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Novel extracts from *Callyspongia siphonella* and *Negombata magnifica* sponges from the Red Sea, induced antiproliferative and proapoptotic activity in HepG-2, MCF-7, and Caco-2 cancer cell lines

Islam Rady[1,2]*, Mansour A.E. Bashar[1]

1. Zoology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt.
2. Masonic Cancer Center, University of Minnesota, Minnesota, Minnesota, USA.

*** Corresponding Author: islamrady@azhar.edu.eg**

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ABSTRACT

Marine organisms derived extracts have been shown to exert multiple anti-cancer activities. Two marine sponge species; finger-sponge "*Negombata magnifica* (*Nm*)" and tube-sponge "*Callyspongia siphonella* (*Cs*)" were collected during summer 2020 from the Gulf of Aqaba (Red Sea, Egypt). Each sponge macerated with methylene chloride (CH_2Cl_2), ethyl acetate ($C_4H_8O_2$), acetone (C_3H_6O), and chloroform ($CHCl_3$) separately into four different crude extracts for each sponge species and eight extracts as a total for both marine species, where each extract was *in vitro* assessed for its antiproliferative and proapoptotic activity in HepG-2, MCF7, and Caco-2 cancer cell lines. *Cs*-CH_2Cl_2, *Cs*-$C_4H_8O_2$, *Cs*-C_3H_6O, *Cs*-$CHCl_3$, *Nm*-CH_2Cl_2, *Nm*-$C_4H_8O_2$, *Nm*-C_3H_6O, and *Nm*-$CHCl_3$, each in a dose-dependent manner inhibited the growth of HepG2 cancer cells within IC_{50} values 17.53, 11.18, 9.97, 19.21, 9.14, 10.94, 8.78, and 7.23 µg/ml, respectively, MCF-7 cancer cells within IC_{50} values of 19.48, 15.34, 11.76, 13.62, 7.65, 6.18, 11.82, and 8.26 µg/ml, respectively, and Caco-2 cancer cells within IC_{50} values of 10.17, 14.87, 18.35, 17.12, 12.67, 9.27, 8.37, and 10.68 µg/ml, respectively. In addition, all extracts were found to induce apoptosis in HepG2, MCF-7, and Caco-2 cancer cells *via* an increase of proapoptotic protein Bax and caspase-3 and decrease of anti-apoptotic protein Bcl-2. Current data introducing multiple extracts from two marine sponges as promising sources for cancer therapeutic agent(s) to be further developed for cancer control outcome.

INTRODUCTION

Cancer is a group of diseases associated with abnormal cell growth with potentiality to invade or spread to other parts of the body **(Rady *et al*., 2018)**. It is considered as one of the major ailments threatening the humankind **(Rady *et al*., 2018a)**. The current available cancer statistics are indicating that the number of new cancer patients has been reached over 10 million cases associated with over 6 million deaths representing roughly 12% of worldwide mortality **(Rady *et al*., 2017, 2018a)**. Moreover, over 20 million new cancer cases are anticipated to be diagnosed in the year 2025 **(Zugazagoitia *et al*., 2016; Rady *et al*., 2017)** and potentially will be increased to 21.7 million with about 13 million cancer deaths by the year 2030 **(Torre *et al*., 2015; Rady *et***

***al*., 2017)**. In Egypt, according to national incidence rates and proportions, the crude incidence rates on the national level for all sites, excluding nonmelanoma skin cancer (C44) were 113.1/100,000 (both sexes), 115.7/100,000 (males), and 110.3/100,000 (females), where the age-standardized rates (world) were 166.6/100,000 (both sexes), 175.9/100,000 (males), and 157.0/100,000 (females) **(Ibrahim *et al*., 2014)**. Those high rates of cancer morbidity and mortality are required more ways of cancer control, therapy or management where the current treatment modalities are mainly radiation-based therapy and chemotherapy **(Rady *et al*., 2017, 2018)**, although their applicability to human is associated with toxic properties causing hair loss with some other serious adverse effects **(Tsuda *et al*., 2004)**. Natural products have been used to help mankind sustain its health since the dawn of medicine **(Moghadamtousi *et al*., 2015; Rady *et al*. 2017, 2018)**. Nowadays, just like in ancient times, utilization of complementary and alternative medicines is gaining more popularity as an important and promising strategy for human diseases prevention and therapy **(Peter and Horvath, 2017; Rady *et al*., 2018)**. Marine natural products are regarded as a rich and renewable source for novel therapeutics **(Jiménez, 2018)**. Marine derived bioactive compounds are attractive candidates for cancer therapy 50 years ago and in recent past there are like 3000 new marine compounds that have been assessed for their anticancer activity **(Young *et al*., 2006)**. Beside the anticancer effects of marine organism constituents, a lot of modern studies suggested that some bioactive compounds isolated from marine organisms have been shown to exhibit anti-microbial, anti-fungal, anti-inflammatory, and other pharmacological activities **(Venkateswara-Rao *et al*., 1998; Wali *et al*., 2019)**. Likewise, in clinical research, there are many marine anticancer bioactive compounds that have been isolated, characterized, identified, and preclinically assessed; now they are under clinical trials for human use **(Wali *et al*., 2019)**.

Marine fauna for instance sponges, echinoderms, mollusks, ascidians, and coral reefs are accounting for more than 90% of the total oceanic biomass where those organisms possess a variety of pharmacological bioactive compounds that can be used in drug discovery for cancer control **(Franscesco, 1997)**. Likewise, a variety of bioactive metabolites and compounds have isolated from marine organisms were found to exert antitumor, antimicrobial, and anti-inflammatory **(Shnit-Orland *et al*., 2008)**. **El-Gamal *et al.* (2004)** isolated five bioactive substances from CH_2Cl_2 extracts of Taiwan soft coral of *Nephthea armata*, which have showed significant cytotoxicity to human lung adenocarcinoma and human colon adenocarcinoma and mouse lymphocytic leukemia. In addition, the soft corals of the genus *Xenia* are rich in terpenoids and steroids since six sesquiterpenoids were isolated from CH_2Cl_2 extracts of the soft coral of *Xenia peurto-galera* that have founded to induce cytotoxicity against cancer cells **(Duh *et al*., 2002)**. Also, lemnalol is a bioactive antitumor agent has been isolated from the Japanese soft coral *Lemnalia* sp. **(Kikuchi *et al*., 1983)**. As part of research on the bioactive material from marine organisms, the Formosan soft coral *Cespitularia hypotentaculata* (family: Xeniidae) was studied because its CH_2Cl_2 extract showed significant cytotoxicity to human lung adenocarcinoma and human colon adenocarcinoma and mouse lymphocytic leukemia cells **(Duh *et al*., 2002a)**. Moreover, two bicyclic cembranolides have been isolated from *Sarcophyton* sp. were found to exert cytotoxicity towards MCF7 cancer cells **(Gross *et al*., 2004)**. Furthermore, among the groups of marine organisms, sponges are the most diverse and abundant, due to their soft bodies and sedentary lifestyles

(Moitinho-Silva *et al*., 2014). Marine sponges (Phylum: Porifera) are a large phylum belongs to the animal Kingdom and regarded as prolific factories for bioactive natural products **(Blunt *et al*., 2016, 2017, 2018)**. Therefore, sponges are suggested to provide a drug discovery for multiple diseases, such as cancer and viruses **(Faulkner, 2000; Perdicaris *et al*., 2013)**. Red Sea is rich with sponges such as that of genus *Negombata*, which produce latrunculins that are known for their antimicrobial and antiviral activity **(Eid *et al*., 2011)**. A variety of natural products from the marine sponges have been found to exhibit remarkable antitumor and anti-inflammatory activities **(Edrada *et al*., 2002)**. **Pawlik and McMurray (2020)** have also confirmed that marine sponges are containing variety of natural products than any other marine phylum and those products have bioactivities including anticancer, antimicrobial, and anti-inflammatory activities, and are often applicable for medical use. Moreover, some of the most potential sponge-derived bioactive molecules include the anti-inflammatory compound manoalide from the palauan sponge *Luffariella variabilis* **(De-Silva and Scheuer, 1980)**. Here, the current study assesses cytotoxicity, viability, and primary apoptotic effects of novel multiple extracts derived from two sponges *Callyspongia siphonella* and *Negombata magnifica* collected from Red Sea, Egypt in HepG2, MCF-7, and Caco-2 cancer cell lines.

MATERIALS AND METHODS

1- Sampling and identification of specimens

Specimens were collected during summer 2020 along Gulf of Aqaba at different depths by SCUBA diving. Immediately upon collection, the samples were cleaned with seawater and preserved in ice-box at -20°C. The taxonomy details were studied, and the two voucher specimens were deposited at Marine laboratory, Department of Zoology, Faculty of Science, Al-Azhar University, Cairo, Egypt, with a registration number of MZ1047 and MZ1048. The identification of the specimens has been carefully checked based on Porifera morphological characters according to Systema Porifera **(Hooper, 2000)** associated with the most recent update undertaken in the World Porifera Database **(Van Soest *et al*., 2008)** and at the end the taxonomy is directed by **Ruggiero *et al*. (2015)**.

2- Preparation of extracts

The frozen sponge specimens were left to defrost and then broken down into small pieces. 10 g of macerated tissues were extracted by soaking in 50 ml of different four absolute solvents of (CH_2Cl_2, $C_4H_8O_2$, C_3H_6O, and $CHCl_3$) for 24 h at room temperature. Extractions were repeated three times until no color was obtained to ensure complete extraction. The combined extracts were filtered through Whatman no.1 filter paper and dried at 40°C using a rotary evaporator.

3- Cell culture

HepG-2, MCF7, and Caco-2 cancer cell lines and HFB-4 cell line (normal control cells) were obtained from American Type Culture Collection (Manassas, VA, USA) and (VACSERA Co., Cairo, Egypt). HepG-2, MCF7 and Caco-2 cancer cells were cultured in DMEM obtained from Corning Thomas Scientific (Swedesboro, NJ, USA). DMSO was purchased from (Sigma-Aldrich, St. Louis, USA), while FBS was purchased from Hyclone (Pittsburgh, PA, USA) and PSA was obtained from Mediatech Inc. (Herndon, VA, USA). Cancer cells were cultured in DMEM supplemented with 5% heat inactivated

FBS and 1% PSA at 37°C in 5% CO_2 incubator **(Chamcheu *et al*., 2018)**. Similarly, HFB-4 cells were maintained in RPMI-1640 medium (Thermofisher Scientific Co., Waltham, MA, USA) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 **(Amin *et al*., 2012)**.

4- Measurement of Cytotoxicity by MTT assay

The cytotoxicity of each extract against HepG2, MCF-7, and Caco-2 cancer cells was measured by Cell Proliferation Kit I MTT (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer instructions. Briefly, cells were seeded in 96 well culture plates at 5000/well for 24 h. Then, cells were incubated with different concentrations of each extract (0, 12.5, 25, and 50 µg/ml) for 24 hours in 37 °C and humidified 5% CO_2 incubator. Treated and untreated cells were incubated with MTT powder in the detection day for 2 hours after until violet crystals were formed at different color hue indicating cell metabolic activity. Colorimetric absorbance was measured at 620 nm (A620) and 570 nm (A570) by Synergy™ 2 Multi-Mode Microplate Reader (BioTek Inc., Vermont, USA). The cell viability was calculated as previously reported by **Cho *et al*., (2015)** using the following equation:

$$\text{Percentage of viability} = \frac{\text{A570} - \text{A620 of UA}}{\text{A570} - \text{A620 of the Control (0µM)}} \text{X } 100$$

The IC_{50} of each extract was calculated by the IC_{50} calculator (AAT Bioquest, Inc., CA, USA) as had been previously described by **Luparello *et al*., (2019)**. Treatment and analysis protocols were carried out at least three times.

5- Determination of SI

SI indicates the cytotoxic selectivity (i.e. Safety) of the crude extract against cancer cells versus normal control cells **(Prayong *et al*., 2008)**. SI= IC_{50} of the extract in the normal control cells / IC_{50} of the same extract in the cancer cell line **(Bézivin *et al*., 2003)**.

6- Determination of the active Caspase-3

Caspase-3 level was measured by using the Invitrogen Caspase-3 (active) Human kit from (Thermofisher Scientific Co., Waltham, MA, USA). Briefly, after washing the cells with PBS, they collected and lysed by adding the extraction buffer containing 1 mM PMSF (stock is 0.3 M in DMSO) and Protease inhibitor cocktail, e.g., Sigma-Aldrich Cat. # P-2714 (St. Louis, USA). Later, 500 µl per 5 ml cell extraction buffer-protease inhibitors (1 mL per 1 x 107 cells) were added. Next, the lysate was diluted immediately prior to the assay. At the end, OD of each well was determined within 30 minutes using a microplate reader AC3000 (Azure Biosystems, Inc, CA , USA) set at 450 nm.

7- Determination of Bax and Bcl-2

Cancer cells were grown in DMEM containing 5% FBS at 37°C and after treatment with present marine extracts, cancer cells suspension was tested for Bax and Bcl-2 using lysed cell extraction. This cell lysate was diluted in standard diluent buffer over the range of the assay and measured for human active Bax and Bcl-**2** content using Bax ELISA (EIA-4487) kit (DRG Instruments, Ma, Germany) and Zymed Bcl-2 ELISA Kit (Thermofisher Scientific Co., Waltham, MA, USA).

RESULTS

1- Specimens identification

The collected sponges were identified according to **Hooper (2000)**, **Van Soest *et al.* (2008)** and **Ruggiero *et al.* (2015)** as two marine sponge species; tube-sponge "*Callyspongia siphonella*" and finger-sponge "*Negombata magnifica*". Both sponge species were classified as follows:

Superkingdom: Eukaryota Chatton, 1925
Kingdom: Animalia (Metazoa) Linnaeus, 1758
Subkingdom: Eumetazoa Buetschli, 1910
Phylum: Porifera Grant, 1836
Class: Demospongiae Sollas, 1885
Subclass: Heteroscleromorpha Cárdenas, Pérez & Boury-Esnault, 2012
Order: Haplosclerida Topsent, 1928
Family: Callyspongiidae de Laubenfels, 1936
Genus: *Callyspongia* Duchassaing & Michelotti, 1864
Species$_1$: *Callyspongia siphonella* Lévi, 1965
Order: Poecilosclerida Topsent, 1928
Family: Podospongiidae de Laubenfels, 1936
Genus: *Negombata* de Laubenfels, 1936
Species$_2$: *Negombata magnifica* Keller, 1889

Both sponges were collected exactly from Dahap area, Red Sea but differ in depth range where *Callyspongia siphonella* was between 10 - 37m depth range and *Negombata magnifica* was at 7 - 14 m. *Callyspongia siphonella* was like tube forming clusters of vertical tubes from a common base. Those tubes can be reach 60cm in height and have a smooth consistency due to the absence of spicules. Color of *Callyspongia siphonella* is usually pale purple or pink or reddish-brown. It lives on hard surfaces and shelters towards reef slopes. *Negombata magnifica* is Reddish-brown color and narrow crooked branched sponge. It is live and grows between the shallow coral reefs and rocks.

2- *Cs* and *Nm* derived extracts, each inhibited cell proliferation, Viability of HePG2, MCF-7, and Caco-2 cancer cells

All extracts inhibited cancer cells growth (HepG2, MCF-7, and Caco-2 cancer cell lines) in dose-dependent manner with remarkable cell viability decrease associated with cytotoxicity increase **(Figures 1-12)**. The IC_{50} values between all extracts are different in range between 6.18— 19.48 μg/ml. The IC_{50} ranges for all extracts on HepG-2, MCF-7, and Caco-2 cancer cells were 7.23 — 19.21 μg/ml, 6.1 8 — 19.48 μg/ml and 8.37 — 18.35 μg/ml, respectively. Therefore, among all extracts, the lowest IC_{50} was for *Nm*-$C_4H_8O_2$ on MCF-7 cancer cells within a value of 6.18 μg/ml and the highest IC_{50} was for the *Cs*-CH_2Cl_2 on the same cancer cell line within a value of 19.48 μg/ml. Similarly, in HepG2 cancer cells, *Nm*-$CHCl_3$ inhibited proliferation within the lowest IC_{50} value of 7.23 μg/ml, while the highest IC_{50} was for *Cs*-$CHCl_3$ within a value of 19.21 μg/ml for its proliferation inhibition on HepG-2 cancer cells, whereas, in MCF-7 cancer cells, *Nm*-$C_4H_8O_2$ obtained the lowest IC_{50} value of 6.18 μg/ml and *Cs*-CH_2Cl_2 existed the highest IC_{50} value of 19.48 μg/ml and in Caco-2 cancer cells, *Nm*-C_3H_6O obtained the lowest IC_{50} value of 8.37 μg/ml and *Cs*-C_3H_6O exerted the highest IC_{50} value of 18.35 μg/ml.

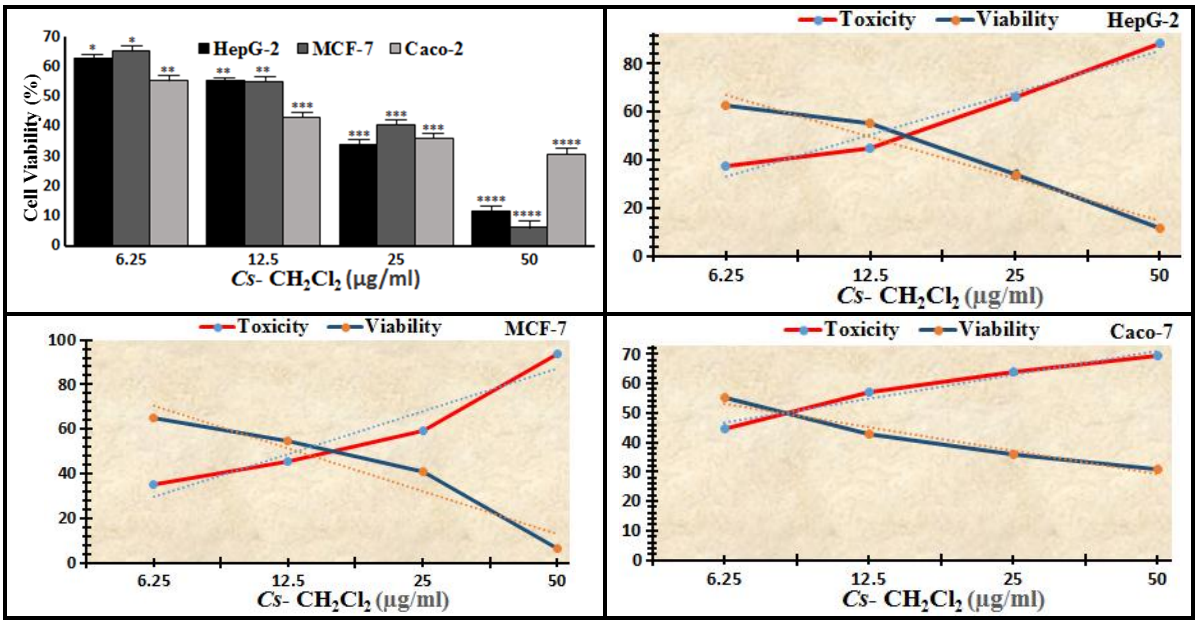

Figure (1): Effects of *Cs*-CH_2Cl_2 on HepG2, MCF-7, and Caco-2 on cell toxicity and viability after 24 h**.** Cells were incubated with the indicated concentrations of *Cs*-CH_2Cl_2, while the percentage of cytotoxicity and viability were determined by MTT. Values used for plotting are means of experiments performed three times, with each concentration tested in 7–8 wells. Statistical differences from control cultures are shown as bar graphs with error bars representing the means ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ *vs*. control cells.

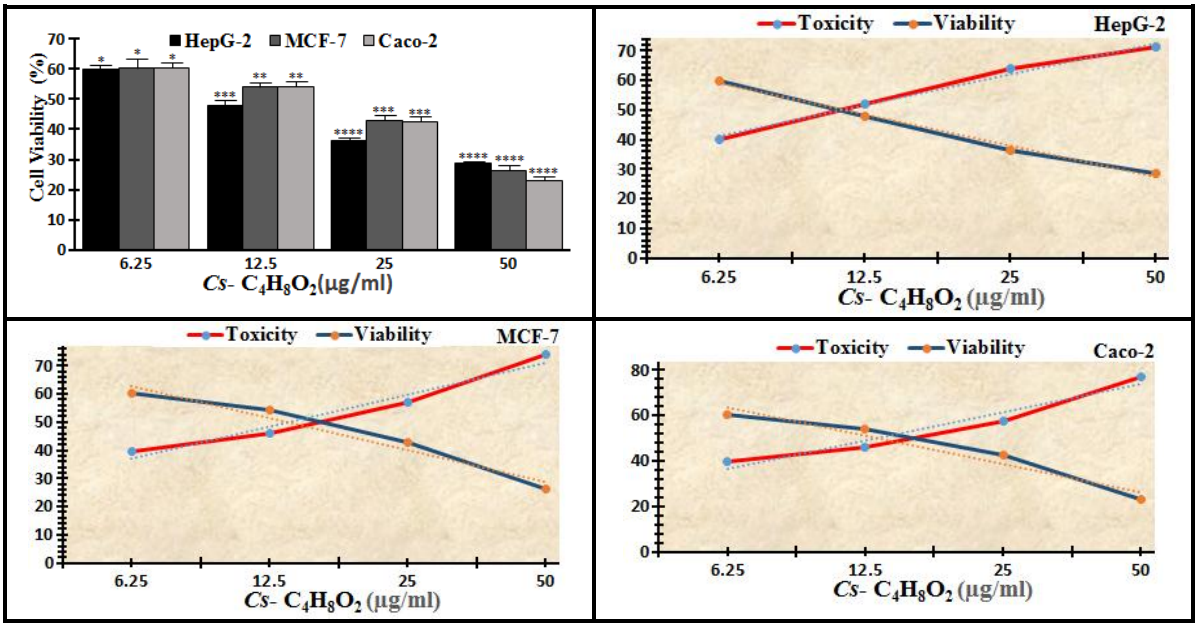

Figure (2): Effects of *Cs*-$C_4H_8O_2$ on HepG2, MCF-7, and Caco-2 on cell toxicity and viability after 24 h. Cells were incubated with the indicated concentrations of *Cs*-$C_4H_8O_2$ while the percentage of cytotoxicity and viability were determined by MTT. Values used for plotting are means of experiments performed three times, with each concentration tested in 7–8 wells. Statistical differences from control cultures are shown as bar graphs with error bars representing the means ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ *vs*. control cells.

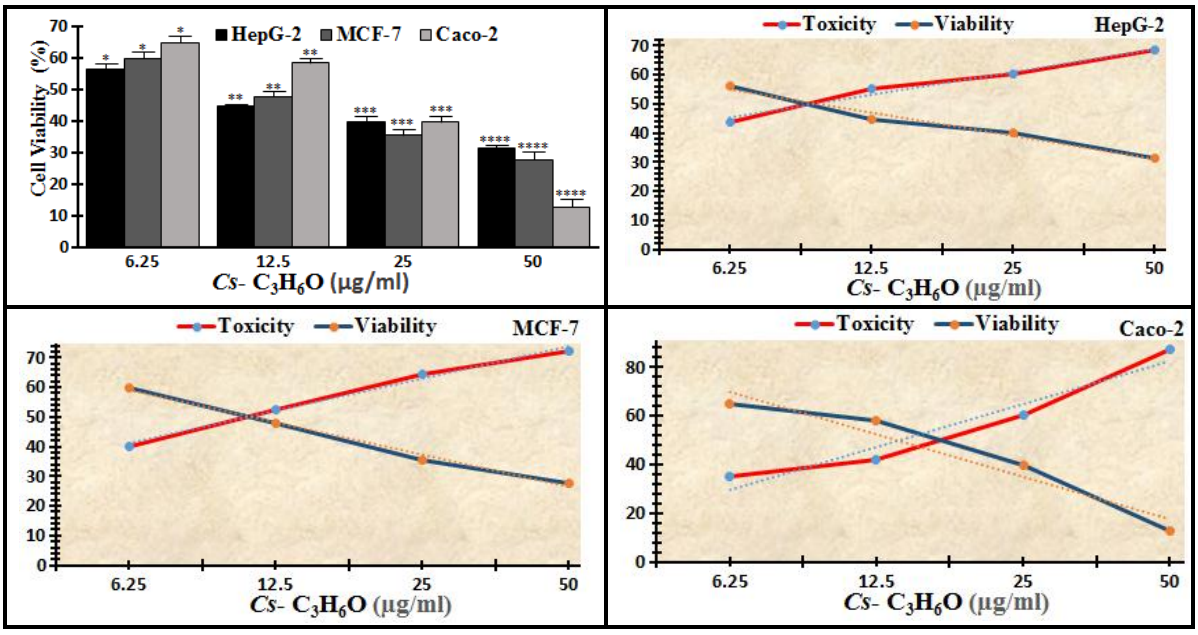

Figure (3): Effects of *Cs*-C_3H_6O on HepG2, MCF-7, and Caco-2 on cell toxicity and viability after 24 h. Cells were incubated with the indicated concentrations of *Cs*-C_3H_6O while the percentage of cytotoxicity and viability were determined by MTT. Values used for plotting are means of experiments performed three times, with each concentration tested in 7–8 wells. Statistical differences from control cultures are shown as bar graphs with error bars representing the means ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ *vs*. control cells.

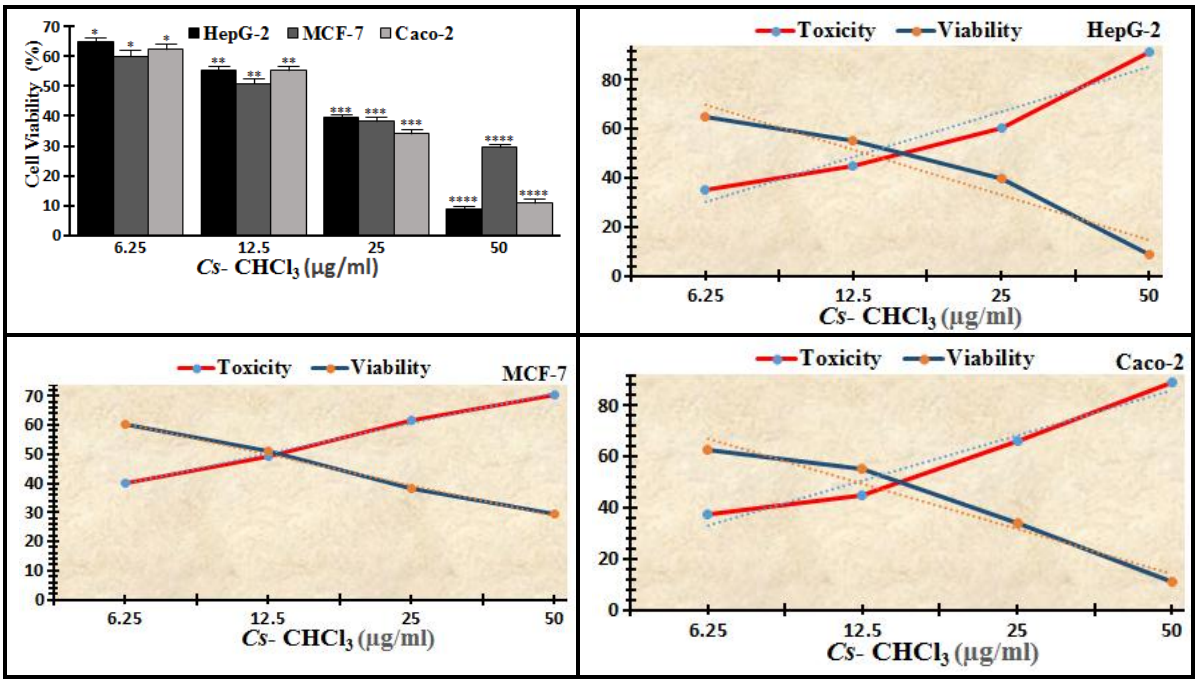

Figure (4): Effects of *Cs*-$CHCl_3$ on HepG2, MCF-7, and Caco-2 on cell toxicity and viability after 24 h. Cells were incubated with the indicated concentrations of *Cs*-$CHCl_3$ while the percentage of cytotoxicity and viability were determined by MTT. Values used for plotting are means of experiments performed three times, with each concentration tested in 7–8 wells. Statistical differences from control cultures are shown as bar graphs with error bars representing the means ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ *vs*. control cells.

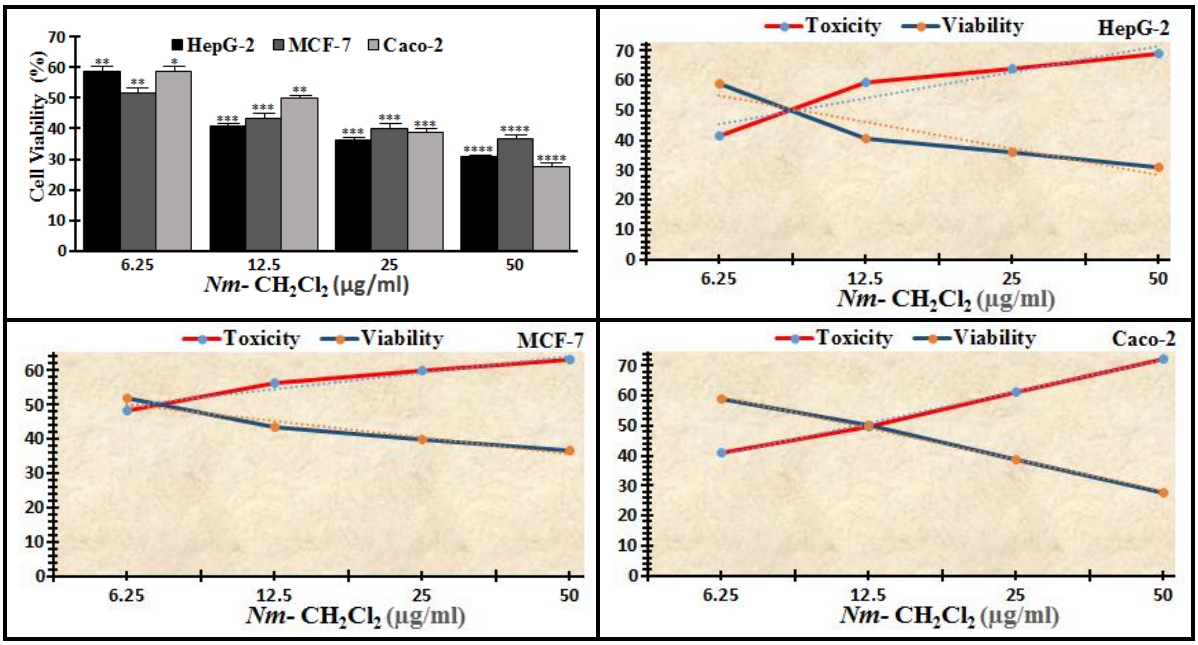

Figure (5): Effects of *Nm*-CH_2Cl_2 on HepG2, MCF-7, and Caco-2 on cell toxicity and viability after 24 h. Cells were incubated with the indicated concentrations of *Nm*-CH_2Cl_2 while the percentage of cytotoxicity and viability were determined by MTT. Values used for plotting are means of experiments performed three times, with each concentration tested in 7–8 wells. Statistical differences from control cultures are shown as bar graphs with error bars representing the means ± SD; * p < 0.05, ** *p* < 0.01, *** *p* < 0.001 and **** *p* <0.0001 *vs*. control cells.

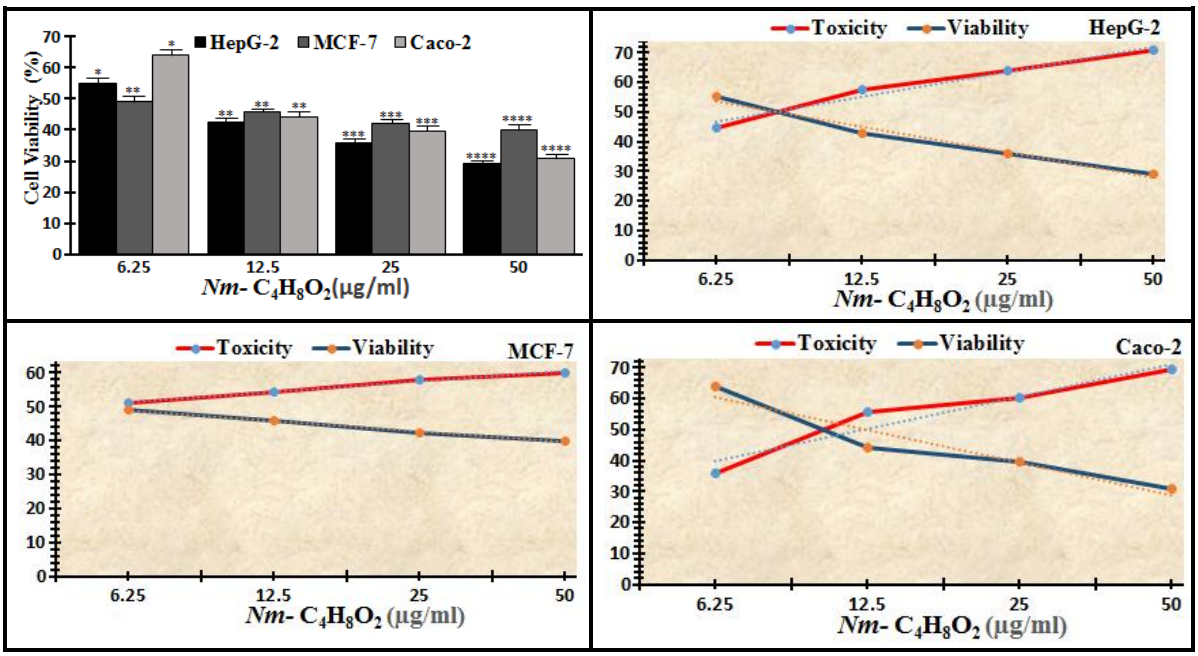

Figure (6): Effects of *Nm*-$C_4H_8O_2$ on HepG2, MCF-7, and Caco-2 on cell toxicity and viability after 24 h. Cells were incubated with the indicated concentrations of *Nm*-$C_4H_8O_2$ while the percentage of cytotoxicity and viability were determined by MTT. Values used for plotting are means of experiments performed three times, with each concentration tested in 7–8 wells. Statistical differences from control cultures are shown as bar graphs with error bars representing the means ± SD; * p < 0.05, ** *p* < 0.01, *** *p* < 0.001 and **** *p* <0.0001 *vs*. control cells.

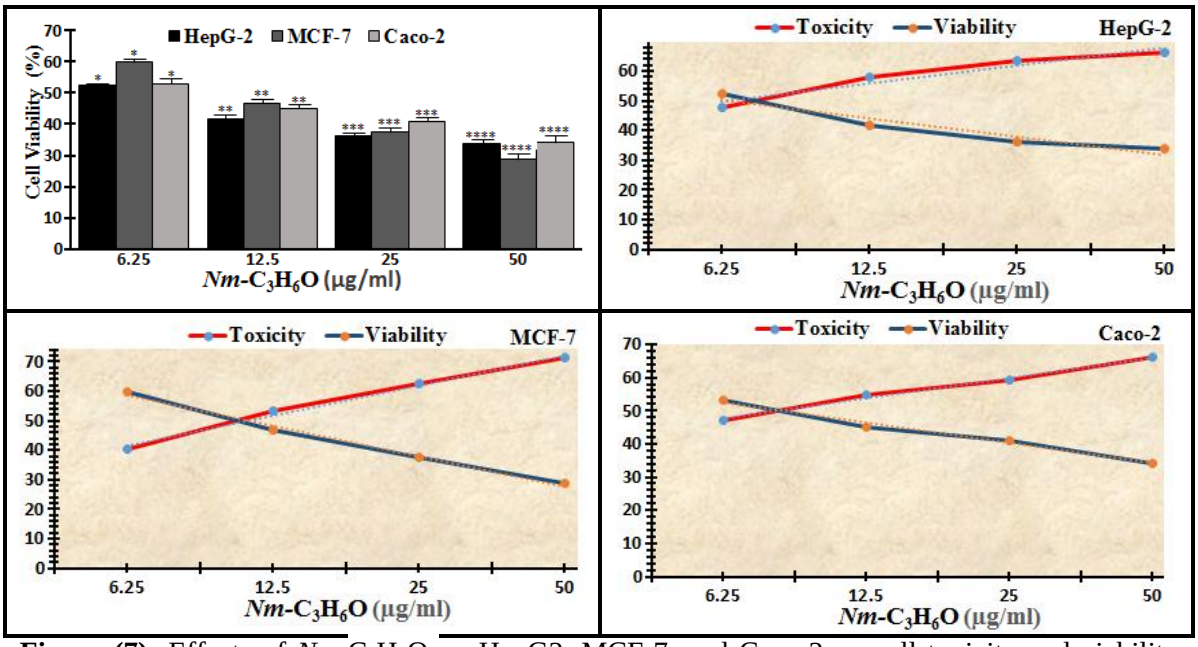

Figure (7): Effects of *Nm*-C_3H_6O on HepG2, MCF-7, and Caco-2 on cell toxicity and viability after 24 h. Cells were incubated with the indicated concentrations of *Nm*-C_3H_6O while the percentage of cytotoxicity and viability were determined by MTT. Values used for plotting are means of experiments performed three times, with each concentration tested in 7–8 wells. Statistical differences from control cultures are shown as bar graphs with error bars representing the means ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ *vs*. control cells.

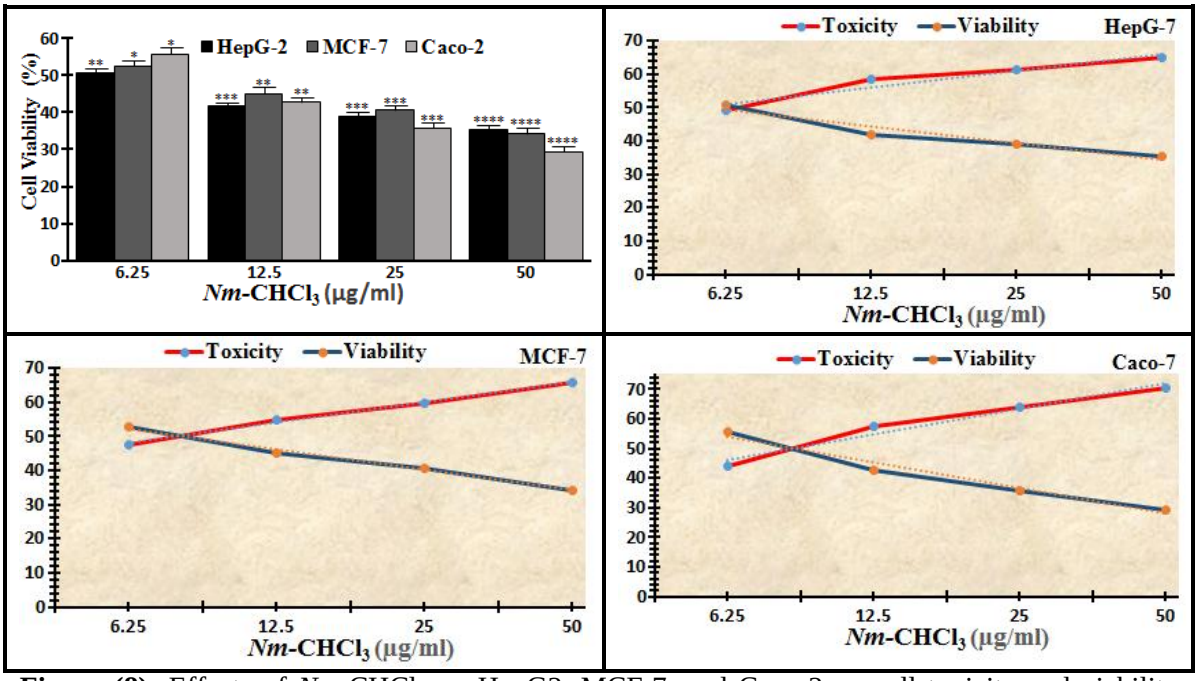

Figure (8): Effects of *Nm*-$CHCl_3$ on HepG2, MCF-7, and Caco-2 on cell toxicity and viability after 24 h. Cells were incubated with the indicated concentrations of *Nm*-$CHCl_3$ while the percentage of cytotoxicity and viability were determined by MTT. Values used for plotting are means of experiments performed three times, with each concentration tested in 7–8 wells. Statistical differences from control cultures are shown as bar graphs with error bars representing the means ± SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ *vs*. control cells.

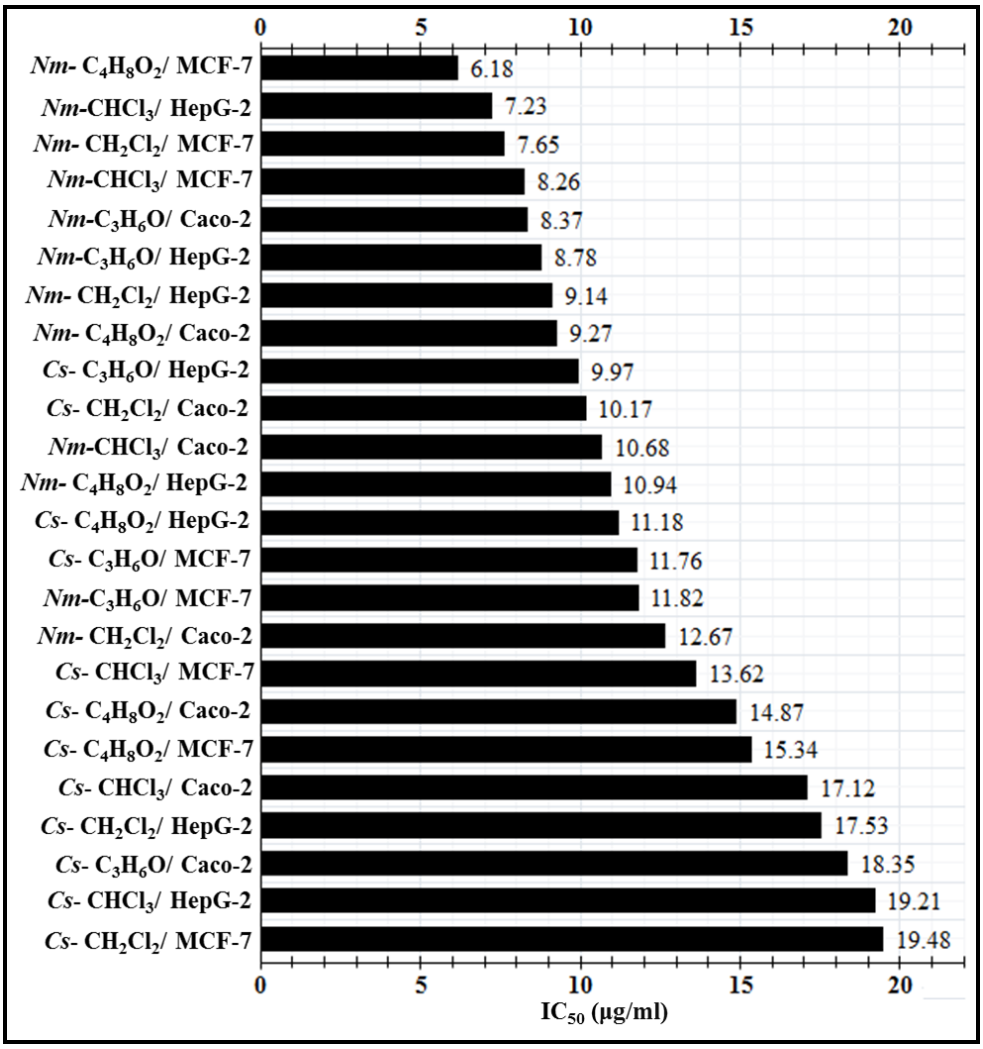

Figure (9): Comparative IC_{50} for all current investigated extracts on HepG-2, MCF-7, and Caco-2 cancer cells. IC_{50} values are shown as bar graphs.

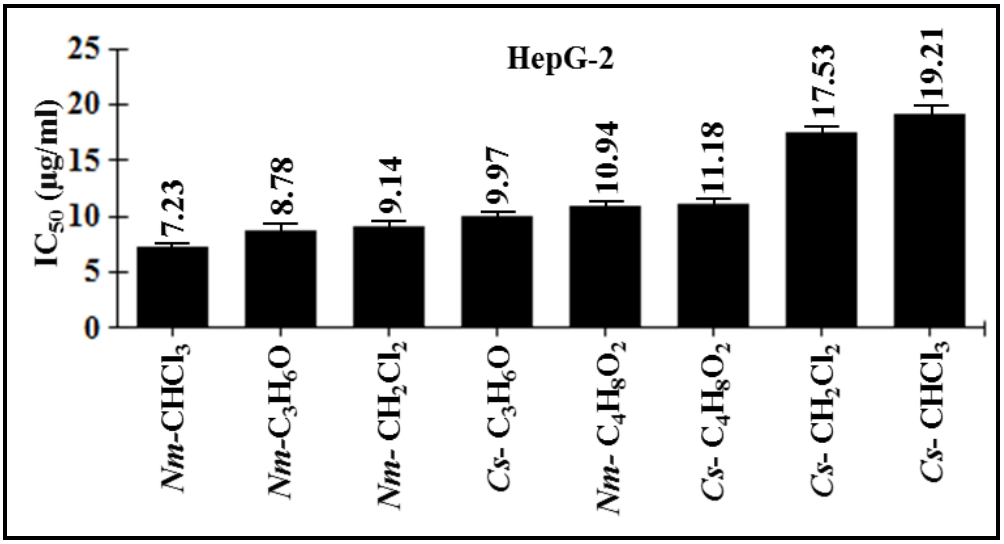

Figure (10): Comparative IC_{50} for all current investigated extracts on HepG-2 cancer cells. IC_{50} values are shown as bar graphs with error bars representing the means ± SD.

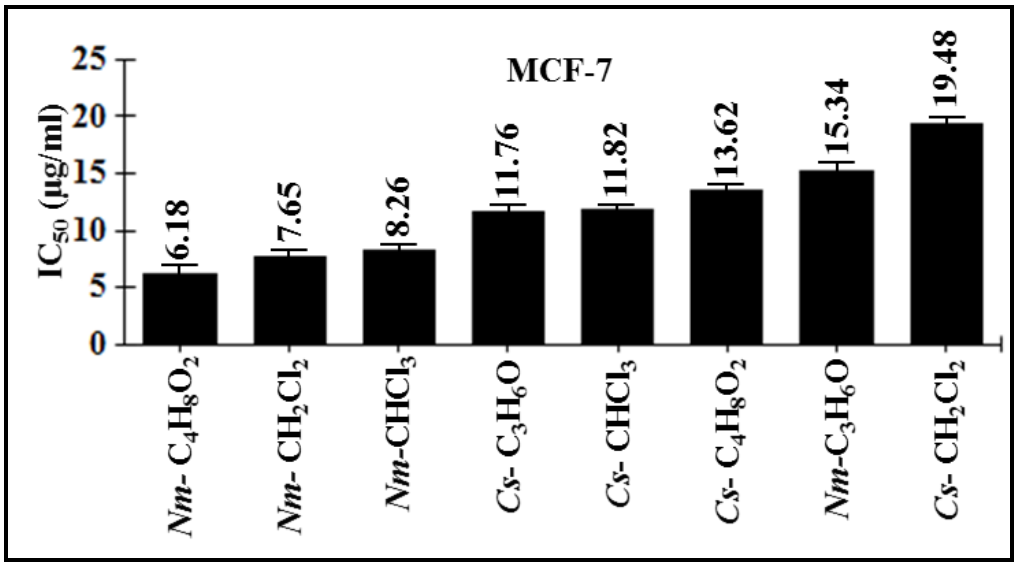

Figure (11): Comparative IC_{50} for all current investigated extracts on MCF-7 cancer cells. IC_{50} values are shown as bar graphs with error bars representing the means ± SD.

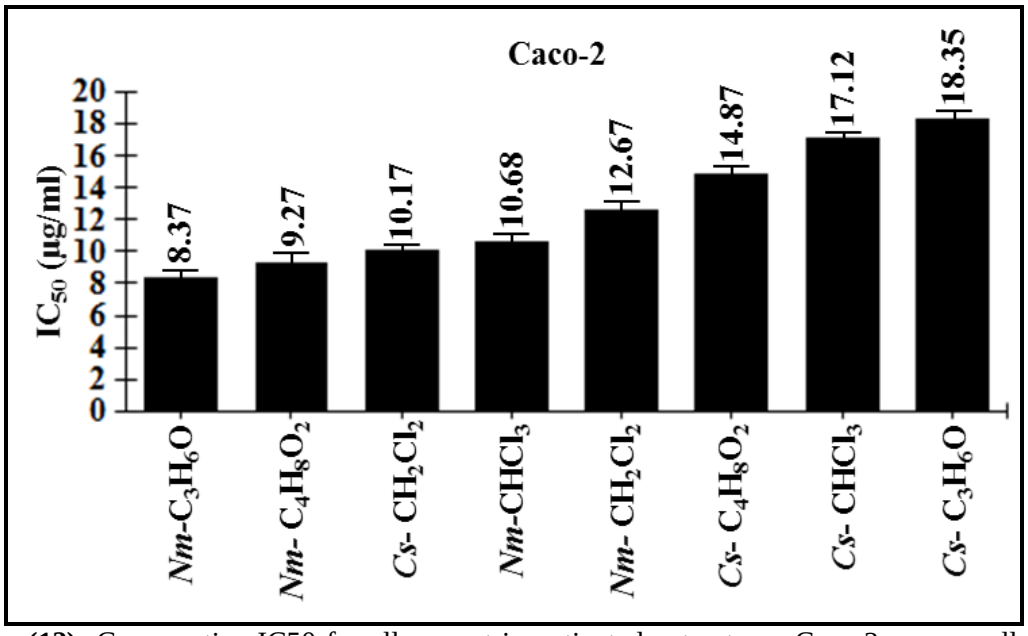

Figure (12): Comparative IC50 for all current investigated extracts on Caco-2 cancer cells. IC_{50} values are shown as bar graphs with error bars representing the means ± SD.

Likewise, the SI results were synergistically matched extremely the same previously mentioned arrangements in HepG2, MCF-7, and Caco-2 cancer cells **(Figures 13-16)**. In addition, SI between all extracts are different in range between 2.23— 4.56. The SI ranges for all extracts on HepG-2, MCF-7 and Caco-2 cancer cells were 2.39 — 4.48, 2.23 — 4.56 and 2.47 — 4.16, respectively. Therefore, among all extracts, the highest SI was for *Nm*-$C_4H_8O_2$ on MCF-7 cancer cells within a value of 4.56 and the highest SI was for the *Cs*-CH_2Cl_2 on the same cancer cell line within a value of 2.23. Similarly, in HepG2 cancer cells, *Nm*-$CHCl_3$ inhibited proliferation within the highest SI value of 4.48, while the lowest SI was for *Cs*-$CHCl_3$ within a value of 2.39 for its

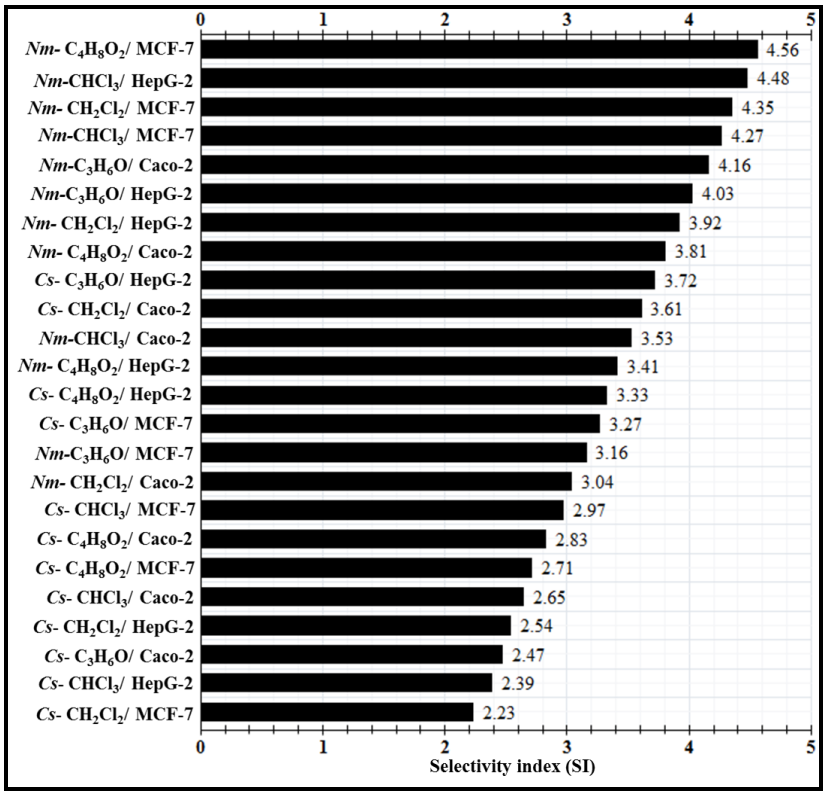

Figure (13): Comparative SI for all current investigated extracts on HepG2, MCF-7, and Caco-2 cancer cells.

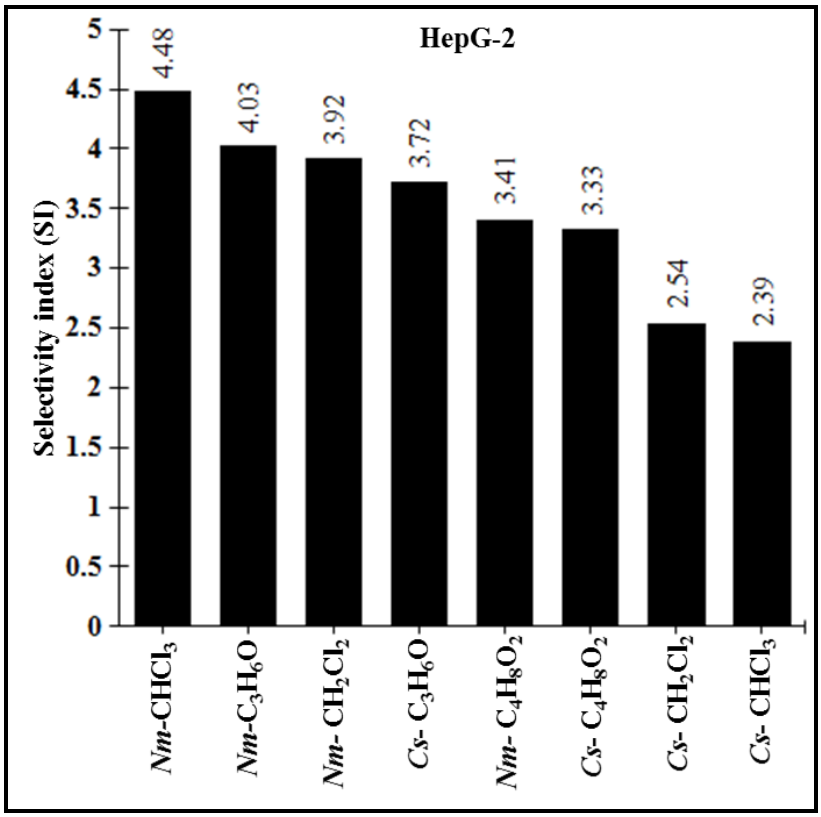

Figure (14): Comparative SI for all current investigated extracts on HepG2 cancer cells.

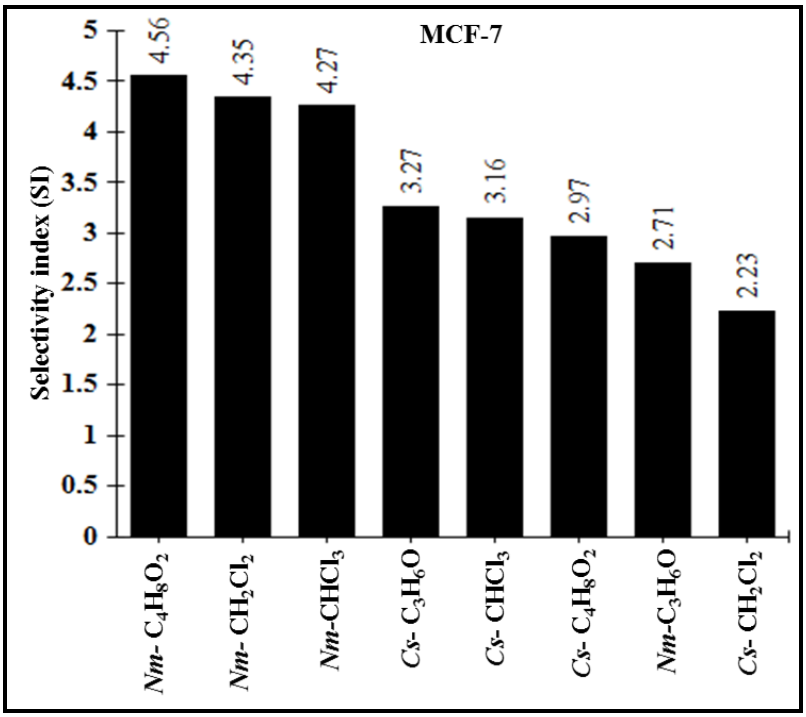

Figure (15): Comparative SI for all current investigated extracts on MCF-7 cancer cells.

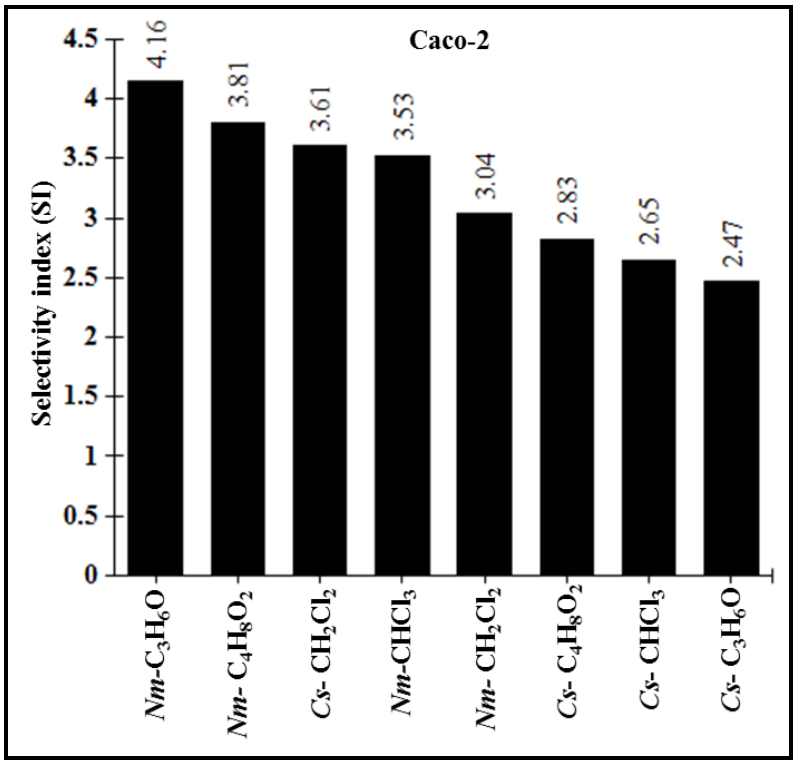

Figure (16): Comparative SI for all current investigated extracts on Caco-2 cancer cells.

proliferation inhibition on HepG-2 cancer cells, whereas, in MCF-7 cancer cells, *Nm*-$C_4H_8O_2$ obtained the highest SI value of 4.56 and *Cs*-CH_2Cl_2 existed the lowest SI value of 2.23 and in Caco-2 cancer cells, *Nm*-C_3H_6O obtained the highest SI value of 4.16 and *Cs*-C_3H_6O exerted the lowest SI value of 2.47.

3- *Cs* and *Nm* derived extracts, each induced apoptosis and increased Bax and caspase-3 expressions in cancer cells

All extracts caused apoptotic effect through the expression of some regulatory proteins. Each extract increased the level of the pro-apoptotic protein Bax and caspase-3, while it decreased the level of the anti-apoptotic protein Bcl2 in HePG2, MCF-7, and Caco-2 cancer cell lines **(Figures 17-20)**. The Bax values between all extracts are different in range between 61.95 — 455.81 ng/ml. The Bax ranges for all extracts on HepG-2, MCF-7 and Caco-2 cancer cells were 83.26 — 436.35 ng/ml, 61.95 — 455.81ng/ml and 95.47 — 384.63 ng/ml, respectively. Therefore, among all extracts, the lowest Bax expression was for *Cs*-CH_2Cl_2 on MCF-7 cancer cells within a value of 61.95 ng/ml, and the highest Bax expression was for the *Nm*-$C_4H_8O_2$ on the same cancer cell line within a value of 455.81 ng/ml. Similarly, in HepG2 cancer cells, *Nm*-$CHCl_3$ induced cellular apoptosis within the highest Bax value of 436.35 ng/ml **(Figure 21)**, while the lowest Bax expression was for *Cs*-$CHCl_3$ within a value of 83.26 ng/ml for its apoptosis on HepG-2 cancer cells, whereas, in MCF-7 cancer cells, *Nm*-$C_4H_8O_2$ obtained the highest Bax value of 455.81 ng/ml **(Figure 22)** and *Cs*-CH_2Cl_2 existed the lowest Bax value of 61.95 ng/ml and in Caco-2 cancer cells, *Nm*-C_3H_6O obtained the highest Bax value of 384.63 ng/ml **(Figure 23)** and *Cs*-C_3H_6O exerted the lowest Bax value of 95.47 ng/ml. The Bcl-2 values between all extracts are different in range between 2.47 — 8.81 ng/ml. The Bcl-2 ranges for all extracts on HepG-2, MCF-7 and Caco-2 cancer cells were 2.54 — 8.51 ng/ml, 2.47 — 8.81 ng/ml and 3.58 — 8.24 ng/ml, respectively. Therefore, among all extracts, the highest Bcl-2 expression was for *Cs*-CH_2Cl_2 on MCF-7 cancer cells within a value of 8.81 ng/ml and the lowest Bcl-2 expression was for the *Nm*-$C_4H_8O_2$ on the same cancer cell line within a value of 2.47 ng/ml. Similarly, in HepG2 cancer cells, *Nm*-$CHCl_3$ induced cellular apoptosis within the lowest Bcl-2 value of 2.54 ng/ml, while the highest Bcl-2 expression was for *Cs*-$CHCl_3$ within a value of 8.51 ng/ml for its apoptosis on HepG-2 cancer cells, whereas, in MCF-7 cancer cells, *Nm*-$C_4H_8O_2$ obtained the lowest Bcl-2 value of 2.47 ng/ml and *Cs*-CH_2Cl_2 existed the highest Bcl-2 value of 8.81 ng/ml and in Caco-2 cancer cells, *Nm*-C_3H_6O obtained the lowest Bcl-2 value of 3.58 ng/ml and *Cs*-C_3H_6O exerted the highest Bcl-2 value of 8.24 ng/ml. The Caspace-3 values between all extracts are different in range between 98.04 — 518.34 ng/ml. The Caspace-3 ranges for all extracts on HepG-2, MCF-7 and Caco-2 cancer cells were 116.45 — 500.08 ng/ml, 98.04 — 518.34 ng/ml and 133.58 — 445.24 ng/ml, respectively. Therefore, among all extracts, the lowest Caspace-3 expression was for *Cs*-CH_2Cl_2 on MCF-7 cancer cells within a value of 98.04 ng/ml and the highest Caspace-3 expression was for the *Nm*-$C_4H_8O_2$ on the same cancer cell line within a value of 518.34 ng/ml. Similarly, in HepG2 cancer cells, *Nm*-$CHCl_3$ induced cellular apoptosis within the highest Caspace-3 value of 500.08 ng/ml, while the lowest Caspace-3 expression was for *Cs*-$CHCl_3$ within a value of 116.45 ng/ml for its apoptosis on HepG-2 cancer cells, whereas, in MCF-7 cancer cells, *Nm*-$C_4H_8O_2$ obtained the highest Caspace-3 value of 518.34 ng/ml and *Cs*-CH_2Cl_2 existed the lowest Caspace-3 value of 98.04 ng/ml and in Caco-2 cancer cells, *Nm*-C_3H_6O obtained the highest Caspace-3 value of 445.24 ng/ml and *Cs*-C_3H_6O exerted the lowest Caspace-3 value of 133.58 ng/ml.

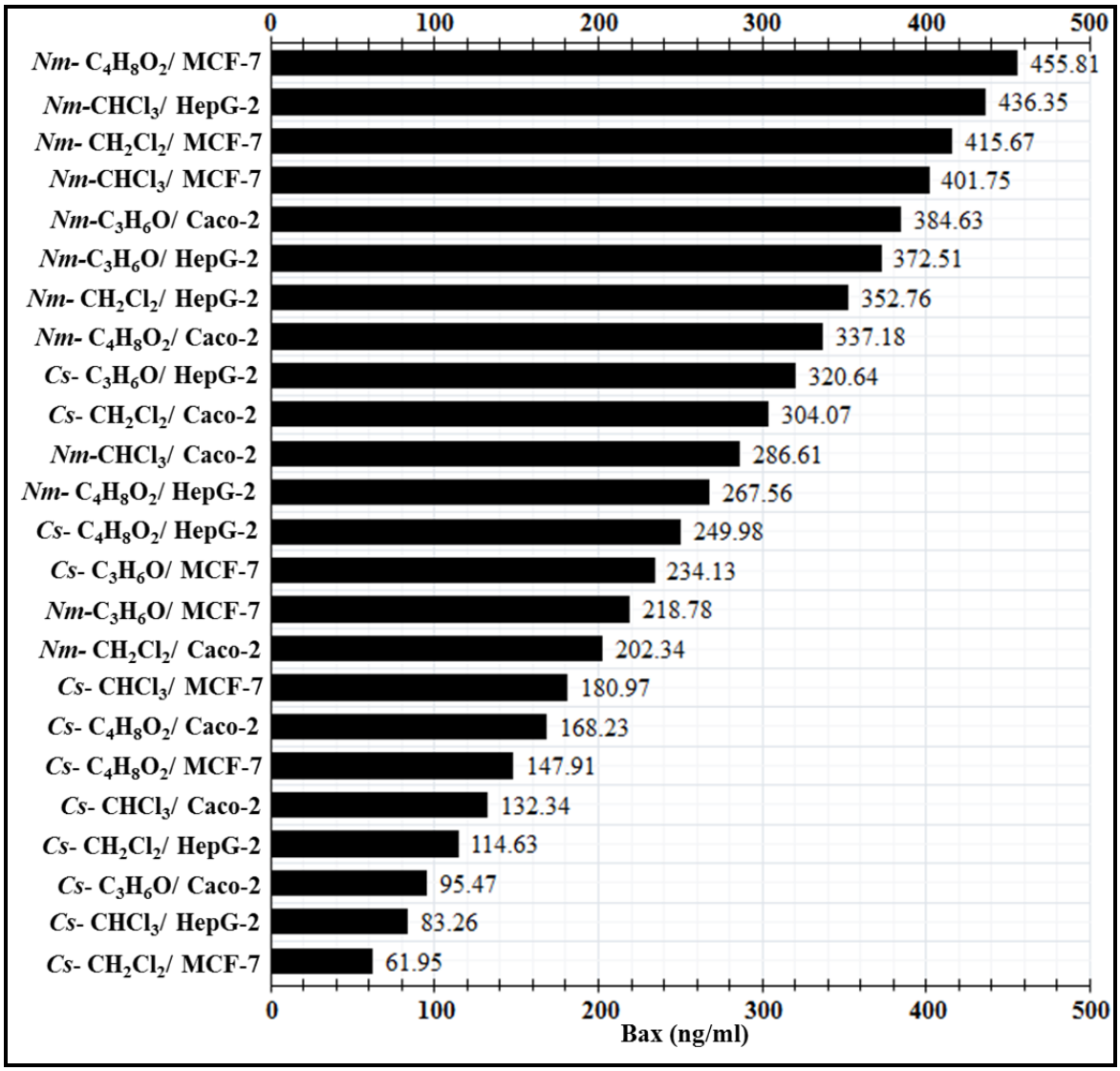

Figure (17): Comparative modulation of Bax protein expressions in HepG-2, MCF-7 and Caco-2 cells were treated with current studied extracts and harvested 24 h after treatments.

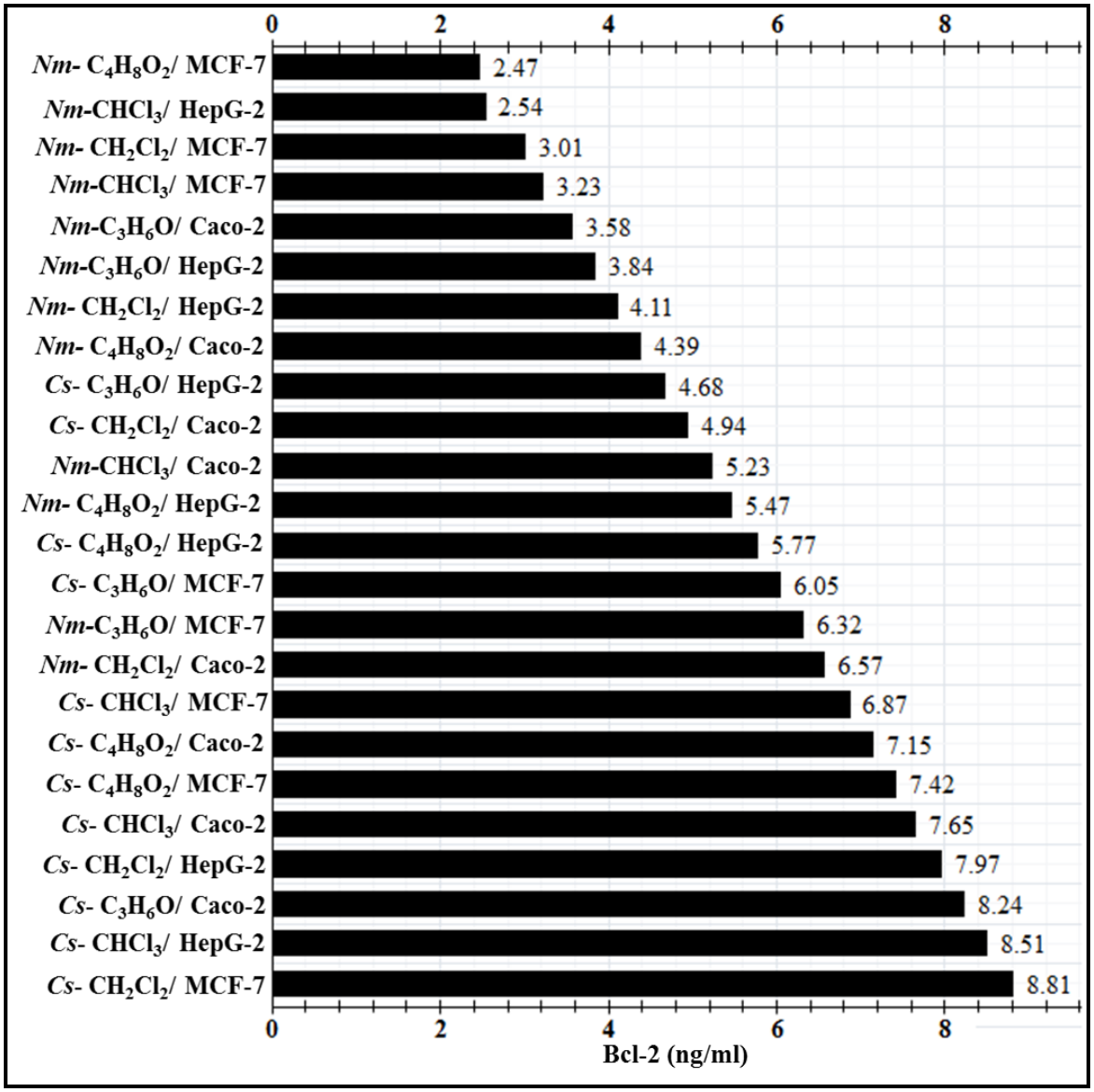

Figure (18): Comparative modulation of Bcl-2 protein expressions in HepG-2, MCF-7 and Caco-2 cells were treated with current studied extracts and harvested 24 h after treatments.

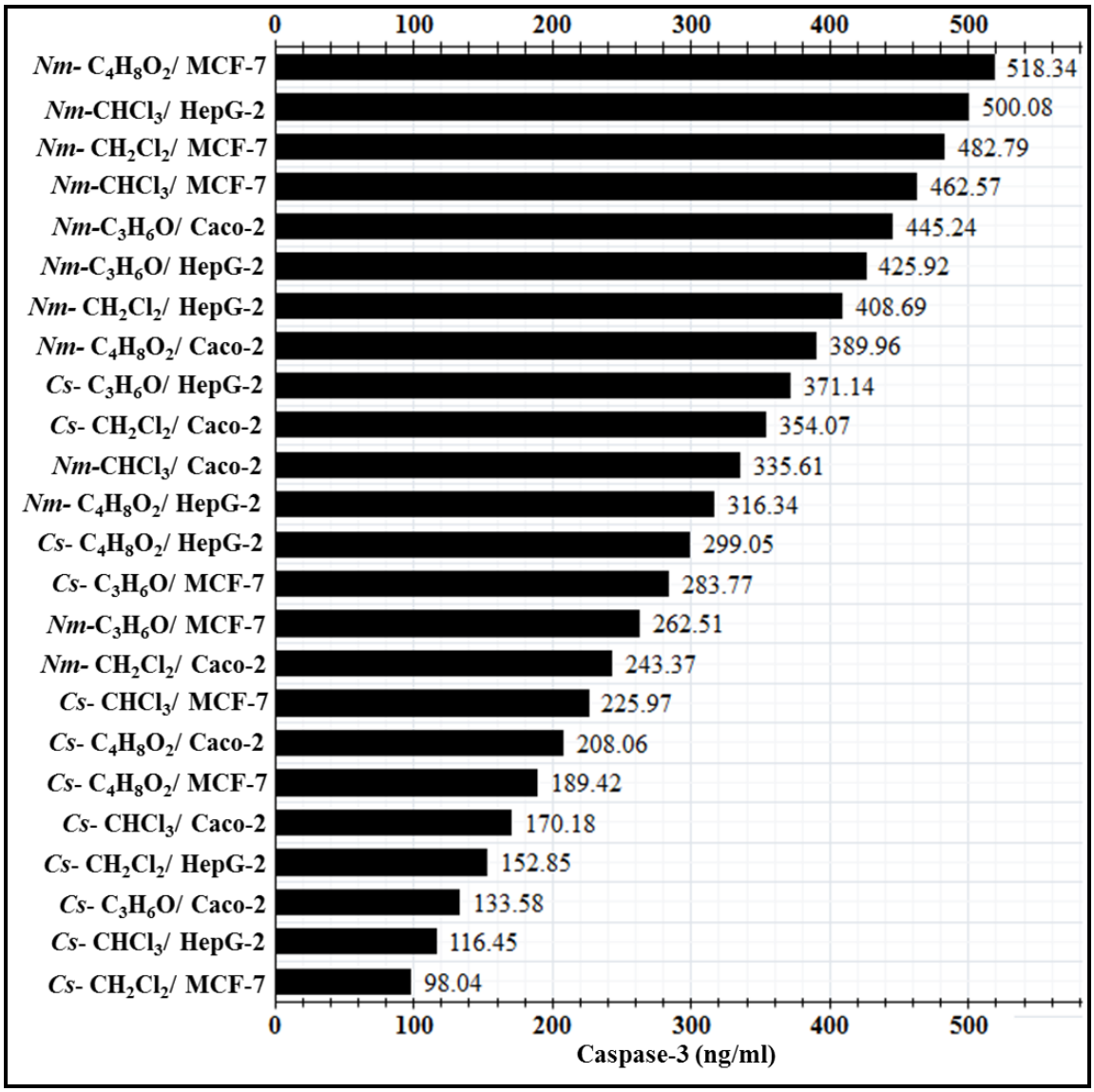

Figure (19): Comparative modulation of Caspase-3 protein expressions in HepG-2, MCF-7 and Caco-2 cells were treated with current studied extracts and harvested 24 h after treatments.

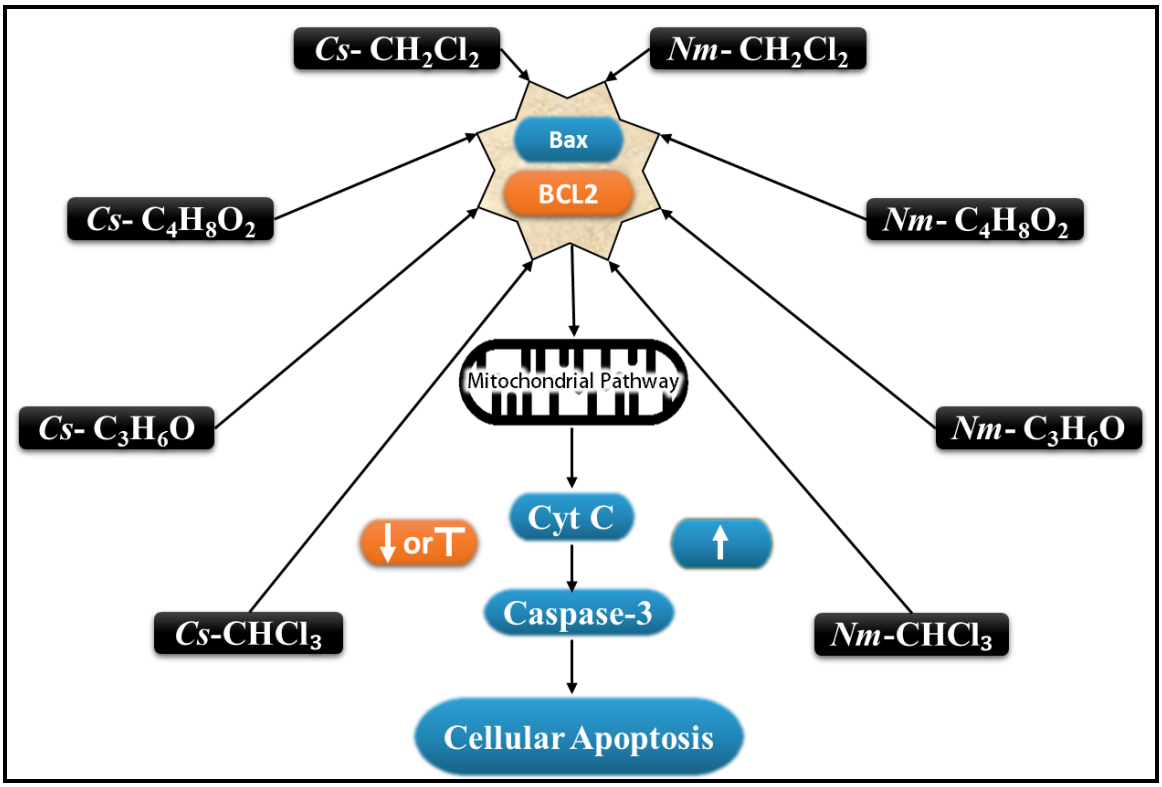

Figure (20): Schematic drawing of the mechanism of action of current studied extracts. This carton is based on the current available data throughout the present study.

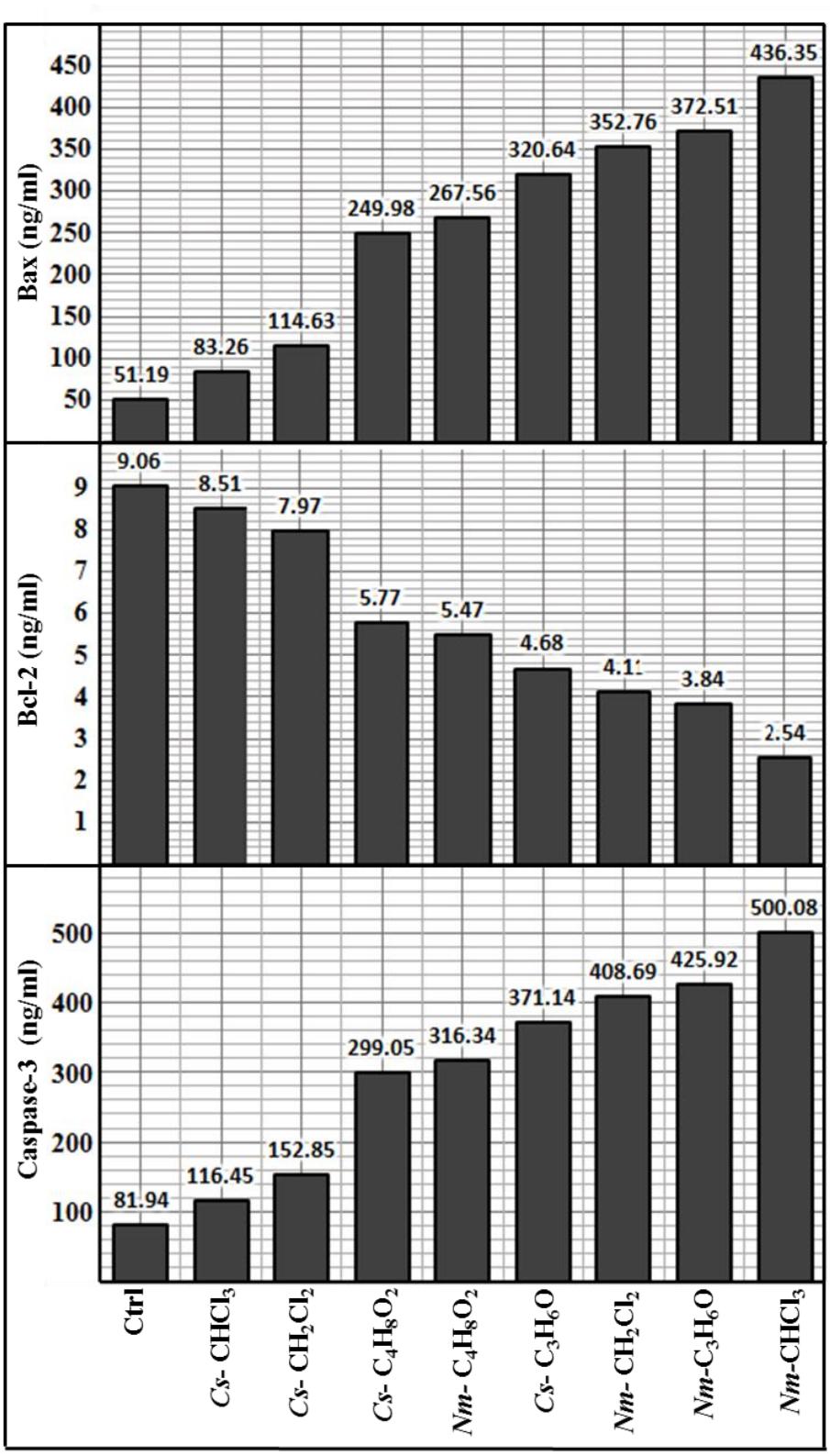

Figure (21): Comparative modulation of Bax, Bcl-2 and Caspase-3 protein expressions in HEPG-2 cells were treated with current studied extracts and harvested 24 h after treatments.

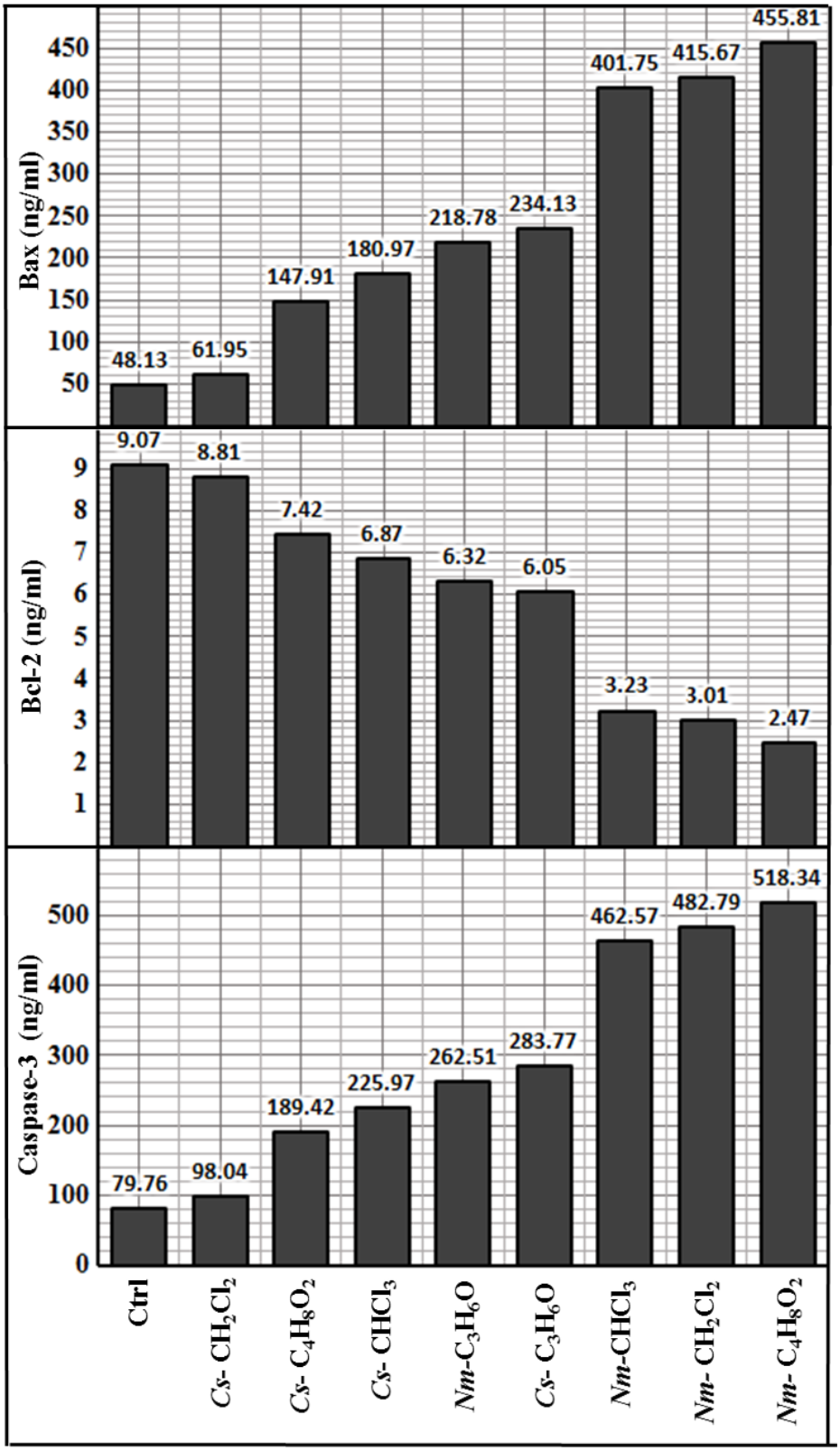

Figure (22): Comparative modulation of Bax, Bcl-2 and Caspase-3 protein expressions in MCF-7 cells were treated with current studied extracts and harvested 24 h after treatments.

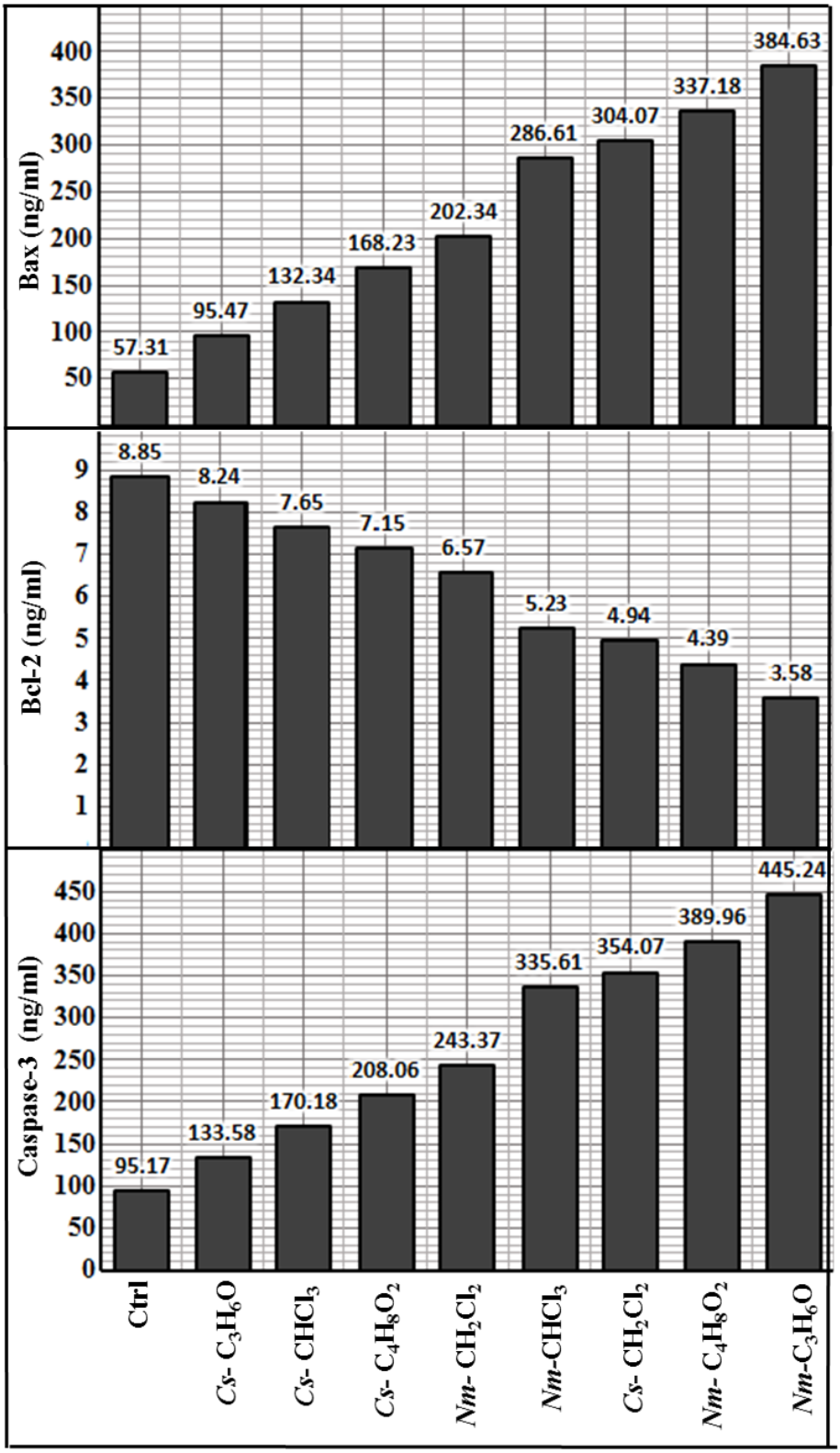

Figure (23): Comparative modulation of Bax, Bcl-2 and Caspase-3 protein expressions in Caco-2 cells were treated with current studied extracts and harvested 24 h after treatments.

DISCUSSION

Marine organisms are a rich source of structurally novel and biologically active metabolites **(El-Damhougy *et al*., 2017; Hasaballah and El-Naggar, 2017; Ibrahim *et al*., 2017; El-Damhougy *et al*., 2019)**. Recently, studies have suggested that some bioactive compounds isolated from marine organisms have been shown to have anti-cancer, activity **(Borowitzka and Borowitzka, 1992; Mayer and Hamann, 2005; Blunt *et al*., 2005; Somnath and Ghosh, 2010)**. According to many previous studies, those bioactive constituents of marine organisms are attractive candidates for cancer chemoprevention and therapy. Therefore, investigations are still required to gain more details about the biological activity of marine organism compounds although scientists have known a lot about nutritional and economical importance of many marine organisms. Here, eight marine organism extracts *Cs*-CH_2Cl_2, *Cs*-$C_4H_8O_2$, *Cs*-C_3H_6O, *Cs*-$CHCl_3$, *Nm*-CH_2Cl_2, *Nm*-$C_4H_8O_2$, *Nm*-C_3H_6O, and *Nm*-$CHCl_3$ are introduced to study their anticancer effects such as many marine organism derived compounds and extracts that have shown to exert multiple anticancer effects, for instance, Sphingolipids/glycosides of sea cucumber *Stichopus japonicus* **(Kariya *et al*., 2004)**, TBL12 **(Chari *et al*., 2018)**, *Holothuria parva* methanolic extract **(Salimi *et al*., 2017)**, *Apostichopus japonicus* extracts **(Kim *et al*., 2017)** Philinopside E **(Tian *et al*., 2005, 2007)**, *Acaudina molpadioides* cerebrosides and *Asterias amurensis* cerebrosides **(Du *et al*., 20012)**. The present study has utilized HepG-2, MCF7, and Caco-2 cancer cell lines, each was subjected to *Cs*-CH_2Cl_2, *Cs*-$C_4H_8O_2$, *Cs*-C_3H_6O, *Cs*-$CHCl_3$, *Nm*-CH_2Cl_2, *Nm*-$C_4H_8O_2$, *Nm*-C_3H_6O, and *Nm*-$CHCl_3$ dose independent treatments differ from the other previously investigations that have used Skin melanoma cancer cell line-2 **(Kim *et al*., 2017)**, murine sarcoma cancer cell line-180 **(Tian *et al*., 2005, 2007; Du *et al*., 20012)** and hepatoma 22 cancer cells **(Tian *et al*., 2005, 2007)** that have been exposed to previous bioactive compounds of different prior studied marine organisms. Compared to the anticancer effect of the ethanolic crude extract of the same sponge studied here *Callyspongia siphonella* in two cancer cellines MCF-7 and Caco-2 **(Ibrahim *et al*., 2017)**, the present *Cs*Es showed lower anticancer activity where the ethanolic *Callyspongia siphonella* crude extract inhibited MCF-7 and Caco-2 cancer cells within IC_{50} 1.39 and 5.57 µg/ml, respectively. Therefore, ethanol is completely more efficient than current *Cs*Es to induce better *Callyspongia siphonella* antiproliferative activity output on MCF-7 and Caco-2 cancer cells. Similarly, the ethanolic crude extract of *Negombata magnifica* **(El-Damhougy *et al*., 2017)** induced more intense anticancer activity with lower IC_{50} values of 0.37 and 1.09 µg/ml against MCF-7 and Caco-2 cell lines, respectively, compared to current *Nm*Es, but the differences between IC_{50} values overall indicated that both ethanolic and current crude extracts of *Negombata magnifica* are extremely potential for anticancer properties although ethanolic extract remain require more information about its effects on cancer cell induced apoptosis.

So, beside the proliferation inhibitory effect for the present *Cs*Es and *Nm*Es in HepG-2, MCF7, and Caco-2, *Cs*Es and *Nm*Es were also founded to induce apoptosis in the same cancer cell lines while some other marine bioactive compounds that had previously studied *in vitro* suppressed the angiogenesis and osteoclastogenesis and also rose up the cytotoxicity **(Kariya *et al*., 2004)**. Similarly, cerebrosides of both *Asterias amurensis* and *Acaudina molpadioides* inhibited *in vitro* cell proliferation and induced

apoptosis in murine sarcoma cancer cell line-180 **(Chari *et al*., 2018)**, *Holothuria parva* methanolic extract induced *in vitro* apoptosis in Chronic lymphocytic leukemia B-lymphocytes **(Salimi *et al*., 2017)**, *Apostichopus japonicus* extracts from low-temperature ultrasonification process was *in vitro* inhibited skin melanoma cancer cell line-2 proliferation and metastasis **(Kim *et al*., 2017)** and philinopside E was fund to *in vitro* inhibit the murine sarcoma cancer cell line-180 and hepatoma proliferation and induced apoptosis and anti-angiogenic activity **(Tian *et al*., 2005, 2007)**.

However, the *in vitro* anticancer effects of current *Cs*Es and *Nm*Es were supported by Bcl-2 decrease along with Bax and caspase-3 increase in HepG-2, MCF7, and Caco-2 cancer cells, the anticancer effects of the other previously investigated bioactive compounds of marine organisms were resulted in Bcl-2, STAT3, and MMP-9 decrease **(Kim *et al*., 2017)**. Additionally, there is an *in vivo* prior study mentioned have used quantitative real-time PCR analysis which revealed that the administration of cerebrosides of either *Asterias amurensis* or *Acaudina molpadioides* decreased the expression of both Bcl-2 and Bcl-xL while increased Bax, Cyt c, caspase-3, and caspase-9 of the murine sarcoma cancer cell line-180 ascites **(Du *et al*., 20012)** compared to the present *Cs*Es and *Nm*Es, which were studied *in vitro,* where it suppressed the expression of Bcl-2 and increased the expression Bax and caspase-3 in HepG-2, MCF7 and Caco-2 cancer cell lines, unlike another *in vivo* investigation indicated philinopside E anti-angiogenic activity associated with downturn of VEGFR2 signaling in murine sarcoma cancer cell line-180 **(Tian *et al*., 2005, 2007)**. Despite of the clinical research about the anticancer efficacy of bioactive compounds of marine organisms is very limited, there is an available investigation of a total 20 patients with high risk asymptomatic multiple myeloma were given TBL12, the TBL12 was well tolerated and 9 (45%) patients remain on treatment with one minimal response noted **(Chari *et al*., 2018)**.

Altogether, the data generated during the current study is emphasize our hypothesis of *Cs*-CH_2Cl_2, *Cs*-$C_4H_8O_2$, *Cs*-C_3H_6O, *Cs*-$CHCl_3$, *Nm*-CH_2Cl_2, *Nm*-$C_4H_8O_2$, *Nm*-C_3H_6O, and *Nm*-$CHCl_3$, each has anticancer properties that can be promising and developed in the future as anticancer drug for cancer therapy outcome.

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ABBREVIATIONS

***Aa*C:**	*Asterias amurensis* cerebrosides
***Aj*Es:**	*Apostichopus japonicus* extracts
***Am*C:**	*Acaudina molpadioides* cerebrosides
ASx:	Asymptomatic
Bax:	B-cell lymphoma 2 associated X protein
Bcl-2:	B-cell lymphoma 2
Bcl-xL:	B-cell lymphoma extra-large
C_3H_6O:	Acetone
$C_4H_8O_2$:	Ethyl acetate
Caspase-3:	Cysteine proteases with aspartate specificity 3
Caspase-9:	Cysteine proteases with aspartate specificity 9
CH_2Cl_2:	Methylene chloride
$CHCl_3$:	Chloroform
CLLB:	Chronic lymphocytic leukemia B-lymphocytes
***Cs*E:**	*Callyspongia siphonella* crude extract
Ctrl:	Control
Cyt C:	Cytochrome complex
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dimethyl sulfoxide
FBS:	Fetal bovine serum
HepG-2:	Human hepatocellular-carcinoma cell line
HFB-4:	Normal human melanocytes cell line
***Hp*E:**	*Holothuria parva* methanolic extract
IC_{50}:	50% inhibitory Concentration
MCF7:	Mammary gland breast cancer cell line
MM:	multiple myeloma
MMP-9:	Matrix metalloproteinase-9
MTT:	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
***Nm*E:**	*Negombata magnifica* crude extract
OD:	Optical density
PE:	Philinopside E
PMSF	Phenylmethylsulfonyl fluoride
PSA:	Penicillin-streptomycin-amphotericin B
rPCR:	Real-time polymerase chain reaction
RPMI-1640:	Roswell Park Memorial Institute series no. 1640
S180:	Murine Sarcoma cancer cell line-

	180
SCUBA	Self-contained underwater breathing apparatus
SI:	Selectivity index
***Sj*SG:**	Sphingolipids/glycosides of sea cucumber Stichopus japonicus
SK-MEL-2:	Skin melanoma cancer cell line-2
STAT3:	Signal transducer and activator of transcription 3
TBL12:	Drug extract of sea cucumber
VEGFR2:	Vascular endothelial growth factor-2
***Xp*-CH_2Cl_2:**	*Xenia peurto-galera* methylene chloride extract
***Xp*:**	*Xenia peurto-galera*