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Original Article

ANALYSIS OF FATTY ACIDS FROM OIL OF GREEN TEA (*CAMELLIA SINENSIS L*) BY GAS CHROMATOGRAPHY COUPLED WITH FLAME IONIZATION DETECTOR AND ITS ANTICANCER AND ANTIBACTERIAL ACTIVITY *IN VITRO*

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ABSTRACT

Objective: Tea is a widely consumed beverage worldwide. The effect of green tea is mainly due to its high polyphenols-(-) epigallocatechin-3-gallate (EGCG) content in the culture of cancer cell and bacterial cells. The present work was carried out to investigate the efficacy of green tea oil (GTO) against cancer cells and bacterial cells.

Methods: In this study green tea oil was prepared from green tea for different experiment and determination of fatty acids profile from green tea oil. In the present study, peripheral blood lymphocyte (PBL) was chosen as human peripheral blood lymphocytes and blood cancer MCF-7 cells were chosen as human cancer cells. To fulfill our aims and also to evaluate the activity of this phytomedicine against normal lymphocytes and cancer cells the cell samples were divided into 26 experimental groups in the following ways. Each Petri dish contains 2 X 10^5 cells.

Results: GTO shows a potent anticancer agent but nontoxic to normal cells. The GTO decreases the reduced glutathione (GSH) level and increase the oxidized glutathione (GSSG) level significantly ($P<0.05$) in MCF-7 cells. But in lymphocytes the GSH level and GSSG level were almost the same with the control group but doxorubicin (DOX) significantly decreased the GSH and increase the GSSG level. Green tea oil treatment causes generation of reactive oxygen species (ROS) in MCF-7 cells revealed by $DCFH_2DA$ staining. Agar diffusion test shows the GTO is effective against multi-drug resistant bacteria.

Conclusion: This phytomedicine has a potent anticancer activity without damaging the normal lymphocytes. So, this drug can be used for further treatment of anticancer and antibacterial.

Keywords: Green tea, Green Tea Oil, Polyphenols-(-) Epigallocatechin-3-gallate, Cancer, Phytomedicine

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INTRODUCTION

Cancer is the second foremost life-threatening disease worldwide causes of human death. In spite of substantial advancement in biomedical researches on cancer biology, identifications of cancer biomarkers, different surgical procedures, antibody therapy, radiotherapy, and chemotherapy, the overall survival rate of cancer patients have not significantly improved in the last few decades [1]. In the present scenario, different pathogenic bacteria particularly multidrug-resistant bacteria also have become national and international concern [2]. According to the centers for disease control (CDC) report, there has a possibility of a post-antibiotic world, where a minor cut or a small injury could be fatal because the antibiotic efficacies will loss. Among them, the most problematic pathogens are *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus*, all the bacterial strains show multidrug resistance. Natural assets or resources including plant derived molecules have been used to prevent human diseases for thousands of years. The therapeutic benefits of several plant products, as well as phyto-chemicals, have become one of the major areas of interest in cancer therapy and control of drug-resistant bacteria. Natural substances show a rising role in different biomedical applications. Severe side effects of various traditional and conventional chemotherapeutic drugs are one of the major problems for cancer therapy. Thus, to overcome this important issue, phytochemical based antimicrobial and anticancer drug development becomes the major thrust area of many researchers. Green tea (*Camellia sinensis* L.) contain various phytocomponents such as polyphenols (catechins), caffeine (called theine), tannin (flavonols), theophylline, theobromine, saponins, essential oils, carotene, and nutrients are vitamin C, B_1, B_{12}, K, flouride, iron, magnesium, calcium, strontium, copper, nickel, zinc, and trace elements like molybdenum and phosphorus, which have been found to protective effect against bacterial infections[4] and viral infections[5] as well as anticarcinogenic [6] and antimutagenic activities [7], inflammation [8], platelet aggregation [9] and elevation of vascular reactivity [10]. We consume mainly tea aqueous beverage for health benefits comes from mainly polyphenols consumption include catechins 45.5% (-)-epigallocatechin gallate, 18.2% (-)-epicatechin gallate, 10.1% (-)-epicatechin and 4.2% (-)-epigallocatechin [3] There is no data regarding green tea oil (GTO) which is considered a kind of edible oil because the predominant fatty acids (FAs) are the monounsaturated fatty acid (MUFA) i.e., oleic acid and the polyunsaturated fatty acid (PUFA) i. e, linoleic acid, they are essential in human nutrition and helps to reduce levels of low-density lipoprotein (LDL) cholesterol, total cholesterol and the triglycerides. Besides, it is most important that GTO contains mono, di, trichosanic acid (conjugated mono and polyunsaturated fatty acid). The present study was carried out to evaluate the GTO mediated selective cytotoxicity on cancer cells *in vitro* experimental settings. Doxorubicin (DOX) was taken as a positive control for the study as it is a potent chemotherapeutic agent. Simultaneously, human peripheral blood lymphocyte (PBL) was taken as a normal cell and any toxicity due to GTO treatment was carefully investigated.

MATERIALS AND METHODS

Culture media and chemicals

Histopaque 1077, DMEM, penicillin, streptomycin, pentoxifylline (POF), Doxorubicin, were procured from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GIBCO/Invitrogen, MTT reagent. Dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), Tris–HCl, Tris buffer, Titron X-100, Sodium dodecyl sulphate (SDS), ethanol (C_2H_5OH),

iso-amyl alcohol, were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. Di-methyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was procured from Hi-media, India. All other chemicals were purchased from SRL, India and MERCK, India. sd FINE-CHEM Limited, India, HiMedia Laboratories Pvt. Ltd. Mumbai. India and Crest Biosystems Goa, India.

Collection of green tea leaves

Green tea leaves was collected from heritage tea garden, Gopali, Indian Institute of Technology, Kharagpur (Geographical extension between 22 ° 28' 59" North to 22 ° 29' 19" North latitude and 87 ° 30' 20" East to 87 ° 30' 35" East), Paschim Medinipur District of West Bengal, India. The plants were identified by a renowned taxonomist (CNH/27/2017) in the Department of Botany, Raja Narendra Lal Khan Women's College, Midnapore and also the voucher specimens were deposited in the Department of Botany, Raja Narendra Lal Khan Women's College.

Green tea oil preparation

The oil content was extracted from green tea leaves by soxhlet apparatus using petroleum ether as a solvent for 6 h according to the american oil chemists' society (AOCS) method [11]. At first 1 kg green tea leaves were soaked in warm water at 60 °C for 5 minute and discard the water for removing the water dissolving compounds from the tea leaves. Then the wet tea leaves was dried at 40±1 °C in an incubator and the dried parts were crushed in an electric grinder machine and fine dust (800 gm) was collect for the experimentation [12]. Dried and finely powdered dust (800 gm) was dissolved in hexane: isopropanol (3:2; v/v) on an airtight glass jar for 24 h and allow it for shaking incubation at 37 °C and filtered through Whatman No.1 filter paper and the filtrate was collected. Then filtrate was evaporated by a rotary evaporator at 40 °C. The collected part was content total oil.

Determination of oil content

The ether was removed from the oil by rotary evaporator under pressure and the oil content was calculated from the weight of oil and dusts using the formula [13].

$$\text{Oil content (\%)} = \frac{\text{Oil weight}}{\text{Tea dust weight}} \times 100$$

Determination of fatty acid profile by GC-FID

The fatty acid profile of edible oils and fats were determined according to the method of O'Fallon, Busboom, Nelson, and Gaskins (2007) and association of official analytical chemists (AOAC) official method 996.06 [14]. Fatty acid methyl esters (FAMEs) were prepared from the oils and fat samples by direct trans-esterification using 2% sulphuric acid in methanol. Separations of FAMEs were done and quantified it by Gas Chromatography coupled with flame ionization detector (FID) (Agilent Series, 7890 Series, USA). Briefly, forty milliliter (40 ml) of oil or 50 mg of fat sample was weighed and taken in a screw-capped Pyrex culture tube and 1 ml of C17 (1 mg/ml; Heptadecanoic acid; Sigma H3500) was added as internal standard. Potassium hydroxide (0.7 ml) and 5.3 ml of methanol with 0.05% of butylated hydroxyl toluene (BHT) were added to the tube and incubated in a boiling water bath set at 55 °C for 90 min with vigorous shaking of 20 s for every 20 min. After incubation, the tubes were cooled under tap water followed by the addition of 2% sulphuric acid and incubation at 55 °C for 90 min. Further, n-Hexane (3 ml) was added to the tube, vortexed and centrifuged for 5 min at 2000 rpm. The n-Hexane layer was collected in a 5 ml test tube having 0.1 g of sodium sulphate. The tubes were again vortexed and the solvent was evaporated under a gentle stream of nitrogen. 1.5 ml of dichloromethane was added to the tube and mixed thoroughly. 0.5 ml of the dichloromethane with FAME was filtered through 0.22 mm PVDF syringe filter and injected to the gas chromatograph. SP 2560 (75 m x 0.18 mm x 0.14 mm) column was used for the gas chromatographic separation with following instrumental conditions: Injector temperature: 250 °C; Carrier gas: Hydrogen @ 0.6 ml/minute; Split Ratio: 1:100; Oven Program: 140 °C (Hold 1.5 min) to 220 °C @ 3 °C (Hold 1.0 min) to 230 °C (Hold 3 min); Detector: Flame Ionization Detector; Temperature: 260 °C; H2: 40 ml/minute; Zero Air: 400 ml/min; Injection Volume: 1 ml. The fatty acids present in the samples were quantified by area percentage calculation using Supelco 37 FAME Mixture (Sigma Cat. No. 47885-U) as the reference standard. Results were expressed as % fatty acids of oil. Internal standard, as well as Standard Reference Material (SRM)-1544, was used for analytical quality assurance.

Selection of human subjects for the collection of lymphocytes

Lymphocytes were collected from 6 healthy human subjects, abstaining from any kind of disease (hereditary disease, chronic disease) drug addiction and medication. After regular routine checkup and all the participants were belonging to the same geographical area was another protocol for the study. The study protocol approved by the Institutional Ethical committee (1905/PO/Re/S/2016/CPCSEA) of Raja Narendra Lal Khan Women's College was in accordance with the Helsinki protocol previously reported from our laboratory [15].

Isolation of peripheral blood lymphocytes

Blood samples were collected from six healthy human subjects in 5 ml heparin coated Vacutainers by the vein-puncture method according to the method of Hudson and Hay [16]. After diluting 5 ml blood 1:1 with phosphate buffered saline (PBS), Histopaque 1077 (Sigma) was used for density gradient centrifugation at 1500 rpm for 40 min at room temperature using a Pasteur pipette. The upper monolayer of the buffy coat which comprises Lymphocytes were collected and washed three times in balanced salt solution. The PBL were re-suspended in RPMI complete media supplemented with 10% FBS and incubated for 24 h in a 95% air/5% CO_2 atmosphere in CO_2 incubator at 37 °C.

Cell culture and maintenance

Breast cancer cell line MCF-7 which was gifted from Jadavpur University West Bengal. Breast cancer cell line MCF-7 and normal lymphocytes were cultured in a DMEM complete medium with 10% FBS, 2 mmol L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin under 5% CO_2 and 95% humidified atmosphere at 37 °C in CO_2 incubator.

Drug preparation

The green tea oil was dissolved in 0.5% DMSO in distilled water and the working concentration of the drug was 1 mg/ml.

Experimental groups for *in vitro* cytotoxicity study

In the present study, PBL was chosen as human peripheral blood lymphocytes and MCF-7 cells were chosen as human cancer cells. To fulfill our aims and also to evaluate the activity of this phytomedicine against normal lymphocytes and cancer cells the cell samples were divided into 26 experimental groups in the following ways. Each Petri dish contains 2 X 10^5 cells.

Group I: Control i.e., PBL Cells+culture media, Group II: Control i.e., MCF-7 Cells+culture media, Group III: PBL Cells+5 µg/ml Doxorubicin in culture media, Group IV: PBL Cells+5 µg/ml GTO in culture media, Group V: MCF-7 Cells+5 µg/ml Doxorubicin in culture media, Group VI: MCF-7 Cells+5 µg/ml GTO in culture media, Group VII: PBL Cells+10 µg/ml Doxorubicin in culture media, Group VIII: PBL Cells+10 µg/ml GTO in culture media, Group IX: MCF-7 Cells+10 µg/ml Doxorubicin in culture media, Group X: MCF-7 Cells+10 µg/ml GTO in culture media, Group XI: PBL Cells+25 µg/ml Doxorubicin in culture media, Group XII: PBL Cells+25 µg/ml GTO in culture media, Group XIII: MCF-7 Cells+25 µg/ml Doxorubicin in culture media, Group XIV: MCF-7 Cells+25 µg/ml GTO in culture media, Group XV: PBL Cells+50 µg/ml Doxorubicin in culture media, Group XVI: PBL Cells+50 µg/ml GTO in culture media, Group XVII: MCF-7 Cells+50 µg/ml Doxorubicin in culture media, Group XVIII: MCF-7 Cells+50 µg/ml GTO in culture media, Group XIX: PBL Cells+100 µg/ml Doxorubicin in culture media, Group XX: PBL Cells+100 µg/ml GTO in culture media, Group XXI: MCF-7 Cells+100 µg/ml Doxorubicin in culture media, Group XXII: MCF-7 Cells+100 µg/ml GTO in culture media, Group XXIII: PBL Cells+200 µg/ml Doxorubicin in culture media, Group XXIV: PBL Cells+200 µg/ml GTO in culture media, Group XXV: MCF-7 Cells+200 µg/ml Doxorubicin in culture media, Group XXVI: MCF-7 Cells+200 µg/ml GTO in culture media, after the

treatment schedule the cells were collected from the Petri dishes separately and centrifuged at 2200 rpm for 10 min at 4 °C to separate cells and supernatants [10]. The cells were washed twice with 50 mmol PBS, pH 7.4. A required amount of cells were lysed using hypotonic lysis buffer (10 mmol TRIS, 1 mmol EDTA and Titron X-100, pH 8.0) for 45 min at 37 °C and then processed for the biochemical estimation. Intact cells were used for ROS, and different microscopic observations.

Cellular toxicity assay

After the completion of treatment with tea oil at different concentrations, the treated cells such as PBLs and MCF-7 cells were washed with PBS (1X) for three times using centrifugation (2200 rpm for 3 min/wash) and the cells were prepared for cytotoxicity study. The cell viability was estimated quantitatively by nonradioactive, colorimetric assay systems using tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenil-tetrazolium bromide (MTT) according to the method of the previous report [17]. The percentage of proliferation was calculated by using the following equation:

$$\% \text{ of viable cells} = \frac{(\text{OD sample} - \text{OD control})}{\text{OD control}} \times 100$$

Intracellular redox balance

Determination of reduced glutathione (GSH)

Quantization of GSH estimation in cell lysate (1 x 10^6 cells/ml) was performed according to the standard method [18]. After the treatment schedule with tea oil at different concentrations, an aliquot of the each sample was mixed with 25% of TCA and centrifuged at 2000×g for 15 min to settle down the precipitated proteins. The supernatant was aspirated and diluted to 1 ml with 0.2 M sodium phosphate buffer (pH 8.0). After that, 2 ml of 0.6 mmol DTNB was added. After 10 min of the mixing, the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as µg of GSH/mg protein.

Determination of oxidized glutathione (GSSG)

After derevatization of GSH with 2-vinylpyidine, the GSSG level was measured according to the method of Kar Mahapatra *et al.* [19]. After the treatment schedule with tea oil at different concentrations, the PBLs and MCF-7 cells were washed three times and cell lysate was prepared. In brief, 2 µL of 2-vinylpyidine was added to a 0.5 ml of test sample and incubated for 1 hour at 37 °C. The reaction mixture was then deprotenized with 4% sulfosalicylic acid and centrifuged at 1000 × g for 10 min to settle the precipitated proteins. The supernatant of the solution was collected and the GSSG level was estimated with the reaction of DTNB at the absorption of 412 nm and calculated with a standard GSSG curve. The level of GSSG was calculated with the standard GSSG curve. The levels of GSSG were expressed as µg of GSSG/mg protein.

Determination of lipid peroxidation

Lipid peroxidation in terms of malondialdehyde (MDA) level was estimated by the method of Das *et al.* [20]. In brief, after the treatment schedule with drug and DOX, cell lysate (1 x 10^6 cells/ml) of PBLs and MCF-7 were prepared. This cell lysate was used as the sample for MDA level estimation. The levels of lipid peroxidation were expressed in terms of nmol/mg protein.

Intracellular reactive oxygen species (ROS) measurement

Measurement of intracellular ROS generation was performed using H_2DCFDA according to our previously reported method [17]. In brief, after the treatment schedule with drug and DOX on PBLs and MCF-7, the cells were washed with culture media followed by incubation with 1 mg/ml H_2DCFDA for 30 min at 37 ℃ Then, the cells were washed three times with fresh culture media. As a positive control, PBLs were incubated with H_2O_2 (100 mmol) for 30 min prior to the analysis [21]. After H_2DCFDA exposure, DCF fluorescence images were observed by a fluorescence microscope (NIKON ECLIPSE LV100POL). All experiments were done in triplicate.

Protein estimation

Protein estimation was measured by using a commercially available standard kit (AGAPPE) by semiautoanalyser (AGAPPE) by a standard protocol for photometric determination [22].

Antimicrobial activity

Disc agar diffusion (DAD)

Susceptibility of GTO to different multidrug resistant bacteria was determined by the DAD technique according to Bauer *et al.* 1966. The test indicator bacterium (Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 25093 and Escherichia coli ATCC 8739) taken from an overnight culture (inoculated from a single colony) was freshly grown for 4 h having 10^6 CFU/ml were standardized against MacFarland's standard. With this culture, a bacterial lawn was prepared on Mueller-Hinton agar. 6-mm sizes well are prepared and used to observe drugs susceptibility patterns against GTO. GTO and standard antibiotics solutions were prepared. The diameter of zone of bacterial growth inhibition surrounding the well (including the well), was measured [15].

Statistical analysis

All the parameters were repeated at least three times. The data were presented as mean±SEM (n = 6). Performing one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060, USA) the means of control and treated group were compared by multiple comparison t-tests, $P<0.05$ as a limit of significance.

RESULTS

Total oil content (%)

The total oil content from green tea was 40 ml. The total oil content 5% in green tea.

Fatty acid composition of GTO

Data revealed that GTO contained a very different fatty acid profile with the maximum amount of polyunsaturated fatty acids (60.04%). Among the polyunsaturated fatty acid, linolenic acid was found to be the highest amount (39.01%). Results show in table 1.

Table 1: Fatty acid profile of GTO

Fatty acids (% of FAMEs)	GTO
C14:0 (Myristic)	1.81
C16:0 (Palmitic)	23.36
C18:0 (Stearic)	4.08
C16:1 (Palmitoleic)	1.18
C18:1n9c (Oleic)	9.53
C18:2n6c (Linoleic)	21.03
C18:3n3 (Linolenic)	39.01

Cell death assay by MTT

The cytotoxicity of the GTO and DOX was checked towards normal lymphocytes (PBL) and MCF-7 cell lines *in vitro*. The tea oil was charged in these cells for cell death assay at different concentrations (1µg/ml, 5µg/ml, 10µg/ml, 25µg/ml, 50µg/ml, 100µg/ml and 200µg/ml). After 24 h treatment schedule the % of MCF-7 breast cancer cells were killed by 18.20%, 23.16%, 33.81%, 45.05%,

74.81%, 81.12%, 93.84% at the dose 1µg/ml, 5µg/ml, 10µg/ml, 25µg/ml, 50µg/ml, 100µg/ml and 200µg/ml respectively whereas DOX killed 11.2%, 58.19%, 83.82%, 91.89%, 95.29%, 96.64%, 96.91% at their respective doses. The lymphocytes were killed by 2.83%, 6.42%, 9.15%, 11.72%, 13.13%, 15.1%, 20.86% due to GTO treatment whereas DOX killed 10.2%, 48.19%, 76.82%, 88.89%, 91.29%, 95.64%, 97.91% at the dose 1µg/ml, 5µg/ml, 10µg/ml, 25µg/ml, 50µg/ml, 100µg/ml and 200µg/ml respectively. From this study it has been clear that the effective dose of GTO was 50µg/ml. In this dose the cancer cell killing is significant and this dose was biocompatible to normal cells. So, this dose was selected for the rest of the experiments.

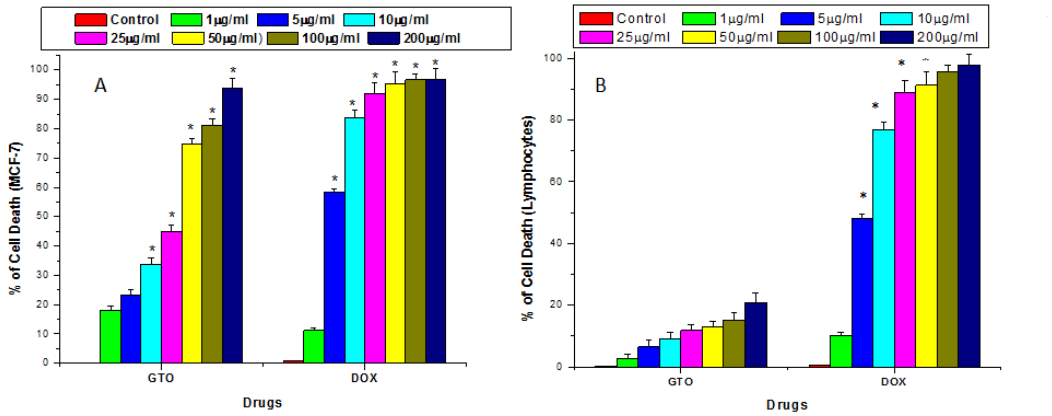

Fig. 1A and 1B: *In vitro* cell death assay of drug treated, DOX-treated MCF-7 and Lymphocytes were measured by the MTT method as described in materials and methods, values are expressed mean±SEM (n=6), symbol indicates the difference significantly each other

Estimation of reduced glutathione level

The GSH level was decreased in case of MCF-7 cells by 24.95% significantly (P<0.05) compared to the control, when treated with the drug. But in case of lymphocytes, the GSH level was almost same with the control group but DOX significantly (P<0.05) decreased the GSH level compared to the control group and drug-treated group at the dose of 50µg/ml.

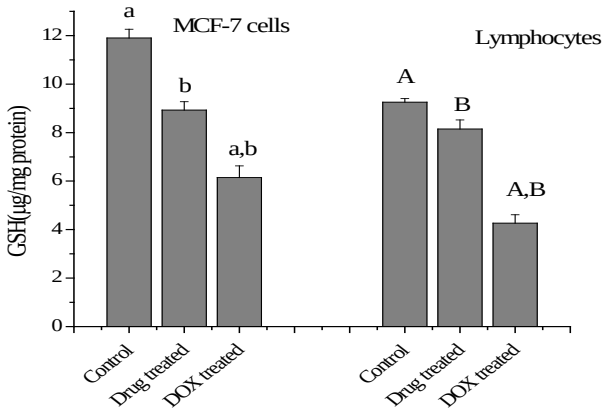

Fig. 2: Intracellular reduced glutathione (GSH) levels of drug-treated and DOX-treated MCF-7 cells and PBLs. The levels of GSH were expressed as mg of GSH/mg protein. Values are expressed as mean±SEM (n=6) of three experiments; superscripts indicate significant difference (P<0.05) compared with the control group

Estimation of oxidized glutathione level

The GSSG level of drug-treated MCF-7 group was elevated significantly (P<0.05) by 130.17% compared with the control of MCF-7 cells. The level of DOX-treated MCF-7 cells increased by 143.62% compared to the control at the dose 50µg/ml. But in case of lymphocytes, the GSSG level was little bit decreased compared to the control group whereas DOX-treated group, in case of Lymphocytes the GSSG level was increased by significantly (p<0.05) 142.6% compared to the control group.

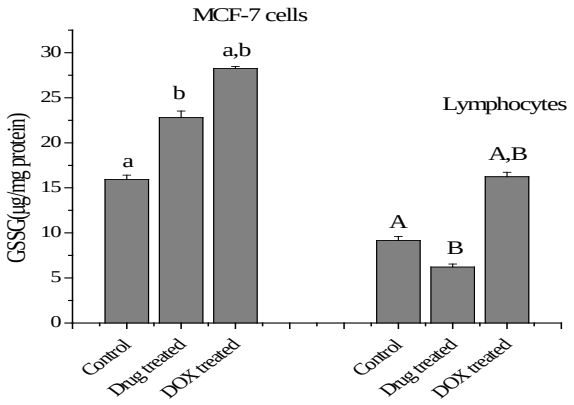

Fig. 3: Intracellular oxidized glutathione (GSSG) levels of drug-treated, DOX-treated, MCF-7 cells and lymphocytes. The levels of GSSG were expressed in term of mg of GSSG/mg protein, values are expressed as mean±SEM (n=6) of three experiments; superscripts indicate significant differences (P<0.05) compared with the control group

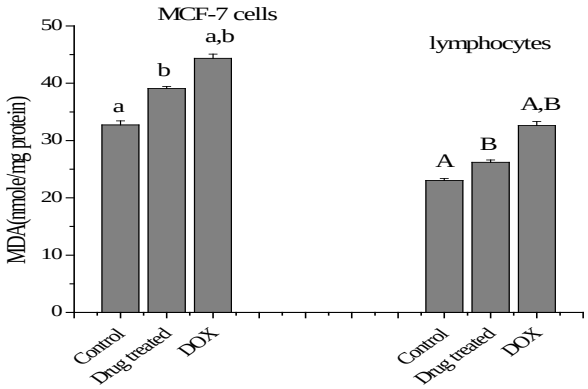

Fig. 4: Estimation of MDA levels of drug-treated and DOX-treated MCF-7 cells and PBLs. MDA level in terms of lipid peroxidation was expressed as n mole/mg protein, values are expressed as mean±SEM (n=6) of three experiments; superscripts indicate significant differences (P<0.05) compared with the control group

Estimation of lipid peroxidation

In the case of MCF-7 cells the MDA level was elevated significantly (P<0.05) by 16.29% in GTO treated group (50µg/ml) compared to the control group. But in the case of DOX-treated MCF-7 cells showed that the MDA level increased by 26.22% significantly (P<0.05) compared with the control group. Green tea oil (GTO) treated Lymphocytes increased the MDA level by 10.09% whereas DOX increased the MDA level by 31.47% significantly (P<0.05) compared to the control group at the dose of 50µg/ml.

Estimation of intracellular ROS measurement

The generation of ROS has been shown to contribute to GTO-triggered cytotoxicity in cancer cells. In this study, we measure the intracellular ROS, 2,7-dichlorofluorescein-diacetate ($DCFH_2$-DA) was used as an intracellular ROS indicator for the GTO and DOX-treated MCF-7 cells and normal Lymphocytes. After exposure, the cells were stained with $DCFH_2DA$ for 30 min. We found that the GTO and DOX-treated MCF-7 cells became DCF+, indicating that ROS were generated and participated in the GTO mediated cell death (fig. 5). In contrast, after the same procedure without GTO and DOX, no fluorescent cells or mild fluorescent cells were found, indicating no or less ROS generation. The ROS production, as well as DCF fluorescence intensity, was altered significantly in both types of cells in DOX treatment. Densitometry study revealed that the GTO and DOX treatment has not significantly altered ROS level in human peripheral blood lymphocyte (PBL), but in MCF-7 cells the intracellular ROS level was highly significant (P<0.05). GTO and DOX treatment were able to significantly (p<0.05) increased ROS level compared with the control group.

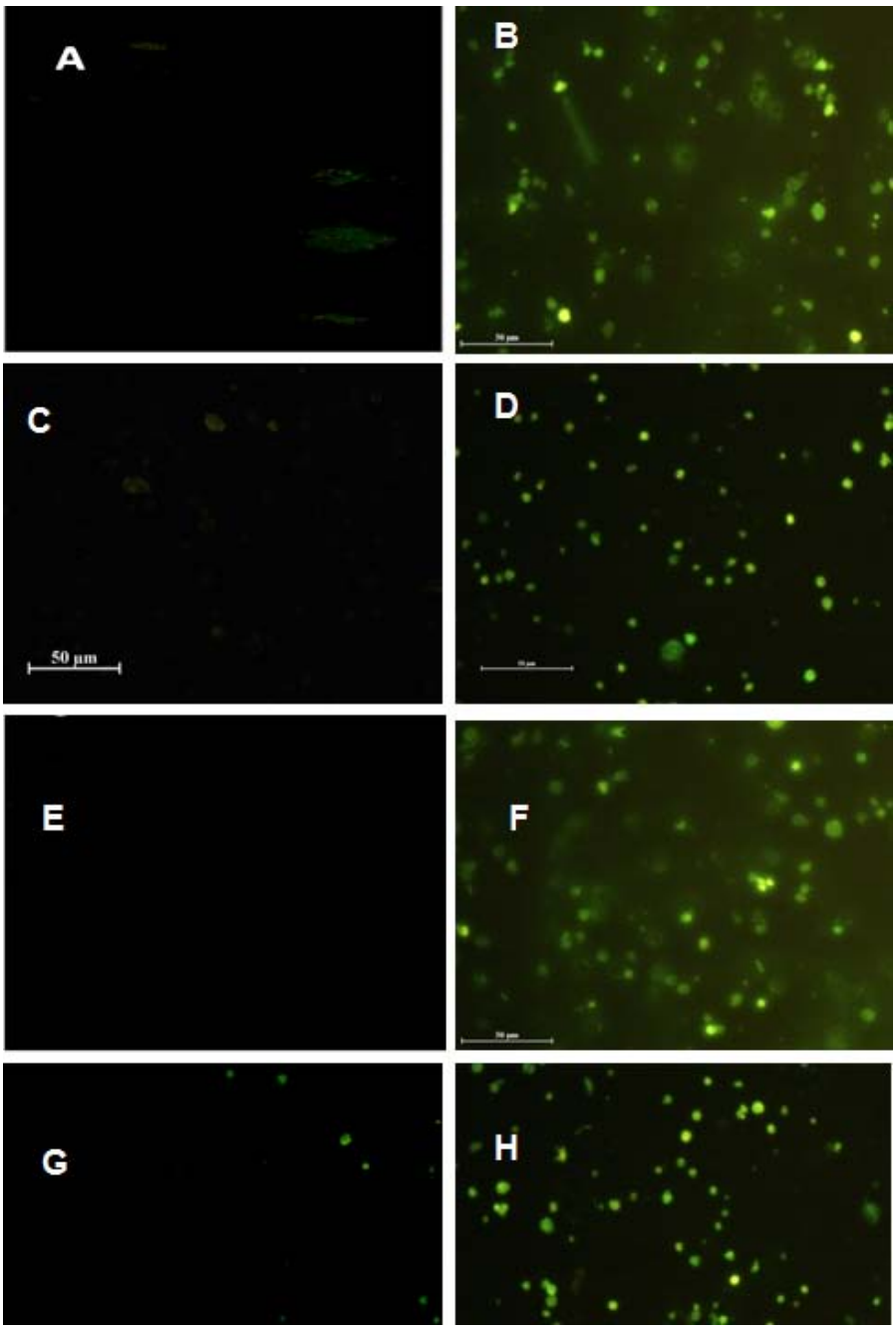

Fig. 5: Intracellular reactive oxygen species measurement was done from GTO treated MCF-7 cells and lymphocytes. The levels of ROS was considered as DCF fluorescence intensity and recorded by Fluorescence microscopic images Here A) Control MCF-7 cells B) GTO treated MCF-7 cells C) control lymphocytes D) GTO treated lymphocytes

Intracellular reactive oxygen species measurement was done from DOX-treated MCF-7 cells and lymphocytes. The levels of ROS was considered as DCF fluorescence intensity and recorded by Fluorescence microscopic images Here E) Control MCF-7 cells F) DOX-treated MCF-7 cells G) Control lymphocytes H) DOX-treated lymphocytes.

Antimicrobial activity

This was evident from the study that the values of the diameter of the zone of inhibition obtained during the evaluation of antibacterial activity. The zones of inhibition of *S. aureus, P. aeruginosa and E. coli* against GTO and standard antibiotics are shown in fig. 7 and table 2. In the agar diffusion test, the diameter of the inhibition zone of GTO (25µg) and vancomycin (25µg) toward *S. aureus* are 22 mm and 25 mm respectively. The diameter of the inhibition zone of GTO (25µg) and Imipenem (25µg) toward *E. coli* are 27 mm and 29 mm respectively and the zone of inhibition of GTO (25µg) and amikacin (25µg) toward *P. aeruginosa* are 30 mm and 23 mm respectively. A plant-derived oil showed potent function in the inhibition of growth of well-known pathogenic bacteria. This green tea oil has really proved to be beneficial to minimize the total microbial growth inhibition.

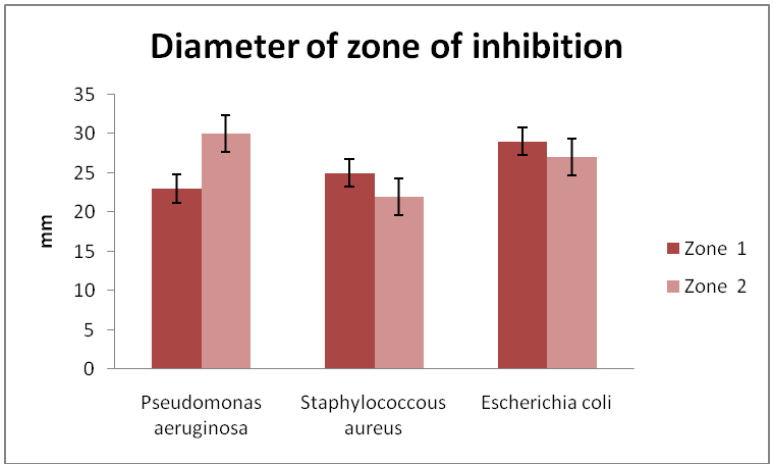

Fig. 6: Bar diagram represents the diameter of zone of inhibition on nutrient broth. Here, Zone 1: Standard antibiotic and Zone 2: green tea oil

DISCUSSION

More than 25% of new drugs are derived from natural products or their synthetic analogues. Plant-derived chemicals or phytochemicals and different microbial extracts from various biological origins are the main source of new drug development [23]. The phyto-components of green tea are polyphenols, carotene, caffeine, flavonols, theophylline, the bromine, saponins, essential oils, and some vitamins (vitamin C, B_1, B_{12}) and minerals (K, F, Fe, Mg, Ca, Cu, Ni, Zn, Mo,P etc), possess a variety of biological activities, including anticarcinogenic, antibacterial, antiviral, anti-inflammatory etc [4,5,6,8]. The present study showed that green tea oil (GTO) significantly killed the MCF-7 cells selectively in a dose-dependent manner, with no significant toxic effects on PBLs (fig. 1A) at the dose 50 µg/ml. Very less amount of lymphocytes were dead at this dose. So, this plant-derived single compound (GTO) is nontoxic to the normal healthy cells (lymphocytes) and highly active against cancer cells (MCF-7). Cytotoxicity of a chemical compound has been defined as the independent cellular killing property and the mechanism of cell death differ from the existing one [24]. So, 50µg/ml dose was selected as a biological safe dose and it was used for further experiments. Anticancer agent DOX killed the lymphocytes significantly ($P<0.05$) at the selected dose for GTO. Comparative cytotoxicity study of green tea oil with doxorubicin, the most potent anticancer drug showed that though GTO killed a lower amount of cancer cells as compared to doxorubicin but GTO showed good compatibility with PBL.

Glutathione is an important cellular reluctant, involved in protection against different cellular peroxides, free radicals, and toxic compounds in the cell [25, 26]. This study showed that GSH level in normal cells decreased slightly which was not significantly ($p<0.05$) when treated with GTO, but decreased significantly ($p<0.05$) when treated with DOX. These results revealed that the GTO maintains the normal cellular redox balance in PBL which is very much necessary for the normal cellular metabolic process. From our result, it was also found that GSH levels in cancer cell line were decreased significantly ($p<0.05$) when treated with GTO and DOX. In case of cancer cell lines, GTO can alter the cellular redox balance and helps these cells towards the oxidative damage. From the experiment, it was observed that GSSG level in normal cells slightly (not significantly) increased when treated with GTO, but increased significantly ($p<0.05$) when treated with DOX. So, results revealed that the GTO is less toxic to normal cells than DOX. From this experiment, it was observed that GSSG level in cancer cells increased significantly ($p<0.5$) when treated with GTO and DOX indicate the killing of MCF-7 cells. So, this phytomedicine performed as a better anticancer agent without hampering normal cells. These results we observed due to alteration of the membrane structure. In normal cells, the stability of the membrane is much higher compared to the cancer cells, leading to the different cellular uptake of the GTO. The uptake of GTO is higher in cancer cell causes cancer cell killing and due to the stable structure of normal cells restricts to uptake the GTO results lower amount of cell killing [27]. Lipid peroxidation in term of MDA is another marker of an oxidative stress response, which higher in cancer tissues that leads to cell membrane damage [25]. In our study, an elevated level of MDA in DOX and GTO treated cells suggested the DOX-induced cell damage (fig. 4).

Intracellular reactive oxygen species (ROS) are the important biomolecules and ions containing unpaired electrons and acts as a free radical and shows an important role in cell signaling processes, leading to oxidative cellular damage and cell death [28]. In the normal physiological system, a lower amount of ROS was formed during metabolism, which is effectively quenched by several antioxidant enzymes of the glutathione system. In addition, cellular ROS production takes place by the mitochondrial respiratory chain reaction, superoxide-generating enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and arachidonic metabolic reaction [29, 30]. From the microscopic image showing in fig. 5 revealed that the ROS generation inside the MCF-7 cells and lymphocytes. The fluorescence intensity of drug-treated MCF-7 cells was more intense compared to the control MCF-7 but in the case of lymphocytes that kind of phenomenon was not observed. The fluorescence intensity from the lymphocytes after the drug treatment was very poor and the ROS generation inside the MCF-7 cells was high after the treatment with DOX compared to the control. In the case of lymphocytes, the cells became ruptured and the ROS

generation was observed inside the lymphocytes. This increased the levels of ROS stimulate the release of different pro-inflammatory cytokines like TNF-α. This TNF-α activates nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (c-Jun NH_2-terminal kinase, JNK), as well as ultimately produces cell death by apoptotic and necrotic pathways [31]. The GTO can induce oxidative stress in the cancer cells which was the key factor of the toxicity. High amounts of reactive oxygen species (ROS) can cause oxidative damage to cellular lipids, proteins and DNA [32]. In lowered concentration of the GTO the generation of ROS was low which implies the toxicity should be stumpy, but at higher concentration, the generation of ROS was higher along with the high toxicity towards MCF-7 cells.

To date, different studies have evaluated the antimicrobial effects of tea oil and demonstrated that tea oils were active to kill certain bacterial species [33, 34]. Antimicrobial activity of our synthesized green tea oil against gram-negative *P. aeruginosa*, *E. coli* and gram-positive *S. aureus* microorganisms showed that they revealed a strong antimicrobial activity against the test microorganisms (fig. 6). It was found that in low concentration of green tea oil possesses a higher reduction in microbial growth in both the cases. Green tea oil was observed to exhibit more antimicrobial activity on gram-negative microorganism than gram-positive ones. These results suggest that growths were inhibited due to the penetration of tea oils into the bacterial cell and acts as a bactericidal agent followed by the bacteriostatic activity. These results can be explained on the basis of the differences in the cell wall of each strain; the cellular wall of gram-positive strains is wider than the cell wall of gram-negative strains [35]. All the gram-negative bacteria have an outer membrane outside the peptidoglycan layer of cell wall, which is lacking in gram-positive organisms. This outer membrane acts as a selectively permeable barrier which protects bacteria from harmful agents, like different detergents, toxins, drugs, several degradative enzymes and penetrating nutrients to sustain bacterial growth. The lipid bilayer present in this outer membrane is asymmetric in type: the inner leaflet contains close-packed phospholipid chains, while the outer leaflet is composed of the lipopolysaccharide (LPS) molecules. Different genetic and chemical experiments have revealed that the LPS layer of the outer membrane plays an essential role in providing a selective permeability barrier for *E. coli* and other gram-negative bacteria [36]. Here again, the cellular outer membrane plays an important role in those results.

CONCLUSION

In conclusion, green tea oil showed potent anticancer activity towards MCF-7 cell lines without showing toxic impact on human blood lymphocytes (PBL). Selectivity and biocompatibility are the two basic requirements of the anti-cancer drug. Considering this fact, we found that GTO showed selective cytotoxicity on both cancer cells occurred by the alteration of cellular redox balance, disruption of cellular oxidative stress and thereby induction of apoptosis. It was also manifested that green tea oil showed the potent antibacterial activity against *E. coli, P. aeruginosa* and *S. aureus* bacterial strains. Thus this molecule can be used as a lead molecule for the generation of drugs in the treatment and management of cancer and bacteria.

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ABBREVIATIONS

AOAC-Association of official analytical chemists, AOCS-American oil chemists' society, ATCC-American type culture collection, BHT-Butylated hydroxyl toluene, CDC-Centers for disease control, CNH-Collection Number Herbarium, DAD-Disc agar diffusion, DMSO-Di-methyl sulfoxide, DOX-Doxorubicin, FAMEs-Fatty acid methyl esters, FID-Flame ionization detector, GSH-Reduced glutathione, GSSG-Oxidized glutathione, GTO-Green tea oil, LDL-Low density lipoprotein, MDA-Malondialdehyde, MTT-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, MUFA-Monounsaturated fatty acid, PBL-Peripheral blood lymphocyte, PBS-Phosphate buffered saline, POF-Pentoxifylline, PUFA-Polyunsaturated fatty acid, ROS-Reactive oxygen species, SDS-Sodium dodecyl sulphate, SRM-Standard reference material

AUTHORS CONTRIBUTIONS

Meghamala Mandal has made a contribution to design, analysis and conceptualizes the work. Sreenivas R J has made a contribution to the estimation of fatty acid profile. Dr. Koushik Das, Dr. Dilip Kumar Nandi and Dr. Balaram Das reviewing the article content critically. All authors read manually and approved the final manuscript.

CONFLICT OF INTERESTS

There was no conflict of interests of the researchers

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