Extraction and identification of flavonoids of the Prosopis farcta (L.) leaf extract and study of the anti-inflammatory and protective effect on DNA damage.

Zainab Tuama Al-Dallee1, Ula M. Noor Al- Mousawi2, Kawther T. Khalaf3

1,2 Department of pharmacognosy , Collage of pharmacy, University of Basrah, Basrah Iraq.
3Clinical laboratory science Department, College of Pharmacy, University of Basrah, Basrah, Iraq

Abstract

Background: Plant extracts have been used as therapeutic substances and have gained importance for human health around the world. The current study aims to identify flavonoids in the leaves of Prosopis farcta and to verify the anti-inflammatory efficacy of the leaf methanol extract, in addition to its ability to protect radiation-induced DNA damage in the presence of H2O2.

Methods: The reflux method was applied to extract the flavonoids in leaves of P. farcta. The type of flavonoids present in the methanol extract was fixed by thin-layer chromatography (TLC) and high-performance-liquid chromatography (HPLC) depending on the reference markers, the role of the extract as an anti-inflammatory substance was studied using the denaturation method of bovine serum albumin. The activity of different concentrations (250, 500 and 1000) micrograms/ milliliter, of P. farcta extract in protecting DNA damage induced by radiation with hydrogen peroxide and ultraviolet radiation was determined laboratory. **Results:** The TLC and calibration plot of standard luteolin, hesperidin and rutin showed that the alcoholic extract of P. farcta was found to contain luteolin, hesperidin and rutin, with the retardation factor 0.83, 0.4, 0.56 respectively. Methanolic extract of leaves at 500 µg/ml had anti-inflammatory action similar to that standard medicine aspirin. The P. farcta extract possesses protective properties against DNA oxidative stress at concentration of 1000 µg/ml.

Conclusion: Three flavonoids were found in refluxed P. farcta leaves with 80% methanol and it can be used as a potential source of natural anti-inflammatory and DNA damage protecting.

Keywords: Prosopis farcta, anti-inflammatory activity, flavonoids, and DNA damage protection

استخلاص وتحديد الفلافونويدات من مستخلص أوراق نبات Prosopis farcta (L.) ودراسة التأثير المضاد للالتهابات والحماية من تلف الحمض النووي.

زينب طعمت خلف 1 , علا محمد 2 , كوثر طعمت خلف

1,2 فرع العقاقير، كلية الصيدلة، جامعة البصرة، البصرة، العراق.
3فرع العلوم المختبرية السريرية، كلية الصيدلة، جامعة البصرة، البصرة، العراق

الخلاصة

الخلفية: تم استخدام المستخلصات النباتية كمواد علاجية واكتسبت أهمية لصحة الإنسان في جميع أنحاء العالم. تهدف الدراسة الحالية إلى تحديد الفلافونويدات في أوراق Prosopis farcta والتحقق من فعالية مستخلص الأوراق الميثانولي المضاد للالتهابات، بالإضافة إلى قدرته على حماية تلف الحمض النووي الناجم عن الإشعاع في وجود H2O2.

طريقة العمل : تم تطبيق طريقة الارتجاع لاستخراج الفلافونويدات في أوراق P. farcta. تم تثبيت نوع الفلافونويدات الموجودة في مستخلص الميثانول بواسطة كروماتوغرافيا الطبقة الرقيقة (TLC) وكروماتوغرافيا السائل عالية الأداء (HPLC) اعتمادًا على العلامات المرجعية، تمت دراسة دور المستخلص كمادة مضادة للالتهابات باستخدام طريقة تحلل ألبومين مصل البقر. تم تحديد نشاط تركيزات مختلفة (250 و 500 و 1000) ميكروجرام / مل من مستخلص P. farcta في حماية الحمض النووي من التلف الناتج عن الإشعاع ببيروكسيد الهيدروجين والأشعة فوق البنفسجية معملياً. **النتائج:** أظهرت TLC ومخطط المعايرة للوتولين القياسي والهسبيريدين والروتين أن المستخلص الكحولي لـ P. farcta يحتوي على اللوتولين والهسبيريدين والروتين، مع عامل تباطؤ 0.83 و 0.4 و 0.56 على التوالي. كان للمستخلص الميثانولي للأوراق عند 500 ميكروجرام / مل تأثير مضاد للالتهابات مماثل لذلك الذي يحدثه دواء الأسبرين القياسي. يمتلك مستخلص P. farcta خصائص وقائية ضد الإجهاد التأكسدي للحمض النووي عند تركيز 1000 ميكروجرام / مل.

الاستنتاجات: تم العثور على ثلاثة فلافونويدات في أوراق P. farcta المرتجعة مع 80٪ من الميثانول ويمكن استخدامها كمصدر محتمل لمضادات الالتهاب الطبيعية وحماية الحمض النووي من التلف.

الكلمات المفتاحية: Prosopis farcta، النشاط المضاد للالتهابات، الفلافونويدات، وحماية الحمض النووي من التلف

Introduction

The Prosopis farcta is counted as one of the most interest medicinal plants belonging to the Fabaceae family, due to its content of variety of compounds known for their biological and medicinal activity **[1].** Although there are few sources regarding optimal growing conditions for P. farcta, Seemingly this plant grows well in dried areas. Its native habitat is North Africa, Southwest Asia, Iraq, Turkey, Kuwait, and Iran **[2]** and to a large degree is spread throughout the Middle East and North Africa, mostly in the dry areas of most African and Asian countries **[3]**. P. farcta has been traded in traditional medicine to treat infections, diabetes, and measles, in addition to using it to relieve heart or chest pain. Some studies have shown the antitumor activity of P. farcta and its antioxidant and antimicrobial activity in relation to Prosopis species **[4,5].** P. farcta is also considered a medicinal plant rich in flavonoids, so it can be considered as a valuable and useful source for extracting flavonoids **[6]**.Flavonoids, which fall within the class of polyphenols, have attracted ever-increasing interest due to their widespread nature in plants, in addition to their biological efficacies, making them a sensational target for many scientific researches **[7].**

Flavonoids are secondary metabolites of plants their skeleton structure a benzo-γ-pyrone, resulting by difference synthesis ways, namely the phenylpropanoid way, the shikimate way, and the flavonoid way **[8].**

Not only do flavonoids play important ecological and physiological roles in plants, but their wide applications in the food and pharmaceutical industry make them of obvious commercial value **[9, 10].** Many studies have addressed the broad applications of these compounds on the medical scale as antibacterial, antioxidant, anti-inflammatory, antitumor, and antigenotoxic agents. In addition to its role in protecting DNA from oxidative damage **[11].** This study aims to investigate the flavonoids types that found in P. farcta plant extract and measure their anti-inflammatory and damage-DNA -protective effects.

Methods

1. Plant Sampling

Al-garma city in Basrah, Iraq the area from which samples of Prosopis farcta leaves were collected. Asst. Prof. Dr. Dr. Ula Almousawi Pharmacognosy Department, Pharmacy College, Basrah University diagnosed and verified the plant species. Fresh material of the plant sample was collected in August, cleaned, shade

dried for seven days at room temperature and crushed to medium-milled pieces and stored in airtight bottles.

2. Extraction Method

The powdered plant material 20 g was defatted using hexane, for two hours, filtration with filter paper, and the collected plant was dried at room temperature. Hot solvent extraction was used as methods of extraction using a reflux apparatus for 2 h at 60 °C using 80% methanol (solvent: plant sample ratio of 10:1 mL/g). The resulting extract was collected in a glass container and stored in the refrigerator at 4°C until the next use **[12]** with some modifications.

3. Qualitative Assessment of flavonoids in P. farcta Extract

Based on the favonoids Shinoda test, it was performed by adding conc. Hydrochloric acid to 1 ml of P. farcta methanol extract containing part of the magnesium chip where a positive result gives a pink color **[13].**

4. Thin Layer Chromatography (TLC)

The TLC was performed with two different mobile phase systems as in **(Tab.1),** standard solutions were prepared by dissolving one milligram of Luteolin, Hesperidin and Rutin standards in 2 milliliters of methanol. All plates were visualized at 366 nm under ultraviolet light, presence of luteolin, hesperidin and rutin were determined by contrasting Rf values with their respective standards.

Mobile phase	Percent
n-butanol: acetic acid: water	6 : 1.5 :7.5
Tolune : ethylacetate :acetic acid	5 : 4 : 1

Table1: mobile phases were tested

5. High Performance Liquid Chromatography for the Flavonoids Detection in Crude Extract

HPLC analysis carried out using German HPLC Knauer systems elements. Relying on Seal **[14]** as a reference. Before the final validation and real samples analysis the sample preparation, mobile phase, and stationary phase conditions were optimized.

Solutions for each of the luteolin, hesperidin and rutin standards were prepared, 10µl of each standard solution was prepared a mixture of the three standards was prepared, and a mixture of three standards solution with crude extract was injected into the HPLC system.

The detection was accomplished through elution with a mobile phase of 1% aq. acetic acid solution (Solvent A) and acetonitrile (Solvent B) (90%:10% v/v), a flow rate was 1.0 ml/min, and UV detection at 272, 280, 310 and 28°C. HPLC analysis resulted in the detection of luteolin, hesperidin and rutin together in a crude extract by comparison of the retention time to the retention time of the standard solution.

6. Anti-inflammatory in Vitro Assay

The anti-inflammatory potential of P. farcta methanolic extract was tested in vitro according to Dharmadeva et al. **[15].** Serial dilutions (250 µg/mL, 500 µg/mL, and 1000 µg/mL) were carried out on the plant extract and the reference drug (aspirin). 5.0 ml of the reaction mixture was prepared by adding phosphate buffered saline (pH 6.4) 2.8 ml, bovine serum albumin 0.2 ml and 2 ml of each different concentration from extract, the mixture were mixed gently. Aspirin was treated in the same procedure as above. Using distilled water as negative control, the reaction mixtures were placed in a water bath for 20 min at 37°C, and later, the reaction was maintained for 5 min with heating at 70°C, then, the reaction mixture was allowed to cool for 15 min at room temperature. Reaction absorbance after denaturation was recorded for each concentration at 680 nm. The percentage of protein inhibition was determined depending on absorbance of sample with respect to control as the following formula.

$$\% \text{ inhibition} = 100 \times ([Vt/Vc] - 1).$$

Vt = absorbance of test extract, Vc = absorbance of control.

7. Assay DNA Damage Protection

DNA damage study was evaluated by using blood human genomic DNA. DNA extraction kit (Geneaid) was used, to extract the DNA and we putted up steps the extraction according to the prescribed instructions by the company.

The P. farcta methanol extract protective ability was achieved according to modified method described by Al-Dallee et al. [16]. DNA with the presence of H2O2 for 20 min at room temperature had been placed directly under the UV using a UV transilluminator (300 nm), each tube contains 5 µL of 20 µg/ml of human DNA, extract in different concentrations (250, 500, and 1000 µg /ml) and 5 µL of 30% H2O2. A tube was irradiated control which did not contain extract, 1 µL aliquot genomic DNA in another tube was placed and act as a no irradiated control. All tubes were examined on 1% agarose gel and then photographed.

Results

1. Flavonoids Shinoda's- assay

Alcoholic solution of the crude extract expose the presence of flavonoids by the appearance of an orange to red color **(Fig.1).**

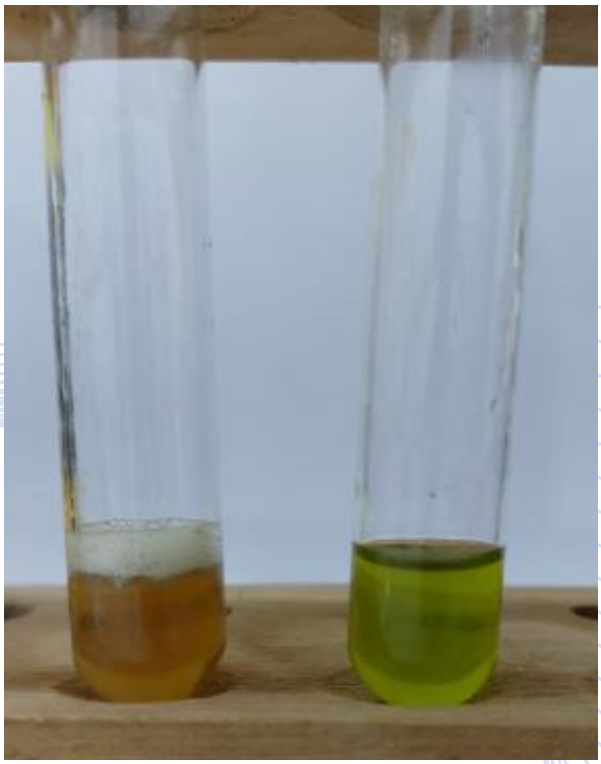

Figure1: Shinoda test result: A: positive result B: P. farcta methanol extract

2. Thin Layer Chromatography (TLC)
Chromatogram (TLC) was utilized to identify types of flavonoids in the methanol extract of P. farcta leaves, the result shown the presences of luteolin, hesperidin and rutin under UV light (365 nm). The Rf values of the identified flavonoids compared to standard are show in **Fig 2 and Tab. 2.**

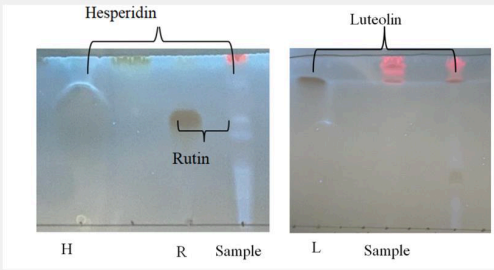

Figure2: Identification the flavonoids types in P. farcta leaves extract with TLC at UV 365 nm. Hesperidin and Rutin mobile phase toluene: ethyl acetate: acetic acid (5:4:1); Luteolin mobile phase n-butanol-acetic acid – water (6:1.5:7.5) L: Luteolin, H: Hesperidin, and R: Rutin standards

Mobile phase	Type of flavonoid	Rf value of sample	Rf value of standard
n-butanol: acetic acid: water	Luteolin	0.83	0.84
Ethylacetate : methanol: water	Hesperidin	0.4	0.45
Ethylacetate : methanol: water	Rutin	0.56	0.53

Table2: Rf value of flavonoid identified in extract and standards

3. High Performance- Liquid Chromatographic -Analysis (HPLC) for the of Flavonoids Detection in Methanol Extract
HPLC analysis revealed the presence of luteolin, hesperidin and rutin together in methanol extract of P. farcta leaves and this was done by comparing the retention time of the mentioned flavonoids with the retention time of the standard solution **(Fig.3&4).**

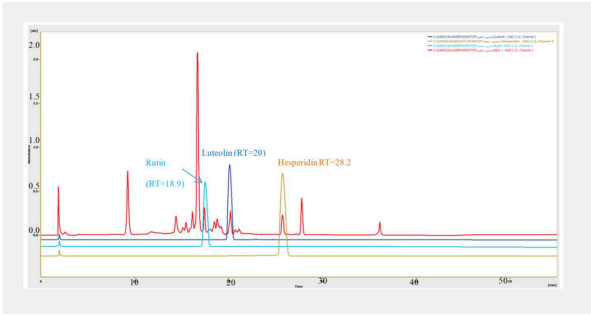

Figure3: HPLC chromatogram of of luteolin, hesperidin, and rutin standard solution with P. farcta methanol extract

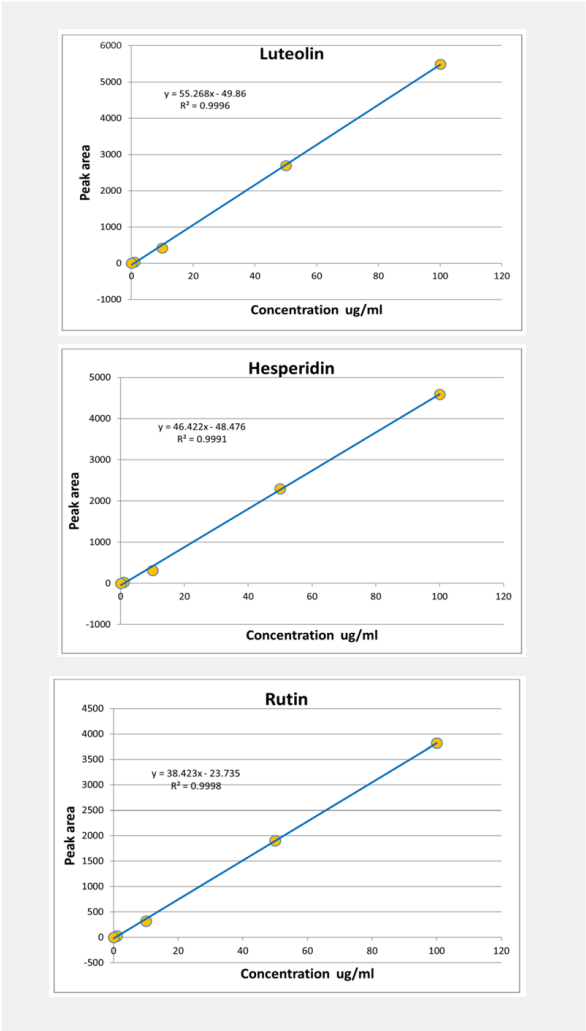

Figure 4: Calibration curve of Luteolin, Hesperidin, and Rutin standards

4. Antinfamatory activity

Effect of P. farcta methanol leaves extract at different concentrations on inhibiting albumin denaturation it is shown in **(Fig.5),** the results showed that the extract had an inhibitor activity 30%, 60% and 80% respectively, while aspirin apperences inhibitory activity 40%, 60% and 96% respectively, By comparing the results between the extract and aspirin, we notice that they are equally effective at a concentration of 500 µg/ml.

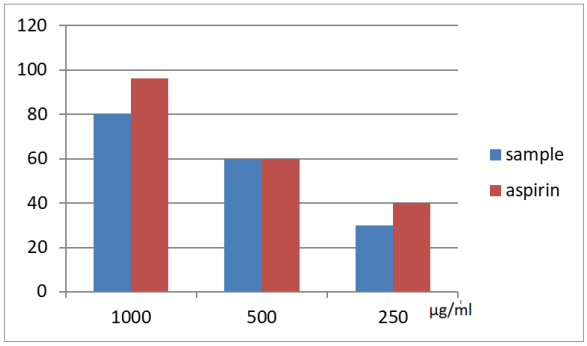

Figure 5: denaturation inhibition of P. farcta leaves methanol extract.

5. DNA Damage Protection

Reveals **(Fig 6)** DNA electrophoresis exposure to ultraviolet radiation in the presence of H2O2, whether added or not different concentrations of methanol extract. It was shown that DNA is not exposed to radiation appeared bright on the agarose gel (lane 1). From 3 to 5, the DNA was exposed to ultraviolet radiation in addition to H2O2 in the presence of different concentrations of the extract. We not there is a great deal of preservation at a concentration of 1000 µg/ml of the extract compared to bands 2 and 6 in the presence of water instead of the extract and the absence of the extract.

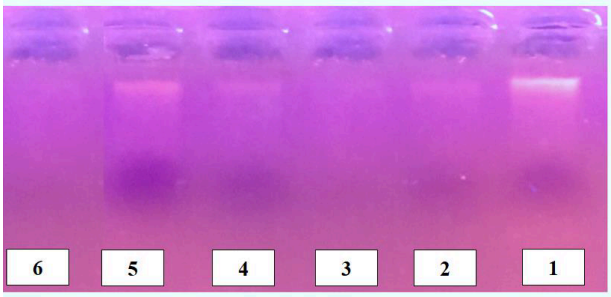

Figure 6: Electrophoregram of DNA after ultraviolet-photolysis of H2O2 (30%) in the presence or absence of the extract. Lane 1: un irradiated DNA, Lane 2: irradiated DNA + H2O2 lane 3: irradiated DNA + H2O2 + 250 µg/ml extract, lane 4: irradiated DN + H2O2 + 500 µg/ml, lane 5: irradiated DNA + H2O2 + 1000 µg/ml extract, lane 6: double distilled water

Discussion

1. Phytochemical Analysis of P. farcta Leaves Extract

The result of chemical analysis of the leaves extract of P. farcta given in (Fig.1) demonstrated the presence of flavonoids which were identified using the Shinoda test. In this test, concentrated acid plays a role in hydrolyzing flavonoid glycosides into flavonoid aglycones, which give a red or orange color after forming a complex with magnesium [17]. Among the many flavonoids identified in the leaves of medicinal plants, luteolin, hesperidin, and rutin were among the flavonoids whose presence was identified in P. farcta leaves extract. These compounds were characterized by comparing their TLC and HPLC with the propriety and reference compounds. Thin layer-chromatography is compatible for inception detection of materials before resorting to more proceeding analysis using sophisticated analytical tools **[18, 19].**

In the current study, TLC enabled the concurrent detection of luteolin, hesperidin, and rutin in P. farcta with 80% methanol extract the three different Rf flavonoids were compared with the respective flavonoid standards found in **Tab.1.** The results of HPLC analysis showed the presence of luteolin, hesperidin and rutin in the methanol extract, which means that 80% methanol is a suitable choice of solvent for extracting flavonoids. The results of this study are consistent with **[20],** which show that methanol has polar organic properties and is the best solvent to use in extracting flavonoids that are most soluble in polar solutes.

Calibration curves for each standard solution in the range of 1 to 100 µg/mL were relied upon to determine flavonoid content, and were scaled linearly for all standards over the showed concentration range. Fig. 4 display the frequency calibration curves for luteolin, hesperidin, and rutin. The process of proteins losing their quaternary, tertiary, and secondary structures is called protein denaturation. It is a process that occurs by the action of compounds or the implementation of external stress which generally leads to a loss of their biological functions **[21].**

One of the symptoms of inflammatory diseases and arthritis is protein denaturation, which is the result of the presence of auto-antigens for proteins in tissues, which is the case in some joint diseases. **[22],** as a part of this study, the potential of the leaves methanol extract to have anti-inflammatory activity was investigated, and the ability of the methanolic extract of P. farcta to inhibit protein denaturation was determined. The leaves extract showed an inhibitory activity, in comparable to the anti-inflammatory aspirin treatment; P. farcta extract has the same inhibitory activity as anti-inflammatory drugs (aspirin) at a concentration of 500 µg/ml Fig.5. This study also investigates the ability of P. farcta methanol extract to stimulate DNA repair and protect against the effects of H2O2-induced DNA damage **Fig. 6.** It is possible that the activity of P. farcta extract is due to its luteolin, hesperidin, and rutin content. Many studies indicate the biological activity possessed by these flavonoids. **[23, 24, 25].**

Conclusions

P. farcta using thin layer chromatography proved to contain flavonoid fractions in leaves. The HPLC analyses confirmed the presence of the luteolin, hesperidin, and rutin in methanol extract. The results of the present study show that the methanolic extract of P. farcta leaves possesses anti-inflammatory activity evidenced by inhibiting albumin denaturation in vitro and protects DNA against H2O2 induced oxidative damage.

Acknowledgment

We extend our sincere thanks to Central laboratory management in the College of Pharmacy, University of Basrah for the opportunity to work in the PCR laboratory

References

1- Townsend, C.C. and Guest . Flora of Iraq. Vol.3,1974 ministry of Agriculture 7& Agrarian reform. Rebublic of Iraq. P. 38- 42.

2- Gishini, M F, Azizian A, Alemzadeh A, Shabani M, Amin S, Hildebrand D. Response of Prosopis farcta to copper and cadmium stress and potential for accumulation and translocation of these heavy metals. bioRxiv .2020; 2020-11.

3- Sharifi-Rad J, Hoseini-Alfatemi S M, Sharifi-Rad M, Miri A. Phytochemical screening and antibacterial activity of different parts of the Prosopis farcta extracts against methicillin-resistant Staphylococcus aureus (MRSA). Minerva Biotecnologica. 2014; 26(4): 287-293.

4- Asadollahi K, Abassi N, Afshar N, Alipour M, Asadollahi, P. Investigation of the Effects of Prosopis farcta Plant Extract on Rat's Aorta. Journal of Medicinal Plants Research. 2010; 4(2): 142-147.

5- Al-Aboudi A, Afifi FU. Plants Used for the Treatment of Diabetes in Jordan: A Review of Scientific Evidence. Pharmaceutical Biology. 2010; 49 (3): 221-239. https://doi.org/10.3109/13880209.2010.501802.

6- Dheeb B I, Hashim S S, Hameed R K, Hasan S.W. Phytochemistry and biological activity of Prosopis farcta. Journal of Biotechnology Research Center. 2020; 14(1): 25-33.

7- Liga S, Paul C, Péter F. Flavonoids: Overview of biosynthesis, biological activity, and current extraction techniques. Plants. 2023; 12(14): 2732.

8- Alcaraz M, Olivares A, Achel D G, García-Gamuz J, Castillo J, Alcaraz-Saura M. Genoprotective effect of some flavonoids against genotoxic damage induced by X-rays in vivo: Relationship between structure and activity. Antioxidants. 2021; 11(1): 94.

9- Dias M C, Pinto D C, Silva A M. Plant Flavonoids: Chemical Characteristics and Biological Activity. Molecules. 2021; 26(17): 5377. https://doi.org/10.3390/molecules26175377.

10- Ortuno A, Arcas M C, Botia J M, Fuster M D, Del Río J. A. Increasing resistance against Phytophthora citrophthora in tangelo Nova fruits by modulating polymethoxyflavones levels. Journal of Agricultural and Food Chemistry. 2002; 50(10): 2836-2839.https://doi.org/10.1021/jf011382a

11- Alcaraz M, Olivares A, Achel D G, García-Gamuz J A, Castillo J, Alcaraz-Saura M. Genoprotective effect of some flavonoids against genotoxic damage induced by X-rays in vivo: Relationship between structure and activity. Antioxidants. 2021; 11(1): 94. doi: 10.3390/antiox11010094

12- Abidin L, Mujeeb, M, Mir S R, Khan S A, Ahmad A. Comparative assessment of extraction methods and quantitative estimation of luteolin in the leaves of Vitex negundo Linn. by HPLC. Asian Pacific Journal of Tropical Medicine. 2014; 7: S289-S293. doi: 10.1016/S1995-7645(14)60248-0

13- Jaradat N, Hussen F, Al Ali A. Preliminary phytochemical screening, quantitative estimation of total flavonoids, total phenols and antioxidant activity of Ephedra alata Decne. J. Mater. Environ. Sci, 2015; 6(6): 1771-1778.

14- Seal, T. Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves, Sonchus arvensis and Oenanthe linearis of North-Eastern region in India. Journal of Applied Pharmaceutical Science. 2016; 6(2): 157-166. doi: 10.7324/JAPS.2016.60225.

15- Dharmadeva S, Galgamuwa L S, Prasadinie C, Kumarasinghe N. In vitro anti-inflammatory activity of Ficus racemosa L. bark using albumin denaturation method. Ayu. 2018; 39(4): 239.

16- Al-Dallee Z T, Khalaf, K T, Dawood Y A, Ramadhan U H Phytochemical analysis of Portulaca oleracea L. leaves extract and study the role in protecting genomic human DNA from UV damage. J. Basrah Researches Sci. 2022; 48(1): 1-9.

17- Saptarini N, Herawati IE, Permatasari U. Total flavonoids content in acidified extract of flowers and leaves of gardenia (Gardenia jasminoides Ellis). Asian Journal of Pharmaceutical and Clinical Research. 2016; 9(1):213-5.

18- Phadungrakwittaya, R, Chotewuttakorn S, Piwtong P, Thamsermsang O, Laohapand T, Akarasereenont P.. Identification of apigenin and luteolin in Artemisia annua L. for the quality control. Siriraj medical journal. 2019;71(3): 240-245. http://dx.doi.org/10.33192/Smj.2019.37.

19- Baranowski R, Kabut J, Baranowska I. RP-HPLC and RP-TLC in the analysis of flavonoids. Chemia analityczna. 2005; 50(1): 301.

20- Agrawal P K, Agrawal C, Blunden G. Pharmacological significance of hesperidin and hesperetin, two citrus flavonoids, as promising antiviral compounds for prophylaxis against and combating COVID-19. Natural Product Communications. 2021; 16(10); 1934578X211042540. https://doi.org/10.1177/1934578X211042540.

21- Chigayo K, Mojapelo P E L, Mnyakeni-Moleele S, Misihairabgwi J M. Phytochemical and antioxidant properties of different solvent extracts of Kirkia wilmsii tubers. Asian Pacific Journal of Tropical Biomedicine.2016; 6(12): 1037-1043. https://doi.org/10.1016/j.apjtb.2016.10.004

22- Angel G R, Vimala B, Nambisan B. Antioxidant and anti-inflammatory activities of proteins isolated from eight Curcuma species. Phytopharmacology. 2013; 4(1):96-105.

23- Ruiz-Ruiz J C, Matus-Basto A J, Acereto-Escoffié P, Segura-Campos, M. R. Antioxidant and anti-inflammatory activities of phenolic compounds isolated from Melipona beecheii honey. Food and Agricultural Immunology. 2017; 28(6): 1424-1437. https://doi.org/10.1080/09540105.2017.1347148;

24- Ullah A, Munir S, Badshah S L, Khan N, Ghani L, Poulson B G, Jaremko M. Important flavonoids and their role as a therapeutic agent. Molecules.2020; 25(22): 5243. https://doi.org/10.3390/molecules25225243

25- Shanmugasundaram D, Roza J. M. Assessment of anti-inflammatory and antioxidant activity of quercetin–rutin blend (SophorOx™)–an in vitro cell based assay. Journal of Complementary and Integrative Medicine.2022; 19(3): 637-644. https://doi.org/10.1515/jcim-2021-0568