Research Square

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In vitro antioxidant and anti-inflammatory activity of Secondary Metabolites, Fractions and Extracts from Allium subhirsutum L

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Abstract

Allium subhirsutum L. is known as a wild garlic and edible plant. Since few studies on this plant are present, we aimed to make comprehensive phytochemical study and test two biological activities (antioxidant, anti-inflammatory) on it's extracts, fractions of *Allium subhirsutum* L. We have screened all extracts, fractions obtained from the extracts using the open normal phase silica gel column (NP-CC) and vacuum liquid reverse phase silica gel column chromatography (VLC-RP), and some compounds isolated from some fractions applying preparative thin layer chromatography in the first step of our study. According to their amounts and thin layer chromatography (TLC) profile, their antioxidant and anti-inflammatory activities have been tested. We have used chromatographic techniques for the separation and puirification of the extracts, DPPH scavenging assay for the antioxidant test, egg albumin denaturation and lipoxygenase inhibition assays fort he anti-infalmmatory activity. The most active samples (extracts, fractions, and compounds) have been assessed for showing more than 70% inhibition at minimum concentration. We have found PAC_{13}, a compound from the CH_2Cl_2 extract- as the most antioxidant sample; PAB_{12}, a fraction from the n-BuOH extract- as the most anti-inflammatory sample in the preventing the egg albumin denaturation method; AB-n-BuOH extract- as the most anti-inflammatory sample in the lipoxygenase inhibition method. With this investigation, we conducted a complete phytochemical analysis and antioxidant (DPPH), anti-inflammatory (preventing egg albumin denaturation and Lipoxygenase inhibition) activity analysis. This work will guide our continuing research on *Allium subhirsutum* L.

1. INTRODUCTION

Living beings are complex creatures that undergo many enzymatic reactions needed for oxygen. Therefore, molecular oxygen(O_2) is the main component of energy production. Still, this molecule forms short-life, very reactive derivatives such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH). These molecules cause cell damage by affecting DNA and unsaturated fatty acids in cell membranes (Wickens, 2001). The organisms improve the antioxidant defense systems to deal with this damage. Besides, the effectiveness of these protective systems reduces with age, and accumulating these derivatives in the body causes emerging illnesses like Alzheimer's (Huang et al., 2016) and diabetes (Ullah et al., 2016). Antioxidant compounds are essential in improving health and preserving foods by inhibiting oxidation and using many food additives, nutraceuticals, and functional foods. The antioxidant activity is measured using many mechanisms, such as hydrogen atom transfer (HAT), single electron transfer (ET), reducing power, and metal chelation.

The DPPH radical scavenging method is the standard and suitable for pre-assessing a sample's antioxidant activity. This method has the hydrogen atom transfer (HAT) mechanism based on single electron transfer (ET) (Prior et al., 2005). DPPH is a stable purple-colored chromogene. The DPPH radical-scavenging method is based on giving electrons of the antioxidant compound to the DPPH

radical to neutralize it. At the end of the reaction, there is a color change at 517 nm, and the fading of the color of the tested solution is an indicator of antioxidant activity (Shahidi and Zhong, 2015).

Inflammation is a situation that results from the injury of living tissue. There are four main signs: ache, redness, increase of heat, and swelling at the region of inflammation. The arteries get enlarged at the moment of the injury anywhere in the body, and since accelerating the bloodstream to injured tissue, this brings about redness (Verma, 2016; Sarveswaran et al., 2017). The mechanism of inflammation includes the series of reactions in the arachidonic acid mechanism, which plays a vital role in the inflammation. While this compound converts to prostaglandins and thromboxane A_2 via cyclooxygenase (COX), eicosanoids convert to leukotrienes via 5-lipoxygenase (5-COX). All these compounds are the chemical mediators of various inflammation reactions (Anoop and Bindu, 2015). Although present anti-inflammatory drugs have serious side effects, they affect by blocking both enzyme activity and reducing inflammation symptoms (Verma, 2016; Sarveswaran et al., 2017). Therefore, new anti-inflammatory drugs must be found with fewer side effects (Sarveswaran et al., 2017).

Many anti-inflammatory activity tests have been conducted in vitro and in vivo. These include tests to inhibit protein denaturation, membrane stabilization methods, hemolysis methods with hypotonic solution and heat, cyclooxygenase, 5-lipooxygenase, proteinase, and hyaluronidase enzyme inhibition tests (Sarveswaran et al., 2017), and tests to measure inflammation mediators like NF-kB transcription factor and tumor necrosis factor (TNF).

The Allium family consists of more than 600 species. The garlic (Allium sativum), onion (Allium cepa), leek (Allium ampeloprasum var. porrum), chives (Allium schoenoprasum), shallot (Allium ascalonicum) are the most studied species, and used as a food (Poojary et al., 2017; Putnik et al., 2019). Allium species have many biological activities such as antimicrobial, antiviral, antidiabetic, antiprotozoal, antioxidant, antispasmodic, anticarcinogenic, antimutagenic, antiasthmatic, anti-amnesia, anti-inflammatory, hepatoprotective, neuroprotective, hypotensive, hypoglycemic, immunomodulatory, urease/xanthin oxidase inhibitör and prebiotic features (Putnik et al., 2019; Lanzotti et al., 2014; Lee et al., 2012; Kumari et al., 1995; Bisen and Emerald, 2016; Nile et al., 2017; Prakash et al., 2006; Santas et al., 2007; Wang et al., 2012; Coreaet al., 2005).

It has been reported that phenolic compounds, flavonoids (Fossen et al., 1998), saponins (Sang et al., 2001; Barile et al., 2006; Dini et al., 2004), phytosterols (Nishimura et al., 2015), organosulphur compounds (Ramirez et al., 2016), anthocyonin glycosides (Fossen et al., 2003) are isolated from the Allium species. The garlic species belonging to the Allium family have vast biological activities partially because of their antioxidant activity. *Allium subhirsutum* L., known as (the wild garlic), is an aromatic (odorous) plant from the old Ages. Potent antioxidant composition, such as flavonoids and polyphenols of the plant, has been investigated (Badraoui et al., 2020; Zammel et al., 2021; Sut et al., 2020). In these publications, it has been reported that *Allium subhirsutum* L. is a potent source of anticancer and antioxidant compounds (Rekik et al., 2019), and it is rich in valuable compounds like polyphenols, vitamins, flavonoids, carotenoids and carbohydrates (Badraoui et al., 2020).

As a result of our literature survey, the most comprehensive paper we found is Badraoui et al.'s paper. It has been performed the determination of the superficial chemical composition of the MeOH extract of *Allium subhirsutum* L by using high resolution-liquid chromatography-mass spectroscopy, comparing the obtained peaks from the extract with the known compounds in the database of the instrument and the measurement of total phenolic and flavonoid content of the extract. Besides, the MeOH extract has been tested for antioxidant activity using the DPPH method and anticancer activity in vitro. As in vivo studies, they investigated the anticancer and angiogenesis activity of the plant extract. Finally, some known compounds determined from the instrument database have been selected and searched for their ADME (absorption, distribution, metabolism, elimination), i.e., pharmacokinetic characteristics using a computer program as in silico research. According to this research findings, Badraoui, R et al. have found high antioxidant and anticancer activity of *Allium subhirsutum* L. MeOH extract and reported that *Allium subhirsutum* L. MeOH extract is a promising plant for cancer treatment.

In another paper by Saodi et al., oil extracts were also studied in addition to the plant MeOH extract. Again, this study tested both extracts' in vitro antioxidant activity, total phenolic and flavonoid contents, and in vivo wound healing activity. Saoudi et al. determined that plant oil extract is mainly used as a wound-healing agent (Saoudi et al., 2021).

There are many studies about *Allium subhirsutum* L. aqueous and alcoholic extracts in the literature. Their antioxidant and antimicrobial activities, superficial phytochemical analysis with the content measurements (total phenolic, flavonoid, etc.), and various mass spectra techniques (HR-LC-MS, LC-ESI-MS/MS, LC-DAD-MS, etc.). In the first part of our study, we extracted, fractionated, isolated, and purified the compounds from the *Allium subhirsutum* L. Then, we tested the antioxidant and anti-inflammatory activity of all extracts, fractions, and pure compounds. We have conducted a more comprehensive study on the plant than the other studies in the literature. In light of the results of our study, we will continue to do in vivo activity studies of the active ones and elucidate the structure of active compounds.

2. MATERIALS AND METHODS

2.1 General

Rotary Evaporator (Heidolph Laboratora 4011-digital), Diaphragm pump (KNF Lab Laboport), Spectrum CF-2 Fraction Collector, Hot plate (Daihan MSH20A), Vortex (MX-S 2500 rpm BIOBASE), 6000 rpm Centrifuge (Hettich EBA 20), Analytical balance (Precisa XB 220A), Lab Water Purification System (Filtek KRS-34), PG Instrument T-80 UV-VIS Spectrophotometry, Maxilab Biotechnology MS4-Maxiread 96 Microplate ELISA Absorbance Reader (400-800nm) were used during all experiment processes. *n*-Hexane (Cat. No: 32293-2.5L-M), *n*-BuOH (Cat No: 537993), methanol (Cat No: 8.48335) acetic acid (Cat No: 338826) were purchased from Sigma-Aldrich to be used in the solvent systems; silica gel 60 (70–230 µm Cat No: 60741, C_{18}-Reversed Phase Silica Gel (230-400 mesh Cat No: 60757), TLC aluminum sheets, Silica gel 60 F_{254} (Cat No: 1.05554), HPTLC aluminum sheets, silica gel 60 (Cat No: 1.05547), LuxPlate™ TLC plates, Silica gel 60 F_{254} (Cat No: 1.05805) were purchased from Merck Supelco to be

used as an media for open and VLC column chromatography, thin layer chromatography (TLC), and preparative chromatography (Prep-TLC); dichloromethane (CAS No: 75-09-2) was purchased from Isolab, and ethyl acetate (Cat. No: 1.00864) form SAFC as solvents. The spray reagent for TLC and Prep-TLC was 1% of vanilline H_2SO_4 reagent. 760700 codded Lipoxygenase Inhibitory Screening Assay Kit was purchased from CaymanÒ to be conducted the lipoxygenase activity tests. 2,2-diphenyl-1-picrylhydrazyl (DPPH) cat. no. D 9132 was purchased from Sigma-Aldrich for antioxidant tests.

2.2 Plant Material

In January 2022, Allium subhirsutum was purchased from the local market and dried in an oven at 40^0C. 2.5 kg of dried plant material was then ground.

2.3 The Extraction & Fractionation

One kg of powder plant was extracted with CH_2Cl_2 and MeOH in several batches using the Soxhlet apparatus. Each extract was evaporated with a rotary evaporator. MeOH extract was dissolved in H_2O and partitioned with *n*-BuOH. H_2O and *n*-BuOH phases were evaporated the same way. At the end of this procedure, CH_2Cl_2 extract (11.2565 g), *n*-BuOH extract (60 g), and H_2O extract (121.88 g) were obtained. All extracts were applied to TLC (Thin Layer Chromatography) and sprayed with the reagents to have a quick idea of their chemical profile.

One g of CH_2Cl_2 extract was submitted to a silica gel-NP (100 g) open column eluted with CH_2Cl_2® CH_2Cl_2: MeOH: 9:1®MeOH, respectively. Four fractions (AC_{1-4}) were obtained after their TLC controls. The fraction AC_2 (62.6 mg) was applied on a silica gel-NP (7 g) column and eluted with the same solvent system. Five sub-fractions (AC_{21-25}) were afforded. The fraction AC_3 (351.9 mg) was applied on silica gel-NP (35 g) column chromatography eluted with CH_2Cl_2® CH_2Cl_2: MeOH:9:1®MeOH solvent systems, respectively. Sixteen fractions were obtained (AC_{31-39}). The fraction AC_4 (83.1 mg) was submitted to silica gel-NP (8 g). Elution System: CH_2Cl_2® CH_2Cl_2: MeOH: 9:1®MeOH. Nine sub-fractions were obtained (AC_{41-49}). The sub-fraction AC_{47} (56.6 mg) was applied on Sephadex-LH 20 (7 g) column chromatography. CH_2Cl_2 did elution as an isocrotic system. Two sub-fractions ($AC_{471-472}$) were afforded.

The *n*-BuOH extract (5 g) was submitted on silica gel-RP (500 g). Eluted with H_2O® H_2O: MeOH: 9:1®MeOH. Ten fractions (AB_{1-10}) were obtained. The fraction AB_6 (556.2 mg) was applied to silica gel-RP (50 g). Eluted with H_2O® H_2O: MeOH: 9:1®MeOH. Ten sub-fractions were obtained (AB_{61-610}).

The H_2O extract (5 g) was submitted using silica gel-RP (500 g) column chromatography. Eluted with H_2O® H_2O: MeOH: 9:1®MeOH. Fifteen fractions (AW_{1-15}) were obtained.

2.4 Isolation with Preparative TLC

The fractions containing dense and clear bands in their TLC profile were selected. They were applied with a capillary tube on 20x20 sized NP-Silica gel-coated TLC plates and developed in the solvent system at the chromatography chamber. First, the TLC plate was evaluated under UV light; if the UV-active compound was present, marked, and scratched, then 1 % vanillin H_2SO_4 was sprayed on a 2 cm part of the plate and heated. The colored bands are scraped off and dissolved in a proper solvent to apply TLC. $AC_{21,}$ AC_{22}, $AC_{23,}$ AC_{24}, AC_{25}, AC_{33}, AC_{34}, $AC_{36\text{-}38}$, AC_{471}, and AC_{472} were applied to preparative plates and developed in an n-hexane: EtOAc: 8: 2 solvent system; the bands were scratched, and the solution of the bands was applied to TLC using the same solvent system. AB_9 and AB_{10} were applied to preparative plates and developed in an n-BuOH: AcOH: H_2O: 4: 1: 5 solvent system. The bands were scratched, and the solution of the bands was applied to TLC with the same solvent system.

2.5 The Antioxidant Activity (The DPPH Method)

The antioxidant activity was measured using the modified method of Shimada et al. (Shimada et al., 1992). One hundred µL sample solutions are mixed with equal volume (Chacha et al., 2005) of DPPH solution [(%0.004 (m/V)] in MeOH (Guleria et al., 2022). The concentrations of the extracts were prepared as CH_2Cl_2 (AC-5.8 mg/ml), n-BuOH (AB-5.0 mg/ml), and H_2O (AW-6.7 mg/ml). The other fractions and compounds were dissolved in one ml of appropriate solvent. Prepared mother test solutions were diluted six times by 1/2. Quercetin solution (5.3 mg/ml) was used as a positive control. All reaction mixtures stayed in the dark for thirty minutes. Then, the absorbance values were measured with a UV spectrometer. All analyses have been done triple. The percentage of inhibition has been calculated using the equation below.

The percentage of inhibition (%) = [(Ab − As)/Ab] × 100

In the equation,

Ab =The absorbance of blank

As = The absorbance of the sample

The maximum inhibition and concentration values of each sample are presented in Table 1-4. We evaluated the samples divided into three classes according to the balance of their maximum inhibition percent and concentration. The first class is composed of the samples that have the maximum inhibition percent at mg/ml level (Table 1, Graph 1); the second class includes the samples that have the maximum inhibition percent at 0-10 mg/ml (Table 2-3, Graph 2-3), and the third class contains the samples that have the maximum inhibition percent at 10-70 mg/ml (Table 4, Graph 4). When applying the samples to the wells, the solvent dissolves the samples into the blank wells. Since the CH_2Cl_2 extract sourced samples were dissolved in the mixture of EtOAc: MeOH, the n-BuOH extract sourced samples were dissolved in MeOH, the H_2O extract sourced samples were dissolved in the mixture of MeOH: H_2O, two blank wells were placed in ELISA plates for each type of sample. The absorbance of the blank is

found by calculating the arithmetic mean of two wells. Six dilutions (1/2-1/4-1/8-1/16-1/32) were tested for each sample.

2.6 The Anti-Inflammatory Activity (The Egg Albumin Method)

The egg albumin method has been used according to 35. 5.8, 5.0, and 6.7 mg/ml of the extract solutions were prepared respectively from the CH_2Cl_2 (AC), n-BuOH (AB) ve H_2O (AW) extracts. The other fractions and compounds were dissolved in one ml of appropriate solvent. Prepared mother test solutions were diluted six times by 1/2. Diclofenac sodium solution (5.2 mg/ml) was used as a positive control. 78 mL of sample solutions were mixed with 124 mL of egg albumin-phosphate buffer (pH: 6.4). All test samples were heated at 37 ± 2 °C for 15 minutes, then continued to heat above 70 °C for 5 minutes. The sample solutions were cooled at room temperature, and their absorbance was measured at 660 nm using a UV spectrometer. All analyses have been done triple. The percentage of inhibition has been calculated using the equation below.

% İnhibition = [As /Ab]-1 x 100 (Guleria et al., 2022; Chandra et al., 2012; Banerjee et al., 2014).

In the equation,

Ab =The absorbance of blank

As = The absorbance of the Sample

The maximum inhibition and concentration values of each sample are presented in Table 5-8, Graph 5-8. We evaluated the samples divided into three classes according to the balance of their maximum inhibition percent and concentration, the same as the assessment of antioxidant activity. The first class is composed of the samples that have the maximum inhibition percent at mg/ml level (Table 5, Graph 5); the second class includes the samples that have the maximum inhibition percent at 0-10 mg/ml (Table 6-7, Graph 6-7), and the third class contains the samples that have the maximum inhibition percent at 10-20 mg/ml (Table 8, Graph 8). Samples were applied to the wells in the same manner as for the antioxidant activity assay. Dilutions, blank preparations, and numbers are also performed with the antioxidant activity.

2.7 Lipoxygenase Inhibition Method

The measurements were done with a 760700 codded CaymanÒ Lipoxygenase Inhibitory Screening Assay Kit (Gaffney, 1996). Only twelve samples, which include all extracts (AC, AB, AW), five fractions (PAC_{22}, PAB_3, PAW_1, PAW_2, PAW_3), and four compounds (PAC_1, PAC_6, PAB_1, PAB_2) were chosen according to having high anti-inflammatory activity and good TLC profile. Nordihydroguairetic acid (NDGA) was used as a positive control inhibitör. Arachidonic acid was used as a substrate. 10 ml Sample and 90 ml 15-Lipoxygenase enzyme were pipetted to all wells. They were incubated for five minutes at room temperature. Then, 10 ml substrate solution was added to all wells to initiate the reaction, and the 96-well plate was placed on a shaker for ten minutes. 100 ml chromogen solution was

added to all wells to stop enzyme catalysis and develop a reaction. One more time, the 96-well plate was placed on a shaker for five minutes. Their absorbance was read at 490 nm using a plate reader.

We evaluated the samples divided into two classes according to the balance of their maximum inhibition percent and concentration, the same as the assessment of antioxidant activity. The first class is composed of the samples with the maximum inhibition percent at 0-10 mg/ml (Table 9, Graph 9); the second class includes the samples with the maximum inhibition percent at higher than 10 mg/ml (Table 10, Graph 10)

3. RESULTS AND DISCUSSION

Based on the extracts, we found that the most antioxidant-active one is CH_2Cl_2 extract (AC) (187,26 ± 42,59-1,45 mg/ml), the least one is H_2O extract (AW) (67,72 ± 2,09-6,7 mg/ml). Although antioxidant compounds generally have polar characteristics and are usually alcoholic, water extracts have higher antioxidant activity. In many studies, apolar extracts like dichloromethane can show higher activity than the polar extracts. Dichloromethane is a non-polar solvent, making it well-suited for the extraction of lipophilic compounds. These lipophilic (fat-soluble) compounds exhibit antioxidant activity by inhibiting lipid peroxidation, a chain reaction that can cause damage to cell membranes (Rapinel, 2018). A study suggested synergistic antioxidant effects of lipophilic and hydrophilic components (Li et al., 2008). Therefore, due to its sensitivity the steady diamagnetic radical (DPPH) is frequently used to assess the antioxidant capabilities of both lipophilic and hydrophilic substances (Arika et al., 2019).

PAC_{13} (78,68 ± 0,27-6,25 µg/ml) is the most active fraction, which shows high activity at µg/ml level (Table 1, Graph 1). We have four fractions (PAC_8, PAC_{11}, PAC_{12}, PAC_{13}) with an activity above 70% (Table 1, Graph 1). All of them belong to the CH_2Cl_2 extract. Quercetin, our positive control, can only exhibit high activity at 2,65 mg/ml of concentration with 186,70 ± 16,15 inhibition percent (Table 2, Graph 2). Among the samples that have the activity between 1-10 mg/ml of concentrations (total 33 samples, three of them extracts, seventeen of them CH_2Cl_2 fractions, eight of them *n*-BuOH fractions, five of them H_2O fractions, CH_2Cl_2 extract (AC) (187,26 ± 42,59-1,45 mg/ml) which has better activity than the positive control, is the most active one (Table 2, Graph 2). Thirteen of these thirty-three samples can exhibit activity above 70% (Table 2-3, Graph 2-3).

We comprehensively evaluated the anti-inflammatory activity of all samples using the egg albumin denaturation method. After analyzing the TLC profile and anti-inflammatory activity results, we selected all extracts and some fractions for further testing using the Lipoxygenase inhibition method. This thorough approach ensures the validity and reliability of our findings.

After evaluating the result of the egg albumin method, we determined that PAB_{12}, which is a *n*-BuOH fraction, and PAB_{32}, which is a H_2O fraction, have the highest inhibition values at the low concentration (31,25 µg/ml) (Table 5, Graph 5). The values (145,69 ± 11,21 for PAB_{12} and 96,39 ± 11,93 for PAB_{32}) display PAB_{12} as the most active fraction (Table 5, Graph 5). One CH_2Cl_2 fraction (PAC_{12}), five n-BuOH

fractions (PAB_2, PAB_3, PAB_{12}, PAB_{18}, PAB_{21}), two H_2O fractions (PAB_{29}, PAB_{32}) have the above 70% activity at µg/ml level. These samples have higher activity at lower concentrations than the positive control: sodium diclofenac (126,88 ± 31,17-1,0 mg/ml) (Table 6, Graph 6).

Among the samples demonstrating activity at 1-10 mg/ml (comprising forty-seven samples, including three extracts, twenty-five CH_2Cl_2 fractions, nine *n*-BuOH fractions, and eleven H_2O fractions), one stands out as the most active-a CH_2Cl_2 fraction known as PAC_{22} (132,22 ±8,38-0,125 mg/ml) (Table 6-7, Graph 6-7). Notably, PAC_{22} exhibits even better activity than the positive control (sodium diclofenac). Thirty-four of these samples show above 70% inhibition, but it is PAC_{22} that piques our interest.

In the lipoxygenase inhibition method, the results reveal unique findings. None of the tested samples can surpass the activity of the positive control, Nordihiydroguiaretic acid (NDGA). NDGA exhibits high activity (186,70±16,15) at a concentration of 2,65 mg/ml, with the sample closest to this value being the *n*-BuOH extract codded AB (129,67 ± 2,03-5 mg/ml) (Table 9, Graph 9). Only one sample, the PAW_2- H_2O fraction (109,01±0,12-7,90 mg/ml), shows above 70% activity at 1-10 mg/ml (Table 9, Graph 9). The samples show maximum inhibitions at 10-100 mg/ml, with four samples exhibiting very high inhibitions (Table 10, Graph 10). However, one sample, PAW_1, stands out with its high inhibition at a low concentration (293,31 ±62,91-19,4 mg/ml) (Table 10, Graph 10).

Comparing the egg albumin denaturation results with Lipoxygenase inhibition results, AB has 150,85 ±34,45 % inhibition at 0,63 mg/ml; PAW_2 has 151,74 ± 2,47 % inhibition at 1,98 mg/ml; PAC_{22} has 132,22 ± 8,38 % inhibition at 0,125 mg/ml; PAB_3 has 85,31 ± 8,90 % inhibition at 62,5 µg/ml; PAW_1 has 156,40 ± 15,62 % inhibition at 1,21 mg/ml; PAW_3 has 148,26 ± 5,76 % inhibition at 4,54 mg/ml in the egg albumin denaturation method. These results have significant implications as they demonstrate that the samples show higher anti-inflammatory activity at lower amounts in the egg albumin denaturation method. This indicates that their anti-inflammatory activity results from preventing the egg albumin denaturation mechanism, not the lipoxygenase inhibition mechanism, which could lead to the development of more effective anti-inflammatory drugs.

The CH_2Cl_2 extract, a non-polar oil extract, emerged as the most potent antioxidant in our study. This finding is significant as it aligns with Saoudi's investigation (Saoudi et al., 2021), reinforcing the robustness of our results and contributing to the growing body of knowledge on the antioxidant activities of plant extracts.

Badraoui's report (Badraoui et al., 2020), which focused solely on the MeOH extract, revealed its unique antioxidant potential. Their comprehensive chemical profile analysis using HR-LCMS unveiled a rich composition, including amino acid derivatives like Methyl N-(amethylbutyryl) glycine, alkaloid like Cepharanthine, sterol lipids like 6-Deoxo castasterone, sixteen tri- and dipeptids. Although we have CH_2Cl_2 fractions (PAC_8, PAC_{11}, PAC_{12}, PAC_{13}) as the most antioxidant active samples at µg/ml level, also we found some *n*-BuOH and H_2O fractions have more antioxidant active than quarcetin.

As it comes to anti-inflammatory activity, PAC_{22}, which is originated from the CH_2Cl_2 extract, is to be found active in two methods (Lipoxygenase and egg albümin denaturation). This finding is particularly promising as it suggests the potential of PAC_{22} as a natural anti-inflammatory agent, which could have significant implications in pharmacology and biochemistry. Saoudi et al. (Saoudi et al., 2021) also found the oil i.e non-polar extract of A. subhirsitum L. has more wound-healing properties than the MeOH extract.

In summary, we have thoroughly investigated the antioxidant and anti-inflammatory activity of *Allium subhirsutum* L. Our aim was to complete a bio-activity screen of all *A. subhirsutum* L. extracts, some fractions, and compounds with a good TLC profile and enough amount. Additionally, we have enlightened the relationship activity and purity of each sample. For example, PAC_{22}, a second NP-CC fraction from CH_2Cl_2 extract, is a mixture that showed anti-inflammatory activity in the prevention of egg albumin denaturation and the inhibition of lipoxygenase. Besides, we have active pure compounds like PAC_8 (antioxidant-active), PAC_{11} (antioxidant-active), PAC_{12} (antioxidant,anti-inflammatory-active), PAC_{13} (antioxidant-active), PAB_2 (anti-inflammatory-active). Our research has uncovered that most anti-inflammatory *Allium subhirsutum* L. samples show their activity in preventing egg albumin denaturation. The next step of our study will be active structure determination of pure active compounds, and in vivo activity testing of active extracts, fractions, and pure compounds, i.e, producing new antioxidant and anti-inflammatory agents.

Abbreviations

ADME
absorption,distribution,metabolism,elimination
COX
cyclooxygenase
DPPH
2,2-diphenyl-1-picrylhydrazyl
ET
single electron transfer
HAT
hydrogen atom transfer
HR-LCMS
High Resolution-liquid chromatography mass spectrometry
NDGA
Nordihydroguairetic acid
NP-CC
normal phase silica gel column chromatography
Prep-TLC
preparative chromatography

TLC
thin layer chromatography
TNF
tumor necrosis factor
VLC-RP
vacuum liquid reverse phase silica gel column chromatography.

Declarations

Author Contributions

We confirm contribution to the paper as follows: study conception and design: Dr. Sibel AVUNDUK; data collection: Dr. Sibel AVUNDUK, Dilara Nur ABACI; analysis and interpretation of results: Dr. Sibel AVUNDUK; draft manuscript: Dr. Sibel AVUNDUK, Dilara Nur ABACI. All authors reviewed the results and approved the final version of the manuscript.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process.

We confirm that no generative or assistive artificial intelligence tools were utilized at any stage during the development of this manuscript.

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Conflicts of interest the authors declare no conflicts of interest.

Consent for Publication

The manuscript includes no individual's data, so there is no need consent for publication.

Availability of Data and Materials

We confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Tables

Table 1. The antioxidant activity values at mg/ml level

Sample	% Inhibition	µg/ml
PAC_8	80,08	43,75
PAC_{11}	77,64	62,5
PAC_{12}	77,26	6,25
PAC_{13}	78,68	6,25
PAC_{15}	2,92	50
PAC_{17}	2,11	28,13

Table 2. The antioxidant activity values with above 50% inhibitory percent at 0-10 mg/ml level

Sample	% Inhibition	mg/ml	Sample	% Inhibition	mg/ml
Quarcetin	186,7	2,65	PAC_{10}	77,17	0,31
AB	82,46	5	PAB19	131,41	7,7
AC	187,26	1,45	PAB30	109,27	0,5
PAC_4	85,54	0,79	PAW_1	162,89	2,43
PAC_5	79,83	0,19	PAW_2	125,74	3,95
PAC_6	88,1	0,78	AW	67,72	6,7
PAC_7	79,76	0,2	PAB9	77,15	9,63
PAC_9	80,91	0,8	PAB11	52	6,85

Table 3. The antioxidant activity values with below 50% inhibitory percent at 0-10 mg/ml level

Sample	% Inhibition	mg/ml	Sample	% Inhibition	mg/ml
PAC_1	35,33	1,63	PAC_{23}	2,54	0,16
PAC_2	17,38	1,8	PAC_{27}	2,08	0,6
PAC_3	37,07	1,65	PAB_1	26,31	9,2
PAC_{14}	5,15	0,3	PAB_2	15,63	6,4
PAC_{16}	1,52	0,12	PAB4	19,99	2
PAC_{18}	7,18	0,4	PAB5	37,85	2
PAC_{19}	10,3	0,4	PAB17	36,25	3,75
PAC_{20}	7,31	0,5	PAB25	20,9	3,75
PAC_{21}	7,86	0,28	PAB26	32,79	1,03

Table 4. The antioxidant activity values of the samples that have the maximum inhibition percent at 10-70 mg/ml

Sample	% Inhibiton	mg/ml	Sample	% Inhibition	mg/ml
PAC_{22}	113,99	69,6	PAB_{21}	146,18	42,5
PAB_3	119	33,8	PAB_{22}	103,38	54,6
PAB_8	9,53	36,62	PAB_{23}	93,71	30
PAB_{10}	129,21	24,1	PAB_{24}	42,01	15
PAB_{12}	32,54	35,42	PAB_{27}	79,12	39,6
PAB_{13}	59,93	11,15	PAB_{28}	49,3	30
PAB_{14}	12,4	17,2	PAB_{29}	13,79	13,85
PAB_{15}	42,63	39,5	PAB_{31}	115,86	21,1
PAB_{16}	43,65	35,65	PAB_{32}	80,49	20,1
PAB_{18}	110,25	16,15	PAB_{33}	166,39	30
PAB_{20}	125,86	28,9	PAW_3	81,53	36,3

Table 5. The anti-inflammatory activity values of the samples that have the maximum inhibition percent at mg/ml (Egg denaturation method

Samples	% Inhibition	µg/ml
PAC_{12}	100,65	50
PAC_{30}	38,65	1,3
PAB_{2}	60,8	62,5
PAB_{3}	85,31	62,5
PAB_{12}	145,69	31,25
PAB_{16}	65,13	62,5
PAB_{18}	84,05	62,5
PAB_{21}	137,83	62,5
PAB_{24}	64,13	15,6
PAB_{29}	157,83	62,5
PAB_{32}	96,39	31,25

Table 6. The anti-inflammatory activity values of the samples that have have above 80% inhibition percent at 0-10 mg/ml (Egg denaturation method)

Samples	% Inhibition	mg/ml	Samples	% Inhibition	mg/ml
SODIUM DICLOFENAC	126,88	1	PAB_{11}	120,6	3,43
AW	88,76	0,25	PAB_{13}	91,61	0,5
AB	150,85	0,63	PAB_{15}	104,79	9,9
AC	178,29	0,36	PAB_{19}	99	3,85
PAC_2	98,29	0,45	PAB_{20}	135,67	1,81
PAC_3	186,29	0,2	PAB_{27}	137,75	4,95
PAC_{11}	157,81	1	PAB_{28}	98,68	3,75
PAC_{13}	184,87	0,2	PAB_{30}	82,78	0,94
PAC_{22}	132,22	0,125	PAB_{31}	99,31	2,64
PAC_{33}	98,48	1,2	PAB_{33}	88,89	1,88
PAC_{38}	82,04	0,7	PAW_1	156,4	1,21
PAB_1	121,68	0,125	PAW_2	151,74	1,98
PAB_9	117,15	4,82	PAW_3	148,26	4,54

Table 7. The anti-inflammatory activity values of the samples that have have below 80% inhibition percent at 0-10 mg/ml (Egg denaturation method)

Samples	% Inhibition	mg/ml	Samples	% Inhibition	mg/ml
PAC_4	61,6	6,3	PAB_{23}	39,11	1,88
PAC_6	16,02	3,1	PAC_{25}	59	1,3
PAC_{10}	57,76	0,5	PAC_1	79,19	0,2
PAC_{19}	25,52	0,4	PAC_{34}	70,93	2
PAC_{21}	22,86	1,1	PAC_{39}	71,82	1,4
PAC_{25}	59	1,3	PAC_{41}	75,21	0,25
PAC_{26}	38,89	0,15	PAC_{43}	73,66	0,25
PAC_{27}	39,46	0,15	PAB_{10}	78,99	0,25
PAC_{40}	57,33	0,7	PAB_{22}	73,06	2
PAC_{42}	34,22	1,2	PAB_{25}	75,74	7,5
PAC_{45}	24,11	0,35	PAB_{26}	71,78	8,23
PAC_{46}	50,42	1			
PAC_{47}	16,36	0,5			

Table 8. The anti-inflammatory activity values of the samples that have the maximum inhibition percent at 10-20 mg/ml (Egg denaturation method)

Samples	% Inhibition	mg/ml
PAC_{31}	72,72	15,16
PAB_{17}	171,29	15

Table 9. The anti-inflammatory activity values of the samples that have the maximum inhibition percent at 0-10 mg/ml (Lipoxygenase inhibition method)

Sample	% Inhibition	mg/ml
Nordihydroguairetic acid (NDGA)	186,7	2,65
AW	47,37	6,7
AB	129,67	5
AC	65,55	5,8
PAC_1	66,03	6,5
PAC_6	9,09	3,1
PAB_1	23,26	9,2
PAB_2	22,19	6,4
PAW_2	109,01	7,9

Table 10. The anti-inflammatory activity values of the samples that have the maximum inhibition percent at higher than 10 mg/ml (Lipoxygenase inhibition method)

Sample	% Inhibition	mg/ml
PAC_{22}	103,8	69,6
PAB_3	189,18	33,8
PAW_1	293,31	19,4
PAW_3	146,81	36,3

Graphs

Graphs 1 to 10 are available in the Supplementary Files section.

Supplementary Files

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