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Phenolic Extract from Cell Suspension Culture of Strawberry (Fragaria SP) and Its Cytotoxic Study

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ABSTRACT: The aim of the present research work describes the effects of Ben-Zyl aminopurine (BAP)/2-4dichlorophenoxyacetic acid (2-4D) ratio on callus induction, total phenol extraction production and antioxidant activates in the leaf of Fragaria sp (Strawberry). The supplementation of 25 mg/L 2,4D and 25 mg/L BAP to MS medium was found to be the most efficient for callus induction The result of biochemicals analysis showed that the higher total phenolic contents were obtained in callus cultured on MS medium. Then Phenol assay, Tannin assay, Flavonoid assay are used for the estimation test. Then the callus culture is subculture in the liquid media to extract phenolic metabolites and phenolic metabolites are purified by using TLC, PTLC and HPTLC. And the cytotoxicity study of strawberry callus extract.

KEYWORDS: Callus, Suspension culture, phenolic compounds, cytotoxicity studies.

I. INTRODUCTION

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The strawberry was first bred in Brittany, France in 1750s via cross of Fragaria chil0ensis from Chile and Fragaria virginiana bought from North America. Woodland strawberry was the first strawberry species cultivated in17th century. The strawberry fruit was mentioned in ancient Roman literature with reference to its mediational use . In 15 th century European monks were used strawberry in their manuscripts. Strawberry are used to treat depressive illness Strawberry in Italian, German art and in Flemish and English miniatures In 16 th century Strawberry cultivation become more common. People being to use Strawberry fruit as medicinal properties. Two subspecies of F. vesca , F. sylvestris Semper Florens identified. The introduction F. virginiana from North America to Europe is an Important part in strawberry evolution and that give rise to modern strawberry. The Indians of Chile cultivated the female strawberry. The F. ananassa was determined by Duchesne. Fragaria ananassa was a hybrid of F. virginiana and F. cheilosis's. The Fragaria ananassa produce giant fruit. F.ananassa is smell and taste like pineapple and in berry shape. Strawberry has 200 seeds on its outer surface. The strawberry cultivars widely in size, colour, flavour, degree of fertility, ripening season and liability to disease. In strawberry breeding was conducted to increase fruit size and taste. The strawberry can also grow in indoor gardens but during the winter season strawberry are not grow well in indoor gardens. Strawberry belonged to Rosaceae family, grown in different climatic area of the world. Strawberry known for its aroma. It is cultivated in 73 countries on 200,000-hectare area to produce 31 lakh metric tons of strawberry. The strawberry are commercially cultivated in India, USA, Spain, Japan, Italy, Poland, Germany, Thailand, Korea. But in India strawberry are cultivated in very small scale. Strawberry grows in temperate climate. Spring season is best season for planting strawberry and every Spring strawberry produce three periods of buds, flowers and fruit. These plants take about one year to give good fruit. Some cultivars grow in sub-tropical climate and moderate temperature are important for bud and flower formation. It needs light period of 12 hrs for grow. In India Mahabaleshwar is major produce of strawberry it accounts for above 85 percent of the strawberry produced in India. Strawberry season in Nainital, Kashmir and Mahabaleshwar start during the month of May to June and it was repens during February last or April first. The fruit are harvested when it was looking for like firm and in developed colour. A Strawberry plant can live up to 4-6 years and it take 3 months to bear fruit . This three

months include the whole growing process to harvest time. The fruit is usually ready for harvesting after 4 to 6 weeks and it take 60 to 90 days for plant to mature from seed to a strawberry fruit. The strawberry are mostly used in food and cosmetic industry and it also used in pharma industry to give flavour to medicament. In food industry it is used to prepare ice cream, juice, jams and jelly. Salicylic acids are rich in strawberry so salicylic acid is used as ingredient in skin care product. This reduces acne scars and acne in human body surface. And it also used to reduce dark spot and hyperpigmentation on the face. Micropropagation is artificial way to producing plants vegetatively by cell culture or tissue culture techniques. It multiplies plant material by growing plantlets in tissue culture to produce bulk: number of progeny plants. Micropropagation also known a Tissue culture. Plant tissue culture is a technique used to grow or maintain Malat cell, organs, tissues under the sterile conditions by using the nutrient culture media of suitable composition. Phenolic componds are part of secondary metabolites most commonly found in all plant species The Solvent, such as ethanol, acetone, Ethyle acetate, methanol has been used for the extraction of phenolics from plant materials. Three common methods for extraction of phenolic compounds using LLE method. LLE method include maceration, Soxhlet extraction, hydro distillation methods. Phenolics are mostly found in fruits, vegetables, wine, coffee. Phenolics compounds are response for the bitterness of many fruits due to its interaction with glycoprotein. Phenolic also give a natural colour to many fruits and vegetables. Including in plants phenolics are phenolics acids, tannins acid, flavonoids. Flavonoids are the most common polyphenols in human diet. The flavonoids are composed of flavan nucleus with 14 to 15 carbon atoms arranged in 3 rings and labelled AB and C. There are six subgroups of flavonoid. These are anthocyanins, flavones, flavanols, flavanones, and isoflavones. The common flavonoid are catechin, naringenin, quercetin, and daidzein. Tannins are polyphenolic biomolecules and it is a class of astringent they are subdivided into 2 group they are Hydrolysable and condensed tannins. Central glucoses are present in Hydrolysable tannins. Condensed tannins are known as Proanthovanidins. Tannins are soluble polyphenols that are present in most of the plant. Tannins result in feed efficiency, decreases in feed consume, growth rate, and help in food and protein digestibility in human. It as lower cholesterol level, and stimulate the immune system and has antibacterial properties

II. **Results and Discussion**

1.1. Callus and cell suspension culture induction

For the induction of callus, leaf fragments from in vitro growing shoots of strawberry were cultured on a MS medium. The medium is supplemented with 0.02 g of BAP, 0.02 g of 2,4 dichlorophenoxyaceticacid and 2g of agar was added as a solidifying agent in MS medium. Calli were produced from the explant. The most effective medium for callus induction and suitable for further suspension culture established in the MS liquid broth. The callus produced from the medium was diminutive in appearance, and crumb green, yellow in colour, calli in MS slant medium was suitable for further initiation of sub- culture.

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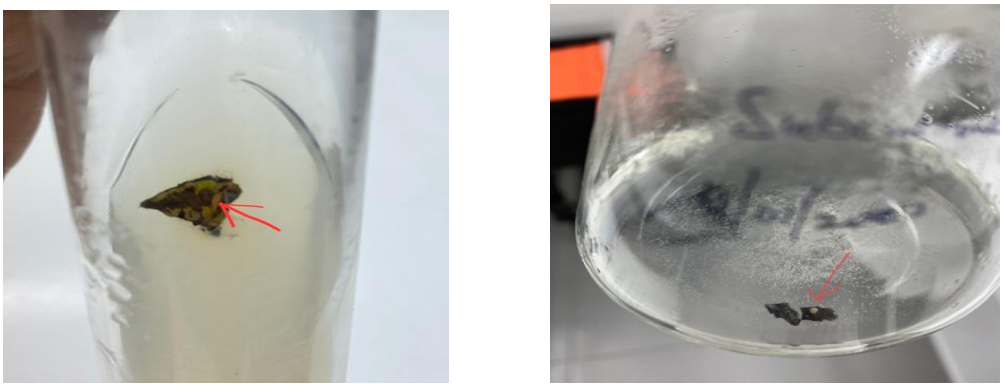


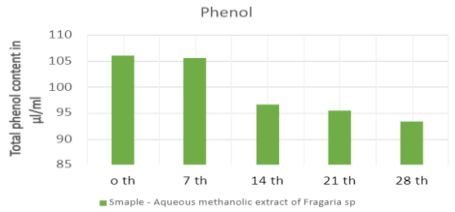
(a) (b)

Figure 1. (a) Formation of callus tissue in MS slant medium (b) callus tissue was transferred liquid broth for the mass production of callus.

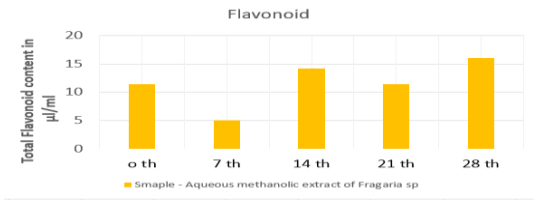
Crumb green yellow and diminutive parts of calli from MS medium were sub-cultured every five weeks in a fresh medium to obtaining a homogenous callus line, which was used for the further process of cell suspension culture in the presence of 0.02 g of BAP, 0.02 g of 2,4- dichloro phenoxy acetic acid (2,4-D). The initiated callus is sub-cultured in liquid medium at suitable environment. After subculturing, 1mL of sub cultured sample from liquid medium is used for the suspension culture with 2 different hormones in five different conical flask and 1mL sub-cultured sample from liquid medium is added in a five different conical in the one-week time interval Then the suspension culture kept in the suitable environment for the production of phenolic metabolites.

1.2. Preliminary Investigations

Extract from cell suspension culture of strawberry was investigated by using colorimetric assay to detect the presence of polyphenols such as Flavonoid, Tannin. .The total phenolic content of aqueous methanolic extraction of Fragaria sp Show higher concentration of phenolic content was observed in 0th day sample with the concentration of 106 l/ml. In Tannin content of aqueous methanolic extraction of Fragaria sp Show higher concentration of Tannin content was observed in 21 th day sample with the concentration of 13.3 µl ml.



(a)



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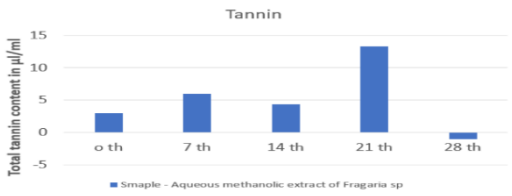
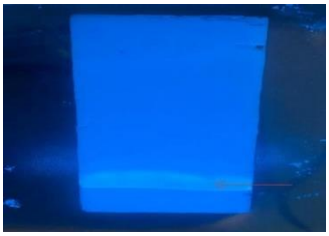


(c)

Figure 2. (a) Flow chart of phenol assay this Figure Represent that, total phenolic content of aqueous methanolic extraction of Fragaria sp Show higher concentration of Phenolic content was observed in 0 th day sample with the concentration of 106 µl/ml and lower concentration of phenolic content was observed in 28 th day sample with the concentration of 93.3 µg/ml (b) Flow chart of Flavonoid assay this Figure Represent that, Total Flavonoid content of aqueous methanolic extraction of Fragaria sp Show higher concentration of Flavonoid content was observed in 28 th day sample with the concentration of 16 µl/ml and lower concentration of phenolic content was observed in 7 th day sample with the concentration of 5 µg/ml; (c) Flow chart of Tannin assay This figure Represent that, total Tannin content of aqueous methanolic extraction of Fragaria sp Show higher concentration of Tannin content was observed in 21 th day sample with the concentration of 13.3 µl/ml and lower concentration of Tannin content was observed in 28 th day sample with the concentration of -1 µg/ml.

1.3. Chromatographic Analysis

The preparatory thin layer chromatography (PTLC) is used for the purification of samples in the separable mixture. The chromatogram profiles of the lyophilized extracts, dissolved in three different alcohols and water in the ratio of (7:2:1) indicate that the presence of Phenolic acid, Flavones, and absent of anthocyanins in the extracts. Then UV spectroscopy method to determine the phenolic content present in sample. After the UV light treatment, the result show that the extracted Supernatant sample contain Flavonoids.



(a)

Figure 3. PTLC UV chromatogram determined the presence of Flavonoid in the extract of strawberry callus with a concentration of 447µg/ml and flavonoid scrapped from the plate.

1.4. Investigation of cytotoxic activity of fruit extract by MTT assay

Brest cancer cell line is used for this cytotoxicity study this cell lines were treated with extract obtained from Flavonoid in the extract of strawberry callus. The cytotoxic potential of Flavonoid was evaluated in human Brest cancer cell lines for the inhibition of cell proliferation and to identify the decrease of cell viability. The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product. It reflects the normal function of mitochondria and measuring thecytotoxicity cell and viability of the extract.

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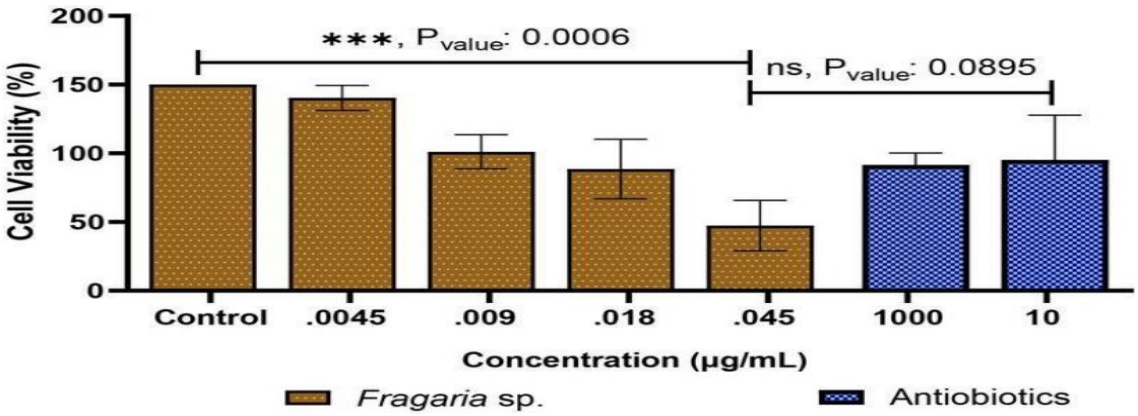


(a) **Figure 4.** (a) Percentage cell viability obtained from recovered extract of Fragaria sp this figure show that concentration of extract increases, there is a decrease in the % cell viability, which depicts the efficiency of the extract on cancer cells. The extracts were also compared with the standard antibiotic which eventually showed decrease in % cell viability with increase in concentration.

III. Materials and Methods

2.1 Media preparation

Media	Volume
MS 1	25mL
MS 2	10mL
MS 3	10mL
MS 4	10mL
MS 5	5mL
Sucrose	30mL
Myoinositol	100mg

All the above different media and solution are added in volumetric flask and the flask is mixed to dissolve all the ingredients together after that 980ml of distilled water is added into flask and mixed well. Myo inositol is added in media as it breaks down into ascorbic acid and pectin and is incorporated into phosphoinositide and phosphatidyl-inositol, it is thought to be involved in cell division. The preparation of MS media involves inorganic nutrient, organic nutrient, growth hormones and gelling agents. Take 400ml double- distilled water in 1L beaker. Weight the macronutrients and dissolve them completely in wates. Take 100mg Myo-inositol and dissolve it in a mixture and add 30g sucrose to the same mixture. Then add 800ml of double- distilled water in the beaker next to adjust the pH of the media to 5.7. Transfer the previous mixture to 1L beaker and make up the final volume to IL then keep the prepared solution in the refrigerator.

2.2 Surface sterilization and Inoculation of explant

Before starting the inoculation process, we want to sterilize all the equipment which are used for the tissue culture process. previously prepared stock solution was taken in the quantity of 200ml then in was transferred to conical flask and keep in the autoclave at 120 °C for 20 minutes. Then collected strawberry leaves were washed in the running tap water to remove the soil and other dust particles then the leaves are transferred to petri plate and kept in laminar air flow chamber. The UV light of laminar air flow chamber is switched on for 4-

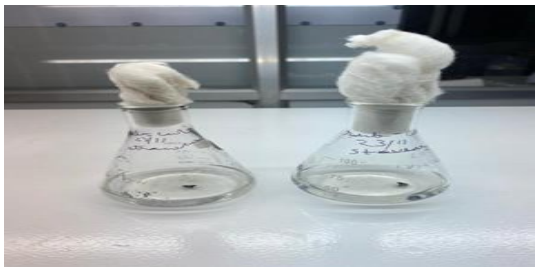
5 minutes to sterilize the leaves and the inner interior of laminar air flow chamber to avoid contamination. In the initial sterilization a collected strawberry leaves surface sterilized by using 0.1% mercuric chloride. for 1 minutes by vigorous shaking. Surface sterilization with mercuric chloride will remove the surface microorganism then it was washed with double distilled water then it was surface sterilized by using 4 % sodium hypo chloride for 2 minutes then it was washed with double distilled water for 1 minute's addition to it then it was surface sterilised by using 0. 1 % of Nystatin dihydrate for 1 minutes to remove the surface contamination such as endophytic bacteria. Fragaria ananassa is collected from the nursery, Medium was prepared with the supplementation of 0.02g of BAP,0.02g of 2-4D and 2g of agar was added as a gelling agent. Then mixture is heated in oven for 60 sec. Then it is transferred into a test tube and autoclaved at 121°c for 20 minutes. Surface sterilization of the explants was achieved using 0.1% mercuric chloride, Nystatin dihydrate and sodium hypochlorite. After that, the explant was cut into suitable sized and injected into the medium. The inoculated cultures were maintained in a room at 24 ° 16 h light and 8 h dark, after 4 Weeks the callus tissue was formed in the slant medium.

(a)

Figure 5. (a) surface sterilization of explant strawberry leaf

2.3 Subculture of strawberry

Subculture is transferring of plant tissue or plant cell to new fresh media for the purpose of mass production. This process helps the plant cell to reproduce. After the formation of callus tissue, the callus is cut into four and the each, piece. of callus are inoculated in the slant media and liquid broth for the mass production of callus with the same growth regulator BAP and 2,4-D with the same concentration. Once the callus tissue is formed the newly formed tissue is transferred on to the new media (Slant media and liquid broth). Liquid media is also called liquid broths. The liquid broth allows the uniform growth of callus tissue. After 21 days strawberry callus is formed in liquid broth from the callus tissue.



(a)

Figure 6. (a) subculture of callus for the mass production **.**

2.4 suspension culture of strawberry

International Journal of Advances in Engineering and Management (IJAEM)
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The initiated callus is subculture in liquid media at suitable environment. After subculturing, 1mL of sub cultured sample from liquid media is used for the suspension culture with 2 different hormones in 5 different conical flasks the suspension culture kept in the suitable environment for the production of phenolic metabolites.

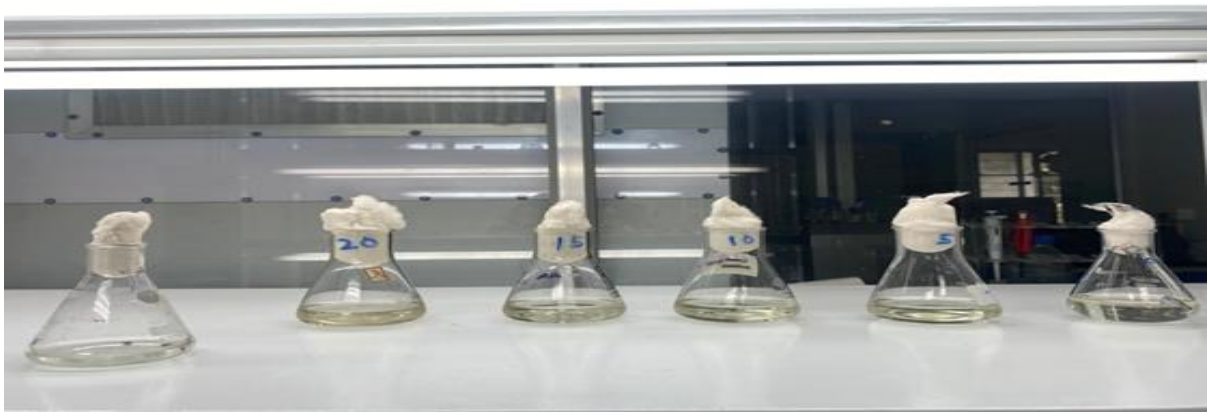

Figure 7. Suspension culture of subculture sample.

2.5 Assay procedure

(a) Estimation of Phenolic assay: Gallic acid equivalent was used to determine the total phenolic content. extract, then 50µl of water, 3600 l of the Folin - Ciocalteu reagent, and vertexing were performed. After adding 680µl of a 7.5% sodium carbonate solution, the mixture was vortexed and incubated for 90 minutes at room temperature. A spectrophotometer was used to measure the absorbance at a wavelength of 750 nanometre (nm). 13µl of water was used in place of extract in the blank tube. A standard graph is used to compare the graph. Three separate experimental repetitions involved the preparation of each solution in triplicate.

(b) Estimation of Tannin assay: By using the Folin-Ciocalteu method. The procedure was modified as follows: 50µl of the extract, 240µl Folin-Ciocalteu reagent,3700 l of distilled water, adding 1000ml of a 17.5% sodium carbonate solution, the mixture was vortexed and incubated for 30 minutes at room temperature. The absorbance at 725nm is measured. 13µl of water was used in place of extract in the blank tube. A standard graph is used to compare the graph. Three separate experimental repetitions involved the preparation of each solution in triplicate.

(c) Estimation of flavonoid assay: First 500µl of the extract was added then 120ml of water, 150ml of 5% sodium nitrite, after 5min 150µl of 10%aluminium chloride and vertexing were performed. The absorbance at 510 nanometres was measured using a spectrophotometer (nm) 100ml of water was used in place of extract in the blank tube. A standard graph is used to compare the graph. Three separate experimental repetitions involved in the preparation of each solution in triplicate. Then total flavonoids content was determined by using rutin equivalent.

2.6 phytochemical extraction from callus

Sub cultured callus was taken out from the culture media and kept in the petri plate. Then the callus was cut into two pieces then transferred to the motor for crushing process. The callus sample was extracted by using 70 % methanol by pulverizing the sample by using of pestle and motor and the extraction was centrifuge for 5 minutes for 3000 rpm for the production of supernatant. Then the Supernatant liquid extract was stored in a vacutainer and kept in the freezer for the future use.

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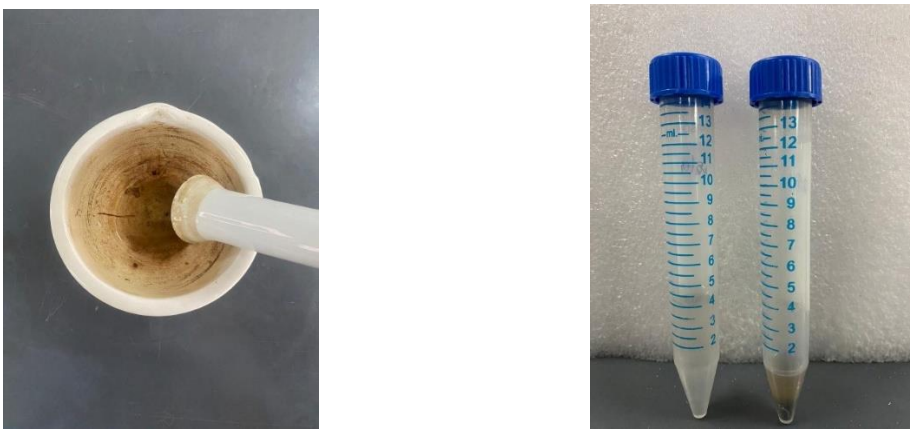

(a) (b)

Figure 8. (a) Callus pulverization by using pestle (b) Extract supernatant liquid extract.

1.7 Preparatory thin layer chromatography: Preparatory thin layer chromatography (PTLC) is used for the purification of samples in the separable mixture. In Mobile phase first to make a solvent mixture in the ratio of 7:2:1. For 200 ml solvent mixture we should add 140 ml Toluene, 40 mi Ethyl acetate and 20 ml formic acid in the glass container and close the top lid of the container for 1 hour after 1 hour. In Stationary phase to make a slurry of PTLC. slurry is prepared by using Silica with water in a conical flask by vigorous shaking for three minutes. Usually, 40 g of Silica is mixed with 80 ml of H2O to make 1 plates of thickness 2mm Then place the PTLC spreader on the PTLC plate and pour the slurry in the chamber. Daw the spreader over the glass plates. And cover the glass plate with Silica slurry. Then transfer the glass plate to oven and keep the slurry mixture in the oven for 2 to 3 hours. After drying keep the slurry plate in the table for loading the sample. By using the pencil first to make a spot in the glass plate for depositing the sample. In 20 CM × 20 CM slurry plate make a four spot. Then start loading the sample of the supernatant in the spot in the volume of 10mµl pre spot for 4 time in the time interval of 5 minutes. After loading the supernatant in the spot then transfer the slurry plate to the solvent mixture container for the determination of Phenolic content in the sample after 1 hour the slurry plate was read more than 80 % so take away the slurry plate from the solvent solution. Then kept under the UV light to determine the phenolic content present in our sample. After the LIV light treatment, the result show that the extracted Supernatant sample contain Flavonoids.

(a)

Figure 9. (a)Flavonoid are determined by using PTLC method.

2.8 INVESTIGATION OF CYTOTOXIC ACTIVITY OF FRUIT EXTRACTS OF SELECTED CULTIVARS and Determination of cell viability by using MTT assay

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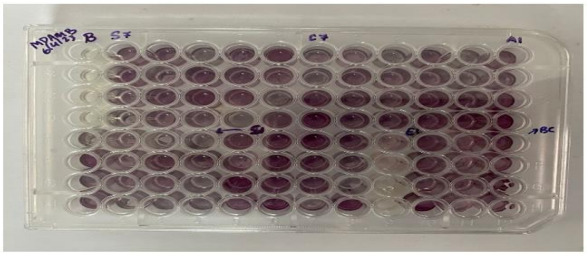
MDA-MB 231 (breast cancer cell lines) was obtained from Avinashi Lingam Institute for Home Science and Higher Education for Women, Coimbatore and cultured in DMEM media with 10% Fetal Bovine Serum (FBS). On reaching appropriate confluence, the cells were passaged and cultivated in sterile T-25 culture flasks. The cells were counted using a haemocytometer and cell viability was determined by trypan blue test. Cells from log phase is seeded in 100 pl of DMEM medium supplemented with 10% fetal bovine serum per well of 96-well flat-bottom culture plates. Cells were incubated with various concentration of the methanol and aqueous methanol extracts of Fragaria sp. for a defined time (12, 24 and 48 hours. Proliferative response and cell death of the extracts-treated cells was determined by MTT assay. The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product. It reflects the normal function of mitochondria and and measuring the cytotoxicity cell and viability of the extract. 1.64×10S viable cells/well was plated into the 96-well tissue culture plates and then incubated at 37°C overnight. The next day when the confluence of the cells has reached >80%, the media was replaced with 200 ml of fresh complete medium containing O (ascontrol), 200, 400, 600, 800 and 1000 g/ml concentrations of crude extract (methanol and aqueous methanol extracts of Fragaria sp. There was no extract was added to the negative control well. After 12 hours, the supernatants were removed, and cell layers are washed with phosphate buffered saline (PBS) and incubated with MTT (50ml and 3 mg ml) in PBS for 3 hours in a humidified atmosphere at 37°C. Sulof 5mg/ml MTT was added to each well. The plate was covered by aluminium foil and incubated at dark for 2 to 4 hours at 37°C.The media and MIT were removed from each well and the remaining MIT- formazan crystals were dissolved by adding 50ul of DMSO to all wells. The recorded absorbance at 590m by using a micro- plate reader. The percentage of cell viability = OD of treated cells/OD of control cells× 100.

2.9 Percentage viability obtained from the extract of Fragaria sp;100 µl of treated cells were incubated with 50ml of MTT for 3 hours at 37°C Following the incubation period, 200ml of PBS was added to all the samples and thoroughly aspirated to eliminate any excess MTT. 200 µl of acid propanol were added and left to solubilize overnight in the dark. In a microtiter plate reader, the absorbance was measured at 595nm. The control cells optical density was set to 100% viable, and the percent viability of the cells in the other treatment groups was computed using the formula.

$$\text{Percent viability} = \frac{\text{Control OD - Sample OD}}{\text{Control OD}} \times 100$$



(a)

Figure 10. (a) 96 well plate with MTT after incubi

VI. CONCLUSION

In this study leaf explant were used to induce callus by using MS media. The MS media is supplemented with BAP and 2,4-D with concentration of 0.02mg/L. After 21 days callus induction was observed. From the callus, Phytochemical extraction was made by using aqueous methanol in 1:1 ratio for extraction of total phenol, flavonoid and tannins. From this studies flavonoid shows higher concentration 447 ug/ml. Compared to

tannins and phenol. Further total flavonoid was separated and partially purified by TLC and PTLC. Separated flavonoid are isolated from silica gel and again total flavonoid estimated. Flavonoid content time PTLC fraction is 447 g/ml. In anticancer study, the investigation of cytotoxic activity was found by MIT assay. The result showed that, when concentration of the extract increases, there is a decrease in the % cell viability, which depicts the efficiency of the extract on cancer cells. From the PTLC analysis of the Methanolic extract of Fragaria sp under hot extraction condition showed that yellowish coloured fluorescence zone at UV366nm mode were present in the tracks, it was observed from the chromatogram after derivatization, which confirmed the presence of phenol in the extract. By PTLC, the recovered sample was scrapped and used for further investigation.

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