Research Article

Biological activity of phytoconstituents isolated from *Cinnamomum zeylanicum* and *Punica granatum*

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Received: 13 May 2018 Revised: 8 June 2018 Accepted: 17 June 2018

Abstract

Background: Ayurvedic preparations consist of a variety of medicinal plants with a range of properties. *Cinnamomum zeylanicum* Blume and *Punica granatum* L. are two plant species included in most of the preparations. The antioxidant property elicited by plant species has a full range of perspective applications in human healthcare. Plant-based risk-free, compounds with specific antioxidants and anticancerous activity is a continuous search. **Objective**: The present study aims to evaluate the purified extracts of the barks of *C. zeylanicum* and fruits peel of *P. granatum* so that they can be a potential source of molecules which has free radical scavenging or medicinal properties. **Materials and methods**: Plant extracts from *C. zeylanicum* bark and *P. granatum* fruits purified by using chromatographic methods. Structural elucidation of isolated compounds done by IR, HPLC and LC-MS analysis. The plant extracts were also evaluated for *in-vitro* antioxidant activity using 2,2'-AzinoBis [3-ethylbenzoThiazoline-6-Sulfonic acid]-di-ammonium salt (ABTS), nitric oxide and hydrogen peroxide radical with different concentrations of *C. zeylanicum and P. granatum* extract. **Results and conclusion**: These assays revealed that the plant extracts possess inhibitory activity in a dose-dependent manner with IC_{50} values ranging from 19.32±1.62 to182.62 ± 0.38 µg/ml for ABTS assay, 771.10 ± 0.21 to > 1000µg/ml for nitric oxide assay and 278.64 ± 3.68 to 894.90± 4.38 µg/ml for hydrogen peroxide assay. *In-vitro* cytotoxicity of the purified compounds from plant extracts was tested against Vero (African green monkey kidney), A549 (Human lung carcinoma), MCF–7(Human breast cancer) and $CaCo_2$ (Human colon carcinoma) cell lines. The result showed that CTC50 value of 61.55±4.8, 148.56±5.0, 75.11±3.4 and 113.00±2.5 by *C. zeylanicum* and 322.73±4.0, 599.01±4.0, 581.69±1.4 and 67.49±4.3 by *P. granatum* on Vero, A549, MCF – 7 and $CaCo_2$ cell lines respectively.

Keywords: *Cinnamomum zeylanicum* Blume, *Punica granatum*, antioxidant, hydrogen peroxide, cytotoxicity

Introduction

Plant-based foods provide, essential nutrients needed for life, and also many active compounds for the promotion of health and prevention of diseases. Purificaion and standardization is an essential tool for the quality, purity, and authenticity. Over the last decades, there is an enormous growth of worldwide interest in herbal medicine in both the developed and developing countries. The increasing market for plant products has attracted much interest of some pharmaceutical companies, which in turn motivated pharmacological studies as well as controlled and randomized clinical trials to prove the safety of herbal products (Calixto, 2000). In addition to pharmacological activities, the pharmacokinetic properties are key factors in determining whether a compound could be a viable medicinal product (Sy and Derendorf, 2014; Sy et al., 2014). According to the World Health Organization (WHO), 70-95% of the world's population relies on traditional medicine for their primary health care, and most of these practices include the use of plant extracts or their active components (Sardesai, 2002).

In the cellular system Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the system. Increased oxidative stress helped to generate free radicals which play important role in the process of aeging and cause numerous diseases like

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DOI: https://doi.org/10.31024/ajpp.2018.4.4.11

atherosclerosis, arthritis, ischemia, cancer and diabetes mellitus (Kekuda et al., 2009; Kohen and Ernst, 2010). Improved antioxidant status helps to minimize the oxidative damage and thus can delay or decrease the risk of developing many chronic age-related, free radical-induced diseases.

Cinnamomum zeylanicum tree belongs to the family, Lauraceae, is most noted for its bark, which provides the world with the commonly known culinary spice, Cinnamomum. The plant is valuable for its medicinal property and has been used to treat gastrointestinal complaints and other ailments (Lansky and Newman, 2007). It possesses antiallergenic, anti-inflammatory, anti-ulcerogenic, anti-pyretic, antioxidant, anesthetic activities (Cao and Anderson, 2011; Lin et al., 2003). Antioxidant studies with *Cinnamomum zeylanicum* bark showed better free radical scavenging capacity against a battery of free radicals (Varalakshmi et al., 2012).

Punica granatum is globally distributed and is known as dalima in Sanskrit and finds application in all traditional medical practices *ie.,* Ayurveda, Folk, Homeopathy, Unani and Siddha medicine. In Ayurvedic medicine, pomegranate is considered "a pharmacy unto itself" and is used as an antiparasitic agent, a blood tonic and to heal diarrhoea, and ulcers. *P. granatum* is also served as a remedy for diabetes in the Unani system of medicine practiced in the south East Asia. The extracts of plant parts are a rich source of anthocyanidins, cyanadines, delphinidrine, phenolic acids like caffeic acid, chlorogenic acid and tannic acid like gallic acid, ellagic acid which has the curative effect on dysentery, AIDS, Ulsers, skin lesions and cancer (Orhan et al., 2007). Extracts from seeds also confirmed the presence of a wide variety of constituents such as flavonoids, glycosides, tannins, anthocyanins and ascorbic acid. The plant has been shown to possess strong antioxidant, anti-inflammatory, and antitumorigenic property. In fact, the antioxidant activity of pomegranate is shown to be higher than that of red wine and green tea (Sharma et al., 2017).

Polyphenol components present in fruits and vegetables have antibacterial activities. Phenolic compounds have an ability to denature enzyme (Furneri et al., 2002) but they can also bind to substances such as minerals, vitamins, and carbohydrates to make them unavailable for microorganisms (Hugo and Bloomfield, 1971). Phenols can be absorbed by the cell wall, resulting in a disruption of the membrane str ucture and function. Due to the presence of a large variety of phenolics and their biological activities and uses in medicines, pomegranate fruit is of great interest to be studied for its phenolic contents. Several studies have shown that certain phenolic compounds attenuate neuronal cell death induced by oxidative stress (Parihar and Hemnani, 2003).

In *Cinnamomum zeylanicum* cytotoxic activity of the essential oil was evaluated in H-ras active-rat fibroblasts (5RP7) and normal rat fibroblasts (F2408) by 2,5-diphenyltetrazolium bromide assay (Unlu et al., 2010) . Constituents of the oil that may interfere with ras transformation were indicated by the cytotoxic activity (Zeytinoglu et al., 2003). Isoprenylation of proteins was inhibited by many monoterpenes from essential oils of *Cinnamomum* oil such as limonene and geraniol and 20-benzyloxy-cinnamaldehyde (Crowell, 1999; Gelb et al.,1995; Moon et al., 2006; Gould et al., 1994; Carnesecchi et al., 2004) .

The present work intended to carry out the antioxidant and anticancerous activity of purified extract from *C. zeylanicum* bark and *P. granatum* fruit rind and its structural elucidation by IR, HPLC and LC-MS analysis.

Materials and methods

The *Cinnamomum zeylanicum* bark was collected from plantation, dried under shade, powdered and stored in a sterile container until use. Fresh fruits of *Punica granatum* were picked from orchards and brought to the laboratory. These fruits were washed, peeled and rinds were cut into smaller pieces and then washed thoroughly with distilled water. It was then dried under shade, the pericarp was then kept in hot air oven at 80°C for 2-3 days. Dried pericarp was then ground to get a fine powder. The solvents and chemicals used were of analytical grade. The standard of ellagic acid, cinnamaldehyde, linalool, and eugenol were procured from Sigma-Aldrich.

Isolation of phytochemicals from *C.zeylanicum* bark and *P. granatum* fruit rind

To each 100g of powdered materials, 600 ml of purified water was added and pH was adjusted to 9 using 10% of potassium hydroxide and allowed to stand for an hour and the pH was adjusted to 7 using 5% hydrochloric acid during which precipitate was formed. To this 4 L of methanol was added with manual stirring and allowed to stand for 30 minutes. The methanol soluble fraction was filtered through ordinary filter paper and concentrated using water bath at 90°C under a fume hood. The obtained extracts were used for further studies.

Chromatographic separation was carried out by reverse-phase chromatography using the gradient elution. Chromatography was performed on Hypersil ODS column (5μ,250 x 4.6mm). The mobile phase consisted of acetonitrile as component B and water with 0.1% Phosphoric acid as component A. Gradient elution program was used from 0.01min:5% B; 10.0min:15% B; 15min:30% B; 18.0min:50% B; 20.0min:5% B; 22.0min:5% B; flow rate 1 mL/min and peaks were measured at 254 nm.

Chromatographic separation was performed using HPLC equipped with a degasser (DGU-20A5), binary pump (LC-20AD), auto sampler (SIL-20ACHT) and diode array detector (SPD-M20A) of Shimadzu. The data were acquired and processed using LC solutions software version 1.2.

The preliminary investigations revealed the nature of the compounds present in *Cinnamomum zeylanicum bark* as volatile and further analyzed using Gas chromatography. Compound separation was carried out using ZB-5column (30mx0.25mm, 0.25µfilm thickness and nitrogen as carrier gas with flowrate of 1.0ml per minutes) and Flame ionization detector.

In-vitro antioxidant activity was evaluated using different methods such as 3-ethylbenzoThiazoline-6-Sulfonicacid (ABTS), nitric oxide, and hydrogen peroxide.

Evaluation of antioxidant activity and determination of Minimum inhibitory concentration

Preparation of samples for ABTS assay

In 1ml of methanol, 21mg of the extracted samples were dissolved to obtain stock of 21mg/ml concentration. The stock solutions were serially diluted to get lower concentrations (62.5, 125, 250, 500 and 1000 µg/ml).

Preparation of standard for ABTS assay

In 0.95ml of methanol, 10mg of rutin was dissolved to obtain stock of 10.5 mg/ml concentration. The stock solution was serially diluted to get lower concentrations (62.5, 125, 250, 500 and 1000 µg/ml).

Preparation of ABTS reagent

In 5ml of distilled water, 5.48 mg of ABTS was dissolved to get 2mM concentration and to this solution 17 mM potassium persulphate was added. The reaction mixture was left to stand at room temperature overnight in dark before use and is prepared fresh for each trial.

ABTS inhibition assay:

A test was conducted by taking 0.2 ml of various concentrations of the Plant extracts and standard, 1.0 ml of phosphate buffer saline and 0.16 ml of ABTS solution were added to get a final volume of 1.36 ml in Eppendorf tubes and mixed using cyclomixer.Control and control blank were run parallel to test where methanol was taken instead of test sample. Similarly, test blank and control blank were carried out where distilled water was replaced with ABTS reagent. The reactions were carried out in triplet. After 20 minutes of incubation, 0.1 ml of the reaction mixture was pipetted to microtitre plate in triplets for the test, control, and singlet for test blank and control blank. The absorbance was measured in ELISA reader at 734 nm and the values were recorded.

Preparation of sample for nitric oxide assay

In 1ml of DMSO, 21mg of the samples was dissolved to obtain stock of 21mg/ml concentration. The stock solutions were serially diluted to get lower concentrations (62.5, 125, 250, 500 and 1000 µg/ml).

Preparation of standard for nitric oxide assay

In 0.95 ml of DMSO, 10 mg of ascorbic acid was dissolved to obtain stock of 10.5 mg/ml concentration. The stock solution was serially diluted to get lower concentrations (62.5, 125, 250, 500 and 1000 µg/ml).

Antioxidant activity by nitric oxide assay:

Sodium nitroprusside (10 mM, 0.4 ml), phosphate buffer saline (PBS, pH 7.4, 0.1 ml) 0.1ml of Plant extract and standard of various concentrations were incubated at 25°C for 15 minutes. To the reaction mixture, instead of the Plant extract, 0.1ml of DMSO is used for control and control blank. For test blank and control blank, in place of sodium nitroprusside, distilled water is taken. Followed by incubation, 0.05 ml of all reaction mixtures containing nitrite ion was pipette out from centrifuge tubes into micro titer plate in triplets for test and control. Control blank and test blank are taken in the inglet. Sulphanilic acid reagent, 0.1 ml each, was added to all the wells, mixed and allowed to stand for 5 minutes for completion of diazotization. Then, 0.1 ml of N-(1-Naphthyl) Ethylenediamine was added to all reaction mixtures and allowed to stand for 30 minutes in diffused light. The absorbance of these solutions was measured at 540nm using ELISA reader.

Preparation of sample for hydrogen peroxide assay

In 1ml of distilled water, 10mg of Plant extracts was dissolved to obtain stock of 10mg/ml concentration. The stock solutions were serially diluted to get lower concentrations (62.5, 125, 250, 500 and 1000 µg/ml).

Preparation of standard for hydrogen peroxide assay

In 1ml of distilled water, 10mg of ascorbic acid was dissolved to obtain stock of 10mg/ml concentration. The stock solution was serially diluted to get lower concentrations (62.5, 125,250, 500 and 1000 µg/ml).

Antioxidant activity by hydrogen peroxide assay

Sample and standard were taken as 1 ml each of various concentrations (62.5, 125, 250, 500 and 1000 µg/ml) for the analysis. To the samples, 0.6 ml of hydrogen peroxide solution was added and allowed to react. The test control was substituted with distilled water for the reaction. For test blank and control blank phosphate buffer was used instead of the sample. The above mixture was incubated for 15 minutes at 27°C. The absorbance of hydrogen peroxide was measured at 260 nm using spectrophotometer.

Evaluation of *in-vitro* cytotoxicity of the extracts

The in vitro cytotoxicity was performed on Vero (African

green monkey kidney), A549 (Human lung carcinoma), MCF – 7 (Human Breast Cancer) and $CaCo_2$ (Human colon carcinoma) cell lines to find out the toxic concentration of the sample.

Preparation of samples

For cytotoxicity studies, 10 mg of extract were dissolved and volume was made up with MEM/DMEM-HG supplemented with 2% inactivated Fetal Bovine Serum (FBS) to obtain a stock solution of 1 mg/ml concentration and sterilized by 0.22µ syringe filtration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

Cell line and culture medium

Vero (African green monkey kidney), A549 (Human lung carcinoma), MCF-7 (Human breast cancer) and $CaCo_2$ (Human colon carcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in their respective media *viz*., Minimum Essential Media (MEM)/ Dulbecco's Minimum Essential Media (DMEM-HG) supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 iu/ml), streptomycin (100µg/ml) and amphotericin B (5µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in $25cm^2$ culture flasks and all experiments were carried out in 96 well microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Cytotoxicity studies

In both the cell lines, the monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using respective media viz., MEM/DMEM-HG containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension was added. After 24hours, when a partial monolayer was formed, the supernatant was flicked off, monolayer washed once with medium and 100 µl of different test concentrations of Plant extracts were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 72 hours in 5% CO_2 atmosphere. The supernatant was removed and 100µl of propanol was added and the plates

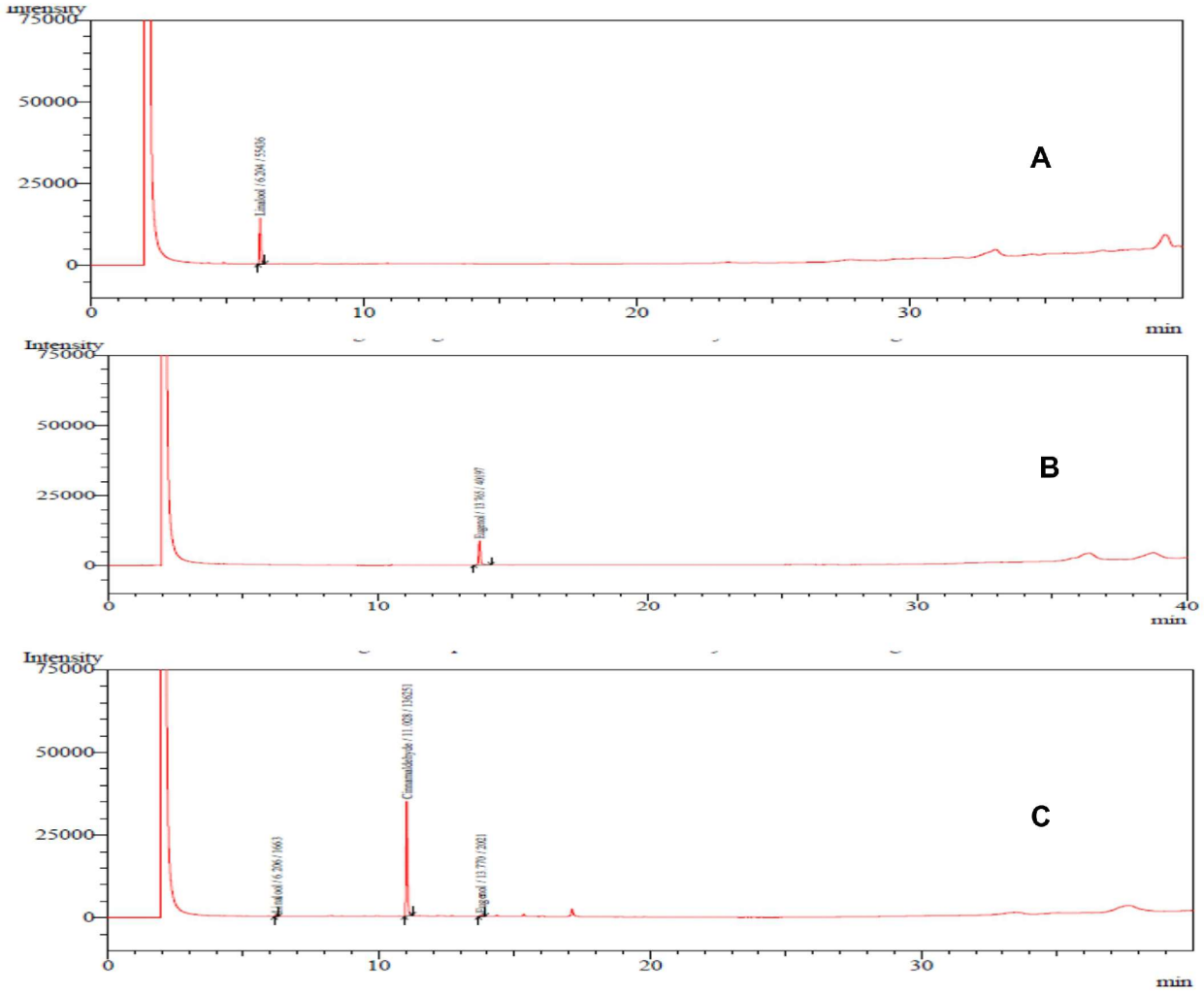

Figure 1. Chromatograms of Standards of Linalool (A), Eugenol (B) and Extract of *Cinnomon zeylanicum* barks (C)

were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the standard formula and concentration of Plant extracts needed to inhibit cell growth by 50% values was generated from the dose-response curves for each cell line.

Statistical analysis

The results were expressed as mean ± SD. Descriptive statistics was used to analyse the mean, standard deviation, variation and level of statistical significance between groups.

Results

Herbal products are used as a constituent in the formulation of more than 50% of modern drugs which possess pronounced biological activity without any harmful effect. It is essential to confirm the biological properties of the phytoconstituents. Determination of radical scavenging activity was carried out for the extracts purified from *Cinnamomum zeylanicum* and *Punica granatum* by using ABTS, Nitric oxide and hydrogen peroxide methods. Evaluation of toxicity level on cell lines is also essential to analyse the potential compound as a drug. The major phyto-compounds in the spectrum of *C. zeylanicum* bark *and P. granatum* fruit rind extract were identified as cinnamaldehyde, eugenol, linalool and ellagic acid, gallic acid respectively**.** The quantity of cinnamaldehyde, eugenol and linalool was calculated from the respective peak areas according to individual standard curves and the assay was 52 % w/w, 1.05% w/w and 0.68% w/w respectively (Figure 1A-C). The phyto compounds from *P. granatum*, ellagic acid, the major constituent, was quantified was 25.0 % w/w (Figure 2A- B).

In-vitro ABTS, nitric oxide and hydrogen peroxide antioxidant assay of the sample exhibited inhibitory activity in dose dependent manner with IC_{50} values ranging from 19.32± 1.62 to182.62 ± 0.38 µg/ml for ABTS assay, 771.10 ± 0.21 to > 1000µg/ml for nitric oxide assay and 278.64 ± 3.68 to 894.90± 4.38 µg/ml for hydrogen peroxide assay (Figure 3-4).

The cytotoxicity, *In-vitro* activity, of *P.granatum* and *C. zeylanicum* extracts were analyzed against different cancer cell lines namely Vero (African green monkey kidney), A549 (Human lung carcinoma), MCF–7 (Human breast cancer) and CaCo2 (Human colon carcinoma) and the result showed a significant inhibitory activity in the extracts. The *P. granatum* fruit rind extract and *C. zeylanicum bark* extract were taken at concentrations ranging from 1000 µg/ml to 62.5µg/ml to determine the percentage growth inhibition on the cell lines Vero, A549, MCF–7 and $CaCo_2$. The activity observed as growth inhibition with the extract from *P. granatum* showed CTC_{50} value of 322.73±4.0, 599.01±4.0, 581.69±1.4 and 67.49±4.3 on Vero, A549, MCF–7 and CaCo2 respectively (Figure 5-6). The cell lines treated with the extract from *C. zeylanicum* exhibited a lower CTC_{50} value than the former plant extract, *i.e.,* 61.55±4.8, 148.56±5.0, 75.11±3.4 and 113.00±2.5 on Vero, A549, MCF– 7 and $CaCo_2$ cell lines respectively.

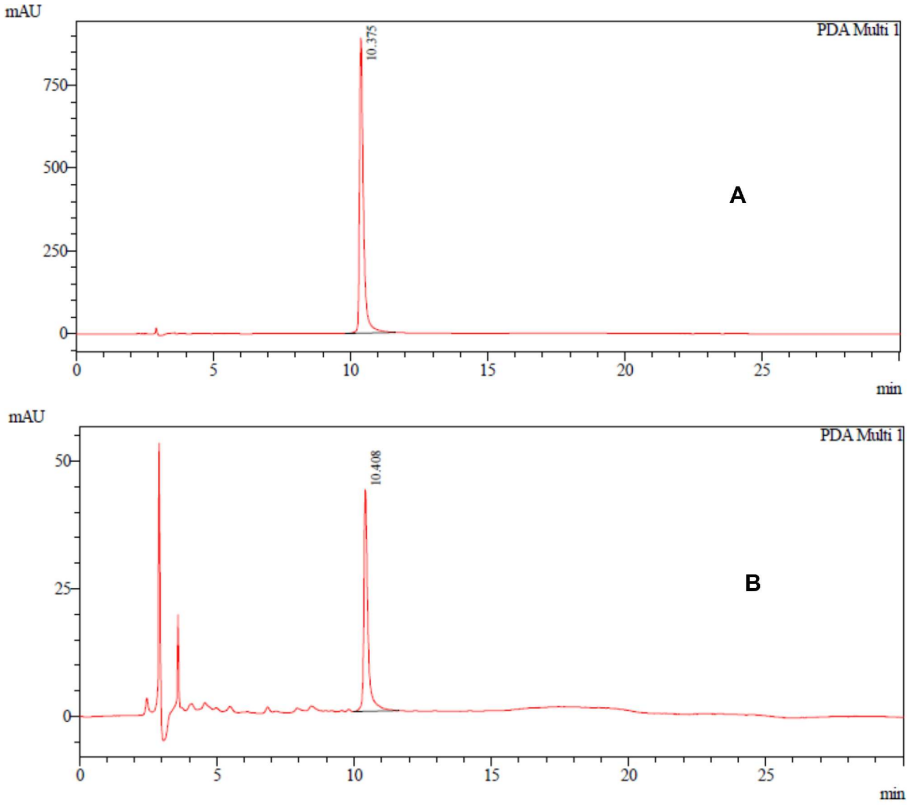

Figure 2. Chromatograms of Standard of Ellagic acid (A) and *Punica granatum* fruit rind extract (B)

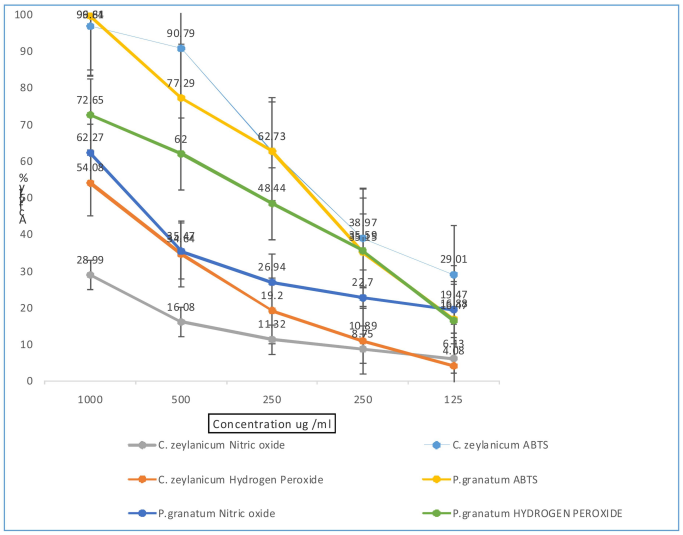

Figure 3. Antioxidant activity of C. *zeylanicum* and *P. granatum* extracts

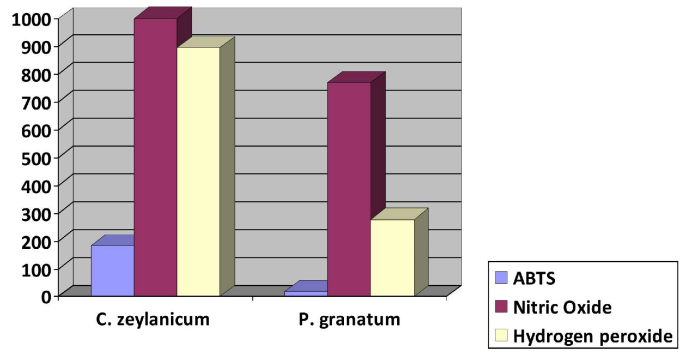

Figure 4. IC_{50} of *C. zeylanicum* and *P.granatum* extract on antioxidant activity

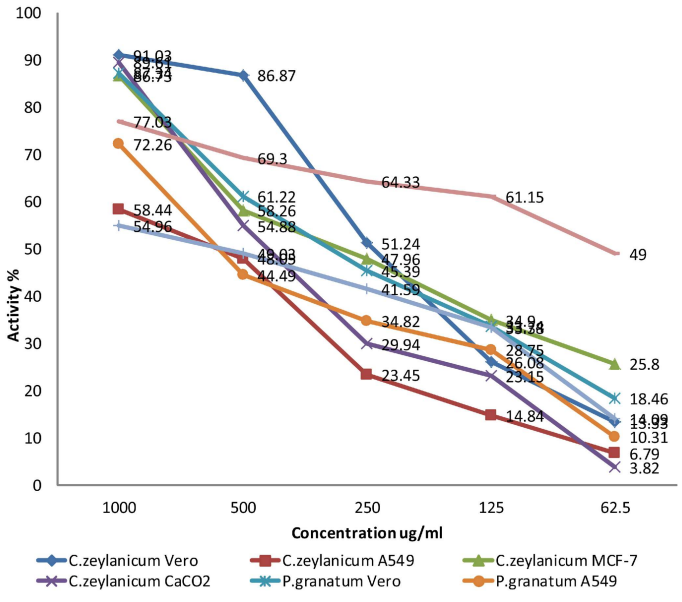

Figure 5. Effect of C. *zeylanicum* and *P.granatum* extracts on cell line

The experimental studies revealed that the phytoconstituents identified in the above plant extracts prevent the development of cancer probably through their ability to function as cytotoxic agent. The treatment of Vero cell line, A549 cell line, MCF - 7 cell line and CaCo2 cell line with the methanolic extracts of fruit rind of *P. granatum* and bark of *C.zeylanicum* conform the highest percent of apoptosis. The antioxidant property of the plants might be incremental advantages which contribute to the cell protection. The antioxidant activity of *C. zeylanicum* and *P. granatum* are probably due to the high content of cinnamaldehyde and ellagic acid respectively along with other minor phytoconstituents.

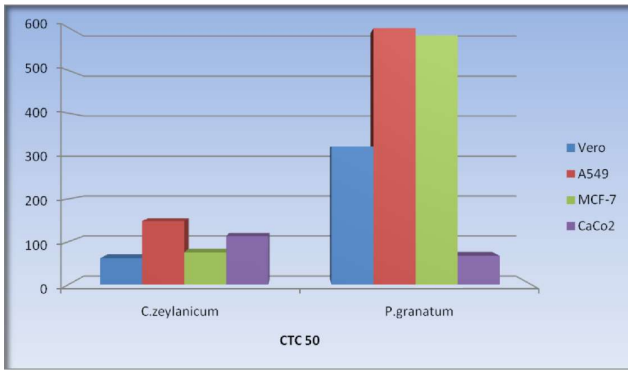

Figure 6. CTC_{50} of C. *zeylanicum* and *P.granatum* extracts on cell line

Discussion

Plant extracts have been used for many years in pharmaceuticals, herbal preparation and in natural therapies. Several phytoconstituents have been identified as potential chemo-preventive agents based on their inhibitory effect on cancer cell proliferation.

The analysis revealed the presence of phenolic compounds in both the selected plant extracts. Evaluation of these extracts is essential to identify the intrinsic toxicity while employing it for treatments.

The mechanism includes the inhibition of cancer cell growth by interfering with growth factor receptor signaling and cell progression, by blocking the cellular differentiation. Radical scavenging is very essential due to the toxic role of free radicles in the biological system. Scavenging activity of the purified plant extract, essentially qualifying it as a compound of preference as potential biological actives. Cytoprotective vs. cytotoxic effects of these phenolic compounds is beneficial as anti-carcinogenic agents of natural origin (Brglez et al., 2016).

Cinnamon is traditionally used as a spice for flavoring foods and also as a natural remedy for many years. *C. zeylanicum* and *P. granatum* possess many specific

functional properties like anti helminthic, antifungal activity and curative effect (Rakhshandehroo et al., 2016; Shahina et al., 2018; Orhan et al., 2007). Phenolic compounds which are present in plants are responsible for a variety of pharmacological activity (Kumar and Pandey, 2013). The secondary metabolite from plants form diverse group of compounds which are active as Chemo-preventive agents with different modes of action can lead to apoptosis. Cell cycle disruption and arresting the proliferation of cancer cells is one of the key roles. The study also supported by earlier reports on induced cell cycle arrest with extract of medicinal plants where they are used in cancer prevention therapy (Abdolmohammadi et al., 2008; Singh et al., 2002).

Conclusion

The present study is a search for phytochemical compounds which can serve as a biomolecule that can be added to the list of effective cytotoxic agent with less or no side effects, cost effective and reliable source of treatment. The significances of these plants are essentially a part of the day to day life as it serves as dietary supplements. Extraction and purification of the phytoconstituents enable these compounds to utilize as anti-cancerous and cyto protective agents. These properties that can further be explored to obtain and use its formulations as a cure for various ailments especially as specific anti-cancer drug. The study also showed that these two plant extracts can be considered as a good source of natural antioxidant and can be used as natural food additives.

Conflicts of interest

The authors confirm that this article content has no conflicts of interest.

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