Phytochemical Profiling and Biological Effects of *Glycyrrhiza glabra* L.

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Abstract
Glycyrrhiza glabra L. commonly known as licorice belonging to the family Leguminosae is an ayurvedic medicinal plant having substantial pharmacological properties such as anti-inflammatory and anticancer effects. Analysed ethnopharmacological studies show phytochemical and biological activities of the plant extract and serve as a resource for studying anti-endometriosis. According to WHO, 176 million women in the world are affected with endometriosis condition and up to 30-50% of women may experience infertility.

Our study aims to evaluate in vitro activity of ethanolic extract of Glycyrrhiza glabra in different cell lines, DPPH assay and HPLC studies in order to propose it to be an effective remedy in endometriosis treatment. In vitro cytotoxic assessment of the extract was determined in estrogenic (MCF 7), non-estrogenic (MDAMB 231) and endometrial cancer cell lines (Ishikawa) along with its safety assessment in normal cell lines (L929). The extract of G. glabra exhibited DPPH radical scavenging activity with 77.09% inhibition in 100 µg concentration. Cytotoxic activity of extract in MCF 7, MDAMB 231, endometrial cancer cells- Ishikawa and significant cell viability in normal L929 cell line shows promising effect of the extract and suggests it as a possible therapeutic adjuvant for treating endometriosis.

Keywords: Endometriosis, *Glycyrrhiza glabra*, Phytochemical, Cell lines

Introduction

Glycyrrhiza glabra L. is a traditional medicinal plant with broad healing capabilities. This plant has been recommended for relieving problems related to uterine pain and used as folk medicine to treat dysmenorrhea[10]. The active ingredients such as flavonoids and triterpenoids in it show varieties of biological activities including anti-microbial, anti-carcinogenic and anti-allergic properties[7]. It has been traditionally used to improve fertility and to treat menstrual irregularities[4]. It has been reported that the active compounds in this plant extract can resolve hormonal imbalance by regulating the level of sex hormones[28]. As estrogen is the key cellular process regulator involved in the maintenance of female reproductive system; reduced level of it may give several side effects such as uterine bleeding, increased risk of breast cancer etc.[21] The potential benefit of herbal medicine are the antagonistic and synergistic interactions between compounds in such plants that could substitute estrogen as per the requirement.

Glycyrrhiza glabra (*G. glabra*) is one of the most widely used herbal plants with phyto-estrogenic property. This plant contains triterpene, saponins, flavonoids, isoflavonoids, hydroxycoumarins and steroids. This plant is also referred to as liquorice and mainly seen in southern Europe, India and some parts of Asia[15]. It has been widely used for its roots containing multi active glycosides named glycyrrhizin. Among the various biological constituents present in licorice, glycyrrhizin also known as glycyrrhizic acid is the major constituent (10-25%) extracted from the root of plant[9] which has been reported to have anti-inflammatory and anti-oxidative effects[5,8].

The pharmacological targets of herbal medicines have anti-proliferative properties with estrogenic and antiestrogenic effects which can mimic the action of endogenous estrogen and thereby can act as antagonistic to tissue specific estrogen production[25]. It is reported that phyto-estrogenic compounds in this plant can form a structural similarity to 17-beta estradiol and can act in a similar manner[11]. They are reported to be the inhibitors of estrogen mediated tumorigenesis. Endometriosis is such a disease which is characterised by the presence of estrogen responsive functional endometrium like tissue seen outside the uterus[18]. Even though several theories have been proposed to explain the pathogenesis of this disease, excess estrogen seems to play a critical role and a causative reason of the disease. The increased local estradiol synthesis with reduced ability of its inactivation promoting the local estrogen in excess would result in proliferation of ectopic endometrium leading to endometriosis condition[27]. *Glycyrrhiza glabra*, a herbal and traditional medicinal plant with broad healing capabilities has been administrated as a plant to relieve uterine pain[13]. Being a selective estrogen receptor modulator (SERM) plant, it has been demonstrated that antiangiogenic property[3] of *G. glabra* would inhibit anti-tumour characteristics too. As this is one of the most important medicinal plants mentioned in many medical textbooks to treat menstrual illness, the present study was to investigate its *in vitro* effect on estrogen-responsive human breast cancer cell lines (MCF7 and MDAMB 231) along with endometrial cancer cell line (Ishikawa) over a range of concentrations. The cytotoxicity of the extract was also determined in normal cell line L929 to evaluate its safety.

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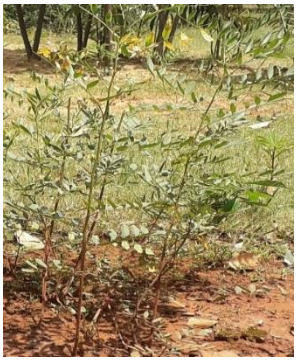

Glycyrrhiza glabra

Dried root

Fig. 1: *Glycyrrhiza glabra* used for the study

Glycyrrhiza species are present in menopausal dietary supplements and are better choices in terms of estrogenic efficacy and safety. Keeping this view in mind that the quality and standardization parameters of the herbal drug need to be of great importance, the study also aims for HPLC profiling and its bioactivity studies in different cell lines.

Material and Methods

Collection and extraction of plant materials: Roots of *G. glabra* (Fig. 1) (Family: Fabaceae & Genus: *Glycyrrhiza*) were collected from Foundation for Revitalisation of Local Health Traditions (FRLHT), Bangalore and were thoroughly washed with distilled water for removal of adhered dust particles. It was oven dried at 40ºC for 7 days and then ground into fine powder using mixer grinder. The samples were powdered and stored in an air-tight container in the dark at room temperature for further experiments.

Soxhlet extraction procedure was done for its relatively high extraction efficiency. 24 gm powdered sample was precisely weighed in weighing balance and inserted in to a Soxhlet apparatus with 250 ml of ethanol and extracted for 8 hrs. The solvent was then evaporated under reduced pressure using a rotary evaporator at 40ºC (Buchi Labor technik AG, Flawil, Switzerland) R-210 set at 4ºC. The pressure gradient about 300 mbar to 0 mbar was given for evaporation. The dried extract was weighed and stored for further studies of phytochemical characterisation, determination of antioxidant activity and for identification of marker compound glycyrrhizin. Later on, it was screened for cytotoxicity and cell viability assay in different cell lines. The percentage yield was calculated using the following formula[1]:

Yield (%) = (Weight of the extract obtained) / (Total weight of the sample loaded) x 100

Phytochemical screening of *G. glabra* ethanolic extract: The *G. glabra* extract consist of several secondary metabolites like alkaloids, tannins, saponins, terpenoids, flavonoids etc. These compounds might be responsible for the therapeutic effects. To check its presence or absence, the extract was subjected to various phytochemical tests[14].

Antioxidant assay: Antioxidant potential was determined by DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) assay (Alfa Aesar). It is a dark-coloured crystalline powder composed of stable free-radical molecules. DPPH assay was carried out as per the standard procedure[22]. Briefly, the electron donating abilities of the corresponding extracts and standard ascorbic acid were measured with the colour change of purple-colour methanol solution of DPPH to yellow. DPPH was prepared by dissolving 3.96 mg DPPH in 100 ml MeOH. Ascorbic acid and methanol were used as standard and blank respectively. 1mg/ml concentration of all the samples and standard ascorbic acid was prepared as stock.

Working standards of ethanolic extracts with concentration (10 to 100 µg /ml) were prepared. Ascorbic acid standard (Sigma Aldrich) was prepared in concentration ranging from 2 to 20 µg/ml. 1 ml of each concentration of samples was mixed with 2 ml of DPPH. The absorbances of standard, samples and control were recorded at 517 nm after an incubation of 30 min at room temperature. The decreased absorbance value of reaction mixture indicated an increased percentage of free radical scavenging activity. All the experiments were done in triplicate. The percentage inhibition or free radical scavenging activity was calculated by using the following formula:

% Inhibition = [(Control absorbance – Test absorbance) / Control absorbance] x 100

Upon reduction, the colour of the solution faded from violet to pale yellow. % inhibition was plotted against respective concentrations used and IC_{50} was calculated by using ascorbic acid.

Thin layer chromatography (TLC) and Column chromatography: The chromatographic techniques used in this work focused to extract and isolate glycyrrhizin as an antioxidant[19], anti-inflammatory[17] and anti-cancerous[6] compound from *G. glabra* root extract. The isolation of marker compound using conventional column chromatography procedure was done using 60-120 mesh size silica gel. 1 gm of ethanolic extract was charged into the

column and eluted with solvents of chloroform and methanol. Fractions were collected (50 ml each) and pooled on basis of TLC chloroform: methanol: water (6:3.5:0.5) (Merck TLC Silica gel 60 F 254 plates) and concentrated under high vacuum to isolate pure compounds. It was further identified by HPLC to confirm the presence of marker compound.

Profiling method for RP-HPLC analysis: Ethanolic extract of *G. glabra* and standard glycyrrhizin were dissolved in methanol to obtain 5 mg/ml and 1 mg/ml concentrations respectively. It was passed through 0.2 mm NY-membrane (Sartorius, Germany) before injecting into HPLC. The extract and reference standard glycyrrhizin (Tokyo Chemical Industry) were subjected to HPLC analysis (HPLC; LC Agilent, Agilent Technologies, Boblingen, Germany) using a reverse phase sun fire column (4.6 ×150 mm, C18 5µm; Waters Corporation, Milford, USA). HPLC solvents were purchased from Merks, Mumbai, India. The gradient solvent system was acetonitrile: 0.1% orthophosphoric acid 20% at 8 min, 30% at 10 min, 35% at 12 min, 50% at 14 min, 60% at 16 min, 80% at 18 min, 90% at 28 min and 100% at 35 min with a flow rate of 0.5 ml/min. The peaks were detected at 254 nm.

The crude extract was evaluated for their *in vitro* biological activities in estrogenic (MCF7) and non-estrogenic (MDAMB 231) cell lines as well as in endometrial cancer cell line (Ishikawa). The safety assessment of the extract was carried out in L929 normal cell line. The partially purified glycyrrhizin rich fraction was again tested for cytotoxicity assay in order to prove its efficacy in endometrial cancer cells.

Chemicals used in cell culture: Dulbecco's modified eagle medium (DMEM F12) was purchased from Thermo Fisher. Fetal bovine serum (FBS), antibiotic-antimycotic was obtained from Gibco, USA. All other chemicals were purchased from Sigma Aldrich.

Cell culture for measurement of cell viability by MTT assay: *In vitro* assessment of *G. glabra* extract as estrogen modulators was done based on MCF7, MDAMB 231 cell line study. MCF7 is estrogen +ve, progesterone receptor +ve, HER2 +ve, perfect model for hormone therapy while MDAMB 231 is triple negative and perfect model for chemotherapy. The cell viability with L929 and cytotoxicity with Ishikawa cells were performed by MTT assay procedure. All the cell lines were originally obtained from NCCS, Pune. Ishikawa cells were originally endometrial adenocarcinoma cells with estrogen and progesterone receptors and are widely used in the areas of reproductive biology.

The tests were done in accordance to ISO 10933-5-2009 standards. Cell lines at exponential growth phase were washed, trypsinized and resuspended in fresh medium. The cell lines were cultivated in DMEM with phenol red, except ishikawa cells in DMEM F12, supplemented with antibiotics (penicillin and streptomycin) and10% FBS at 37ºC in an atmosphere of 5% CO_2 and 95% air under saturating humidity. The cell lines (5x 10^3cells/well) were added to 96-well plates and the culture was propagated at 37ºC under 5% CO_2. The cells were allowed to attach for 24 hr and the crude *G. glabra* extracts dissolved in DMSO were added at different concentrations of 500, 250, 125 and 62.5 µg/ml, each in triplicate on to the wells. After 48 hr of incubation, MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetra zolium bromide] (Sigma) solution was added to each well to obtain the final concentration of 100 μg/ml and then further incubated at 37°C in a CO_2 incubator for 3 hrs.

The MTT solution was removed from cells by aspiration. This was followed by adding DMSO with 2 hrs incubation period. The plates were shaken and estimated by measuring the absorbance at 570nm in an Elisa plate reader and percentage of cytotoxicity was calculated. *In vitro* analysis of crude ethanolic extract was analysed in Ishikawa cell lines for its anti-cancerous activity and IC_{50} values were calculated.

Results and Discussion

The percentage yield of ethanolic extract of *G. glabra* was calculated and found to be 20.3%. The extract was dark brown in colour. The phytochemical characteristics of ethanolic extract of the plant were investigated and are summarized in table 1 indicating the presence of various secondary metabolites like tannins, saponins, terpenoids, flavonoids and are evidenced for various pharmacological applications as anti-oxidant agents.

Table 1
Phytochemical Analysis of *G. glabra* ethanolic extract

Tests	Ethanolic extract
Tannins	+
Saponins	+
Flavanoids	+
Qunine	+
Terpenoid	+
Glycoside	-
Cardiac glycoside	-
Triterpenoid	-
Phenols	+
Steroids	+
Carbohydrates	+
Protein	-
Alkaloid	+

The absorbance produced by reduced DPPH was used to evaluate the ability of *G. glabra* ethanolic extract to act as free radical scavengers. It shows that DPPH radical scavenging activity was in dose dependent manner (Fig. 2).

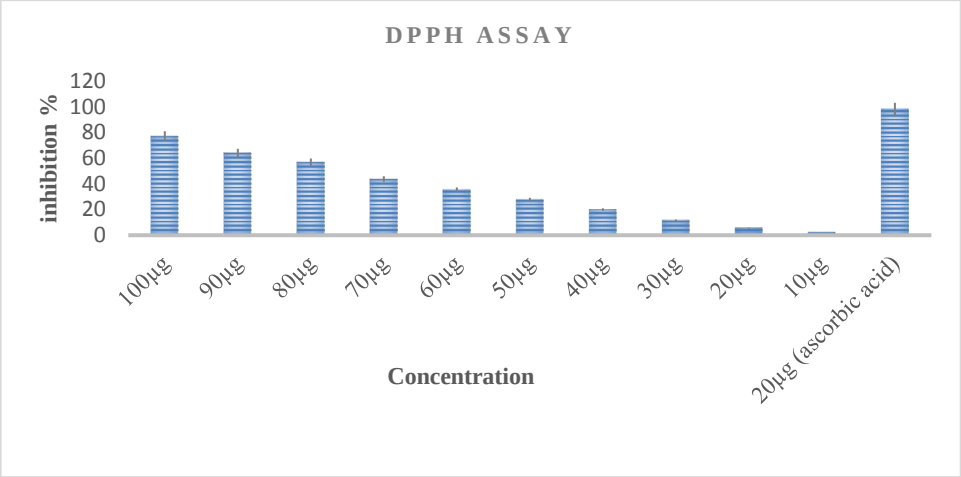

Fig. 2: Antioxidant assay of *G. glabra* ethanolic extract

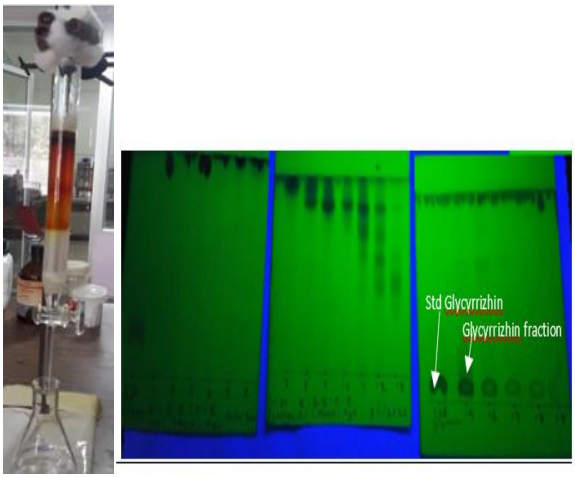

Fig. 3: Column chromatography and TLC of *G. glabra*

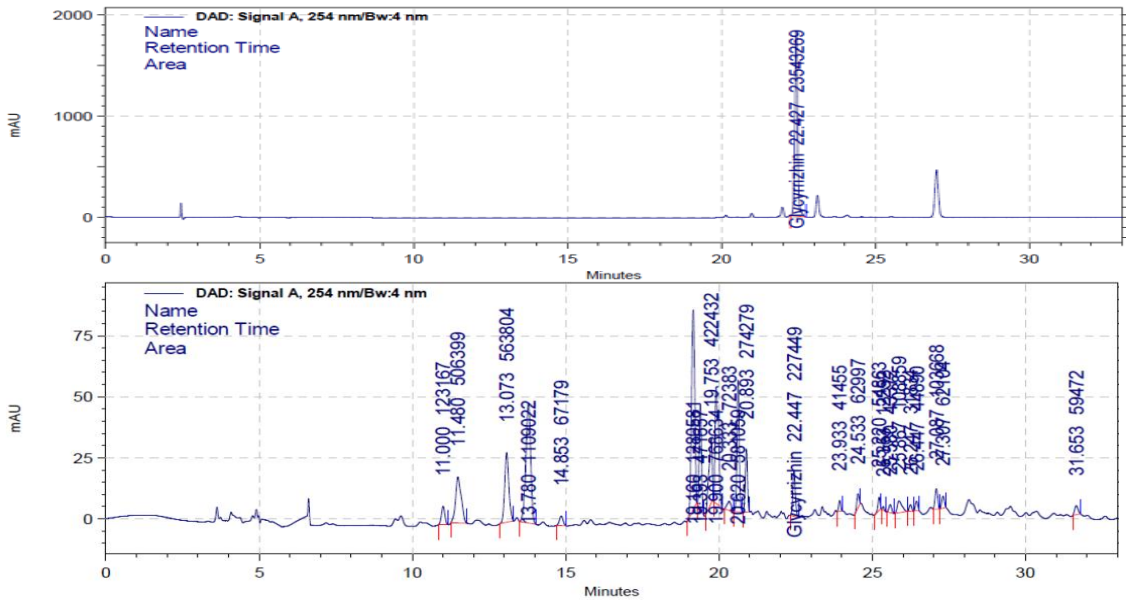

Fig. 4: HPLC profile of standard glycyrrhizin and *G. glabra* ethanolic extract

The ethanolic extract of *G. glabra* at 100 µg concentration exhibited 77.09% inhibition with IC50 value of 74.30 when compared with standard ascorbic acid with 98% inhibition at 20 µg concentration. Various factors affect the antioxidant properties of medicinal plants such as its variety, environmental conditions, seasonal and climatic variations, geographical regions of growth, degree of maturity, growing practices and postharvest treatments etc.[12]

In the present work, the results obtained from chemical tests were confirmed by isolating the marker compound glycyrrhizin, which is one of the major ingredients of the plant and has been reported to exhibit anti-inflammatory effect [23]. TLC of the extract (Fig. 3) was performed and the Rf value was shown as 0.4cm in chloroform: methanol solvent system. The major chemical constituent glycyrrhizin and the crude extract were applied to TLC plates coated with silica for the identification of bioactive compounds in our extract. The crude extract and standard were compared and characterized through HPLC (Fig. 4) coupled with a photo-diode array (PDA) detector and the same reference standard was confirmed by comparing retention time and UV spectra.

Chromatographic peak with various chemical constituents in the crude extract with RT at 22.47 in 254 nm represents the marker compound. The phytochemical profiling and TLC made the confirmation. To demonstrate the potential biological activity of the extract, we carried out the screening of the extract in MCF 7 (ER +ve) and MDA MB 231 (ER -ve) breast cancer cell lines along with endometrial cancer cell line ishikawa (Fig. 5).

The cytotoxic effect revealed that ethanolic extract of *G. glabra* exhibited dose dependent activity with high activity against MCF7 with 59.47% and moderate activity in MDA MB 231 with 41.35% at 500 µg concentration in 48 hrs incubation while the extract has shown 91.2% activity in the same concentration with ishikawa cell lines. The result of current study revealed that cytotoxic effects of *G. glabra* ethanolic extract on normal cell line (L929) were significantly important as they have shown 78.50% cell viability even in 500 µg concentration (Fig. 6) in 48 hr incubation. The present study shows similar results as compared to results of Nazmi et al[16] supporting the data that *G. glabra* extract has antiproliferative effect on MCF 7. Data were obtained from three separate experiments and presented as the mean + standard deviation.

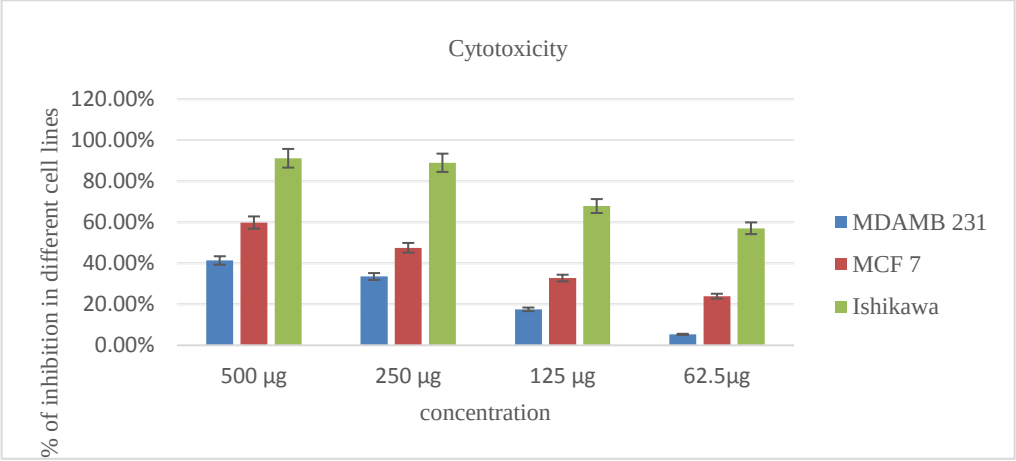

Fig. 5: Effect of *G. glabra* extract on MDA MB 231, MCF-7 and Ishikawa cell lines after 48hr incubation

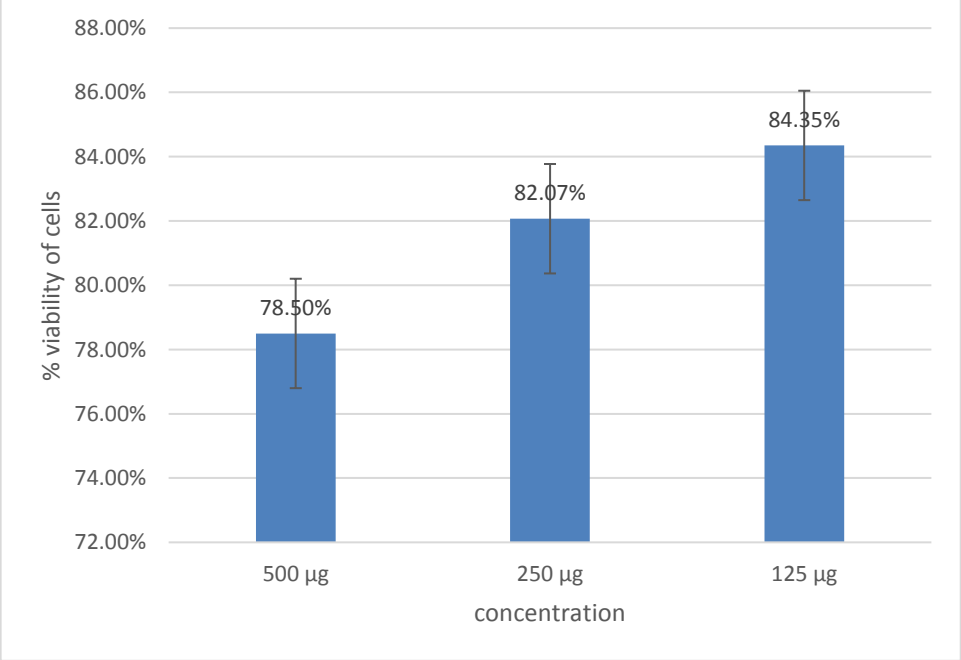

Fig. 6: L929 Cell viability with *G. glabra* ethanolic extract

MDAMB 231 cells have shown IC50 value above 500 µg concentration while in MCF 7, it was 260µg. At 24 hr, the IC 50 value of the extract was lesser for all cell lines. When the incubation was extended for 48 hrs, the cells exhibited promising IC 50 value. Crude extract of *G. glabra* plant showed prominent IC50 value with 28.5µg in Ishikawa cells with significant antiproliferative effect. Ethanolic *G. glabra* extract in MDAMB231 cells induced a lower increase in cytotoxicity compared to MCF 7 demonstrating its use as a safe estrogen replacement therapy. MTT assay showed that *G. glabra* has cytotoxic effects on MCF7, MDA MB 231 and Ishikawa cells but was non-toxic to normal L929 cells in a time-dose dependent manner.

The semi purified fraction from column chromatography was studied in order to evaluate the anti-endometrial activity of the identified compound. It was further subjected to antiproliferative assay in Ishikawa cell line. It has been shown that at 50µg concentration, 76.2% of Ishikawa cells were inhibited and have shown better activity in a dose dependent manner. Activity of glycyrrhizin with anticancer properties[2] is also in agreement with our studies and proves its efficacy and focus for this herbal plant toward medicinal research. The results indicate that the cytotoxic influence of *G. glabra* on MCF 7, MDAMB 231 and ishikawa cell lines has influence against the extract and is able to prevent proliferation of these particular cell lines depending on time and dose of incubation. The anticancer properties of *G. glabra* along with biological and toxicological studies can serve as a resource for future clinical studies and data presented here indicates and establishes similar activity of this plant in recent findings[24].

Besides these findings, molecular studies have to be carried out to identify the mechanism and mode of action of the drug. Reports show that phytoestrogens are also found to be inhibitors of estrogen-mediated tumorigenesis[20]. Increased oxidative stress with high levels of reactive oxygen species (ROS) and increased cell proliferation were mainly seen in peritoneal environment of women with endometriosis[26]. So, the *in vitro* studies in the present study support that *G. glabra* could potentially serve as a chemoprotective agent and its major chemical constituents may have the ability to treat endometriosis.

Further investigation and additional studies are need to identify the molecular mechanism underlying endometriosis treatment. Researchers should pay special attention on *in vivo* animal model to prove the anti-endometrial activity of the plant.

Conclusion

Medicinal plants are safe, economical and show better results for long time usages. The potencies of SERM categorised *Glycyrrhiza glabra* plant extract were evaluated in estrogen receptor (ER) positive cell line (MCF-7) and (ER) negative cell line (MDA MB-231) using MTT assay for cytotoxicity. As phytochemical and pharmacological effect of the phytoestrogens may have a complex role in tissue specific action, estrogen agonistic/ antagonistic nature of this medicinal plant can be used for endometriosis treatment.

The TLC and HPLC profiling used to play as an important role in characterization of phytochemicals that may block estrogen dependent growth of endometriosis and thereby reducing inflammation. The results show that the extract is cytotoxic to endometrial cancer cells which show more predictable activities in endometriosis disease management. Considering the prevalence of endometriosis and its treatment in women's health, this study proves the therapeutical efficacy of the extract and may pave the way for curative management of endometriosis.

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