Evaluation of in vitro anti-cancer effects of *Styphnolobium japonicum* root extract in human colon (HT-9), brain (U-87), and prostate (PC-3) cancer cell lines

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**ABSTRACT**

**Background and Aims:** *Styphnolobium japonicum* (L.) Schott. (*Sophora japonica*) is a medicinal plant applied for various diseases, in the traditional medicine field. The evaluation of methanol extract of *S. japonicum* root derived from the Pharma Grade plant drug, was performed in terms of various *in vitro* biological activities.

**Methods:** The LC-MS analysis was used for the chemical characterization of the methanol extract. The anti-cancer activity was evaluated in colon (HT-9), brain (U-87), and prostate (PC-3) cancer cells by Cell Titer Glo viability assay (Promega) and western blot analysis of PARP (Poly ADP-ribose polymerase) cleavage.

**Results:** The relative amounts of matrine and oxymatrine in the extract were found as 0.49±0.006 mg/mL and 0.27±0.016 mg/mL, respectively. The *S. japonicum* extract showed 53.17±0.97 mg of gallic acid (GA)/g corresponding to the total phenolic amounts, resulting in relatively moderate antioxidant activity (1.94±0.23 and 2.79±0.15 mg/mL) on the *in vitro* 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS•) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assays. Treatment with 10 mg/mL *S. japonica* root extract for 24h resulted in a significant decrease in cell viability. The cell viability of U-87, HT-29, and PC-3 cancer cell lines was determined as 35±2.21%, 14±2.11%, and 46±5.67%, respectively. The extract showed 5.104, 5.012 and 0.555 mg/mL IC50 values for HT-29, U-87, and PC-3 cell lines, respectively. Particularly, the IC50 value of PC-3 cancer cell line was significantly lower than the healthy human fibroblast cells. In further, the apoptosis in *S. japonicum* root extract treated PC-3 cells was detected through flow cytometry analysis of Annexin V positive cells and western blot analysis of PARP cleavage.

**Conclusion:** It can be concluded that the methanol extract in determined doses induces the apoptosis of the PC-3 cancer cells, without any significant cytotoxic effect on healthy human fibroblast cells. In addition, the LCMS analysis showed the presence of matrine and oxymatrine, which are known for their anticancer activity. To the best of our knowledge, these are the first preliminary results indicating the possible use of *S. japonicum* root extract. Thus, the methanol extract can be further studied for its therapeutic potential of primarily prostate and other cancer types.

**Keywords:** Cytotoxicity, *Styphnolobium japonicum* (*Sophora japonica*), antioxidant, cancer cell lines, western blotting, flow cytometry analysis

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INTRODUCTION

Cancer is a serious health burden and is responsible for the second leading cause of death worldwide. A World Health Organization (WHO) report in 2003 stated that cancer rates could further increase by 50% to 15 million during the year 2020 (Verma & Singh, 2020). Traditional medicinal plants and their natural components have been under special scientific research interests during recent years. Application of aromatic and medicinal plants in phytotherapy is typically due to their numerous biological activities such as antiviral, antibacterial, antitumorogenic, and antioxidant properties. (Nasrollahi, Ghereishi, Ebrahimabadi, & Khooobi, 2019). Naturally derived anticancer chemotherapeutic products being currently used in cancer management such as vincristine, vinblastine, irinotecan, etoposide, paclitaxel, and epipodophyllotoxin occupy a crucial position because of their limited side effects and anti-multidrug resistance (Cragg & Newman, 2005; Nobili et al., 2009).

*Styphnolobium japonicum* (Sophora japonica) is a plant known as Chinese Scholar Tree which also grows in Asian countries such as Korea, and Japan. It belongs to the *Fabaceae* family and is used in Traditional Chinese Medicine. Monographs of the herbal drug have been published in the Materia Medica as well as the European Pharmacopoeia. Fruits, roots, and bark preparations are commonly used to treat haemorrhoids, haematuria, arteriosclerosis, headache, hypertension and as well as a haemostatic agent in Korean traditional medicine (He et al., 2016; Kim & Yun-Choi, 2008).

More than 150 chemical compounds have been characterised from *S. japonicum* such as isoflavonoids, flavonoids, alkaloids, triterpenes, and other compounds (He et al., 2016). Especially the roots are rich in quinolizidine alkaloids. Matrine and oxymatrine are the characteristic constituents of the root extract and were reported for their diverse biological and pharmacological activities (Pelletier, 1991). There are studies on the sedative, inotropic, antiprettic, antitumor effects, antinociceptive activity among others (Ding, Liao, Huang, Zhou, & Chen, 2006; Higashiyama et al., 2005; Ma et al., 2008). Clinically, oxymatrine is known to be more active than matrine. In previous studies, it has been recorded that oxymatrine can regulate cardiac arrhythmias. Matrine is used against eczema, psoriasis, and neurodermatitis in combination with other anti-inflammatory combinations (Ting, Ruwei, Guoyong, Meizhen, & Songhua, 2002). In previous studies, matrine showed in vitro activities in cervical cancer research (Zhang, Jiang, Yan, Liu, & Zhang, 2015). In another study, it was found that matrine inhibited the growth of MCF-7 breast cancer cells with MTT assay. It was determined that the MCF-7 cell cycle changed 48 hours after the administration of matrine and is more effective in S-G0-G1 phases in this cell cycle (Shi, Shen, Fang, Xu, & Hu, 2015).

The aim of the present study is to detect possible anticancer activity of the methanol extract of *S. japonicum* root. For this aim, in the present study, the extract was analysed by LC techniques to confirm its matrine and oxymatrine content. *S. japonicum* root methanol extract was evaluated in respect to its in vitro cytotoxic, apoptotic and antioxidant activities. To analyse cell death, upon treatment with *S. japonicum* root extract, the viability was measured in vitro in various cancer cell lines including colon (HT-9), brain (U-87), and prostate (PC-3) cancer cells in comparison with healthy human fibroblasts. Furthermore, apoptosis was analysed by Annexin V/PI staining and western blot detection of PARP cleavage as a final downstream biochemical indicator of apoptosis (Fischer, Jänicke, & Schulze-Osthoff, 2003; Kaufmann, Desnoyers, Ottaviano, Davidson, & Poirier, 1993; Tewari et al., 1995).

MATERIAL AND METHODS

Materials

The standard chemicals were provided from Sigma Chemical Co. (USA) and the HPLC-grade solvents were obtained from Merck.

Plant material and extraction

The Pharma Grade *Styphnolobium japonicum* root was acquired from Germany. For the extraction procedure, the roots were ground to a powder (100 gr), and then macerated with methanol (3x 100 mL) for 48 h. The extract was filtered, and the filtrate was concentrated using a rotary evaporator (Heidolph, Germany). The prepared extract (21g) was stored at 4°C until the experiments.

Antioxidant activity

**DPPH**• scavenging assay

The antioxidant capacity of the extract was detected using DPPH• by its capability to bleach the stable radical (Blois, 1958). The reaction mix contained 100 µM DPPH• in crude extract and methanol. After 30 min, absorbances were measured at 517 nm by using a UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 25±2°C and the radical scavenging activity (RSA) was determined as follows:

\[
\text{DPPH}^-\text{RSA} \% = \left( \frac{\text{Absorbance control} - \text{Absorbance test sample}}{\text{Absorbance control}} \right) \times 100
\]

**ABTS**• scavenging assay

The other method for determining antioxidant capacity of the extract was the ABTS• method (Re et al., 1999). ABTS• solution (7 mM) was mixed with potassium persulfate (2.45 mM). The mixture was kept for 12-16 h in the dark at 25±2°C. The antioxidant capacity of the extract was detected using the ABTS• scavenging assay as follows:

\[
\text{ABTS}^-\text{RSA} \% = \left( \frac{\text{Absorbance control} - \text{Absorbance test sample}}{\text{Absorbance control}} \right) \times 100
\]

Total phenolic content of the extract

Folin-Ciocalteu method was used for determination of total phenolics content. Folin-Ciocalteu’s reagent (0.25 mL) and Na₂CO₃ (0.2 mL) were mixed with the extract (5 mL) and allowed to incubate at 45°C for 15 min. The absorbance was determined at 765 nm at 25±2°C. The total phenolic content was measured from a linear calibration curve (R² = 0.9892) (Spanos & Wrolstad, 1990).
**LC-MS analysis**

The LC analyses were studied on a Shimadzu (LC 2040c, Japan). LC was run on an Agilent C18 column (4.6 x 250 mm x 5 μm, Zorbax, Agilent, Japan) and the column temperature was kept at 40°C. The mobile phase was methanol/water/diethylamine (50:50:0.07, v/v/v) at pH 10.5. The flow rate was 0.8 mL min⁻¹. Injection volume was 50 μL and total run time was 22 min for each test sample.

An MSD mass spectrometer system (Shimadzu 2020, America) was equipped with electrospray ionisation (ESI) source for the mass analysis and detection. MS data were acquired in the positive mode with selective ion monitoring (SIM). The drying gas (nitrogen) flow rate was 12.0 L min⁻¹ and gas temperature was maintained at 300°C (Wu, Chen, & Cheng, 2005). Matrine and oxymatrine were analysed by matching their retention times and mass spectra against those of the standards analysed under the same conditions.

**Preparation of stock solutions**

For the preparation of calibration curves of matrine and oxymatrine, 2 mg of each compound was dissolved in water and filtered. By diluting the stock solutions, three different (0.7, 0.4, 0.1 mg/mL) concentrations of matrine and oxymatrine were prepared. Each concentration was applied triplicate to the system. The regression equation of the calibration curve was obtained as 0.9927.

**Cell culture**

HT-29; colon cancer, U-87-GBM; brain cancer (ATCC, #HTB-14), (ATCC, #HTB-38), PC-3; prostate cancer (ATCC, #CRL-1435) and human primary dermal fibroblast cells (HDFa) (ATCC, #CRL-PCS-201-012) were purchased from ATCC (U.S.). Then the cells were grown and expanded in DMEM (Gibco), 10% fetal bovine serum (Gibco), 1% antibiotics (penicillin/streptomycin) at 37°C in a 5% CO₂ incubator. The cells were incubated at 37°C in 5% CO₂ for 24 h. Then the culture medium was discarded, and cells were treated with 0 mg/mL (control) and 1 or 5 mg/mL, of S. japonicum extract. (Control wells were treated with an equal amount of extract solvent; DMSO). After 24 h treatment with S. japonicum extract, Annexin V-FITC/Propidium Iodide (PI) early apoptosis double staining protocol was applied according to manufacturer’s instructions (CST #6592 Annexin V-FITC Early Apoptosis Kit). Then, the percentages of apoptotic cells were determined by flow cytometry analysis.

**Statistics**

Statistical comparisons were performed by unpaired Student’s t-test assuming equal variance. Differences were considered as statistically significant at 0.001<p*<0.005; p**<0.0005; and p***<0.0001. Data are the mean ± standard error (SE).

**RESULTS AND DISCUSSION**

According to our results, the S. japonicum methanol extract showed relatively less antioxidant activity against DPPH (IC₅₀=2.79±0.15 mg/mL) and ABTS (IC₅₀=1.94±0.23 mg/mL) radicals compared to the standards ascorbic acid and trolox, respectively. The total phenolic content (TPC) of the S. japonicum MeOH extract was measured by using the Folin-Ciocalteu technique and calculated as a gallic acid (GA) equivalent amount. The S. japonicum extract showed 53.17±0.97 mg of GA/g corresponding to the total phenolic amounts, resulting in relatively moderate antioxidant activity on the *in vitro* ABTS- and DPPH- assays.

Our results also indicate that the S. japonicum root extracts have remarkable antioxidant activity. In previous antioxidant activity studies on *S. japonicum* extracts, it was observed that *S. japonicum* extracts showed different and varying results. It can be concluded that this may be due to the differences in the locations and extraction methods of the plant material (Mihaaylova & Schalow, 2013; Tang, Li, Hu, & Lou, 2002). The results suggest that TPC is present in a relatively good amount in the extract. Based on the data obtained from performed experiments, a high correlation was found between the total phenolic content and antioxidant activity for methanol extract of *S. japonicum*.

As shown in Figures 1-3, matrine and oxymatrine standards and crude methanol extract were analysed and quantified by LCMS. According to the results obtained from LCMS analysis,
Figure 1. MS Chromatogram of Matrine Standard.

Figure 2. MS Chromatogram of Oxymatrine Standard.

Figure 3. MS Chromatogram of *S. japonicum* root extract.
matrine (0.49±0.006 mg/mL) and oxymatrine (0.27±0.016 mg/mL) were determined in *S. japonica* root methanol extract. Therefore, it can be asserted that matrine and oxymatrine alkaloids in the extract are responsible for the anticancer activity of the extract. It can be thought that the extract shows a significant cytotoxic effect against the tested cell lines due to these alkaloids (Li et al., 2011; Ma et al., 2008; Shi et al., 2015; Yu et al., 2009; Zhang et al., 2015).

In previous studies, *S. japonicum* leaf and bud extracts have been investigated for their anti-cancer activity against breast and colon cancer cell lines (Abdelhady, Kamal, Othman, Mubarak, & Hadda, 2015; Lee et al., 2015). The obtained results were quite significant in terms of anti-cancer activity. Besides, matrine and its derivatives are molecules for which their anticancer activity is well documented (Li et al., 2011; Yu et al., 2009); matrine is the main alkaloid of *S. japonicum* root extracts. In past studies, matrine and oxymatrine have been assessed for their remarkable anticancer activity. Matrine and its derivatives have been reported as antineoplastic agents since they can inhibit proliferation and induce apoptosis of cancer cells. Besides, matrine could synergistically improve the efficacy of chemotherapy when it is used in combination with other anticancer drugs (Rashid, Xu, Muhammad, Wang, & Jiang, 2019).

Cancer is defined as uncontrolled or abnormal growth and proliferation of cells as a result of errors in DNA division during cell division (Hassanpour & Dehghani, 2017). In this present study, the effect of *S. japonicum* root MeOH extract on the viability of various cancer cell lines was investigated. The cytotoxic effects of the root of *S. japonicum* methanol extract was tested on HT-29, U-87 and PC-3 cell lines by measuring metabolically active cells using a luciferase-based assay, Cell Titer Glo (Promega). According to the obtained results, the cell viability was decreased at varying concentrations (1 mg/mL, 5 mg/mL, and 10 mg/mL) in a concentration-dependent manner for all tested cancer cell lines. The results are in accordance with previous studies (Chang et al., 2013; Cousset et al., 2018; Huang et al., 2018; Rodenhizer, Dean, Xu, Cojocari, & McGuigan, 2018). Previous studies have shown the superior effects of matrine on the growth of HT29 cell lines, and the expression of the related proteins. MTT assay indicated that matrine considerably inhibited the HT29 cells proliferation in vitro in a dose- and time-dependent manner. MTT assay was used to study the inhibitory effects of matrine on the proliferation of HT29 cells; the treatment of cells was performed using different concentrations (2–32 mg/mL) for 24, 36 and 48 h. Consequently, when the matrine dose increased, the proliferation of the HT29 cells was significantly suppressed in vitro in a dose- and time-dependent way. In conclusion, matrine has strong antitumor activity against HT29 cells and can act as an alternative agent to treat colon cancer (Chang et al., 2013). Huang et al. studied the efficacy of matrine on prostate cancer lines (DU145 and PC3 cell lines). The results showed that matrine and GADD45B overexpression synergistically inhibited the proliferation, migration, and invasion of prostate cancer cells. Additionally, the apoptosis of prostate cancer cells was also synergistically enhanced by matrine and GADD45B overexpression (Huang et al., 2018).

Treatment with 10 mg/mL *S. japonicum* extract resulted in a 35±2.21% viability of cells on U-87 cells, while PC-3 cells displayed 46±5.67% cell viability, respectively. Additionally, the extract showed the lowest viability against the HT-29 colon cancer cell line as 14%. Cell viability of *S. japonicum* root extract-treated HT-29, U-87 and PC-3 cell lines at 24 hours are given in Figure 4 A-C. Accordingly, the tested methanol extract showed 5.104 and 5.012 mg/mL IC$_{50}$ values for HT-29, and U-87 cell lines, respectively (Figure 4A and Figure 4B) while the IC$_{50}$ value of PC-3 cell line was determined as 0.555 mg/mL (Figure 4C). Furthermore, among all the cancer lines tested, at the lowest dose of 1 mg/mL of extract treatment, only the PC-3 cell line showed significant cell death with a decreased viability to 64% (Figure 4C).

To analyse the anti-cancer effects of *S. japonicum* root extract, as a healthy control, human primary fibroblast cells were included in cell viability experiments. As a result, fibroblasts showed 1.320 mg/mL IC$_{50}$ value which is approximately two-fold higher than the IC$_{50}$ of PC-3 cancer line and therefore, fibroblasts did not show a significant decrease in cell viability when treated with 1mg/mL (Figure 4D).

This data indicated the PC-3 cancer cell line could be treated with *S. japonicum* root methanol extract while the same dose is not cytotoxic to the healthy cells. Then, further analysis of the cell death was followed with biochemical verification of cell death and staining of apoptosis in PC-3 prostate cancer cells upon treatment with *S. japonicum* root extract for 24h. For detection of apoptosis, we stained the extract-treated PC-3 cells (0, 1 and 5 mg/mL) with Annexin V and Propidium Iodide (PI) then analysed by flow cytometry. According to our results, 1 mg/mL and 5 mg/mL extract treatments showed increased apoptosis in PC-3 with 16.3% and 99.6% respectively while untreated cells showed 1.89% apoptotic cells (Figure 5). This data indicates that *S. japonica* root extract treatment causes enhanced apoptotic cell death following increased doses.

Poly (ADP-ribose) polymerases (PARPs) are enzymes that can catalyse the transfer of ADP-ribose to target proteins. They take an important role in various cellular processes, including transcription, replication, recombination, and DNA repair. Caspase mediated apoptosis occurs through the cleavage of several key proteins required for cellular functioning and survival (Fischer et al., 2003) and PARP-1 cleavage by caspases is considered to be a hallmark of apoptosis (Kauffman et al., 1993; Tewari et al., 1995). One of the main biochemical indicators of cell death is the cleavage of PARP (Boulares et al., 1999). For this reason, we analysed cPARP levels of extract-treated PC-3 cells (1mg/mL), as well as DMSO treated control cells (DMSO was added instead of an equal volume of the extract). The results indicated significantly increased cPARP (89kda) levels in the extract-treated PC-3 cells (p<0.0002) (Figure 6). This reveals that cell death is triggered upon treatment and possibly by the matrine and oxymatrine compounds residing in *S. japonicum* extract.

In conclusion, the results revealed that *S. japonicum* root methanol extract could trigger apoptosis in cancer cells and it is likely that cell death is initiated by the cleavage of PARP, which plays a pivotal role in orchestrating the apoptotic pathway.
Figure 4. In vitro anti-cancer effects of *S. japonicum* root extract in various cancer cell lines and relative IC₅₀ graphs. Effects of *S. japonicum* root extracts in cell viability were tested on A) HT-29 (colon cancer), B) U-87 (brain cancer; glioblastoma) C) PC-3 (prostate cancer), cell lines and D) Human primary fibroblasts (healthy control) and their IC₅₀ calculations were plotted. Data are expressed as ± SE and considering the differences, statistical significance was determined as 0.001 < p* < 0.005; p** < 0.0005; and p*** < 0.0001.
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Highly cytotoxic to the PC-3 prostate cancer cell line while cells of healthy tissue (human fibroblasts) were not affected at the same concentrations.

To sum up, the obtained methanol extracts from *S. japonicum* roots can be recognized as a potential anticancer candidate, especially against prostate cancer while sparing healthy tissue. The obtained results are quite remarkable and the in vitro and in vivo anti-cancer effectiveness of *S. japonicum* extracts can be further studied, in detail. Therefore, this study can be considered as the first alternative report focusing on a pharma-grade *S. japonicum* root extract in the cancer field. The preliminary data could be used to demonstrate the potential of the *S. japonicum* root and can lead the way to future studies.

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REFERENCES


