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Tea Root Extract (*Camellia sinensis*) and its tritepenoidsaponin induce apoptosis and cell cycle arrest in HL60 cell lines .

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Abstract

The presence of triterpenoidsaponins in tea root has been reported.The anticancer activity of this extract on U937 and K562 cell lines had been previously reported by us. However, no studies concerning biological activity of the plant extracts on HL60 cells have been done so far. In the present study, the anti-leukemic activity of the methanol extract of tea root and two of its saponins TS1 and TS2 have been investigated, and efforts have been made to understand the molecular cause of the such positive effect. The effect of the respective compounds on normal blood cells have been also estimated. The anti-leukemic activity of ME(methanol extract), TS1 and TS2, was investigated on leukemic cells using cell viability and **MTT assays**. The molecular pathways leading to the activity of tea root extract ,TS1 and TS2 were examined by **confocal microscopy and flow-cytometric analysis.** Tea root extract and its saponins TS1 and TS2 inhibited cell growth and produced significant cytotoxicity in HL60 cells. TRE and its compound produced apoptotic cell death which resulted in the subsequent **accumulation** of cells in **the sub-G0 phase** followed by DNA fragmentation.Tea root extract and its triterpenoidsaponins possesses significant anti-leukemic activity on HL60 cells . Since the necrotic body formation is low after treatment and apoptosis was not induced in normal cell, the occurrence of inflammation in *in vivo* systems could be reduced, which represents a positive indication in view of therapeutic application.

Keywords:

promyelocytic leukemia;
Tea Root Extract;
TriterpenoidSaponins;
Apoptosis;
Cell Cycle Arrest.

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1. Introduction

Bioactive natural products lead to structures that became major sources of novel agents withpharmacological promise .The anti-inflamatory and anti tumour activity of saponins are well known [1].Green tea polyphenols showed cancer chemopreventive effect both in vivo and in vitro [2]. Since it was found that tea root extract (TRE) inhibited Ehrlich Ascites Carcinoma (EAC) in mice [3] and induced apoptosis in K562 and U937 cell line [4], so in the present study we have investigated anticancer effect of the methanol - water extract of tea root and two of its saponins purified and characterized for their anticancer effect on promyelocytic leukemic cell line HL60 .

The saponins of TRE are different from those of tea leaves. They were found to have a disaccharide chain, whereas the saponins from tea leaves and seeds have a tetrasaccharide chain. These saponins, TS1 and TS2, are naturally occurring triterpenoid compounds with 23 carboxyl and 15, 16-dihydroxy substitutions in the olean series.

It is now well recognized that apoptosis is a mode of cell death used by multicellular organisms to eradicate cells in diverse physiological and pathological settings. Recent evidence has also shown that suppression of apoptosis by tumor promoting agents in preneoplastic cells is an important mechanism in tumor promotion [5]. In this context, it is noteworthy that the apoptosis inducing ability seems to have become a primary factor in considering the efficacy of chemopreventiveagents . The present study was designed to investigate TRE as a new herbal product with less toxicity but with potent anti-cancer activity.Tobe more precise and rational, studies were conducted on normal WBC isolated from healthy patient to conclude the toxicity of the extracts

2. Research Method

Materials

Cell lines: Human leukemic cell line HL60 were purchased from National Facility For Animal Tissue and Cell Culture, Pune, India. Cells were routinely maintained in RPMI 1640 medium supplemented with 10% heat inactivated FCS. Cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO_2 in
air.

Plant Material

The roots of Camellia sinensis var assamica (clone TV-1, planted in 1964) were collected and supplied by Tocklai Experimental Station, Jorhat, Assam, India. A voucher specimen (TR-004) is deposited at the Tea Research Association, Kolkata, India.

Method of preparation of crude tea root extract and its saponons named TS1 and TS2

The tea root (1 kg) was cut into small pieces, dried, ground and soaked in 3 litres of 50% aqueous methanol for one week at room temperature (20-30° C) with occasional shaking. The solvent was filtered and to the residue 1 litre of 50% aqueous methanol was added and kept for another one week at the end of which the whole solvent was evaporated by a rotary evaporator to get 12 g of dark brown mass. This was kept at 4°C in a closed container and was designated as tea root extract (TRE) for our experiments. TRE was dissolved in normal saline at a concentration of 100 µg/ml and doses of 5µg, 10µg and 15µg were used after standardization for all the study TS1 and TS2, two saponins isolated from the n-butanol fraction of tea root by repeated column chromatography, were also used for anticancer study by dissolving them in 5% DMSO and the concentrations used were 10,20 and 30µg/ml respectively. 17.5 gm of the butanolic part was subjected to column chromatography over silicic acid (80 g). Graded elution was effected with chloroform followed by chloroformmethanol (95:5, 90:10, 85:15, 80:20 and 70:30) and chloroform-methanol-water (65:35:10, upper layer).Fractions of similar T.L.C spots were combined.The major fraction TS-1 was then passed through Sephadex LH-20 column (70 X 1.5 cm) using MeOH as eluent.Thesemipurified material thus obtained, was again column chromatographed over silicic

acid and finally subjected to Sephadex LH-20 column twice (MeOH as eluent) to yield 5mg of white solid.

Cell count study

Log phase cells HL60 and normal WBC at a concentration of 10^5 /ml in RPMI 1640 (with 5% FCS) were taken in multiple well sterile plastic plates where in each plate 100ul of cell suspension was given. TRE was added at different concentrations (5, 10 ,15 µg/ml respectively) ,TS1 and TS2 were also added at a concentration of 10, 20 and 30 µg/ml to the cells separately and viable cells were counted by trypan blue exclusion principle [8] after 24 hrs.

Cytotoxicity assay (MTT assay)

After 24 hours treatment in the same method used in cell count, MTT was added to each well to a final concentration of 400µg/ml and incubated again at 37˚C in 5% CO2 incubator for 3 hrs. 100µl of DMSO was added to each well to dissolve the incorporated MTT crystal [9]. The optical density at 540nm was recorded using microtitre plate reader.

Actually the chemosensitivity of the leukemic cells was determined by this quantitative assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a substrate which is being reduced by the mitochondrial dehydrogenase of intact cells to a purple formazan product[6].

Detection of Nuclear fragmentation by Confocal microscopy

HL60 cells were grown to about 70% confluence and then 10^5 cells were taken and treated with 10 µg/ml TRE for 24 hrs. The cells were then washed with PBS and stained with 10 µg/ml propidium iodide (PI) and 10µg/ml hoescht 33342 separately for 5 min and a 10 µl cell suspension was taken on slide and fluorescent images were scanned using confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystem, Heidelberg, Germany) installed with an inverted microscope (Leica DM-7RB) [7]. Images of PI and hoescht 33342 were acquired from argon/krypton laser and UV laser line using 590 nm long pass filter for PI and 450 nm band pass filter for UV images. The diameter of the detection pinhole 121corresponded to one airy unit of 512x512 and pixel images were obtained and rescaled identically in Adobe Photoshop 6.0.

Flowcytometry and apoptosis study

Dot plot assay & quantitative estimation of apoptosis

Apoptotic cell detection was performed using FITC conjugated Annexin-V Fluos and PI. 10^5 HL60 cells were treated with 10 and 15 µg/ml TRE for 18 hrs and .Cells were precipitated by centrifugation and were resuspended in 0.5ml of binding buffer(Annexin-V binding buffer containing 10mM HEPES,150mM NaCl and 2.5mM CaCl2 2H2O; pH7.4) and again centrifuged. Pellets were dissolved in100 µl of the same buffer containing 5 µl of 2µg/ml annexin-V FITC and then to it 1 µg of PI was added. Cells were incubated in dark for 15 min and were then analyzed by flow-cytometry [8].

All data were acquired with a Becton-Dickinson FACS caliber single laser cytometer.

Although cytometer settings vary, the ranges for cytometer settings during acquisition were FSC ((Voltage-1, Amp/Gain 3.0, linear scale), FL1 voltage 450-500 mV, FL2 voltage 450-500 mV, compensation FL1-(0.8-1.3%) FL2, FL2-(25-30%) FL1 and threshold (FSC at 52). Flow cytometric reading was taken using 488 nm excitation and band pass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection). For each type of cell, appropriate electronic compensation of the instrument was performed to avoid overlapping of the two emission spectra. Live statistics were used to align the X and Y mean values of the annexin-V FITC or PI stained quadrant populations by compensation. Data analysis was performed with cell Quest (Macintosh platform) program.

Cell Cycle Phase Distribution Analysis by Flow-Cytometry

HL60 cells ($10^{5/}$ml) were treated with TRE at a dose of 10 ug/ml for 18 hrs and harvested, washed and fixed overnight in ethanol, washed twice in PBS, treated with RNase and stained with 69 µM propidium iodide in 38 mM sodium citrate buffer for 1 hr in dark . Cell cycle phase distribution of nuclear DNA was determined on FACS, fluorescence detector equipped with 488 nm argon laser

light source and 623 nm band pass filter (linear scale) using Cell Quest software (Becton Dickinson).A total of 10,000 events was acquired and analysis of flowcytometric data was performed using ModFitsoftware.A histogram of DNA content (x-axis, PI-fluorescence) versus counts (y-axis) has been displayed [9].

Statistics

Student's t-test was applied and in each case comparison was made with control and significance level was considered at $p<0.05$.

3. Results and Analysis

Effect on cell growth

TRE, TS1 and TS2 inhibited the growth of leukemic cells HL60concentration-dependent manner. TRE caused 52.24, 64.55% and 75.12 % inhibition of HL60 cells.TS1 and TS2 also showed similar concentration-dependent cell killing effect after 24 hrs treatment. TS1 caused 60.64% inhibition at a concentration of 30ug/ml respectively and TS2 caused 60.99% inhibition in the same concentrations. Fig-1 represents the effect and the inhibitory effect was statistically significant in all the cases.In all cases the effect of Ara-C, the reference agent, is also shown.[Fig.1a]

Cytotoxicity study/MTT assay(effect on cellular metabolism)

In the MTT assay it was found that TRE, TS1 and TS2 possessed marked cytotoxic effect and caused significant inhibition of metabolic activity of HL-60 cell line.The optical density (O.D) value of the control group was found to be significantly higher than treated group after 24 hrs treatment and it was found that with the increase in concentration of the drugs as well as with increase in time [Fig.2]

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Confocal Microscopy and Morphological Analysis of Nuclear fragmentation

Morphological investigation of apoptosis by confocal microscopy revealed that TRE induced cell death in HL60 cell lines by apoptosis. The treated cell showed typical features of apoptosis revealed by cell shrinkage, chromatin compaction, plasma membrane blebbing, DNA fragmentation and formation of apoptotic bodies. Fig. 3a and 3bshowedthe morphology of control or untreated and treated HL60 cells respectively stained with either Hoescht 33342 or PI (morphology of corresponding control and treated cells is also shown). The confocal images showed that the untreated cells possess intact nuclei [fig.3]

Flowcytometric detection of apoptosis in HL60 cell line

Double labeling technique using annexin-V FITC and PI was used to understand the effect of TRE to be apoptotic or necrotic. Phosphatidylserine is a protein that reside on the cytosolic face of plasmamembrane of a live and healthy celland possesses a high affinity for annexinV.The translocation of phosphatidylserine (PS), from inner to the outer plasma membrane leaflet is a very early event in the apoptotic cells, occurring long before the classical appearance of DNA fragmentation [15] .Propidium iodide is a nuclear staining dye.So by dual staining of the cells and their quantification provides a tool to distinguish apoptotic and necrotic cells. During necrosis, since the cell membrane is ruptured, these cells take up both the fluorochromes. Our flow-cytometric data revealed that, in comparison to control untreated HL60 cells, TRE treated unfixed cells howed that most of the cells were bound to annexin-V FITC but not to PI and very few with both indicating that the mode of cell death was apoptosis. The results and respective percentage of cells in apoptotic and necrotic regions for HL60 cells are given in Fig-4.. Dual parameter dot plot

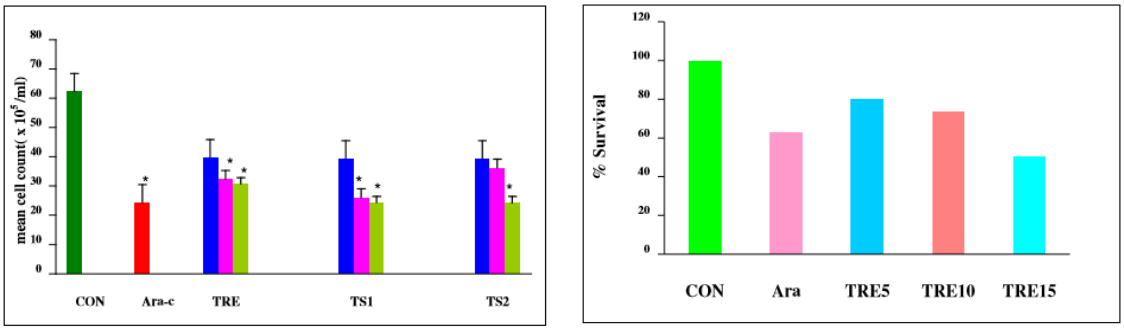
of FITC – fluorescence (x-axis) versus PI –fluorescence (y-axis) shows logarithmic intensity. Percentage of apoptotic nuclei were, for HL60; control cells – 0.38%, TRE (15µg) – 40.07%, Ara-C – 38.87%

Flowcytometric analysis of Cell Cycle Phase Distribution of HL-60 Cells

To investigate the cause of apoptosis induced by TRE in HL-60 cells we have done flowcytometric analysis of cell cycle phase distribution . After 24hrs treatment with TRE at a concentration of 15 µg /ml , the content of hypoploid DNA was increased ,untreated control 35.57% versus treated 70.63%, DNA content in G0/G1 decreased in treated. These results suggested the breakdown of the DNA resulted in cell killing and the breakdown was caused due to cell cycle arrest. [Fig.5]

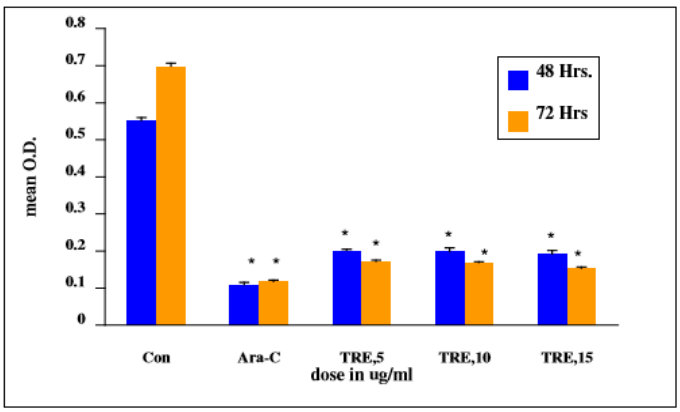
Effect on Normal White Blood Cells of Healthy Human Vokunteers

TRE at the concentrations of 5 & 10 & 15 µg/ml did not cause any significant reduction in cell number as well as cellular metabolism reflecting comparatively less toxic effect of it on normal W.B.C. specifically at the concentrations used in this study[Fig.1b]



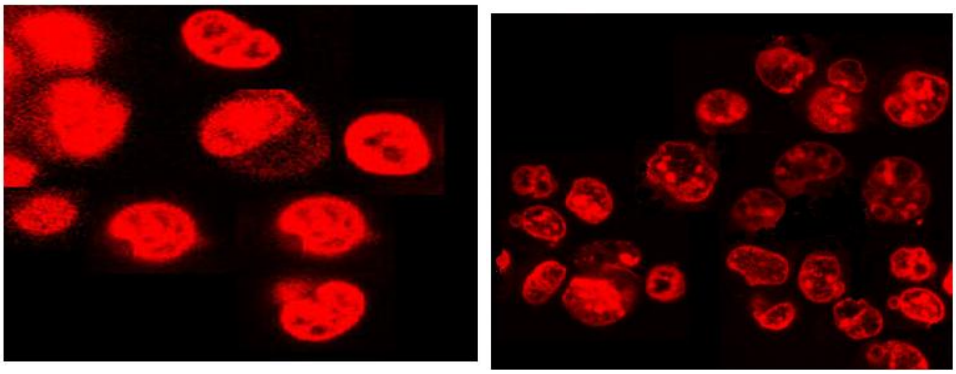
(a) (b)

Fig-1a) Showing the mean **cell count** in control (without treatment), after treatment with Ara-C (standard drug at a concentration of 2 mg/ml),TRE at concentrations of 5,10 & 15 µg/ml respectively and TS1 and TS2 at concentrations of 10.20 & 30 µg/ml respectively from left to right .* denotes significant reduction from control ($p < 0.05$).1b count of WBC



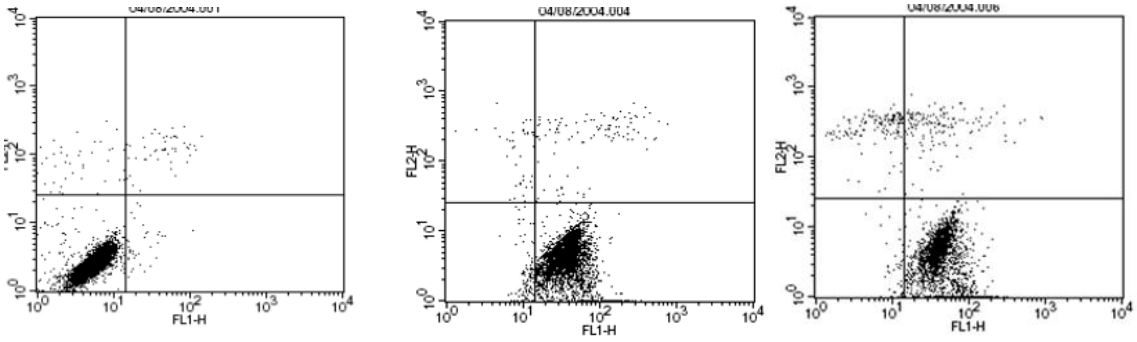
Fig,2 Showing the mean O.D. reading at 540 nm wavelength (in an Elisa reader) of **MTT assay** after 48 and 72 hrs culture of log phase cells in control (without treatment), after treatment with

Ara-C (standard drug at a concentration of 2 mg/ml), TRE at concentrations of 5,10 & 15 µg/ml respectively .*denotes significant reduction from control ($p<0.05$).



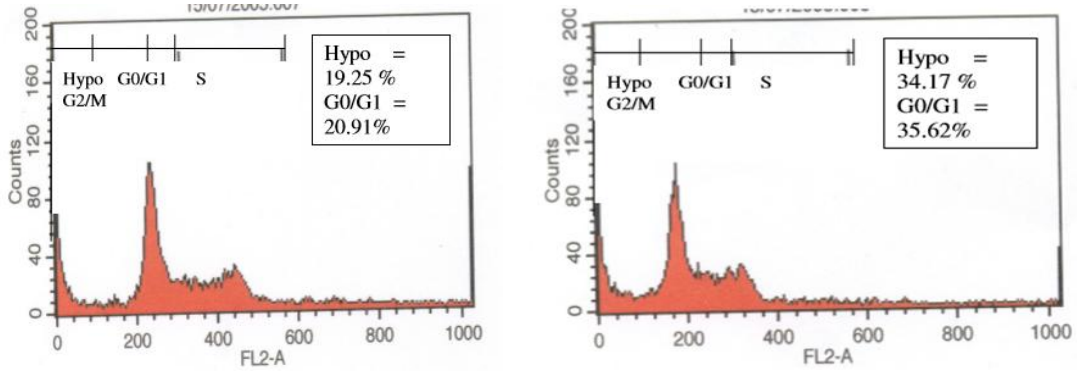
3a. Control (Intact nuclei) **3b. TRE treated (nuclear fragmentation)**

Fig3.Confocal microscope images showing apoptotic bodies in TRE treated HL-60 cells using nuclear staining dye propidium iodide



(a) (b) (c)

Fig.4 a,b,cFig-21: Flow-cytometric analysis of HL-60 cells by double 27abeled27 with annexin-V FITC and PI. Unfixed cells from control and treated were 27abeled with annexin-V FITC and PI and then fixed and analyzed on a flow-cytometer. Dual parameter dot plot of FITC- fluorescence (x-axis) versus PI-fluorescence (y-axis) shows logarithmic intensity. Quadrants: lower left – live cells; lower right – apoptotic cells and upper right – necrotic cells. Percentage of apoptotic nuclei are: (a). control cells – 0.38%, (b). TRE (15 µg) – 40.07%, (c) Ara-C – 38.87%,



(a) (b)

Fig.5. Flowcytometric analysis of cell cycle phase distribution of HL-60 (a-control, b- treated) cells after 18 h treatment with 15 µg/ml TRE, determined by single label
system.Histogram display of DNA content (x-axis, PI fluorescence) vs.counts (y-axix) has been shown.

4. Conclusion (12pt)

Research in cancer chemoprevention provides convincing evidence that increased intake of vegetables and fruits may reduce the risk of several human malignancies. Phytochemicals present therein provide beneficial anti-inflammatory and antioxidant properties that serve to improve the cellular microenvironment. Compounds known as flavonoids categorized anthocyanidins, flavonols, flavanones, flavonols, flavones, and isoflavones have shown considerable promise as chemopreventive agents. Apigenin (4', 5, 7-trihydroxyflavone), a major plant flavone, possessing antioxidant, anti-inflammatory, and anticancer properties affecting several molecular and cellular targets used to treat various human diseases [10]
Epigallocatechin-3-gallate (EGCG) is a component of green tea with anticancer effects that have been demonstrated in multiple types of cancer by inducing apoptosis.EGCG treatment induced apoptosis, decreased the mitochondrial membrane potential and increased caspase-3 protein expression levels. PCR-TRAP argentation analysis revealed that EGCG inhibited telomerase activity [11].
Epigallocatechin-3-gallate (EGCG) is an antioxidant agent derived from green tea. Because it has chemopreventive and anti-invasive effect against various cancer cells, EGCG can be used to inhibit proliferation and invasion of cholangiocarcinoma (CCA) cells. (final ref.2).Certain phytochemicals, particularly several steroidal saponins from roots of different plants, possess marked cancer chemopreventive properties [12,13].

Vinorelbine (NVB) is a semi-synthetic vinca alkaloid that is approved for the clinical therapy of lung cancer [14].Timosaponin A-III (TSA-III), a saponin isolated from the rhizome of Anemarrhenaasphodeloides, exhibits potent cytotoxicity and has the potential to be developed as an anticancer agent [15]Ten compounds were isolated and identified from green vegetable soya beans, of which five are new triterpenoidsaponins having potent anti-inflammatory activity [16].Some anti-inflammatory chemopreventive agents have been found to suppress growth and proliferation of transformed or malignant cells through induction of programmed cell death or apoptosis .

Recent advances in the development of anti-inflammatory agents have improved their therapeutic outcome in inflammatory bowel disease (IBD), however, the presence of side effects and limited effectiveness hinder their widespread use. Therefore, novel compounds with strong anti-inflammatory efficacy are still required. Makino saponins (GpS), a major component of the herbal medicine widely used in Asian countries. In *in vitro* studies it was established thatGpS dose dependently suppressed activation of macrophages, one of the main effectors in IBD. GpS also suppressed cytokine production and the activation of NF-κB and STAT3 signaling in lipopolysaccharide-induced macrophages, without affecting their viability [17,18].

In the present study, the morphological investigation for apoptosis by confocal microscopy clearly demonstrated the formation of apoptotic bodies by TRE treatment in HL60 cells. Flowcytometry allows a simultaneous estimation of apoptosis and cell cycle arrest induced by Tea Root Extract.The two saponins from TRE showed much better cytotoxic effect as compared to crude TRE. Since in our previous study [19] it was reported that TRE possessed significant anti-inflammatory activity and that it inhibited both the cyclooxygenase and lipooxygenase pathways of arachidonic acid metabolism, one of the plausible mechanisms that could account for the chemopreventive activity of TRE may be its ability to inhibit cyclooxygenase (COX) activity, because suppression of prostaglandin biosynthesis through selective inhibition of COX is now regarded as an important cancer chemopreventive strategy [20-25]. Our previous work has shown that TRE possessed super oxide radical inhibitory effect [26]. Since super oxide anions produce other kind of cell damaging free radicals and oxidizing agents , the potential scavenging of super

oxide anions by TRE suggests thatthe anticancer potential of TRE may be associated with its anti-oxidant property. So the implication of our work is that the triterpenoid saponins in tea root, which generally do not possess any industrial application, possess strong activity against human leukemia cells, suggesting the potential use of these compounds for treatment of leukemia. Moreover TRE did not produced statistically significant cytotoxicity in normal healthy white blood cells that enhances the consideration of TRE as potential therapeutic agent The findings suggest that the cytotoxic effect and apoptosis inducing capacity of TRE, reflects its importance as a potential chemopreventive agent and its use in cancer therapy in future after some more detailed investigations.

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