Journal of Research in Pharmacy

Research Article

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Anti-proliferative activity and phytochemical study of the methanolic extract from the pods of *Gleditsia caspica* Desf.

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Received: 22 August 2023 / Revised: 23 October 2023 / Accepted: 23 October 2023

ABSTRACT: *Gleditsia caspica* Desf. (Caesalpinaceae) is traditionally used to treat diverse diseases. This research paper aims to investigate anti-proliferative properties and phytochemical compounds of methanolic extract from the pods of *G. caspica*. MTT test was used to measure in vitro anti-proliferative effect of the soxhlet-extracted methanolic extract of *G.caspica* pods with the concentration ranging from 25-1000 µg/ml against A549 cell line. The extract was applied to SPE, HPLC preparative system, and spectral analysis (^{1}HNMR, ^{13}CNMR, and UV) for fractionation, purification, and identification of phytoconstituents, respectively. The results indicated that the extract prevented proliferation of A549 cell line dependent on time and concentration. After 48 h and 72 h, there was a significant decrease in cell viability initiated from 100 and 500 (µg/ml) concentrations in comparison to untreated control. Phytochemical assessment of the extract resulted in two alkaloids, locustoside A and saikachinoside A, and four flavonoids quercetin-3,7-diglucoside, luteolin-4',7–diglucoside, quercetin-3-glucoside, and quercetin. This experiment was an activity-guided phytochemical study. In prior studies, quercetin and quercetin-3-β-D-glucoside showed in vitro inhibitory effect on proliferation of A549 cell line. However, the cytotoxic properties of the other identified compounds have not been reported. Cytotoxic properties of *G.caspica* was previously attributed to saponins, while isolated flavonoids and alkaloids could be responsible for the extract's cytotoxic effects. This paper first explored cytotoxic activity of methanolic extract from the pods of *G.caspica* on A549 cell line and identified flavonoids and alkaloids of this plant.

KEYWORDS: *Gleditsia caspica*; Phytochemistry; Pod; Human lung cancer; A549 cell line; Apoptosis.

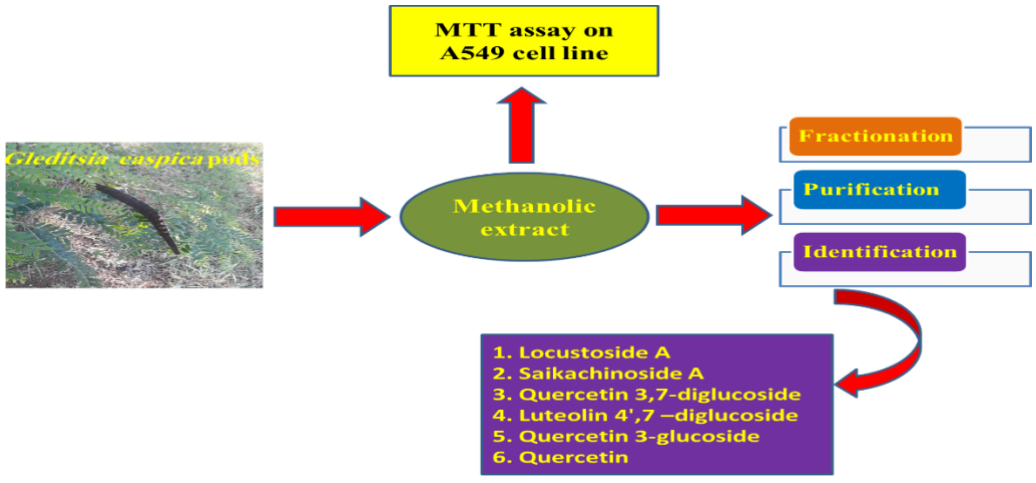

Figure 1. Graphical abstract

How to cite this article: Mirzaeidarband S, Samadishams S, Hamedeyazdan S, Eskandari Z, Ebrahimi A, Bamdad—Moghaddam S, Barar J, Omidi Y, Khodaie L, Nazemiyeh H Anti-proliferative activity and phytochemical study of the methanolic extract from the pods of Gleditsia caspica Desf.. J Res Pharm. 2024; 28(1): 304-312.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.697

1. INTRODUCTION

Cancer as a dominant health care issue triggers significant morbidity and mortality throughout the world. Cancer chemotherapy agents cause several side effects. Thus, research is undertaken to discover new chemotherapy agents with less side effects [1]. Non-small cell lung cancer is a leading cause of death and a challenging ailment to be cured [2]. The statistics indicates that the survival rate of lung cancer is low. Therefore, it necessitates to discover novel, safe, and efficient medications [3]. A549 is a commonly-used cell line explanted from the tumor in lung tissue. These cells are cultured in vitro and applied as models to explore and develop drugs against it. Medicinal plants are precious resource of drug discovery and development. Also, plant-derived constituents are the outstanding source of clinically-used anti-cancer remedies. Because of the increasing number of cancer-diagnosed cases and development of new types of cancer, further endeavor is required to assess anti-proliferative and cytotoxic herbal extracts to identify active phyto-constituents. Owing to diversified structures of herbal bioactive ingredients indicating various mechanism of action it is worth to investigate in order to unravel new plant-derived anti-cancer agents [4].

Gleditsia genus (Caesalpinaceae), with common English names of "honey locust" and "three-thorned acacia" and common Persian name of "Leilaky", comprises 14 species, 3 of which grow in Iran [5]. They are distributed in various Asian as well as American countries. Different species of this genus have been traditionally and locally utilized to cure diverse bacterial and inflammatory diseases. Mainly, mature pods and fruits as well as seeds of different species, both singly and in formulations, are mainly used to treat diarrhea, constipation, dysentery, pharyngitis, cough, asthma, and ulcers [6]. According to the findings of modern pharmacological experiments, the plants of this genus were bactericidal and fungicidal. Furthermore, they reduce inflammation, allergy, mutagenicity, and analgesia. Hence they have indicated anti-proliferative, anti-cancer, anti-tumor, and anti-mutagenic effects. The important phytoconstituents previously isolated from various species of this plant were triterpenoid saponins (a chemotaxonomic marker of the genus). Besides, sterols, flavonoids, phenolics, galactomannans, neolignans, and alkaloids were characterized from different species of *Gleditsia* [3-7]. *Gleditsia caspica* Desf. known as the "Caspian honey locust", is a deciduous tree growing up to 12 m (Figure 2). This temperate-zone legume species are found in forests of the Caspian Sea as well as Southeastern Azarbaijan (Iran) (1). According to the results of previous studies, the extract from the thorns of *G. sinensis* indicated anti-tumor effects on diverse cancer cells and animal models. Hence, isolated phytochemicals from mentioned extract induced apoptosis, inhibited cancer cell lines, and proliferation [7]. Also, prior studies showed that triterpenoid saponins from *G.sinensis* had anti-proliferative activities. Saponins related to triterpenoid group isolated from the fruits of *G.caspica* decreased cell viability, caused apoptosis, and arrest in the activities of cell cycle. So far, however, to our understanding, there was no study regarding the cytotoxicity and anti-cancer activities of methanolic extract from the pods of *G.caspica* as well as isolation and identification of the flavonoids and alkaloids of this plant. Thus, this study aimed to focus on assessment of anti-proliferative and anti-cancer potential of this herb on A-549 and isolation of flavonoids as well as alkaloids from the methanolic extract of its pods.

Figure 2. *Gleditsia caspica* Desf. known as the Caspian honey locust.

2. RESULTS

2.1. MTT assay

Figure 3 indicates the results of MTT assay to evaluate anti-proliferative activity of different concentrations from the MeOH extract of the pods of *G. caspica* on A549 cell line.

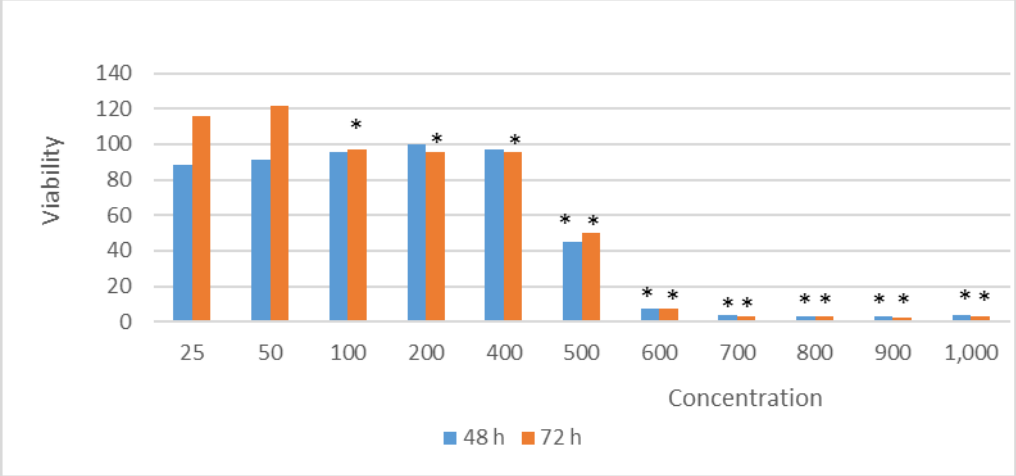

Figure 3. A549 cell viability after their exposure to MeOH extract within 48 and 72h.

* Statistical significance was set at $p < 0.05$.

The results demonstrated concentration and time-dependent anti-proliferative activity of MeOH extract on A549 cell line. After 48 h, cell viability was significantly reduced from the concentration of 500 µg/ml and higher than that when compared to control. In the concentration of 500 µg/ml, approximately 50% inhibitory effect could be seen on the proliferation of A549 cell lines. After 72h, cell viability was significantly declined from 100 µg/ml and higher than that compared to control. At the concentrations equal and higher than 700 µg/ml, the cell viability was reduced to almost 3% compared to untreated control cells after both 48 and 72h.

2.2. Phytochemical study

The results of MTT assay led to phytochemical study of methanolic extract of this herb. The results of phytochemical screening of the fractions of methanolic extract was indicated below.

2.2.1. Compound 1 Locustoside A

Locustoside A (5 mg) (Figure 4) was cream amorphous solid. ^{1}HNMR (CD3OD, 200 MHz) data of its aglycone moiety were δ 8.14(1H, S, H-8), δ 5.40 (2H, t, J=7.1Hz, H-2'), δ 4.6 (1H, d, J=7Hz, H-1'), δ 4.7 (1H, d, J=7Hz, H-1'), δ 1.80 (3H, S, H-5'), δ 1.37 (3H, S, H-4'). 1HNMR data of glucose were δ 5.57 (1H, d, J = 8.4 Hz, H-1''), 3.2-3.8 (overlapped peaks for H2''-H3''-H4''-H5''-H6''). ^{13}CNMR (50 MHz, CD3OD as solvent) data of aglycone were δ 155.4(C-6), δ 154.1 (C-4), δ 153.4 (C-2), δ 143.6 (C-8), δ 105.2 (C-5), δ 139.8(C-3'), δ 120.9 (C-2'), δ 40.1(C-1'), δ 20.3 (C-4'), and δ 20.3 (C-5'). 13CNMR data of glucose moiety were δ 87.4 (C-1''), δ 79.7 (C-5''), δ 76.5 (C-3''), δ 72.9 (C-2''), δ 68 (C-4''), and δ 60.4(C-6''). UV, 1H NMR, and 13C NMR data were in agreement with the published data [10].

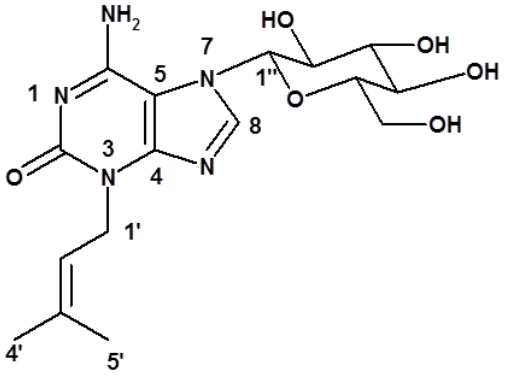

Figure 4. Locustoside A

2.2.2. Compound 2 Saikachinoside A

Saikachinoside A (4 mg) (**Figure 5**) was cream amorphous solid. Data elucidated from ^{1}HNMR (CD3OD, 200 MHz) of aglycone moiety were δ 8.14 (1H, S, H-8), δ 5.26 (2H, t, J=7.1Hz, H-2'), δ 4.6 (2H, d, J=7Hz, H-1'), δ 4.32 (2H, S, H-4'), δ 1.37 (3H, S, H-5'). 1HNMR data of glucose were δ 5.43 (1H, d, J= 8.3 Hz, H-1''), 3.2-3.8 (overlapped signal patterns for H2''-H3''-H4''-H5''-H6''). ^{13}CNMR (CD3OD, 50 MHz) data of aglycone were δ 155.4(C-6), δ 154.1 (C-4), δ 153.4 (C-2), δ 143.6 (C-8), δ 105.2 (C-5), δ 139.8(C-3'), δ 120.9 (C-2'), δ 40.1(C-1'), δ 58.9 (C-4') δ 29.3 (C-5'). 13CNMR data of glucose moiety were δ 87.4 (C-1''), δ 79.7 (C-5''), δ 76.5 (C-3''), δ 72.9 (C-2''), δ 68 (C-4''), δ 60.4(C-6''). UV, 1H NMR, and 13C NMR data were in agreement with the published data [10].

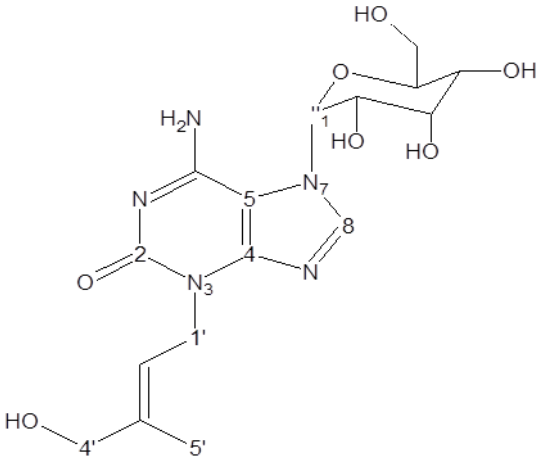

Figure 5. Saikachinoside A

2.2.3. Compound 3 Quercetin 3,7-diglucoside

Quercetin-3,7-diglucoside (4 mg) (**Figure 6**) was yellow amorphous solid. ^{1}HNMR (CD3OD, 200 MHz) data of aglycone were δ 7.75 (1H, dd, J = 8.20, 2 Hz, H-2'), δ 7.65 (1H, dd, J = 8.5, 2.1 Hz, H-6'), δ 6.90 (1H, d, J = 8.40 Hz, H-5'), δ6.43 (1H, d, J = 1.9 Hz, H-8), δ 6.24 (1H, d, J= 2Hz, H-6). ^{1}HNMR data of glucose moiety were δ 5.29 (1H, d, J = 7.8Hz, H-l''), δ5.20 (1H, d, J = 7.4Hz, H-l'''), δ 3.2-4.2 (overlapped peaks for H2''-H3''-H4''-H5''-H6''). 13CNMR (CD3OD, 50 MHz) data of aglycone were δ 181.39 (C-4), δ 163.85 (C-7), δ 164.62 (C-2), δ 161.77 (C-5), δ 158.13 (C-9), δ 148.44 (C-4'), δ 144.90 (C-3'), 133.01 (C-3), δ 121.98 (C-1'), δ 118.88 (C-6'), δ 115.37 (C-5'), δ 112.74 (C-2'), δ 102.93 (C-10), δ 98.71 (C-6), δ93.59 (C-8). 13CNMR data of first glucose were 101.3 (C-l''), 74.5 (C-2''), 76.7 (C-3''), 70.1(C-4''), 77.4 (C-5''), and 61.3 (C-6''). ^{13}CNMR data of second glucose were 100.4 (C-l'''), 73.7 (C-2'''), 75.5 (C-3'''), 70.3 (C-4'''), 77.5 (C-5'''), and 61.0 (6'''). Ultraviolet spectra of this compound solved in MeOH exhibited band II and band I with λmax of 271 and 350 nm respectively. After addition of NaOMe, λmax of band II and band I were 266 and 405 nm, successively; + NaOMe after 5 min, 265, 404 nm; + AlCl3

276, 390 nm; + AlCl3/HCl 275, 385 nm; after addition of NaOAC 269, 365 nm; + NaOAC/H3BO3 270, 348 nm. The spectrum of ultraviolet radiation of compound 3 solved in MeOH is indicative of flavonol derivatives. Studying UV spectra data indicated 4'-OH, since NaOMe yielded bathochromic shift of 55 nm in band I. 25 nm band I bathochromic shift was resulted by adding AlCl3. When HCl was added, it triggered return of band I to the last pattern. Therefore, it could be ascribed to two hydroxyl groups in the ortho position. Bathochromic shift in band II was noticed after addition of NaOAC, which indicated glycosylation at 7-hydroxyl group.

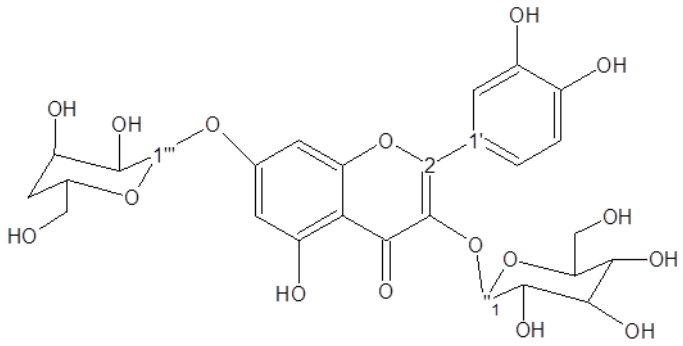

Figure 6. Quercetin-3,7-diglucoside

2.2.4. Compound 4 Luteolin -4',7-diglucoside

Luteolin-4',7-diglucoside (6 mg) (**Figure 7**) was a yellow amorphous solid. Data extracted from ^{1}HNMR (CD3OD, 200 MHz) of aglycone were δ 7.48 (1H, dd, J = 8.20, 2 Hz, H-2'), δ 7.46 (1H, dd, J = 8.5, 2.1 Hz, H-6'), δ 6.90 (1H, d, J = 8.40 Hz, H-5'), δ6.43 (1H, d, J = 1.9 Hz, H-8), δ 6.24 (1H, d, J= 2Hz, H-6). Data of 1HNMR of glucose moiety were δ 5.47 (lH, d, J = 7.4Hz, H-l''), 3.1-4.2 (owing to overlapping, peaks were not clear for H2''-H3''-H 4''-H5''-H6''). 1HNMR data of another glucose were δ 5.39 (lH, d, J = 7.4Hz, H-l''), 3.2-4.2 (over lapping peaks for H2''-H3''- H4''-H5''-H6''). Data elucidated from ^{13}CNMR (CD3OD, 50 MHz) of Aglycone were δ 181.99 (C-4), δ 163.95 (C-7), δ 163.89 (C-2), δ 161.69 (C-5), δ 156.95 (C-9), δ 148.95 (C-4'), δ 144.01 (C-3'), 142.01 (C-3), δ 122.10 (C-1'), δ 118.75 (C-6'), δ 115.42 (C-5'), δ 112.74 (C-2'), δ 102.93 (C-10), δ 102.33 (C-3), δ 98.71 (C-6), δ93.59 (C-8). 13CNMR data of first glucose were 101.3 (C-l''), 74.5 (C-2''), 76.7 (C-3''), 70.1(C-4''), 77.4 (C-5''), 61.3 (C-6''). 13CNMR data of second glucose were 100.4 (C-l'''), 73.7 (C-2'''), 75.5 (C-3'''), 70.3 (C-4'''), 77.5 (C-5'''), 61.0 (C-6'''). Regarding ultraviolet spectra, λmax of bands II and I were as follows (MeOH as solvent): 277 and 342 nm, respectively. + NaOMe 278, 398; + NaOMe after 5 min, 280, 402; + AlCl3 274, 355; + AlCl3/HCl 275, 352; + NaOAC 276, -; + NaOAC/H3BO3 275, 343. UV spectrum of compound 4 solved in MeOH is indicative of flavone derivatives. For the next step, NaOMe was added and 58nm bathochromic shift in band I was identified. This result showed presence of hydroxyl on B ring. Addition of AlCl3 produced 15 nm bathochromic shift in band I. Addition of HCl did not cause any return to last pattern. Therefore, it could be elucidated that there is no 3', 4' ortho-dihydroxy moiety on B ring. NaOAC resulted no bathochromic shift in band II, indicative of 7-OH glycosylation.

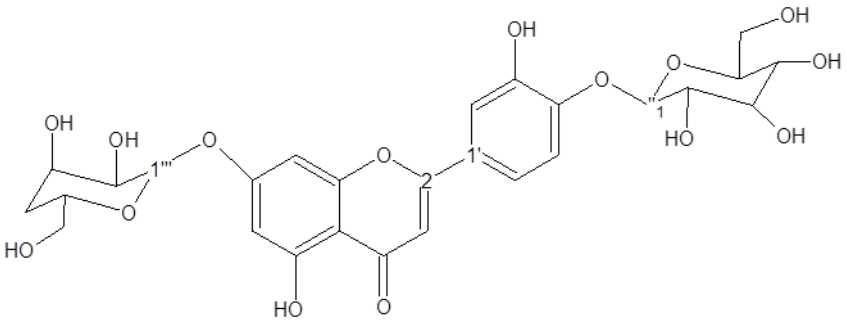

Figure 7. Luteolin-4',7–diglucoside

2.2.5. Compound 5 Quercetin 3-glucoside

Quercetin-3-glucoside (10 mg) (**Figure 8**) was yellow amorphous solid. Data extracted from 1H NMR (CD3OD, 200MHz) for aglycone were δ 7.78 (H, d, J = 2.1 Hz, H-2'), δ 7.62 (H, dd, J = 8.4, 2.1 Hz, H-6'), δ 6.96 (1H, d, J = 8.4 Hz, H-5'), δ 6.43 (1H, d, J = 2.0 Hz, H-8), δ 6.24 (1H, d, J = 2.1 Hz, H-6), δ 5.20 (1H, d, J = 6.6 Hz, H-1''), δ 3.20–4.20 (owing to overlapping, peaks were not clear for H2''-H3''-H 4''-H5''-H6'').13CNMR (CD3OD, 50 MHz) data of aglycone were δ 177.4 (C-4), δ 164.10 (C-7), δ 156.3 (C-2), δ 161.10 (C-5), δ 157.3 (C-9), δ 150.8 (C-4'), δ 146.01 (C-3'), 134.5 (C-3), δ 121.0 (C-1'), δ 123.2 (C-6'), δ 115.0 (C-5'), δ 117.2 (C-2'), δ 102.93 (C-10), δ 98.70 (C-6), δ93.60 (C-8), 104.1(C-10), δ 98.71 (C-6), δ93.59 (C-8). 13CNMR data of glucose were 104.0 (C-l''), 76.0 (C-2''), 78.9 (C-3''), 71.4(C-4''), 78.5 (C-5''), 62.5 (C-6''). Ultraviolet spectra of this phytochemical, solved in methanol resulted bands II and I with λmax of 257 and 350nm, respectively; + NaOMe 268, 409; + NaOMe after 5 min, 272, 402; + AlCl3 274, 429; + AlCl3/HCl 275, 401; + NaOAC 277, 390; + NaOAC/H3BO3 273, 385. Ultraviolet spectra of methanolic solution of compound 5 was characteristic of flavonol derivatives. After addition of NaOMe and observation of 59nm bathochromic shift in band I, existence of OH on B ring was elucidated. AlCl3 yielded 79 nm bathochromic shift of band I. When HCl was added, it triggered band I hypsochromic shift compared to AlCl3. Thus, it could be elucidated that there are both 3', 4'- ortho-dihydroxy and 5-OH moieties in this structure. Inclusion of NaOAC resulted 20nm bathochromic shift in band II, indicative of 7-OH structure.

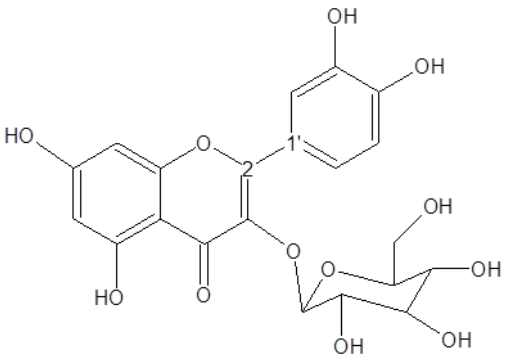

Figure 8. Quercetin-3-glucoside

2.2.6. Compound 6 Quercetin

Quercetin (11 mg) (**Figure 9**) was amorphous yellow solid. Data elucidated from 1HNMR (CD3OD, 200MHz) were δ 7.75 (H, d, J = 2.1 Hz, H-2'), δ 7.59 (H, dd, J = 8.4, 2.1 Hz, H-6'), δ 6.89 (1H, d, J = 8.4 Hz, H-5'), δ 6.39 (1H, d, J = 2.0 Hz, H-8), δ 6.20 (1H, d, J = 2.1 Hz, H-6). Data extracted from 13CNMR (CD3OD, 50 MHz) were δ 178.2 (C-4), δ 164.8 (C-7), δ 160.3 (C-2), δ 162.10 (C-5), δ 158.3 (C-9), δ 149.7 (C-4'), δ 144.57 (C-3'), 133.0 (C-3), δ 121.0 (C-1'), δ 123.3 (C-6'), δ 115.0 (C-5'), δ 116.2 (C-2'), δ 104.93 (C-10), δ 98.70 (C-6), δ93.60 (C-8). Ultraviolet spectra of this component solved in methanol resulted bands II and I with λmax of 256 and 350nm, respectively. + NaOMe 260, 400; + NaOMe after 5 min, 272, 402; + AlCl3 274, 420; + AlCl3/HCl 271, 398; + NaOAC 280, 390; + NaOAC/H3BO3 273, 385. Ultraviolet spectra of compound 6 (solved in MeOH) were indicative of flavonol derivatives. Inclusion of NaOMe and 50nm band I bathochromic shift elucidated presence of hydroxyl group on B ring. When AlCl3 was added, 70 nm bathochromic shift of band I was produced. When HCl was added, band I showed hypsochromic shift compared to +AlCl3. Thus, it could be elucidated that there are both 3', 4'- ortho-dihydroxy and 5-OH moieties in this structure. Inclusion of NaOAC showed 23nm bathochromic shift in band II, indicative of 7-OH structure.

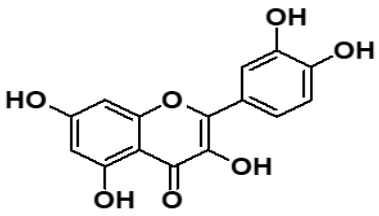

Figure 9. Quercetin

3. DISCUSSION

SPE pursued by a reversed-phase prep-HPLC analysis of the F1-20%, and F2-40% yielded two alkaloids, including lucostoside A and saikachinoside A, and four flavonoids namely, quercetin-3,7-diglucoside, luteolin-7,4'-diglucoside, quercetin-3-glucoside, and quercetin. The isolated phytoconstituents' chemical structure was determined via UV spectroscopy, ^{1}HNMR, and ^{13}CNMR. To the best of our perception, neither of the mentioned phytochemicals was previously identified from this species. Thus, this report could be deemed the first report of the mentioned phytochemicals. In previous manuscripts, oleanane type triterpenoid saponins, seven bisdesmosidic triterpenoid saponins including caspicaosides E–K, as well as sapiens C', E', and Gleditsioside I, B, C, and Q were purified from MeOH extract of fruits of *G. caspica* [13-15]. In previous manuscripts, alkaloids and phenolic compounds were characterized from various species of Gleditsia. Phytochemical study of the MeOH extract of *G. japonica* seeds yielded isoguanine glycosides with an N3-prenyl group, including locustoside A and B, saikachinoside A, B, and C [10]. Furthermore, phytochemical assessment of ethyl acetate extract of the thorns of *G. sinensis* brought about the purification of ellagic acid glycosides, flavanocoumarin, and flavonoids, namely quercetin and luteolin [16]. Another phytochemical study on the spines of *G. sinensis* revealed the presence of phenolics, such as ethyl gallate, caffeic acid, dihydrokaempferol, eriodictyol, quercetin, 3,3',5',5,7-pentahydroflavanone and (-)-epicatechin [8]. Chemical investigations on phytoconstituents of hydroalcoholic extract of the leaves of *G. triacanthos* brought about purification of eight flavone glycosides, namely vicenin-I, vitexin, isovitexin, orientin, isoorientin, luteolin-7-O-ß-glucopyranoside, luteolin-7-O-ß galactopyranoside, apigenin-7-O-ß-glucopyranoside, as well as two flavone aglycones including luteolin and apigenin [3]. Even though phenolic compounds and flavonoids are distributed in various species of the Gleditsia genus, to our knowledge, quercetin-3,7-diglucoside, luteolin-7,4'-diglucoside, and quercetin-3-glucoside were purified for the first time from the Gleditsia genus in this phytochemical study. However, locustoside A and saikachinoside A were identified from *G. japonica* seeds [10] and quercetin was isolated from the spines of *G. sinensis* in previous manuscripts [8].

MTT assay was employed to demonstrate the cytotoxicity of the MeOH extract of the pods of *G.caspica*. This approach is a technically simple, accessible, and reliable assay to measure and screen cell viability and cytotoxicity of various agents. The results of this assay led to phytochemical study of methanolic extract, which could be basis for introduction of effective chemotherapeutic agents. Results of this study indicated the dose and time-dependent anti-proliferative activity of the methanolic extract of *G.caspica* pods and reduction of the viability of A549 cell line.

Since this experiment is an activity-guided phytochemical study, cytotoxic activity of the methanolic extract led phytochemical study of it and isolation of six phytochemicals. The cytotoxic activity of the phytochemicals was investigated from the databases. Previous studies indicated that quercetin, a flavonol, showed an in vitro cytotoxic effect on A549 cell line. Quercetin declined growth of the cells and increased the level of apoptosis. Also, it triggered cell cycle arrest in subG1 phase [9]. Moreover, according to the prior studies, quercetin-3-β-D-glucoside showed growth inhibitory activity on A549 cells. Its growth inhibitory activity was more potent than quercetin itself, indicating that glucose moiety increased the anti-proliferative activity of quercetin [10]. On the other hand, investigation of previous publications indicated that there was no evidence regarding anti-proliferative activity of the quercetin-3,7-diglucoside and luteolin-4',7–diglucoside. However, results of this study could encourage further investigations on anti-proliferative activities of two aforementioned flavonoids. Moreover, there are some evidence illustrating that combination of flavonoids are more effective in reduction of cell proliferation in different cell lines [11]. Yet, combination of the identified flavonoids, both glycosylated and aglycons, has not been investigated, however it could be assessed in future studies. Hence, there was no evidence regarding anti-proliferative activity of the isolated alkaloids, Locustoside A and Saikachinoside A. According to prior investigations, caspicaosides L, M and N, which are triterpenoid saponins, identified from *G. caspica* pods indicated anti-proliferative effect against various cell lines. While, in this experiment, methanolic extract of *G.caspica* pods containing alkaloids and flavonoids illustrated anti-proliferative activity which could be indicative of the role of the aforementioned phytoconstituents on cytotoxic activity of the herb.

4. CONCLUSION

To conclude, in this study, the methanolic extract of *G.caspica* pods indicated anti proliferative activity against A459 cell line. That result led to phytochemical study of the mentioned extract and flavonoids and alkaloids were identified. Although in previous experiments the cytotoxic effects of *G.caspica* were attributed to triterpenoid saponins, the results of this study illustrated that alkaloids and flavonoids are also responsible

for anti-proliferative activity of this herb. This paper first explored cytotoxic activity of methanolic extract from the pods of *G.caspica* on A549 cell line and identified flavonoids and alkaloids of this plant. Further investigations are required to assess cytotoxic activities of the isolated compounds, especially glycosylated flavonoids and alkaloids on A459. Thus, the relation between identified compounds, traditional uses and cytotoxic activities would be elucidated.

5. MATERIALS AND METHODS

5.1. Plant material and extraction

Gleditsia capsica Desf. pods were assembled from Tabriz, East Azerbaijan province, Iran, in 2018. A sample (TBZFP 1139) for this collection was preserved at the herbarium of the faculty of pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. Pods of *G.caspica* (250 g) were air-dried, crushed, and extracted by soxhlet apparatus using solvents of hexane (8 h), dichloromethane (8 h), and methanol (MeOH) (8 h), respectively. Rotary evaporator was employed to obtain concentrated extracts at temperature around 45°C. This process resulted in 8.01 g n-hexane, 3.93 g dichloromethane, and 21.94 g MeOH extracts.

5.2. Cell viability assessment using MTT assay

Cell growth inhibition by methanolic extract was assessed by MTT assay against A549 cell line. MTT assay is a colorimetric test in which tetrazolium salts are reduced by the dehydrogenase enzymes of the active cells. After solubilization of formazan and its quantification by spectrophotometer the viability of cells and their normal function is revealed [8].

A549 cell line is obtained from lung tumor and models non-small cell lung cancer. It was acquired from National Cell Bank of Pasteur Institute of Iran. The cultivation of cells was performed in RPMI 1640 growth medium (Gibco, UK) enriched with 10% FCS (fetal calf serum). For the inhibition of contamination, 100 units/ml penicillin and 100 µg/ml streptomycin were added to culture container incubated at 37°C in humidified condition and supplemented with 5% CO_2. When 70-80% of cultured containers were covered with cells (70-80% confluency), the cells were released from the container using trypsin/EDTA (0.05%, 1ml). They were seeded 15000 cells per well at 96-well plate. After 24h, increasing concentration of the extract ranging from 25-1000 µg/ml was added to the plates. They were prepared by adding 1% methanol and were incubated for 24, 48, and 72 hours. This assay was carried out twice in triplicate. Negative control groups were treated with methanol 1%, which was the solubilizing agent.

Data were expressed as the mean ± SEM of results obtained from three independent experiments. P-values less than 0.05 were considered statistically significant. SPSS version 20 software was used for statistical analysis. Doxorubicin was used as positive control.

5.3. Fractionation of MeOH extract

This stage was carried out by solid phase extraction (SPE) technique using C18 cartridge (Sep-Pak, Vac 35 cc, 10 g). 2 g dried MeOH extract of pods was applied for the mentioned extraction. The chosen solvent system was gradient elusion using 200 ml of MeOH-water mixtures including 10: 90 (F1-10%), 20: 80 (F2-20%), 40: 60 (F3-40%), 60: 40 (F4-60%), 80: 20 (F5-80%) and 100: 0 (F6-100%). For removal of solvents of eluted fractions, rotary evaporator was used at a temperature around 45°C. For increasing the amount of accessible MeOH-water fractions, fractionation was carried out with an extra 2 g of MeOH extract resulting 5.90 g (F1-10%), 0.39 g (F2-20%), 0.27 g (F3-40%), 0.09 g (F4-60%), 0.10 g (F5-80%), and 0.18 g (F6-100%).

5.4. Purification and identification of phytochemicals

Purification of phytochemicals of 20% and 40% SPE fractions was carried out by subjecting them to preparative HPLC (Reversed-phase, Shimadzu, 2100 pump), equipped with a PDA detector (Shimadzu SPD-M10A) as well as a column of YMC-Pack pro-ODS preparative (250mm*20mm, S-5µm, 12µm). (A) MeOH and (B) water were two components of the mobile phase. To isolate lucostoside A (Figure 3), saikachinoside A (Figure 4), and 3,7-glucosyl quercetin (Figure 5) from F2-20% fraction, an ensuing program of mobile phase was carried out over 64 min run: 10% B changed to 30% within 50min and maintained there for 12 min, then 30% changed to 10% within 2 min. For separation of 7,4'-glucosyl lutein (Figure 6), 3-glucosyl quercetin (Figure 7), and quercetin (Figure 8) from F3-40% SPE fraction, a mobile-phase program over a 64 min run time was used: 30% B surged to 50% within 20 min, it maintained there for 12 min, then B was decreased to 30% within 2 min. The flow rate was 15 ml/min and the injection volume was and 1 ml. ^{1}H and ^{13}C NMR spectra were obtained by a 200MHz Bruker-spectrospin instrument to determine atom and bond arrangement of the

phytoconstituents, and UV-visible (Shimadzo2100). Furthermore, a comparison was carried out with the previously published data of respective compounds [11-12]. Besides, shift reagents were utilized to establish the site of attachments and more confirmation. Shift reagents included NaOMe (Merck), $AlCl_3$, $AlCl_3$/HCl (Merck) NaOAC, and NaOAC/H_3BO_3 (Merck).

Acknowledgements: This research was performed as part of a Pharm D thesis at the Pharmacy faculty of Tabriz University of Medical Sciences with Grant No. 60884. The authors confirm that the data supporting the findings of this study are available within the mentioned thesis.

Author contributions: Concept – H.N., J.B, Y.O.; Design – H.N., J.B., Y.O.; Supervision – H.N., J.B, Y.O; Resources – H.N., J.B, Y.O.; Materials – H.N., J.B, Y.O; Data Collection and/or Processing – S.H., A.E., S.B.M., Z.E.; Analysis and/or Interpretation – H.N., J.B, Y.O.; Literature Search – H.N., L.Kh.; Writing – L.Kh.; Critical Reviews – H.N., J.B, Y.O. L.Kh.

Conflict of interest statement: there is no conflict of interest.

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