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Estimation of Secondary Metabolites from Methanolic Extract of Treated and Non-Treated Callus of *Glycine max* (L.) Merrill using Thin Layer Chromatography and High Performance Liquid Chromatography

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ABSTRACT:

The secondary metabolites have a lot of economic importance in the plant breeding, plant defense, pollination, ecological effects and others. The present study has been undertaken to estimate the secondary metabolites (phenolic compounds) from methanolic extracts of UV-B treated and non-treated eight weeks old callus of *Glycine max* leaf explants. Qualitative SMs profiling was done through TLC while quantification was carried out through HPLC. TLC and HPLC results showed that the major secondary metabolites were detected in UV-B treated callus extract as compared to the non-treated callus extract. UV-B radiation (for short period) enhanced the production of Secondary metabolites in *Glycine max* callus.

***Keywords*: High Presser Liquid Chromatography, Secondary metabolites, Thin Layer Chromatography, UV radiation, *Glycine max* (GM).**

1. **Introduction:** The *Glycine max* belongs to the family Fabaceae is a major annual crop. It is a major source of vegetable protein and edible oil. The nutritional quality of these seed components depends upon the relative abundance of specific proteins and fatty acids. Many bioactive compounds such as the phenolic compounds, Isoflavones, soya saponins, tocopherols etc have antioxidant properties in *Glycine max*. Soy Isoflavones used for the potential in lowering cholesterol levels, preventing prostate and breast cancers, osteoporosis, and cardiovascular disease as well as relieving menopausal symptoms (Bennett J.O., 2005 and Lien, D. T. P. *et al*., 2016). Soybean is an economically important plant and their regeneration from tissue culture has been difficult and recently it became routine. Plant regenerated from tissue culture have exhibited various morphological and biochemical variation due to mutations which is termed as soma clonal variation. *Glycine max*, as a component of daily diet, has gained worldwide importance as functional food that can

provide protection against free radicals and oxidative damage, which are cause of diseases of our time, such as atherosclerosis, diabetes and cancer (Radhakrishnan R. and Ranjitha Kumari B.D., 2008 and Josipovic *et al*., 2016). It is the source of an excellent vegetable oil used in the manufacturer of various domestic and pharmaceutical products like paints, plastics, soaps, cosmetics, plastics, clothing, glycerine and solvent and recently used as biofuel crop to meet global energy demands. Being devoid of starch, *Glycine max* are also recommended to diabetic patient. *Glycine max* cake is an excellent time stock food especially for poultry. The haulms provide good food for sheep and goats Olhoft, P. M. & D. A. Somers, 2007). Isoflavones and saponins comprise the majority of secondary metabolites in *Glycine max*. Isoflavones, the important secondary metabolic compounds of *Glycine max*, may play essential roles in preventing certain cancers and reducing the risk of cardiovascular diseases, improving bone health, inhibiting the growth of human breast cancer and prostate cancer cell lines in culture, and possessing anti-estrogenic activity (Lee, S. J., *et al*, 2003). Isoflavonoids are a sub-class of plant flavonoid metabolites exclusively foundin legumes,where they are important compounds mediating multiple plant-mcrobial interactions.The multiple health-promoting effects of isoflavonoids, especially those of typical soybean isoflavones genistein and daidzein against hormone related cancer,osteoporosis, menopausal symptoms, and cardovascular disease have been studied intensively. A high consumption of *Glycine max* derived food correlates to a low incidence of such dseases, and the health-protective activities are as decribed to isoflavonoids(Liu, R. *et al*., 2007). Isoflavones (such as genistein & daidzen) and saponins isolated from *Glycine max* inhibit growth & spread of various cancers such as cancers of the breast, uterus, cervix, ovary, lung, stomach, colon, pancreas, liver, kidney, urinary bladder, prostate, testis, oral cavity, larynx and thyroid. *Glycine max* is also effective in nasopharyngeal carcinoma, skin cancer, malignant lymphoma, rhabdoyosarcoma, neuroblastoma, malignant brain tumours and leukaemia. Isoflavones & saponins isolated from *Glycine max* possess wide ranging anticancer properties such as inhibition of cancer cell proliferation, promotion of cell differentiation and induction of apoptosis. *Glycine max* protect against many cancers including that of the colon, lung and ovary (M. Umadevi *et al*., 2013).

Plant tissue culture systems have been developed for *Glycine max*. For shoot morphogenesis, either cotyledonary node or primary leaf tissue was used to obtain cultures, which formed shoots when placed on a medium containing benzyladenine. For somatic embryogenesis, the starting material was the immature zygotic embryo. The explant was the intact zygotic embryo, the excised embryo axis, or the excised cotyledons (Finer, J. J. & A. Nagasawa, 1988).

Cell and organ culture have been used for production of secondary metabolites; however, yield of with anolide metabolites have been low. Attempts to increase levels of secondary metabolites in cell and organ cultures should be pursued as these metabolites can be produced in shorter periods of time. (Shivanadhan G. et al., 2012). Over the past several years, tissue culture technology has been exploited as an efficient and useful tool for production of commercially important metabolites, biotransformation of intermediates in to pharmaceutically important products and genetic enhancement of medicinal plants (Arya D.

et al., 2013). Secondary compounds responsible for different biological properties. Hence, increasing levels of secondary metabolites in UV-B exposed plants play an important role in plant function. The common response to UV-B stress in plant consequently activation of the genes of phenyl propanoid pathway (aromatic amino acids- phenylalanine and tyrosine) producing phenolic compounds. These phenolic compounds can mitigate the UV-Induced damage by protecting the photosynthetic pathway and cellular components. Increase in concentration of UV-B absorbing phenolic acid and flavonoids compounds are the most consistent response of Plants to UV-B supplementation. Phenolic compounds may have a multifunction role in UV-B defense (Kshama Rai and S.B. Agrawal, 2017).

2. **Materials and methods:** Different phytochemical tests were conducted for the qualitative and quantitative analysis of secondary metabolites (SMs). Analysis was done using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) methods.

A. Plant materials

Ten callus culture groups were made on the basis of different combinations and concentrations of Plant Growth Regulators (PGRs) and each group have two UV-treated and non-treated sets. In both sets MS media was supplemented with different concentrations of PGRs. The non-treated sets of each group was maintained for 8 weeks without UV radiation treatment but in treated sets the callus was maintained for 7 weeks without UV radiations and in the 8th week UV radiation were given for a period of 2 hours / day. Among all groups the best response were found in non- treated set of callus in MS media supplemented with, 4 mg/L NAA, 4 mg/L 2,4-D and 10 ml/L coconut water. The UV treated set of callus in MS media supplemented with 4 mg/L NAA, 2 mg/L 2,4-D and 10 ml/L CW was found to be the best responsive so both sets (Treated and non-treated callus) were taken for phytochemical studies.

B. Preparation of callus extracts (sample)

Extraction is the separation of bioactive compounds from plants tissues using selective solvent in standard extraction procedures. The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction thereby increasing the rate of extraction and solvent to sample ratio of 10:1 (v/w) solvent to dry weight ratio has been used (Tiwari P. et al., 2011). Eight weeks old callus was transferred to 10 ml separate volumetric flask and dissolved in methanol. The solution was grinded for 20 min and filtered through Whatman filter paper no. 41 and the filtrate was used in TLC and HPLC for further studies.

C. Thin layer chromatography

1) TLC Principle

TLC is a chromatographic technique which is used for the separation of mixture of compounds. The Separation of compounds is based on the differential adsorption as well as partitioning of analysis between the liquid stationary phase and mobile solvent phase.

2) Mobile phase

Mobile phase consists of chloroform: Ethyl acetate in the 60:40 ratio in SS-I and chloroform: Acetone: Formic acid in the 75:16.5:8.5 ratio in SS-II, both mobile phase were used for the separation of the phenolic compounds (Kathiresan, Prabhu et al., 2011 and Mohammed, S.S.A. et al., 2003).

3) Stationary phase

Stationary phase was done using TLC plate coated with silica gel, which are commercially available 60 F254 (Merck silica gel 60 F254 plate).

4) Procedure: A TLC plate coated with silica gel G was taken and gently draw a straight line across the TLC plate approximately 1 cm from the bottom. Methanolic callus extract was loaded on the TLC plate (silica gel-G) as a single spot at the center of the TLC plates and put in TLC chamber, covered with a lid. The sample was run until the solvent front reaches the top-end border of the plates. The developed plate was taken out from the chamber and the solvent front was marked and allowed to air dry at room temperature for few minutes. The TLC plates were observed under UV light. The spots were visualized in the UV inspection cabinet (254 nm to 365 nm) and the distances of separated spots were marked and separated distance was measured. The Rf value calculated by following formulas (Biradar, S. R. & B. D. Rachetti, 2013, Asante, I. K. et al., 2016).

$$\textbf{Rf value} = \frac{\textbf{Distance travelled by the solute}}{\textbf{Distance travelled by solvent}}$$

D. High performance liquid chromatography

1) Basic principle

HPLC stands for High performance liquid chromatography (sometimes also referred to as High Pressure Liquid Chromatography). HPLC is a chromatographic technique used in analytical chemistry and analytical recent biochemistry to separate a mixture of compounds for the purpose to identify, quantify and purify the individual specific components of the complex mixture. Separation is based on the polar (hydrophilic) or non-polar (hydrophobic) tendency of analytic between two liquid phases.

2) HPLC equipment

HPLC estimation of SMs was performed on Water modular HPLC system, equipped with UV detector. For estimation, Thermo C18 (Dimension 250 x 4.6) RP column, pump 715, software Data Ace, Injector and the mobile phase with the mixture of Acetonitrile: Methanol (50:50) at a flow rate of 1.0 ml/min and the column temperature at 25° C was used. The wavelength of UV detector was set at 254 nm. The HPLC was run and sample detected at 254 nm. The sample injection volume was 20µl and total 20 minutes run time.

3) Preparation of standard stock solution

10 mg of tannic acid was weighed accurately and transferred to separate 10ml volumetric flask, and the volume was adjusted to the mark with the methanol to give a stock solution of 1000 ppm.

4) Preparation of working standard solution (mobile phase)

From stock solutions of tannic acid 1 ml was taken and diluted up to 10 ml with this solution, 1.0, 1.5ml solution was transferred to 10 ml volumetric flasks and make up the volume up to 100 ml with methanol, gives standard drug solution of 10 µg/ ml concentration. The Methanolic extract of *Glycine max* leaf explants callus was prepared and subjected to HPLC with Acetonitrile: Methanol (50: 50) as mobile phase and the peak for SMs was obtained in retention time

3. Results and discussion:

I. Thin Layer Chromatography (TLC) of Glycine max callus extract- Four sample were used for TLC of Glycine max. MeOH extracts of GM1-C callus (Non-treated Glycine max callus) sample A, GM1-UV callus (Treated *Glycine max* callus) sample B, showed following fractions in SS-I mobile phase.

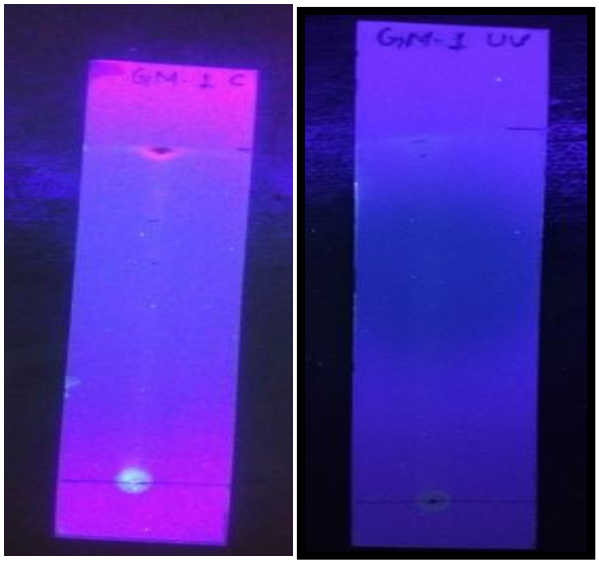

Photo plate 1: TLC of MeOH callus extracts of Glycine max (sample A & B with SS-I) under UV light

MeOH extracts of GM1-C callus showed four fractions of phenolic compounds in SS-1 with Rf values between 0.106 to 0.969. GM1-UV, MeOH extract (set of treated callus of Glycine max) showed two fractions with Rf value 0.922 & 0.974 in SS-1 mobile phase on TLC plate. Rf values visible in Specific UV inspection cabinet at 254 to 365 nm **(Photo plate 1 and Table 1 & 2).**

Table 1: TLC of GM1-C callus group(sample A), using SS-I

TLC Solvent system –I (Chloroform: Ethyl acetate (60:40))				
No. of spots	**Colour when viewed in UV**	**Distance travelled by solvent**	**Distance travelled by solute front**	**Rf value of GM 1-Control(sample A)**
1.	Pale green	6.6	0.7	0.106
2.	Pale green	6.6	4.3	0.651
3.	Pale green	6.6	5.1	0.772
4.	Pale green	6.6	6.4	0.969

TLC Solvent system - Chloroform: Ethyl acetate (60:40)				
No. of spots	**Fluorescence in UV light**	**Distance travelled by solvent**	**Distance travelled by solute front**	**Rf value of GM 1-UV (sample B)**
1.	Pale green	7.7	7.1	0.922
2.	Pale green	7.7	7.5	0.974

Table 2: TLC of GM1-UV callus group(sample B), using SS-I

TLC analysis of MeOH extract of GM2-C (sample C, set of non-treated callus) and GM2-UV (sample D, UV treated Glycine max callus) performed in SS-2 on TLC plate and observed under UV inspection cabinet.

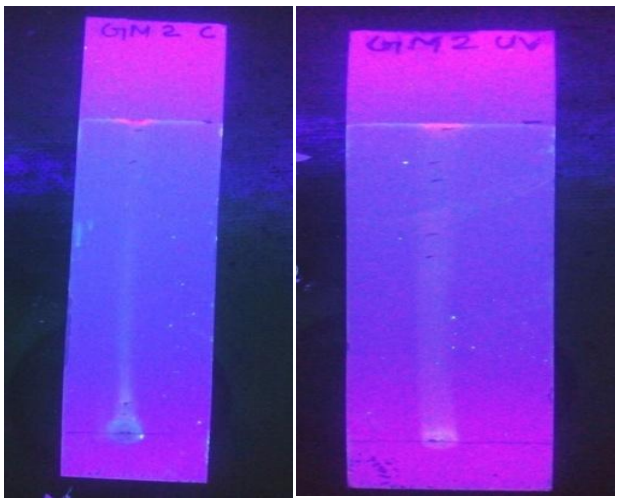

Photo plate 2: TLC of MeOH callus extracts of Glycine max

(sample C & D with SS-II) in UV light

TLC analysis of MeOH extract of GM2-C or sample C (set of non-treated callus) revealed that three spots were obtained in SS-2 phase. The fraction obtained have Rf values of 0.097, 0.847 and 0.958 when a solvent phase SS-2 of Chloroform: Acetone: Formic acid (75:16.5:8.5) was used. GM2-UV (UV treated set of *Glycine max*) showed six fractions with Rf values between 0.380 to 0.971 in SS2 phase on TLC plate shown in the Table of 3 & 4 and photo plate 2.

Among all of these groups and solvent system, the best result was found in Chloroform: Acetone: Formic acid (75:16.5:8.5) or SS-2 of UV treated *Glycine max* callus extract or sample D visualized six spots on TLC plate. Flavonoids may be appearing as dark spots under UV-365 nm.

Table 3: TLC of GM2-C Callus Group (Sample C), using SS-II

TLC Solvent system - Chloroform: Acetone: Formic acid (75:16.5:8.5)				
Number of spots	**Fluorescence in UV light**	**Distance travelled by solvent**	**Distance travelled by solute front**	**Rf value of GM 2-C(sample C)**
1.	Green pale	7.2	0.7	0.097
2.	Green pale	7.2	6.1	0.847
3.	Green pale	7.2	6.9	0.958

Table 4:TLC of GM2-UV callus group (sample D), using SS-II

TLC Solvent system - Chloroform: Acetone: Formic acid (75:16.5:8.5)				
No.of spots	**Fluorescence in UV light**	**Distance travelled by solvent**	**Distance travelled by solute front**	**Rf value of GM 2-UV (sample D)**
1.	Pale green	7.1	2.7	0.380
2.	Pale green	7.1	4.2	0.591
3.	Pale green	7.1	4.7	0.661
4.	Pale Purple	7.1	5.8	0.816
5.	Pale Purple	7.1	6.2	0.873
6.	Pale Purple	7.1	6.9	0.971

II. High Performance Layer Chromatography (HPLC) of Glycine max callus extract- Injected amount of sample detect peak area at a range of 5.299 to 1439.812 (total area 2068.181), average recovery was 100%.HPLC analysis of GM control Methanolic extract revealed 3 peaks on the basis of their retention time 2.866 min (Table 5 and photo plate 3). GM UV Methanolic extract revealed 4 peaks on the basis of their Retention time 3.389 min (Table 6 and photo plate 4).

Table 5: Results for HPLC analysis of *Glycine max* non-treated callus

Peak Number	**Retention time (min)**	**Area**	**Area %**
1.	1.696	5.299	0.256
2.	2.415	1439.812	69.617
3.	2.866	623.07	30.126
Total		2068.181	100

Table 6: Results for HPLC analysis of *Glycine max* UV-treated callus

Peak Number	Retention time (min)	Area	Area %
1.	1.867	169.228	5.534
2.	2.419	1081.49	35.368
3.	2.871	1715.402	56.098
4.	3.389	91.723	3.000
Total		3057.843	100

The UV-treated samples of plants were compared with control samples it showed that the numbers of peaks of UV-treated samples were higher than control samples. UV-B radiation (for short period) enhanced the production of compounds in GM.

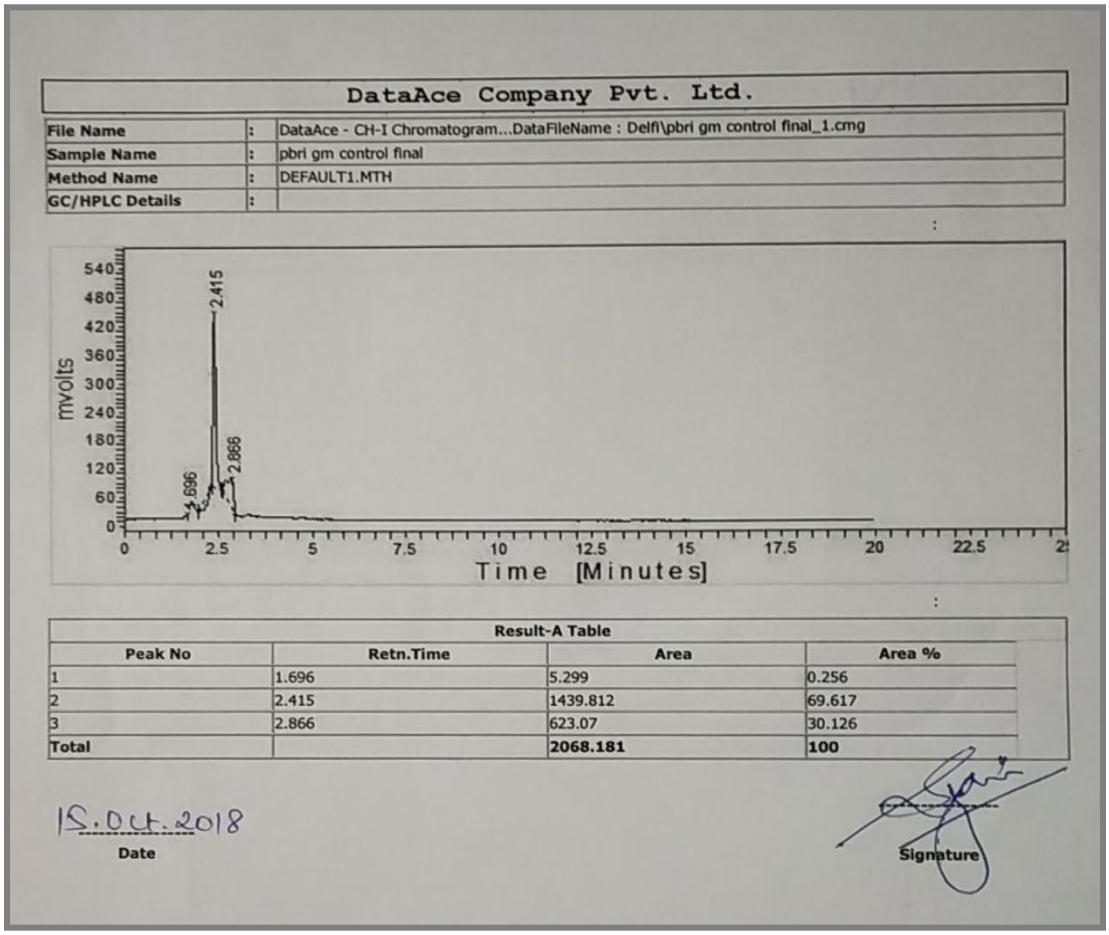

Photo plate 3: HPLC chromatogram of *Glycine max* control callus extract(non-treated)

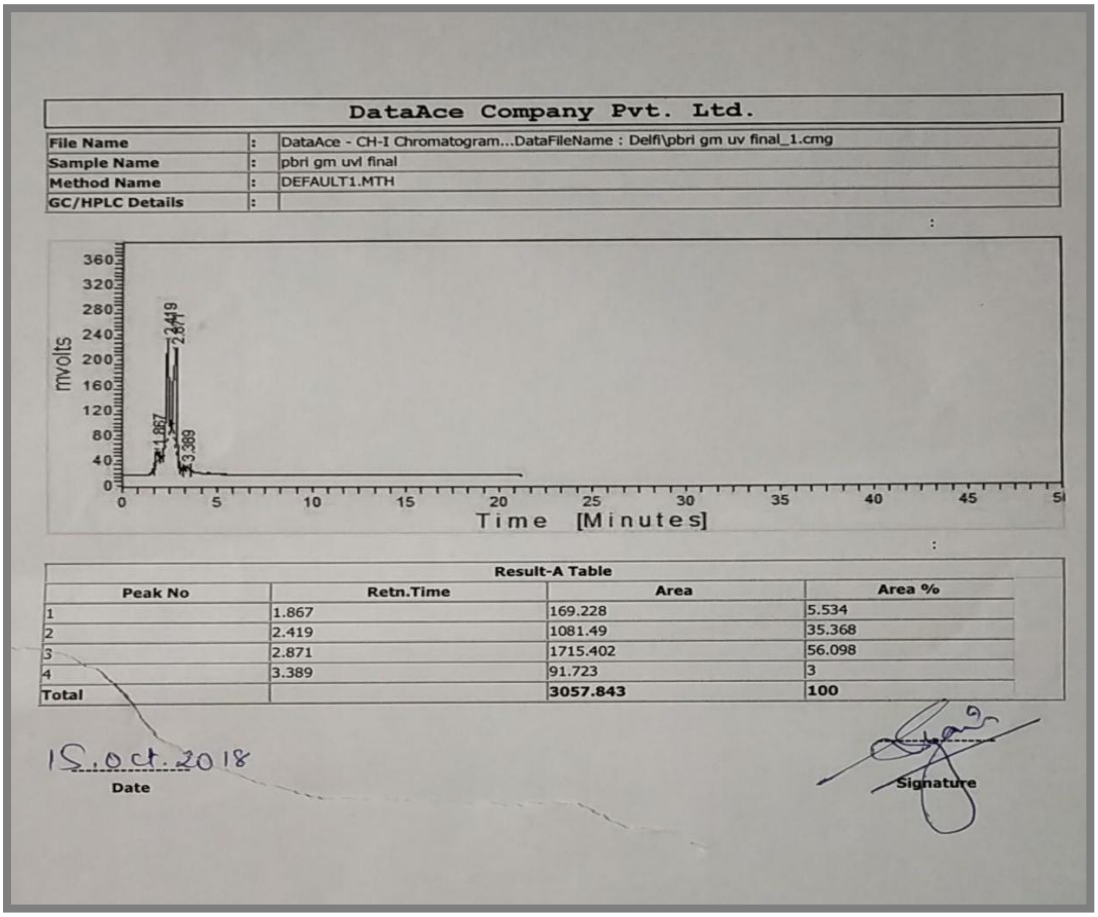

Photo plate 4: HPLC chromatogram of *Glycine max* UV treated callus extract

4. Conclusion: TLC- The methanol (MeOH) with SS-II phase was found effective for the analyses of large number of SMs compounds. Many researchers have proved that Methanol extract was more suitable than ethanol, acetone, dimethylformamide (DMF) extract for analysis of SMs. In UV treated callus extract were found maximum fraction with Rf values in SS-II phase as compared to the non-treated callus extract. These studies indicated that the UV treated callus extract with SS-II phase gave best response for analysis of SMs compounds. In SS-I phase was found highest number of friction with Rf values in Non-treated callus extract of *Glycine max* as compared to the SS-II phase.

HPLC- The UV treated sample of callus extract obtained the higher number of peaks than non-treated sample of callus extract of *Glycine max*. UV-B radiations (for short period)could enhance the number of peak, it was shown by UV treated sample as compared to the non-treated sample. UV treated sample of GM callus extract showed increased peaks number as compared to the non-treated sample. Previous studies have indicated that increased UV-B radiation have positive, neutral and negative effects on plant growth, chlorophyll content, cell size, Growth index, Callus induction rate. Exposure of UV-B radiations for short period on callus, cause increased stress in the callus, and then callus produces SMs for defense. Therefore, UV-B treatment may be beneficial for enhancing the SMs production and also proved our studies, moreover long period exposure of UV-B radiations may be harmful for

callus as it causes death of the cells.These results showed that the PTC with UV supplementation has possibility for commercial and medicinal exploitation of secondary metabolites.

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