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Maclura pomifera (Rafin.) Schneider %80 metanol ekstresinin *in vitro* yaşlanma karşıtı potansiyelinin değerlendirilmesi ve kantitatif HPTLC analizi

In vitro anti-aging potential evaluation of *Maclura pomifera* (Rafin.) Schneider 80% methanol extract with quantitative HPTLC analysis

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ABSTRACT:
Objectives: *Maclura pomifera* (Rafin.) Schneider is a widespread species all around the world, which is also cultured commonly for ornamental purposes. Previous studies revealed that *M. pomifera* fruits are rich in prenylated isoflavonoids, exhibit noteworthy biological activities and have probable benefits, particularly when applied topically. Considering that phenolic compounds are important sources in the development of anti-aging cosmetic products, the present study aimed to investigate the anti-aging potential of Maclura pomifera 80% methanolic extract (MPM) via evaluating the antioxidant and extracellular matrix (ECM) degrading enzymes inhibiting activity.

Materials and Methods: For this study, the inhibitory potential of 80% methanolic extract of *Maclura pomifera* fruits (MPM) against different enzymes associated with aging process was evaluated. Given the unequivocal role of oxidative stress in aging, *in vitro* antioxidant tests were employed as well. Moreover, osajin was determined as the major bioactive isoflavonoid of the sample via high performance thin layer chromatography (HPTLC) analysis.
Results: Results of the mechanistically different antioxidant assays exhibited notable antioxidant potential of the extract. Inhibition potential of MPM against hyaluronidase, collagenase and elastase enzymes, which are directly linked to acceleration of aging process, were investigated and results revealed that MPM inhibited aforementioned enzymes explicitly. MPM had notable phenolic and flavonoid content, 113.92 ± 2.26 mg GAE/g and 66.41 ± 0.74 mg QE/g respectively. When total antioxidant capacity assays were considered, it is possible to suggest that MPM may be a promising antiaging agent.
Conclusion: As a result, this study disclosed that extracts of fruits of *M. pomifera* has significant anti-aging potential and may be used for this purpose.
Keywords: *Maclura pomifera*; anti-aging; antioxidants; HPTLC; osajin.
Özet
Amaç: *Maclura pomifera* (Rafin.) Schneider, süs amaçlı da çoğunlukla kültürü yapılan, tüm dünyada yaygın bir türdür. Önceki çalışmalar*, M. pomifera* meyvelerinin prenillenmiş izoflavonoidler açısından zengin olduğunu, kayda değer biyolojik aktiviteler sergilediğini ve özellikle topikal olarak uygulandığında muhtemel faydaları olduğunu ortaya koymuştur. Yaşlanma karşıtı kozmetik ürünlerin geliştirilmesinde fenolik bileşiklerin önemli kaynaklar olduğu göz önüne alındığında, bu çalışma, *Maclura pomifera* %80 metanolik ekstresinin (MPM) yaşlanma karşıtı etkisini antioksidan potansiyeli ve ECM parçalayıcı enzim inhibe edici aktiviteyi değerlendirerek araştırmayı amaçlamıştır.
Gereç ve Yöntemler: Bu çalışma için, *Maclura pomifera* meyvelerinin (MPM), %80 metanol ekstresinin yaşlanma süreci ile ilişkili farklı enzimlere karşı inhibitör potansiyeli değerlendirildi. Oksidatif stresin yaşlanmadaki kesin rolü göz önüne alındığında, *in vitro* antioksidan testleri de kullanıldı. Ayrıca, osajin, yüksek performanslı ince tabaka kromatografisi (HPTLC) analizi yoluyla numunenin ana biyoaktif izoflavonoidi olarak belirlendi.
Bulgular: Mekanistik olarak farklı antioksidan testlerinin sonuçları, ekstrenin kayda değer antioksidan potansiyelini sergiledi. MPM'nin yaşlanma sürecinin hızlanması ile doğrudan bağlantılı olan hiyalüronidaz, kollajenaz ve elastaz enzimlerine karşı inhibisyon potansiyeli araştırılmış ve sonuçlar MPM'nin yukarıda bahsedilen enzimleri açıkça inhibe ettiğini ortaya koymuştur. MPM, sırasıyla 113.92 ± 2.26 mg GAE/g ve 66.41 ± 0.74 mg QE/g olmak üzere kayda değer fenolik ve flavonoid içeriğine sahipti. Toplam antioksidan kapasite analizleri göz önüne alındığında, MPM'nin umut verici bir yaşlanma karşıtı ajan olabileceğini önermek mümkündür.
Sonuç: Sonuç olarak, bu çalışma *M. pomifera* meyvele ekstresinin önemli bir yaşlanma karşıtı potansiyele sahip olduğunu ve bu amaçla kullanılabileceğini ortaya koymuştur.

Anahtar Kelimeler: *Maclura pomifera*; yaşlanma karşıtı; antioksidanlar; HPTLC; osajin.

Introduction

Likewise all organs, human skin undergoes various physiological changes with advancing age.[1] There are two classes of ageing, intrinsic ageing is controlled by genetics and extrinsic aging is natural result of physiological modifications due to detrimental effects of environmental factors like UV radiation, chemical toxins and smoking.[1,2] Vascular and glandular structure degradation, fibrous tissue loss and decreasing cell regeneration are fundamental factors of ageing.[3] which lead to increment in tissue degeneration, wrinkles and decrement of extracellular matrix (ECM).[4]

As the largest organ, the skin has several roles such as protection, regulation of the body temperature and detection of senses.[5] The skin consists of epidermis, dermis and subcutaneous tissue and is the first barrier between human body and outer environment. [6,7] The ECM is the largest unit of the dermis and supports growth and elasticity by presenting a structural scaffold.[8] Collagen, elastin and fibronectin, which are formed by dermal fibroblasts, constitute the ECM, being fused with proteoglycans [5].Collagen is a basic protein composing approximately 25-35% of all protein content in the body, which is found in the extracellular space of various types of animal connective tissues.[3] One of the major reason of skin ageing and wrinkle formation is the alterations of collagen structure.[1]Elastin is a protein which confers a unique physiological elasticity to the skin and is present in several connective tissues.[6] Skin hydration, keeping the skin smooth, moist and lubricated are important factors to prevent skin ageing and a major glycosaminoglycan (GAG), hyaluronic acid, plays a vital role in these activities.[8] These pivotal constituents are degraded by hyaluronidase, collagenase and elastase enzymes thus lead to acceleration of skin ageing. Moreover, exposure to microorganisms, pollution, ionizing radiation, chemicals, and toxins lead to the formation of reactive oxygen species (ROS) and its deleterious consequences accelerate skin ageing.[9] ROS can initiate complex molecular pathways and as a result collagenase, elastase and hyaluronidase activity may be increased, leading to detectable ECM breakdown and skin texture modifications.[10] For the reasons mentioned, novel natural agents, which decline ROS formation and inhibit ECM degrading proteases may delay skin-ageing process.[11]

Maclura pomifera (Rafin.) Schneider belongs to the Moraceae or the mulberry family, and is also known as the Osage orange tree, cultivated almost all around the world. [12] *M. pomifera* has several biological activities such as antibacterial, antifungal, antiviral, cytotoxic, antitumor, estrogenic and antimalarial[13] due to its prenylated isoflavones, osajin and pomiferin, which are considered as major metabolites of fruits.[14] In anti-aging cosmetic production, phenolic compounds are significant natural sources. Thus, there is a growing interest to discover phenolic compound rich plants such as *Maclura pomifera* for such activities. In previous studies, it is found that isoflavones of *Maclura* increases the expression levels of collagen, elastin and fibrillin comparable or superior to equivalent concentrations of retinol. Hence, it may be assumed that *Maclura* isoflavones are potent extracellular matrix protein stimulants.[15] In the light of these data, aim of this study is to investigate the anti-

aging potential of *Maclura pomifera* 80% methanolic extract (MPM) via exploring its potential for antioxidant bioactivity and inhibiting ECM degrading enzymes. In addition, quantitative analysis of the major bioactive component of the extract, osajin, was measured via high performance thin layer chromatography (HPTLC) and total phenolic content and total flavonoid content assays were conducted for more accurate understanding of the phenolic profile. Results showed that *M. pomifera* might be a valuable source for anti-aging products.

Material and methods

Chemicals

All enzymes, chemicals and references employed in the tests were afforded from Sigma Chemical Co. (St. Louis, MO, USA). The quality of all chemicals was of analytical grade.

Plant material

The fruits of *M. pomifera* were collected from Uşak, Turkey in May 2020. Dr. Hilal Bardakcı carried out the botanical identification procedure of the plant samples. A voucher sample of the plant was deposited at Acibadem University Faculty of Pharmacy Herbarium. (ACUPH 00002)

Preparation of extract

The fruits were separated into small pieces and passed through a blender. 6.45 kg of fruits were macerated with 3125 mL of 80% methanol by using shaking device for three days at room temperature in a dark place. The macerate was filtered and this procedure was repeated twice. The filtrates obtained were gathered together, and then methanol was evaporated in rotary evaporator. The crude methanolic extract was lyophilized (the yield was 204.37 g, 3.16%) and stored at −20°C (MPM).

Quantification procedure of the major bioactive compound by HPTLC

All chemicals and reagents used were of analytical grade. Chloroform, and ethyl acetate, were purchased from Sigma-Aldrich. Commercially available standard osajin was purchased from Sigma-Aldrich (SMB 00092). HPTLC analyses were performed on 20cm × 10cm glass HPTLC silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). Osajin content in MPM was determined by CAMAG HPTLC analytical system. Mobile phase used in the current study was previously described by Bozkurt et al. [16] during the isolation of active principles of *M. pomifera*. 10 mg/mL (MeOH) extract was used for analysis as test solution. A standard stock solution (0.5 mg/mL) of osajin was prepared using 2 mL of acetone. Working solution with 50 µg/mL concentration of standard compound was prepared by dilution with acetone from the stock solution. Each sample were filtered through a 0.45 µm syringe filter. 10 µL of plant extract along with at least five different concentrations of the standard solution (3.3-4.7 µL) were applied in the form of bands with 8 mm length on silica gel glass HPTLC plates 60 F254 with CAMAG Automatic TLC Sampler IV. Developments were carried out in CAMAG Automatic Developing Chamber (ADC-2) and the mobile phase was $CHCl_4$:EtOAc [8:2 (v/v)]. Chamber was saturated for 10 min and the plate was preconditioned for 5 min before the development. The humidity was controlled by ADC-2 using $MgCl_2$ (33% RH) for 10 minutes. Densitometric evaluation was carried out by using Camag TLC Scanner IV in fluorescence mode. The slit dimension was kept at 5×0.2mm, micro and the scanning speed was set at 20 mm/s. Standard contents were afforded

by comparing AUCs with the calibration curve of standards at 280 nm. The presence of standards in extracts was assured by comparison of both retention factors (R*f*) and overlaying UV spectra of each extract and standards. Quantity of PA osajin was determined by comparison of the intensity of diffusely reflected light from extract and fractions with the standard compound.
Osajin content in crude plant extract was measured by using HPTLC-densitometry. The R*f* value of osajin standard was found to be 0.556. The occurrence of osajin in test samples was verified by comparison of R*f* values as well as overlapping their UV spectra (Figure 1). Quantification was afforded by comparing AUCs of samples with the calibration curve obtained with the standard compound osajin. The calibration function was y=2,268*10-8x. The correlation coefficient (R) and the coefficient of variation of the calibration function was 0.998% and 1.06%, respectively. HPTLC analysis showed that MPM contains 0.22% (w/w) osajin. The results of HPTLC study were given in Table I.

In vitro Phenolic Profile Assay

Total Phenolic Content Assay

The assay was performed to evaluate total phenolic contents of the samples in accordance with the Folin-Ciocalteu method, as previously used by Kurt-Celep et al.[17] 20 µL of freshly diluted sample solutions were mixed with 75 µL Na_2CO_3 (20%) and 100 µl of FCR (Folin-Ciocalteu reagent) diluted with H_2O (1:9). After incubated for 30 min at 45°C, the absorbance of the mixtures was read spectrophotometrically at 765 nm. The results were expressed as mg gallic acid equivalents (GAE) per g extract.

Total Flavonoid Content Assay

Measurement of total flavonoid contents of the fractions was done as a method previously reported by Bardakci et al.[18] Concisely, the freshly prepared 1M CH_3COONa and 10% $AlCl_3$ were mixed with samples. Then, 30 min of incubation of the mixtures was performed at room temperature and in the dark. After the incubation process, the absorbance was calculated at 415 nm. The results were asserted as mg quercetin equivalents (QE) in 1 g sample.

2.6. Determination of in vitro *antioxidant activity*

DPPH radical-scavenging activity test

To determine DPPH (2.2-diphenyl- 1-picrylhydrazyl) radical-scavenging activity, combination of freshly diluted sample solutions (Various concentrations prepared from 1 mg/ml stock solution) and methanolic DPPH solution (100 mM) were done. After the incubation interval at room temperature for 45 min, the absorbance was calculated at 517 nm. Butylated hydroxy toluene (BHT) was used as the reference compound to acquire a calibration curve. IC_{50} values of results were stated as µg/mL.[19]

Ferric reducing antioxidant power (FRAP) test

To obtain FRAP reagent, 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ (2,4,6- Tris(2-pyridyl)-s-triazine) and 2.5 mL of $FeCl_3.6H_2O$ (20 mM) were mixed. Thereafter, 10 mL of samples were added to 260 mL of FRAP reagent, and diluted to 300 mL with distilled

water in a 96 well-plate. After incubating for 30 min at 37°C, the measurement of absorbance was performed at 593 nm. BHT was utilized as a reference compound. Ferrous chloride solution (0.252 mM) was used to develop a standard curve and the results were given as mM $FeSO_4$ in 1 g dry extract.[20]

Cupric reducing antioxidant capacity (CUPRAC) test

CUPRAC test was estimated according to the method described before by Barak et al. [21] Equal volumes of 10 mM $CuSO_4$, neocupraine and ammonium acetate buffer (85 mL) were mixed in a 96-well plate. After that, 51 mL of distilled water and 43 mL of sample solutions were added to the mixture respectively. After incubating for 20 min, the absorbance was calculated at 450 nm. The results were stated as mg ascorbic acid equivalent in 1 g dry extract.

Determination of total antioxidant capacity test

The total antioxidant capacity test was calculated according to the phosphomolybdenum method explained earlier by Barak et al. [22] Firstly, in order to obtain TOAC solution; 28 mM sodium phosphate monobasic, 4 mM ammonium molybdate and 600 mM H_2SO_4 were mixed. Then, 300 µL of TOAC solution were mixed with 30 µL of sample solutions in a 96-well plate. After the incubation period at 95°C for 90 min. The absorbance was read at 695 nm. Ascorbic acid was used to obtain a standard curve and the results were calculated as mg Trolox equivalents in 1 g dry extract.

Inhibitory activity on skin aging related enzymes

Anti-collagenase activity

To measure the anticollagenase activity of the MPM, 50 mM tricine buffer solution (pH: 7.5) was prepared. (400 mM NaCl and 10 mM $CaCl_2$). *Clostridium histolyticum* (ChC - EC. 3.4.23.3) was used as the source of collagenase enzyme which was dissolved in the 50mM tricine buffer solution to achieve an initial concentration of 0.8 U/mL. 2mM of N- [3- (2-furyl) acryloyl] -Leu-Gly-Pro-Ala (FALGPA) dissolved in tricine buffer was used as substrate. The extracts were incubated with collagenase enzyme in buffer solution for 15 min before adding the substrate to commence the reaction. Final reaction mixture comprised a total volume of 150 µL; tricine buffer, 0.8 mM FALGPA, 0.1 units of ChC and 25 µL of MPM. Water was used for blank results. After the addition of the substrate, measurement of absorbance was done immediately. Positive controls were performed with EGCG. [23]

Anti-elastase activity

Evaluation of MPM for anti-elastase activity was carried out by using 0.2 mM tris-HCl buffer solution (pH: 8.0). A stock solution of elastase enzyme (P.E.-E.C. 3.4.21.36) obtained from porcine pancreas was prepared with distilled water at a concentration of 3.33 mg/mL. N-succinyl-Ala-Ala-p-nitroanilide (AAAPVN) to be used as substrate was dissolved in buffer solution (1.6 mM). MPM extract was incubated with the 1 µg/mL of PE enzyme for 15 min. at 37^0C prior to substrate addition. At the end of 15 min pre-incubation, 0.8 mM AAAPVN substrate was added to the enzyme mixture containing 1 mg/mL plant extract and incubation was performed again for 15 min. at 37^0C. While using 0.25

mg/ml EGCG as a positive control, this test sample contains the same volume of EGCG instead of MPM, and the test setup was repeated. Following the incubation periods, measurements were taken at 4 different time points for 5 to 30 minutes by Thermo Scientific Multiskan SkyHigh Microplate Spectrophotometer at 365 nm excitation and 410 nm emission.[24, 25]

Anti-hyaluronidase activity

Anti-hyaluronidase enzyme activity was performed by modifying the method described by Kolayli et al. [26] and Lee et al.[3] Firstly, the commercially-purchased hyaluronidase enzyme (EC 3.2.1.35 - Sigma-Aldrich) was dissolved in 0.02 M phosphate buffer (pH 3.5) containing NaCl and bovine serum albumin (BSA). Then, hyaluronic acid, a proper substrate of the enzyme, was prepared in acetate buffer (0.1M, pH 3.5) and made ready for use. The assay mixture consisting of 20 µL of MPM at a concentration of 1 mg/mL, 10 µL of hyaluronidase enzyme and 60 µL of 0.1M acetate buffer was pre-incubated for 20 min at 37°C. After the incubation time, 10 µL of hyaluronic acid was added to the mixture and incubated again at 37°C for 20 min. At the end of the total incubation time, measurements were made at different time points by Thermo Scientific Multiskan SkyHigh Microplate Spectrophotometer at 600 nm. The blank group did not contain enzymes in the experimental setup, while the control group did not contain plant extract. The per cent of anti-hyaluronidase activity was calculated using the following equation:

Anti−aging activities (%) = [(Abs of Control − Abs of Sample)/ Abs of Control]×100

Statistics

The anti-elastase, anti-collagenase and anti-hyaluronidase enzyme activity experiments included in this study were repeated three times independently. The statistical difference was analyzed using the t-test of the GraphPad Prism 8 software program ($p \leq 0.05$).

Results & Discussion

Determination of anti-ageing potential

Elastin, collagen and hyaluronic acid are known contents of ECM, which have pivotal roles for young appearance of the skin. Elastin is a vital protein for maintaining elastic properties of the skin, consequently decrement of elastin in ECM leads to acceleration in aging process. [27] Previous literature clearly indicated the direct link between wrinkling and aging of skin with lowered amount of elastin.[28] Hyaluronic acid is a hydrophobic glycosaminoglycan molecule which is depolymerized via hyaluronidase enzyme. Hyaluronic acid is crucial to keep the skin smoothness and moisture stable; hence, it has been shown that excessive breakdown leads to drying and wrinkling to the skin.[29] Through aging process with time, decreased level of collagen causes thinning on dermis, which is considered as a distinctive indication under microscopic examination.[30] It was precisely indicated that delaying breakdown of collagen via collagenase inhibitors consequently intervals the wrinkling and aging of skin structure.[5] In the light of this information, it is apparent that substances that inhibits elastase, collagenase and hyaluronidase have noteworthy potential for anti-aging products. Previous studies have demonstrated that various isoflavonoids exhibited significant inhibition bioactivity against aforementioned enzymes. Addotey et al. [31] showed that four different isoflavonoids inhibited hyaluronidase enzyme up to 61.2%. Kim et al. [32] have shown that an isolated isoflavonoid from *Glycyrrhiza uralensis*, licoricidine, has significant elastase inhibition activity.

IC_{50} value of licoridine was calculated as 61.2 ± 4.2 µM while oleanolic acid was calculated 131.4 ± 11.4 as reference compound. Results indicated that isoflavonoids might inhibit elastase enzyme. Consistent with aforementioned study, Kim et al.[33] studied nine different prenylated isoflavonoids, exceedingly related structures with osajin, isolated from roots of *Flemingia philippinensis*. Researchers reported that five of the prenylated isoflavones have potent inhibition activity against neutrophil elastase, IC_{50} values diversify between 1.9-12.0 µM, while IC_{50} value of oleanolic acid was 28.4 µM. In another study, Ergene-Öz et al.[34] investigated *in vitro* inhibition activity of five isoflavonoids isolated from roots of *Ononis spinoza* L. against hyaluronidase, collagenase, elastase enzymes. Hyaluronidase inhibition activity of isoflavones were reported between 22.08-45.58% while in the same concentration, tannic acid showed 88.32% inhibition. Collagenase inhibition results calculated between 20.41-28.49% and elastase inhibition was measured as 20.47-46.88%. Epigallocatechin gallate was used as reference compound for both assays and inhibition activity in the same concentration was measured as 41.18% and 84.64% respectively. Another study investigated topical treatments of pomiferin directly isolated from *M. pomifera*.[15] Pomiferin is a prenylated isoflavonoid which can be found in *M. pomifera* fruits and its molecular structure exceedingly resembles to osajin. Investigators reported that pomiferin exhibited potent ECM protein stimulation activity via increasing collagen and elastin which is superior or equivalent to reference compound retinol. All mentioned studies revealed that isoflavones are moderate to potent inhibitors of these enzymes and they have significant potential as natural anti-aging materials.
In this study, *in vitro* hyaluronidase, collagenase, elastase inhibition activities of MPM were investigated for determination of antiaging potential. A comparative assay was conducted for collagenase inhibition assay in two time points, 20 and 40 min, for both MPM and reference compound EGCG. Results demonstrated that collagenase inhibition activity increased with time. 1 mg/mL MPM showed 84.55% ± 1.99 inhibition while 250 µg/ml EGCG showed 84.66% ± 1,83 subsequent to 20 min of incubation. Inhibition bioactivity was amplified through time, after 40 min MPM and EGCG inhibited collagenase 94.68% ± 2.42 and 94.98% ± 2.81, respectively. In consistent with the literature, MPM exhibited significant inhibition activity against elastase enzyme. Results were measured for four time points (5, 10, 20 and 30 min) and demonstrated increment through time (Figure 2). 250 µg/mL EGCG was used as reference compound and inhibition activity increased in every time point (44,07% ± 0.00, 52.19% ± 0.00, 64.69% ± 0.00 and 86.21% ± 0.00, respectively). Meanwhile, MPM in 1 mg/mL concentration exhibited higher enhancement and inhibition activity which raised from 34,70% ± 0,57 to 97.40 % ± 1,04 from 5 min to 30 min. In a similar manner, 1 mg/mL MPM inhibited hyaluronidase enzyme significantly after 40 min incubation. 83.91% ± 2.36 inhibition was measured following 40 min, thereafter 80 min of incubation, inhibition rate amplified to 97.19 % ± 0.45. When entire enzyme inhibition assays were taken into account, results noticeably demonstrated that MPM may be a valuable natural anti-aging agent and may be used in the contents of various anti-aging products thus *M. pomifera* may gain extra economic importance.

Determination of antioxidant potential

It is well known that numerous exogenous and endogenous factors lead to skin aging via various mechanisms. Majority of these factors are directly or indirectly affected by ROS formation in the ECM of the skin.[35] Since the skin covers the outer part of our body, it encounters with significant amount of UV irradiation in our daily life. Thus, majority of the skin problems such as sunburn, hyperpigmentation, skin carcinogenesis etc. are originated or related to direct effects of UV radiation. Likewise, photoaging is an additional consequence of its hazardous properties.[36] Moreover, it is well known that UV light generates ROS formation and consequently oxidative stress in skin tissue which is the one of the most main mechanism leads to photoaging.[37] It was hypothesized that since excessive ROS formation grounds premature skin aging, agents with a significant antioxidative capacity may be a valuable tool against perilous effects of UV radiation. Accordingly, clinical studies showed that topical antioxidant utilization has a protective effect on the skin.[38]

Subsequent to interlink between topical antioxidant utilization and postponement of skin aging which is well established in previous literature, examining *in vitro* antioxidant potential of MPM provides valuable information for its anti-aging potential when applied topically. Earlier literature clearly demonstrated that extracts with high amount of isoflavonoids might be valuable antioxidants. In a previous study, isoflavonoid-rich extract of *Flemingia macrophylla* extract reduced UVB-induced skin damage via scavenging ROS.[39] Santos and Silva[40] indicated that prenlylated isoflavonoids have important antioxidant potential due to their flavonoid moiety and additive effect of prenyl sidechain. Antioxidant potential of extracts and isoflavonoids of *M. pomifera* were evaluated in a previous study. Results demonstrated that, hydroalcoholic extract and pure osajin showed significant activity on DPPH, FRAP and TOAC assays, even though pomiferin and ethly acetate fraction showed higher activity.[41] For this study, DPPH radical scavenging activity, FRAP, CUPRAC and TOAC assays were conducted for comprehensive determination of *in vitro* antioxidant potential of MPM (Table II). MPM exhibited significant DPPH radical scavenging activity, where IC_{50} value was measured 1998.86 ± 0.02. FRAP and TOAC assays also resulted with notable metal reducing activity, 0.191± 0.01 mM $FeSO_4$/DE and 114.43 ± 0.02 AAE/g DE, respectively. These findings were consistent with the previous study published by Erdoğan-Orhan et al. [41]. CUPRAC assay was conducted on *M. pomifera* fruit extracts for the first time to our knowledge. Correspondingly, MPM showed noteworthy copper reducing activity in CUPRAC assay, results measured as 73.928 ± 0.01 AAE/g DE. When total antioxidant capacity assays were considered, it is possible to suggest that MPM may be a promising antiaging agent.

Phenolic profile and HPTLC analysis

Isoflavonoids are phenolic substances, which are known as plant constituents responsible for various noteworthy biological activities such as antioxidant, anticancer and against gynecological problems.[42] Previous studies evidently demonstrated that prenylated isoflavonoids are major phenolic compounds in *M. pomifera* fruits. [43] Numerous studies identified osajin and pomiferin as the major ingredients of *M. pomifera* fruits,

which are primarily accountable for its biological activities.[12] Osajin and pomiferin are highly similar prenylated isoflavonoids that only differ with one hydroxyl group.[44] Previous reports demonstrated conflictual results for osajin and pomiferin content of *M. pomifera* fruits. Kartal et al.[45] developed an LC-MS method for determination of osajin and pomiferin from *M. pomifera,* which were collected from Ankara province of Turkey. Results demonstrated that pomiferin content was slightly higher than osajin content in different parts of the fruit samples. In another study, *M. pomifera* fruit samples were collected from different regions of Midwest and Southern United States and osajin and pomiferin contents were measured via a novel HPLC analysis method. Results showed that geographic differences lead to significant alteration for isoflavonoid amounts of samples. [46] Tsao et al.[47] determined osajin and pomiferin content of fruits collected from Canada and found that pomiferin content is slightly higher than osajin content. On the contrary, Hwang et al.[48] summarized several studies which found osajin amount higher than pomiferin amount in various extracts. It can be claimed that osajin and pomiferin contents of *M. pomifera* fruits are exceptionally variable with geographic differences and extraction techniques due to their decidedly analogous chemical structure. For this study, osajin amount of MPM was measured via HPTLC analysis, for the first time to our knowledge. Results of the analysis showed that osajin is the predominant ingredient of MPM which were collected from Uşak province, 0.22% of the sample consists of osajin (Table 1). Furthermore, for achieving further assessment of phenolic profile of MPM, total phenolic and total flavonoid content assays were conducted. Results exhibited that MPM had notable phenolic and flavonoid content, 113.92 ± 2.26 mg GAE/g and 66.41 ± 0.74 mg QE/g respectively. Result of phenolic profile evaluation showed that MPM might be prominent candidate as a novel natural anti-aging agent.

Conclusion

Anti-aging market in the world is increasing extremely, forecasts about future of the market are expecting more than 300 billion USD in sales by the year 2025.[49] For this reason, scientific studies aiming to determine mechanisms underlying skin aging are increasing radically for discovering novel approaches for improved treatment options against dermatological concerns.[50] Even though, studies investigating topical implementation of *M. pomifera* fruits are relatively new, attention in this manner is increasing with encouraging reports. Therefore, this study is aiming to describe a comprehensive evaluation of possible anti-aging potential of *M. pomifera* fruit extract. HPTLC analysis used for *M. pomifera* fruits for determination of isoflavonoid content for the first time to our knowledge along with *in vitro* studies for determining total phenolic profile. Results showed that osajin is the major ingredient of the samples. In addition, *in vitro* antioxidant potential was assessed with four different assays and results demonstrated significant antioxidant potential of MPM. Additionally, inhibition activity against enzymes which are related to aging process was measured and it was seen that MPM has notable enzyme inhibition bioactivity. In conclusion, this study provides information which may lead to production of novel skincare products.

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References

1. Hwang E, Park SY, Yin CS, Kim HT, Kim YM, Yi TH. Antiaging effects of the mixture of Panax ginseng and Crataegus pinnatifida in human dermal fibroblasts and healthy human skin. J Ginseng Res. 2017;41:69–77.
2. Ganceviciene R, Liakou AI, Theodoridis A, Makrantonaki E, Zouboulis CC. Skin anti-aging strategies. Dermatoendocrinol. 1980;4(3):318-319.
3. Lee H, Hong Y, Tran Q, Cho H, Kim M, Kim C, Kwon SH, Park S,Park J, Park J. A new role for the ginsenoside RG3 in antiaging via mitochondria function in ultraviolet-irradiated human dermal fibroblasts. J Ginseng Res. 2019;43:431–441.
4. Rouvrais C, Bacqueville D, Patrick B, Haure MJ, Duprat L, Coutanceau C, Castex-Rizzi N, Duplan H, Mengeaud V, Bessou- Touya S. Assessment of antiaging properties of retinaldehyde delta-tocopherol glucoside/glycylglycine oleamide combination. J Invest Dermatol. 2017;137(5):S95.
5. Bravo K, Alzate F, Osorio E. Fruits of selected wild and cultivated Andean plants as sources of potential compounds with antioxidant and anti-aging activity. Ind Crops Prod. 2016; 85:341–352.
6. Yepes A, Ochoa-Bautista D, Murillo-Arango W, Quintero-Saumeth J, Bravo K, Osorio E. Purple passion fruit seeds (Passiflora edulis f. edulis Sims) as a promising source of skin anti-aging agents: Enzymatic, antioxidant and multi-level computational studies. Arab J Chem. 2021;14(1):102905.
7. Rittié L, Fisher GJ. UV-light-induced signal cascades and skin aging. Ageing Res Rev. 2002;1(4):705–720.
8. Duque L, Bravo K, Osorio E. A holistic anti-aging approach applied in selected cultivated medicinal plants: A view of photoprotection of the skin by different mechanisms. Ind Crops Prod. 2017; 97:431–439.
9. Manjia N. J, Njayou N. F, Joshi A, Upadhyay K, Shirsath K, Devkar V. R, Moundipa F. P. The anti-aging potential of medicinal plants in Cameroon - Harungana madagascariensis Lam. and Psorospermum aurantiacum Engl. prevent in vitro ultraviolet B light-induced skin damage. Eur J Integr Med. 2019; 29:100925.
10. Pujimulyani D, Suryani CL, Setyowati A, Siwianti Handayani RA, Arumwardana S, Widowati W, Maruf A. Cosmeceutical potentials of Curcuma mangga Val. extract in human BJ fibroblasts against MMP1, MMP3, and MMP13. Heliyon. 2020;6(9):e04921.
11. Stavropoulou MI, Stathopoulou K, Cheilari A, Benaki D, Gardikis K, Chinou L, Aligiannis N. NMR metabolic profiling of Greek propolis samples: Comparative evaluation of their phytochemical compositions and investigation of their anti-ageing and antioxidant properties. J Pharm Biomed Anal. 2021;194:113814.
12. Filip S, Djarmati Z, Lisichkov K, Csanadi J, M.Jankov R. Isolation and characterization of Maclura (Maclura pomifera) extracts obtained

by supercritical fluid extraction. Ind Crops Prod. 2015;76:995–1000.
13. Saloua F, Eddine NI, Hedi Z. Chemical composition and profile characteristics of Osage orange *Maclura pomifera* (Rafin.) Schneider seed and seed oil. Ind Crops Prod. 2009;29:1–8.
14. Veselá D, Kubínová R, Muselík J, Zemlicka M, Suchy V. Antioxidative and EROD activities of osajin and pomiferin. Fitoterapia 2014;75(2):209–211.
15. Gruber JV, Holtz R, Sikkink SK, Tobin DJ. *In vitro* and ex vivo examination of topical Pomiferin treatments. Fitoterapia 2014;94:164–171.
16. Bozkurt İ, Dilek E, Erol HS,Çakır A, Hamzaoğlu E, Koç M, Keleş ON, Halıcı MB. Investigation on the effects of pomiferin from *Maclura pomifera* on indomethacin-induced gastric ulcer: An experimental study in rats. Med Chem Res. 2017;26:2048–2056.
17. Kurt-Celep İ, Celep E, Akyüz S, Inan Y, Barak TH, Akaydın G, Telci D, Yeşilada E. *Hypericum olympicum* L. recovers DNA damage and prevents MMP–9 activation induced by UVB in human dermal fibroblasts. J Ethnopharmacol. 2020;246:112202.
18. Bardakci H, Cevik D, Barak TH, Gozet T, Kan Y, Kırmızıbekmez H. Secondary metabolites, phytochemical characterization and antioxidant activities of different extracts of Sideritis congesta P.H. Davis et Hub.-Mor. Biochem Syst Ecol. 2020;92:104120.
19. Celep E, Seven M, Akyüz S, İnan Y, Yeşilada E. Influence of extraction method on enzyme inhibition, phenolic profile and antioxidant capacity of Sideritis trojana Bornm. South African J Bot. 2019;121:360–365.
20. Bardakci H, Barak TH, Özdemir K, Celep E. Effect of brewing material and various additives on polyphenolic composition and antioxidant bioactivity of commercial *Tilia platyphyllos* scop. Infusions. J Res Pharm. 2020;24:133–141.
21. Barak TH, Celep E, İnan Y, Yesilada E. Influence of in vitro human digestion on the bioavailability of phenolic content and antioxidant activity of *Viburnum opulus* L. (European cranberry) fruit extracts. Ind Crops Prod. 2019;131:62–69.
22. Barak TH, Celep E, İnan Y, Yeşilada E. *In vitro* human digestion simulation of the bioavailability and antioxidant activity of phenolics from *Sambucus ebulus* L. fruit extracts. Food Biosci. 2020;37:100711.
23. Ersoy E, Eroglu Ozkan E, Boga M, Yılmaz MA, Mat A. Anti-aging potential and anti-tyrosinase activity of three Hypericum species with focus on phytochemical composition by LC–MS/MS. Ind Crops Prod. 2019;141:111735.
24. Lee KK, Kim JH, Cho JJ, Choi JD. Inhibitory effects of 150 plant extracts on elastase activity, and their anti-inflammatory effects. Int J Cosmet Sci. 1999; 21(2):71–82.
25. Itoh S, Yamaguchi M, Shigeyama K, Sakaguchi I. The Anti-Aging Potential of Extracts from *Chaenomeles sinensis*. Cosmetics 2019;6(1): 21.
26. Kolayli S, Can Z, Yildiz O, Şahin H, Alpay-Karaoglu S. A comparative study of the antihyaluronidase, antiurease, antioxidant, antimicrobial and physicochemical properties of different unifloral degrees of chestnut (Castanea sativa Mill.) honeys. J Enzyme Inhib Med

Chem. 2016;31:96–104.
27. Korkmaz B, Horwitz MS, Jenne DE, Gauthier F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev. 2010;62(4):726–759.
28. Akazaki S, Nakagawa H, Kazama H, Osanai O, Kawai M, Takema Y, Imokawa G. Age-related changes in skin wrinkles assessed by a novel three-dimensional morphometric analysis. Br J Dermatol 147(4):689–695.
29. Barla F, Higashijima H, Funai S, Sugimoto K, Harada N, Yamaji R, Fujita T, Nakano Y, Inui H. Inhibitive effects of alkyl gallates on Hyaluronidase and collagenase. Biosci Biotechnol Biochem. 2019;73:2335–2337.
30. Chung JH, Kang S, Varani J, Lin J, Fisher GJ, Voorhees JJ. Decreased extracellular-signal-regulated kinase and increased stress-activated MAP kinase activities in aged human skin *in vivo.* J Invest Dermatol 115(2):177–182.
31. Addotey JN, Lengers I, Jose J, Gampe N, Beni S, Petereit F, Hensel A. Isoflavonoids with inhibiting effects on human hyaluronidase-1 and norneolignan clitorienolactone B from Ononis spinosa L. root extract. Fitoterapia 2018;130:169–174.
32. Kim KJ, Xuan SH, Park SN. Licoricidin , an isoflavonoid isolated from Glycyrrhiza uralensis Fisher , prevents UVA-induced photoaging of human dermal fibroblasts. Int. J. Cosmet. Sci. 2017;39(2):133–140.
33. Kim JY, Wang Y, Uddin Z, Song YH, Li ZP, Jenis J, Park KH. Competitive neutrophil elastase inhibitory isoflavones from the roots of Flemingia philippinensis. Bioorg Chem. 2018;78:249–257.
34. Ergene Öz B, Saltan İşcan G, Küpeli Akkol E, Süntar İ, Bahadır Acıkara Ö. Isoflavonoids as wound healing agents from Ononidis Radix. J Ethnopharmacol. 2018;211:384–393.
35. Azevedo Martins TE, Sales de Oliveira Pinto CA, Costa de Oliveira A, Robles Velasco MV, Gorriti Guitiérrez AR, Cosquillo Rafael MF, Huamani Tarazona JP, Retuerto-Figueroa MG. Contribution of topical antioxidants to maintain healthy skin—A review. Sci. Pharm. 2020;88(2):27.
36. Krutmann J, Schroeder P. Role of mitochondria in photoaging of human skin: The defective powerhouse model. J Investig Dermatology Symp Proc. 2009:14:44–49.
37. Dong KK, Damaghi N, Picart SD, Markavo NG, Obayashi K, Okana Y, Masaki H, Grether Beck S, Krutmann J, Smiles KA, Yarosh DB. UV-induced DNA damage initiates release of MMP-1 in human skin. Exp Dermatol. 17(12):1037–1044.
38. Oresajo C, Pillai S, Manco M, Yatskayer M, McDaniel D. Antioxidants and the skin: Understanding formulation and efficacy. Dermatol Ther. 2012;25:252–259.
39. Chiang HM, Chiu HH, Liao ST, Chen YT, Chang HC, Wen KC. Isoflavonoid-rich Flemingia macrophylla extract attenuates UVB-induced skin damage by scavenging reactive oxygen species and inhibiting MAP kinase and MMP expression. Evid. Based Complement. Alternat. Med. 2013.

40. Santos CMM, Silva AMS. The Antioxidant Activity of Prenylflavonoids.Molecules 2020;25(3):696.
41. Orhan IE, Senol FS, Demirci B, Dvorska M, Smejkal K, Zemlicka M. Antioxidant potential of some natural and semi-synthetic flavonoid derivatives and the extracts from *Maclura pomifera* (Rafin .) Schneider (osage orange) and its essential oil composition. Turkish J. Biochem. 2016;41(6):403–411.
42. Krızová L, Dadáková K, Kašparovská J, Kašparovský T. Isoflavones. Molecules 2019; 24(6):1076.
43. Su Z, Wang P, Yuan W, Grant G, Li S. Phenolics from the fruits of *Maclura pomifera*. Nat Prod Commun. 2017;12:1743–1745.
44. Orazbekov Y, Ibrahim MA, Mombekov S, Srivedavyasasri R, Datkhayev U, Makhatov B, IChaurasiya ND, Tekwani BL, Ross SA. Isolation and Biological Evaluation of prenylated flavonoids from *Maclura pomifera*. Evid. Based Complement. Alternat. Med. 2018.
45. Kartal M, Abu-asaker M, Dvorska M, Orhan I, Zemlicka M. LC – DAD – MS Method for Analysis of Pomiferin and Osajin , Major Isoflavones in *Maclura pomifera* (Rafin .) Schneider. Chromatographia 2009;69(3):325–329.
46. Darji K, Miglis C, Wardlow A, Abourashed EA. HPTLC determination of isoflavone levels in osage orange from the midwest and southern United States. J Agric Food Chem. 2013;61:6806–6811.
47. Tsao R, Yang R, Young JC. Antioxidant isoflavones in osage orange, *Maclura pomifera* (Raf.) Schneid. J. Agric. Food Chem. 2003; 51(22):6445-6451.
48. Hwang HS, Winkler-Moser JK, Tisserat B, Harry-O'kuru RE, Berhow MA, Liun SX. Antioxidant activity of Osage orange extract in soybean oil and fish oil during storage. J Am Oil Chem Soc. 2021;98(1):73-87.
49. Dong X, Zeng Y, Liu Y, You L, Yin X, Fu J, Ni J. Aloe-emodin: a review of its pharmacology, toxicity, and pharmacokinetics. Phytother Res. 2020; 34(2):270-281.
50. Fachel FNS, Schuh RS, Veras KS, Bassani VL, Koester LS., Henriques AT, Braganhol E, Teixeira HF. An overview of the neuroprotective potential of rosmarinic acid and its association with nanotechnology-based delivery systems: A novel approach to treating neurodegenerative disorders. Neurochem Int.2019;122:47–58.

Table I. Spectrophotometric determination of phenolic profile and HPTLC quantification of MPM

	Total phenolic content[A]	**Total flavonoid content[B]**	**Osajin Content (%)[C]**
MPM[D]	113.92 ± 2.26	66.41 ± 0.74	0.22 ± 0.01

[A] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg gallic acid equivalents (GAE) in 1 g sample.

[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg quercetin equivalents (QE) in 1 g sample.
[C] Quantification data of Osajin in MPM[D] by HPTLC analysis.
[D] MPM: 80% methanolic extract of *Maclura pomifera* (Rafin.) Schneider.

Table II. *In vitro* antioxidant activity of MPM

	DPPH scavenging act.[A]	***FRAP*** [B]	***CUPRAC*** [C]	***Total antioxidant capacity*** [C]
MPM[D]	1998.86 ± 8.02	0.191 ± 0.01	73.928 ± 0.004	114.43 ± 0.02

[A] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and IC_{50} value of the reference compound "butylated hydroxytoluene (BHT)" in DPPH scavenging activity is found to be 975,11 ± 4.16
[B] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as Mm $FeSO_4$ equivalents in 1g sample.
[C] Result was expressed as the mean of triplicates ± standard deviation (S.D.) and as ascorbic acid equivalents (AAE) in 1 g sample.
[D] MPM: 80% methanolic extract of *Maclura pomifera* (Rafin.) Schneider.

Figure 1.
[A] HPTLC chromatogram of standard osajin and test samples at 280 nm.
[B] Overlapped UV spectra osajin and *Maclura pomifera* extract.
[C] HPTLC chromatogram of standard osajin and test samples at 280 nm.

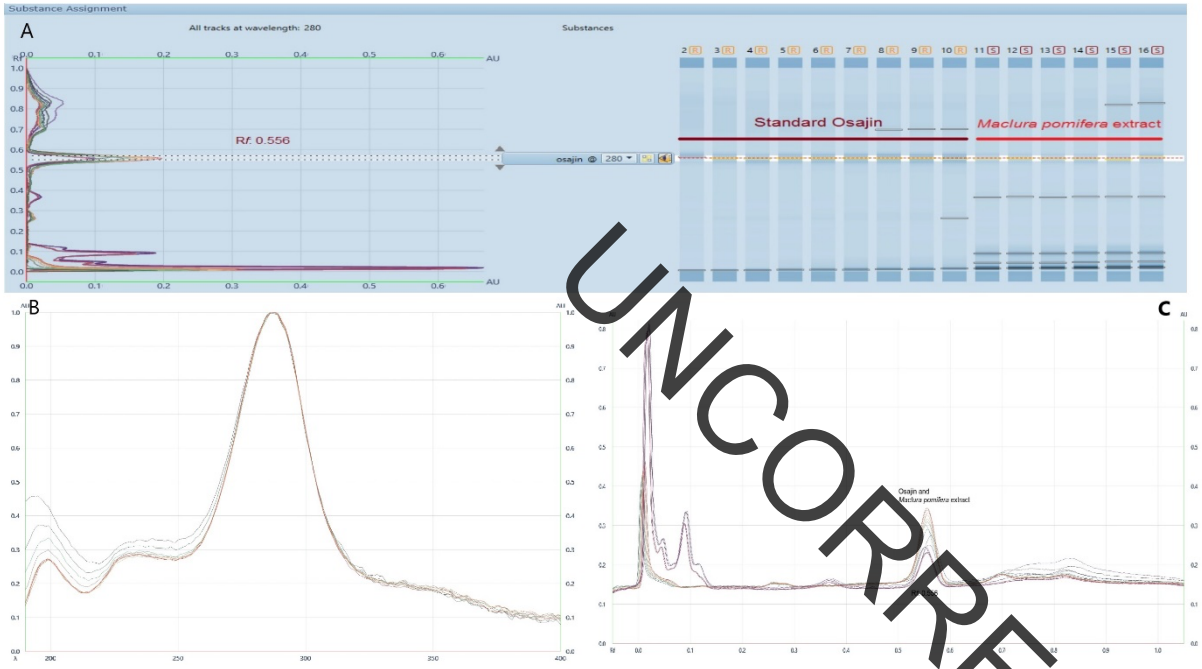

Figure 2.
In **Figure 2A**, the per cent anti-collagenase enzyme activity of MPM at a 1 mg/mL concentration at the end of 20 and 40 minutes is presented. In the statistical analysis, the $P \leq 0.01$ value was symbolized with **. % of the anti-elastase activity of 1mg/mL MPM was shown in **Figure 2B**, and **** was meant $P \leq 0.0001$. EGCG was used for positive control. EGCG was used as a positive control in anti-collagenase and anti-elastase assays (Figure 2A and B). **Figure 2C** was shown that % of the anti-hyaluronidase activity of 1mg/mL MPM at two different time points (at the end of the 40 and 80 minutes). $P \leq 0.001$ value was symbolized with ***.