IDENTIFICATION AND ISOLATION OF BIO-ACTIVE CONSTITUENTS FROM AERIAL PARTS OF *GOMPHRENA GLOBOSA* FOR CANCER THERAPY

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INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled division of cells in the part of the body and is able to invade to other tissues or parts of the body through blood and lymph system. Cancer is of different types that based on the organ or type of cell in which they start. Different cancer types like cancer of lung, esophagus, stomach, oral, pharynx, colon and skin, are affecting the human population. In the developed world, cancer is the leading cause of death whereas in the developing world it is the second leading cause of death as evidenced by the projected number of new cases and death worldwide[1].

About 12.7 million people were suffering from cancer and 7.6 million people died of cancer in 2008 worldwide. Of the above estimate 56% of the cancer cases and 64% of the deaths were reported in the developing countries. Cancer burden in India is lower than that of developed countries like United States[2], and WHO, projected the dramatic rise in cancer burden to 20 million by 2020 with 70% in the developing countries[3]. The cause for higher cancer burden in developing countries is due to population growth and ageing that would have massive impact on the number of new cancer cases and deaths. In India, the more prevalent cancers in men are lung, esophagus, stomach, oral and pharynx, while in women, breast and cervix cancers followed by stomach and esophagus. Recently it has been reported that there were more than 5 lakes people died of cancer in India in the year 2010, and in female most deaths were due to breast cancer.

The causes for cancer are either due to environmental factors with 90-95% of cases and due to heredity with 5-10% of the cases. The environmental factors causing cancer include tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation, lack of physical activity and environmental pollutants[4]. These environmental factors cause or enhance abnormalities in the genetic material of the cells[5].

There is no simple cure for cancer due to the multiplicity of causes or interactions. The treatment options for cancers include radiation therapy, chemotherapy, surgery, immunotherapy and biologic therapy as a combination with a goal to reduce the neoplastic cell burden. However, the treatment plan for cancer is based upon the type of cancer, how far it has spread, age and general health of the patient.

Currently available anticancer drugs are either of synthetic or of plant origin that produce side effects or toxic effects, such as myelosuppression, suppression of both cellular and humoral immunity, oral mucosal ulceration and intestinal denudation, pulmonary fibrosis,

venoocclusive disease (lead to renal failure), alopecia, mucositis, thrombocytopenia, pneumonities, hepatic fibrosis, cirrhosis etc[6], and as such no anticancer drug is free of toxicity.

More than 60% of all modern drugs in clinical use are of natural products, many of which have been recognized to have the ability to induce apoptosis in various tumor cells. Many of the plants are still used traditionally as herbal drugs against various types of tumors such as sarcoma, lymphoma, carcinoma and leukemia. Traditionally over the years herbal medicine have been used in their crude form; however its safety and efficacy remains a concern, due to lack of knowledge about the bio-active and non bio-active constituent's role in treating the diseases including cancer. Therefore the scientists continue their focus in identifying the active constituent and isolating them and using them for the treatment of cancer in order to exclude the participation of non bio-active constituents. And thus came, the plant derived lead molecules with anticancer property such as vinblastine, vincristine, camptothecin derivatives like topotecan and irinotecan, etoposides derived from epipodophyllotoxin, and paclitaxel (taxol). However, these plant derived lead molecules with anticancer properties are not free of side effects or toxicities like any other anticancer drugs. Despite the above said points, researchers continue their work on plants for searching a lead molecule that can be used in its original form or as a derivative in order to overcome the drugs induced toxicity.

Based on the above consideration, the present study initially attempted to identify plants with anticancer property. It has been documented that plants of different species, but belonging to same genus may have the biological activity in common [7]. Applying this factor, a preliminary investigation on the search for plants, revealed that plants belonging to Amaranthaceae family have been reported for anticancer property. They are *Celosia argentia Linn, Gomphrena macrocephala* and *Gomphrena martiana*[8,9,10]. On further search, two plants, *Celosia cristata* and *Gomphrena globosa belonging* to the family amaranthaceae, were randomly selected and subjected to preliminary investigation for anticancer property by *in-vitro* model and the results were positive. And also, no reports are available for the anticancer property of these plants. Therefore these two plants were selected in the present study.

Traditionally these plants have been used for dysmenorrhea, gonorrhea, dieresis, conjunctivitis, cough, dysentery, diarrhea, etc. In yet other studies, the presence of moroidin

in *Celosia argentia Linn,* for antimitotic activity, and 5,6,7-trisubstituted flavones in *Gomphrena martiana* for antitumor property have been documented[8, 11].

Thus, the study focused on investigation of the plants, *Celosia cristata and Gomphrena globosa* for anticancer property, and selection of the plant with potent anticancer property for further studies. The study included isolation of the active constituents, investigation of anticancer activity of the extract(s) and isolated constituents, investigation of mechanism of action, and structural elucidation of isolated constituents from the selected plant. The findings of the present study may provide scope for lead molecules with proven anticancer property with least or no side effects or toxicity.

REVIEW OF LITERATURE

Cancer is a second leading cause of death in developing countries, with uncontrolled division of cells in the part of the body[12]. The cancer causes are grouped into two main types in which environmental causes with 90-95%, that include tobacco, poor diet and obesity, infection, radiation, lack of physical activity and environmental pollutants. And the second cause is hereditary with 5-10%. The cancer burden in developing countries is raising as a result of increasing population aging and growth, and adaptation of cancer-associated lifestyle choices including smoking, physical inactivity, and 'Westernized' diets[4].

The standard methods used to cure or control the cancer is radiation therapy, chemotherapy, surgery, immuno therapy, and biological therapy with a goal to reduce cancer cell burden without causing excessive damage to normal cell. This ideal property is difficult, or perhaps impossible to attain, because of the side effects of the treatments of cancer. It has been well recognized that allopathic drugs exhibit severe toxicity on normal tissues. Therefore, worldwide research is going on to investigate the best effective antitumor agents from different sources.

Among the different sources, herbal research remains important in identifying lead molecule in the plants with proven anti cancer property that ultimately occupies the platform for clinical use. More than 60% of the currently used effective anti-cancer agents are plant based lead molecules or their analogs, have come into clinical practice and so remains promising for the built of more drug molecules that are few of toxicities for better management of cancer. These include camptothecin, vincristin, vinblastin, taxol, phodophyllotoxin, combretastatins etc. Though plants have given beneficial molecules for cancer therapy, their toxicities become a concern for effective management of cancer. This can be evidenced by number of antitumor ethnomedicinal plants reported and the numbers of known lead molecules as a drug of choice in the treatment of cancer. The numbers of ethnomedicinal plants reported in India are around 62, with the commonest phytochemical constituents like alkaloids, glycosides, terpenoids, stearic, oleic, palmitic acid, flavonoid, β-sitosterol, amino acids, saponins etc. Therefore, researchers continue their focus on identifying safer anticancer molecules from the plants.

Traditional medicines, including Chinese herbal formulations, can serve as the source of potential new drugs, and also, the anti cancer activity of certain natural products and their analogs can be enhanced by synthesizing new derivatives based on active pharmacophore

models. Drug resistant, solubility, and metabolic limitations can be overcome by appropriate molecular modifications; and new biological properties or mechanisms of action can be added by combining other functional groups or molecules. These can be evidenced with few examples, in which plant lead molecule that were derivatives for better result in the treatment of cancer.

Podophyllotoxin, a bioactive moiety, isolated from *Podophyllum peltatum Linnaeus*, is a strong cytotoxic agent against the treatment of wilms tumors, various genetal tumors, non-Hodgkin's and other lymphomas and lung cancer[13, 14]. But it was unsuccessful as a whole in the treatment of human neoplasia, because of its complicated side effects, such as nausea, vomiting, damage of normal tissues etc [15, 16]. These side effects have been overcome by etoposide and teniposide. These semi synthetic agents are used widely for the treatment of lymphomas, acute leukemia, testicular cancer, small cell lung cancer, ovarian cancer, bladder cancer, brain cancer etc [17].

Taxotere, is a semi-synthetic, structurally related analog of taxol, which was isolated from pacific yew, *Taxus brevifolia* has better pharmacological properties such as improved water solubility. [18,19,]. Camptothecin, a naturally occurring alkaloid was first extracted from the stem wood of the Chinese ornamental tree *Camptotheca acuminata* during the screening of several plants in a search for steroids [20, 21]. Preliminary studies revealed a substantial antitumour activity in standard *in-vitro* test system as well as in mouse leukaemia cells.

To date, the only CPT analogues, such as topotecan and irinotecan are approved for clinical use. They are found to be the most promising anticancer against the treatment of ovarian and colon cancer [22, 23].

Under multi-institutional project, 35 lead molecules were identified so far, for different pharmacological activities like, anti-cancer, anti-diabetic and Immunomodulatory after the investigation of bioactive-based *in-vitro* screening. The above mentioned evidence explained the isolation and characterization of lead molecule from medicinal plants, used in Indian traditional system of medicine, are in progress. Despite the rise of combinatorial chemistry as an integral part of lead discovery process, the natural products still play a major role as starting material for drug discovery [24]. Historically, many useful drugs were developed from lead molecules, which were isolated from medicinal plants.

Previous studies reported the presence of the presence of moroidin in *Celosia argentia Linn,* for antimitotic activity, and 5,6,7-trisubstituted flavones in *Gomphrena martiana* for antitumor property[8, 11]. It was also found that other species belonging to the same genus of

the above said plants were identified with anticancer property. They are *Celosia argentia Linn, Gomphrena macrocephala* and *Gomphrena martiana*[8, 9, 11]. It has been documented that plants of different species, but belonging to same genus may have the biological activity in common. And based on this consideration, the plants *Celosia cristata* and Gomphrena globosa

Celosia cristata and allied species

Celosia cristata [Celosia in Greek means burning] is a member of the genus *Celosia*, and is commonly known as cockscomb, since the flower looks like the head on a rooster (cock). It is called Chi Kuan in China. Caryophyllales (Order), Amaranthaceae (Family), *Celosia* (Genus), C. cristata (Species).

Water extracts (infusions) from *Matricaria chamomilla L.*, potmarigold calendula, *Celosia cristata L.*, *Plantago lanceolata L. et Plantago major L., Symphytum officinale L.*, *Capsella bursa pastoris L.*, *Hypericum perforatum L.*, were studied in terms of their activity enhancing the uterine tonus of an isolated rabbit and guinea pig uterine horn [25]. Extracts of *Celosia argentea L.* (CAE) and Cucurbita moschata Duch (CME), are Chinese herbal medicines, examined for the effects on anti-DNP antibody responses in mice [26]. Hepatoprotective effect of celosian, an acidic polysaccharide isolated from the water extract of the seed of *Celosia argentea*, was investigated using chemical and immunological liver injury models. Celosian inhibited the elevation of serum enzyme (GPT, GOT, LDH) and bilirubin levels on $CC1_4$-induced liver injuries in rat [27]. Anti-metastatic and immunomodulating properties of the water extract of *Celosia argentea* seeds were demonstrated [28].A unique bicyclic peptide, moroidin, from the seeds of *Celosia argentea* strongly exhibited antimitotic activity by inhibiting the polymerization of tubulin [29]. Alcoholic extract of *Celosia argentea* seeds was found to reduce the increase of blood glucose in alloxan-induced diabetic rat. Also the extract prevented a decrease in body weight in alloxan-induced diabetic rats [30]. Seventy-two extracts (methanol) obtained from the leaves, barks, and roots of 50 plant species used in the traditional medicine of Perak, Peninsular Malaysia, have been screened for antibacterial and antifungal activities. *Celosia argentea* along with other plants have displayed the broadest spectrum of activity [31]. *Celosia argentea* and *Talinum triangulare* were demonstrated for their pro-oxidants property [32]. The chloroform, methanol and aqueous extracts of *Celosia cristata* were reported the presence of alkaloids, flavonoids, mucilages, triterpenoids and the anthelmintic activity of the chloroform, methanol and aqueous extracts of *Celosia cristata* were reported

with maximum activity in aqueous followed by methanol. And no activity in chloroform activity. [33].

Gomphrena globosa and allied species

Commonly known as Globe Amaranth or Bachelor Button, is an annual plant that grows up to 24 inches in height. The true species has magenta bracts, and cultivars have colors such as purple, red, white, pink, and lilac. Its native range is in Brazil, Panama and Guatemala. Caryophyllales (Order), Amaranthaceae (Family), *Gomphrena* (Genus), G. globosa (Species)

The antimicrobial activity of extracts and constituents of *Gomphrena martiana* and *Gomphrena boliviana* were determined [34]. An *in-vivo* antitumor screening of extracts of *Gomphrena martiana* indicated positive activity in the petroleum ether extract, and its further bioactivity-directed fractionation resulted in a lipophilic flavonoid fraction. Upon inoculation of various doses of 5,6,7-trisubstituted flavones on two murine tumor lines, Sarcoma 180 and Ehrlich's carcinoma, a decrease of tumor growth was observed. An *in-vitro* KB cultured cell screen indicated cytotoxicity [10]. A new aurone and two known substances, aurantiamide acetate and tiliroside, were isolated from ethanolic extract of *Gomphrena agrestis*. The ethanolic extract of *G. agrestis* and compounds 1, 2, and 3 were shown to be active mainly against *Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa* [35]. Ethanolic extract and pure compounds of *Gomphrena celosioides* have showed significant activity against *Staphylococcus aureus* and *Salmonella typhi* [36]. Phytochemical screening of the roots of *Gomphrena macrocephala*, has resulted in the isolation of two new oleanane glycosides and a new taraxerane glycoside. The aglycones with an epoxy group showed cytotoxic activity against HSC-2 human oral squamous carcinoma cells [37]. *Gomphrena arborescens,* along with 7 other plant species, was reported to possess anti-malarial activity [38]. Phytochemical screening of the ethanol extract from leaves of *Gomphrena globosa L.,* has indicated the presence of saponins, alkaloids, reducing sugars and coumarins, and reported the antihypertensive activity [39].

The above mentioned literature clearly indicates that *Celosia cristata* and Gomphrena globosa were not studied in detail for their anticancer property except for the presence of glycosides, alkaloids, flavanoids, terpenoids etc. Therefore, these plants were selected in the present study for investigation.

AIM AND OBJECTIVES

The present study aimed to investigate the plants *Celosia cristata and Gomphrena globosa,* for anticancer property by *invitro* method and select the best plant with potent antitumor property and investigate the plant in detail.

The objectives of the study are as follows:

- Authentication of the selected plant
- Successive extraction of the plant material using non-polar to polar solvents
- Screening of the plant extracts for anticancer property by *in-vitro*
- Selection of the plant extract with potential anticancer property.
- Toxicity study on the selected plant extract
- Screening of the selected plant extract for anticancer property by *in-vivo*
- Phytochemical screening of the selected plant extract
- Isolation and structural elucidation of the active constituent(s) from the extract
- Screening of the active constituent(s) for anticancer property by *in-vitro* and *in-vivo*
- Studying the mechanism of anticancer activity of the active constituent

MATERIALS AND METHODS

Materials

The selected plants, *Celosia cristata* and *Gomphrena globosa* are widely distributed in and around Tiruchengode of Namakkal district. They were freshly collected in the month of June and authenticated **(**Specimen No. 1367, 1368**)** by Dr. G. V. S. Murthy, Joint Director, Botanical Survey of India, Coimbatore. The aerial parts of both the plants were taken for the present study.

Extraction

Aerial parts of *Celosia cristata and Gomphrena globosa* were selected for preliminary screening of anticancer property by *in-vitro*. Both the plants were dried separately under shade and then powdered to coarse. Powdered material (500 g) of both the plants was separately extracted with chloroform, ethanol and water by continuous hot extraction method for 48 h by using Soxhlet apparatus [40]. The extracts were concentrated to a dry mass by vacuum distillation. After complete drying the extracted materials were weighed and their extractive value in percentage was calculated with reference to the air dried sample. All these extracts were subjected to *in-vitro* anticancer study by Trypan blue method.

Pharmacological Screening

Preliminary screening of in-vitro cytotoxic study of extracts:

Preliminary investigation of *in-vitro* cytotoxic study was performed for chloroform, ethanol and water extracts of *Celosia cristata and Gomphrena globosa* using Daltons Lymphoma Ascites (DLA) and Ehrlichs Ascites Carcinoma (EAC) cell lines. DLA and EAC cell lines were aspirated from the peritoneal cavity and washed 3 times with phosphate buffer solution. One million cells were incubated with various concentrations (61.25 - 1000 mcg/ml) of the individual extract in a total volume of 1 ml for 3 h at 37ºC. After incubation, the viability of the cells was determined by Trypan blue dye exclusion method [41]. The percentage of dead cells was calculated from which the IC_{50} concentration was determined.

Based on the results of *in-vitro* cytotoxic activity on various extracts of the selected plants, the chloroform extract of *Gomphrena globosa* (CEGG) was found to be possessing potent activity as compared to other extracts and hence, it was chosen for further study.

Toxicological studies of CEGG

Animals

Albino mice of Swiss strain and Albino rats of Wistar strain were used for toxicological studies. The ethical clearance for animal experiments was obtained from the Institution Animal Ethical Committee, Swamy Vivekanandha College of Pharmacy (889/ac/05/CPCSEA dated 29th April 2005). Female mice selected were nulliparus and non-pregnant. Female mice weighing 25 to 30 g and rats of either sex weighing 125 to 150 g were used for the study. Each animal, at the commencement of its dosing, was between 8 and 12 weeks old and their weight variation was within ± 20% of the mean weight of any previously dosed animals. The temperature in the experimental animal room was 22ºC (± 3ºC) and the relative humidity was between 50-60%. These animals were fed with pellet diet manufactured by Amrut laboratory, Animal Feed Company, Sangli, Maharashtra and drinking water *ad libitum*. They were kept in 12 h/12 h light/dark cycle and maintained for at least 5 d prior to dosing to allow for acclimatization to the laboratory conditions.

Acute oral toxicity study

The acute toxicity study was carried out on albino mice as per the guidelines No: 423 given by the Organization for Economic Co-operations and Development, Paris [42]. Three Albino mice were fasted over night and the test sample CEGG was given orally at a starting dose of 5 mg/kg b. wt. p.o. Animals were observed for a period of 2 h, then occasionally for 4 h for severity of any toxic signs and mortality. Since no mortality was observed, same dose was repeated with another group of animals. The procedure was repeated for doses of 50, 300 and 2000 mg/kg. p.o. in separate group of animals. The maximum dose of 2000 mg/kg did not produce any mortality and toxic symptoms. So, for further studies 1/10th and 1/5th of the maximum dose (2000 mg/kg. p.o) values were taken as treatment dose [43, 44]. Behavior as well as other toxic symptoms if any was observed for 24, 48 and 72 h [45]. The animals were kept under observation up to 14 d after drug administration to find out delayed mortality if any [46].

Repeated oral toxicity study

The repeated oral toxicity study was done on Wistar strain albino rats as per methods previously reported [47]. Albino rats of either sex were divided into 2 groups of 10 animals each (5 males and 5 females). First group served as solvent control and was given normal saline (1 mL/kg. p.o) and the other group was administered CEGG at the dose of 1000 mg/kg, p.o. The test extract was given once daily orally for 90 d. All the rats were observed for any physiological and behavioral changes and mortality if any. Food and water consumption was checked daily. Body weight was recorded on 1st and 90th day.

Haematological parameters such as total red blood corpuscles (RBC), white blood corpuscles (WBC), differential count (DC) and haemoglobin were estimated on the next day of the prescribed period and all the animals were sacrificed and the blood samples were collected from each rat individually into non-heparinised tubes and were allowed to coagulate. Serum was separated by centrifugation and glucose, cholesterol, urea, creatinine, proteins, albumin, total bilirubin, acid phosphatase, alkaline phosphatase, SGOT and SGPT were analyzed. Vital organs such as liver, kidney, spleen, brain, lungs and stomach were removed and the color of the organs was observed for gross pathological changes. Liver and kidney were fixed in Bouin's fixative and processed for histopathological examination. The slides were stained with hematoxylin and eosin and observed under low and high power microscope for pathological changes if any.

The hematological parameters like Hemoglobin, Total RBC count, Total WBC count and Differential count were estimated by standard procedures [48]. The biochemical parameters like glucose [49], cholesterol [50], urea [51], creatinine [52], total proteins [53], albumins [54], total bilirubin [55], acid phosphatase [56], alkaline phosphatase [57], SGOT [58] and SGPT [59] were analyzed as per the reported methods.

Screening of *in-vivo* anticancer activity of CEGG

Dalton Lymphoma Ascites (DLA) and Ehrlich Ascites Sarcoma (EAC) cells were obtained from Amala Cancer Research Center, Thrsissur. They were maintained by weekly intra-peritoneal inoculation of 10^6cells/mouse. The doses 200 mg/kg.p.o, and 400 mg/kg.p.o of chloroform extract of *Gomphrena globosa* were selected for the study based on the acute toxicity study.

Mean survival time

Animals were inoculated with 1 X 10^6 cells/i.p per mouse on day 0 and treatment with CEGG started 24 h after inoculation at a dose of 200 mg/kg.p.o and 400 mg/kg/ day p.o. The control group was treated with the same volume of 0.9% sodium chloride solution. All the treatments were continued for 10 d and observation was carried out for 45 d. The mean survival time of each group consisting of 6 mice were noted and changes in body weight also recorded. The antitumor efficacy of CEGG was compared with that of 5-fluorouracil (20 mg/kg, p.o) [60]. The MST of the treated group was compared with that of the control group using the following formula.

$$MST = \frac{T - C}{C} \times 100$$

T = Treated group; C = Control group

Hematological parameters

In order to observe the effects of CEGG on the hematological parameters of DLA and EAC bearing mice, comparison were made against four groups of mice each group consisting of six animals, on the 14^{th} d after inoculation. The four groups comprised of (I) Tumor bearing mice (II) and (III) tumor bearing mice treated with CEGG 200 mg/kg.p.o and 400 mg/kg/day p.o. respectively and (IV) normal mice. Blood was drawn from each mouse from the retro-orbital flexes under sterilized condition and the Red Blood Cell Count (RBC), Hemoglobin content (Hb) and White Blood Cell Count (WBC) were studied using cell diluting fluids and hemocytometer. Differential Cell Count (DC) was carried out from Leishman stained blood smears [61].

Solid tumor

Mice were divided into three groups, each group consisting of six animals, and tumor cells 1 X 10^6 cells per mouse were injected into the right hind limb (thigh) of all the animals intramuscularly. The group (I) was tumor control, group (II) and (III) received CEGG 200 and 400 mg/kg.p.o respectively and given for 10 d continuously. Tumor mass was measured

from 7th d of tumor inoculation. The measurement was carried out every 6th d for a period of 48 d [62].

Histopathological Analysis of the ascitic tumor

The ascitic fluid was collected from peritoneal cavity once on 10th and 20th day from each animal and observed for appearance, color and cell count. A small amount of the ascitic fluid (tumor) was withdrawn aseptically from the mice. A drop of the fluid was placed on the slide and made into smear as said earlier. The slide was kept aside and the smear was fixed with methanol for half an h. Then a few drops of maygrunwalds's reagent were added to the smear. The slide was allowed to stay for three minutes and was washed with excess of distilled water. Then the slide was allowed to stand for half an h. It was fixed with xylene and examined under the microscope. Photographs were taken focusing the appropriate regions in the smear.

Phytochemical study of CEGG

The CEGG was subjected to qualitative chemical tests for the detection of various plant constituents like carbohydrates, glycosides, proteins and amino acids, fixed oils and fats, gums and mucilage, alkaloids, phytosterols, flavonoids, tannins and phenolic compounds, saponins, triterpenoids, etc [63-65].

Chromatographic analysis of CEGG

The CEGG was subjected to column chromatography for the separation of phytoconstituents. A column of suitable size (1 m x 1.5 inch) was chosen and packed with silica gel 60-120 mesh by adding slurry of the adsorbent in hexane. CEGG was dissolved in chloroform, and mixed with silica gel (60-120 mesh) and fed to the column through a funnel. Hexane was added to the column and kept aside without disturbance for overnight for the settlement of the extract. Maximum precautions were taken to remove the air bubbles. The column was eluted with different organic solvents in the order of increasing polarity (hexane, petroleum ether, chloroform, methanol and water). All the fractions were subjected to TLC studies. Fractions showing similar R_f value, melting point and identification test, were pooled together and solvents evaporated to get residues.

The extracts as well as the eluant of column chromatography were subjected to thin layer chromatographic analysis[66]. Various trials have been made and few solvent systems that were given useful information are:

Chloroform: methanol (4.5: 0.1),

Chloroform: acetone (4.8: 0.1),

Hexane: ethyl acetate (5.6: 1),

Chloroform: petroleum ether: acetic acid (3.5: 1: 0.5),

Chloroform: methanol: acetic acid (2.9: 2.9: 0.3),

Ethyl acetate: methanol (4.5: 0.5),

Chloroform: methanol: glacial acetic acid (2.9: 1.4: 0.1)

Chloroform: methanol: 10% ammonia (5.3: 2.6: 1)

Chloroform: methanol: water (4.3: 1.6: 0.6)

Ethyl acetate: pyridine: water (1.5: 0.5: 1.5)

Hexane: isopropanol (5: 0.2)

In-vitro cytotoxic activity of CEGG and isolated compounds (MTT Assay)

The study was performed on CEGG and its isolated compounds (two) using human cell lines. The selected human cell lines were breast cancer (MCF7), prostate cancer (DU-145), Hela (human epithelial carcinoma cell line), and A-431(human epidermoid carcinoma) and normal cell lines, which were obtained from National Centre for Cell Sciences, Pune, India, and all other chemicals and reagents were of appropriate grades. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine serum (FBS). To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in

microtitre plates. The plates were then incubated at 37^{o} C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of 3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT) in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^{o} C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan [67]. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

From the results compound 1 was taken for studying mechanism of anticancer activity and structural elucidation.

Mechanism of anticancer activity of compound - I

Antioxidant activity

Scavenging of nitric oxide

Weighed accurately 21 mg of compound I and was dissolved in 1 ml of distilled DMSO separately to obtain 21 mg/ml. This solution was serially diluted separately to obtain lower concentrations.

Weighed accurately 10 mg of ascorbic acid and rutin and dissolved in 1 ml of DMSO separately. From these solutions, serial dilutions were made to obtain lower concentrations using DMSO.

The reaction mixture (6 ml) containing sodium nitro prusside (10 mm, 4 ml), phosphate buffer saline (PBS, pH 7.4, 1 ml) and 1ml each of compound I and standards of various concentration (1000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/ml) in DMSO was

incubated at 25º C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was pipetted out and 1 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min for completion of diazotisation. Then 1 ml of naphthyl ethylene diamine dihydrochloride (NEDD) was added, mixed and allowed to stand for 30 min in diffused light. A pink coloured chromophore was formed. These solutions were measured at 540 nm against corresponding blank solutions. The results were recorded as IC_{50} value [68, 69], the absorbance is the concentration of the sample required to inhibit 50% nitric oxide radical.

Scavenging of hydrogen peroxide

30 mg each of compound I and the standards, ascorbic acid and rutin were accurately weighed and separately dissolved in 10 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations (1000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/ml) of 1 ml of the compound I and standards in methanol were added to 2 ml of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm, after 10 min against a blank solution that contained the compound I in PBS without hydrogen peroxide. IC_{50} values were calculated from the absorbance [70].

DPPH assay (2, 2-diphenyl-1-picryl hydrazyl)

21 mg of the compound I was dissolved in distilled DMSO separately to obtain a solution of 21 mg/ml concentrations. These solutions were serially diluted with DMSO to get lower concentrations.

The assay was carried out in a 96 well microtitre plate. To 200 µl of DPPH solution, 10 µl of the compound I and the standard solution were added separately in wells of the microtitre plate. The final concentration of the test and standard solution used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/ml. The plates were incubated at 37º C for 30 min and the absorbance of each solution was measured at 490 nm, using ELISA reader against the corresponding test and standard blanks and the remaining DPPH was calculated. IC_{50} is the concentration of the sample required to scavenge 50% of DPPH free radicals [71,72]. The following formula was used

DPPH radical scavenging activity (%) = $\frac{\text{OD control – OD sample}}{\text{OD control}}$ X 100

LDH leakage assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different compound I concentrations (1000, 500, 250, 125, 62.5, 31.25 and 15.625 µg/ml) were added to the cells in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, microscopic examination was carried out and observations were recorded every 24 h. After 72 h, the supernatant was collected from individual wells, centrifuged at 2000rpm for 10 min.Collected the supernatant into clean vials. 100µl of each sample was transferred to a fresh clean 96 well plate, added 100µl of reaction mixture of the kit and incubated at room temperature for 30 min in dark. The absorbance was measured using microplate reader at a wavelength of 490 nm. The percentage activity was calculated over the untreated control samples [73].

DNA fragmentation study

Cells (3 x 10^6 /ml) were seeded into 6 well plates and incubated at 37°C with 5% CO_2 atmosphere for 24 h. The cells were washed with medium and were treated with different doses (50 and 100 µg/ml) of the compound I, standard drug (campothecin, 3µg/ml) and incubated at 37ºC, 5% CO_2 for 24 hrs. At the incubation time ended, the chromosomal DNA of cancer cells was prepared with apoptotic DNA ladder kit (Apoptic DNA ladder kit was purchased from Roche, Germany). Briefly, cells were harvested and lysed with lysis buffer for 10 min. Then the samples were mixed with isopropanol before passing through the filter and washed. The DNA was eluted from the filter and treated with RNAse at 37ºC for 30 min before loading on to 2% agarose gel electrophoresis and run 50 V/cm for 3 hrs. The gel was visualized under UV transilluminator and photographed.

Effect on capsase – 3 activity

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using medium containing 10% FBS. To each well of the 6 well plates, 2ml of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and different compound I concentrations (25 and 50 µg/ml) prepared in DMEM were added to the cells. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, microscopic examination was carried out and observations were recorded every 24 h. After 72 h, cells were scrapped and centrifuged at 2000rpm for 10 min to separate the pellet. The pellet was resuspended in 50 µl of chilled cell lysis buffer and incubated cells on ice for 10min. Centrifuged at 15000rpm for 1 min and supernatant was transferred to a fresh tube. The vial was maintained in ice and protein concentration of the samples were measured by Bradford assay. The samples were diluted to get 4 mg per ml. 50µl of samples was mixed with 50µl of 10mM DTT, followed by 5µl of 4 mM DEVD-*p*-nitroanilide and incubated at 37^0C for 120 min. The absorbance was measured using microplate reader at a wavelength of 405nm [74].

Statistical Analysis

All values were expressed as mean ± SEM. The data were statistically analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test. P values <0.05 was considered significant.

Spectral characterization

IR spectra of compound I were recorded using a Fourier Transformed-Infra Red (FT-IR) spectrophotometer of Jasco make and model of 4100. IR values are measured in cm^{-1}. IR is used to probe bond vibrations and bending in molecules and to reveal the types of functional groups present in compound. Functional group region is in the range from 4000-1600 cm^{-1} and Finger print region is from 1550-660 cm^{-1}.

NMR spectra of compound I were recorded using a Bruker Avance-111, 400MHz spectrometer 9.4 Tesla super- conducting magnet equipped with a BBO 400MHz, with Z-gradient nucleus probe, operating temperature range 360^0. NMR is an important spectroscopic method and a premier organic spectroscopy to determine the detailed

chemical structure of the chemicals they were isolating from natural products. The structure of isolated compounds were elucidated by ^{1}H-NMR and ^{13}C-NMR (BRUKER Avance111 400MHZ) analysis. The sample was dissolved in $CDCl_3$ and value was measured in δ ppm.

LC-ESI-MS and LC-ESI-MS/MS analysis was conducted on Agilent 6520 accurate mass Q-TOF LC/MS coupled with Agilent LC 1200 equipped with extend C18 column of 1.8 μm, 2.1×50 mm. Gradient elution was performed with methanol (solvent A, 70%) and 0.1% formic acid (solvent, 30%) at a constant flow rate of 0.3 ml/min. Column temperature was maintained at 30^{0}C. The MS analysis was performed using ESI in the positive mode. The condition for mass spectrometry were as follows: drying gas (nitrogen) flow 5 L/min; nebulizer pressure 50 psig; drying gas temperature 325^{0}C; capillary voltage + 3000 V; fragmentor volt 250 V; Oct Rf Vpp 750 V.

Mass spectrometry provides both molecular weight and fragmentation pattern of the compound. It relies of production of ions from a parent compound and the subsequent characterization of the pattern that are produced.

RESULTS

Preliminary screening of *in-vitro* cytotoxic study of extracts

From the results it was observed that the chloroform extract of *Gomphrena globosa* , produced cytotoxic activity with inhibitory concentration (IC_{50}) of 45.94 µg/ml for DLA and 56.87 µg/ml for ECA. And the effect was relatively more potent than other extracts of the same plant or of *Celosia cristata* and therefore the chloroform extract of *Gomphrena globosa* was selected for further studies.

Toxicological studies of CEGG

There was no significant change in the behavioral response of the animal at the different doses of chloroform extract tested (2000, 300, 50 and 5 mg/kg. p.o). No mortality of the animals was observed even in the maximum dose of 2000mg/kg.p.o. In repeated oral toxicity, the extract (1000 mg/kg. p.o) did not produce any significant change in the body wt, food and water intake. There were no significant changes in the hematological parameters such as hemoglobin (Hb), red blood cell count (RBC), white blood cell count (WBC), lymphocyte, neutrophil and monocyte count in the extract treated group. No changes were observed in the levels of blood glucose and cholesterol, SGOT, SGPT, BUN, total protein and creatinine as compared to normal. The histopathological study did not show significant changes in the appearance of the cells of various organs investigated as compared to normal. There was no significant weight loss of vital organs of the animals treated with extract when compared with control animals.

***In-vivo* anticancer activity**

Mean survival time (MST)

The CEGG extract in both doses (200 and 400 mg/kg p.o) increased significantly the MST to 42.00±0.88 ($p<0.001$) days in lower dose treated group and 42.83±0.70 ($p<0.001$) days in higher dose treated group as compared to tumor control (21.17±0.70 days) of DAL and the results are comparable with the MST of the standard, 5-Fluoro Uracil, which was found to be 43.17±0.79. The mean survival time of CEGG in both doses (200 and 400 mg/kg p.o) on ECA were increased significantly to 36.50±1.06 ($p<0.001$), 37.83±0.67 ($p<0.001$) respectively as compared to tumor control (20.50±0.99) and the effects were almost close to that of the standard (5-Fluoro Uracil, 40.67±0.88).

Hematological parameters

The CEGG in both doses (200 and 400 mg/kg p.o) reversed the changes in the hematological parameters induced by tumor to normal values in both cell lines of DAL and EAC.

Solid tumor

The solid tumor mass of DAL and EAC of the CEGG in both doses (200 and 400 mg/kg p.o) was reduced significantly. The reduction of the solid tumor of the CEGG in both doses (200 and 400 mg/kg p.o) for DLA was found to be 1.01±0.47 ($p<0.05$) and 0.25±0.38 ($p<0.001$) respectively, as compared to tumor control (2.99±0.54) on 35th d. And for EAC, the results were found to be 1.41±0.64 ($p<0.05$) and 0.47±0.58 ($p<0.001$) for the doses of 200 and 400 mg/kg p.o respectively, as compared to tumor control (3.09±0.74) on 35th d The degree of reduction in the solid tumor was significantly greater in higher dose ($p<0.001$) as compared to lower dose.

Phytochemical studies of CEGG:

The CEGG was subjected to qualitative chemical tests and found out the presence of alkaloids, glycosides and presence terpenoids.

Chromatographic analysis of CEGG

CEGG was subject to column chromatography and TLC of the fractions were carried out and the fractions with the similar Rf, melting point and identification tests were pooled together. Two major isolations were obtained, and denoted as compound I and compound II. Compound I was collected from the fractions of petroleum ether: chloroform (70: 30), (60: 40) and (50: 50). The fractions of chloroform: methanol (80: 20), (70: 30) and (50: 50) yielded compound II.

Out of the various trials made in TLC, for the mobile phase, Chloroform: methanol (8: 2) was found to be effective.

Physical examination of isolated compound I

Colour: Pale yellow

Stationary phase: Silica gel G

Mobile phase: Hexane: ethyl acetate (8.5: 0.7)

Melting point: $85^{o}C$

Solubility: $CHCl_3$, petroleum ether

Yield: 600 mg/80gm of extract

***In-vitro* cytotoxic activity of CEGG and isolated compounds**

The IC_{50} values against the cell lines MCF7 (breast cancer), DU-145 (prostate cancer), Hela (human epithelial carcinoma cell line) and A-431(human epidermoid carcinoma) were 100µg/ml, 115g/ml, 210µg/ml and 190µg/ml respectively. And Among the two isolated compounds of the CEGG, compound I produced significant cytotoxic effect on all four human cancer cell lines used and the cytotoxic effect of compound II was found insignificant on all four human cancer cell lines. The IC50 values of compound I against MCF7, DU-145, Hela and A-431 were 81, 90, 100 and 90µg/ml respectively. And IC50 of compound I against normal cell line was >350 mg/ml. These relative cell lines survival progress decreased in a dose dependent manner.

Mechanism of anticancer activity of compound - I

Antioxidant activity

Scavenging of nitric oxide

Nitric oxide radical inhibiting activity was found out using sodium nitro prusside in aqueous solution at physiological pH, which was measured by a modified Griess-Illosvoy method. The IC_{50} value of the compound I was found to be 192.24 $\pm$ 0.24µg/ml), and of rutin, the reference compound was 189.47$\pm$ 0.24µg/ml.

Scavenging of hydrogen peroxide

The IC_{50} value for the inhibition of hydrogen peroxide was calculated as 40.44 $\pm$ 0.24µg/ml for the compound I, whereas 38.63$\pm$ 0.24µg/ml and 29.13$\pm$ 0.43µg/ml were required for rutin and butylated hydroxyl anisole (BHA) respectively which were used as reference compounds.

DPPH assay (2, 2-diphenyl-1-picryl hydrazyl)

DPPH assay of the compound I was calculated as IC_{50} value, and found out 4.36 $\pm$ 0.21, and for the rutin and ascorbic acid, which were used as standards, were found out as 8.54$\pm$ 0.46µg/ml and 4.68$\pm$ 0.35µg/ml respectively.

LDH leakage assay

Quantification of plasma membrane damage of compound I was measured by lactate dehydrogenase (LDH) release assay. The absorbances measured at a wavelength of 490 nm were calculated over the untreated control samples to find out the percentage activity. From the calculation it revealed that the compound I produced high LDH activity (73%) at high concentration (1000 µg/ml), starting from 62.5 µg/ml with (20 %) in a dose dependant manner.

DNA fragmentation study

Apoptosis by DNA fragmentation analysis indicated, from the photograph, that the treatment with the compound I caused DNA damage. It was observed that samples treated with the compound I and standard drug campothecin showed induced apoptosis of cells.

Effect on capsase – 3 activity

In Capsase-3 analysis, assay results indicated that treatment with compound I increased the Capsase-3 activity (1.45, 1.80) in a dose dependant manner (25, 50 µg/ml respectively). Standard drug, Campothecin increased the Capsase-3 activity significantly (2.15 for 5 µg/ml) compared to tumor control (0.54). These observations, confirm that the extract induced apoptosis in A-431 cells involves the activation of caspase-3.

Spectral characterization

The structure of isolated compound **1** was elucidated by Fourier Transformed Infra Red (FT-IR) spectrophotometer in KBr –pellet method (Jasco-4100). IR values are measured in cm^{-1}. IR spectral result is shown below.

IR Spectra:

(3490, b) O-H str of Free OH, (2940, s) C-H str (Asym), (2890, s) C-H str (Sym), (1710, s) C=O str, (1480, s) C-H def (Asym), (1390, s) C-H def (sym). The IR spectra are consistent

with the presence of phenolic group at 3380 cm-1, carbonyl groups and olefinic bonds at 1670-1720 cm-1, and aromatic ring absorption at 1490cm-1.

NMR spectroscopy

The structure of isolated compound 1 was elucidated by ^{1}H-NMR and ^{13}C-NMR (BRUKER Avance-111 400 MHZ) analysis. The sample was dissolved in $CDCl_3$ and value was measured in δ ppm. The value of the assigned structure was interpreted as follows.

^{1}H NMR:

δ 1.01 (d, J = 5.2 Hz, 3*H*, CH_3 of CH-CH_3), δ 0.980 (t, J = 5.2 Hz, 2H, CH2 of Phenethoxy), δ 1.072 (s, 1H, OH of Pyranose), δ 1.204 (s, 1H, OH of Pyranose)

δ 1.254 (s, 1H, OH of Pyranose), δ 1.304 (s, 1H, OH of Pyranose), δ1.53 – 1.58 (m, 4H, CH of Pyranose)., δ 2.31 (s, 3H, Acetate), δ 3.62 (s, 3H, carbomethoxy), δ 4.20 (t, J = 12 Hz, 1H, CH of CH-CH_2-COO), δ 4.07 (d, J= 6.8 Hz, 1H, CH of Pyranose)

δ 7.524 (m, 3H, ArH of Dihydroxy phenol), δ 7.694 (s, 1H, CH of Pyran)

δ 8.09 (s, 2H, Phenolic OH).

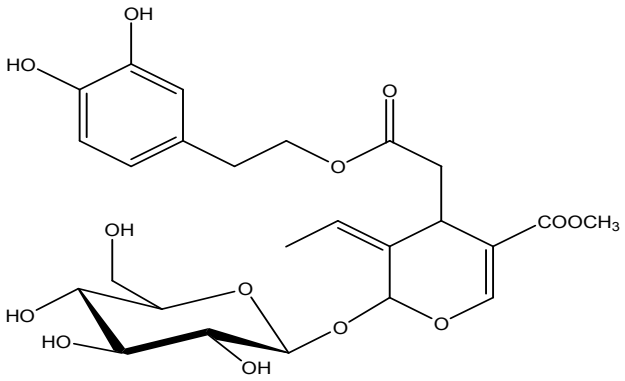
^{1}H NMR spectra confirm the occurrence of three components as deduced from the presence of one doublet at δ 1.01 of CH3 group, carbomethoxy group at δ 3.62, one singlet at δ 8.09 of phenolic OH group.

^{13}C NMR:

14.09, 22.68 (CH3), 25.74, 29.35, 31.92, 32.83 (CH2), 44.83, 47.09, 52.9, 55.4, 56.1 (CH), 63.12, 93.9, 94.09(C). ^{13}C NMR spectra indicated the occurrence of chemical shift at 44.83 (CH), 47.09 (CH), 52.9(CH), 55.4 (CH), 56.1(CH) could correspond to the presence of five olefinic carbon signals were observed. Moreover two anomeric signals almost superimposable at 94 and 93.9 were observed, indicating that a β-glucopyranosyl moiety must be attached to the hydroxyl group at 6th position of pyran-oxy nucleus.

Mass spectroscopy

The structure of isolated compound 1 was elucidated by LC-MS and LC-MS-MS represented in Fig. 1 as shown below,



Molecular formula $C_{25}H_{32}O_{13}$,

Exact Molecular Mass = 540.514,

Mass Spectra: ESI MS,

$(M + H)^+$ = 541.2665.,

The LC-MS results showed high intense molecular ion peak at 541.22 which translated to a compound with molecular formula $C_{25}H_{32}O_{13}$.

The TLC, IR, ^{1}H NMR, ^{13}C NMR and LC - MS spectra data with a molecular mass 541.22 recorded elucidated the isolated compound I to be as (E)-methyl 4-(2-(3,4-dihydroxyphenethoxy)-2-oxoethyl)-5-ethylidene-6-(3,4,5-trihydroxy-6 (hydroxymethyl)-tetrahydro-2H-pyran-2-yloxy)-5,6-dihydro-4H-pyran-3-carboxylate with molecular formula $C_{25}H_{32}O_{13}$ (Oleuropein).

DISCUSSION

The plant with proven biological activity need to be identified by its anatomical characteristics. Keeping this in view, the plants were authenticated **(**Specimen No. 1367, 1368**)** by Dr. G. V. S. Murthy, Joint Director, Botanical Survey of India, Coimbatore.

A preliminary anti-cancer activity of the Chloroform, ethanol and water extracts of aerial parts of *Celosia cristata and Gomphrena globosa* was performed by Trypan blue method[75]. From the results it was observed that chloroform extract of *Gomphrena globosa*, produced significant cytotoxic effect with inhibitory concentration (IC_{50}) of 45.94 µg for DLA and 56.87µg for ECA. The results suggest a detailed investigation of the chloroform extract of *Gomphrena globosa* for anticancer activity and therefore this plant was selected for further studies. It is also understood that the phytoconstituents with anticancer property may be lipophillic in nature as they are present in non polar solvent (chloroform).

The CEGG was subjected to qualitative chemical tests and found out the presence of alkaloids[21] glycosides[76] and terpenoids[77]. Various pharmacological studies were reported for the above phytochemical constituents including anticancer activity and therefore indicates that the CEGG reporting the above constituents may answer for anticancer property.

The toxicity study performed for the CEGG indicated that the extract was safe and did not produce significant changes in body weight and various hematological and biochemical parameters during the 90 days treatment.

The anti-cancer activity was assessed by mean survival time (MST), hematology, solid tumor and histopathological parameters. Prolongation of MST is a critical factor determined in the anti-cancer activity of the drug. The MST was extended nearly two fold by the extract treatment as compared to tumor control, and the MST of extract treatment was almost comparable to that of 5 –fluorouracil, which reveals that the plant has potential anti- cancer property.

Tumor growth normally affects various hematological parameters and the anti-cancer activity is generally assessed by restoration of the changes in these parameters to normal and most significantly in decreased WBC and increased RBC, lymphocyte and hemoglobin content as compared to tumor control. The acceptance criteria for determining the antitumor activity of a compound is the determination of circulating WBC[78] and the life span

prolongation[79]. The CEGG significantly decreased WBC Count and the effect increased proportionately with the dose of the extract. The changes in hemoglobin, RBC, neutrophil, monocyte and lymphocyte brought about by the induced cancer reversed values close to normal. Further the anticancer activity appears to increase proportionately with the dose of the extract.

From the above mentioned discussion, it is obvious that the plant *Gomphrena globosa* promises for phytoconstituents, that are soluble in chloroform, for its potential anticancer activity. Based on this consideration CEGG that showed better anticancer activity was subjected to column chromatography. Among the seven fractions only two fractions were found to be similar by TLC study indicating each fraction contain one compound, as only single spot was observed.

Out of the various trials made in TLC, for the mobile phase, Chloroform: methanol (8: 2) was found to be effective.

These two compounds were studied for their anticancer activity by *in-vitro* method using human cell lines including of normal cell lines for standard. The results of the study revealed that among these two compounds (compound I and compound II) only compound I showed potential anticancer property.

The TLC, IR, ^{1}H NMR, ^{13}C NMR and LC-MS spectra data with a molecular mass 541.22 recorded elucidated the isolated compound I to be as (E) - methyl 4- (2- (3, 4- dihydroxy phenethoxy) -2- oxoethyl) -5- ethylidene - 6 - (3, 4, 5 - trihydroxy - 6 (hydroxyl methyl) - tetrahydro -2H- pyran -2- yloxy) - 5, 6 - dihydro -4H- pyran -3- carboxylate with molecular formula $C_{25}H_{32}O_{13}$ (Oleuropein), is a phenolic secoiridoid glycoside. Iridoids are known to have variety of biological effects like antimicrobial[80], cardioprotective and neuro protective activity [81].

Oleuropein aglycone is the most potent phenolic compound in decreasing breast cancer cell viability[82]. HER2 oncogene-amplified SKBR3 cells were ~5-times more sensitive to oleuropein aglycone than HER2-negative MCF-7 cells[82]. The secoiridoids deacetoxy oleuropein aglycone, ligstroside aglycone and oleuropein aglycone induce strong tumoricidal effects within a micromolar range by selectively triggering high levels of apoptotic cell death in HER2-overexpressing breast carcinomas. These compounds markedly depleted HER2 protein and reduced HER2 tyrosine autophosphorylation in a dose- and time-dependent manner [83].

The 200 µg/mL of oleuropein remarkably reduces the viability of MCF-7 cells and decreases the number of MCF-7 cells by inhibiting the rate of cell proliferation and inducing cell apoptosis. Additionally, oleuropein exhibited a statistically significant block of G1 to S phase transition, which was manifested by the increase in the number of cells in the G0/G1 phase[83]. The antiproliferative activity of crude extracts olive plant and phytochemicals (the dominant compound of the extracts is oleuropein) against cell lines at low micromolar concentrations. These extracts inhibit cell proliferation of human breast adenocarcinoma (MCF-7), human urinary bladder carcinoma (T-24) and bovine brain capillary endothelial (BBCE) [84].

From the above reported data it is very clear that oleuropein is a powerful anticancer agent against breast cancer though in our findings we observed the activity against prostate cancer, epithelial carcinoma and epidermoid carcinoma which were not reported earlier.

Oleuropein has both the ability to scavenge nitric oxide and to cause an increase in the inducible nitric oxide synthase (iNOS) expression in the cell[85]. NO has been shown to be part of the oxidative war chest of the immune system by virtue of involvement in anti-tumor and anti-pathogen host response[86]. NO and NO-derived chemical species can inhibit enzyme function, alter DNA and induce lipid peroxidation, NO has antioxidant properties and the ability to protect cells against cytokine induced injury and apoptosis. Therefore, nitric oxide scavenging effect observed with compound I in the present study clearly suggest its role for anticancer activity.

Excessive concentrations of reactive oxygen species in the human body can be involved in a number of pathological events [87-90] Hydrogen peroxide (H_2O_2) when present in excess, is one of the many compounds that can be injurious for the cells [91]. The efficiency of phenolic compounds as anti-radicals (DPPH) and antioxidants is diverse and depends on many factors, such as the number of hydroxyl groups bonded to the aromatic ring, the site of bonding and mutual position of hydroxyls in the aromatic ring. For example, Rice-Evans *et al*., (1996)[92], Joyeux *et al*., (1995)[93], described the advantageous effect of an O-hydroxyl substitution in the aromatic ring for the anti-radical and antioxidant activity of phenolic acids. The data obtained with scavenging of hydrogen peroxide and DPPH assay clearly indicates that the compound I (oleuropein) reported in the study did produce anticancer activity through antiradical and antioxidant mechanisms due to the presence of O-substituted – OH group in the compound I.

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme in every cell and releases its enzyme activity when the cell death occurs. The enzyme activity detected in the culture supernatant correlates with the proportion of lysed cells. Detection of LDH in the supernatant of target cells can be used to measure cell-mediated cytotoxicity [94]. Lytic and apoptotic cell death have been suggested as mechanisms by which cytotoxic T lymphocyte (CTL) and Natural killer (NK) cells may kill target cells [95-99]. The present study of LDH leakage assay, of compound I suggest the lytic and apoptotic cell death, which occurs in a dose dependent manner.

DNA gel electrophoresis was performed in order to verify DNA fragmentation [100]. The criteria for assessment of apoptosis generally include morphologic changes examined by light or electron microscopy and DNA fragmentation detected by colorimetric assay or visualization of fragmented DNA ladder patterns by agarose gel electrophoresis [101]. The visualized photography of compound I from DNA fragmentation indicates that the apoptosis of the compound I is in a dose dependent manner.

The efficacy of cancer therapy might be determined by the ability to induce caspase-dependent apoptosis. Caspase – 3, apoptosis-related cysteine peptidase encoded by *CASP3* gene, plays a central role in the execution-phase of cell apoptosis. It has been found to the peptide sequence DEVDG (Asp-Glu-Val-Asp-Gly) with the cleavage occurring on the carboxy side of the second aspartic acid residue (between D and G). We observed the Caspase-3 activity with compound I in a dose dependent manner, thus establishing the apoptosis induced by compound I is through activation of Caspase-3.

Thus, the present study revealed the presence of the compound, oleuropein in CEGG which showed significant anticancer activity through antioxidant, antiradical and cell apoptosis mechanisms.

SUMMARY AND CONCLUSION

- Two plants, *Celosia cristata and Gomphrena globosa,* were initially studied for anticancer activity by *in-vitro* method against DLA and ECA cell lines.
- The chloroform extract of *Gomphrena globosa* was found to be potential cytotoxic activity.
- The CEGG was further screened in detail for anticancer activity against *in-vitro* human cell lines and *in-vivo* DLA and ECA cell lines.
- The compound possessing potent anticancer activity against human cell lines MCF – 7, DU 145, Hela and A-431 was isolated and structurally elucidated by TLC, IR, ^{1}H NMR, ^{13}C NMR and LC-MS spectral data.
- The isolated compound was found to be Oleuropein.
- The compound Oleuropein was found to possess anticancer activity through antioxidant, antiradical and cell apoptosis mechanism.

In conclusion, the results of the study demonstrated that the plant, *Gomphrena globosa* possess anticancer activity and the compound responsible for this activity was found to be Oleuropein. Further studies are recommended in humans for effective use of this compound in cancer.

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