortexing, the tubes were incubated in the dark for 20 minutes. Absorbance was measured at a wavelength of 510 nm. The results were reported in mg Fe^{2+}/g dry extract as iron II sulfate equivalents.

2.4.3 α *-amylase inhibition assay* (AAI)

A modified starch iodine protocol was used for this investigation (Ademiluyi & Oboh, 2013). To make an iodine solution, 0.1 g I_2 and 1.0 g KI were dissolved in 50 mL of distilled water. The starch solution was made by dissolving 1.0 g of starch in 50 mL of distilled water, slowly heating, cooling, and completing to 100 mL with distilled water. The amylase solution was made by mixing 15 mg of α-amylase suspension with 30 mL of phosphate buffer (pH 6.9). Briefly, 0.25 mL α-amylase was incubated with 0.25 mL (3 mg/mL) of the extracts for 15 minutes at 37 °C. After that, 0.25 mL of starch solution was added and the mixture was re-incubated for 30 minutes. The process was stopped by adding 0.1 mL of HCl (1.0 M). After vortexing extensively, 1.0 mL of iodine reagent was added, followed by 3.0 mL of distilled water. A spectrophotometer was used to measure the absorbance at 580 nm. To adjust the background absorbance, individual blanks were also studied. The tassels extracts were replaced with 0.25 distilled water in the controls, which were carried out in the same way. As a positive control, acarbose, a well-known anti-diabetic drug was used. The inhibition % was determined using the following formula:

$$\% \text{ Inhibition} = [(A_c - A_S)/A_c] \times 100$$

A_c-absorbance for control; A_s-absorbance for standard

2.5. Gas-Chromatographic Determinations of Methanolic Extracts

From the dried sample detailed in the section 2.2, 0.03 g of extract was dissolved in 10 mL of ethanol and used for GC-MS (Shimadzu OP2010 ultra GCMS) determinations.

3. RESULTS

The quantitative phytochemical analysis shows that corn tassels extracts include considerable amounts of phenolic, flavonoids, and flavonol content, indicating its powerful antioxidant capacity. Polyphenolic compounds and their functional derivatives have an aromatic benzene ring with substituted hydroxyl groups and so, they can be effective as reducing agents, hydrogen donors, and metal chelators (Rice-evans *et al*., 1995). They are capable of absorbing free radicals (radical scavengers) and chelating metal ions that may stimulate the development of reactive oxygen species (ROS), which promotes lipid peroxidation (Iqbal *et al*., 2015); (Kessler, Ubeaud, and Jung, 2003).

Among polyphenols, flavonoids are particularly beneficial since they are powerful antioxidants and aid the human body in fighting disease. They are found in plants as glycosides,

commonly (Rajanandh & Kavitha, 2010). The most prevalent flavonol with antioxidant activity is quercetin, which possesses all of the necessary structural characteristics for free radical scavenging activity (Kalita *et al*., 2013). The TPC values for corn tassels reported in the literature are in a wide range of 15-45 mg/g dw (Aziz *et al*., 2021; Taha *et al*., 2015). This high range may be attributed to the grown conditions of the plant as well as the extraction conditions such as solvent species, extraction type, extraction time, temperature, ratio of sample/solvent and measurement conditions. From the Table 1 and Figure 1, it is seen that the obtained TPC values (40 mgGA/g) in this study are in the high region of the reported in the literature. So, the added solvent can highly extract the phenolic compounds due to the defatting from the matrix. The TFC values (13 mg Q/g) obtained (Table 1) are in the ranges of the literature (3.3-21 mg Q/g) for corn tassels (Aziz *et al*., 2021; Taha *et al*., 2015).

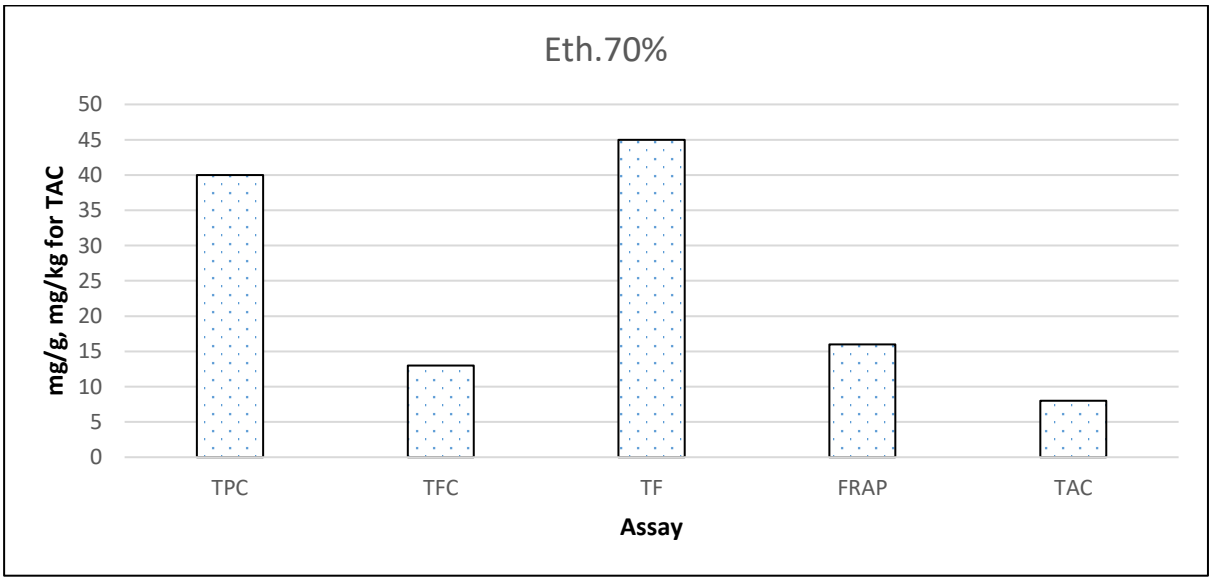
Interestingly, the obtained TF values (45 mg R/g) were found to be higher than the TFC values (13 mg Q/g) for the same samples (Table 1).

Table 1. Biochemical assays and antioxidant activities of the corn tassels extracts.

Assay	70%ethanol as extract
TPC (mg GA/g)	40±4
TFC (mg QE/g)	13±±2
TF (mg R/g)	45±5
Anthocyanin Monomeric (mg cy-3-glu/kg)	0.5±0.1
Anthocyanin Total (mg cy-3-glu/kg)	8±1
Antioxidant activity	
FRAP **(**mg $FeSO_4$/g extract)	16±2
MCC **(**mg Fe^{2+}/g dry extract)	217±19
Amylase inhib. %	90±10

GA= gallic acid equivalent, QE= Quercetin equivalent, R= Rutin equivalent
Data are expressed as mean ± standard deviation (n=3)

Figure 1. TPC, TFC, TF, FRAP and TAC values for the corn tassels extracts



3.1. Total Anthocyanin Contents

Anthocyanins may act as antioxidants and may provide additional health benefits (Hosseinian *et al*., 2008). Antioxidant power is widely recognized for anthocyanins (phenolic chemicals) that are responsible for the red, blue, or purple coloring of many plants. Because it is a quick and simple operation, the pH differential approach is the most commonly used method for quantifying anthocyanins (Lee *et al*., 2005). The monomeric and total Anthocyanins of the extract have been expressed in mg cy-3-glu/kg (Table 1). It was found that the monomeric and total anthocyanin contents are 0.3 and 1.1, respectively (Table 1).

3.2 Antioxidant capacities

3.2.1. *Ferric reducing antioxidant power* (FRAP)

The ability of extracts of corn tassels to reduce ferric ions was assessed using the FRAP test. It was found that the extracts of corn tassels have a significant antioxidant capacity (Table 1) that the obtained values (16 mg/g) are in ranges of the literature values (Aziz *et al*., 2021). The reducing power assay is frequently used to assess a natural antioxidant's ability to donate an electron or hydrogen, which is a key mechanism of phenolic antioxidant action (Dorman *et al*., 2003).

3.2.2. *Metal chelating capacity* (MCC)

By-products of mitochondrial electron transport and other metabolic activities constitute the majority of reactive oxygen species (ROS). As a result of metal-catalyzed oxidation processes, ROS are generated as essential intermediates. Using electron gain or loss, the transition metal ion Fe^{2+} can keep generating free radicals in the solution. So, chelating metal ions with chelating agents reduces the generation of reactive oxygen species. Therefore, the chelation ability of crude extracts was assessed using a chelation power test (Sudan *et al*., 2014). The results of this study (217 mg/g) showed extracts of corn tassels to be highly capable of chelating metals (Table 1) and may therefore protect against oxidative damage caused by metal-catalyzed degradation processes. The obtained high MCC values (217 mg/g) reveal that corn tassels have high antioxidant capacity.

3.2.3. *α -amylase inhibition assay*

Some studies have shown that plants used as conventional medicine are also useful in the treatment of diabetes, whereas synthetic anti-diabetic drugs can produce serious side effects such as hypoglycemic coma and liver and kidney disorders (Shabab *et al*., 2021).

α-Amylase is one of the most important enzymes in the human body, and it is responsible for the breakdown of starch into more simple sugars. Inhibitors of this enzyme can cause a delay in carbohydrate digestion and a reduction in the rate of glucose absorption. As a result, Amylase inhibitors have the potential to lower attenuated postprandial plasma glucose levels and enhance glucose tolerance in diabetic individuals (Nickavar & Yousefian, 2011). Inhibitors of alpha Amylase, also known as starch blockers, work by preventing dietary starch absorbed by the body and as a result, provide lower postprandial blood glucose levels. In persons with diabetes, slowing the digestion and breakdown of starch may have favorable benefits on insulin resistance and glycemic index control (Uddin *et al*., 2014). From the obtained results in Table 1 and Figure 2, it can be concluded that corn tassels is more effective as antidiabetic because amylase inhibition up to 90% was observed.

3.3. Individual Components of Corn Tassels by GC-MS

The obtained GC-MS chromatogram for the extract of corn tassels was given in Figure 3. From the library of GC-MS device, the evaluated components depending on the peaks in this chromatogram were given in Table 2. It was seen that the highest ratio of the compound (area%-

R. time) is the Dihydrofuran-2-one, 4-(3,4-dimethoxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)- known as Arctigenin (69.60%-51.102 min).

The values given in Table 2 were analyzed against the GC-MS library. The percentage of the compound Dihydrofuran-2-one, 4-(3,4-dimethoxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)dihydrofuran-2(3H)-one known as Arctigenin that considered in this study was found to be % 89.

Figure 2. MCC and Alfa amylase values for the corn tassels extracts.

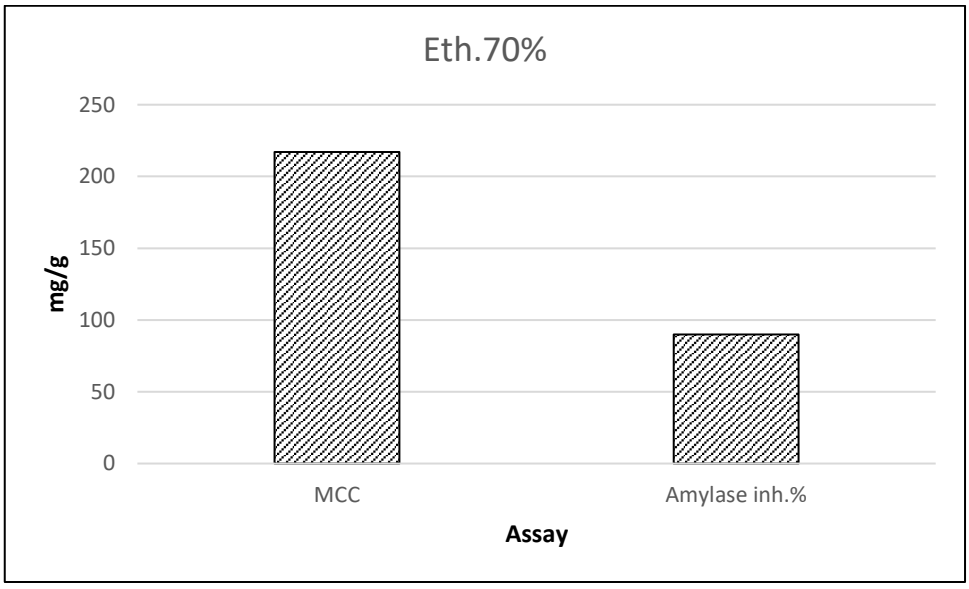


Figure 3. GC-MS chromatogram of the corn tassels extracts.

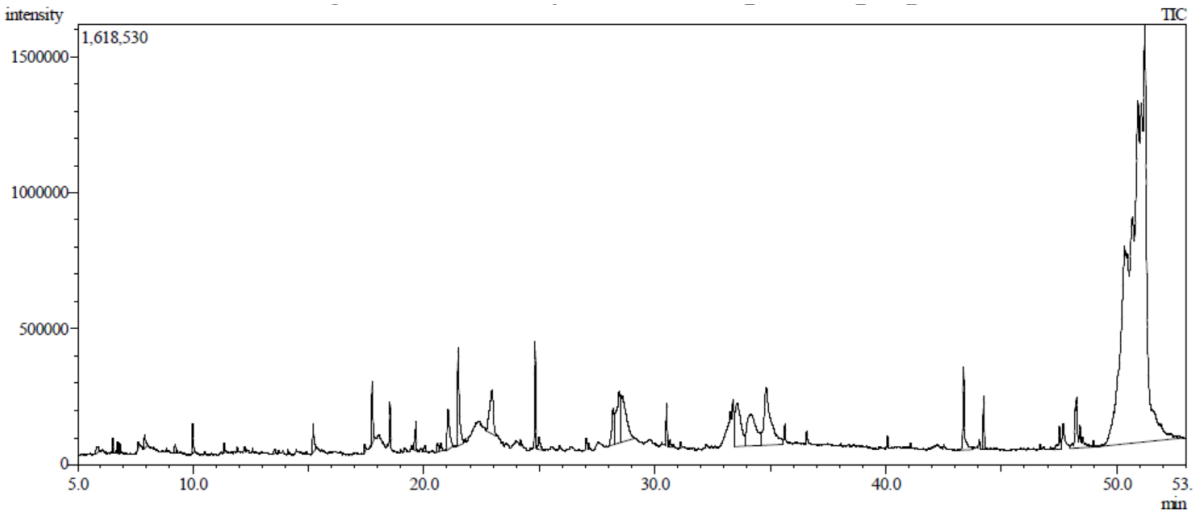


Table 2. GC-MS analysis of the corn tassels extracts.

Peak	R. Time	Area	Area%	Name
1	3.548	271560	0.26	1,2,3-Propanetriol (CAS)
2	3.973	223376	0.22	2-Propenoic acid, methyl ester (CAS)
3	4.015	247654	0.24	(R)-alpha-Methyl-beta-alanine
4	4.137	613917	0.60	N-methyl-N-(methyl-d3)aminoheptane
5	5.886	163529	0.16	dl-Glyceraldehyde
6	6.541	117138	0.11	Silane,(2-methoxyethyl)trimethyl-
7	6.740	100520	0.10	1,3-Dioxolane-4-methanol,2-ethyl-
8	6.845	88743	0.09	2-Furanmethanol (CAS)
9	7.617	102419	0.10	Butanoic acid, 2-ethyl-methyl ester (CAS)
10	7.911	213794	0.21	dl-Glyceraldehyde dimer
11	9.222	127222	0.12	2-Hydroxy-2-cyclopenten-1-one
12	9.977	307960	0.30	2,5 Furandione,3-methyl-
13	11.362	105084	0.10	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
14	13.521	77747	0.08	4-Methylenecyclohexanone
15	15.212	453877	0.44	1.3.5-Triazine-2,4,6-triamine
16	17.446	83402	0.08	2-Propanamine, N-methyl-N-nitroso-(CAS)
17	17.765	793893	0.77	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
18	19.650	485945	0.47	4-Methyl itaconate
19	20.058	94741	0.09	1,3-Benzenediol (CAS)
20	20.582	183087	0.18	Tetrahydrofuran-5-oı-2-methanol,alpha-[alpha-methoxy-(tetrahydrofuran-5-on-2-ylmethoxy)]-
21	20.730	144493	0.14	2,3-Dihydro-Benzofuran
22	21.048	1083282	1.05	5-hydroxymethylfurfural
23	21.489	1834159	1.78	1,2,3-Propanetriol,1-acetate (CAS)
24	22.938	1767818	1.72	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one
25	24.172	92124	0.09	Guaiacol<4-vinyl->
26	24.986	284003	0.28	Butanedioic acid, 2-hydroxy-2-methyl-,(S)-
27	27.150	78510	0.08	Tetradecane
28	28.201	929739	0.90	Alpha-d-Lyxofuranoside,methyl
29	28.457	2222580	2.16	Benzaldehide,2-hydroxy-4-methyl-
30	28.550	2762467	2.68	1,3-Propanediol,2-(hydroxymethyl)-2-nitro-
31	30.667	120044	0.12	1-Dodecanamine,N,N-dimethyl- (CAS)
32	31.121	76991	0.07	2,5-Pyrrolidinedione,3-(1-aminoethylidene)-4-methyl-(CAS)
33	33.581	2647741	2.57	Quinic acid
34	34.189	3410195	3.31	3-Deoxy-d-mannoic acid
35	34.836	4311118	4.19	Beta-D-Glucopyranose, 4-O-beta,-D-galactopyranosyl-
36	43.378	1414390	1.37	Palmitic acid
37	44.265	637855	0.62	Hexadecanoic acid, ethyl ester (CAS)
38	47.543	472057	0.46	9,12-Octadecadienoic acid (Z,Z)-
39	48.266	2035793	1.98	Ethyl linoleate
40	49.015	115219	0.11	Stearate <ethyl->
41	51.102	71638186	69.60	Dihydrofuran-2-one, 4-(3,4-dimethoxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)-
		102934372	100.00	

4. DISCUSSION

In the literature, there is not any information about the arctigenin in the corn tassel. It was reported that the seeds of *Arctium lappa* (Chinese medicinal plant) had high concentration of arctigenin (Gao *et al*., 2018).This study is the first on the high arctigenin levels in the corn tassel. Related with the biochemical assays, the obtained TF values were found to be higher (45 mgR/g) that is higher than the TFC values of 13 mgQ/g for the same samples. Due to the obtained high concentration of flavonol in the studied samples, it can be concluded that some flavonols do not signal at the TFC determination step.

The MCC results (217 mg/g) showed that the extracts of corn tassels have high capable of chelating metals and therefore protect against oxidative damage caused by metal-catalyzed degradation processes.

In the literature, it was reported that arctigenin stimulates glucose reuptake in cultured L6 skeletal muscle cells and AMPK (AMP-activated protein kinase) phosphorylation. The AMPK is striking target to treat type 2 diabetes (Srivastava & K, 2017). Briefly, arctigenin activates AMPK by inhibiting respiratory complex I and play antidiabetic role.

The obtained results in the current study showed that corn tussels is highly effective as antidiabetic because amylase inhibition was found to be 90%. The high actinic concentrations in the extract of corn tussels are in accordance with amylase inhibition.

Further, the arctigenin compound has anti-inflammatory, antidepressant and anticancer effects (Gao *et al*., 2018; Shabgah *et al*., 2021; Xu *et al*., 2020; Zhong *et al*., 2019). Due to the anti-inflammatory effects, it is also suggested in combination of drugs used for rheumatoid arthritis (Chakraborty *et al*., 2021).

In a behavior study, the antidepressant effect of arctigenin was shown and mechanism of this effect was described as suppressor effect on both HMGB1/TLR4/NF-κB and TNF-α/TNFR1/NF-κB signaling pathways (Xu *et al*., 2020). ATG may be useful in diabetic kidney disease by enhancing PP2A activity, reducing p65 NF-κB-mediated inflammatory response and also high glucose-induced migration in podocytes via interaction with Drebrin-1 (Zhong *et al*., 2019). ARG inhibits inducible nitric oxide synthase (INOS). Hence, ARG has potent anti-inflammatory effects in acute and chronic auto inflammatory diseases (Gao *et al*., 2018). Arctigenin can trigger apoptosis and necrosis and prevent drug resistance in tumor cells by stimulating apoptotic signaling pathways, caspases and cell cycle arrest. Anti-inflammatory properties of Arctigenin provide the inhibition of inflammation in the tumor microenvironment. Arctigenin also prevents tumor metastasis and angiogenesis downregulating some factors such as N-cadherin, TGF-β, and VEGF (Shabgah *et al*., 2021).

5. CONCLUSION

In this study, the antioxidant capacity as well as biochemical assays and GC-MS analysis were performed in the extract of corn tassels. The phytochemical examination of the sample is critical for determining the sample's antioxidant capacities. The amount of total phenolic was approximately three-times higher than the amount of total flavonoid. Thus, a significant amount of phenolic compounds is responsible for their high antioxidant and free radical scavenging ability and they could be used as a natural source of antioxidants to treat oxidative stress-related disorders. Again, the obtained total flavonol content is higher more than three times than TFC. This is considered that some flavonol compounds in the corn tassels could not be determined by the known TFC method. The effect of natural extracts as antioxidants was evaluated using FRAP-MCC, which demonstrated that these plants have a high capacity for scavenging free radicals. The extract evaluated for α-amylase inhibitory capacity had the highest level of inhibition, which may be a result of the synergistic action of the phytochemical ingredients present. GC-MS analysis revealed the presence of valuable compounds such as Arctigenin

because this compound has antidepressant, antiviral, anti-inflammatory, and anticancer effects as well as anti-rheumatic effects.

The findings of this research will assist future research in identifying, isolating, and characterizing the specific molecule responsible for increased antioxidant activity.

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Declaration of Conflicting Interests and Ethics

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

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REFERENCES

Abraham, A.M., Quintero, C., Carrillo-Hormaza, L., Osorio, E., & Keck, C.M. (2021). Production and Characterization of Sumac PlantCrystals: Influence of High-Pressure Homogenization on Antioxidant Activity of Sumac (Rhus coriaria L.). *Plants (Basel, Switzerland)*, *10*(6). https://doi.org/10.3390/PLANTS10061051

Ademiluyi, A.O., & Oboh, G. (2013). Aqueous Extracts of Roselle (Hibiscus sabdariffa Linn.) Varieties Inhibit α-Amylase and α-Glucosidase Activities In Vitro. *Journal of Medicinal Food*, *16*(1), 88–93. https://doi.org/10.1089/JMF.2012.0004

Aziz, M., Saeed, F., Ahmad, N., Ahmad, A., Afzaal, M., Hussain, S., Mohamed, A.A., Alamri, M.S., & Anjum, F.M. (2021). Biochemical profile of milk thistle (Silybum Marianum L.) with special reference to silymarin content. *Food Science & Nutrition*, *9*(1), 244–250. https://doi.org/10.1002/FSN3.1990

Chakraborty, D., Gupta, K., & Biswas, S. (2021). A mechanistic insight of phytoestrogens used for Rheumatoid arthritis: An evidence-based review. *Biomedicine and Pharmacotherapy*, *133*. https://doi.org/10.1016/J.BIOPHA.2020.111039

Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., & Ju, Y.H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. *Journal of Food and Drug Analysis*, *22*(3), 296–302. https://doi.org/10.1016/J.JFDA.2013.11.001

Dorman, H.J.D., Peltoketo, A., Hiltunen, R., & Tikkanen, M.J. (2003). Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. *Food Chemistry*, *83*(2), 255–262. https://doi.org/10.1016/S0308-8146(03)00088-8

Duangpapeng, P., Ketthaisong, D., Lomthaisong, K., Lertrat, K., Scott, M.P., & Suriharn, B. (2018). Corn Tassel: A new source of phytochemicals and antioxidant potential for value-added product development in the agro-industry. *Agronomy*, *8*(11), 242. https://doi.org/10.3390/agronomy8110242

Gao, Q., Yang, M., & Zuo, Z. (2018). Overview of the anti-inflammatory effects, pharmacokinetic properties and clinical efficacies of arctigenin and arctiin from Arctium lappa L. *Acta Pharmacologica Sinica*, *39*(5), 787–801. https://doi.org/10.1038/APS.2018.32

Hosseinian, F.S., Li, W., & Beta, T. (2008). Measurement of anthocyanins and other phytochemicals in purple wheat. *Food Chemistry*, *109*(4), 916-924. https://doi.org/10.1016/J.FOODCHEM.2007.12.083

Iqbal, E., Salim, K.A., & Lim, L.B.L. (2015). Phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of Goniothalamus velutinus (Airy Shaw) from

Brunei Darussalam. *Journal of King Saud University - Science*, *27*(3), 224–232. https://doi.org/10.1016/J.JKSUS.2015.02.003

Işil Berker, K., Güçlü, K., Tor, I., Demirata, B., & Apak, R. (2010). Total antioxidant capacity assay using optimized ferricyanide/Prussian blue method. *Food Analytical Methods*, *3*(3), 154–168. https://doi.org/10.1007/S12161-009-9117-9/TABLES/7

Kalita, P., Tapan, B.K., Pal, T.K., & Kalita, R. (2013). Estımatıon of total flavonoıds content (TFC) and antı oxıdant actıvıtıes of methanolıc whole plant extract of bıophytum sensıtıvum lınn. *Journal of Drug Delivery and Therapeutics*, *3*(4), 33-37. https://doi.org/10.22270/jddt.v3i4.546

Kessler, M., Ubeaud, G., & Jung, L. (2003). Anti-and pro-oxidant activity of rutin and quercetin derivatives. *Journal of Pharmacy and Pharmacology*, *55*(1), 131–142.

Lee, J., Durst, R.W., Wrolstad, R.E., Barnes, K.W., Eisele, ; T, Giusti, ; MM, Haché, ; J, Hofsommer, ; H, Koswig, ; S, Krueger, D. A., Kupina, ; S, Martin, ; S.K., Martinsen, ; B.K., Miller, T. C., Paquette, ; F, Ryabkova, ; A, Skrede, ; G, Trenn, ; U, & Wightman, J.D. (2005). Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines by the pH Differential Method: Collaborative Study. *Journal of AOAC International*, *88*(5), 1269-1278. https://doi.org/10.1093/JAOAC/88.5.1269

Mihailović, V., Kreft, S., Benković, E.T., Ivanović, N., & Stanković, M.S. (2016). Chemical profile, antioxidant activity and stability in stimulated gastrointestinal tract model system of three Verbascum species. *Industrial Crops and Products*, *89*, 141–151. https://doi.org/10.1016/J.INDCROP.2016.04.075

Mohsen, S.M., & Ammar, A.S.M. (2009). Total phenolic contents and antioxidant activity of corn tassel extracts. *Food Chemistry*, *112*(3), 595-598. https://doi.org/10.1016/J.FOODCHEM.2008.06.014

Nickavar, B., & Yousefian, N. (2011). Evaluation of α-amylase inhibitory activities of selected antidiabetic medicinal plants. *Journal Fur Verbraucherschutz Und Lebensmittelsicherheit*, *6*(2), 191–195. https://doi.org/10.1007/S00003-010-0627-6

Rajanandh, M.G., & Kavitha, J. (2010). Quantitative estimation of β-sitosterol, total phenolic and flavonoid compounds in the leaves of Moringa oleifera. *International Journal of PharmTech Research*, *2*(2), 1409–1414.

Rice-evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M., & Pridham, J.B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research*, *22*(4), 375–383. https://doi.org/10.3109/10715769509145649

Shabab, S., Gholamnezhad, Z., & Mahmoudabady, M. (2021). Protective effects of medicinal plant against diabetes induced cardiac disorder: A review. *Journal of Ethnopharmacology*, *265*, 113328. https://doi.org/10.1016/j.jep.2020.113328

Shabgah, A.G., Suksatan, W., Achmad, M.H., Bokov, D.O., Abdelbasset, W.K., Ezzatifar, F., Hemmati, S., Mohammadi, H., Soleimani, D., Jadidi-Niaragh, F., Ahmadi, M., & Navashenaq, J.G. (2021). Arctigenin, an anti-tumor agent; a cutting-edge topic and up-to-the-minute approach in cancer treatment. *European Journal of Pharmacology*, *909*. https://doi.org/10.1016/J.EJPHAR.2021.174419

Srivastava, D., & K, S. (2017). Arctigenin, A Plant Lignan With Tremendous Potential: A Review. *International Journal of Current Research*, *9*(05), 50232–50237.

Sudan, R., Bhagat, M., Gupta, S., Singh, J., & Koul, A. (2014). Iron (FeII) chelation, ferric reducing antioxidant power, and immune modulating potential of Arisaema jacquemontii (Himalayan Cobra Lily). *BioMed Research International*, *2014*. https://doi.org/10.1155/2014/179865

Taha, M.N., Krawinkel, M.B., & Morlock, G.E. (2015). High-performance thin-layer chromatography linked with (bio)assays and mass spectrometry - A suited method for

discovery and quantification of bioactive components? Exemplarily shown for turmeric and milk thistle extracts. *Journal of Chromatography A, 1394*, 137-147. https://doi.org/10.1016/J.CHROMA.2015.03.029

Uddin, N., Hasan, M.R., Hossain, M.M., Sarker, A., Nazmul Hasan, A.H.M., Mahmudul Islam, A.F.M., Chowdhury, M.M.H., & Rana, M.S. (2014). In vitro α-amylase inhibitory activity and in vivo hypoglycemic effect of methanol extract of Citrus macroptera Montr. fruit. *Asian Pacific Journal of Tropical Biomedicine*, *4*(6), 473. https://doi.org/10.12980/APJTB.4.2014C1173

Wang, L.C., Yu, Y.Q., Fang, M., Zhan, C.G., Pan, H.Y., Wu, Y.N., & Gong, Z.Y. (2014). Antioxidant and antigenotoxic activity of bioactive extracts from corn tassel. *Journal of Huazhong University of Science and Technology - Medical Science*, *34*(1), 131–136. https://doi.org/10.1007/s11596-014-1244-x

Xu, X., Piao, H.N., Aosai, F., Zeng, X.Y., Cheng, J.H., Cui, Y.X., Li, J., Ma, J., Piao, H.R., Jin, X., & Piao, L.X. (2020). Arctigenin protects against depression by inhibiting microglial activation and neuroinflammation via HMGB1/TLR4/NF-κB and TNF-α/TNFR1/NF-κB pathways. *British Journal of Pharmacology*, *177*(22), 5224-5245. https://doi.org/10.1111/BPH.15261

Yefrida, Suyani, H., Alif, A., Efdi, M., & Aziz, H. (2018). Modification of phenanthroline method to determine antioxidant content in tropical fruits methanolic extract. *Research Journal of Chemistry and Environment*, *22*(4), 28–35.

Zhong, Y., Lee, K., Deng, Y., Ma, Y., Chen, Y., Li, X., Wei, C., Yang, S., Wang, T., Wong, N.J., Muwonge, A.N., Azeloglu, E.U., Zhang, W., Das, B., He, J.C., & Liu, R. (2019). Arctigenin attenuates diabetic kidney disease through the activation of PP2A in podocytes. *Nature Communications*, *10*(1). https://doi.org/10.1038/S41467-019-12433-W

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Research Article

Combined effect of nitrogen and phosphorus on growth and biochemical composition of Tetradesmus obliquus (Turpin) M.J. Wynne

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Abstract: Microalgae have many biotechnological applications in various industries including food and feed, fertilizer, biofuel, cosmetics, pharmaceutics, and wastewater treatment. Since hey produce secondary metabolites under stress conditions such as pigments, carotenoids, hydrocarbons, and vitamins, investigating the effects of stress factors on growth parameters and biochemical composition of microalgal biomass is needed in producing bioproducts.

In this paper, the combined effects of nitrogen and phosphorus on growth and the protein/amino acid and Lipid-FAMEs profiles of microalgae *Tetradesmus obliquus* (MAKUMACC-037) were investigated.

Nitrogen and phosphorus deficiency reduced the algal growth. Biochemical composition was changed in a nitrogen and phosphorus dependent manner.

High concentration of protein and lipid were associated with increased nitrogen and phosphorus concentration However, the FAMEs profiles were changed depending on only the nitrogen concentration.

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1. INTRODUCTION

Microalgae are unicellular aquatic organisms, ranging in size from 1 µm to 2 mm. They are photoautotrophic microorganisms that produce their own food utilizing light, CO_2, and H_2O and thus primary producers in the aquatic system (Sirakov *et al.*, 2015; Maizatul *et al.*, 2017). Microalgae can be cultivated and can produce sufficient biomass for use in different biotechnological applications under optimum conditions. These microorganisms can survive in a diverse set of environmental habitats such as salt water, inland water, and wastewater. Their growth rates, biomass productivity, and biochemical compositions are affected by different cultivation conditions (Yaakob *et al.*, 2021).

Microalgal biomass is commonly used in food, feed, cosmetic, bioenergy, pharmaceutical, biofertilizer, and wastewater treatment sectors. Microalgae contain 28–70% of protein (dry biomass), 10–20% of lipids, and 10–50% of carbohydrates (Amaro *et al.*, 2011). Because of

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their high macromolecule rates, they are quite suitable for fish, shrimp, and livestock feed. Moreover, biomass can be used for human consumption with ease. FAO's report on potential food supplies and agricultural products emphasizes importance of finding alternative food sources (FAO, 2017). The high oil content of these organisms also makes them important for use in biofuel production. Also, they produce many other metabolites including pigments and secondary metabolites which are valuable in producing cosmetic and pharmaceutical components. (Zachleder *et al.*, 2014; Fernandes *et al.*, 2013).

Regarding the biochemical capacities of microalgae mentioned above, the following research question has been raised, "In which culture medium and conditions do they produce these valuable metabolites the most?" In order to answer this question, maximum biomass efficiency studies have been carried out by changing growth parameters such as nutrients, temperature, light intensity, and quantity (Zarrinmehr *et al.*, 2013). Since the most important ones among those are the nitrogen (N) and the phosphorus (P) concentrations of the culture media, studies mainly focus on their impact on growth (Xin *et al.*, 2010).

Nitrogen is the basic element for constructing cell structure and producing physiological molecules such as enzymes and hormones. On the other hand, phosphorus has importance in terms of the structure of membranes, RNA, DNA, and ATP. Additionally, the amounts and types of N and P have found to promote microalgal growth, and biomass productivity. Changing the N and the P amounts of the culture media stresses the algal cells and causes a modification in the biochemical composition (Beuckels *et al.*, 2015).

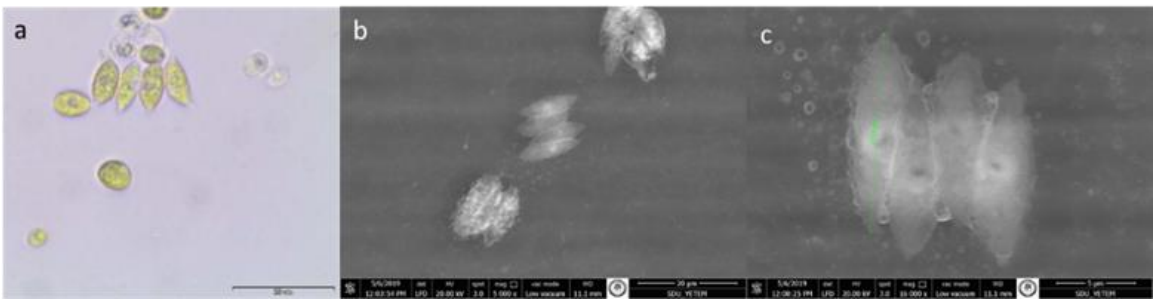
This study primarily focused on the combined effects of N and P on growth and biomass productivity of *Tetradesmus obliquus.* Secondly, the coupling effects of N and P on biochemical composition such as total protein, amino acid, lipid, and FAMEs (Fatty Acids Methyl Esters) of *T. obliquus* were addressed.

2. MATERIAL and METHODS

2.1. Algal Strain and Culture Conditions

In this study, *Tetradesmus obliquus* (MAKUMACC-037), which was isolated from the Ergene River Basin (Turkey) and deposited in the Mehmet Akif Ersoy University Microalgae Culture Collection (MAKUMACC) was used. It was characterized according to the ITS gene region as *Acutodesmus obliquus* (NCBI-KF470790) in 2014. Then this microalga was reclassified as *Tetradesmus obliquus* (Turpin) M. J. Wynne, so the current name is used in this paper (Wynne *et al.*, 2015). Figure 1 shows the light and scanning electron micrographs of the study material, *T. obliquus.*

Figure 1. Light and scanning electron micrographs of *T. obliquus*; Light micrograph (a), scale bar: 20 µm; general view (b), scale bar: 20 µm; length of a cell (c), scale bar: 5 µm.



To obtain the stock culture, BG11 medium (Rippka *et al.*, 1979) was used for culturing until the exponential phase under 16:8 h light: dark photoperiod (70 µmol $m^{-2}s^{-1}$) at 25±2°C and pH 7.2 as a first step. The culture was semi-continuously aerated (1 L/minute). The BG 11 medium was consisting of (in mgL^{-1} H_2O) $NaNO_3$, 1500; K_2HPO_4, 40; $MgSO_4.7H_2O$, 75; $CaCl_2.2H_2O$, 36; citric acid, 6; ferric ammonium citrate, 6; Na_2EDTA, 1; and Na_2CO_3, 20. 1 mL trace solution was added, its composition (in gL^{-1}) is H_3BO_3, 2.86; $MnCl_2$, 1.81; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.39; $CuSO_4.5H_2O$, 0.079; and $Co(NO_3)2.6H_2O$, 0.0494.

The second step of the study is harvesting and transferring microalgae into 3 different media that have been modified according to N and P concentrations as follows: BG11 (Control Medium); %50N+%50P+ (Medium I); %50N-%50P- (Medium II). [N+P+: contains more N and P than BG11; N-P-: contains less N and P than BG11]. The starter OD (Optical Density) value of all cultures is 0.09 and the cell number value is $3.6x10^5$ cells/mL. The cultures were grown under the same conditions as stock cultures.

2.2. Determinations of Culture Growth Parameters

Cell number (CN), optical density (OD), and dry weight (DW) values were used as culture growth parameters. The R^2 correlation values of these data were detected with Microsoft Office Excel 2007 (Microsoft, USA). The CN (cell mL^{-1}) was determined every other day by counting three replicate samples in a Thoma haemocytometer under a light microscope. To detect cell density, the OD at 680 nm was measured every other day by a UV/visible spectrophotometer (Shimadzu UV-1650) (Santos-Ballardo *et al.*, 2015). DW was determined according to Boussiba *et al.* (1992) where 10 mL of culture was filtered through a filter (Whatman GF/C, 1.2 lm, UK) and dried at 105°C for two hours and weighed.

All culture growth measurements were conducted to detect the stationary phase. After reaching the stationary phase the cultures were harvested to obtain dry biomass.

The specific growth rate (µ), Doubling Time (DT); Biomass productivity ($P_{Biomass}$) values were estimated according to these formulas.

$$\text{Specific growth rate } (\mu): \mu = \ln (x_2-x_1)/(t_2-t_1).$$

Where x_2 and x_1 are the cell density at t_2 and t_1, respectively (Chia *et al.*, 2013).

$$\text{Doubling Time (DT): } DT = 0.6931/\mu.$$ (Godoy-Hernández *et al.*, 2006).

Biomass productivity ($P_{Biomass}$): the dry biomass produced per day ($gL^{-1}day^{-1}$). It was calculated using $P_{Biomass}$ ($gL^{-1}day^{-1}$) = (X_2-X_1) x $(t_2-t_1)^{-1}$.

where X_1 and X_2 were the biomass concentrations (gL^{-1}) on days t_1 (starting point of cultivation) and t_2 (endpoint of cultivation), respectively (Hempel *et al.*, 2012). The results were converted to mg $L^{-1}day^{-1}$.

2.3. Determining the Protein-Amino acid and Lipid-FAMEs Profiles

To obtain the dried biomass, all the cultures were centrifuged after the stationary phase. Then the cultures were washed with distilled water, and dried in an oven at 40°C for 48 hours. Protein content was detected by using a CHNS elemental analyzer (Perkin- Elmer Model 2 400, USA) with the total N content. The crude protein contents of all groups were estimated following the equation below (Lopez *et al.*, 2010).

$$\text{Protein content} = \text{Nitrogen content} \times 6.25.$$

The results were expressed as a percent of dry weight. Amino acid compositions were detected by using the HPLC method (Köse *et al.*, 2011). The dried samples were hydrolyzed at 110°C for 24 hours with 6.0 M hydrochloric acid. Hydrolysates were filtered through a 0.20 µm PTFE syringe filter and then all the hydrochloric acid was evaporated. The levels of amino

acids were measured using EZ: fast kits (EZ: fast GC/FID Protein Hydrolysate Amino Acid Kit) by gas chromatography.

To detect the lipid amount, at least 100 mg of dry biomass was used (Bligh & Dyer (1959)). Profiles of FAMEs were analyzed with the method of Metcalf *et al.* (1966) through gas chromatography (Agilent 5975 C, Agilent 7890 A GC) with column DB WAX (50*0.20 mm, 0.20 μm).

2.4. Statistical analysis

All experiments were conducted with three replicates (n=3). The values were reported as the mean ± standard deviation (SD) of three replicates. Data were analyzed with ANOVA (one-way analysis of variance) in Minitab Statistical Software 2016 2 (Microsoft, USA). A difference at a significant level is at $p < 0.001$.

3. RESULTS

3.1. Combined Effect of Nitrogen and Phosphorus on Culture Growth of *T. obliquus*

For determining the culture growth of *T. obliquus*, cell number (CN), optical density (OD) and dry weight (DW) values were detected every other day under stable cultivation. A robust linear correlation was obtained between CN and OD measurements and OD and DW (Table 1).

Table 1. R^2 correlation values between CN-OD and OD-DW.

Media	CN-OD	OD-DW
BG 11 (Control Medium)	0.949	0.972
%50 N+ %50P+ (Medium I)	0.940	0.966
%50 N-%50P- (Medium II)	0.949	0.932

In the control medium (BG11) the highest CN ($2.4x10^7$ cell/mL); OD (1.05) and DW (0.041 mg/L) values are detected on the 24th, 26th, 28th days of culture, respectively (Figure 2, Figure 3, Figure4). At the same culture days; Medium II has the lowest CN ($0.8x10^7$ cell/mL); OD (0.73) and DW (0.029 mg/L) (Figure 2, Figure 3, Figure 4).

Figure 2. Cell numbers (CN) of *T. obliquus* in 3 media. The SD of three replicates for each media were shown with error bars ($n = 3$).

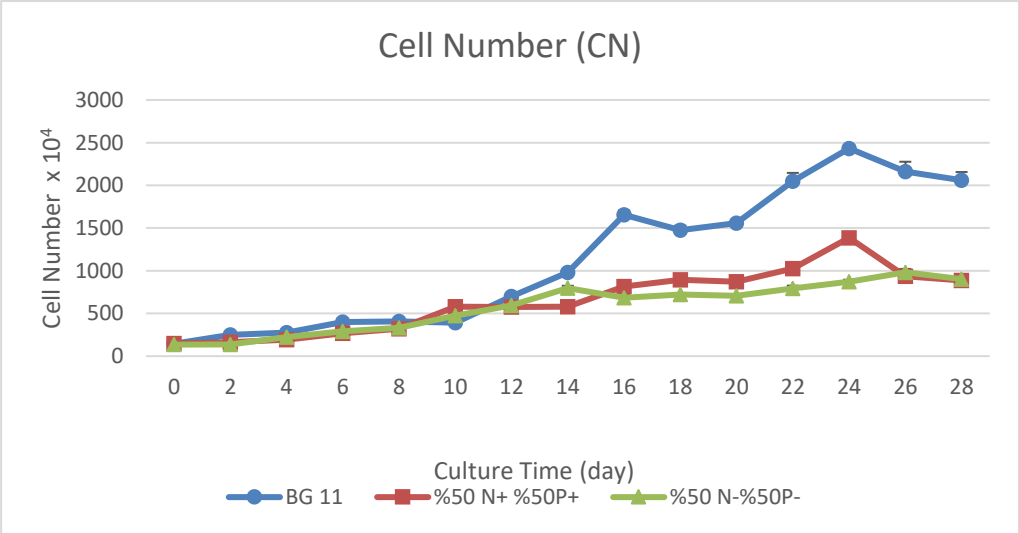


Figure 3. Optical Density (OD) of *T. obliquus* in 3 media. The SD of three replicates for each media were shown with error bars (n = 3).

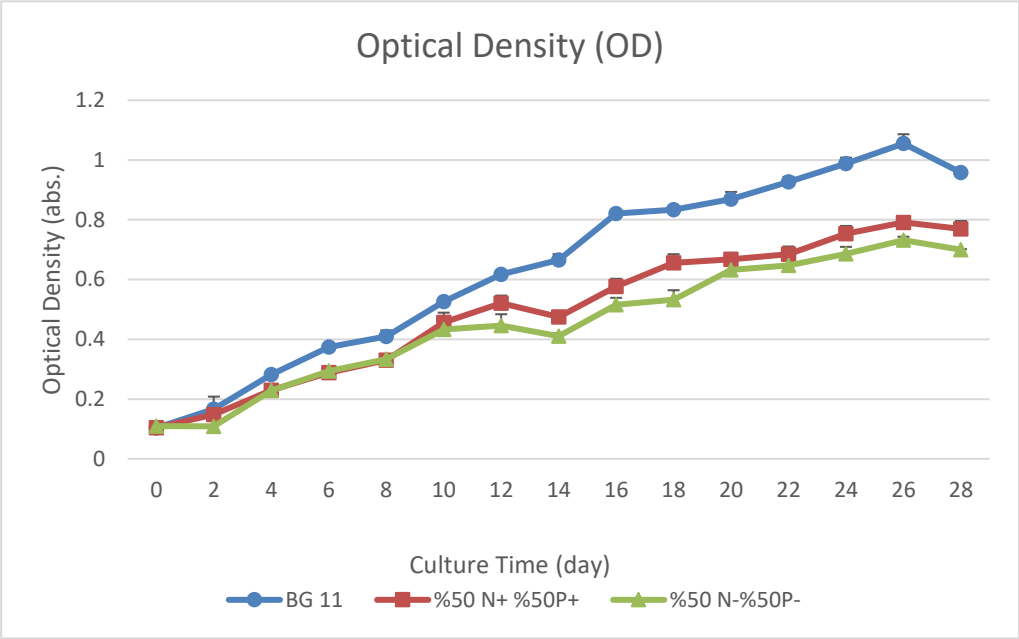
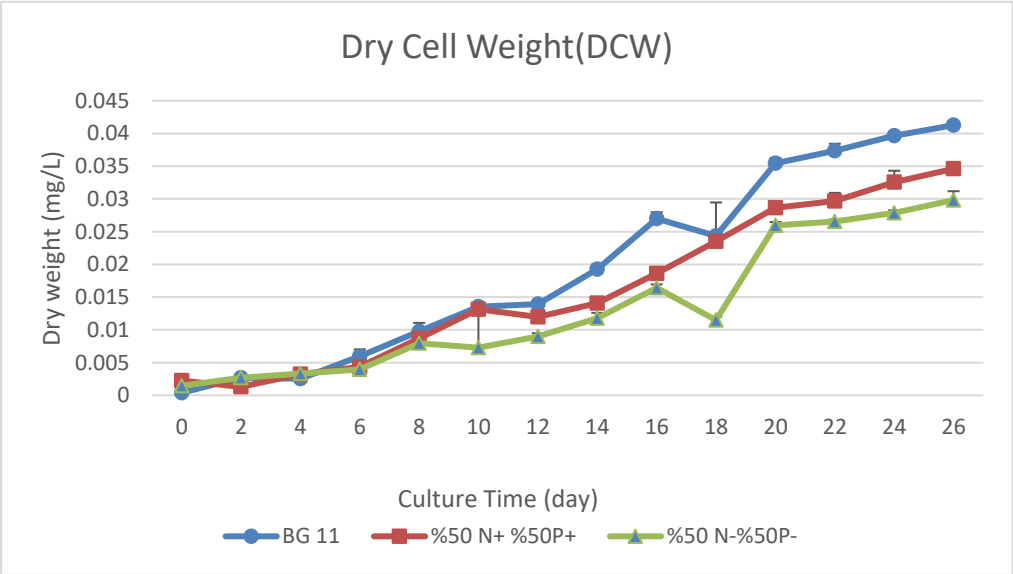


Figure 4. Dry cell weight (DCW) of *T. obliquus* in 3 media. The SD of three replicates for each media were shown with error bars (n = 3).



If Medium I and Medium II are compared between themselves; it is shown that Medium I has higher values than Medium II in all parameters. Same results can be seen in Table 2 which shows the Specific Growth Rate (SGR), Doubling Time (DT) and Biomass Productivity ($P_{Biomass}$) values. The highest SGR and $P_{Biomass}$ values are in the Control medium, Medium I and Medium II, respectively. Of course, there was an inverse order in the DT values, as expected.

Table 2. Culture growth parameters of *T. obliquus* in different media.

Media	SGR (μ)	Doubling Time (DT)	Biomass Productivity ($P_{Biomass}$)
BG 11 (Control Medium)	0.214±0.013	3.246±0.012	0.0008±0.008
%50 N+ %50P+(Medium I)	0.197±0.011	3.509±0.017	0.0007±0.005
%50 N-%50P-(Medium II)	0.183±0.010	3.781±0.015	0.0006±0.004

When all the results are evaluated together, it is seen that N or P deficiencies cause a decrease in culture growth. Many studies have stated that depletion of N in culture medium causes a decrease in growth (Vooren et al., 2012; Zhu et al., 2014; Ji et al., 2011). Anand & Arumugam researched the effects of N concentrations on the growth of *Scenedesmus quadricauda* and they declared that N enrichment causes high growth and N deficiency causes the opposite. So, it can be detected previously, N concentration of culture media is the principal factor in culture growth (Zhu et al., 2014; Breuer et al., 2012; Delgado et al., 2020; Procházková et al., 2013).

Also, P is another essential nutrient for microalgae growth, it can be used for the the synthesizing of cellular components such as components of the cell membrane, DNA, RNA and ATP (Atiku et al., 2016). The papers aim to research the effects of P concentrations on algal growth show that P deficiency causes a decrease in algal growth (Xin et al., 2010; Procházková et al., 2013; Lin et al., 2016).

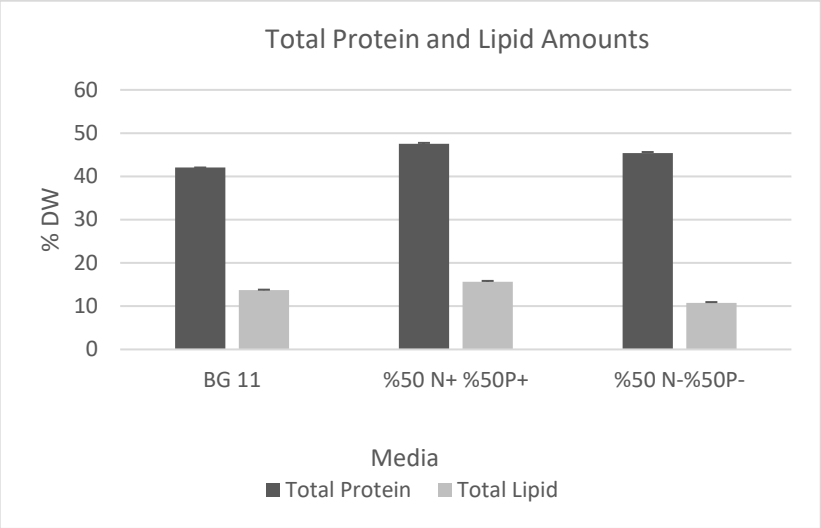
In addition, investigating the effects of N and P on culture growth separately, there are many studies investigating the combined effects of N and P (Singh et al., 2015; Zhuang et al., 2018). Huang et al., (2021) stated that the N-P coupling effect was significant on algal nutrient uptake, dry mass and pigments. Bongiovani et al. (2020) indicated that in their study about the effects of Nitrate or Phosphate Deprivation on *Nannochloropsis oceanica.*

3.2. Combined Effects of Nitrogen and Phosphorus on Protein-Aminoacids and Lipid-FAMEs Profiles of *T. obliquus*

N is the most important macronutrient for microalgal growth and an essential building molecule of proteins, carbohydrates and lipids (Yodsuwan et al., 2017; Zarrinmehr et al., 2019). N concentration affects the culture growth parameters as well as the biochemical composition of microalgal biomass (Vooren et al., 2012). Beside this, the biochemical composition of microalgae depends on the nutrient usability, type and concentrations of nutrients (Gao et al.,2018; Rani et al., 2020)

The biomass obtained from Medium I has the highest protein content (47.53 %DW) followed by Medium II (45.42 %DW), Control medium (42.03 %DW), respectively (Fig. 5). These results show the N and P are very important for synthesizing protein. So high proteins content was obtained in the medium that has high N and P concentrations. On the other hand, Medium II has higher protein content than Control Medium because of low growth rate of Medium II. Studies have shown that N deficiency generally pauses protein synthesis and shifts photosynthesis reactions towards lipid synthesis (Chu et al., 2014; Ho et al., 2014). However, our results contradict this. But these effects are very different than the mutual effect of N and P (Ji et al., 2013; Cao et al., 2014). So, the recent studies have focused on mutual effects of nutrients like ours.

Figure 5. Total protein and lipid amount of *T. obliquus* biomass harvested from 3 different media (p<0.001).



Total amino acid contents do not change according the modification of media, but amino acid composition and amount of amino acid types change (Table 3). In the Medium I the most abundant amino acid is Arginine (40.65 %DW) and least abundant amino acid is Phenylalanine (0.18 %DW). Whereas the control medium has different concentrations of Arginine (15.09 %DW) and Phenyl alanine (8.41 %DW). On the other hand, the highest amount of amino acid is Leucine (25.63 %DW) and lowest amount of amino acid is Lysine (0.1 %DW) in the Medium II. It can be understanding due to these results that N and P concentrations change the compositions and amounts of amino acids strongly. Total aa amounts of *T. obliquus* are the highest in control medium and lowest in Medium II. These results are not surprised because of N starvation causes to reduce the amino acid contents in the microalgae cells (Salbitani et al., 2020).

Table 3. Amino acids composition and amounts (mgg^{-1} DW) of *T. obliquus* biomass harvested from 3 different media.

	BG 11 (Control Medium)	%50 N+ %50P+ (Medium I)	%50 N-%50P- (Medium II)
Arginine	15.09±0.11	40.65±0.11	6.859±0.10
Serine	2.048±0.09	0.759±0.05	1.367±0.09
Glycine	1.057±0.08	1.435±0.06	0.537±0.07
Alanine	1.082±0.04	0.454±0.04	0.724±0.04
Proline	2.435±0.09	0.638±0.09	1.315±0.03
Valine	8.982±0.08	2.159±0.10	1.534±0.05
Threonine	2.209±0.07	0.997±0.09	4.128±0.01
Methionine	4.214±0.05	0.975±0.04	1.893±0.06
Isoleucine	1.646±0.06	0.788±0.03	1.551±0.04
Leucine	2.611±0.08	0.628±0.04	25.63±0.02
Phenylalanine	8.415±0.12	0.18±0.05	3.795±0.03
Tyrosine	3.301±0.11	0.198±0.07	4.909±0.10
Aspartic acid	3.61±0.13	4.331±0.08	0.725±0.11
Glutamic acid	3.799±0.14	1.929±0.09	0.674±0.12
Histidine	1.009±0.06	1.785±0.10	0.148±0.13
Lysine	1.005±0.06	4.24±0.02	0.162±0.11

Data were expressed as mean ± *SD*. n=3. p<0.001.

Also, some researchers stated that the types of N sources are affecting the assimilation of N by microalgae cells and synthesizing of amino acids (Imamura et al., 2010). Since this situation directly affects the synthesis of photosynthetic pigments and enzymes, changes occur in the metabolic pathway (Vona et.al.,1999; Mark et al., 2007; Hockin et al., 2012).

Our lipid analysis results (Fig. 5) show that Medium I (oversupplied both N and P) have the highest lipid content (15.6 %DW), Control medium (13.71 %DW), Medium II (10.72 %DW); respectively. Synthesizing the lipids and depositing the FAME are regarding to supplying of both N and P, directly. Many studies declared that when the culture medium has the sufficient N amount, carbohydrate accumulation shifts to depositing of lipid and protein because of specific metabolic pathway of microalgae (Li et al., 2019; Ross et al., 2018).

In N deprived conditions, high lipid accumulations were reported in *Scenedesmus* species (Radakovits et al., 2010). Besides, these same inferences were declared for *Neochloris oleobundans* and *Nannochloropsis* sp. (Courchesne et al., 2009; Gao et al., 2013). However, our results conflict to these papers since The Medium II (N and P depletion) had the lowest lipid content. The effects of N or P's deficiency separately are decreasing the culture's growth and increasing the lipid accumulation, however we researched the combined effects of N and P, so our results may be different from the general trend. The impact of our study can be understandable with this output.

According to our FAMEs results (Table 4) the highest Palmitic acid (C16:0) concentration (21.11 %FAME) is detected in Medium II and the highest total SFAs concentration (24.22 %FAME) in same medium. In every case, many studies declared that the N starvation causes producing higher SFAs. (Zhu et al., 2014; Yodsuwan et al., 2017; Breuer et al., 2012; Anand et al., 2015; Singh et al., 2015; Cointet at al., 2019).

Table 4. FAMEs percentages (% of total fatty acids) of *T. obliquus* biomass harvested from 3 different media.

Name of Fatty Acids	BG 11 (Control Medium)	%50 N+ %50P+ (Medium I)	%50 N-%50P- (Medium II)
Saturated fatty acids (SFAs)			
C14:0 (myristic acid)	2.208±0.02	2.349±0.01	2.097±0.05
C16:0 (palmitic acid)	19.342±0.01	19.211±0.01	21.111±0.01
C18:0 (stearic acid)	1.073±0.02	1.142±0.01	1.019±0.01
Total SFAs	22.623±0.03	22.702±0.01	24.227±0.01
Monounsaturated fatty acids (MUFAs)			
C16:1 (9-hexenoic acid)	2.156±0.05	2.294±0.04	2.048±0.05
C18:1 (oleic acid)	12.513±0.03	13.316±0.02	12.879±0.01
C18:1 t10, t11e 12	4.498±0.04	4.786±0.01	4.273±0.01
Total MUFAs	19.167±0.05	20.396±0.01	19.200±0.03
Polyunsaturated fatty acids (PUFAs)			
C18:2 (linoleic acid, c9 c12)	12.348±0.04	11.589±0.02	11.732±0.01
C18:3 (linolenic acid, c9, c12, c15)	36.291±0.03	36.217 ±0.01	35.582±0.02
Total PUFAs	48.639±0.02	47.806±0.04	47.314±0.04
Others	7.789±0.04	8.291±0.04	7.401±0.03

Data were expressed as mean ± SD. n=3; p<0.001.

Results of the total MUFAs values show that the highest MUFAs concentration (20.39; 19.20; 19.16 %FAME) were in the Medium I, Medium II and Control medium respectively.

Microalgae can deposit high levels of SFAs and MUFAs with a low growth rate (Ji et al., 2011). Our results were compatible with SFAs but not with MUFAs.

Control medium has higher PUFAs concentrations (48.63 %FAME) than Medium I (47.80 %FAME) and Medium II (47.31 %FAME). When comparing of the growth rate and PUFAs concentrations, the results were similar to study of Pribly et al., (2014) which declared the high growth rate causing high PUFAs concentration.

4. CONCLUSION

The aim of this study is to give an idea of the combined effects of N and P in microalgae culture growth parameters and protein-amino acid; lipid-FAMEs profiles of *T obliquus*. Microalgae has high growth rates in different culture conditions and their biomasses might be an alternative source for bioproducts because of their secondary metabolites contents in the cells. Also, microalgae can be used as an alternative sustainable green energy source due to their high lipid content. In the same time, the culture media with high amount of N and P accelerate the culture growth and nutrient deficient cultures are more suitable for lipid and fatty acids production. However, physical factors such as light quantity and intensity, temperature, pH should be evaluated together with nutrient concentrations. This phenomenon is known well but the reactions of different microalgae with same conditions may change. The species variety is as important as cultural conditions. Therefore, the number of studies conducted for this purpose should be increased and studies with different species should be brought to the literature.

For that purpose, *T. obliquus,* on which the combined effects of N and P had not been investigated before, was chosen as the study material. All data which obtained from this study and the studies should be used in the future to commercialize algae for better usage in biotechnological sustainable applications.

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Declaration of Conflicting Interests and Ethics

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

Authorship contribution statement

Fusun Akgul: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Riza Akgul:** Investigation, Methodology, Supervision, and Validation.

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REFERENCES

Amaro, H.M., Guedes, A.C., & Malcata, F.X. (2011). Advances and perspectives in using microalgae to produce biodiesel. *Applied Energy*, *88*(10), 3402-3410. https://doi.org/10.1016/J.APENERGY.2010.12.014

Anand, J., & Arumugam, M. (2015). Enhanced lipid accumulation and biomass yield of *Scenedesmus quadricauda* under nitrogen starved condition. *Bioresource Technology*, *188*, 190–194. https://doi.org/10.1016/J.BIORTECH.2014.12.097

Atiku, H., Mohamed, R., Al-Gheethi, A., Wurochekke, A., & Kassim, A.H.M. (2016). Harvesting of microalgae biomass from the phytoremediation process of greywater.

Environmental Science *and Pollution Research*, *23*(24), 24624-24641. https://doi.org/10.1007/S11356-016-7456-9

Beuckels, A., Smolders, E., & Muylaert, K. (2015). Nitrogen availability influences phosphorus removal in microalgae-based wastewater treatment. *Water Research*, *77*, 98–106. https://doi.org/10.1016/J.WATRES.2015.03.018

Bongiovani, N., Popovich, C.A., Martínez, A.M., Constenla, D., & Leonardi, P.I. (2020). Biorefinery Approach from Nannochloropsis oceanica CCALA 978: Neutral Lipid and Carotenoid Co-Production Under Nitrate or Phosphate Deprivation. *Bioenergy Research*, *13*(2), 518–529. https://doi.org/10.1007/S12155-019-10045-2/TABLES/2

Boussiba, S., Fan, L., & Vonshak, A. (1992). Enhancement and determination of astaxanthin accumulation in green alga *Haematococcus pluvialis*. *Methods in Enzymology*, *213*(C), 386–391. https://doi.org/10.1016/0076-6879(92)13140-S

Breuer, G., Lamers, P.P., Martens, D.E., Draaisma, R.B., & Wijffels, R.H. (2012). The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology*, *124*, 217 226. https://doi.org/10.1016/J.BIORTECH.2012.08.003

Bligh, E.G., & Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911-917.

Cao, J., Yuan, H.L., Li, B.Z., & Yang, J.S. (2014). Significance evaluation of the effects of environmental factors on the lipid accumulation of *Chlorella minutissima* UTEX 2341 under low-nutrition heterotrophic condition. *Bioresource Technology*, *152*, 177–184. https://doi.org/10.1016/J.BIORTECH.2013.10.084

Chia, M.A., Lombardi, A.T., & Melão, M. da G.G. (2013). Growth and biochemical composition of *Chlorella vulgaris* in different growth media. *Anais Da Academia Brasileira de Ciencias*, *85*(4), 1427–1438. https://doi.org/10.1590/0001-3765201393312

Chu, F.F., Chu, P.N., Shen, X.F., Lam, P.K.S., & Zeng, R.J. (2014). Effect of phosphorus on biodiesel production from *Scenedesmus obliquus* under nitrogen-deficiency stress. *Bioresource Technology*. *152*. 241–246. https://doi.org/10.1016/J.BIORTECH.2013.11.013

Cointet, E., Wielgosz-Collin, G., Bougaran, G., Rabesaotra, V., Gonçalves, O., & Méléder, V. (2019). Effects of light and nitrogen availability on photosynthetic efficiency and fatty acid content of three original benthic diatom strains. *Plos One*, *14*(11), e0224701. https://doi.org/10.1371/JOURNAL.PONE.0224701

Courchesne, N.M.D., Parisien, A., Wang, B., & Lan, C.Q. (2009). Enhancement of lipid production using biochemical. genetic and transcription factor engineering approaches. *Journal of Biotechnology*, *141*(12), 31 41. https://doi.org/10.1016/J.JBIOTEC.2009.02.018

FAO. 2017. *The future of food and agriculture – Trends and challenges*. Rome.

Fernandes, B., Teixeira, J., Dragone, G., Vicente, A. A., Kawano. S., Bišová. K., Přibyl. P., Zachleder. V., & Vítová. M. (2013). Relationship between starch and lipid accumulation induced by nutrient depletion and replenishment in the microalga *Parachlorella kessleri*. *Bioresource Technology*, *144*, 268–274. https://doi.org/10.1016/J.BIORTECH.2013.06.096

Gao, B., Liu, J., Zhang, C., & Van de Waal, D.B. (2018). Biological stoichiometry of oleaginous microalgal lipid synthesis: The role of N:P supply ratios and growth rate on microalgal elemental and biochemical composition. *Algal Research*, *32*, 353-361. https://doi.org/10.1016/J.ALGAL.2018.04.019

Gao, Y., Yang, M., & Wang, C. (2013). Nutrient deprivation enhances lipid content in marine microalgae. *Bioresource Technology*, *147*, 484-491. https://doi.org/10.1016/J.BIORTECH.2013.08.066

Godoy-Hernández, G., & Vázquez-Flota, F.A. (2006). Growth Measurements. *Methods in Molecular Biology*, *318*, 51–58. https://doi.org/10.1385/1-59259-959-1:051

Hempel, N., Petrick, I., & Behrendt, F. (2012). Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. *Journal of Applied Phycology*, *24*(6), 1407-1418. https://doi.org/10.1007/s10811-012-9795-3

Ho, S.H., Chan, M.C., Liu, C.C., Chen, C.Y., Lee, W.L., Lee, D.J., & Chang, J.S. (2014). Enhancing lutein productivity of an indigenous microalga *Scenedesmus obliquus* FSP-3 using light-related strategies. *Bioresource Technology*, *152*, 275-282. https://doi.org/10.1016/J.BIORTECH.2013.11.031

Hockin, N.L., Mock, T., Mulholland, F., Kopriva, S., & Malin, G. (2012). The response of diatom central carbon metabolism to nitrogen starvation is different from that of green algae and higher plants. *Plant Physiology*, *158*(1), 299-312. https://doi.org/10.1104/PP.111.184333

Huang, Y., Lou, C., Luo, L., & Wang, X.C. (2021). Insight into nitrogen and phosphorus coupling effects on mixotrophic *Chlorella vulgaris* growth under stably controlled nutrient conditions. *Science of the Total Environment*, *752*, 141747. https://doi.org/10.1016/j.scitotenv.2020.141747

Imamura, S., Terashita, M., Ohnuma, M., Maruyama, S., Minoda, A., Weber, A.P.M., Inouye. T., Sekine. Y., Fujita. Y., Omata. T., & Tanaka. K. (2010). Nitrate assimilatory genes and their transcriptional regulation in a unicellular red alga cyanidioschyzon merolae: genetic evidence for nitrite reduction by a sulfite reductase-like enzyme. *Plant and Cell Physiology*, *51*(5), 707–717. https://doi.org/10.1093/PCP/PCQ043

Ji, C.F., Yu, X.J., Chen, Z.A., Xue, S., Legrand, J., & Zhang, W. (2011). Effects of nutrient deprivation on biochemical compositions and photo-hydrogen production of *Tetraselmis subcordiformis*. *International Journal of Hydrogen Energy*, *36*(10), 5817–5821. https://doi.org/10.1016/J.IJHYDENE.2010.12.138

Ji, F., Hao, R., Liu, Y., Li, G., Zhou, Y., & Dong, R. (2013). Isolation of a novel microalgae strain *Desmodesmus* sp. and optimization of environmental factors for its biomass production. *Bioresource Technology*, *148*, 249–254. https://doi.org/10.1016/J.BIORTECH.2013.08.110

Köse, S., Kaklikkaya, N., Koral, S., Tufan, B., Buruk, K.C., & Aydin, F. (2011). Commercial test kits and the determination of histamine in traditional (ethnic) fish products-evaluation against an EU accepted HPLC method. *Food Chemistry*, *125*(4), 1490–1497. https://doi.org/10.1016/J.FOODCHEM.2010.10.069

Li, K., Liu, Q., Fang, F., Luo, R., Lu, Q., Zhou, W., Huo, S., Cheng, P., Liu, J., Addy, M., Chen. P., Chen, D., & Ruan, R. (2019). Microalgae-based wastewater treatment for nutrients recovery: A review. *Bioresource Technology*, *291*, 121934. https://doi.org/10.1016/J.BIORTECH.2019.121934

Lin, H., Shavezipur, M., Yousef, A., & Maleky, F. (2016). Prediction of growth of *Pseudomonas fluorescen*s in milk during storage under fluctuating temperature. *Journal of Dairy Science*, *99*(3), 1822–1830. https://doi.org/10.3168/JDS.2015-10179

López, C.V.G., del Carmen Cerón García, M., Fernández, F.G.A., Bustos, C.S., Chisti, Y., & Sevilla, J.M.F. (2010). Protein measurements of microalgal and cyanobacterial biomass. *Bioresource Technology*, *101*(19), 7587-7591. https://doi.org/10.1016/J.BIORTECH.2010.04.077

Maizatul, A.Y., Radin Mohamed, R.M.S., Al-Gheethi, A.A., & Hashim, M.K.A. (2017). An overview of the utilisation of microalgae biomass derived from nutrient recycling of wet market wastewater and slaughterhouse wastewater. *International Aquatic Research*, *9*(3), 177–193. https://doi.org/10.1007/S40071-017-0168-Z/TABLES/8

Merzlyak, M.N., Chivkunova, O.B., Gorelova, O.A., Reshetnikova, I.V., Solovchenko, A.E., Khozin-Goldberg. I., & Cohen. Z. (2007). Effect of nitrogen starvation on optical properties.

pigments. and arachidonic acid content of the unicellular green alga *Parietochloris incisa* (trebouxiophyceae. Chlorophyta)1. *Journal of Phycology*, *43*(4), 833 –843. https://doi.org/10.1111/J.1529-8817.2007.00375.X

Metcalfe, L.D., Schmitz, A.A., & Pelka. J.R. (1966). Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Analytical chemistry*, *38*(3), 514-515

Přibyl, P., Cepák. V., & Zachleder, V. (2014). Oil Overproduction by Means of Microalgae. *Algal Biorefineries*, *1*, 241–273. https://doi.org/10.1007/978-94-007-7494-0_10

Procházková, G., Brányiková, I., Zachleder, V., & Brányik, T. (2014). Effect of nutrient supply status on biomass composition of eukaryotic green microalgae. *Journal of Applied Phycology*, *26*(3), 1359–1377. https://doi.org/10.1007/S10811-013-0154-9/TABLES/2

Radakovits, R., Jinkerson, R.E., Darzins, A., & Posewitz, M.C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell*, *9*(4), 486–501. https://doi.org/10.1128/EC.00364-09/ASSET/CFE6817C-EED9-4151-8ABD-4A098B6BE4CF/ASSETS/GRAPHIC/ZEK9990935090003.JPEG

Rani, S., Chowdhury, R., Tao, W., & Srinivasan, A. (2020). Tertiary treatment of municipal wastewater using isolated algal strains: treatment efficiency and value-added products recovery. *Chemistry and Ecology*, *36*(1), 48-65. https://doi.org/10.1080/02757540.2019.1688307/SUPPL_FILE/GCHE_A_1688307_SM0210.DOCX

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., & Stanier, R.Y. (1979). Generic Assignments. Strain Histories and Properties of Pure Cultures of Cyanobacteria. *Microbiology*, *111*(1), 1–61. https://doi.org/10.1099/00221287-111-1-1

Ross, M.E., Davis, K., McColl, R., Stanley, M.S., Day, J.G., & Semião, A.J.C. (2018). Nitrogen uptake by the macro-algae *Cladophora coelothrix* and *Cladophora parriaudii*: Influence on growth. nitrogen preference and biochemical composition. *Algal Research*, *30*, 1–10. https://doi.org/10.1016/J.ALGAL.2017.12.005

Salbitani, G., & Carfagna, S. (2020). Different Behaviour between Autotrophic and Heterotrophic *Galdieria sulphuraria* (Rhodophyta) Cells to Nitrogen Starvation and Restoration. Impact on Pigment and Free Amino Acid Contents. *International Journal of Plant Biology*, *7*, *11*(1), 8567. https://doi.org/10.4081/PB.2020.8567

Santos-Ballardo, D.U., Rossi, S., Hernández, V., Gómez, R.V., del Carmen Rendón-Unceta. M., Caro-Corrales. J., & Valdez-Ortiz. A. (2015). A simple spectrophotometric method for biomass measurement of important microalgae species in aquaculture. *Aquaculture*, *448*, 87–92. https://doi.org/10.1016/J.AQUACULTURE.2015.05.044

Singh, P., Guldhe, A., Kumari, S., Rawat, I., & Bux, F. (2015). Investigation of combined effect of nitrogen. phosphorus and iron on lipid productivity of microalgae Ankistrodesmus falcatus KJ671624 using response surface methodology. *Biochemical Engineering Journal*, *94*, 22–29. https://doi.org/10.1016/J.BEJ.2014.10.019

Sirakov, I., Velichkova, K., Stoyanova, S., & Staykov, Y. (2015). The importance of microalgae for aquaculture industry. Rewiev. *International Journal of Fisheries and Aquatic Studies*, *2(4)*, 31-37.

Tarazona Delgado, R., Guarieiro, M., dos, S., Antunes, P.W., Cassini, S.T., Terreros, H.M., & Fernandes, V. de O. (2021). Effect of nitrogen limitation on growth. biochemical composition. and cell ultrastructure of the microalga *Picocystis salinarum*. *Journal of Applied Phycology*, *33*(4), 2083–2092. https://doi.org/10.1007/S10811-021-02462-8

Van Vooren, G., Le Grand, F., Legrand, J., Cuiné, S., Peltier, G., & Pruvost, J. (2012). Investigation of fatty acids accumulation in *Nannochloropsis oculata* for biodiesel application. *Bioresource Technology*, *124*, 421–432. https://doi.org/10.1016/J.BIORTECH.2012.08.009

Vona, V., Di Martino Rigano, V., Esposito, S., Carillo, P., Carfagna, S., & Rigano, C. (1999). Growth. photosynthesis. and respiration of *Chlorella sorokiniana* after N-starvation.

Interactions between light. CO2 and NH4+ supply. *Physiologia Plantarum*, *105*(2), 288–293. https://doi.org/10.1034/J.1399-3054.1999.105214.X

Wynne, M.J., & Hallan, J.K. (2015). Reinstatement of *Tetradesmus* G.M. Smith (Sphaeropleales. Chlorophyta). *Feddes Repertorium*, *126*(3-4), 83-86. https://doi.org/10.1002/FEDR.201500021

Xin, L., Hong-ying, H., Ke. G., & Ying-xue, S. (2010). Effects of different nitrogen and phosphorus concentrations on the growth. nutrient uptake. and lipid accumulation of a freshwater microalga *Scenedesmus* sp. *Bioresource Technology*, *101*(14), 5494–5500. https://doi.org/10.1016/J.BIORTECH.2010.02.016

Yaakob, M.A., Mohamed, R.M.S.R., Al-Gheethi, A., Ravishankar, G.A., & Ambati, R.R. (2021). Influence of nitrogen and phosphorus on microalgal growth. biomass. lipid. and fatty acid production: An Overview. *Cells*, *10*(2), 393.https://doi.org/10.3390/CELLS10020393

Yodsuwan, N., Sawayama, S., & Sirisansaneeyakul, S. (2017). Effect of nitrogen concentration on growth. lipid production and fatty acid profiles of the marine diatom Phaeodactylum tricornutum. *Agriculture and Natural Resources*, *51*(3), 190-197. https://doi.org/10.1016/J.ANRES.2017.02.004

Zarrinmehr, M.J., Farhadian, O., Heyrati, F.P., Keramat, J., Koutra, E., Kornaros, M., & Daneshvar, E. (2020). Effect of nitrogen concentration on the growth rate and biochemical composition of the microalga. Isochrysis galbana. *Egyptian Journal of Aquatic Research*, *46*(2), 153–158. https://doi.org/10.1016/j.ejar.2019.11.003

Zhu, S., Huang, W., Xu, J., Wang, Z., Xu, J., & Yuan, Z. (2014). Metabolic changes of starch and lipid triggered by nitrogen starvation in the microalga Chlorella zofingiensis. *Bioresource Technology*, *152*, 292–298. https://doi.org/10.1016/J.BIORTECH.2013.10.092

Zhuang, L.L., Azimi, Y., Yu, D., Wu, Y.H., & Hu, H.Y. (2018). Effects of nitrogen and phosphorus concentrations on the growth of microalgae Scenedesmus. LX1 in suspended-solid phase photobioreactors (ssPBR). *Biomass and Bioenergy*, *109*, 47–53. https://doi.org/10.1016/j.biombioe.2017.12.017

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Research Article

The phenolic profile and biological activities of common *Scorzonera* species from Eastern Anatolia

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Abstract: The present study focused on chemical composition and base therapeutic potential of common three *Scorzonera* species (*Scorzonera mollis* M.Bieb. subsp. *mollis*, *Scorzonera papposa* DC. and *Scorzonera semicana* DC), which have been utilized as food and medicine by local people of Eastern Anatolia for a long time. Comparative analytical studies were performed on ethanol-based extracts and traditional preparations (infusions) through chromatographic (HPLC-MS/MS) and reagent-based antioxidant and enzyme inhibitory assays. Results revealed that leaf extracts were rich in phenolics, particularly hydroxycinnamic acids that were confirmed by HPLC-MS/MS, chlorogenic acids and luteolin were the major phytochemical compounds of extracts. With regards to biological activities findings, it was determined that ethanol-based extracts showed better antioxidant activities and effectively suppressed the activities of α-glucosidase. In addition, both of the extracts were found as strong suppressive agents of pancreatic lipase activity so *Scorzonera* species were rich sources of bioactive compounds that able to deactivate reactive oxygen species and free radicals and as well as suppress the activities of α-glucosidase and pancreatic lipase. Finally, obtained findings reveal base data of *Scorzonera* species for researches that focused on novel candidates of nutraceuticals and biotherapeutics.

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1. INTRODUCTION

Food and medicinal plant sources have been examined by mankind through the process of trial and error for a long time. It was documented that 5000 years ago Sumerian clay slab described twelve recipes from nearly two hundred fifty plants for food and drug preparation, which are still in use today (Petrosvka, 2012). Recently, the trend of natural alternatives usage as a source of new commercial products has become very popular. Although, the plant use in medicine is

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frequently undervalued, the active components of the plants, which allow them to perform the healing process, should be valued more (Reid *et al.*,, 2018).

Several researchers in Turkey have thoroughly investigated the use of wild medicinal plants over the last decade. Because of its varied weather and environmental zones, the eastern part of Anatolia has a plethora of the flora. This variety supplies an abounding source of medicinal plants (Özgökçe & Özçelik 2004; Ergün 2021). Medicinal plants are acknowledged to have antioxidant activities along with multiple bioactivities; for instance, antimicrobial, antiinflammatory, and antiallergic activities (Gülçin *et al.*, 2010).

Asteraceae is one of major flowering plants families globally, which chemically contained significant biologically active compounds (Hattori & Nakajima 2008; Funk *et al.*, 2009). *Scorzonera* genus is belong to Asteracea family among the most utilized species in food and medicinal plant culture in Turkey and there is a need to investigate their biologically active chemical compounds bioactivities to contribute the scientific literature of plant-based pharmaceuticals .

The main purposes of the present study were to (i) detect the biotherapeutic potential and (ii) chemical composition of leaf organs of three *Scorzonera* taxa (*Scorzonera mollis* M. Bieb. subsp. *mollis*, *Scorzonera papposa* DC. and *Scorzonera semicana* DC. Therefore, ethanol-based extracts and water-based infusion preparations obtained from plant leaf matrix were assayed for antioxidant and enzyme inhibitory activities. Major contributors of the activities were analysed by reagent-based spectrophotometric assays and HPLC-MS/MS.

2. MATERIAL and METHODS

2.1. Plant Materials

Leaf samples of *Scorzonera* species (*S. mollis*, *S. semicana* and *S. papposa*) were collected from the Van/Türkiye (Table 1). Plant samples were transferred to the laboratory at the appointed time (within 2 hr). The identification of the plant samples was done at Van Yuzuncu Yil University, Faculty of Pharmacy. Voucher specimens were placed at Van Pharmaceutical Herbarium. The plant materials were cleaned properly for dust and contaminants and air-dried in the dark at room temperature (RT) (22±2 °C) for 96 hr. Subsequently, they were ground into a fine powder using a grinding mill.

Table 1. Location and herbarium details of *Scorzonera* species.

Species	Location/GPS/Date	Herbarium Code
Scorzonera mollis	Van/Türkiye, Çatak, Konalga village, steppe/ 37° 50' 255"N 043° 09' 857"E; 2258 m/ 08.05. 2019,	Herbarium code: VPH553; Collector code: DM350.
Scorzonera semicana	Van/Türkiye, Çatak, Konalga village, steppe/ 37º50'191"N 043º08' 185"E, 2290m/ 09.05.2019	Herbarium code: VPH355; Collector code: DM301
Scorzonera papposa	Van/Türkiye, Çatak, Konalga village, steppe/ 37º 51' 555" N, 43º 08' 984" E, 1808m/ 12.05. 2019	Herbarium code: VPH356; Collector code: DM302

For extraction of plant samples two different extraction methods (aqueous extraction and herbal infusion) were preferred. The plant samples were prepared for in accordance to Dalar *et al.*, 2012 with slight adjustments. In brief, 1 g of pulverized plant materials were thoroughly mixed with 10 mL of ethanol (80%) then set approximately 5 minutes for shaking at RT. Subsequently, the solutions were sonicated at 37 °C for 40 minutes then incubated for 24 hr at 4 °C. Extracts were centrifuged at 15,300 x g for 20 minutes at 4 °C, aliquots of the supernatants were collected kept at -20 °C to be subsequently analyzed.

Herbal infusion extracts preparation was done according to Baytop 1999, from the pulverized plants. In brief, plant material was mixed with a 20-fold volume (g/mL) of pre-boiled

distilled water then left for incubation for 10 min followed by sonication for 10 minutes at 50 °C for homogenizing. The solutions were left for overnight incubation at 4 °C, Next the mixtures were filtered by cotton and vacuum filtering. Supernatants were stored (-20 °C) until analyses.

2.3. Determination of Antioxidant Capacities

In ABTS capacity the extracts were evaluated for their antioxidant activities by the decolorization of ABTS radical (Re *et al.*, 1999). The resulting color changes were measured using spectrophotometer and the results were expressed as percent inhibition of absorbance at 734 nm compared with the ascorbic acid regression curve.

Determination of total reducing capacity of the extracts were done using the FRAP assay (Benzie & Strain, 1996) with minor modifications as described by Dalar *et al.*, (2012) and the reducing capacity of the extracts were expressed as mmol of iron (Fe^{2+})/g dw according to iron sulphate standard (Fe_2SO_4) curve against a blank control. The analyses were performed in triplicate. Folin-Ciocalteu reducing (FCR) assay was performed as described formerly by Ainsworth and Gillespie (2007), with minor modifications as described by Dalar *et al.*, (2012). Shortly, plant extracts already prepared in the selected solvent for extraction were mixed with Folin-Ciocalteu reagent which had been previously diluted with MilliQ water in 96-multiwell microplates. The absorbance was measured at 600 nm for ascorbic acid correction. Next, the addition of Na_2CO_3 to the microplate was done. The absorbance was measured once more at 600 nm. The results were expressed as mg gallic acid (GA) E/g dw according to GA standard curve. The analyses were performed in triplicate

The assay of oxygen radical absorbance capacity (ORAC) was done using the ORAC assay according to Prior *et al*. (2005) with some modifications as described previously by Dalar *et al.*, (2012). The ORAC capacities of the samples was expressed as mmol Trolox equivalents (E)/g dw according to Trolox standard curve.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity was performed according to Konczak *et al.*, (2003). In brief, the prepared sample solution in the solvent used for extraction, 4-morpholineethanesulfonic acid (MES) buffer, and DPPH solution were combined and set for 2 minutes at RT. The decline in absorbance of DPPH at 520 nm was measured employing the microplate reader. The measurements were assessed in triplicate. The relative DPPH inhibitions (%) were calculated applying the equation of; [(Abs Control-Abs Sample) /Abs Control]*100

2.4. Enzyme Inhibitory Activity

Pancreatic lipase inhibitory activity was performed as described by Dalar & Konczak (2013) 4-methylumbelliferyl oleate was used as a substrate, except for porcine pancreatic lipase (Sigma type II). Sample solutions were prepared employing citricphosphate buffer. 4-Muo and the enzyme solution were placed in a 96-multiwell microplate and incubated for 20 minutes at 37°C. The reaction was ended with the addition of hydrochloric acid and sodium citrate, respectively. The lipase inhibition activity was relatively calculated applying the formula: % Inhibition = (1- (FS-FSB) / (FC-FCB)) x100. While the FS and FC were the sample values as well as negative control measured fluorometrically at an emission wavelength of 460 nm and excitation of 320 nm, respectively.

The inhibition of α-glucosidase enzyme, the assay was assessed with mild adjustment. Dalar & Konczak (2013). Sucrose was applied as a substrate. The enzymatic reaction was ended by increasing the temperature of the mixture to 100 °C by heating for 10 minutes and the absorbance was measured at 505 nm.

2.5. Antimicrobial Activity Test

The antimicrobial capacity of each of the extracts (ethanol and water) were examined against six different bacterial pathogen applying disc diffusion method. Pencilline was the positive control. This test was assessed as formerly described by Boussaada *et al.*, (2008) with mild adjustment.

2.6. Phytochemical Profile

Total phenolics levels of the extracts were done by high-performance liquid chromatography as described previously, (Dalar & Konczak 2013). The levels of total phenolics were expressed as mg chlorogenic acid/g extract. Total flavonoid content **(**TFC) was determined according to previous description by Dalar *et al.,* (2012) with minor changes. In brief, plant extracts prepared in the solvent were placed in 96-multiwell microtiter plates. Next, addition of dH_2O and $NaNO_2$ (1:20 w/v in water) took place, then incubation was followed further $AlCl_3$ was added. The samples were set to be incubated for 6 minutes at RT then NaOH was added and mixed thoroughly till samples turned pinkish into a pinkish color. The absorbance was measured at 510 nm. The extracts were tested in triplicates and the results were expressed as mg rutin (R) E/g dw, The Total hydroxycinnamic acids (THA) content THA contents were determined According to Dalar *et al.*, (2012). In brief, plant extracts were placed in 96-multiwell microplate and HCl in 95% ethanol was added, followed by addition of HCl. The solution was mixed thoroughly and left ~15 minutes at RT.

2.7. Identification and Quantification of Phenolic Compounds

Identification and quantification of phenolic compounds were done using high performance liquid chromatography-diode array detector (HPLC-DAD) and liquid chromatography-photo-diode array–mass spectrometry (LC-DAD–MS/MS) on a Quantum triple stage quadrupole mass spectrometer equipped with a quaternary solvent delivery system, a column oven, a photo-diode array detector and an auto sampler (Thermo Fisher Scientific, Waltham, MA, USA) as described previously (Dalar & Konczak, 2013). An aliquot (3 µl) of each sample solution prepared in the solvent used for extraction was chromatographed on a 150 x 2.1 mm, 5 µm Luna Synergy Hydro column (Phenomenex, Torrance, CA, USA) which was heated to 30 °C. Analytes were separated using 0.5% formic acid and acetonitrile with a flow rate of 200 µl/min. The photodiode array detector was used to acquire data from 190-520 nm.

The composition of phenolic compounds was characterized based on their UV spectrum, retention time, co-chromatography with commercial standards, when available, and MS/MS fragmentation patterns. Mass spectrometry data were obtained using an electrospray source in either the positive (quercetin 3-glucoside) or the negative (chlorogenic acid) modes. MS experiments in the full scan (parent and product-specific) and the selected reaction monitoring (SRM) mode were done. The quantification of phenolic compounds was done using the HPLC-DAD system, which consisted of two LC-10ADVP pumps, SPD-M10ADVP diode array detector, CTO-1-ADVP column oven, DGU-12A degasser, SIL-10ADVP auto injector, and SCL-10A system controller equipped with an Atlantis column (dC_{18}, 4.6 x 100 mm, 5 µm particle size) (Waters Associates, Milford, MA, USA). Analytical HPLC was run at 30 °C and monitored at 250, 280, 320, 370 and 520 nm. Injection volume was 10 µl. The levels of phenolic compounds were quantified as authentic standard E/g dw based on a calibration curve.

To assist the spectrophotometric analysis of phenolic composition, the extracts were hydrolysed according to Pinto et al. (2008). Briefly, lyophilized plant extracts (10 mg) were added to 2 mL of 2N trifluoroacetic acid (TFA) in pyrex glass tube. The tube was placed into a heating instrument and maintained at 120 °C for 2 h. Subsequently, the solution was transferred into 5 mL volumetric flask and 80% methanol was added to adjust the volume. The extract was then directly analysed by HPLC-MS/MS as described above.

3. RESULTS

3.1. Antioxidant and Enzyme Inhibitory Activities

Antioxidant abilities of the extracts were presented in Table 2 through complementary methods including FCR, FRAP, ORAC, DPPH, and ABTS assays. Findings revealed a pattern of *S. papposa* ≥ *S. mollis* > *S. semicana* in ethanol extracts. A similar pattern was observed in infusion preparations with slight differences in ORAC and DPPH results, where *S. semicana* exhibited pronounced activities (Table 2). Ethanol-based extracts had higher antioxidant values which were approximately double that of the infusion preparations, and therefore it can be concluded that ethanol solvent had more effective than water solvent in terms of extracting of antioxidative compounds (Table 2).

Table 2. Antioxidant capacities of ethanol-based extractions and infusion preparations

Taxa	FCR [1]		FRAP [2]		ORAC [3]		DPPH (%) [4]		ABTS(%)[5]	
	Ethanol	Infusion	Ethanol	Infusion	Ethanol	Infusion	Ethanol	Infusion	Ethanol	Infusion
SP	15.6±1.1^{a}	8.3±0.7^{a}	796.3±26.6^{a}	318.9±25.8^{b}	1573.1±77.0^{a}	801.1±43.6^{b}	73.9±1.0^{a}	35.7±1.4c	98.92±6.3^{b}	90.15±8.9^{a}
SM	17.5±1.2^{a}	8.6±0.3^{a}	780.6±35.8^{a}	378.4±8.6^{a}	1483.1±68.9^{a}	768.9±22.9^{b}	73.1±1.1^{a}	58.8±1.7^{b}	93.54±8.2^{a}	83.53±4.5^{b}
SS	9.4±0.4^{b}	6.9±0.2^{b}	409.7±14.3^{b}	308.6±4.9c	1256.8±21.9^{b}	885.6±33.3^{a}	69.9±1.6^{b}	66.4±2.7^{a}	94.15±7.9^{a}	84.61±5.2^{b}

Means with different letters in the same column were significantly different at the level (p<0.05); n=9. [1] Folin-Ciocalteu reducing-total phenolics content–mg Gallic acid Eq. /g dw, [2] Ferric reducing antioxidant power – µmol Fe $^{2+}$ /g dw, [3] Oxygen radical absorbance capacity - µmol Trolox Equivalent/g dw, [4] DPPH radical scavenging activity and [5] ABTS % inhibition at 1 mg/mL concentration. (Taxa: SP: *S. papposa*, SM: *S. mollis;* SS: *S. semicana*)

With regards to enzyme inhibitory activities, suppressive abilities of the extracts were measured towards pancreatic lipase and alpha-glucosidase (Table 3). Similar to antioxidant findings, ethanol extracts had superior activities than infusion preparations. All species showed similar and pronounced antilipase activities. With regards to glucosidase enzyme, *S. papposa* in ethanol extracts and *S. semicana* in infusion preparations had better inhibitory activities (Table 3).

Table 3. Enzyme inhibitory activities of ethanol-based extractions and infusion preparations.

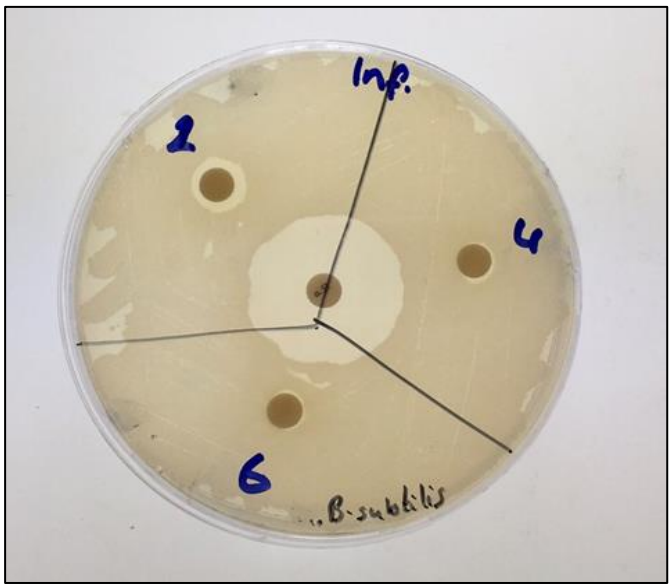
Taxa	Pancreatic Lipase (%)*		Alpha Glucosidase (%)*	
	Ethanol	Infusion	Ethanol	Infusion
S. papposa	96.5±0.9^{a}	74.2±6.0^{a}	82.1+1.0^{a}	62.1±1.1^{b}
S. mollis	96.3±0.6^{a}	72.9±2.3^{a}	78.2±0.8^{b}	60.2±1.0^{b}
S. semicana	96.4±0.8^{a}	71.5±3.0^{a}	79.5±1.3^{b}	71.3±0.9^{a}

Means with different letters in the same column were significantly different at the level (p<0.05); n=3.
*Inhibition of samples at 1 mg/mL concentration.

3.2. Antimicrobial Activity

The antimicrobial activity was tested for each ethanol extracts and infusion extracts against pathogenic bacteria. The obtained results from the ethanol extract of *S. papposa*, *S. mollis* and *S. semicana* showed no significant effect against *P. aeruginosa*, *S. aureus*, *B. cereus B. subtilis*, *E. faecalis* or *E. coli*. However, for the water extracts *S. papposa* displayed a converse effect as opposed to *S. mollis* and *S. semicana.* Unpredictably the zone of inhibition of approximately 5mm was obtained against *Bacillus subtilis* (Figure 1). Evaluation of antibacterial activity was extended further when the extracted were lyophilized and the antimicrobial test was repeated after the lyophilizing, yet no significant difference in the results were obtained.

Figure 1. Antimicrobial results of ethanol-based extractions and infusions



3.3. Phytochemical Profile

Preliminary studies of phytochemical profile were conducted through reagent-based spectrophotometric assays following mass data based chromatographic studies by LC-MS/MS. The preliminary studies showed that the extracts were rich in total phenolics and phenolics subgroups including flavonoids and particularly hydroxycinnamic acids. The similar pattern of *S. papposa* ≥ *S. mollis* > *S. semicana* observed in total phytochemical content determination (Table 4).

Table 4. Phytochemical profile of ethanol-based extractions and infusion preparations

Taxa	Total phenolics[1]		Total flavonoids[2]		Total hydroxycinnamic[3]	
	Ethanol	Infusion	Ethanol	Infusion	Ethanol	Infusion
S. papposa	30.1±1.0^{a}	14.1±0.8^{a}	6.7±0.2^{b}	2.4±0.2b	22.8±1.5^{a}	9.6±0.8^{a}
S. mollis	32.4±1.2^{a}	14.7±1.0^{a}	8.9±0.4^{a}	2.9±0.4^{a}	24.2±1.1^{a}	9.2±1.0^{a}
S. semicana	16.5±0.9^{b}	11.9±0.5^{b}	3.4±0.3^{c}	3.0±0.2^{a}	11.6±0.9^{b}	7.3±0.2^{b}

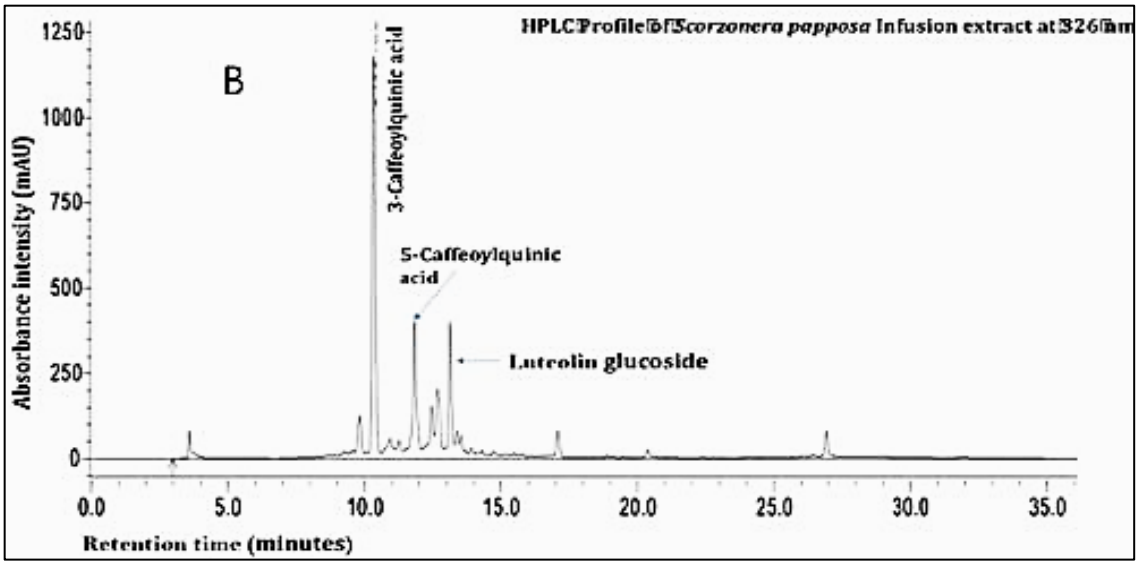
Means with different letters in the same column were significantly different at the level ($p< 0.05$); n=3. [1] mg Chlorogenic acid Eq./g dry weight at 326 nm by HPLC, [2] mg Rutin Eq./g dry weight, [3] mg Chlorogenic acid Eq./g dry weight.

Subsequently, HPLC-MS/MS studies were performed to confirm preliminary phytochemical studies. According to MS/MS data three major phenolics were found in the extracts (Figure 2). Caffeoylquinic acids were the dominant phenolics followed by luteolin glucoside, which confirms preliminary chemical content results (Table 5).

Table 5. Phenolic composition of *Scorzonera* species from Eastern Anatolia by HPLC-MS/MS profile.

MS/MS	3-Caffeoylquinic acid		5-Caffeoylquinic acid		Luteolin glucoside	
-/[M-1]$^-$	-/353		-/353		449/447	
Fragments (m/z) (+/-)	-/191, 179		-/191, 179, 173		287/285	
Concentration (mg/g)	Ethanol	Infusion	Ethanol	Infusion	Ethanol	Infusion
S. papposa	16.6±0.9^a	7.5±0.4^a	3.5±0.2^a	2.2±0.1^a	5.2±0.3^b	2.1±0.1^b
S. mollis	17.3±0.8^a	7.7±0.1^a	3.8±0.1^a	1.7±0.1^b	6.9±0.1a	3.3±0.1^a
S. semicana	8.4±0.2^b	5.1±0.2^b	2.7±0.1^b	1.7±0.0^b	2.9±0.0^c	3.1±0.0^a

Figure 2. Representative HPLC profile of *Scorzonera* species



4. DISCUSSION and CONCLUSION

Investigating various food sources and medicinal plants in order to obtain effective antioxidants and enzyme inhibitors are extremely accredited by researchers for the effective controlling and (optionally management) avoidance of many illnesses (Ak *et al.*, 2020). The recovery rate of phytochemicals varies depending on the type of extraction and solvent applied, solubility and chemical nature of phenolic compounds targeted and/or present in the matrix. A major factor which is critical for the compound's solubility in the plant samples is the polarity rate of used solvent (Luthria & Biswas 2007; Ergün 2022). Extraction of *Scorzonera veratrifolia* by different solvent and method exhibit variable results comparing to our study (Taskın *et al.*, 2021)

The limited scientific record related to the antioxidant capacity of *Scorzonera* species in the literature provoked the need to perform this study in order to provide up to dated data about the selected species. In order to accomplish the successive evaluation of the antioxidant capacity of *S. papposa*, *S. mollis* and *S. semicana* extracts, four bioassays were conducted. Since different methodologies are required to comprehend the biological activity of multipart mixture of secondary metabolites (Gironés *et al.*, 2012).

The antioxidant capacities of extracts from three different species of plants from the Asteraceae family were detected by applying DPPH, FCR, FRAP, ABTS, ORAC assays to

confirm the consistency of the results. Generally, the results from all of the following assays, DPPH, FRAP, ABTS and ORAC displayed a high antioxidant capacity. Interpreting the results of FRAP its indicated that the ethanol extract of *S. papposa* and *S. mollis* had the highest scavenging activity. This suggests the suitability of ethanol in extraction procedure of *S. papposa* and *S. mollis*. This result is in line with Kenny *et al.,* (2014) and Moe *et al.,* (2018) who recorded similar concept about the effectiveness of ethanol use as a solvent. While for infusion extracts there was variability in the results which by *S. mollis* exhibited the highest antioxidant activity following *S. papposa* and lastly *S. semicana*, the variation could be because of each method of antioxidant capacity detection has a different mechanism for scavenging the radicals.

Moreover, the obtained antioxidant activity from FCR, DPPH, FRAP, ABTS and ORAC displayed variability in the antioxidant potential. The variability might be resulted from diverse mechanism of action of different assays and to the variety of chemical constituents. On the other hand, the aerial parts of plants which are rich in antioxidant compounds infusion extracts seems to be verified to be effectual for recovering effective compounds.

A linear relation between the antioxidant potency and TPC reveal the outcome of higher phenolic content provides the higher antioxidant potency. Due to the significantly high levels of phenolic compounds in the particular species of *Scorzonera* in our study, the species might be counted as a supply for natural antioxidants. There are multiple previous studies assessed assays about antioxidant abilities of some other species of *Scorzonera* L. which in accordance with our findings. Milella *et al.*, (2014) reported a high antioxidant potential of both *S. papposa* and *S. judaica*, which then lead to find a base for additional investigation on dihydroisocoumarins and phthalates. In addition, it can be argued that ethanol has better extraction capability than water due to its effectiveness capability of isolation of phenolic compounds from plant matrix and polarity index, which provides to extract utmost of the polar and relatively polar constituents (Mükemre *et al.,* 2020).

A major enzyme for absorbing lipid by hydrolysis of total dietary fats is pancreatic lipase enzyme. Hence, obesity which is one of the common diseases suggested to be controlled by regulating the pancreatic lipase activity inhibition. Pancreatic lipase (triacylglycerol acylhydrolase) is critical enzyme for absorbing triglycerides gained from nutrition (Slanc *et al.,* 2009). The results of both (ethanol and infusion) extracts revealed a high activity of pancreatic lipase, which implies that these herbs appear to be a powerful source for the inhibition of pancreatic lipase enzyme and therefore could be used as a safe natural product for the management of obesity.

Searching through literature, several species belonging to the same family (Asteraceae) have been investigated previously however, it is noticed that the *Scorzonera* species investigated in this study haven't been examined before for their anti-lipase activity. For comparison, both of the *Cnicus benedictus* L. and *Bellis perennis* L. belong to Asteraceae showed 40–70% pancreatic lipase inhibitory activity. However, *Arctium lappa* L extract from the same family showed pancreatic lipase inhibitory activity under 40% reported by Slanc *et al.,* (2009). While our results of the pancreatic lipase enzyme inhibition ranged (71.5-96.5%). Furthermore, from the same study the inhibition activity of glycosidases (α-amylase, α and β-glycosidases) which are major enzymes involved in the pathology of diabetes were detected from the lyophilized methanol extracts. Detected results revealed inhibition of α and β-glycosidases unlike amylase which showed no significant inhibition (Souza *et al.,* 2011). In our study, we also detected the inhibition of α-glucosidase activity. Since α-glucosidase is a major enzyme involved with declining the blood glucose in diabetics (Kwon *et al.,* 2008; Agarwal & Gupta 2016). Overall, comparing our results for the α-glucosidases inhibition activity, the found results showed a significantly high α-glycosidases inhibition activity. The results ranged between 60.2-82.1%

with ethanol extract of *S. papposa* being the highest 82.1% and infusion extract of *S. mollis* being the lowest 60.2%. While Ak *et al.,* (2020) reported their study on *S. hispanica* showed a poor inhibitory activity for both (a-amylase and a-glucosidase. Similarly, a study report by Dall'Acqua *et al.,* (2020) detected a modest α -amylase 0.08 mmol (ACAE)/g) and α glucosidase 0.09 mmol (ACAE/g) inhibitory activity of *Scorzonera tomentosa* L. (Dall'Acqua *et al.,* 2020).

Thus, the revealed results from our study gives credit to the popular use of the studied *Scorzonera* species for treating diabetes. The α-glucosidase inhibition rate was positively correlated with TPC, the higher the phenolic content the higher α-glucosidase inhibitory activity. Our results are in line with Mai *et al.* (2007); Wongsa *et al.*,, (2012) detected a strong linear relation between caffeic acid content and α amylase inhibitory activity ($r = 0.68$, $p < 0.05$), whereas, the correspondence relation between caffeic acid content and p-coumaric acid content with inhibition against α -glucosidase was 0.28 and 0.33 respectively.

Current research has examined the antimicrobial activity against Gram-negative bacteria *P. aeruginosa* and *E. coli* as well as Gram-positive bacteria *E. faecalis B. cereus S. aureus,* and *B. subtilis* using disc diffusion method and Penicillin was applied as positive control. The results from the ethanol extracts had not shown a substantial antimicrobial activity on the gram negative or positive bacteria. Results from our study are in accordance with (Vergun *et al.*,, 2018; Sarı *et al.*,, 2019; Şahin *et al.*,, 2020). Sarı *et al.,* (2019) stated that no antimicrobial activity was detected against *S. aureus, E. coli and P. aeruginosa* apart from a weak activity against *E. faecalis*. Moreover, Şahin *et al.,* reported that fractions from *S. pygmaea* displayed no particular antimicrobial activity against tested bacteria or fungi. Boussaada *et al.,* 2008 investigated *Scorzonera undulata* subsp. *deliciosa* oil and detected that the antimicrobial capacity of the oil. This indicates that environmental factors such as soil composition, region, climate, humidity, can alter the structure of the bioactive compounds that are in control of the antimicrobial activity.

Many different compounds such as dihydro-isocoumarins, flavonoids, lignans, phenolic acid have been previously identified through chemical composition analysis of the *Scorzonera* genus (Abd el Raheim, 2016). Considering the results from the HPLC-MS/MS profile, three major phenolic compounds 3-Caffeoylquinic acid, 5-Caffeoylquinic acid and Luteolin glucoside were isolated from both infusion and ethanol extracts. Similar results were observed regarding 3-Caffeoylquinic acid among all of the investigated *Scorzonera* species in this study meaning that both ethanol and infusion are effective for extracting these phenolic compounds. This result is contrary to several studies in the literature which mostly indicates that a solvent with a higher polarity such as ethanol or methanol are better solvent for extracting phenolic content rather than water (Dall'Acqua *et al.,* 2020).

The results from our study are compatible with previous studies about various *Scorzonera* species that have found similar compounds (Granica *et al.,* 2015) has detected several chlorogenic acid isomers including 4-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, 5-O-feroylquinic acid, 5-O-caffeoylquinic acid. It has been reported that luteolin (Rees and Harborne, 1984), luteolin 3'-(6-E-p-coumaroyl-beta-d-glucopyranoside) (Jiang *et al.,* 2007) has been detected in members of the *Scorzonera* genus. Additionally, number of mono and di-caffeoylquinic acids, hydroxycinnamates, and luteolin glycosides have been found as well as two luteolin-7-O-glycosides (Schütz *et al.,* 2005). Furthermore, our results were also in line with Şahin *et al.,* (2020), who used a spectroscopic technique in order to identify the presence of the derivatives of 3 phenolic acid (chlorogenic acid methyl ester, 3,5-di-O-caffeoylquinic acid, and chlorogenic acid). Comparing the species, ethanol and infusion extracts of *S. papposa, S. mollis* contained the highest 3-Caffeoylquinic and 5-Caffeoylquinic acid compound compared to *S. semicana*. This could be related with the fact that those methods of extraction

are most appropriate for isolating most of the phenolic compounds in those species. However, both of the ethanol and infusion extracts of *S. mollis* seemed to have the highest luteolin glucoside content interestingly same result applied to *S. semicana*.

While contrary ethanol extracts of *S. semicana* exhibited the lowest luteolin glucoside content. This could be due to genetic variation among the species and their correspondence to the polarity of the different kind of solvents that is applied for extracting them. There is a link between phenolic compounds and suppression of several enzyme activities which involved in metabolic disorders as previously reported by (Mai *et al.*, 2007; Gonçalves & Romano, 2017). There are several amino acids of various enzyme's protein which some phenolic compounds attach to (Oboh *et al.*, 2015). For instance, CA attaches to Phe78, Pro181, His264, Tyr115, and Ser153A.A in pancreatic lipase (Martinez *et al.*, 2017).

This is the first report of antioxidant testing and phytochemical composition of native botanicals including *S. papposa, S. mollis, and S. semicana.* In conclusion, the obtained results highlighted the potential benefits of the three species of *Scorzonera* as sources of bioactive constituents; it is thought that they play important role in managing metabolic disorders. Thereby, the use of these plants in pharmaceutical industry is strongly recommended due to the effectivity of their antioxidant, key enzyme inhibition potential and rich phenolic compound constituent. Among native botanicals investigated within this study, *Scorzonera mollis* might serve bioactive antioxidant both for industrial and pharmaceutical use. Nevertheless, more detailed research work must be perfomred to evaluate and optimize the extraction methods and detection of specific mechanism of action.

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Declaration of Conflicting Interests and Ethics

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

Authorship Contribution Statement

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REFERENCES

Abd El Raheim, M.D. (2016). Phytochemical and pharmacological studies on *Scorzonera alexandrina* Boiss. *Journal of Saudi Chemical Society*, *20*, S433-S439. http://dx.doi.org/10.1016/j.jscs.2013.01.001

Agarwal, P., & Gupta, R. (2016). Alpha-amylase inhibition can treat diabetes mellitus. *Research & Reviews: Journal of Medical and Health Sciences*, *5*(4), 1-8.

Ainsworth, E.A., & Gillespie, K.M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols*, *2*(4), 875-877. https://doi.org/10.1038/nprot.2007.102.

Ak, G., Dall'Acqua, S., Sut, S., Ferrarese, I., Yıldıztugay, E., Mahomoodally, M.F., & Zengin, G. (2020). Chemical characterization and bio-pharmaceutical abilities of five different solvent extracts from aerial parts and roots of *Scorzonera hispanica* L. *South African Journal of Botany, 133*(1), 212-221. https://doi.org/10.1016/j.sajb.2020.08.003

Baytop, T. (1999). *Therapy with Medicinal Plants in Turkey (Past and Present)*. Publication of the Istanbul University, 312, Türkiye.

Benzie, I.F.F., & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Analytical Biochemistry, 23*(1)*9*, 706. https://doi.org/10.1006/abio.1996.0292.

Boussaada, O., Saidana, D., Chriaa, J., Chraif, I., Ammar Mahjoub, M.A., & Helal, A.N. (2008). Chemical composition and antimicrobial activity of volatile components of *Scorzonera undulata*. *Journal of Essential Oil Research, 20*(4), 358-362. https://doi.org/10.1080/10412905.2008.9700030

Dalar, A., & Konczak, I. (2013). Phenolic contents, antioxidant capacities and inhibitory activities against key metabolic syndrome relevant enzymes of herbal teas from Eastern Anatolia. *Industrial Crops and Products, 44*(1), 383-390. https://doi.org/10.1016/j.indcrop.2012.11.037

Dalar, A., Türker, M., & Konczak, I. (2012). Antioxidant capacity and phenolic constituents of *Malva neglecta* Wallr. and *Plantago lanceolata* L. from Eastern Anatolia Region of Turkey. *Journal of Herbal Medicine, 2(2),* 42-51. https://doi.org/10.1016/j.hermed.2012.03.001

Dall'Acqua, S., Ak, G., Sut, S., Zengin, G., Yıldıztugay, E., Mahomoodally, M.F., & Lobine, D. (2020). Comprehensive bioactivity and chemical characterization of the endemic plant Scorzonera hieraciifolia Hayek extracts: A promising source of bioactive compounds. *International Food Research Journal, 137*(1), 109371. https://doi.org/10.1016/j.foodres.2020.109371

Ergün, F. (2021). *Cotoneaster transcaucasicus* Pojark. Determination of bioactive component amounts and antioxidant activities in fruit extracts. *Food Science and Technology, 9*(7), 1258-1263.

Ergün, F. (2022). The Effects of Drying Methods on total phenolic and flavonoid substances and antioxidant capacity of redstem filaree *Erodium cicutarium*. *Applied Ecology and Environmental Research, 20*(1), 499-509.

Funk, V.A, Anderberg, A.A., Baldwin, B.G., Bayer, R.J., Bonifacino, J.M., Breitwieser, I., & Crawford, D.J. (2009). Compositae Metatrees: The next generation. systematics, evolution, and biogeography of Compositae. Institute of Botany, University of Vienna, Rennweg/Austria.

Gironés-Vilaplana, A., Valentão, P., Andrade, P.B., Ferreres, F., Moreno, D.A., & García-Viguera, C. (2012). Phytochemical profile of a blend of black chokeberry and lemon juice with cholinesterase inhibitory effect and antioxidant potential. *Food Chemistry, 134*(4), 2090-2096. https://doi.org/10.1016/j.foodchem.2012.04.010

Goncalves, S., & Romano, A. (2017). Inhibitory properties of phenolic compounds against enzymes linked with human diseases. Phenolic compounds-biological activity. London: IntechOpen, 99-118.

Granica, S., & Zidorn, C. (2015). Phenolic compounds from aerial parts as chemosystematic markers in the Scorzonerinae (Asteraceae). *Biochemical Systematics and Ecology, 58*(1), 102-113. https://doi.org/10.1016/j.bse.2014.11.005

Gülçin., İ., Huyut, Z., Elmastaş, M., & Aboul-Enein, H.Y. (2010). Radical scavenging and antioxidant activity of tannic acid. *Arabian Journal of Chemistry, 3*(1), 43-53. https://doi.org/10.1016/j.arabjc.2009.12.008

Hattori, E.K., & Nakajima, J.N. (2008). A família Asteraceae na estação de pesquisa e desenvolvimento ambiental galheiro, perdizes, Minas Gerais, *Brasil. Rodriguésia. 59*(4), 687-749. https://doi.org/10.1590/2175-7860200859405

Jiang, T.F., Wang, Y.H., Lv Z.H., & Yue, M.E. (2007). Determination of kava lactones and flavonoid glycoside in Scorzonera austriaca by capillary zone electrophoresis. *Journal of Pharmaceutical and Biomedical Analysis, 43*(3), 854-858. https://doi.org/10.1016/j.jpba.2006.08.024

Kenny, O., Smyth, T.J., Walsh, D., Kelleher, C.T., Hewage, C.M, & Brunton N.P. (2014). Investigating the potential of under-utilised plants from the Asteraceae family as a source of natural antimicrobial and antioxidant extracts. *Food Chemistry, 161*(1), 79-86. https://doi.org/10.1016/j.foodchem.2014.03.126

Konczak, I., Yoshimoto, M., Hou, D.X., Terahara, N., & Yamakawa, O. (2003). Potential chemopreventive properties of anthocyanin-rich aqueous extracts from *in vitro* produced tissue of Sweetpotato (*Ipomoea batatas* L.). *Journal of Agricultural and Food Chemistry, 51*(1), 5916-5922. https://doi.org/10.1021/jf030066o

Kwon, Y.I, Apostolidis, E., & Shetty, K. (2008). Inhibitory potential of wine and tea against α-Amylase and α-Glucosidase for management of hyperglycemia linked to type 2 diabetes. *Journal of Food Biochemistry, 32*(1), 15-31. https://doi.org/10.1111/j.1745-4514.2007.00165.x

Luthria, D.L., Biswas, R., & Natarajan, S. (2007). Comparison of extraction solvents and techniques used for the assay of isoflavones from soybean. *Food Chemistry, 105*(1), 325-333. https://doi.org/10.1016/j.foodchem.2006.11.047

Mai, T.T., Thu, N.N., Tien, P.G., & Van Chuyen, N. (2007). Alpha-glucosidase inhibitory and antioxidant activities of Vietnamese edible plants and their relationships with polyphenol contents. *Journal of Nutritional Science and Vitaminology, 53*(3), 267-276. https://doi.org/10.3177/jnsv.53.267

Martinez-Gonzalez, A.I., Alvarez-Parrilla, E., Díaz-Sánchez, Á.G., de la Rosa, L.A., Núñez-Gastélum, J.A., Vazquez-Flores, A.A., & Gonzalez-Aguilar, G.A. (2017). In vitro inhibition of pancreatic lipase by polyphenols: A kinetic, fluorescence spectroscopy and molecular docking study. *Food Technology and Biotechnology, 55*(4), 519-530. https://doi.org/10.17113/ftb.55.04.17.5138

Milella, L., Bader, A., De Tommasi, N., Russo. D., & Braca, A. (2014). Antioxidant and free radical-scavenging activity of constituents from two *Scorzonera* species. *Food Chemistry, 160*, 298-304. https://doi.org/10.1016/j.foodchem.2014.03.097

Moe, T.S., Win, H.H., Hlaing, T.T., Lwin, W.W., Htet, Z.M., & Mya, K.M. (2018). Evaluation of in vitro antioxidant, antiglycation and antimicrobial potential of indigenous Myanmar medicinal plants. *Journal of Integrative Medicine, 16*(5), 358-366. https://doi.org/10.1016/j.joim.2018.08.001

Mukemre, M., Kończak, I., Uzun, Y., & Dalar A. (2020). Phytochemical profile and biological activities of Anatolian plantain (*Plantago anatolica*). *Food Bioscience, 36*(1), 100658. https://doi.org/10.1016/j.fbio.2020.100658

Oboh, G., Agunloye, O.M., Adefegha, S.A., Akinyemi, A.J., & Ademiluyi, A.O. (2015). Caffeic and chlorogenic acids inhibit key enzymes linked to type 2 diabetes (in vitro): a comparative study. *Journal of Basic and Clinical Physiology and Pharmacology, 26*(2), 165-170. https://doi.org/10.1515/jbcpp-2013-0141

Özgökçe, F., & Özçelik, H. (2004). Ethnobotanical aspects of some taxa in East Anatolia, Turkey. *Economic Botany, 58*(4), 697-704.

Petrovska, B.B. (2012). Historical Review of Medicinal Plants' Usage. Pharmacognosy Reviews, *6* (11), 1-5. https://doi.org/0.4103/0973-7847.95849

Pinto, M.S., Lajolo, F.M., & Genoves, M.I.G. (2008). Bioactive compounds and quantification of total ellagic acid in strawberries (*Fragaria* x *ananassa* Duch.). *Food Chemistry, 107*(1), 1629-35. https://doi.org/10.1016/j.foodchem.2007.10.038

Prior, R.L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*, 4290–302. https://doi.org/10.1021/jf0502698

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*(9-10), 1231-1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Rees, S., & Harborne, J. (1984). Flavonoids and other phenolics of Cichorium and related members of the Lactuceae (Compositae). *Botanical Journal of the Linnean Society, 89*(4), 313-319. https://doi.org/10.1111/j.1095-8339.1984.tb02563.x

Reid, A.M., Oosthuizen, C.B., Fibrich, B.D., Twilley, D., Lambrechts, I.A, de Canha, M.N., & Lall, N. (2018). Traditional Medicine: the Ancient Roots of Modern Practice. In medicinal plants for holistic health and well-Being. Academic Press. 1-11.

Şahin, H., Sarı, A., Özsoy, N., Özbek, Ç.B., & Koyuncu, O. (2020). Two new phenolic compounds and some biological activities of *Scorzonera pygmaea* Sibth. & Sm. subaerial parts. *Natural Product Research, 34(5)*, 621-628. https://doi.org/10.1080/14786419.2018.1493585

Sarı, A., Şahin, H., Özsoy, N., & Çelik, B.Ö. (2019). Phenolic compounds and in vitro antioxidant, anti-inflammatory, antimicrobial activities of *Scorzonera hieraciifolia* Hayek roots. *South African Journal of Botany. 125*(1), 116-119. https://doi.org/10.1016/j.sajb.2019.07.009

Sakulnarmrat, K., & Konczak, I., (2012). Composition of native Australian herbs polyphenolic-rich fractions and in vitro inhibitory activities against key enzymes relevant to metabolic syndrome. *Food Chemistry, 134*(2), 1011-1019. https://doi.org/10.1016/j.foodchem.2012.02.217

Schütz, K., Kammerer, D.R., Carle, R., & Schieber, A. (2005). Characterisation of phenolic acids and flavonoids in dandelion (*Taraxacum officinale* WEB. ex WIGG) root and herb by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry, 19*(1), 179-186. https://doi.org/10.1002/rcm.1767

Slanc, P., Doljak, B., Kreft, S., Lunder, M., Janeš, D., & Štrukelj, B. (2009). Screening of selected food and medicinal plant extracts for pancreatic lipase inhibition. *Phytotherapy Research, 23*(6), 874-877. https://doi.org/10.1002/ptr.2718

Souza, S.P.D., Pereira, L.L., Souza, A.A, & Santos, C.D.D. (2011). Inhibition of pancreatic lipase by extracts of *Baccharis trimera* (Less.) DC., Asteraceae: evaluation of antinutrients and effect on glycosidases. *Revista Brasileira de Farmacognosia, 21*(3), 450-455. https://doi.org/10.1590/S0102-695X2011005000049

Taskin, D., Gecim, M., Dogan, A., & Beceren, A. (2021). Polyphenolic composition and Antioxidant Effect of Aerial Parts and Roots Extracts from *Scorzonera veratrifolia*. *International Journal of Secondary Metabolite, 8 (3),* 284–299

Vergun, O., Kačániová, M., Rakhmetov, D., Shymanska, O., Bondarchuk, O., Brindza, J., & Ivanišová, E. (2018). Antioxidant and antimicrobial activity of *Bunias orientalis* L. and *Scorzonera hispanica* L. ethanol extracts *Agrobiodiversity for Improving Nutrition, Health and Life Quality, 5*(2), 29-38.

Wongsa, P., Chaiwarit, J., & Zamaludien, A. (2012). In vitro screening of phenolic compounds, potential inhibition against α-amylase and α-glucosidase of culinary herbs in Thailand. *Food Chemistry, 131*(3), 964-971. https://doi.org/10.1016/j.foodchem.2011.09.088