A study on the targeting of ceramide metabolism by (-)-epicatechin gallate, catechin and quercetin in A-549 lung cancer cell line

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Abstract. Catechin, epicatechin gallate (ECG) and quercetin, as bioactive flavonoids, have been shown to possess anticarcinogenic effects. Ceramide plays an important role in killing tumor cells. Accordingly, the aim of this study was to clarify the involvement of these compounds in ceramide metabolism in A549 cancerous cell line. Spectrophotometer, cell culture and HPLC methods were used. Cell viability index showed different potential of cytotoxicity effect for each of the studied agents, among which ECG was more potent. This index decreased significantly over 100 to 250 µM concentrations of treatment with respect to control. Cell treatments also caused considerable increase in ceramide level of the studied agents, among which ECG was more potent. This index decreased significantly over 100 to 250 µM in comparison with control. Data also showed no significant variation in glycosyl ceramide synthase activity in treated cells with quercetin, whereas the activity decreased significantly by Catechin and/or ECG. It is our conviction that different effects on ceramide metabolism enzymes may be related to various chemical groups on the common structure of the studied compounds. Due to structure-function relationship, these compounds had different effects on ceramide generation. Elevation in ceramide content in A549 cancer cell line induced apoptosis, which led to anti-cancerous effects, as observed in this study.

Keywords. A549 cell line, catechin, ceramide, epicatechin gallate, quercetin
INTRODUCTION
Flavonoids comprise the most common group of consumed natural polyphenols over the world, in which catechin, epicatechin gallate and quercetin are the most important (Munawar et al., 2017; Maurya et al., 2009). They have some chemical properties in common, one of which is the strong radical scavenging ability attributed to their biological activities (Sak et al., 2014; Shyi-Neng et al., 2014). These bioactive compounds have been shown to possess anti-carcinogenic effects and prevent related degenerative syndromes. This potency is mostly due to their anti-oxidative characteristics (Anna et al., 2014; Ki Duk et al., 2004; Galati & O’Brien, 2004).

On the contrary to the beneficial effects of flavonoids, there are some reports that challenged their in vitro efficacy, because of their low bioavailability (Lotito & Feri, 2006). However, most studies on their chemoprevention process in cancerous cases revealed different mechanisms including retardation of the many protein kinases activities such as protein kinase-1 (Ap-1) (Guohua et al., 2014) inhibition of telomerase and reverse transcriptase (Kumar, G. and Baojun, X., 2018). These compounds may also be involved in sphingolipids (SLs) metabolism that act as major signaling molecules in eukaryotic cells. Among different SLs, ceramide is the central molecule that plays an important role in cell differentiation and growth (Krisha et al., 2013). Ceramide can be produced in cells by enovo synthesis or by the degradation of sphingomyelin (SM) pathway, where sphingomyelinase directly hydrolyses SM. Recent studies showed the critical role of ceramide in inducing and apoptosis in vivo in human cancer xenografts (Wanget et al., 2001). Accordingly, extra-cellular agents may induce apoptosis in cancer cells by an elevation in ceramide levels. Thus, targeting the ceramide metabolic pathway is an attractive strategy for the retardation of cancer, particularly by bioactive substances. In this investigation, we studied the ability of three flavonoids to interfere with ceramide level in A-549 lung cancer cell line. The responsible enzymes underlying the ceramide metabolism were also evaluated.

MATERIALS AND METHODS
(-)-Epicatechin gallate, Quercetin, and (-)-Catechin were purchased from Sigma Chemical Co., St. Louis, MO, USA. Stock solutions were made at high concentrations so that, when diluted prior to use, the residual solubilizing ethanol concentration (0.5% and less) was not cytotoxic. Naphthalene-2,3-dicarboxyaldehyde was purchased from Molecular Probes (Eugene, OR, USA), sodium cyanide was from ICN Biomedicals (Aurora, OH, USA), ceramide and sphingosine were obtained from Matreya (Pleasant Gap, PA, USA). All other biochemical reagents, including Igepal CA-630, were obtained from the Sigma Chemical (St. Louis, MO, USA).

Cell culture: The human lung cancer cell line A549 obtained from Pasteur Institute (Medical Research Center). The A549 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Promega), supplemented with 10% of FBS (Promega) and 1% penicillin-streptomycin antibiotics (Promega) and were grown at 37 °C in a humidified atmosphere with 5% CO2.

Cell viability: The effects of drugs on the growth of cells were assessed by trypan blue dye exclusion assay. In brief, A549 cells were seeded onto 24-well plates (50 × 10^4 cells/well) and grown overnight. The cells were then treated with different concentrations of each compound (0, 50, 100, 150, 200 and 250 μM), and incubated for 48 h. After the incubation period, cells were washed twice with phosphate-buffered saline solution and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution at a final concentration of 0.5 mg/ml for 3 h and then lysed in dimethylsulfoxide. Optical density was measured at 540 nm, and the background absorbance measured at 660 nm was subtracted. All samples were assayed at least in three independent experiments in duplicate, and the mean value for each experiment was calculated. The results were given as mean ± S.E.M. and expressed as percentage of control, which was considered to be 100% (Negrão et al., 2010).

Cell apoptosis: A549 (1×10,000 cells/ml) were grown on glass cover slips and incubated with different concentrations of each compound for 24 h. TUNEL assay was performed using the In Situ Cell Death Detection kit (Roche Diagnostics, Switzerland), as reported previously [Negrão et al., 2010; Soares et al., 2004]. The percentage of stained cells was evaluated by counting the cells stained by TUNEL technique (apoptotic cells) divided by the total number of nuclei stained by DAPI (Roche Diagnostics, Switzerland) at a ×200 magnification field. The percentage of apoptotic cells was...
quantified by counting the number of apoptotic cells in a total of 500 cells with a hemacytometer, with the data presented as an apoptotic index.

Caspases activity: The activities of caspase-3, -8, and -9 were measured using colorimetric substrates. Cells were added to a lysis buffer (100 mM HEPES [pH 7.5], 0.1% CHAPS, 1 mM PMSF, 10 mM MDTT, 1 mM EDTA) and placed on ice for 30 min. After the cells were centrifuged at 10,000xg for 10 min at 4 °C, 50 µg of protein from the supernatants was added to each of the caspase substrates. The colorimetric substrates for caspase-3, -8, and -9 were Ac-DEVD-pNA (Asp-Glu-Val-Asp-pNA), Ac-IETD-pNA (N-acetyl-Ile-Glu-Thr-pNA), and Ac-LEHD-pNA (N-acetyl-Leu-Glu-His-Asp-pNA), respectively. After a 2 h incubation, to measure p-nitroanilide, absorbance was determined at 405 nm (Mi Sun et al., 2010).

Preparation of lysates from cell culture: To prepare the lysates, cells attached to the culture plate were scraped off with a cell scraper and collected in a 1.5-ml tube by centrifugation. The cell pellets were rinsed with phosphate-buffered saline (PBS), suspended in sterile water, and then lysed by sonication.

Quantifying ceramide: Sample (3 µl of cell extract cell lysate) was mixed with 3 µl of acid ceramidase assay solution (0.2 M citrate–phosphate buffer, pH 4.5, 0.3 M NaCl, 0.2% Igepal CA-630, 10% FBS, 50 ng/µl acid ceramidase) and incubated at 37 °C for 1 h. The reaction was stopped by adding ethanol (1:5) and centrifuged for 5 min at 13,000g. Then, 10 µl of the supernatant was transferred into 20 µl of 25 mM sodium borate buffer (pH 9.0) containing 1.25 mM sodium cyanide and 1.25 mM NDA. The reaction mixture was incubated at 50 °C for 10 min, diluted with ethanol (1:4), and centrifuged for 5 min at 13,000 g. Then, 50 µl of the supernatant was transferred to an HPLC sampling vial and 5 µl was applied onto a C18 BetaBasic column for analysis. To calculate the final ceramide content of the samples, the background of the endogenous sphingosine (reaction mixture lacking acid ceramidase) was subtracted from the signal obtained in the presence of acid ceramidase. Analysis was based on the principle that one molecule of hydrolyzed ceramide will yield one molecule of sphingosine. Standard calibration curves were generated as described above.

The HPLC system consisted of Waters 600S controller, 616 pump, 474 scanning fluorescence detector, and 717 autosampler (Waters, Milford, MA) and BetaBasic 18 3µm (20 × 4.6-mm) column (Thermo Electron, Bellefonte, PA) which was not temperature-regulated. All chromatography was carried out using a mobile phase of 90% methanol at a flow rate of 1.0 ml/min. The fluorescent derivatives were monitored at the excitation wavelength of 252 nm and the emission wavelength of 483 nm (Xingxuan et al., 2005).

Sphingomyelinase (SMase) activity assays: The activity was evaluated by Amplitex™ Colorimetric Sphingomyelinase Assay Kit (AAT Bioquest®, Inc. product no: 13620). The kit uses Amplitex™ Blue as a colorimetric probe to indirectly quantify the phosphocholine produced from the hydrolysis of sphingomyelin (SM) by sphingomyelinase (SMase). Amplex Red SMase Assay Kit was used to determine both neutral and acid SMases activities. Cells were washed with ice cold PBS and homogenized in neutral lysis buffer (20 mM Tris–HCl pH 7.4, 2 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1% protein cocktail inhibitor, 1 mM sodium orthovanadate) for neutral-SMase assays and in acid lysis buffer (50 mM sodium acetate pH 5.0, 2 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% protein inhibitor cocktail, 1 mM sodium orthovanadate) for acid-SMase assays. Samples were kept on ice for 15 min and centrifuged at 14,000 xg for 20 min at 4 °C. 100 µl of each supernatant fraction were incubated at 37 °C for 1 hour with working solution. Fluorescence was measured with a fluorescence microplate reader by using excitation at 540 nm and detection at 590 nm (Zhou et al., 1997).

Glucosylceramide synthase (GlcT) activity: To determine GlcT activities, the fluorescent acceptor substrates C6-4-nitrobenzo-2-oxa-1,3-diazole (NBD)-ceramide and a normal-phase high-performance liquid chromatography (HPLC) was used. Acceptor substrate, 50 pM of C6-NBD-Cer and 6.5 nM of lecitin were mixed in 100 µl of ethanol, and then the solvent was evaporated. Next, 10 µl of water was added and the mixture was sonicated to form liposomes. For the GlcT assay, 50 µl of reaction mixture contains 500 µM UDP-Glc, 1 mM EDTA, 10 µl C6-NBD-Cer liposome, and 20 µl of an appropriate amount of enzyme in lysis buffer 1. Addition of conduritol B epoxide (CBE) at 2.5 mM is effective in inhibiting the glycosidase activity. Standard assays were carried out at 37 °C for 1 h. The reaction was stopped by adding 200 µl of chloroform/methanol (2:1, v/v). After a few seconds of vortexing, 5 µl of 500 µM KCl was added and then centrifuged. After the organic phase had dried up, lipids were dissolved in 200 µl of isopropyl alcohol/n-hexane/H2O (55:44:1) and then transferred to a glass vial in autosampler. A 100 µl aliquot of sample was automatically loaded onto a normal-
phase column (Intersil SIL 150A-5, 4.6 x 250 mm, GL Sciences, Japan) and eluted with isopropyl alcohol/n-hexane/H₂O (55:44:1) at a flow rate of 2.0ml/min. Fluorescence was determined using a fluorescent detector (Hitachi L-7480) set to excitation and emission wavelengths of 470 and 530 nm, respectively. The fluorescent peaks were identified by comparing their retention times with those of standards (Yasuhiro et al., 2005).

Statistical analysis: Each experiment was replicated separately for three times. The collected values were analyzed independently, presented as mean ± SD and submitted to statistical evaluation. The one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test multiple comparisons was used to indicate the statistical significance of differences between the experimental means. P value < 0.05 was considered significant for all analyses. The data were analyzed using SPSS software (version 19.0).

RESULTS

To precisely identify the percent of cell death, viability test was quantified by measuring MTT assay (Fig.1). Data showed cytotoxicity effect in a dose-dependent manner in which ECG, quercetin and catechin were potent. Evaluation of IC50 revealed that the index was at 150 µM of EGC, 200 µM of quercetin and 250 µM of catechin. Thus, ECG could be grouped as highly toxic, quercetin as moderately toxic and catechin as slightly toxic. We next investigated the apoptotic potential of these compounds. Table 1 demonstrated that the exposure of cells to each of these agents caused significant induction in apoptosis over 100 to 250 µM concentrations with respect to control. We also found that the highest induction in this index (85%) belonged to ECG and the lowest was related to catechin (58% of control). The difference effect between ECG and catechin for each treatment level was significant but it was insignificant between ECG and quercetin and between quercetin and catechin, respectively.

Variations in ceramide content within cells presented in Fig 2. HPLC analysis revealed that the application of ECG, quercetin and catechin caused increase in ceramide of cell extract in a dose-dependent manner. The maximum levels of ceramide were observed by about 2 folds of control in ECG treated at 250 µM. The elevation potential for ceramide was considerable for ECG, quercetin and catechin, respectively. In this pattern, their difference at each treated concentration was significant.

Fig. 1. The effect of cell treatment with three different compounds on cell viability as evaluated by MTT assay. *significant difference with respect to control.
Table 1. Induction of apoptosis in A549 cells by treatment with three different compounds.

<table>
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<tr>
<th>Compound</th>
<th>(µM)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>100 + 4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>100 + 4</td>
</tr>
<tr>
<td>Catechin</td>
<td>100 + 4</td>
</tr>
</tbody>
</table>

Data are expressed as % of control and are mean ± SD (n=3). (*) significant difference with respect to control. P<0.05

Fig. 2. Ceramide content in extract of cells exposed to three different compounds. (*) significant difference with respect to control.

Table 2. Sphingomyelinase activity as delta fluorescence counts in cells treated with three different compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(µM)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>410 + 35</td>
</tr>
<tr>
<td>Quercetin</td>
<td>410 + 35</td>
</tr>
<tr>
<td>Catechin</td>
<td>410 + 35</td>
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</tbody>
</table>

(*) significant difference with respect to control. P<0.05
Table 2 showed sphingomyelinase activity within cell extract in response to treatment with each of the studied compounds. No significant change in this enzyme activity was recorded in cell culture as treated with ECG. On the other hand, the activity increased significantly in treatment with two other substances among which quercetin elevated the activity around 3 folds of control at 250 µM.

However, catechin was able to intensify the activity moderately with respect to quercetin treatment and there was only 75 % increase for catechin at 250 µM treatment with respect to control.

The activity of ceramide catabolic enzyme "acid ceramidase" is represented in Table 3. There was significant inhibition in the enzyme activity of cell extract in response to all three compounds particularly over 100 µM in comparison with control. The pattern of inhibition showed that quercetin was more potent than ECG and catechin. Treatment at 250 µM with quercetin, ECG and/or catechin reduced the activity to 46%, 55% and 66% of control, respectively. There were only significant differences at 250 µM treatment in enzyme activity among the three compounds.

Table 3. Activity of acid ceramidase (% control) in cell extract after treatment with each of the three different compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(µM)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>100</td>
</tr>
<tr>
<td>Quercetin</td>
<td>100</td>
</tr>
<tr>
<td>Catechin</td>
<td>100</td>
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(*) significant difference with respect to control. P<0.05.

Table 4. Glucosylceramide synthase activity (pmol/h) within cell extract after treatment with three different compounds.

<table>
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<th>Compound</th>
<th>(µM)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Epicatechin gallate</td>
<td>34.6 + 2.8</td>
</tr>
<tr>
<td>Quercetin</td>
<td>34.6 + 2.8</td>
</tr>
<tr>
<td>Catechin</td>
<td>34.6 + 2.8</td>
</tr>
</tbody>
</table>

Cleavage of NBD-Ceramide in one hour considered for activity assay.

(*) significant difference with respect to control. P<0.05.

EGC and catechin lowered the enzyme activity over 100 µM and reduced it to 40% and 56 % of control at 250 µM, respectively. There was a significant difference between ECG and catechin effects on enzyme inhibition.

**DISCUSSION**

A comprehensive study on bio-marker compounds of herbal medicines is essential for ensuring their efficiency in therapeutic and medical uses. Catechin, quercetin and epicatechin gallate, as three important low molecular phenolic phytochemicals, are increasingly being associated with antitumor properties (Han et al., 2009; Maurya & Rizvi, 2009). The mechanisms underlying these effects have not yet been clearly elucidated but may involve interaction with ceramide metabolism, which has been considered in this study. Ceramide is the central molecule in sphingolipids and glycosphingolipids biosynthesis and is involved in apoptotic processes, particularly in cancerous cells.

In this investigation, we showed that treatment of A549 human lung carcinoma cells with each of the considered flavonoids had a strong cytotoxic effect,
among which ECG, quercetin and catechin were more efficient. In accordance with our findings, other study on green tea showed that catechin and its derivatives were the main flavanol constituents of green tea. Using MTT assay confirmed that they played an anti-proliferative role against U373MG cancer cell line (Smarajit, 2019; Ki Duk Park et al., 2004). It is our conviction that the observed cytotoxicity in this study, caused by apoptosis induction, confirmed the apoptotic properties of ECG, quercetin and catechin. In accordance with our findings, another investigation which evaluated cell viability of human breast cancer (MCF7) by MTT test in response to quercetin and other flavonoids showed a significant reduction in this index (Kim et al., 2018; Maggioni et al., 2014). Another confirmation document on anticancer activity of such materials was reported while using catechin derivatives against PC3 and SKOV3 cancer cell lines (Ki Duk. et al., 2004). The results showed that the derivation and modification of catechin, particularly in alkylated form, made it more effective than simple molecule to lower cell viability. The authors referred to the role of additional side groups and chemical modifications of catechin on its stability in whole cell culture, leading to stronger anticancer activity (Ki Duk. et al., 2004). In addition, many studies have confirmed the chemopreventative potential of catechins extracted from various green and black teas against several cancerous conditions including cervical, prostate, and hepatic malignancies (Subhadra et al., 2016).

On the other hand, quercetin as the most ubiquitous dietary flavonoid, has been shown to induce cell cycle arrest at G1 or G2/M phase, depending on the category of cell lines (Jeong et al., 2009). It can cause anti-proliferation by increasing p53 as tumor suppressor, activation of caspasases-6, 8, and 9, and suppression of NF-κB, COX-2, and Akt (Subhadra et al., 2016; Chou et al., 2010; Lee et al., 2009).

There is clear consensus on structure-function relationships based on the various substitutions on central ring of the studied compound in this investigation. Some reports have also highlighted the importance of 4-oxo group presence and/or existence of 2,3-double bonds in the central ring of flavonoids on their biological activities. In addition, the number of hydroxyl groups and their substitution position on the A and B-rings, greatly affect the anti-oxidant and anti-cancer activities of these compounds (Chang et al., 2010; Teillet et al., 2008; Benavente-Garcia et al., 2008).

Due to the role of ceramide in chemo-preventive processes of cancerous cells, and to the anti-proliferative effect of our compounds on A549 cells, this study checked ceramide levels in response to ECG, catechin and quercetin.

The administration of these compounds could effectively increase ceramide content within cell extract in a dose-dependent manner. Accordingly, it is our conviction that the application of these materials induced apoptosis in A549 through ceramide up regulation. In agreement with our evidences, many studies demonstrated that the accumulation of endogenous ceramide cause anti-proliferative response through growth arrest and apoptosis (Ogretmen & Hannun, 2004; Lin et al., 2006). Ceramide elevation caused the activation of aspartat protease catepsin D and released it into cytosol, where it triggered the mitochondrial apoptosis pathway. However, the precise mechanisms by which a compound enhanced ceramide production and induced apoptosis seemed to differ according to the specific cells and stimuli characteristics (A.G. Basnakian et al., 2005).

Ceramide generation might occur mainly by sphingomyelinase by a dependent-manner, by which sphingomyelin converted to ceramide (Elisa, 2017).

Ceramide synthase and glucosylceramide synthase which convert ceramide to glucosylceramide.

With regard to these pathways, we found that quercetin amplified sphingomyelinase activity strongly, in spite of ECG which had no effects on this enzyme. On the other hand, quercetin did not show any significant effect on glucosylceramide synthase, whereas there was considerable inhibitory effect on acid ceramidase. In this way, quercetin enhanced ceramide production by elevation in sphingomyelinase activity parallel to lowering ceramide degradation via inhibition of acid ceramidase. ECG and catechin exerted their effects on ceramide levels by inhibition of acid ceramidase and glucosylceramide synthase leading to prevention of ceramide decreasing within cell extract. In accordance with our results, Geraldine et al., (2009) reported that neither acid nor neutral sphingomyelinase-dependent activities varied considerably upon anticancer drug treatments in follicular thyroid carcinoma. They demonstrated that the administration of the glucosylceramide synthase inhibitor PDMP strengthened the effects of camptothecin on follicular thyroid carcinoma cell by increasing ceramide level. Moreover, Krishna, et al., (2007) showed that the inhibition of ceramide catabolism could improve the efficiency of cancer chemotherapy by ceramide elevation. Thus, the inhibition of ceramidase has become a potential
target for cancer therapy. Due to the structure-function relationship, according to our results, there is still no clear consensus based on the various structures subclasses (Aiping et al., 2017; Carlos et al., 2016). However, some hypothesis can be made regarding the different behavior of these compounds.

It is possible that the stereo chemistry of hydroxyl group of ring C (different from that of quercetin) may influence the biological activity of catechin derivatives (Mendoza et al., 2006). Moreover, ECG and catechin do not contain carbonyl group in comparison with quercetin, which may influence their enzyme effects or their permeability across cell membranes (Moretti et al., 2012). It has also been suggested that more hydroxyl groups lead to better anti-proliferative effects (Williams et al., 2004; Benavente-Garcia et al., 2008), which has appeared in this study for ECG with respect to quercetin and catechin.

Conclusion: In conclusion, these flavonoids had cytotoxic effects on A549 cells, resulting from ceramide up-regulation by different inhibitory effects on the known involved enzymes in ceramide metabolism. The generated ceramide may function as a mediator of apoptosis via caspases activation. In addition, different responses of treated cells to these compounds may be associated with structural modifications and the number of hydroxyl groups on their aromatic rings. More studies are clearly needed to resolve the conflicting data, to fully understand the mechanism(s) of the anti-cancer activity of flavonoids, and to evaluate their potential as therapeutic agents.

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REFERENCES

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