Process for Isolating the Active Ingredients from Different Parts of the Unripe Fruit of *Diospyros peregrina* and their Characterization to Define the Cancer Preventive Functions

Jayanta Mondal* and Radhika Shukla

Department of Biotechnology, SRM University, Chennai, Tamil Nadu, India

**Abstract**

*Diospyros peregrina* is a plant indigenous to India, belonging to the family Ebenaceae. It is a medium-sized evergreen tree having ethnomedicinal significance as an aphrodisiac, astringent, bactericide and is used for the treatment of several diseases. Herbal practitioners have indicated that all parts of this plant specially bark, leaves, flowers and fruits are used in different medicinal preparations. Diverse bioactive compounds have been isolated from various parts of this plant. Every year, there are 500,000 cases of cancer, and compounds in this fruit could be an answer to its treatment, saving millions of lives. The Milli-Q water extracts of the epicarp, mesocarp and endocarp of the unripe fruit of *Diospyros peregrina* will be prepared and lyophilized separately. In vitro cytotoxic assay will be performed on different kinds of cancer cell lines using the lyophilized fractions through MTT assay. In *vitro* lymphocytic proliferation will be also tested on the lymphocytes isolated from normal and cancerous subjects using the lyophilized fractions through MTT assay. Cytotoxic T lymphocyte (CTL) assay will be carried out coculturing lyophilized fractions treated lymphocytes (effector cells) and cancerous cells (target cells) and cellular cytotoxicity will be determined by measuring lactate dehydrogenase (LDH) released by target cancerous cells using a commercially available kit (Roche Diagnostics, Mannheim, Germany). Further, methanolic extract of the unripe fruit of *Diospyros peregrina* will be carried out to identify the secondary metabolites which might possess cancer preventive properties. Further the cancer preventive function of the extracts through immunostimulation was confirmed by using the effector cells (Gab extract treated lymphocytes) against COLO 205 (Colonrectal cancer cell lines) by CTL reaction through LDH release assay. Thus, the fractions having the principle active ingredient responsible for the cancer preventive activity will be further confirmed through HPLC and silica gel column purification. This aims to be a significant milestone in finding a non-invasive cure for cancer. The cancer preventive active ingredient from the unripe fruit of *Diospyros peregrina* could revolutionize cancer treatment.

**Keywords:** *Diospyros peregrina*; Antitumour; Anticancer

**Introduction**

India is a rich source of medicinal plants and a number of plant extracts are used against diseases in various systems of medicine such as ayurveda, unani, and siddha. Only a few of them have been scientifically explored. Plant derived natural products have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects. The genus *Diospyros* L. (Ebenaceae), which is distributed throughout the tropics, is characterized by its ability to produce triterpenes of the lupine series. The genus *Diospyros* consists of 240 species, 59 of which are distributed in India, Thailand, Japan, Nigeria, South Africa and Philippines [1,2].

*Diospyros peregrina* can be seen mostly as trees and rarely shrubs. Various bioactive compounds were isolated from this plant. The plant is traditionally used for the treatment of dysentery and menstrual problems. The plant also possesses antifertility activity [3]. This plant has ethno- medicinal significance as an aphrodisiac, astringent, bactericide and for the treatment of diarrhoea, cholaera, dysentery, fever, malaria and diabetes. The maturation of mature fruits is successfully used in costal West Bengal for treatment of diabetes. The alcoholic extract of stem barks of this plant has been reported to possess hypoglycaemic, diuretic and anti-cancer properties [4]. Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives every year [5]. An extremely promising strategy for cancer prevention today is chemoprevention, which is defined as the use of synthetic or natural agents (alone or combination) to block the development of cancer in humans. Cancer therapy in the form of surgery or radiotherapy is effective when the disease is detected early but many cancers are still diagnosed when cells from a primary tumor have already metastasized to other parts of the body and the main form of treatment at this point is chemotherapy.

Chemotherapy entails delivering drugs systemically so that they can reach and kill the tumor cells, but most of these drugs cause severe side effects in patients and, therefore, need to be used at suboptimal levels. According to various researches, the low efficacy of chemotherapy in patients with advanced cancers is reflected in the low 5-year survival rates observed in these patients and the low efficacy of cancer therapy for the treatment of patients with metastasis makes the development of novel chemotherapeutic agents necessary. The major challenge is to design and find new drugs that will be more selective for cancer cells and thus have lesser side effects [6].

Plants have served as a rich source of therapeutic agents for many centuries, being used themselves or as the basis for synthetic drugs, and despite the great developments, a large portion of recent chemotherapeutic drugs are derived from or based upon natural products. Plants, vegetables and herbs used in the folk and traditional medicine have been accepted currently as one of the main source of cancer chemoprevention drug discovery and development. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine.

*Corresponding author: Jayanta Mondal, SRM University, Kattankulathur, Chennai, Tamil Nadu-603 203, India, Tel: 8569264895; E-mail: jayanta_mondal@gmail.com

Received October 03, 2017; Accepted October 17, 2017; Published October 24, 2017

Citation: Mondal J, Shukla R (2017) Process for Isolating the Active Ingredients from Different Parts of the Unripe Fruit of *Diospyros peregrina* and their Characterization to Define the Cancer Preventive Functions. Med Chem 7: 308-313. doi: 10.4172/2161-0444.1000473

Copyright: © 2017 Mondal J, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
Materials and Methods

Aqueous or methanolic extraction

Three different sections of the *D. peregrina* fruit such as Epicarp, Mesocarp and Endocarp were chopped into tiny pieces and shed dried separately. The shed dried materials were kept for overnight shaking at 4°C with addition of miliQ water or methanol. After the overnight incubation the supernatant was obtained by centrifugation.

Lyophilization

The samples extracted using aqueous solution or methanol was subjected to lyophilization. Lyophilization is an operation by which water (or another component) is separated by sublimation from a frozen system or phase. The passage from solid to gas occurs without the appearance of water or the solvent in liquid state. Freezing is the process by which water becomes congealed into ice by cold. Freeze-drying is split into three process stages, freezing, primary drying at lower temperatures when most of the water is removed, and secondary drying at ambient or higher temperatures to minimize the final unbound water content.

**In vitro cytotoxicity assay using MTT**

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically [7]. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. The monolayer of COLO205 cell culture was trypsinized and the cell count was adjusted to 1 × 10⁶/ml using RPMI 1640 medium containing 10% FBS, penicillin (50 U/ml) and streptomycin (50 μg/ml). COLO205 cells were then plated in each well of a 96-well microtitre plate at the concentration of 2 × 10⁴/200 μl complete culture medium and treated with lyophilized samples isolated from aqueous or methanol extracts dissolving in either PBS or DMSO respectively and kept for incubation at 37°C in 5% CO₂ incubator for 24 hours. After 24 hours, 20 μl of MTT solution (5 mg/ml) was added in each well and incubated for 4 hours at 37°C. The medium was removed by aspiration and the purple colored formazan precipitate was dissolved in DMSO (100 μl) and absorbance was measured at 550 nm using a microplate reader (Tecan Spectra, Grodig, Austria). The absorbance obtained was directly proportional to the number of viable cells.

Lymphocyte isolation

Normal and colorectal patient whole blood samples were collected using sterile EDTA anti-coagulated tubes. The samples were equally diluted using 1X PBS and layered on top of FicollPaque Plus gradient solution in 1:2 ratio. It was then subjected to centrifugation at 400 g for 30 minutes at 4°C. The lymphocyte layer formed was then collected separately and undergone a wash with 1X PBS.

Effect cell preparation

T-lymphocytes thus isolated were treated with the lyophilized samples (Epicarp, Mesocarp and Endocarp) for 48 hours at 37°C, 5% CO₂ and 90% humidity along with controls.

Lymphocytic proliferation assay

The lymphocytic proliferation was examined using 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT). The assay is based on the cleavage of the tetrazolium salt, MTT, in a presence of an electron-coupling reagent “Succinate reductase system” by active mitochondria to formazan. The water insoluble formazan salt produces has solubilized with DMSO (100 μl). The effector cells (2 × 10⁴/200 μl complete culture medium) were incubated with 20 μl of MTT solution (5 mg/ml) for 4 hours at 37°C. After the incubation the amount of formazan dye formed directly correlates to the number of metabolically active cells in the treatment. After solubilization the formazan dye is quantified using ELISA reader at 550 nm (Tecan Spectra, Grodig, Austria).

Cytotoxic T-Lymphocytes (CTL) reaction

CTLs are key component of the adaptive immune response. They recognize MHC I associated tumor antigen on target cell receptor (TCR) complex that includes, TCR, CD3, and CD8. Upon recognition, the golgi and the microtubule-organizing center (MTOC) reorient towards the target cell and cytolytic granules move toward the membrane for docking and release. The immunological synapse formed between CTL and target is stabilized by adhesion molecules including αβ2/αβ2-1 and ICAM-1. Key components of the granules include the granzyme protease and the membrane-perturbing protein perforin. Perforin forms pore on the target cell plasma membrane for passive diffusion of cytolytic molecules. Cytotoxic T-lymphocyte reaction was performed with the help Lactate Dehydrogenase (LDH) release assay. Lactate dehydrogenases were isolated in aseptic conditions from colorectal cancer patients. Those lymphocytes were suspended in RPMI-1640 tissue culture medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS and penicillin-streptomycin solution. These effector lymphocytes (2 × 10⁴) were then cultured with or without glib extracts for 48 hours. These effector lymphocytes (E) were co-cultured with target COLO-205 cells (T) at an E:T ratio of 10:1 at 37°C in round bottom, 96-well microtiter plates. Cellular cytotoxicity was determined by measuring lactate dehydrogenase (LDH) released by target cells using a commercially available kit (Roche Diagnostics, Mannheim, Germany). After 4 hours of incubation, 100 μl of supernatant was removed to estimate the release of LDH as per manufacturer’s protocol and the absorbance was noted at 490-492 nm in microplate reader (Tecan Spectra, Grodig, Austria). The amount of spontaneously released LDH was measured in wells that contained only target cells. Total released LDH was assessed by lysing the target cells with 2% TritonX-100. The percentage of lysis was calculated according to the following formula:

\[ \% \text{specific lysis} = \left( \frac{([\text{experimental lysis}-\text{spontaneous lysis}])}{(\text{maximum lysis}-\text{spontaneous lysis})} \right) \times 100 \]

High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is an important analytical method commonly used to separate and quantify components of liquid samples. In this technique, a solution (first phase) is pumped through a column that contains a packing of small porous particles with a second phase bound to the surface. The different solubilities of the sample components in the two phases cause the components to move through the column with different average velocities, thus creating a separation of these components. The pumped solution is called the mobile phase, while the phase in the column is called the stationary phase. During an HPLC experiment, a high-pressure pump takes the mobile phase from a reservoir through an injector. It then travels through a reverse-phase C18-packed column (5 micron pore size) for component separation. Finally, the mobile phase moves into a detector cell, where the absorbance is measured at 218/220 nm, and ends in a waste bottle. The amount of time it takes for a component to travel from the injector port to the detector is called the retention time.
The HPLC apparatus consisted of a Quat single pump and an automated injector equipped with a fluorescence detector (PDA/DAD). The injection volume was 40 microlitre. The mobile phase consisted of 50% methanol and 50% water. The run time was 50 minutes at a flow rate of 1.0 ml/min with a pump operating pressure of approximately 148 bar.

Results and Discussion

Different In vitro assays were performed to ascertain the effect of D peregrina extract on the cancer cells. The effect of the extract on the necrosis of cancer cells and proliferation on lymphocytes has been tabulated.

In vitro cytophotoxicity assay of the fruit extract using MTT

The treatment of the COLO205 cancer cell lines with the extract. Three separate sets of readings were consistent with the decline. Each data represents the mean ± SEM. P-values were calculated using GraphPad Instat software (Table 1; Figure 1). DMSO (Control) vs Epicarp *** P<0.001; DMSO (Control) vs Mesocarp *** P<0.001; DMSO (Control) vs Endocarp *** P<0.001.

The cytotoxicity assay was conducted and the O.D. value at 550 nm was plotted. A decrease in the O.D. value was observed when the cancer cells were treated with the extracts obtained from the different parts of the fruit. Also both methanolic and PBS extracts of the fruit were tested on the cell lines, and results obtained are steady as observed in the graphs below (Figure 2).

The cytotoxicity assay was conducted and the O.D. value at 550 nm was plotted. A decrease in the O.D. value was observed when the cancer cells were treated with the extracts obtained from the different parts of the fruit as well as with increase in the doses of the extract. At different concentrations of 10 µg/ml, 20 µg/ml and 30 µg/ml, constant decrease in the viability of the cancer cells is observed (Figure 3).

Lymphocytic proliferation assay on cancer patient lymphocytes using MTT

As shown in Table 2, the lymphocytic proliferation after treatment with the fruit extract was higher in comparison with the control. Lymphocytes from three different patients (n=3) were isolated and treated with the extract, the results obtained are regular with one another. Each data represents the mean ± SEM. P-values were calculated using GraphPad Instat software (Figure 4).

Media vs DMSO *** P<0.001; Media vs Epicarp *** P<0.001; Media vs Mesocarp *** P<0.001; Media vs Endocarp *** P<0.001; DMSO vs Epicarp *** P<0.001; DMSO vs Mesocarp *** P<0.001; DMSO vs Endocarp *** P<0.001.

Cytotoxic T-lymphocyte reaction through LDH release

Necrosis of cancer cells by the gab extract was estimated through the LDH release assay. It was checked with different samples from three patients and consistent results have been obtained and plotted.

After 48 hours, a CTL assay was performed using these lymphocytes as effector and COLO 205 cells as target using E:T ratio 10:1. Each data represents the mean ± SEM. P-values were calculated using GraphPad Instat software (Tables 3 and 4; Figure 5).

DMSO(Control) vs Epicarp *** P<0.001; DMSO(Control) vs Mesocarp *** P<0.001; DMSO(Control) vs Endocarp *** P<0.001.

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was performed on the lyophilized methanolic extracts of the endocarp of Diospyros peregrina and different fractions were eluted. Further, silica gel column purification needs to be done to identify the exact active ingredient. Since the compounds eluted out are unknown and novel so there are no standards available for comparison. The present investigation indicates that the Diospyros peregrina fruit extract showed significant anticancer activity (Figure 6).

Conclusion

Diospyros peregrina commonly known as Gab has widely spread in the regions of India, Thailand, Japan, Nigeria, South Africa and Philippines. It has high medicinal impact and has been widely used for the treatment of diabetes, diarrhea, cholera, dysentery, fever, malaria and also been reported to possess hypoglycemic, diuretic and anti-cancer property.

Extracts of three different regions (Epicarp, Mesocarp and Endocarp) of the plant were made using aqueous or methanolic solution and lyophilized. These lyophilized components were then treated with T- lymphocytes isolated from the cancer patient blood samples and the proliferative activity was found using MTT.

Further the cancer preventive function of the extracts through immunostimulation was confirmed by using the effector cells (Gab extract treated lymphocytes) against COLO 205 (Colorectal cancer cell lines) by CTL reaction through LDH release assay [8]. Some triterpinoids are found to have promising anticancer and antioxidant activity. Methonolic extracts of Diospyros peregrina (MEDP) shows the presence of tannins and triterpenes which may act as anticancer and antioxidant principles with MEDP. Thus, the fractions having

|       | DMSO (Control) | Epicarp | Mesocarp | Endocarp |
|-------|----------------|---------|----------|----------|
| Set I | 3.861296992    | 2.25659903 | 3.05879988 | 1.79924795 |
| Set II| 3.70320102     | 2.30098881 | 3.08002785 | 1.67591247 |
| Set III|3.89140009     | 2.21683868 | 3.1086102  | 1.8209838  |
| Average(s)| 3.81683397 | 2.25641251 | 3.08247098 | 1.765381408 |
| SEM(s) | 0.058          | 0.025   | 0.014    | 0.045     |

|       | Media          | DMSO   | Epicarp | Mesocarp | Endocarp |
|-------|----------------|--------|---------|----------|----------|
| Patient 1| 0.4442999595  | 0.7245000005 | 1.19609997 | 1.04099977 | 1.427299976 |
| Patient 2| 0.4999589922  | 0.786837 | 1.236956677 | 1.01116448 | 1.47634887 |
| Patient 3| 0.519589922   | 0.7647837 | 1.259999657 | 1.05701645 | 1.49887 |
| Average(s)| 0.4880849624 | 0.7587017902 | 1.231918777 | 1.036621965 | 1.467506282 |
| SEM(s) | 0.022          | 0.018  | 0.019   | 0.013    | 0.021     |

Table 1: In vitro Cytotoxicity assay through methanolic extracts using MTT.

Table 2: Lymphocytic proliferation assay on colorectal cancer patient lymphocytes using MTT.
Table 3: Experimental lysis.

|            | DMSO (Control) | Epicarp | Mesocarp | Endocarp | spontaneous lysis | Maximum Lysis |
|------------|----------------|---------|----------|----------|------------------|---------------|
| Patient 1  | 1.299650955    | 1.515000029 | 1.396700001 | 1.611999989 | 1.783599973 | 1.205000043 |
| Patient 2  | 1.3309552      | 1.590000229 | 1.42854763 | 1.639885559 | 1.835999727 | 1.240429153 |
| Patient 3  | 1.319650955    | 1.5528881  | 1.416293  | 1.649988556 | 1.805999777 | 1.22000422 |

Table 4: % specific lysis.

|            | DMSO (Control) | Epicarp | Mesocarp | Endocarp | %specific lysis | Average(s) | SEM(s) |
|------------|----------------|---------|----------|----------|----------------|------------|--------|
| Patient 1  | 16.36          | 53.56   | 33.13    | 70.34    | 16.20666667    | 0.5480977204 | 1.501003368 |
| Patient 2  | 15.19          | 58.7    | 31.62    | 67.07    | 16.35666667    | 0.5750072463 | 1.821943407 |
| Patient 3  | 17.07          | 56.81   | 33.5     | 73.38    | 16.20666667    | 0.5480977204 | 1.501003368 |

Figure 1: Extracts isolated from 3 different sections (epicard, mesocarp and endocarp) of D. peregrina fruits show in vitro cytotoxicity on COLO205 cancer cells.

Figure 2: Cytotoxicity assay using MilliQ water extracts of Gub.
Cytotoxicity assay using methanolic extracts of Gub at different doses.

Figure 3: Cytotoxicity assay using methanolic extracts of Gub at different doses.

Extracts isolated from 3 different sections (epicard, mesocarp and endocarp) of Diospyros peregrina fruits involve in lymphocytic proliferation.

Figure 4: Extracts isolated from 3 different sections (epicard, mesocarp and endocarp) of D. peregrina fruits involve in lymphocytic proliferation.

Cytotoxic T-lymphocytes (CTL) Reaction through LDH release

Figure 5: Extracts isolated from 3 different sections (epicard, mesocarp and endocarp) of D. peregrina fruits help to generate cytotoxic T lymphocyte (CTL) reaction.
the principle active ingredient responsible for the cancer preventive activity will be further confirmed through HPLC and silica gel column purification.

Acknowledgements

The authors would like to extend our gratitude and heartfelt thanks to SRM University for providing us with the moral support and motivation for carrying out this research to the best of our abilities.

References

1. Chopra RN, Nayar SL, Chopra IC (1956) Glossary of Indian Medicinal Plants. In: Jessil MA (ed.), CSIR Publication, New Delhi, p: 330.
2. Dhar ML, Dhar MM, Dhawan BN (1968) Screening of Indian plants for biological activity. Ind J Exp Biology 6: 232.
3. Mondal SK, Chakraborty G, Gupta H (2005) Synthesis and anticancer activity of some novel 3-(1,3,4-thiadiazol-2-yl)-quinazolin-4(3H)-ones. Drug Resistance 13: 25-29.
4. Alex AT, Nawagamuwa NH, Joseph A, Rao JV (2012) In vitro anti-cancer and antioxidant activity of different fractions of Diospyros peregrina unripe fruit extract. Free Radicals and Antioxidants 46: 49-53.
5. Abdullaev FI, Luna RR, Roitenburd BV, Espinosa AJ (2000) Pattern of childhood cancer mortality in Mexico. Arch Med Res 31: 526-531.
6. Carlo GD, Mascolo N, Izzo AA, Capassao F (1999) Peregrinol, a lupane type triterpene from the fruits of Diospyros peregrina. Life Sci, pp: 265-337.
7. Jeffrey ME, Linda SA, Andrew OM (1998) A rapid and simple MTT based spectrophotometric assay for determining drug sensitivity in monolayer culture. Tissue Cult Meth 11: 15-17.
8. Venu Gopal Y, Ravindranath A, Kalpana G, Raju AB, Reddy PV (2011) Antitumor Activity of Diospyros peregrina on Ehrlich Ascites Carcinoma in Mice. Journal of Scientific Research 32: 413-419.