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EVALUATION OF THE ANTI-INFLAMMATORY AND ANTICANCER ACTIVITIES OF CATECHINS EXTRACTED FROM GREEN TEA (CAMELLIA SINENSIS) IN SOME CANCER CELL LINES

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Abstract

Catechins from Green tea (Camellia sinensis) is widely known for many biological activities, special anti-cancer and anti-inflammatory properties. Catechins were extracted by mechanical blending, and using citric acid, with 2.31% in extraction efficiency. The result of determining by HPLC-UV showed that Catechin< $EC < EGC < ECG < EGCG$ in concentration compared to dry powder, reaching a total content of 634.51 mg/g, higher than tea powder (537.65 mg/g). Then, anti-inflammatory and anticancer activities of these extracts were investigated. Cell viability assay (MTT) showed that catechin extracts were not toxic to Normal Human Dermal Fibroblasts Cells. They even exhibited anti-inflammatory

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activities by suppressing the production of NO and proinflammatory cytokines. The results also showed that the extracted catechin powder also showed good anti-inflammatory activity, at a concentration of 45 ?g/mL, NO proliferation reached 44.4%. Anticancer activities of these extracts were displayed through their ability to inhibit the growth of cancer cell lines: liver cancer cell line (HepG2), lung cancer cell line (A549), leukemia cancer cell line (K562), with IC_{50} values of 181.1 μg/mL, 186 μg/mL, 137.8 μg/mL, respectively. So catechin extracts from green tea may have the potential to be applied in pharmaceutical.

Introduction

Tea (Camellia sinensis) is one of the most popular beverages consumed worldwide, such as green, black, or Oolong tea. Among all of these, the most significant effects on human health have been observed with the consumption of green tea. Most of the green tea polyphenols are flavonols, commonly known as catechins., which may constitute up to 25 -30% of the total dry leaf weight.Catechin includes main compounds such as (+)-catechin (C); ( )-epicatechin (EC); (+)-gallocatechin (GC); ( )-epicatechin gallate (ECG); ( )-epigallocatechin (EGC), v ( )-epigallocatechin gallate (EGCG) [1]. In addition, there is caffeine (3.5%); theobromine (0.15-0.2%), theophylline (0.02-0.04%) and methylxanthines, lignin (6.5%), organic acids (1.5%), chlorophyll (0.5%) and other pigments, theanine (4%) and free amino acids (1-5.5%), and various flavoring compounds [2].

The health benefits of green tea for a wide variety of ailments, including different types of heart disease, and liver disease, with biological activity such as antioxidant, antiangiogenesis, anti-inflammatory activity and antiproliferative assays that are potentially relevant to the prevention and treatment of various forms of cancer.

Recently, many of the aforementioned beneficial effects of green tea were attributed to its most abundant catechin, (-)-epigallocatechin-3-gallate (EGCG) [1]. Green tea extracts are more stable than pure epigallocatechin gallate, one of the major constituents of green tea, because of the presence of other antioxidant constituents in the extract [3]. In general, herbal medicines are complex mixtures of different compounds that often act synergistically to exert their full beneficial effect.

The aim of the study was to evaluation of the anti-inflammatory and anticancer activities of catechins extracted from green tea on leukemia cancer cell line (K562), lung cancer cell line (A549), and liver cancer cell line (HepG2).

2. Materials and Methods

2.1. Materials

Fresh green tea leaves (*Camellia sinensis*) were collected from Bao Loc, Lam Dong Province, Vietnam. Leukemia cancer cell line (K562), lung cancer cell line (A549) and liver cancer cell line (HepG2), mouse macrophage cell line (Raw 264.7) were provided by the Division of Medical and Pharmaceutical Biotechnology, Biotechnology Center Of Ho Chi Minh City.

Fibroblasts (NHDF) were provided by the Division of Medical and Pharmaceutical Biotechnology, Biotechnology Center Of Ho Chi Minh City.

Reference standards: ECGC, EGC, EC, ECG, caffeine (US). Gallic Acid (USA), Na2CO3 (Spain), Methanol (Germany), Ethyl Acetate (Japan), Citric Acid (India), FeCl3 (Germany). Culture medium: MHB, MHA, TSA, TSB (US); DMEM, RPMI, FBS, ampicillin (Vietnam), Penicillin Streptomyces, Trypan blue ...

2.2. Methods

Preparation of catechins extract

Catechins were extracted according to Yukihiko Hara et al. (1985) [4], with improved steps such as mechanical treatment (stomaching or blending), removing the solvent by rotary vacuum evaporation and decaffeination using a citric acid solution. The steps are as follows: Tea leaves after harvest were quickly heated from 5-7 minutes at a temperature of 95 - 100^0C. The leaves were then dried at a temperature of 105^0C for 30 - 40 minutes and grounded into dry powder. The powder sample was stamped in 5 minutes to break the cell, release compounds, and aid in solvent diffusion during extraction. Then, it was extracted in water at 80^0C for one hour. The operation was repeated three times. The extract was centrifuged to obtain the supernatant, added Chloroform at the ratio of 1:1 (v/v). The aqueous layer was obtained and added ethyl acetate at the ratio of 1:3 (v/v). The ethyl acetate layer was obtained then, continue adding citric acid solution at the ratio of 1:1 (v/v), and finally, the citric acid layer was obtained. The extract was then filtered by a filter paper, condensed by a rotary evaporator at 40^0C and vacuum-dried to form dry powder. The extract efficiency was determined.

Determination of catechins content

Contents of catechins and caffeine in the extract powder were subsequently analyzed by the HPLC-UV method according to Wang et al., 2003 [5], the reference standards used were EGCG; ECG; EGC; EC; GA with the following parameters:

Sample: 25mg of catechins powder diluted with Mili-Q to make up to 50 mL; Stationary phase: a C18 reversed-phase column 5 m (150 x 4.6 mm); Mobile phase: 0.1% orthophosphoric acid methanol (v /v); Solvent flow rate: 1 ml/min, sample injection volume: 10 μL, column's temperature: 30^0C; Measurement wavelength: 210-280 nm, UV spectra: 200 - 400 nm.

Evaluation of NO-producing inhibition in macrophages

In this study, catechins were tested for their ability to decrease NO generation (nitric oxide) produced secreted in LPS-stimulated RAW 264.7 cells [6]. The inhibition of NO production was quantified via the Griess reaction (This reaction is based on a chemical reaction using sulfamide and N-1-naphthylenediamide dihydrochloride (NED) under acid conditions (phosphoric acid) to form a pink azo product). The experiment is described briefly as follows: RAW 264.7 cells were inoculated in 96-well plates with a density of 104 cells per well, incubated for 12 hours under 37^0C, 5% CO_2 incubator. After 12 hours incubator, the cell culture medium was removed and containing 1% FBS was added for 6 hours.

Figure 1. Catechins extract

Replace the previous medium with one the medium containing catechins at different concentrations.

Positive control was a sample treated with dexamethasone. Incubated for 1 hour, then added 0.5 μg/mL of LPS and incubated for an additional 24 hours. 50 μL of the supernatant from each well was transferred into another 96-well plate and added 50 μL of Griess reagent. Plates were incubated at room temperature for 10 minutes. The absorbance of each well was then measured using a microplate reader at 550 nm. NO - producing inhibition and cell viability in the study were determined.

Evaluation of anticancer activity

The cancer cell inhibitory activity of catechins was evaluated based on the method of reducing tetrazolium dye (MTT) to create forms of coloured products as described in [7]. Three cancer cell lines were used: Leukemia cancer cell line (K562), lung cancer cell line (A549) and liver cancer cell line (HepG2). The cancer cell lines were cultured in DMEM and RPMI medium supplemented with FBS (10%) and penicillin-streptomycin (100 μg/mL) in conditions of 37oC, 5% CO_2 for 24h. Cells were transferred to 96-well plates at a density of 2x104

cells/mL and treated for 48 hours with catechin at different concentrations (25; 50; 100; 200; 400 μg/mL). Each well was then filled with 30 L MTT solution and cells were further incubated at 37^0C for 4 hours. After that, the cell culture medium was removed, and DMSO (100 ?L) was added to dissolve the formazan precipitate. The 570nm OD measurement determined a 50% inhibitory activity on cell growth (IC50). EGCG with 100 μg/mL concentration was used as a positive control.

Data processing methods

Experimental data were processed according to the biological statistical method by software GraphPad Prism and Excel 2016.

3. Results and Discussion

3.1. Catechins and caffeine derivatives content analyzed by HPLC-UV.

Catechins extract from green tea leaves was obtained at 2.31% in extraction efficiency. The obtained extract was bright yellow, smooth. After determining by HPLC-UV, the content of catechins in the sample was removed from impurities to increase the concentration of catechins and the result showed that Catechin$< EC < EGC < ECG < EGCG$ in concentration compared to dry powder, reaching a total content of 634.51 mg/g, higher than tea powder (537.65 mg/g). In which, EGCG content was the highest, reaching 263.24 mg/g, accounting for 41.49%; groups of ECG, EGC and EC compounds had approximately the same content, reaching 121.51 mg/g, 113.08 mg/g and 103.19 mg/g, respectively, accounting for 19.15%, 17.82% and 16,26%, respectively. The obtained Catechin had a content of 33.49 mg/g, accounting for 5.28%. The caffeine content in the extract obtained only accounted for 2.89%, much lower than the tea powder sample, with a content of 17.33%. From this result, it can be seen that the extraction method by Yukihiko Hara in 1985 improved by mechanical blending and the use of citric acid had optimum efficiency, helping to remove the caffeine content and increased the content of catechins in the sample significantly.

Table 1. Analysis of catechins and caffeine by HPLC-UV method.

Sample	Catechin (mg/g)	EC (mg/g)	ECG (mg/g)	EGC (mg/g)	EGCG (mg/g)	Caffeine (mg/g)
Green tea powder	28,26	85,53	67,77	188,59	167,5	21,12
Catechins extract	33,49	103,19	121,51	113,08	263,24	18,85

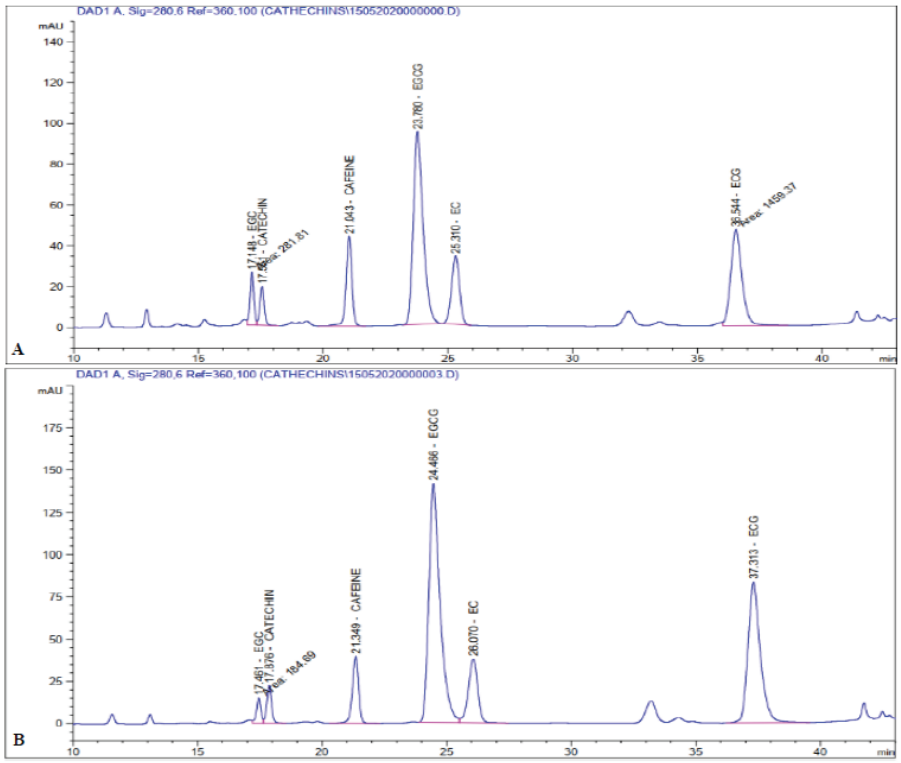

Figure 2. Content of catechins by HPLC – UV method. *(A) Green tea powder, (B) Catechins extract removed caffeine.*

Inhibitory activity of NO production in macrophages

At a 25 μg/mL concentration, catechins did not significantly inhibit NO production; the percentage of inflammatory cells reached 82.2%. However, at a 35 μg/mL concentration, Catechin began to show inhibition of NO. The best inhibitory values at the 45 μg/mL and 75 μg/mL concentration, were above 44.4%, almost equivalent to the positive control. A survey on the concentration affecting cell growth found that, at a concentration of 45μg/mL, RAW 264.7 cell still grew well at 106.3% of the viability, but at a 75 μg/mL concentration, the viability significantly reduced to about 63%. Therefore, Catechin at a 45 μg/mL concentration is considered to have the potential to influence anti-inflammatory activity (Figure 3). From the survey results, it can be concluded that Catechin showed significant anti-inflammatory activity. Evaluation of cytotoxicity to Normal Human Dermal Fibroblasts Cell (NHDF). The cytotoxicity test results on NHDF cell lines of catechins showed that from 25 to 400 μg/mL concentration, the viability was above 100% and equivalent to the negative control (Figure 4). At an above 500 μg/mL concentration began to report a percentage decrease in cell viability. It is proved that that Catechin extracted from green tea leaves was not toxic to the NHDF cells at concentrations below 400 μg/mL.

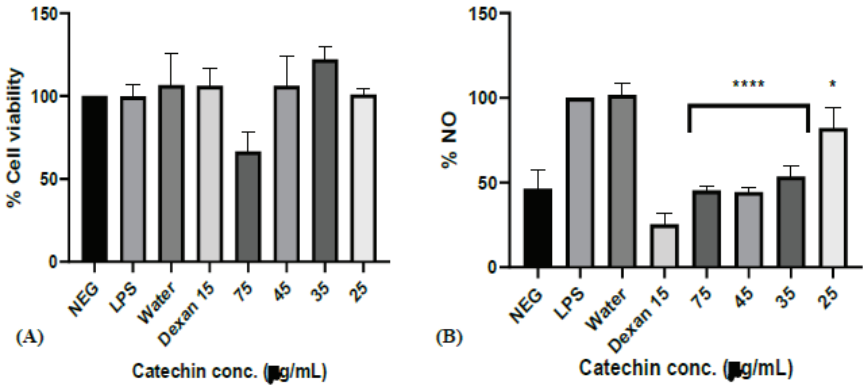

Figure 3. Anti-inflammatory activity of RAW 264.7 cell when treated with catechins at different concentrations. (A) Percentage of cell viability treated with catechins; (B) Percentage of NO-producing by cells. ***** and * represent a significantly difference with P values <0,0001 and 0,05, respectively.*

Effective Growth inhibitory activity on cancer cells

The results of assessing cancer cell inhibitory activity on three cell lines HepG2, A549, K562 showed that catechins inhibited the proliferation of cells gradually increasing with concentration (Figure 5). At a 25 μg/mL concentration, there was almost no inhibitory effect on the proliferation of all three cell lines. However, at the higher concentrations, the viability of cells was significantly reduced. From concentration of 200 μg/mL, all three cell lines had less

than 50% inhibition. The viability was above 100% from all concentrations (25-400 μg/mL): 117%, 80.2%, 70%, respectively for A549 cell lines; 96.7%, 85%, 79.7%, respectively for HepG2 cell lines and less than 50% at 200 and 400 μg/mL concentrations: 40.2% and 31.4%, respectively for A549 cell line; 48.1% and 16.1%, respectively for HepG2 cell line. Leukemia cancer cell line K562 have a viability of over 50% from all concentrations (25-100 μg/mL), 110.8%, 67.5%, 55.7%, respectively and at concentrations of 200 and 400 μg/mL is 44.1% and 19.1%.

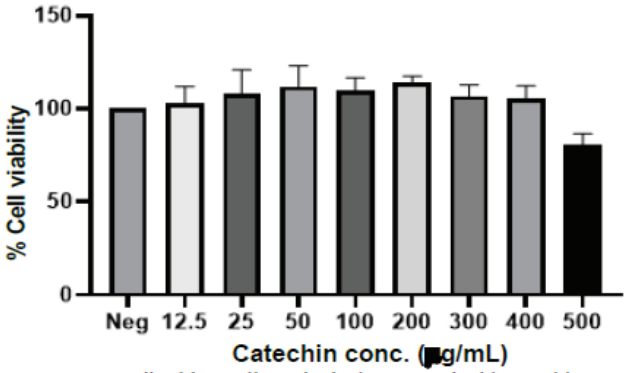

Figure 4. Fibroblast cell survival when treated with catechins at different concentrations for 48 h.

IC50 values on three cell lines HepG2, A549, K562 reached 181.1 μg/mL, 186 μg/mL, 137.8 μg/mL, respectively. The ability to inhibit cell proliferation of obtained catechins at a 100 μg/mL concentration was approximately the same as reference standard ECCG, reaching 70.02%, 79.7%, 55.74% in three cell lines HepG2, A549, K562, respectively. Thus, although it is only in the form of total extract, the obtained catechins showed the ability to inhibit cancer cells almost equivalent to reference standards. Therefore, it can be seen that catechins have potential applications in cancer chemoprevention and treatment. In studies, the results showed a significant reduction in the number of cancer cells over time in contrast to the control with no treatment. In cell morphology, catechins treated human cancer cells caused cell shrinkage, discoloration, chromatin concentration, and loss of intercellular bonds known as apoptosis (Figure 6). In conclusion, catechins that have been shown to have antiproliferative activity against the growth of human cancer cell lines can be considered in clinical situations.

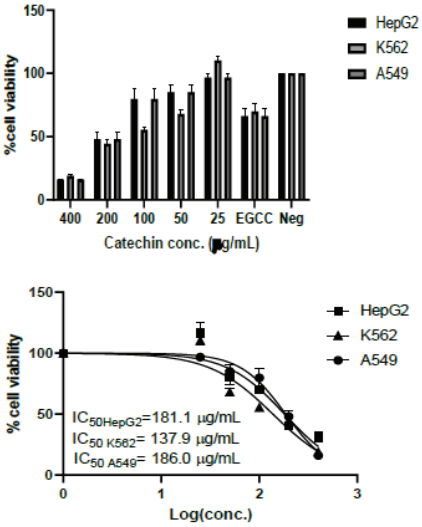

Figure 5. Toxicity assay and IC_{50} value of cancer cell lines A549, HepG2, K562 when treated with catechins at different concentrations for 48h.

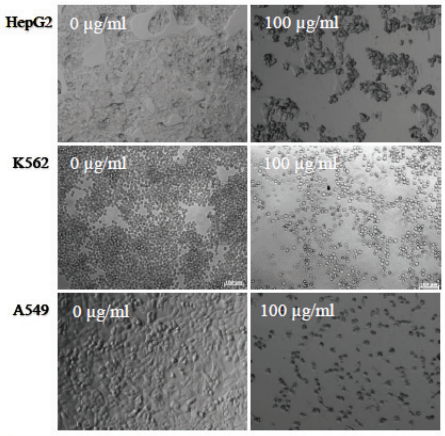

Figure 6. Represent human cancer cell lines incubated in ordinary medium and treated by catechins for 48h. *Scale par 100 µm showing: (0 µg/mL) Human cancer cell lines incubated in ordinary medium as control showed the crowded and contact between cancer cells. (100 µg/mL) human cancer cell lines, HepG2, K562, A549 incubated in 100 µg/mL of catechins, respectively, showing reduction in the number of the cancer cell.*

4. Conclusion

The extraction process improved by mechanical blending, and using citric acid has optimal efficiency, eliminating the caffeine content and increasing the content of catechins. In addition, the extracted catechin powder also showed good anti-inflammatory activity, at a concentration of 45 μg/mL, NO proliferation reached 44.4%, and the test on cell lines often showed that Catechin was extracted from green tea leaves are non-toxic to cells, usually at concentrations below 400 μg/mL. In addition, extracted Catechin has the ability to inhibit cancer cells on three cell lines, HepG2, A459, K462, with IC50 values of 181.1 μg/mL, 186 μg/mL, 137.8 μg/mL, respectively. From the research results, it can be seen that the extracted catechin powder has great potential for product development applications in the field of high-quality medicine and pharmacy.

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