In Vitro Study on Effect of Zinc Oxide Nanoparticles on the Biological Activities of Croton tiglium L. Seeds Extracts

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Abstract

Objective: Croton tiglium L. seeds were studied against colon cancer induced chemically in rats after incorporating silver nanoparticles (Ag-NPs) but the body has no the ability to discrete silver or silver ions. Therefore, the present study was designed to reveal the biological activities of different C. tiglium L. seeds extracts incorporated with zinc oxide nanoparticles (ZnO-NPs). Results: It was found that C. tiglium L. seeds provided with high contents of total protein (27.43 g/100g), carbohydrate (18.29 g/100 g) and lipid (46.31 g/100 g). The chromatographic techniques revealed that concentrations of the predominant compounds increased in all studied extracts (ethanolic, aqueous and petroleum ether) after incorporating ZnO-NPs. The in vitro biological activities showed that the aqueous extract possessed the highest antioxidant and scavenging activities. It exhibited the highest inhibitory effect on α-amylase (41.89%) and acetyl cholinesterase (AChE) (23.00%) in addition to its higher anti-arthritis activity. All the biological activities increased after incorporating ZnO-NPs. It showed the highest cytotoxic activity that increased after incorporating ZnO-NPs against human colon carcinoma (Caco-2) cells. Therefore, this extract was selected for undergoing further studies on Caco-2 cells. The aqueous extract incorporated with ZnO-NPs arrested growth of Caco-2 cells at G2/M and increased percentage of total apoptotic cells and necrosis. The median lethal dose (LD50) showed that the extracts incorporated with ZnO-NPs were safer than the native extracts. Conclusion: The study showed that the aqueous extract was the most active extract that consequently exhibited promising biological activities after incorporating ZnO-NPs.

Keywords: Croton tiglium Seeds- Zinc oxide nanoparticles- Green Nanotechnology- Biological Activity- Toxicity

Introduction

Croton tiglium L. is categorized as the second largest genus under the Family “Euphorbiaceae”. Approximately 1300 species of largely trees, shrubs and herbs that grow under varied environmental conditions in tropical and sub-tropical regions were belonged to this large genus (Torres et al., 2008). Due to the presence of essential oils, the leaves, seeds, stem, roots, and the juice from the bark were widely utilized for medicinal and nutritional purposes among different global communities (Harkat-Madouri et al., 2015; Sai Prassana and Karpaga, 2015). Hu et al., (2010) emphasized that C. tiglium L. seeds are rich in phorbol esters, crotonic acid and fatty acids in addition to active phytoconstituents that are responsible for severe purgative effect of the C. tiglium L. seeds extract. Due to the strong toxic effect of the chemical synthetic products, components of natural essential oil are gaining frequent presence and increasing interest in the recent studies investigating their potential activity and functional utility (Babahmad et al., 2018; Rakmai et al., 2018). These essential oils possessed purgative, analgesic, antibacterial, anti-oxidation, anti-inflammatory and anti-tumor activities. Therefore, they are abundantly utilized in cosmetic industries and indigenous medicines (El Gendy et al., 2015). It was reported that linoleic acid, oleic acid and eicosenoic acid are the most abundant fatty acids exist in a methyl-esterified sample obtained by reflux method (Mei et al., 2012). It increases gastrointestinal motility, has a smoothening effect on skin, reduces inflammation, expectorant and rubefacient. Traditionally, C. tiglium L. is used for kidney stones, bronchial irritation, convulsions and skin disorders. It has been utilized widely among the Asian communities for treating gastrointestinal problems.
Determination of nutritional value of C. tiglium L. seeds
Total protein content was estimated in C. tiglium L. seeds by micro-kjeldahl method as stated by El-Feky et al., (2020). Total carbohydrate content was quantified as glucose by phenol sulfuric acid method suggested by El-Feky et al., (2018). Total lipid content was gravimetrically determined based on the method suggested by AboulNaser et al., (2020). Consequently, the obtained values (proteins, carbohydrates and lipids) were used for calculating the caloric value of C. tiglium L. seeds using the equation suggested by Coelho et al., (2014).

Preparation of different plant extracts
C. tiglium L. seeds were obtained from Agricultural Research Center, Giza, Egypt. The crushed dried seeds were successively extracted in soxhlet apparatus with petroleum ether (60 - 80°C), ethyl alcohol, and then with distilled water for 20 hrs for each solvent, the obtained three extracts were separately filtered then concentrated in a rotary evaporator at 45°C under reduced pressure to dryness.

Isolation and identification of the major compounds from different C. tiglium seeds L. extracts
The petroleum ether extract was chromatographed on silica gel column. Elution was successively carried out by methylene dichloride (CH$_2$Cl$_2$) and increasing the polarity with ethyl acetate. The fractions were successively collected and individually concentrated to 5 ml then screened by thin layer chromatography (TLC) using toluene : ethyl acetate (7:3 v/v) as solvent system. The separated fractions were visualized by spraying with 10% H$_2$SO$_4$ and heating at 110°C for 5 min. The resulting similar fractions were collected from the column together according to Rf values. Structures of the isolated compounds were interpreted based on the spectral analyses (FT-IR, 1H-NMR, 13C-NMR and Mass spectroscopy).

Preparation of zinc oxide C. tiglium L. seeds nano-extract
ZnO-NPs were synthesized using plant extracts as reducing agents in order to prepare ZnO-NPs with optimum yield and particle size. For preparation of Zno-C. tiglium seeds nano-extract, the ZnO-NPs were synthesized by sol-gel method suggested by Bao et al., (2012) with some modifications. The plant extract was added into zinc acetate solution till obtaining the white precipitate that dried then converted into powder to be ready for characterization.

Characterization of the biosynthesized zinc oxide nanoparticles
The ZnO-NPs spectra were assayed by Shimadzu UV-VIS recording spectrophotometer UV-240 at λ 200 - 800 nm after diluting the samples (10-fold) with

Materials and Methods

Determination of nutritional value of C. tiglium L. seeds
Total protein content was estimated in C. tiglium L.
deionized water. The crystalline nature and grain size of the synthesized ZnO-NPs were analyzed by a Philips X-Ray Diffractometer (XRD) (PW 1930 generator, PW 1820 goniometer) equipped with Cu Kα radiation as an X-ray source (45 kV, 40 mA, with λ = 0.15418 nm). Shape and size of the synthesized ZnO-NPs were determined at high resolution level (200 KV) using Transmission Electron Microscope (TEM) (model JEM-1230, Japan) operated at accelerating voltage of 120 kV, with maximum magnification of 600X103 and a resolution until 0.2 nm. The average hydrodynamic size of the synthesized ZnO-NPs was determined by Dynamic Light Scattering (DLS) (Malvern Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, United Kingdom) according to method suggested by Murdock et al., (2008).

**Determination of the phyto-constituents in C. tiglium L. seeds extracts before and after incorporating zinc oxide nanoparticles**

1. **Quantitative determination of major phyto-constituents**

Concentration of total polyphenols was determined using Folin Ciocalteu reagent according to method suggested by Singleton and Rossi (1965). Total tannins contents were determined using tannic acid as a reference compound based on the method described by Broadhurst and Jones (1978).

2. **Investigation of the lipoidal constituents**

The P. ether extracts of C. tiglium L. seeds before and after incorporating ZnO-NPs were subjected to gas chromatograph coupled with a mass spectrometer (GC/MS analysis) (model Shimadzu GC/MS–QP5050A) on an Agilent 6890, 70 eV. MS spectrometer; Finnigan Model 3200, Mass spectrometer at 70 eV. The constituents have been identified by comparison of their spectral fragmentation patterns with those of the available database libraries Wiley (Wiley Int.) USA and NIST (Nat. Inst. St. Technol., USA) and/or published data. Quantitative determination was carried out based on integration of the area under peak according to the method suggested by Elsawi et al., (2020).

3. **Investigation of the phenolics and flavonoidal compounds**

The different phenolic and flavonoidal compounds were identified in the aqueous and ethanolic extracts of C. tiglium L. seeds before and after incorporating ZnO-NPs using high pressure liquid chromatography (HPLC) (Shimadzu-UFLC Prominance) equipped with an auto sampler (Model-SIL 20AC HT) and UV-Vis detector (Model-SPD 20A) (Japan). The separation process was carried out through analytical column of an Eclipse XDBC18 (150 X 4.6 µm; 5 µm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase is consisting of solvent system of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). Before the chromatographic run, all samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI). Each sample (50 µl) was injected automatically by the injector piece. The flow rate was kept at 0.8 m min⁻¹ for a total run time of 70 min and the gradient program was as follows: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min. The peaks were monitored simultaneously at 280 and 320 nm for the benzoic acid and cinnamic acid derivatives, respectively. When the chromatographic run was finished, the peaks were identified by congruent retention times and UV spectra and compared with those of the standards (El-Sayed et al., 2018).

**In vitro study on different C. tiglium L. seeds extracts before and after incorporating zinc oxide nanoparticles**

1. **Antioxidant activity**

Total antioxidant capacity (TAC) and total iron reducing power were assessed according the methods suggested by El-Sayed et al., (1999) and Oyedapo and Famurewa (1996), respectively. The scavenging activities were assessed against 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals according to the methods suggested by Rahman et al., (2015) and Arnao et al., (2001), respectively.

2. **α-amylase inhibitory assay**

It was carried out using acarbose as standard drug for calculating percentage of α-amylase inhibition (%) using 3,5-dinitrosalicylic acid (DNSA) method (Wickramaratne et al., 2016).

3. **Acetyl cholinesterase (AChE) enzyme activity**

It was measured for calculating percentage of AChE inhibition (%) using Ellman’s method (Ellman et al., 1961).

4. **Antiarthritic activity**

4.1. **Protein denaturation**

Percentage of protein denaturation inhibition can be calculated according to the method described by Lavanya et al., (2010) and Das and Sureshkumar (2016). All results were compared with standard (diclofenac sodium) and the control represents 100% protein denaturation.

4.2. **Protease inhibitory activity**

This assay was carried out by calculating percentage of the protease inhibitory activity according to the method suggested by Oyedapo and Famurewa (1995).

5. **Cytotoxic activity**

Cytotoxic activities were determined against human hepatocellular (HEPG-2) and colon carcinoma (CACO-2) cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as suggested by Vichai and Kirtikara (2006). Percent of cell-growth inhibition (%) and protein denaturation were assessed according to the methods suggested by Prieto et al., (1999) and Oyaizu (1986), respectively. The scavenging activities were assessed against 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals according to the methods suggested by Rahman et al., (2015) and Arnao et al., (2001), respectively.

6. **DNA content analysis**

The CACO-2 cells (3×10⁵/well) were seeded into 6-well plates, cultured overnight and treated with different C. tiglium L. extracts and their ZnO nano-extracts individually for 24 hrs. The cells were fixe in 75% ethanol at -4°C overnight then incubated with 50 ng/mL PI staining...
solution and 0.1 mg/mL RNase A in dark place at room temperature for 15 min. The DNA content was quantified in the cells by flow cytometry (BD FASCCalibur-USA).

7. Cell apoptosis

The cell apoptosis was assessed by Annexin V-FITC apoptosis detection kit ( Annexin V-FITC-BD Bioscience PharmingenTM, USA). The CACO-2 cells (3 × 10⁶ well) were seeded into 6-well plates then cultured overnight before the exposure to different concentrations of C. tiglium L. extracts and their ZnO nano-extracts (50, 100, 150, 200 μg/ml). Furthermore, both of each extract and its ZnO nano-extract (100μg /ml) added to CACO-2 cells for different times (24, 36, 48 and 48 hrs). Cells were collected by cold centrifugation at 300 x g for 10 min and consequently washed twice and re-suspended in 500 μl of cold (+4°C) 1X PBS buffer then precipitated again by centrifugation. This step was followed by adding 100 μl 1X Binding Buffer per sample. Annexin V (100 μl) was added to treated cell samples (106 cell/ml) then incubated. Annexin V-FITC (5μl) and propidium iodide (PI) (5μl) were incubated in the dark for 15 min. at room temperature, then 400 μl of 1X binding buffer was added. The cells were immediately analyzed by flow cytometry (BD FASCCalibur-USA) within 1 hr for a maximal signal (Kooiman et al., 1994).

8. Extraction of RNA and quantitative RT-PCR

The CACO-2 cells were cultured in six-well plates and exposed individually to C. tiglium L. extracts and their ZnO nano-extracts (100 μg/ml). Total RNAs were extracted from treated cells using the RNeasy Mini Kit (Qiagen RNA extraction/BioRad syber green PCR MMX) based on the method suggested by Pfaffl (2001). For quantifying expressions of EGFR, Bcl-2 and Casp3 genes, the total RNAs (10 ng) from each sample was processed by reverse transcription for cDNA synthesis using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, USA). Consequently, the cDNA was amplified with the Syber Green I PCR Master Kit (Qiagen RNA extraction/BioRad syber green PCR) in a 48-well plate using the Step One instrument (Applied Biosystems, USA), as a following: 10 min at 95°C for enzyme activation followed by 40 cycles of 15 sec at a temperature of 95°C, 20 sec at 55°C and 30 sec at 72°C for the amplification step. The changes in expression of each target gene were normalized relative to the mean critical threshold (CT) values of GAPDH as a housekeeping gene by the ΔCt method. One μl of both primers specific for each target gene (EGFR (F: 5'-GACTCCGTCCAGTATGTGTCG-3'; 5'-GCCCTCTGGCACTCTTACATT-3'), Bcl-2 (F: 5'-ACTCCTGGCAGTATGTGTCG-3'; 5'-GACCTCTGGCAGTATGTGTCG-3'), Casp3 (F: 5'-TGGTTTGTGTGGTCTTGAGCC-3'; B: 5'-CACGTGGCAGTATGTGTCG-3') and GAPDH (F: 5'-GAAAGTGGAGTGGTCGGAGTCA-3'; B: 5'-TTTGGTTGGCTTGGAGTCA-3') were added. The mRNA levels were quantified using the 2⁻¹ΔΔCt method that was suggested by Wang et al., (2014). GAPDH was used as the internal control. Experiments for each gene were conducted in triplicate.

Median lethal dose of different extracts (LD₅₀)

The different native C. tiglium L. extracts and their ZnO nano-extracts (after incorporating ZnO-NPs) were studied separately for evaluating the LD₅₀. Three hundred and twenty four adult albino mice (weight 20-25 g) were divided into 9 groups (6 mice in each group) for calculating the LD₅₀ of the native ethanolic C. tiglium L. extract, 9 groups for ZnO ethanolic nano-extract, 9 groups for native for native aqueous extract, 9 groups for ZnO aqueous nano-extract, 9 groups for native P. ether extract and 9 groups for ZnO P. ether nano-extract. For each extract, the groups were treated orally by stomach tube with rising doses of 1,000, 2,000, 4,000, 6,000, 8,000, 10,000, 12,000, 14,000 and 16,000 mg/Kg. Number of dead mice was recorded after 24 hrs of extract administration. The LD₅₀ was calculated according to the methods suggested by Paget and Barnes (1964).

Results

The nutritional composition of C. tiglium L. seeds

It was found that C. tiglium L. seeds provided great values of total protein (27.43 g/100g), carbohydrate (18.29 g/100 g) and lipid contents (46.31 g/100 g). Consequently, the caloric value of C. tiglium L. seeds was calculated as 599.67 kcal/100g.

The major compounds isolation from different C. tiglium L. seeds extracts

The steroidal and terpenoidal compounds were isolated from petroleum ether extract and identified by column chromatography technique. The collected fractions were tested using Liebermann–Burchard reagent, then they were spotted on TLC plate and sprayed with 10% H₂SO₄ reagent. The positively tested fractions were applied on TLC silica gel plates against the available authentics. By comparing Rf values and the chromatographic appearance under UV, two steroidal compounds (β-sitosterol and stigmasterol) beside to one pentacyclic triterpenoid (α-amyrin) were identified and isolated in pure form. Stigmasterol was isolated for the first time from C. tiglium L. seeds. It was identified at RF value 0.20 and obtained as white crystalline powder with melting point 170°C. Furthermore, isopimara-7,15-dien-3β-ol was also isolated by silica gel column with CH₂Cl₂:EtOAc (90:10 v/v) as amorphous powder and identified at RF value 0.46 in toluene : ethyl acetate solvent system (7:3 v/v). UV λmax was 226 nm; FT-IR bands at 3320 cm⁻¹ for OH stretching, 2945, 3850 cm⁻¹ for C-H stretching, and 1645cm⁻¹ for –C=C– Stretch. EI-MS 70 eV m/z (rel. int.): 288 [M]+(10) calculated for molecular formula C₂OH₃₂O, 273 [M-Ch]⁺ (15), 270 [M-H₂O]⁺ (8), 255 (30), 245 (13), 213 (16), 200 (22), 185 (25), 171 (29), 145 (51), 132 (69), 129 (100), 119 (81), 105 (70), 91 (25). As well as, stigmast-4-en-3-one was isolated for the first time from C. tiglium L. seeds by silica gel column with CH₂Cl₂:EtOAc (80:20 v/v) as white needles, melting point 155°C. It was identified at RF value 0.63 in toluene
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ethyl acetate solvent system (7:3 v/v). UV λmax was 255 nm; FT-IR bands at 3315 cm⁻¹ for OH stretching, 2927, 3810 cm⁻¹ for C-H stretching, 1715 cm⁻¹ for C=O stretching, and 1,620 cm⁻¹ for C=C– Stretch. EI-MS 70 eV m/z (rel. int.): 412 (35) calculated for molecular formula C29H48O, base peak at m/z 124 (100). The characteristic fragmentations were m/z 43 (55), 55 (40), 71 (25), 95 (28), 134 (10), 150 (15), 178 (12), 213 (20), 229 (35), 255 (20), 273 (11), 329 (16), 381 (9), 396 (17).

Quercetin-7-O-β-D-glucopyranoside was isolated from ethanolic extract with CHCl₃: MeOH (80:20 v/v) as yellow crystals with melting point 220°C. It was identified at Rf value 0.67 and gave positive test for flavonoids. UV λmax in MeOH (256, 269sh, 372), MeOH+NaOMe (244sh, 291, 366, 456), MeOH+AlCl₃ (258sh, 273, 340, 458), MeOH+AlCl₃/HCl (268, 303, 365, 425) stable UV absorbance was observed in band I and II indicating presence of hydroxyl group at C-3 and C-5, MeOH+NaOAc (286, 377, 428sh), MeOH+NaOAc/H3BO3 (261, 290sh, 385) bathochromic shift of band I indicates the presence of 3`, 4`dihydroxy group. 1H-NMR (400 MHz, CD3OD, δ ppm): 6.45 (1H, d, J=2.8Hz, H-6), 6.79 (1H, d, J=2.8Hz, H-8), 7.90 (1H, d, J=2.7Hz, H-2’), 6.93 (1H, d, J=8.5Hz, H-5’), 7.65 (1H, dd, J=2.7, 8.5Hz, H-6’).

Figure 1. Characterization of the Synthesized Zinc Oxide Nanoparticles (ZnO-NPs) Showing. a) Ultraviolet-visible (UV-VIS) spectrum, b) X-Ray Diffraction (XRD) spectrum, c) Transmission Electron Microscope (TEM) image and d) Dynamic Light Scattering (DLS).

Figure 2. Percent of the Inhibitory Effect on Activities of α-amylase and Acetyl Cholinesterase (AChE) for a) native C. tiglium seeds extracts and b) ZnO-C. tiglium L. seeds nano-extracts. Values expressed as mean of three replicates ± SE. * denotes the most effective extract.
### Table 1. GC/MS Analysis of the Lipoidal Constituents in Petroleum ether C. tiglium L. seeds Extract before and after Incorporating ZnO-NPs

| Class          | Compound                        | Mol. Weight | BP | Relative area % | Before | After |
|----------------|---------------------------------|-------------|----|-----------------|--------|-------|
| Hydrocarbons   | 4-Decene                        | 140         | 55 | 0.06            | 0.07   |
|                | 4-Methylnonane                  | 142         | 41 | 1.04            | 1.05   |
|                | 9-methyl-1-decene               | 154         | 56 | 0.97            | 1.11   |
|                | 2-Methydecane                   | 156         | 43 | 1.65            | 2.06   |
|                | 5-Methyl-4-undecene             | 168         | 55 | 0.11            | 0.13   |
|                | 2-Methylundecane                | 170         | 43 | 2.03            | 3.15   |
|                | 2,5,6-Trimethyldecane           | 1184        | 57 | 1.54            | 2.01   |
|                | 2-Methyltridecane               | 198         | 43 | 0.15            | 0.18   |
|                | 2,4,6,8-Tetramethyl-1-undecene  | 210         | 43 | 0.24            | 0.26   |
|                | 2-Methyltetradecane             | 212         | 57 | 1.03            | 1.05   |
|                | n-Hexadecane                    | 226         | 43 | 2.02            | 2.11   |
|                | n-Heptadecane                   | 240         | 43 | 3.34            | 3.37   |
|                | n-Octadecane                    | 254         | 57 | 5.61            | 6.24   |
|                | 6-Methyl octadecane             | 268         | 57 | 2.08            | 2.22   |
|                | n-Docosane                      | 310         | 43 | 3.76            | 4.09   |
|                | Tetracosane                     | 338         | 57 | 6.54            | 6.57   |
|                | Henriacotiane                   | 436         | 57 | 7.04            | 7.12   |
| Fatty alcohols | Hept-4-en-1-ol                  | 114         | 41 | 1.98            | 2.01   |
|                | 2-Octen-1-ol                    | 128         | 57 | 0.93            | 0.96   |
|                | 2-Nonen-1-ol                    | 142         | 57 | 12.25           | 13.02  |
|                | 2-Decen-1-ol                    | 156         | 57 | 2.08            | 2.1    |
|                | 10-Dodecyn-1-ol                 | 182         | 68 | 0.98            | 0.99   |
|                | Dodecanol                       | 186         | 41 | 1.54            | 1.57   |
|                | Tetradecanol                    | 214         | 41 | 1.96            | 2.02   |
|                | 6,9-Pentadecadien-1-ol          | 224         | 67 | 1.14            | 1.14   |
|                | Octadecanol                     | 270         | 41 | 1.04            | 1.06   |
| Aldehydes      | 2,2-Dimethyl-4-pentenal         | 112         | 55 | 0.87            | 1.03   |
|                | Heptanal                        | 114         | 57 | 1.15            | 1.17   |
|                | 2-Isononenal                    | 140         | 43 | 1.01            | 1.04   |
|                | Nonanal                         | 142         | 41 | 0.19            | 0.21   |
|                | 2,4-Decadienal                  | 152         | 81 | 1.06            | 1.07   |
|                | Decanal                         | 156         | 43 | 2.15            | 2.15   |
|                | 9-Octadecenal                   | 266         | 41 | 11.78           | 13.11  |
| Ketones        | 1-Methyl-bicycloheptan-6-one    | 124         | 67 | 5.08            | 5.13   |
|                | 2-Nonanone                      | 142         | 43 | 0.14            | 0.14   |
|                | Campheneronone                  | 220         | 41 | 0.05            | 0.06   |
| Sterols        | Ergosterol                      | 396         | 69 | 0.45            | 0.47   |
|                | β-Sitosterol                    | 414         | 41 | 0.39            | 0.41   |
|                | Total hydrocarbons              |             |    | 41.21           | 42.79  |
|                | Total fatty alcohols            |             |    | 23              | 24.87  |
|                | Total aldehydes                 |             |    | 18.21           | 19.78  |
|                | Total ketones                   |             |    | 5.27            | 5.33   |
|                | Total sterols                   |             |    | 0.84            | 0.88   |
|                | Total identified compounds      |             |    | 88.53           | 93.65  |

BP, Base Peak
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H-6'), 5.00 (H-1`). 13C-NMR (125 MHz, DMSO, δ): 157.53 (C-2), 127.48 (C-3), 180.73 (C-4), 162.14 (C-5), 93.67 (C-6), 161.43 (C-7), 93.76 (C-8), 160.45 (C-9), 99.50 (C-10), 121.47 (C-1`), 103.94 (C-2`), 144.25 (C-3`), 154.33 (C-4`), 101.43 (C-5`), 114.93 (C-6`), 100.00 (C-1``), 72.85 (C-2``), 75.64 (C-3``), 63.80 (C-4``), 75.21 (C-5``), 60.25 (C-6``).

13-O-Acetylphorbol-20-(9Z,12Z-octadecadienoate) was identified at Rf value 0.41 in CHCl₃ : MeOH (70:30 v/v) and isolated as amorphous powder. UV λmax was 245 nm; FT-IR bands at 3390 cm⁻¹ for stretching, 1,720 cm⁻¹ for C=O stretching, 1,695 cm⁻¹ for ester linkage, 1,630 cm⁻¹ for –C=C– stretch. EI-MS 70 eV m/z (rel. int.): 669 (25) calculated for molecular formula C₄₀H₆₁O₈, 632 (37) [M-tiglic acid-H₂O]+, 590 (31) [M-linoleic acid]+ and 388 (28) [M-linoleic acid]⁺. 13-O-Tigloylphorbol-20-(9Z,12Z-octadecadienoate) was identified at Rf value 0.46 in CHCl₃ : MeOH (70:30 v/v) and isolated as amorphous powder. UV λmax was 241 nm; FT-IR bands at 3,420 cm⁻¹ for stretching, 1,715 cm⁻¹ for C=O stretching, 1,700 cm⁻¹ for ester linkage, 1,625 cm⁻¹ for –C=C– stretch. EI-MS 70 eV m/z (rel. int.): 709 (17) calculated for molecular formula C₄₃H₆₅O₈, 590 (29) [M-tiglic acid-H₂O]⁺, 410 (34) [M-linoleic acid]⁺ and 328 (21) [M-tiglic acid-linoleicacid-H₂O]⁺.

The structural properties of prepared ZnO-NPs

The UV-Visible spectroscopy was used for studying the optical properties of the synthesized ZnO-NPs. Data presented in Figure 1a showed that preparation of

Table 2. HPLC Analysis of the Phenolics and Flavonoids in Ethanolic and Aqueous C. tiglium L. seeds Extracts before and after Incorporating ZnO-NPs.

| Compound          | Concentration (µg/g) | Ethanolic extract | Aqueous extract | Ethanolic extract | Aqueous extract |
|-------------------|----------------------|-------------------|-----------------|-------------------|-----------------|
|                   |                      | Before | After | Before | After | Before | After | Before | After |
| Phenolics         |                      |        |       |        |       |        |       |        |       |
| Gallic acid       | 32.715               | 35.278 |       | 27.214 | 29.716 |        |       |        |       |
| Protocatechuic acid | 59.866             | 61.425 |       | 32.086 | 32.981 |        |       |        |       |
| p-Hydroxybenzoic acid | 52.587             | 54.842 |       | 27.264 | 28.094 |        |       |        |       |
| Gentisic acid     | 77.349               | 81.028 |       | 99.581 | 99.984 |        |       |        |       |
| Catechin          | 315.404              | 401.273 |       | 69.793 | 71.428 |        |       |        |       |
| Chlorogenic acid  | 94.764               | 101.254 |       | 112.869 | 117.352 |        |       |        |       |
| Caffic acid       | 50.341               | 51.753 |       | 17.104 | 20.587 |        |       |        |       |
| Syringic acid     | 8.979                | 9.015  |       | 15.889 | 16.102 |        |       |        |       |
| Vanillic acid     | 151.947              | 156.284 |       | 8.171  | 8.987  |        |       |        |       |
| Ferulic acid      | 22.473               | 24.897 |       | 36.934 | 37.891 |        |       |        |       |
| Sinapic acid      | 11.429               | 18.349 |       | 0.000  | 0.000  |        |       |        |       |
| p-coumaric acid   | 13.241               | 19.257 |       | 0.000  | 0.000  |        |       |        |       |
| Rosmarinic acid   | 9.427                | 11.025 |       | 0.000  | 0.000  |        |       |        |       |
| Cinnamic acid     | 8.869                | 10.266 |       | 15.29  | 18.301 |        |       |        |       |
| Flavonoids        |                      |        |       |        |       |        |       |        |       |
| Quercetin         | 27.482               | 32.487 |       | 26.908 | 33.873 |        |       |        |       |
| Kaempferol        | 34.172               | 36.981 |       | 29.941 | 33.427 |        |       |        |       |
| Rutin             | 41.625               | 43.289 |       | 39.233 | 47.814 |        |       |        |       |
| Chrysine          | 27.362               | 29.476 |       | 12.406 | 19.452 |        |       |        |       |

Figure 3. Cytotoxic Activity of the Different C. tiglium L. seeds Extracts before and after Incorporating ZnO-NPs against a) human liver cancer (HEPG-2) and b) human colon cancer (CACO-2)
ZnO-NPs from the biosynthesis route was confirmed from the sharp peak identified in the UV-visible spectrum at 382 nm. Data of the XRD pattern illustrated in Figure 1b and showed that peaks identified at $2\theta = 31.7$, $34.4$, $36.2$, $47.5$, $56.5$, $62.7$, $67.8$ and $68.1$ and assigned to (100), (002), (101), (102), (110), (103), (112) and (201) confirmed formation of ZnO-NPs with high quality. The TEM data were compatible to the UV and XRD data. The TEM analysis was utilized for revealing size and the crystalline characteristics of the biosynthesized ZnO-NPs. As revealed in Figure 1c, the TEM image confirmed synthesis of ZnO-NPs with morphological shape and confirmed formation of ZnO-NPs with high quality. The TEM data were compatible to the UV and XRD data. The TEM analysis was utilized for revealing size and the crystalline characteristics of the biosynthesized ZnO-NPs. As revealed in Figure 1c, the TEM image confirmed synthesis of ZnO-NPs with morphological shape and

Figure 4. Data of DNA Content in a) Control CACO-2, b) CACO-2 treated with doxorubicin, c) CACO-2 treated with aqueous *C. tiglium* L. extract and d) CACO-2 treated with ZnO-Aqueous nano-extract.

Figure 5. Data of Apoptosis Assay with Annexin V-FITC showing a) Control CACO-2, b) CACO-2 treated with doxorubicin, c) CACO-2 treated with aqueous *C. tiglium* L. extract and d) CACO-2 treated with ZnO-Aqueous nano-extract.
hexagonal polycrystalline structure with approximately size ranged from 20 to 70 nm. The catalytic activity of the synthesized ZnO-NPs is strongly related to their hexagonal structure that implies more ionicity. The ZnO-NPs are well dispersed and some of them are irregular in their shapes. Furthermore, it was emphasized that the particles are almost spherical with a slight variation in thickness. The particle size that was determined by TEM technique is almost close to that obtained by XRD analysis. DLS is an accurate procedure to use a monodisperse dilute solution for measuring particle size ranged from 5 nm to 5 mm. Data of the DLS illustrated in Figure 1d, showed that the particle size distribution and the hydrodynamic size of the fabricated ZnO-NPs has main diameter around 164 nm.

The phyto-constituents in C. tiglium L. seeds extracts before and after incorporating ZnO-NPs

Data of GC/MS analysis of petroleum ether C. tiglium L. extract depicted in Table 1 showed that 38 compounds representing 88.53% were identified; where the identified lipoidal components consist of 17 hydrocarbons (41.21%), 9 fatty alcohols (23.0%), 7 aldehydes (18.21%), 3 ketones (5.27%) and 2 sterols (0.84%). It was found that n-octadecane, hentriacontane and tetracosane were the major identified hydrocarbons in petroleum ether extract representing 5.61, 7.04 and 7.59%, respectively.

Table 3. Antioxidant Activity of the Different Extracts of C. tiglium L. seeds before and after Incorporating ZnO-NPs

| Extract       | Total Polyphenols (mg gallic acid/100 gm) | Total Condensed Tannins (μg/mL) | Total Antioxidant Capacity (mg gallic acid/gm) | Iron Reducing Power (μg/mL) |
|---------------|------------------------------------------|--------------------------------|-----------------------------------------------|-----------------------------|
| Before        |                                          |                                |                                               |                             |
| Ethanolic     | 60.02 ± 1.37                             | 22.10 ± 0.18                   | 103.79 ± 1.37                                 | 98.94 ± 1.02                |
| Aqueous       | 124.44 ± 2.19*                           | 26.95 ± 0.12*                  | 140.53 ± 2.00*                                | 138.82 ± 4.51*              |
| P. Ether      | 22.71 ± 1.03                             | -                              | 75.76 ± 1.65                                  | 64.55 ± 1.42                |
| After         |                                          |                                |                                               |                             |
| ZnO-Ethanolic | 114.04 ± 2.61                            | 31.13 ± 0.09                   | 185.78 ± 2.44                                 | 178.09 ± 1.84               |
| ZnO-Aqueous   | 236.44 ± 4.16*                           | 36.93 ± 0.10*                  | 251.55 ± 3.59                                 | 249.88 ± 8.12*              |
| ZnO-P. Ether  | 43.15 ± 1.96                             | -                              | 135.61 ± 2.96                                 | 116.18 ± 2.55               |

* denotes the most effective extract, Values expressed as mean of three replicates ± SE.

Table 4. The 1,1-diphenyl-2-picyrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radicals Scavenging Activity of the Different Extracts of C. tiglium L. seeds before and after Incorporating ZnO-NPs

| Extract       | IC₅₀ (µg/mL) | Inhibition of ABTS Radicals (%) |
|---------------|-------------|--------------------------------|
| Before        |             |                                |
| Ethanolic     | 18.92 ± 0.05| 35.23 ± 0.04                   |
| Aqueous       | 12.91 ± 0.11*| 41.11 ± 0.07*                 |
| P. Ether      | 28.89 ± 0.06| 22.15 ± 0.12                   |
| After         |             |                                |
| ZnO-Ethanolic | 7.17 ± 0.03 | 51.78 ± 0.04                   |
| ZnO-Aqueous   | 3.78 ± 0.02*| 62.91 ± 0.10*                 |
| ZnO-P. Ether  | 11.23 ± 0.04| 38.99 ± 0.09                   |
| Ascorbic acid (Standard) | 3.83 ± 0.02 | 36.78 ± 0.03                   |

* denotes the most effective extract, Values expressed as mean of three replicates ± SE.
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6.54%, respectively. While 2-nonen-1-ol (12.25%) was considered as the main identified fatty alcohol. In addition, 9-octadecenal and 1-methyl-bicycloheptan-6-one are the most common aldehydic and ketonic compounds with the highest concentration representing 11.78 and 5.08%, respectively. Beside to identification of 2 steroidal compounds ergosterol and β-sitostero representing 0.45 and 0.39%, respectively.

It was worthy to mention that the phyto-constituents identified in petroleum ether extract were not changed or missed after incorporating ZnO-NPs, but their values increased. It was noticed that concentration of the total hydrocarbons in that extract elevated from 41.21 to 42.79%, total fatty alcohols increased from 23.0 to 24.87%. Additionally, the total aldehydes and ketones concentration raised from 18.21 to 19.78%, and

Table 5. Anti-Arthritic Activity of the Different C. tiglium L. seeds Extracts before and after Incorporating ZnO-NPs

| Extract          | Proteinase Denaturation (%) | Inhibition of Proteinase Activity (%) |
|------------------|-----------------------------|---------------------------------------|
| Before           |                             |                                       |
| Ethanolic        | 36.99 ± 0.48                | 31.91 ± 0.66                          |
| Aqueous          | 52.44 ± 0.67*               | 52.22 ± 0.42*                         |
| P. Ether         | 26.19 ± 0.66                | 32.28 ± 0.60                          |
| After            |                             |                                       |
| ZnO-Ethanolic    | 67.77 ± 0.39                | 52.46 ± 0.66                          |
| ZnO-Aqueous      | 78.99 ± 0.44*               | 75.17 ± 0.99*                         |
| ZnO-P. Ether     | 45.65 ± 1.11                | 46.75 ± 0.65                          |
| Diclofenac sodium (Standard) | 48.68 ± 0.24 | 40.46 ± 0.73 |

* denotes the most effective extract, Values expressed as mean of three replicates ± SE.

Table 6. Cytotoxic Activity of the Different C. tiglium L. Seeds Extracts against Human Liver (HEPG-2) and Colon Cancer (CACO-2) Cells before and after Incorporating ZnO-NPs.

| Extract          | IC<sub>50</sub> % HEPG-2 | IC<sub>50</sub> % CACO-2 |
|------------------|---------------------------|-------------------------|
| Before           |                           |                         |
| Ethanolic        | 671.3                     | 309.70                  |
| Aqueous          | 546.00*                   | 176.90*                 |
| P. Ether         | 954.3                     | 692.10                  |
| After            |                           |                         |
| ZnO-Ethanolic    | 451.5                     | 252.50                  |
| ZnO-Aqueous      | 314.40*                   | 100.20*                 |
| ZnO-P. Ether     | 902.8                     | 353.30                  |

* denotes the most effective extract, Values expressed as mean of three replicates ± SE.

Table 7. Data of the EGFR, Bcl2 and Casp3 Genes Expression in Human Colon Cancer (CACO-2) Cells Treated with Aqueous C. tiglium L. extract before and after Incorporating ZnO-NPs

| Extract                  | EGFR | Bcl2 | Casp3 |
|--------------------------|------|------|-------|
|                          | Conc. (µM) | GAPDH HC | GEPH TC | ΔΔCT | GAPDH HE | GEPH TE | ΔΔCT | FLD 2<sup>-ΔΔCT</sup> | Eamp=1.849 |
| Aqueous C. tiglium extract | 23.46 | 26.82 | 3.36 | 24.08 | 27.86 | 3.78 | 0.42 | 0.77 |
| ZnO-Aqueous nano-extract  | 23.46 | 26.82 | 3.36 | 23.82 | 28.55 | 4.73 | 1.37 | 0.43 |
| Doxorubicin              | 23.46 | 26.82 | 3.36 | 23.91 | 29.21 | 5.3  | 1.94 | 0.30 |
| Control                  | 23.46 | 26.82 | 3.36 | 23.46 | 26.82 | 3.36 | 0.00 | 1.00 |

| Extract                  | Casp3 | FLD     |
|--------------------------|-------|---------|
|                          | Conc. (µM) | GAPDH HC | GEPH TC | ΔΔCT | GAPDH HE | GEPH TE | ΔΔCT | FLD 2<sup>-ΔΔCT</sup> | Eamp=1.849 |
| Aqueous C. tiglium extract | 23.46 | 33.61 | 10.20 | 24.08 | 32.75 | 8.67 | -1.48 | 2.48 |
| ZnO-Aqueous nano-extract  | 23.46 | 33.61 | 10.20 | 23.82 | 31.62 | 7.80 | -2.35 | 4.24 |
| Doxorubicin              | 23.46 | 33.61 | 10.20 | 23.91 | 30.79 | 6.88 | -3.27 | 7.46 |
| Control                  | 23.46 | 33.61 | 10.20 | 23.46 | 33.61 | 10.2 | 0.00 | 1.00 |
from 5.27 to 5.33%, respectively. Beside to increasing concentration of sterols content from 0.84 to 0.88%. Increasing concentration of the lipoidal components was responsible for improving the biological activities by synergetic mechanism between all compounds of the petroleum ether extract.

Data of HPLC analysis compiled in Table 2 showed that the phenolic compounds that were identified in the Ethanolic and aqueous C. tiglium L. seeds extracts were about 14 and 11, respectively. Moreover, 4 flavonoidal compounds were noticed in both Ethanolic and aqueous extracts. It was noticed that catechin and vanillic acid were the most predominant phenolic compounds identified in the ethanolic C. tiglium L. extract and their concentration increased from 315.404 and 151.947 µg/g to 401.273 and 156.284 µg/g, respectively after incorporating ZnO-NPs. As regard to the aqueous C. tiglium L. extract, gentisic acid and chlorogenic acid were considered as the most common phenolic compounds and their concentrations increased from 99.581 and 112.869 µg/g to 99.984 and 117.352 µg/g, respectively after incorporating ZnO-NPs. On the other hand, rutin was identified as the major flavonoidal compound in both ethanolic and aqueous C. tiglium L. seeds extracts. Its concentration increased from 41.625 and 39.233 µg/g to 43.289 and 47.814 µg/g, respectively after incorporating ZnO-NPs.

In vitro study on different C. tiglium L. seeds extracts before and after incorporating zinc oxide nanoparticles

As presented in Table 3, total polyphenolic compounds and condensed tannins are the major active phyto-constituents in the different C. tiglium L. seeds extracts. The aqueous C. tiglium L. seeds extract contains the highest concentration of total polyphenolic compounds (124.44 ± 2.19 mg gallic acid/100 gm) and condensed tannins (26.95 ± 0.12 µg/mL) more than the other C. tiglium L. extracts. Incorporation of ZnO-NPs into the aqueous C. tiglium L. seeds extract increased concentrations of total polyphenolic compounds and condensed tannins to 236.44 ± 4.16 mg gallic acid/100 gm and 36.93 ± 0.10 µg/mL, respectively.

The biological activities of the different C. tiglium L. seeds extracts were assayed by measuring the total antioxidant capacity and iron reducing power in addition to the scavenging activity against DPPH and ABTS radicals using ascorbic acid as standard. The total antioxidant capacity and iron reducing power are closely related to concentrations of the total polyphenols and total condensed tannins. Therefore, it was noticed that the aqueous C. tiglium L. seeds extract exhibited the highest antioxidant activity (140.53 ± 2.00 mg gallic acid/gm) and iron reducing power (138.82 ± 4.51 µg/mL) as illustrated in Table 3. Incorporation of ZnO-NPs into the aqueous C. tiglium L. seeds extract increased the antioxidant activity and iron reducing power to 251.55 ± 3.59 mg gallic acid/gm and 249.88 ± 8.12 µg/mL, respectively.

As regard to the scavenging activity against DPPH and ABTS radicals, it was found that the aqueous C. tiglium L. seeds extract possessed the highest scavenging activity. Strong scavenging activity against DPPH radical was indicated by low IC\textsubscript{50} value. As presented in Table 4, the lowest IC\textsubscript{50} value (12.91 ± 0.11 µg/mL) was noticed with the aqueous C. tiglium L. seeds extract as compared to the other extracts. Moreover, it showed the highest scavenging activity against ABTS radical (41.11 ± 0.07%) at equal concentrations of all studied extracts, whereas at the same concentration, the standard ascorbic acid was 36.78 ± 0.03%. Incorporation of ZnO-NPs into the aqueous C. tiglium L. seeds extract increased the scavenging activity by lowering the IC\textsubscript{50} value required to inhibit DPPH radical to 3.78 ± 0.02 µg/mL and increasing inhibition of ABTS radical to 62.91 ± 0.10%.

As revealed in Figure 2a, it was found that the aqueous C. tiglium L. seeds extract exhibited the highest inhibitory effect at equal concentrations on α-amylase and AChE activities (41.89 and 23.00%, respectively). Incorporating ZnO-NPs into the aqueous extract increased their inhibitory effect against α-amylase and AChE activities to 67.97 and 49.91%, respectively (Figure 2b).

Data depicted in Table 5 showed percentage of protein denaturation and proteinase inhibitory activity. It was noticed that the aqueous C. tiglium L. seeds extract showed higher protein denaturation (52.44 ± 0.67%) and proteinase inhibitory activity (52.22 ± 0.42%) compared to diclofenac sodium that was used as a powerful non-steroidal anti-inflammatory standard drug. Incorporation of ZnO-NPs into the aqueous extract showed better protein denaturation (78.99 ± 0.44%) and proteinase inhibitory activity (75.17 ± 0.99%) than the reference drug (diclofenac sodium) and the native aqueous extract itself.

Data compiled in Table 6 and illustrated in Figure 3a showed that the aqueous C. tiglium L. seeds extract exhibited the highest cytotoxic activity (IC\textsubscript{50} 546.60 µg/mL) against HEPG-2 cells compared to the other native extracts. The Supplementary 1 showed the maximum (Conc. 1,000 µg/ml) and IC\textsubscript{50} of different native C. tiglium seeds extracts compared to control HEPG-2 cells. It was found that incorporation of ZnO-NPs into aqueous extract increased the cytotoxic activity against HEPG-2 cells compared to native aqueous extract itself (IC\textsubscript{50} 314.40 µg/mL). The Supplementary 2 showed the maximum (Conc. 1000 µg/ml) and IC\textsubscript{50} of different C. tiglium L. seeds extracts after incorporating ZnO-NPs compared to control HEPG-2 cells. As regard to the in vitro cytotoxic activity against CACO-2, data depicted in Table 6 and illustrated in Figure 3b showed that the aqueous C. tiglium L. seeds extract exhibited the highest cytotoxic activity (IC\textsubscript{50} 176.90 µg/mL) against CACO-2 cells compared to the other native extracts. The Supplementary 3 showed the maximum (Conc. 1,000 µg/ml) and IC\textsubscript{50} of different native C. tiglium L. seeds extracts compared to control CACO-2 cells. Incorporation of ZnO-NPs into aqueous extract elevated the cytotoxic activity against CACO-2 cells compared to native aqueous extract itself (IC\textsubscript{50} 100.20 µg/mL). The Supplementary 4 showed the maximum (Conc. 1000 µg/ml) and IC\textsubscript{50} of different C. tiglium L. seeds extracts after incorporating ZnO-NPs compared to control CACO-2 cells. In the present study, it was found that the cytotoxic activity of the aqueous C. tiglium L. seeds extract increased after incorporating ZnO-NPs.

As revealed in supplementary 5, it was found that treatment of CACO-2 cells with the aqueous C. tiglium L. seeds extract exhibited the highest cytotoxic activity (IC\textsubscript{50} 176.90 µg/mL) against CACO-2 cells compared to the other native extracts. The Supplementary 3 showed the maximum (Conc. 1,000 µg/ml) and IC\textsubscript{50} of different native C. tiglium L. seeds extracts compared to control CACO-2 cells. Incorporation of ZnO-NPs into aqueous extract elevated the cytotoxic activity against CACO-2 cells compared to native aqueous extract itself (IC\textsubscript{50} 314.40 µg/mL). The Supplementary 2 showed the maximum (Conc. 1000 µg/ml) and IC\textsubscript{50} of different C. tiglium L. seeds extracts after incorporating ZnO-NPs compared to control HEPG-2 cells. As regard to the in vitro cytotoxic activity against CACO-2, data depicted in Table 6 and illustrated in Figure 3b showed that the aqueous C. tiglium L. seeds extract exhibited the highest cytotoxic activity (IC\textsubscript{50} 176.90 µg/mL) against CACO-2 cells compared to the other native extracts. The Supplementary 3 showed the maximum (Conc. 1,000 µg/ml) and IC\textsubscript{50} of different native C. tiglium L. seeds extracts compared to control CACO-2 cells. Incorporation of ZnO-NPs into aqueous extract elevated the cytotoxic activity against CACO-2 cells compared to native aqueous extract itself (IC\textsubscript{50} 314.40 µg/mL).
L. seeds extract incorporated with ZnO-NPs decreased the fold changes of EGFR and Bcl2 genes (0.43 and 0.41, respectively) compared to the native aqueous extract itself (0.77 and 0.62, respectively). As regard to Casp3 gene, the aqueous C. tiglium L. seeds extract incorporated with ZnO-NPs increased its fold changes (4.24) more than the native aqueous extract itself (2.48). Treatment of CACO-2 cells with the native aqueous C. tiglium L. seeds extract or the aqueous extract incorporated with ZnO-NPs arrested the cell growth at G2/M compared to doxorubicin that arrested the cell growth at G1/S (Supplementary 6). As illustrated in Supplementary 7, treatment of CACO-2 cells with the aqueous C. tiglium L. seeds extract incorporated with ZnO-NPs increased percentage of the total apoptotic cells (29.12%) and increased percentage of the early (4.52%) and late apoptotic cells (13.27%) as compared to control CACO-2 or those cells treated with the native aqueous extract itself. Moreover, percentage of the necrosis increased in the CACO-2 cells treated with ZnO-aqueous nano-extract (11.33%) more than those cells treated with the native aqueous extract (7.26%).

Data presented in Table 7 showed that incorporation of ZnO-NPs into the aqueous C. tiglium L. seeds extract caused on significant changes in expression of the EGFR, Bcl2 and Casp3 genes in the treated CACO-2 cells as compared to control HEPG-2 or those cells treated with the native extract itself.

Data of the flow cytometric analysis presented in Figure 4 and using Annexin V-FITC as shown in Figure 5 showed that treatment of CACO-2 cells with the native aqueous C. tiglium L. seeds extract or the aqueous extract incorporated with ZnO-NPs enhanced apoptosis as compared to control CACO-2 cells.

Toxicity of different C. tiglium L. seeds extracts before and after incorporating ZnO-NPs

It was noticed that the LD_{50} values of ethanolic, aqueous and P. ether extracts were about 7667, 8083 and 5500 mg/Kg, respectively. After incorporating ZnO-NPs, the LD_{50} values increased to 11000, 11333 and 9500 mg/Kg, respectively (Figure 6).

Discussion

The nutritional composition of C. tiglium L. seeds

During the current study, it was noticed that C. tiglium L. seeds provided great values and could be consumed for either nutritional or medicinal purposes which in agreement with Owade et al. (2019) and supported by Aboulthana et al. (2019) who stated that C. tiglium L. seeds have been utilized mainly for its oil, protein and polysaccharide contents.

The major compounds isolation from different C. tiglium L. seeds extracts

During the present study, the steroidal (β-sitosterol and stigmasterol) and the pentacyclic triterpenoid (α-amyrin) compounds were identified and isolated in pure form and this was in agreement with the study carried out by Smina et al. (2011). Their spectroscopical data were in agreement with that reported by El-Feky et al. (2018). Furthermore, isopimara-7,15-dien-3β-ol is diterpene compound isolated from leaves of Croton zambesicus by Block et al. (2004) and not identified before from C. tiglium L. seeds. Although numerous quercetin derivatives were identified by Guerrero et al. (2002) from Croton schiedeanus L. quercetin-7-O-β-D-glucopyranoside was isolated and identified for the first time from C. tiglium L. seeds during the present study. In the current experiment, 13-O-Acetylphorbol-20-(9Z,12Z-octadecadienoate) and 13-O-Tigloylphorbol-20-(9Z,12Z-octadecadienoate) were isolated and identified. These two phorbol esters were previously isolated from C. tiglium L. seeds by El-Mekkawy et al., (2000).

The structural properties of prepared ZnO-NPs

The biosynthesized ZnO-NPs was identified as sharp peak identified in the UV-visible spectrum at 382 nm and this agreed with the experiment carried out by Kavitha et al. (2013) who reported that the peak at 280 nm corresponds to the plant extract. Moreover, the strong absorption band that was assigned to the intrinsic band-gap absorption of ZnO might be attributable to transition of the electron from the valence to conduction band (Khorsand Zak et al., 2013). The DLS showed that the zeta potential denotes repulsion degree between adjacent particles (with similar charges) in dispersions (Hassan et al., 2019). Moreover, the particle size distribution and the hydrodynamic size of the fabricated ZnO-NPs has main diameter around 164 nm. This agreed with Kim et al. (2012) who reported that the synthesized ZnO-NPs can form aggregates (lumps of primary particles held together by strong chemical bonds) or agglomerates (groups of primary particles gathered by weak van der Waals forces) in a liquid dispersion of dry powder.

In vitro study on different C. tiglium L. seeds extracts before and after incorporating ZnO-NPs

Due to the presence of various active bio-components like alkaloids, terpenoids, phenolics, tannins, saponins, polysaccharides, proteins, enzymes and vitamins, plant extract can be used as a potential substitute for reducing agents (Ahmed et al., 2016). It was found that the aqueous C. tiglium L. seeds extract is in the highest concentrations of total polyphenolic compounds and condensed tannins more than the other C. tiglium L. extracts. The phenolic compounds and flavonoids are secondary metabolites exist in almost all medicinal plants. Antioxidative and anti-carcinogenic activities are considered as the most common biological activities of these phyto-constituents in addition to their role as bio-reductants of metallic ions in aqueous medium (Yuvakkumar et al., 2014). This might be attributed to the presence of the functional groups responsible for the bio-reduction and stabilization process during biosynthesis of metal and metal oxide NPs (XiuLan et al., 2017). During the ZnO-NPs biosynthesis, phenol and flavonoids in plant extract react with zinc nitrate by binding zinc surface to control the particle size and this agreed with the experiment carried out by Mekkawy et al., (2000).
It was noticed that aqueous C. tiglium L. seeds extract exhibited the highest antioxidant activity and this might be attributed to the complex nature of the phenolic compounds and the other phytochemicals that have redox properties enable them to act as hydrogen donors and hence to be used as reducing agents. The plant extracts possessed higher scavenging activities against the oxidative stress induced by Reactive Oxygen (ROS) and Nitrogen Species (RNS) due to the presence of these active constituents with high concentrations as suggested by Kalim and Nikolaj (2017).

DPPH and ABTS free radical scavenging assays provide an easy and rapid methods for estimating ability of the antioxidants to scavenge free radical based on quenching stable-colored radicals (Zia-Ul-Haq et al., 2012). During the current study, it was noticed that the scavenging activity of the extract incorporated with ZnO-NPs increased and this was confirmed by decreasing the absorbance at 517 nm as a result of changing color of the reaction product from purple to yellow. This agreed with Nagajyothi et al. (2015) who emphasized that the antioxidant activity of the extract increased after incorporating ZnO-NPs due to presence of the active phyto-constituents that were responsible for imparting antioxidant capabilities and utilized throughout manufacturing of ZnO-NPs for their reduction and stabilization.

α-amylase is the most essential digestive tract enzyme required for hydrolyzing carbohydrates. Therefore, inhibition of this enzyme is one of the most suitable ways for lowering postprandial hyperglycemia (Nair et al., 2013). Moreover, AChE enzyme exhibits its role in tissue synapses or neuromuscular junctions through catalyzing the hydrolysis process during which acetyl choline (a neurotransmitter) is converted into choline and acetic acid. Therefore, activation of this enzyme is one of leading cause of Alzheimer’s disease. Inhibition of this enzyme is one of the suitable ways necessary for Alzheimer treatment (Suganthly et al., 2018). It was found that incorporation of ZnO-NPs into the aqueous extract increased their inhibitory effect against α-amylase and AChE activities and this agreed with Jan et al., (2021) who emphasized that existence of ZnO-NPs in aqueous C. tiglium L. seeds extract showed higher anti-diabetic properties through suppressing α-amylase activity. This might be attributed . The biosynthesized ZnO-NPs exhibited efficient anti-Alzheimer’s activity by inhibiting AChE activity and this agreed with Khalil et al. (2019) who reported that ZnO-NPs belongs to the M-NPs that suppressed AChE followed by cobalt oxide and iron oxide nanoparticles.

Inflammation is a response process by which living tissues react towards injury. It is considered as the most common phenomenon that occurs during arthritis and induced by denaturation of protein. Inflammation and activity of immune response decreased by steroids (Nagajyothi et al., 2014). It was found that the aqueous C. tiglium L. seeds extract showed higher protein denaturation and proteinase inhibitory activity. Incorporation of ZnO-NPs into the aqueous extract showed better protein denaturation and proteinase inhibitory activity than the native aqueous extract itself. This agreed with Senthilkumar et al. (2017) who revealed that the presence of ZnO-NPs enhanced the anti-arthritis activity of the extract due to their capability for inhibiting protein denaturation and proteinase inhibitory activity. This might be related to increasing the scavenging activity against the free radicals that are critically involved in arthritis and inflammation.

Cell viability assays are considered as basic criteria to reveal the cellular response to toxic substances and to elucidate efficiency of the extract against incidence and progression of cancer cells by the MTT assay. A calcein (green fluorescent compound) that produced from calcein AM as a result of role of active esterase exists with intact membranes in living cells serves as a marker for viable cells. Moreover, there is red fluorescent nucleic acid stain called propidium iodide (PI) used for detecting the damaged cells that take up the dye and stain positive and it has no the ability to penetrate normal cells (Sanlioglu et al., 2007). Studying efficiency of the anti-cancer potential of ZnO-NPs against growth and progression of cancer cells belongs to scientific hotspots for cancer therapy (Vimala et al., 2014). During the current study, the in vitro cytotoxicity of different C. tiglium L. seeds extracts was studied after incorporating ZnO-NPs against HEPG-2 and CACO-2 in comparison with native extracts.

Data of the current study showed that the cytotoxic activity of the aqueous C. tiglium L. seeds extract increased after incorporating ZnO-NPs and this agreed with the study carried out by Modena et al. (2019) who emphasized that the cytotoxic activity might be attributed to presence of alcohols and phenolic groups in addition to C-N stretching vibrations of aromatic amines of biomolecules on the surface of the biosynthesized ZnO-NPs that physiologically act on inhibiting growth of cancer cells. Furthermore, the plant-derived ZnO-NPs showed an exciting potential as promising anti-cancer agents by enhancing the cellular death through production of the ROS that disrupt signal transduction pathways (Ismail et al., 2018). When ZnO-NPs get entry into the cancerous cell, they produced ROS species, disturbed depolarization of the membrane and damaged DNA; all these events eventually leads to apoptosis or death of cancer cell (Jan et al., 2020). During the current study, it was noticed that the aqueous C. tiglium L. seeds extract showed better cytotoxic activity against CACO-2 than HEPG-2 cells. Therefore, it was selected for undergoing further studies on CACO-2 cells before and after incorporating ZnO-NPs.

Data of the flow cytometric analysis showed that treatment of CACO-2 cells with the native aqueous C. tiglium seeds extract or the extract incorporated with ZnO-NPs enhanced apoptosis as compared to control CACO-2 cells. This agreed with Jan et al., (2021) who postulated that ZnO-NPs showed prominent apoptosis after for 24h treatment. The cytotoxic activity of ZnO-NPs against growth and progression of human cancer cells occurred through three primary mechanisms including breakdown of the ZnO-NPs into Zn²⁺, ROS production and DNA damage. In addition, the physical properties (size, surface chemistry and dose) of the ZnO-NPs dictate their overall uptake, elimination and antitumor
properties (Chen et al., 2019). Also, ZnO-NPs have the ability to induce apoptosis in CACO-2 cells by oxidative stress leading to cytotoxicity, inflammatory responses, mitochondrial membrane alterations and release of interleukin-8 in cancerous cells (Jain et al., 2018). It was demonstrated that ZnO-NPs showed their anticancer and antiproliferative effect via up-regulating the apoptotic and tumor suppressor genes, down-regulating the antiapoptotic genes, inducing ROS production, DNA fragmentation and caspase-3 enzyme in cancer cells (Ismail et al., 2014; Iswarya et al., 2017).

Toxicity of different C. tiglium L. seeds extracts before and after incorporating ZnO-NPs

It was found that administration of the different C. tiglium L. seeds extracts incorporated with ZnO-NPs orally was safer than the native extracts. This was agreement with Shaban et al., (2021) and supported recently by Aboulthana et al., (2022) who reported that no significant alterations detected in the biochemical markers after oral administration of M-NPs. Biodistribution of the M-NPs in organs and tissues depends highly on size and dose of the M-NPs. Size of the M-NPs is considered as the most important factor affecting their biodistribution. Ratio of the large surface area to volume of the NPs stimulates their interactions with the macromolecules in biological systems (Wang et al., 2020). Biosynthesis of M-NPs by mean of green nanotechnology found with less toxicity and it increased safety of plant extract in which M-NPs by mean of green nanotechnology found with less toxicity and it increased safety of plant extract in which M-NPs incorporated (Aboulthana et al., 2020). This was attributed to efficiency the renal tissue to eliminate the M-NPs with low degradation rate in the body to avoid the undesirable side effects (Aboulthana et al., 2019).

Author Contribution Statement

Wael M. Aboulthana: Writing – original draft, Writing – review & editing. Nagwa I. Omar: Resources, Data curation, Writing – review & editing. Amal M. El-Feky: Methodology, Validation, Formal analysis, Writing – review & editing. Enas A. Hasan: Formal analysis, Writing – review & editing. Noha E. Ibrahim: Literature collection, Writing – review & editing. Ahmed M. Youssef: Conceptualization, Data curation, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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Ethical statement

Handling of the animals and the experimental design were carried out based on the guidelines reported in “Guide for the care and use of laboratory animal” and according to the protocol that approved by Institutional Animal Ethical Committee of National Research Centre, Dokki, Giza, Egypt.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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