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Antioxidant and Anticancer Activities of Extracts and Compounds Isolated from *Terminalia nigrovenulosa* Plant Grown in Vietnam

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

This study was to isolate and identify antioxidant and anticancer compounds from extracts of bark and leaf of *Terminalia nigrovenulosa*. The EtOAc fraction of bark and n-BuOH fraction of leaf exhibited the highest DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) radical scavenging activity. Nuclear magnetic resonance (NMR) and mass spectra results showed that gallic acid, ethyl gallate, ellagic acid, catechin and luteolin isolated in EtOAc and n-BuOH fractions were the main components possessed DPPH radical scavenging activity. These fractions and their isolated compounds reduced human fibrosarcoma (HT1080) cell viability in a dose-dependent manner. In addition, these fractions and their isolated compounds significantly increased caspase-3 activity. Therefore, the reduction of cell viability might be due to the induction of apoptosis via caspase-3 pathway. These findings could be useful for the development of new chemotherapeutic agents for the treatment of malignant cancers from *T. nigrovenulosa* extracts and isolated compounds.

Keywords: T. nigrovenulosa; DPPH; HT1080 cells; caspase-3; WST.

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1. INTRODUCTION

There are about 250 species belonging to the genus *Terminalia* distributed in tropical region of the world. Some of them have been used as a traditional medicine in some Asian countries. Such as *Terminalia catappa* and *Terminalia chebula* in China for diarrhea [1], *Terminalia bellerica, Terminalia chebula* and *Emblica officinalis* (Triphala) in India prescribed for symptoms of inflammation, infection, obesity, fatigue, candida, poor digestion, assimilation, tuberculosis, pneumonia and AIDS [2]. *Terminalia nigrovenulosa* Pierre ex Laness (*T. nigrovenulosa*) is a Vietnamese traditional medicinal plant belonged to *Combretaceae* family and grows wild in deciduous forests in the southern part of Vietnam. The previous researches showed that the extracts of *Terminalia* species possessed a variety of biological activities such as *T. nigrovenulosa* bark and leaf extracts [3]; methanol extracts of *T. chebula* fruits [4,5,6]. Tanaka et al. [7] isolated 12 phenolic compounds from *Terminalia catappa.* L. Pfundstein et al. [8] identified and quantitated 34 phenolic compounds belonged to gallic acid and gallate esters; ellagic acid and its derivatives; chebulic ellagitanins and non-chebulic ellagitanin groups in methanol extracts of fruits of *T. bellerica, T. chebula* and *T. horrid*. Of the compounds isolated from *T. chebula* Retz fruit, chebulic acid was the most growth inhibitory against HOS-1 cell lines [9]. Gallic acid and methyl gallate from *T. superba* showed significant inhibition of α-glucosidase activity [10]. Extract of *T. catappa* L inhibited the growth of LLC cells [11]. However, there have been few data on the biological activities and bioactive components containing in *Terminalia nigrovenulosa* Pierre ex Laness. Therefore, the objective of this study was to figure out the antioxidative and anticancer compounds from *T. nigrovenulosa* extracts in human fibrosarcoma (HT1080) cells.

2. MATERIALS AND METHODS

2.1 Materials and Chemicals

Terminalia nigrovenulosa Pierre ex Laness bark and leaf were collected in Chu Yang Sin National Park, Daklak province, Vietnam. After collection, the different parts of fresh plants were cut and dried at ambient temperature (around 27℃) in a room with active ventilation, packed in PE bags and stored at -80℃ before use.

Sephadex LH-20 resin (25 – 100 µm bead size), DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) and Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) were obtained from Sigma-Aldrich (St. Louis, MO, USA); methanol d_4 (CD_3OD) and DMSO-d_6, silica gel 63 – 200 µm particle size were obtained from Merk (Darmstadt, Germany); ODS-A gel (3 x 40 cm, 120Å pore size) was purchased from YMC Co. LTD (Kyoto, Japan). Other chemicals were of analytical grade.

2.2 Extraction and Isolation of Active Compounds

Dried leaves (3 kg) and bark (1.5 kg) of *T. nigrovenulosa* were separately extracted with MeOH (20 L (leaves) or 10 L (bark) × 3) at room temperature (around 27℃) for 24 h. The combinations of each MeOH extract from bark or leaves were evaporated to produce 300 g extracts. The extracts were then re-suspended in distilled water (3 L for each) and separately partitioned with hexane (2L × 3), chloroform (2L × 3), EtOAc (2L × 3), and n-butanol (2L × 3); detailed in Fig. 1.

The ethyl acetate (EtOAc) fraction of bark (112 g) was chromatographed over a silica gel column (10 x 40 cm; 63 – 200 µm particle size) eluting with a chloroform-EtOAc gradient (10 : 0, 8 : 2, 6 : 4, 5 : 5, 3 : 7; each 3 L) to give 5 fractions (F1: 150 mg; F2: 1 000 mg; F3: 12 000 mg; F4: 10 000 mg; F5: 15 250 mg). Fraction 3 was further chromatographed on a silica gel column (5 x 70 cm; 63 – 200 µm particle size) eluting with chloroform-EtOAc-formic acid (4 : 6 : 0.1). Total 20 sub-fractions of 250 ml each were collected and combined on the basic of TLC spraying with 0.1% of DPPH solution. Sub-fractions 1-10 were then purified on a LH-20 column (4 x 40 cm) eluting with 80% MeOH to yield compound 1 (250 mg). Sub-fractions 13-20 were crystallized in water and then applied to a sephadex LH-20 column (4 x 40 cm) eluting with 70% MeOH to give compound 2 (570 mg). Fractions 4 and 5 were combined and then applied to a ODS-A column (3 x 40 cm, 120Å pore size) eluting with 30% MeOH to give 20 sub-fractions of 200 ml each. Sub-fractions 12-19 were combined on the basic of TLC spraying with 0.1% of DPPH solution and subsequently chromatographed over a silica gel column (4 x 80 cm, 63 – 200 µm particle size) using chloroform-EtOAc-formic acid (8 : 2 : 0.1) to give compound 4 (350 mg). Combination of Sub-fractions 3-8 was further chromatographed on a silica gel column (3 x 80 cm, 63 – 200 µm particle size) using chloroform-

EtOAc-formic acid (6 : 4 : 0.1) to yield compound 3 (250 mg).

The n-BuOH fraction of leaf (150 g) were applied to a silica gel column (10 x 40 cm; 63 – 200 µm particle size) eluting with a hexane-EtOAc gradient (9 : 1 to 1 : 9) to give 9 fractions of 1.5 L each. The combination of fractions 1 – 4 (12 300 mg) was chromatographed on a sephadex LH-20 column (4 x 40 cm) eluting with 70% MeOH to give 10 sub-fractions of 350 mL each. After that, sub-fractions 3 to 4 (1 720 mg) was combined on the basic of TLC and then subjected to column chromatography on a silica gel (4 x 100 cm, 63 – 200 µm particle size) eluting with chloroform-EtOAc-formic acid (6 : 4 : 0.1) to give compound 5 (195 mg). The combination of sub-fractions 8-10 (2 350 mg) was applied to a silica gel column (4 x 100 cm, 63 – 200 µm particle size) eluting with chloroform-EtOAc-formic acid (6 : 4 : 0.1) and then purified in a sephadex LH 20 column (4 x 40 cm) eluting with 80% MeOH to yield compound 1 (320 mg). The main compounds in fractions 5-9 (14 230 mg) were compound 2 (320 mg) and compound 4 (175 mg) which were isolated and purified by the method mentioned above.

2.3 Structure Analysis

Nuclear magnetic resonance (NMR) spectra were obtained on a Varian Unity Inova 500 and 600–MHz spectrometer (Varian, Walnut Creek, CA, USA) with TMS as the standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). The mass spectra were measured by a Micromass mass spectrometer (QTOF2).

2.4 Evaluation of Antioxidant Activity

2.4.1 DPPH radical scavenging activity

Free radical scavenging activity of the extracts against stable DPPH radical (2,2-diphenyl-2-picrylhydrazyl hydrate) was determined by a spectrophotometer using the method described by Nguyen and Eun [3]. Extracted solutions were prepared in a range of concentration (0.075, 0.125, 0.25, 0.5 mg/ml). The solution of DPPH radical in methanol (6×10^{-5} M) was prepared daily before the UV measurements. Three milliliters of this solution was then mixed with 77 µl of extract solution. The samples were kept in the dark for 15 min at room temperature, after which the decrease in absorption was measured. Absorption of a blank sample containing the same amount of methanol and DPPH radical solution was measured daily. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

where A_B and A_A stand for absorption of the blank sample (t=0 min) and absorption of the tested extract solution (t=15 min), respectively. The extract that could scavenge 50% of the DPPH radicals (IC_{50}) was calculated from a plot of scavenging effect versus extract concentration.

2.4.2 Antioxidative activity of fraction in purification procedure

The assay for antioxidative activity of fractions was performed by spraying the 0.1% of DPPH methanolic solution on TLC plate. Each fraction was spotted on TLC plate and developed by a suitable mixture solvent. After spraying the DPPH solution, active fraction will reduce DPPH*, causing a colour change from deep-purple to light yellow.

2.5 Cell Culture

Human fibrosarcoma (HT1080) cells were purchased from the American Type Culture Collection (ATCC, USA). The cells were grown in Dulbecco's modified eagle medium (DMEM; Gibco, USA) containing penicillin (100 U/ml), streptomycin (100 µg/ml) (Sigma-Aldrich, St Louis, MO, USA) and 10% fetal bovine serum (FBS; PAA, Canada) at 37℃ in 5% CO_2 air. The medium was changed 3 times a week.

2.6 Cell Viability Assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8; Dojindo, Japan). The procedure is slightly modified method of the instruction of technical manual. Briefly, HT1080 cells were seeded onto a 96-well culture plate at a density of 10^4 cells/well in 100 µl of DMEM supplemented with 10% fetal bovine serum and 1% streptomycin-penicillin. After 24 h incubation, the media was replaced with 100 µl of fresh medium and treated with the different concentrations of EtOAc bark and n-BuOH leaf fractions, GA, Cat, EG and luteolin (0 - 100 µg/ml), and EA (0 – 2 µg/ml). Afterward, the cells were incubated for 24 h and then 10 µl of CCK-8 was added to each well and incubated for 2 h. Absorbance was measured at 450 nm using a microplate reader.

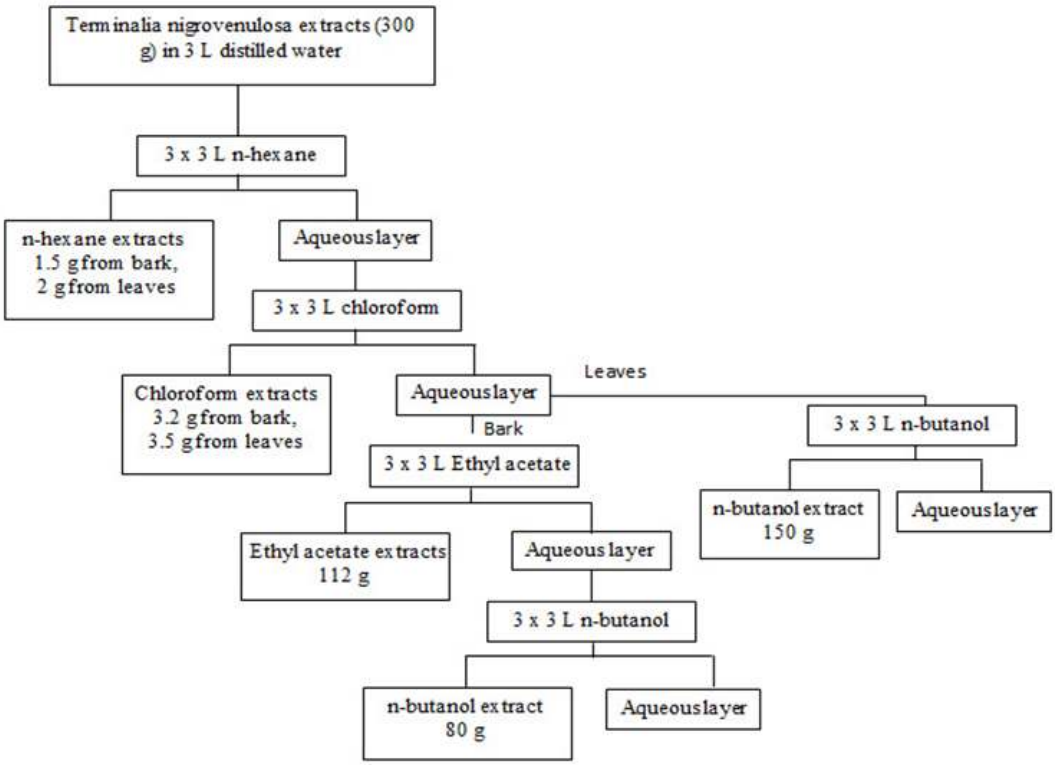


Fig. 1. Extraction fractionation scheme of bark and leaf of *Terminlalia nigrovenulosa*

2.7 Determination of Caspase-3 Activity

The caspase-3 assay was based on the hydrolysis of the peptide substrate N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC). HT1080 cells were seeded onto a cell culture plate at a density of 2 x 10^6 cells/plate in DMEM supplemented with 10% fetal bovine serum and 1% streptomycin-penicillin. After 24 h incubation, the cells were treated with extract fractions and isolated compounds for 24 h. The cells were collected and lysed by 50 µl cold lysate buffer/10^6 cells (130 mM NaCl, 10 mM Tris- HCl, 10 mM phosphate buffer pH 7.4, 10 mM. sodium pyrophosphate, 1% Triton X-100). The cell lysate was incubated on ice for 10 min and then centrifuged at 14,000 × g for 5 min. Supernatant was collected and kept on ice. Addition of 50 µl of 2X reaction buffer (20 mM HEPES (pH 7.5), 10% glycerol, 2 mM DTT) to 50 µl of cell lysate in a 96 wells black plate and then mixed with 5 µl of caspase-3 fluorogenic substrate (Ac-DEVD-AMC: N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin). The mixture was incubated at 37℃ for 1.5 h. The AMC liberated from Ac-DEVD-AMC was measured by a spectro-fluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

2.8 Statistical Analysis

Results were expressed as mean ± standard deviation of three replicated. The significant differences between the means of parameters were determined by LSD test ($p<0.05$) using Statgraphics centurion XV statistical software.

3. RESULTS

3.1 DPPH Radical Scavenging Activity of Extracts and Fractions

The DPPH radical scavenging activity of extracts and various fractions of *T. nigrovenulosa* leaf and bark were presented in Table 1. The results indicated that the effective radical scavengers were concentrated in EtAOc fraction of bark and n-BuOH fraction of leaf. Moreover, the patterns of EtAOc fraction of bark and n-BuOH fraction of leaf on TLC were simpler than that of other fractions (data not shown). Therefore, these fractions were used for isolation of antioxidative compounds.

Table 1. The DPPH radical scavenging activity of extracts and fractions of *T. nigrovenulosa* leaf and bark

Extract and fraction	DPPH radical scavenging activity (IC_{50}) mg/ml
Bark extract	0.273 ± 0.003^{e}
Leaves extract	0.408 ± 0.006^{d}
EtOAc bark fraction layer	0.162 ± 0.039^{g}
n-BuOH bark fraction layer	0.546 ± 0.014^{c}
Water bark fraction layer	0.698 ± 0.006^{a}
n-BuOH leaves fraction layer	0.258 ± 0.016^{e}
Water leaves fraction layer	0.606 ± 0.007^{b}
Vitamin C (Positive control)	0.242 ± 0.001^{f}

Results are means ± SD of triplicate measurements. Different labels (a-g) indicate a significant difference at P<0.05

3.2 Yields and Structure of Isolated Compounds

The structure of compounds showed in Fig. 2 was elucidated by NMR and MS analysis as follows:

Compound 1: Ellagic acid (EA), yellow (Light), **^{1}H-NMR** (300 MHz, CD_3OD): δ 7.46 (2H, s, H-13, H-14), 10.58 (2H, s, OH-19, OH-20), 10.795 (2H, s, OH-21, OH-22). **^{13}C-NMR** (150 MHz, DMSO): δ 107. 69 (C-9, C-4); 110.25 (C-8, C-3); 112.32 (C-13, C-14); 136.39 (C-10, C-5); 139.55 (C-11, C-16); 148.12 (C-12, C-15); 159.15 (C-2, C-7). Electrospray ionization-MS (**ESI-MS)** (negative mode) *m*/*z* 300.9984 $[M - H]^-$.

Compound 2: Catechin (Cat), brown amorphous powder, **^{1}H-NMR** (600 MHz, CD_3OD): δ 2.49 (1H, *J* = 24, dd, H-3), 2.84 (1H, dd, *J* = 18, H-3), 3.97 (1H, m, *J* = 24, H-2), 4.55 (1H, d, *J* = 6, H-1), 5.85 (1H, s, H-6), 5.92 (1H, s, H-8), 6.71 (1H, dd, *J* = 6, H-6'), 6.75 (1H, d, *J* = 12, H-5'), 6.83 (1H, s, H-2'); **^{13}C-NMR** (150 MHz, CD_3OD): δ 28.67 (C-3), 68.96 (C-2), 83 (C-1), 95.62 (C-8), 96.4 (C-6), 100.94 (C-4), 115.38 (C-2'), 116.2 (C-5'), 120.17 (C-6'), 132.35 (C-1'), 146.36 (C-4'), 146.39 (C-3'), 157.05 (C-9), 157.75 (C-7), 157.98 (C-5). **ESI-MS** (negative mode) m/z 289.0710 $[M-H]^-$.

Compound 3: Gallic acid (GA) white amorphous powder; **^{1}H-NMR** (600 MHz, CD_3OD) δ 7.05 (2H, s, H-2, H-6). **ESIMS** (negative mode) *m*/*z* 169 $[M - H]^-$.

Compound 4: Ethyl gallate (EG) white amorphous powder, **^{1}H NMR** (600 MHz, CD_3OD); δ 7.05 (2H, s, H-2, H-6); 4.27 (2H, q, J = 24, CH_2); 1.34 (3H, t, J = 12, CH_3). **ESI-MS** (negative mode) *m*/*z* 197.008 $[M - H]^-$.

Compound 5: Luteolin, bright yellow amorphous powder; **ESI-MS** (negative mode) *m*/*z* 285.021 $[M - H]^-$.

The Table 2 showed that catechin was the highest content in both bark and leaf fractions. Ethyl gallate was found relatively high amount in EtOAc bark fraction, but without in n-BuOH leaf fraction and luteolin was only found in n-BuOH leaf fraction.

3.3 Cell Viability

Cell Counting Kit 8 (CCK-8) was used for determination of toxic concentrations of extract fractions and their isolated compounds to HT1080 cancer cells (Fig. 3). The results showed that EtOAc bark and n-BuOH leaf fractions did not inhibit the viability of HT1080 cells in a concentration range of 0 to 25 µg/mL, however, it significantly decreased the viability of cells of about 22.7 to 50.5% and 13.5 to 51.2% at a concentration range of 50 to 100 µg/mL of bark and leaf fractions, respectively (Fig. 3A). These results were also similar trend in cells treated with Cat, EG and luteolin, with the cell reduction of about 35, 38 and 25% at the treatment concentration of 100 µg/mL, respectively (Fig. 3A). GA inhibited the growth of about 27% of HT1080 cells at a concentration of 25 µg/mL and 64% at 100 µg/mL but without any effect at a concentration ranges of 0 - 12.5 µg/mL (Fig. 3A). EA had no significant cytotoxicity in HT1080 cells up to a concentration of 2 µg/mL (Fig. 3B). The results indicated that extract fractions and their isolated compounds (GA, EG Cat and luteolin) could reduce the viability of HT1080 cells in dose dependent manner.

Table 2. Yields of compounds isolated from bark and leaf fractions of *T. nigrovenulosa* (mg/g dry wt of fractions)

Compounds	Yield (mg/g dry wt of fractions)	
	EtOAc fraction of bark	n-BuOH fraction of leaves
Ellagic acid	2.332	2.133
Catechin	5.089	2.133
Gallic acid	3.125	1.167
Ethyl gallate	2.323	0
Luteolin	0	1.300

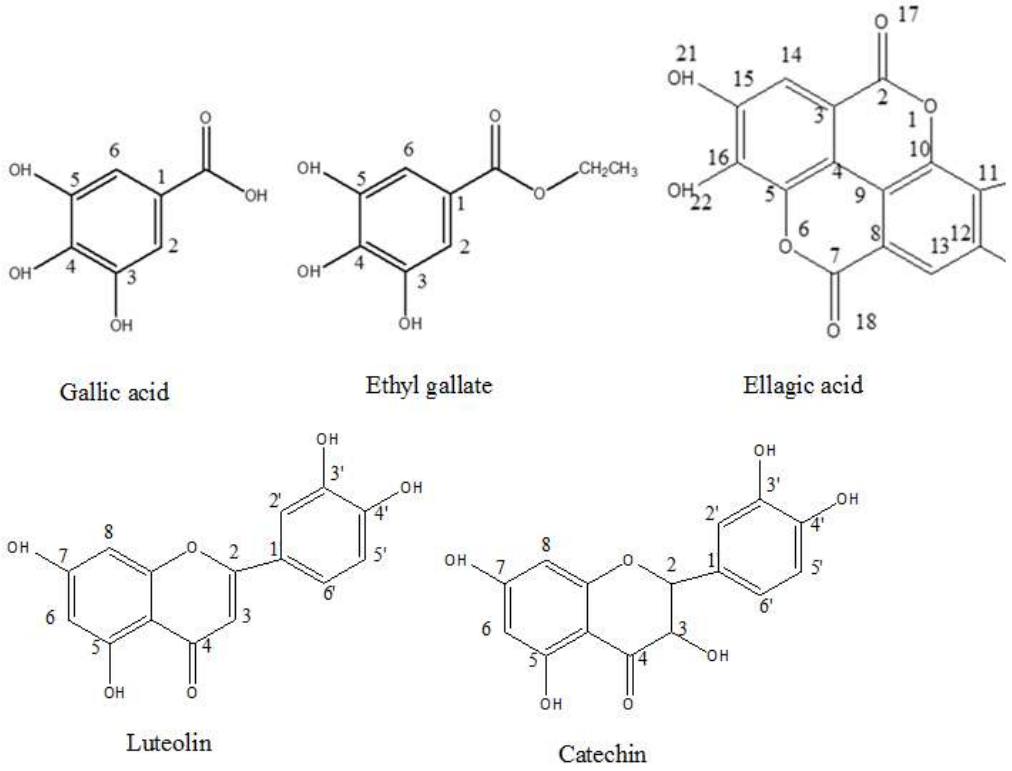


Fig. 2. The molecular structure of isolated compounds from *T. nigrovenulosa* fractions

3.4 Effect of Fractions and Isolated Compounds on Caspase-3 Activity in HT1080 Cells

Recent work has revealed that caspase-3 plays an important role in the signal transduction pathway leading to apoptosis [12,13]. Our data mentioned above exhibited that treatment of HT1080 cells with EtOAc bark and n-BuOH leaf fractions, GA, EG, Cat and luteolin induced cytotoxicity in HT1080 cells (Fig. 4). However, whether this cytotoxicity leads to apoptosis or necrosis, the activity of caspase-3 was measured. The results indicated that the activity of caspase-3 increased together with increasing treatment concentration. At low concentration, there was no significant difference in caspase-3 activity between cells treated with fractions or compounds and control (without any treatment). However, there was a significant increase in the activity of caspase-3 in HT1080 cells treated with EtOAc bark and n-BuOH leaf fractions, EG, Cat, and luteolin at a concentration of 80 µg/mL by about 2.38, 2.69, 2.88, 2.17 and 2.09 fold higher than control cells, respectively. GA made an increase in caspase-3 activity in HT1080 cells by 2.33 fold at the concentration of 30 µg/mL. The data exhibithed that the cytotoxic effect of the fractions and compounds (GA, EG, Cat and luteolin) could induce apoptosis in a population of HT1080 cells.

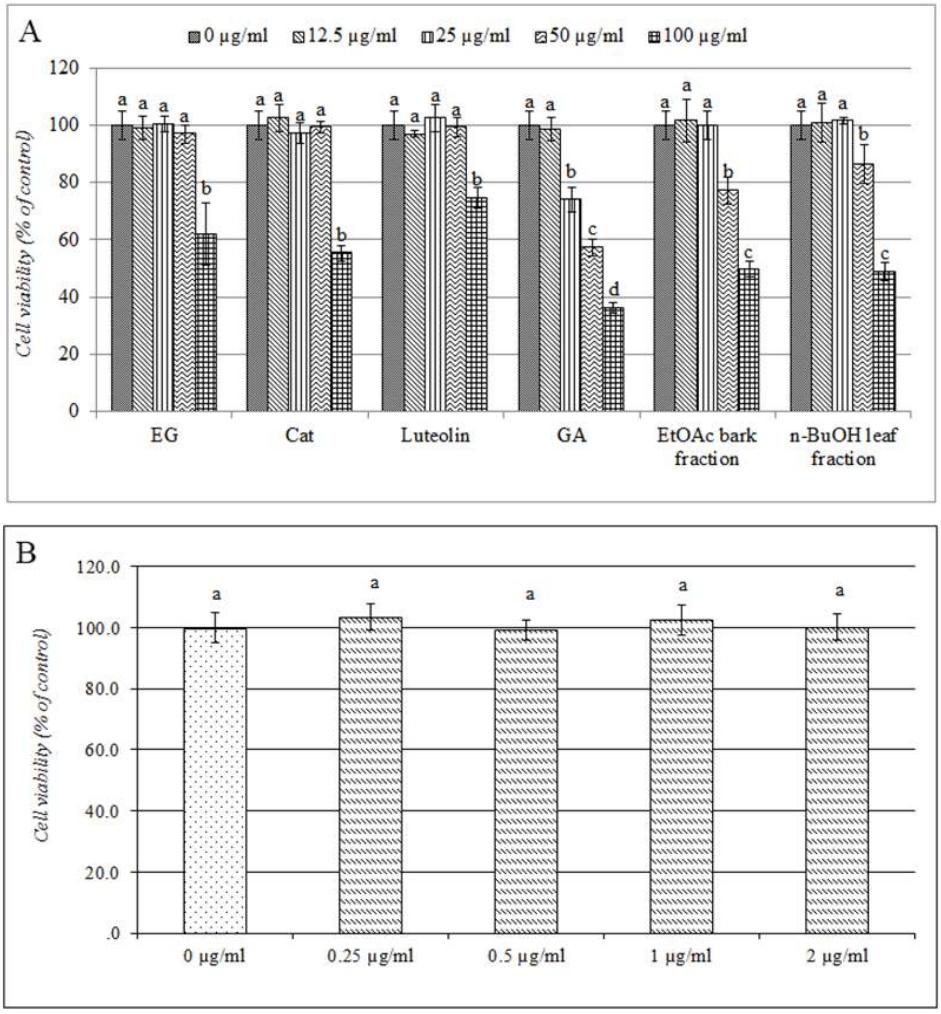
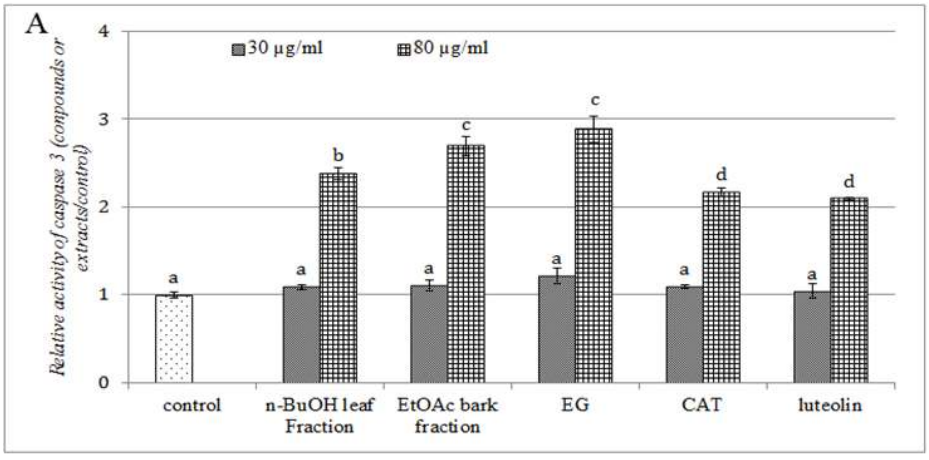


Fig. 3. Effect of EtOAc bark and n-BuOH leaf fractions of *T. nigrovenulosa* extracts, ethyl gallate (EG), catechin (Cat), luteolin, gallic acid (GA) (A) and ellagic acid (B) on viability of HT1080 cells using WST-8 kit

Results are means ± SD of triplicate measurements. Different labels (a-d) above the bars for the same extract or compound indicate a significant difference at P<0.05



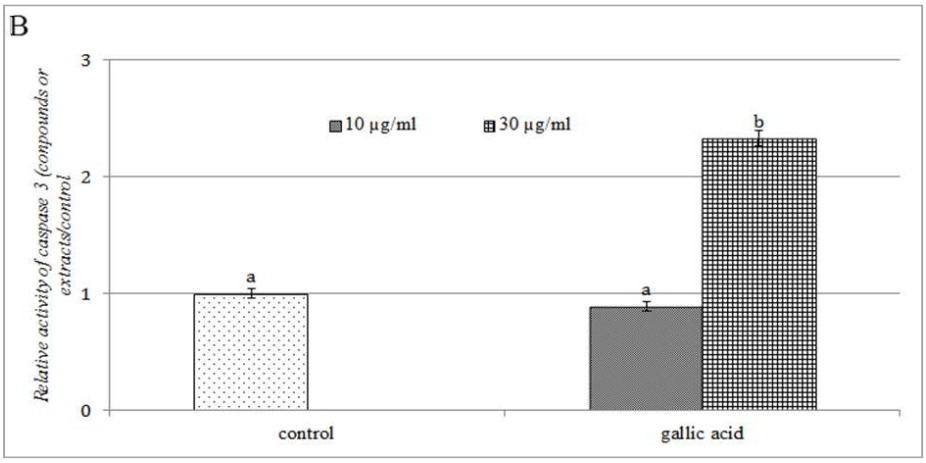


Fig. 4. Effect of EtOAc bark and n-BuOH leaf fractions, ethyl gallate, catechin and luteolin (A) and gallic acid (B) on activity of caspase-3 in HT1080 cells

Results are means ± SD of triplicate measurements. Different labels (a-d) above the bars indicate a significant difference at P<0.05

4. DISCUSSION

Several studies have repoted that the compounds isolated in *Terminalia* species were ellagic acid, luteolin, gallic acid, ethyl gallate, luteolin, tanic acid, catechins and ellagintannins [8,9,14]. The results of the present study demonstrated that the main compounds possessed DPPH radical scavenging activity were GA, EG, Cat, luteolin and ellagic acid concentrated in EtOAc bark and n-BuOH leaf fractions of *T. nigrovenulosa* extracts. These compounds have reported to possess high antioxidant activity [15,16,17,18]. Moreover, our data also showed that EtOAc bark and n-BuOH leaf fractions, GA, EG, Cat and luteolin exerted cytotoxicity to HT1080 cells in a type of fractions or compounds with dose dependent manner (Fig. 3A). As shown in Fig. 4, fractions and their isolated compouds increased caspase-3 activity depending on type of fractions or compounds. Therefore, the reduction of cell viability might be due to the induction of apoptosis via caspase-3 pathway. Previous reports have indicated that several *Terminalia* species induced cytotoxicity via apoptosis in several cancer cells. *T. chebula* retz fruit extract induced the cell death by apoptosis at low concentration and necrosic at high concentration [9]. Acetone extract of Triphala (fruits of *Terminalia bellerica, Terminalia chebula and Emblica officinalis*) induced S115, MCF-7, PC-3 and DU-145 cells death by apoptosis [19]. Moreover, phenolic compounds of plants including phenolic acids and flavonoids are well known as dietary antioxidants. They also exhibit the contrasting pharmacological effects such as prooxidant toxicity at high doses or present of metals ions [20,21] and inducing apoptosis [22,23]. For instance, gallic acid induced apoptosis in A549 cells via intrinsic pathway by caspase-3 induction [24] or in fibroblast cells via both intrinsic and extrinsic apoptotic pathways [25]. The cytotoxic, antioxidant and anticarcinogenic potential of gallic acid and its deviratives are believed due to their three adjacent hydroxyl groups [26]. Luteolin, a common flavonoid that exists in many types of plants including fruits, vegetables, and medicinal herbs, has reported to induce apoptosis in oral squamous cancer cells (OC2) via increasing caspase-3 and -9 [27]. Furthermore, luteolin increased levels of caspase-3 and the expression of the pro-apoptotic protein Bax but decreased the expression of the anti-apoptotic protein Bcl-2 in three human pancreatic carcinoma cell lines [28]. The hydroxyl moieties and 2−3 double bond in structure features of luteolin that are associated with its biochemical and biological activities [29]. Catechin, a natural flavonoid isolated from several plants, especially in tea, has been shown to exhibit cytostatic properties in many tumor cells [30,31]. Alshatwi [32] indicated that catechin hydrate supressed MCF-7 cell proliferation by induction of apoptosis via increasing the expression of caspase-3, -8, -9 and TP53. Therefore, gallic acid, ethyl gallate, catechin and luteolin isolated from EtOAc bark and n-BuOH leaf fractions could be responsible for the reduction of cell viability and the increase in the activity of caspase-3 in HT1080 cells.

5. CONCLUSIONS

Our study found that the antioxidative compounds concentrated in EtOAc bark and n-BuOH leaf fractions of *T. nigrovenulosa* methanol extracts. Gallic acid, ethyl gallate, ellagic acid, catechin and luteolin were the main antioxidative components isolated from these fractions. The fractions, gallic acid, ethyl gallate, catechin and luteolin could reduce the viability of human fibrosarcoma (HT1080) cells. The reduction of HT1080 cell viability might be due to the induction of apoptosis in HT1080 cells via caspase-3 pathway. Further investigation of anticancer effects on HT1080 cells should continue to figure out the relation between the inhibition of cell growth and apoptosis.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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