Effect of *Arbutus andrachne* L. methanolic leaf extract on TRPV1 function: Experimental and molecular docking studies

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**ABSTRACT**  
Currently used analgesics have several side effects urging the need for new natural sources of therapeutics. Transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel and a key receptor in pain. In this study, we explored the effect of a methanolic leaf extract from *Arbutus andrachne* L. (a medicinal plant growing in Jordan) and its mechanism of action on TRPV1 function and expression. Our findings showed that the extract decreased capsaicin-induced cobalt (Co²⁺) influx in dorsal root ganglia (DRG) neurons, an effect that involved cannabinoid receptor 1 (CB1) and TRPV1 but not alpha (α)-2 adrenergic receptors. The extract had no effect on capsaicin-evoked TRPV1 expression in the skin, DRGs, spinal cord, and brain. Importantly, the effect of *A. andrachne* was similar to that observed in the positive control group. Additionally, by conducting molecular docking between the ingredients identified in *A. andrachne* extract and TRPV1 receptor, it was found that α-tocopherol, ursolic acid, and β-sitosterol (similar to TRPV1 antagonist, capsazepine) fit in the same pocket of TRPV1 receptor indicating that these compounds are the active ingredients responsible for the effect of the extract in decreasing capsaicin-induced Co²⁺ influx. *A. andrachne* extract decreased capsaicin-induced Co²⁺ influx significantly and can be a good candidate as an analgesic.

**INTRODUCTION**  
Transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel that is activated by noxious heat (>43°C), anandamide, protons, endogenous activators “endovanilloids,” and the highly selective agonist capsaicin (Caterina et al., 1997). This channel is a key receptor in the detection of different painful stimuli and is widely considered a promising target for pain control (Caterina et al., 1997). TRPV1 is highly expressed in dorsal root ganglia (DRGs), trigeminal ganglia, spinal cord, and other non-neuronal tissues (Caterina et al., 1997, Dinh et al., 2004). Similar to other members of TRP channels, each subunit of TRPV1 has six transmembrane domains with pore-forming hydrophobic stretch linking segment 5 (S5) and S6 (Tominaga and Tominaga, 2005). When TRPV1 is activated, sodium (Na⁺) and calcium (Ca²⁺) channels open leading to ion influx, initiation of depolarization, propagation of action potential into the spinal cord and brain then finally, different sensations such as stinging, burning, itching, or feeling of warmth (Anand and Bley, 2011). Interestingly, there is a crosstalk between TRPV1 and several receptors. A large body of evidence reported the involvement of multiple receptors [e.g., cannabinoid receptor 1 (CB1) and alpha (α)-2 adrenergic receptors] in inducing or inhibiting TRPV1 function. In this context, Wang et al. (2014) revealed the involvement of CB1 in nerve growth factor (NGF)-induced sensitization of TRPV1 in DRG neurons. Also, Alsalem et al. (2016) found that olvanil desensitizes TRPV1 in a CB1-dependent mechanism. The importance of α-2 adrenergic receptor in inhibiting TRPV1 activity was also highlighted previously (Matsushita et al., 2018). As a key receptor in pain processing, it was reported that several analgesic and anti-nociceptive compounds exerted their effects by influencing the trafficking and function of TRPV1.

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or functionality of TRPV1. However, many of these drugs have undesirable effects. Nowadays, there is trend toward finding natural sources that can modulate TRPV1 without producing side effects. In this regard, Abbas (2020) reviewed 137 natural ingredients that affect TRPV1 activity in different in vivo and in vitro assays showing that natural compounds from different origins (e.g., bacterial, fungal, animal, and plant sources) could be potential sources for analgesics.

Arbutus andrachne L. (family Ericaceae) is a flowering strawberry tree growing wild in Jordan in the regions of Jarash, Ajloun, Irbid, Salt, and Amman (Oran, 2015). According to Oran (2015), A. andrachne is an edible plant that is used in traditional medicine in Jordan. Previously, we proved the anti-nociceptive effect of A. andrachne methanolic leaf extract on mechanical, thermal, and chemical models of pain in a mechanism that involves several receptors, mainly TRPV1 and CB1 receptors (Jaffal et al., 2020; 2022). We also demonstrated the anti-inflammatory and antipyretic effects of the plant extract in carrageenan-induced paw edema model and yeast-evoked pyrexia, respectively (Jaffal, 2021a). The aim of this study was to test the effect of A. andrachne methanolic leaf extract on the functionality and expression of TRPV1 using cobalt (Co²⁺) influx and enzyme-linked immunosorbent assay (ELISA), respectively, and to use molecular docking, a valuable tool for enhancing drug discovery, to determine the binding affinity between TRPV1 and each compound identified in the plant extract (Al-Najjar, 2018).

MATERIALS AND METHODS

Materials
Capsazepine, SR141716A, capsaicin, and yohimbine were purchased from Tocris Bioscience, Bristol, UK. Diclofenac sodium was from Novartis, Basel, Switzerland. Collagenase (from Clostridium histolyticum), poly-L-lysine, cobalt chloride (CoCl₂), trypsin, Tween 20, copper sulfate, K⁺-Na⁺ taartrate, and 70 µM cell strainers were from Sigma-Aldrich, St. Louis, MO. Penicillin-streptomycin, phosphate buffered saline (PBS), Dulbecco’s modified eagle medium (DMEM), and DMEM-F12 media were provided by Euroclone, Milano, Italy. Fetal bovine serum (FBS), bovine serum albumin (BSA), and 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) buffer were bought from Biowest, Riverside, CA. Radioimmunoprecipitation (RIPA) lysis buffer (including protease inhibitors) and TRPV1 antibody were ordered from Santa-Cruz Biotechnology, Dallas, TX. 3,3′,5,5′-tetrachloromethylbenzidine (TMB) and stop solution for TMB substrate were from RayBiotech, Norcross, GA. β-mercaptoethanol, diethyl ether, ethanol, methanol, and sodium hydroxide (NaOH) were purchased from Scharlau, Barcelona, Spain. Olive oil was bought from the Department of Agriculture, The University of Jordan, Amman, Jordan. Polystyrene uncoated 96-well plates were purchased from Extragen, Taichung City, Taiwan. Secondary anti-mouse conjugated to horseradish peroxidase (HRP) was from Invitrogen, Carlsbad, CA. Folin–Ciocalteu phenol reagent was from Fluka BioChemika, Buchs, Switzerland, while Na⁺ carbonate and Na⁺ bicarbonate were from Thermo Fisher Scientific, Waltham, MA. Normal saline was from McKesson, Virginia.

Methods

Drugs’ preparation
The stocks of capsazepine (10 mM), capsaicin (1 mM), and SR141716A (1 mM) were initially dissolved in absolute ethanol. Yohimbine and diclofenac sodium were dissolved in normal saline. For the in vivo injection, capsaicin was dissolved in olive oil, while all other drugs and the plant extract were dissolved in normal saline. For the in vitro study, capsaicin was added to 5 mM CoCl₂ solution while the drugs and the extract were solubilized in 10% DMEM.

Collection and identification of the plant
The leaves of A. andrachne were gathered from Jarash, Jordan, in March, 2019, and were authentically identified by a plant taxonomist (Prof. Sawsan Oran) in the Department of Biological Sciences, The University of Jordan, Amman, Jordan. A voucher specimen was deposited at the herbarium, Department of Biological Sciences at The University of Jordan, Amman, Jordan.

Preparing the methanolic extract from A. andrachne leaves
The leaves of A. andrachne were washed, dried, and ground with a blender and then soaked in methanol (10:1 v/w ratio) for 3 days with shaking at room temperature. The extract was filtered by Whatman filter paper and then was evaporated in a rotary evaporator at 45°C under reduced pressure. The extraction was repeated several times. Finally, the extract was preserved in the freezer in an air-tight container.

Animals
Male Wistar rats (200–250 g) were used in this study. All animals were kept in the animal house, The University of Jordan, at a temperature-controlled environment (at 22 ± 1°C) in light and dark cycles (12 hour each). Food pellets and water were provided ad libitum. The animals were allowed to adapt to the experimental conditions before starting the experiments. All experiments were approved by the Animal Ethics Committee at The University of Jordan (Ethical approval number 235/2020/19) in accordance with the “Guide for the Care and Use of Laboratory Animals,” animal research: reporting of in vivo experiments (ARRIVE) guidelines and the guidelines of the International Association for the Study of Pain for animal use in research.

Determining the effect of A. andrachne extract on TRPV1 expression

Tissue collection
In this experiment, the animals were divided into control and experimental groups. Two groups received intraplantar (ipl) injection of 30 µg capsaicin or vehicle (olive oil) into the left hind paw of animals. The dose of A. andrachne extract (100 µg/paw) was chosen in this part of the experiment as it was the effective dose in decreasing capsaicin-induced mechanical allodynia in our previous study compared to the ipl injection of 50 µg or 200 µg (Jaffal et al., 2022). A. andrachne extract (100 µg/paw) or 2.5% diclofenac sodium (positive control) in 50 µl saline were
injected into the left hind paw 30 minutes prior to capsaicin injection, ipsilaterally. The injected animals were kept for 24 hours after capsaicin or vehicle injection (Chen et al., 2008a). After that, the animals were euthanized by diethyl ether to collect different tissues including the spinal cord, DRGs, brain, skin, and its underlying muscles. The tissues were preserved at −80°C for homogenization.

**Protein extraction and quantification**

Tissues were crushed into small pieces before homogenization. All tissues were, then, homogenized in RIPA lysis buffer supplemented with a cocktail of protease inhibitors. The samples were centrifuged at 14,000 g for 10 minutes, 4°C to collect the supernatant. Protein quantification was performed using Hartree–Lowry assay (Hartree, 1972). In brief, BSA was used as a standard to prepare a series of dilutions ranging from 0.03 to 0.15 mg/ml. Three reagents were prepared as the following: reagent A consists of 7 mM K+ Na+ tartrate, 0.81 M Na+ carbonate, and 0.5 N NaOH. Reagent B consists of 70 mM K+ Na+ tartrate and 40 mM copper sulfate, whereas reagent C is a diluent of Folin–Ciocalteau in distilled water at a ratio of 1:15. One milliliter of the standards, protein samples, or buffer was mixed with 0.90 ml of reagent A and incubated at 50°C in a water bath for 10 minutes. After cooling the samples, 0.1 ml reagent B was added to each sample, followed by incubation at room temperature for 10 minutes and then the addition of 3 ml of reagent C to the samples and incubation at 50°C for 10 minutes. The absorbance was measured at 650 nm. Protein levels were quantified according to the absorbance and standard curve.

**ELISA**

TRPV1 expression was tested by ELISA. Protein samples (extracted from different tissues) were diluted to 1 µg/ml final concentration in Na+ bicarbonate buffer (pH 9.6). Polystyrene 96-well plates were coated with the antigens from different samples by adding 100 µl of each diluted sample to a well. After that, the plate was incubated with gentle shaking for 2 hours at room temperature and then was washed thrice using 400 µl PBS containing 0.1% Tween 20. During washes, the excess wash buffer was removed by tapping the plate onto paper towels. 1% BSA was used (A 100 µl per well) to block the remaining binding sites of protein. After the addition of BSA, the plate was incubated with shaking for 2 hours at room temperature. After 3 washes, 100 µl of TRPV1 primary monoclonal antibody dissolved in blocking buffer (1:100) was added to each well and the plate was kept at 4°C overnight. Then, the primary antibody was washed thrice, followed by the addition of 100 µl anti-mouse secondary antibody (conjugated to HRP) in blocking buffer (1:1,000) and incubation for 2 hours at room temperature. Antibodies from a previously utilized rat interleukin-6 ELISA kit (Source: RayBiotech) were used as a positive control to coat part of the wells and ensure the effectiveness of the coating process. The next step was the addition of 100 µl TMB solution per well and incubation of the plate for 30 minutes in the dark at room temperature. Finally, the stop solution (50 µl/well) was added, followed by measuring the absorbance at 450 nm. TRPV1 expression was expressed as a fold change by normalizing data in response to control group.

**Assessing the effect of A. andrachne extract on the functionality of TRPV1 in DRG neurons**

**Isolation and culture of DRG neurons**

The isolation and culture of DRG neurons was performed as reported by Alsalem et al. (2019). All DRG neurons (cervical, thoracic, lumbar, and sacral) were extracted after decapitating the rat. After collection, the DRG neurons were placed at 37°C in DMEM-F12 supplemented with 0.25% collagenase for half an hour with shaking. After that, the neurons were incubated for 7 minutes in DMEM-F12 media containing penicillin/streptomycin (1:200), 1% HEPES buffer, and 0.25% trypsin. The neurons were triturated (50 times) using a Pasteur pipette and then were triturated with thin-flame polished pipette for another 50 times. The neurons were centrifuged (at 400 g for 20 minutes) to collect the pellet. DMEM supplemented with 10% FBS was used to resuspend the pellet, followed by 50 times trituration using thin-flame polished pipette. Finally, the solution that contains DRG neurons was filtered using 70 µM cell strainer and was distributed in the 96-well plate, previously coated with poly-L-lysine. The neurons were cultured at 37°C in a humidified atmosphere containing 5% CO2 for 24 hours.

**Co2+ influx assay**

Colorimetric Co2+ influx assay was used to test the activity of TRPV1 in different groups (Alsalem et al., 2019; Sántha et al., 2010). The principle of this assay is that TRPV1 is a non-selective cation channel that allows influx for cations (including Co2+) when the channel is activated (Wood et al., 1988). 10 µM capsaicin was added to DRG neurons to induce Co2+ influx. Different concentrations of the A. andrachne extract (0.5, 1, and 2 mg/ml) were added to DRG neurons for various periods of time (1, 2, 3, and 4 hour incubation) to determine the effective concentration and incubation period in alleviating capsaicin-induced Co2+ influx. Based on the results, the conditions that were chosen for elucidating the mechanism of action of A. andrachne were 1 mg/ml A. andrachne extract with 1 hour incubation. All antagonists were added 30 minutes prior to 1 mg/ml A. andrachne extract which was added 1 hour before capsaicin. The antagonists included 10 µM capsazepine (a selective TRPV1 antagonist), 10 µM SR141716A or rimonabant (a selective CB1 antagonist), and 20 µM yohimbine (a selective α-2 adrenoceptor antagonist). A positive control group was used for this part of the experiment (10 µM capsazepine + 10 µM capsaicin). In more detail, the protocol was conducted as per the following: capsaicin was dissolved in 5 mM CoCl2 solution and then added to DRG neurons for 2 minutes, followed by the lysis of neurons with a lysis buffer prepared without chelating agents, the addition of 2-mercaptoethanol (5%) to each well and incubation for 5 minutes. The color complex was detected by spectrophotometer at 475 nm wavelength.

**Molecular docking**

The X-ray crystallographic structure of TRPV1 (PDB ID: 5IS0, resolution: 3.43 Å) was selected from Protein Data Bank (Gao et al., 2016). The crystal structure was co-crystallized with capsazepine (Walpole et al., 1994). The molecular docking tool AutoDock 4.2 (The Scripps Research Institute, San Diego, CA) was used to study the intermolecular interactions and binding
energies of the proposed compounds found in A. andrachne leaf extract (Morris et al., 2009).

Ligand and protein preparation

TRPV1 X-ray crystallographic structure was used in this part of the study. The two-dimensional (2D) chemical structures of A. andrachne constituents were generated by available chemical directories (ACD)/ChemSketch software (ACD, 2018). ChemSketch software was used to save all compounds (as .mol files) to be converted into .pdb files that can be uploaded in AutoDockTools (Morris et al., 2009). After the addition of Gasteiger charges, the files were converted into AutoDock.pdbq format to indicate all possible rotatable bonds (Morris et al., 2009). According to the co-crystallized ligand (capsazepine), the intermolecular interactions involved chains B and C but not D and E. Kollman’s charges and polar hydrogens were added to the amino acids and the charged protein was solvated using addsol utility in AutoDockTools (Morris et al., 2009).

Docking simulation

In Jaffal et al.’s (2020) study, the compounds in A. andrachne methanolic leaf extract were identified using liquid chromatography–mass spectrometry (LC–MS) analysis. These compounds were compared for molecular docking studies (Jaffal et al., 2021b). AutoDockTools and AutoGrid 4 were used to limit the search area and to create a set of grid maps, respectively (The Scripps Research Institute, San Diego, CA) (Morris et al., 2009). A grid box (having 22.5, 22.5, and 22.5 Å as x, y, and z dimensions, respectively) was then utilized to select the area to be mapped in the protein structure. Docking simulations were carried out using the Lamarckian genetic algorithm parameters (100 runs per simulation). The output information of the free energy of binding and docked coordinates was obtained from AutoDock in the docking log file (.dlg) format (Morris et al., 2009).

The co-crystallized ligand (capsazepine) was successfully re-docked against 51S0 crystal structure with a root-mean-square deviation (RMSD) of 1.55 Å and free energy of binding of -8.5 Kcal/mol. Molecular docking simulations that showed RMSD values of less than 2.0Å are believed to have performed effectively (*). Similar parameters were used to dock plant constituents within the active site (Hevenet et al., 2009).

Statistical analysis

The normality test was performed using the Shapiro–Wilks test. The statistical significance of difference between groups was examined by one-way analysis of variance (ANOVA), followed by the suitable post-hoc test (Tukey’s or Dunnett’s test) using GraphPad Prism version 7. p < 0.05 was considered significant. Data were presented as mean ± standard error of mean (SEM). The data of using different concentrations and incubation times of A. andrachne extract in capsacin-induced Co²⁺ uptake experiment were analyzed using two-way ANOVA considering concentration and time as main factors. Sidak test was used as a post-hoc test for the two-way ANOVA test

RESULTS AND DISCUSSION

The identification of drug targets is fundamental for the development of novel pharmaceutical compounds. The docking models provide information about the binding energy, binding affinity, orientation of ligand–receptor interactions, and function of compounds. Thus, the aim of this work was to examine the *in vitro* effect of A. andrachne extract on the inhibition of the activity and/or expression of the pain receptor (TRPV1) and to use molecular docking tools to identify the active constituents in this action. The results proved the expected effect and mechanism of action.

Figure 1 shows a fold change in TRPV1 protein expression in different tissues collected from animals that received various treatments. The ipl injection of 30 µg capsacin markedly elevated TRPV1 protein expression in the skin of inflamed paw, DRGs, spinal cord, and brain of animals dissected 24 hour post-capsacin injection. In this context, Jeffry et al. (2009) found that the intrathecal (i.t) injection of the TRPV1 agonist (resiniferatoxin, RTX) for 20 days caused ablation of TRPV1-expressing central nerve terminals in the spinal cord but not DRGs or peripheral terminals in paw skin. Additionally, the ipl injection of 10 µg capsacin caused a reversible reduction in TRPV1-expressing terminals, measured after 3 days of injection (Wang et al., 2020).

The ipl injection of A. andrachne leaf extract prior to capsacin did not change the effect of capsacin on TRPV1 expression in any of the tissues. The effect of the plant extract was similar to the results found in the positive control group that received diclofenac sodium. However, this finding is not necessarily a contradiction to the anti-nociceptive and anti-allodynic effects that were shown by the extract (Jaffal et al., 2020; 2022). The finding that diclofenac sodium exhibited prominent anti-nociceptive effects *in vivo* with no change in TRPV1 expression compared to capsacin-treated group in the current study is a supportive evidence for this point. Furthermore, accumulating evidences revealed that the chronic treatment with morphine increased TRPV1 expression in DRGs, sciatic nerve, and the spinal cord (Chen et al., 2008a; Vardanyan et al., 2009).

On the other hand, capsacin-induced Co²⁺ uptake assay was used to determine the effect of the plant extract on TRPV1 functionality. Figures 2 and 3 show the fold change in Co²⁺ influx in cultured DRG neurons treated with different treatments prior to capsacin. Treating DRG neurons with 10 µM capsacin for 2 minutes produced a 2.7-fold increase in Co²⁺ influx compared to control group. This effect decreased in the group incubated with 10 µM capsazepine prior to capsacin (data not shown).

As illustrated in figure 2, the effect of different concentrations of A. andrachne leaf extract (0.5, 1 and 2 mg/ml) on capsacin-evoked Co²⁺ influx was determined after 1, 2, 3, and 4 hour of adding the extract to DRG neurons (Fig. 2). All concentrations of A. andrachne extract produced a significant reduction in capsacin-induced Co²⁺ influx at all time points. Pre-incubating DRG neurons with 1 mg/ml A. andrachne extract for 1 hour exhibited maximum reduction in capsacin-induced Co²⁺ influx. Additionally, the role of TRPV1, CB1, and α2-adrenergic receptors in mediating the inhibitory effect of A. andrachne extract on capsacin-induced Co²⁺ influx was investigated by applying different antagonists 30 minutes prior to 1 mg/ml A. andrachne extract. The findings of this study showed that the antagonists of CB1 and TRPV1 receptors, but not α2-adrenergic receptor, inhibited the effect of the plant extract on capsacin-induced Co²⁺ influx in DRG neurons (Fig. 3). With respect to TRPV1 functionality, the results of the current study showed...
that *A. andrachne* methanolic extract decreased capsaicin-induced Ca^{2+} influx. The effect of the extract on the activity of TRPV1 can be attributed to the effectiveness of the methanolic solution in extracting several plant constituents that play crucial roles in pain responses, particularly Ca^{2+}-mediated responses (Panda et al., 2009). Many of these compounds are unlikely to be extracted by other solvents like petroleum ether (Panda et al., 2009). Importantly, there are strong lines of evidence that show the involvement of CB1 receptor in the functionality of TRPV1 in different models. Using Ca^{2+} imaging and whole-cell patch clamp techniques, Fenwick et al. (2017) found that the endocannabinoid arachidonylethanolamine displayed excitatory activity on TRPV1 receptor in vagal afferent neurons and that palmitoylethanolamide-evoked Ca^{2+} responses were inhibited.

**Figure 1.** Effect of *A. andrachne* leaf extract on capsaicin (Caps)-induced TRPV1 expression in the skin (a), DRGs (b), spinal cord (c), and brain (d). Data presented as mean ± SEM, *n* = 6. *Significant when compared to the control group. One-way ANOVA, followed by Tukey’s post-hoc test.

**Figure 2.** Effect of *A. andrachne* extract on capsaicin (Caps)-induced Co^{2+} influx in cultured DRG neurons. Data are presented as mean ± SEM, *n* = 8. # Significant when compared to Caps-treated group, *p* < 0.05. Two-way ANOVA, followed by Sidak test.
by TRPV1 antagonist (Fenwick et al., 2017). Also, Patwardhan et al. (2006) found that WIN 55,212 had anti-hyperalgesic effects by inhibiting TRPV1 activity in a Ca\(^{2+}\)-calcineurin-dependent mechanism. Additionally, Mills et al. (2001) reported that the cannabinoids have an inhibitory effect on capsaicin-induced Ca\(^{2+}\) responses in DRG neurons, while Alsalem et al. (2016) found that ovanil desensitizes TRPV1 in a CB1-dependent mechanism.

In other part of the experiments, the 17 compounds that were identified in A. andrachne leaf extract by using LC–MS were docked against TRPV1. The list of top compounds with the lowest binding energies is presented in Table 1 and Figure 4. The results showed that the top compounds with the lowest estimated binding energies were α-tocopherol (a type of vitamin E), ursolic acid (a pentacyclic triterpenoid and a hydroxy-monocarboxylic acid) and β-sitosterol (a phytosterol) having −10.53, −10.49, and −9.76 Kcal/mol binding energies, respectively. A conserved intermolecular interaction was found between these compounds and capsazeine. Importantly, the docked compounds in addition to capsazeine fit in the same pocket of TRPV1 receptor. Figure 5 shows that the docked α-tocopherol performed hydrogen bond interactions with Ser512 and Leu553, whereas ursolic acid exhibited interactions with Glu570, Ala665 amino acids. On the other hand, β-sitosterol had one hydrogen bond interaction with Phe543. Molecular docking tools used in the current study showed that α-tocopherol, ursolic acid and β-sitosterol fit in the same pocket of TRPV1 receptor similar to capsazeine, indicating that these compounds are the active ingredients responsible for the effect of the extract in decreasing capsaicin-induced Co\(^{2+}\) influx.

Similarly, Shukla et al. (2014) reported the interaction between capsazeine or derivatives of capsazeine (CPZ-30, CPZ-33, and CPZ-34) and tumor necrosis factor-alpha (TNF-α). The authors proved the existence of hydrogen bonds between these compounds and TNF-α, indicating the high structural stability and the possibility of an inhibitory interaction. Likewise, the interaction between capsazeine and carbonic anhydrase isoenzymes was identified through molecular docking (Xuan-yi et al., 2015).

Interestingly, previous studies have examined the activity of some compounds against TRPV1 receptor (Crouzin et al., 2007, Zhang et al., 2011). In more detail, it was reported that ursolic acid (100 μM) showed inhibition of capsaicin-induced Ca\(^{2+}\)-flux in TRPV1 in mammalian cells stably expressing human TRPV1 receptor (Zhang et al., 2011). Furthermore, Di et al. (2020) demonstrated that ursolic acid inhibited TRPV1-evoked Ca\(^{2+}\) responses as a defensive mechanism against ototoxicity induced by the anti-tumor agent cisplatin. In addition, the antinoceptive effects of ursolic acid from Agastache mexicana aerial parts involved antagonizing TRPV1 receptor (Verano et al., 2013). Crouzin et al. (2007) demonstrated that α-tocopherol protected against Fe\(^{2+}\)-induced oxidative stress in hippocampal neurons by decreasing Ca\(^{2+}\) entry through TRP channels. Further studies are needed to examine the effect of β-sitosterol on capsaicin-induced Ca\(^{2+}\) entry.

Importantly, the results of this study highlight the mechanism of action of ursolic acid, α-tocopherol and β-sitosterol (compounds on A. andrachne) in exerting their analgesic effects as reported in previous studies. In brief, it was documented that the amelioration of mechanical alldoynia in different animal models was also mediated by α-tocopherol (Tiwari et al., 2009) and ursolic acid (Bhat et al., 2016). Also, both compounds reduced the levels of prostaglandin E\(_2\) (PGE\(_2\)) (Chen et al., 2008b; Sakamoto

![Figure 3. Effect of different antagonists on the decrease of capsaicin (Caps)-induced Co\(^{2+}\) influx mediated by 1 mg A. andrachne extract. Data are presented as mean ± SEM, n = 8. ** Significant when compared to 1 mg/ml A. andrachne-treated group, p < 0.05. One-way ANOVA, followed by Dunnett’s post-hoc test.](image)

| Compound          | Free energy of binding (Kcal/mol) | Residues involved in hydrogen bond interactions | Residues involved in hydrophobic interactions |
|-------------------|----------------------------------|-----------------------------------------------|--------------------------------------------|
| α-Tocopherol      | −10.53                           | Ser512, Leu553                               | Leu515, Met547, Leu553, Ala566, Ile569, Ile573, Leu662, and Ala665 |
| ursolic acid      | −10.49                           | Glu570, Ala665                               | Leu515, Leu553, Ile573, and Ala665         |
| β-sitosterol      | −9.76                            | Phe543                                       | Leu515, Ala546, Met547, Leu553, Ala665, and Leu669 |
| Capsazepine, Ki=120 nM |                                |                                               |                                            |
Moreover, α-tocopherol showed analgesic effects in neuropathic pain, formalin-induced paw licking, and writhing tests (Chen et al., 1998; Kim et al., 2006; Juaira et al., 2018). The analgesic effect of β-sitosterol in tail flick and hot plate was also documented (Sakul and Okur, 2021).

**CONCLUSION**

Taken together, the data of the current research proved that *A. andrachne* methanolic leaf extract decreased capsaicin-induced Co^{2+} influx. Also, molecular docking tools demonstrated that α-tocopherol, ursolic acid, and β-sitosterol (similar to capsazepine) fit in the same pocket of TRPV1 receptor indicating that these compounds are the active ingredients responsible for the effect of the extract in decreasing capsaicin-induced Co^{2+} influx. These findings are promising and can open a gate toward developing new analgesics as *A. andrachne* can be a good candidate.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The Animal Ethics Committee at The University of Jordan (Ethical approval number 235/2020/19) approved the study protocol in accordance with the “Guide for the Care and Use of Laboratory Animals.”

DATA AVAILABILITY

All data generated and analyzed are included within this article.

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