RESEARCH ARTICLE

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Metabolite Profiling and Biological Activity Assessment of *Casuarina equisetifolia* Bark after Incorporating Gold Nanoparticles

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Abstract

Objective: *Casuarina equisetifolia* bark is rich in various active metabolites and selected to be studied due to limitation of the synthetic antioxidants that have adverse side effects. The present study aimed to enhance efficiency of the most effective extract by incorporating gold nanoparticles (Au-NPs). **Methods:** The phytochemical and biological measurements were carried out in total methanolic extract and its successive fractions. Moreover, these measurements were assayed in the most effective extract after incorporating Au-NPs. **Results:** The study revealed that total methanolic extract exhibited the highest biological and cytotoxic activities as compared to other fractions. Therefore, it is considered as good candidate for nano-extract preparation. The methanolic extract incorporated with Au-NPs showed higher antioxidant, scavenging and cytotoxic activities in addition to higher inhibitory effect against α-amylase activity as compared to native extract itself. To pinpoint active agents in total methanolic extract, the secondary metabolite profiling via HPLC-MS showed that 33 and 17 metabolites were annotated in the extract before and after incorporating Au-NPs, respectively. The median lethal dose (LD_{50}) showed that gold total methanolic nano-extract is safer than total methanolic extract. **Conclusion:** This study concluded that total methanolic *C. equisetifolia* bark extract is a valuable bioresource to synthesize an eco-friendly Au-NPs with health-enhancing effect as antioxidant, antidiabetic and cytotoxic agents. The present study is considered as the first report on utilization of *C. equisetifolia* bark in synthesis of Au-NPs by mean of green nanotechnology and investigation of its biological activity in relation to its metabolite fingerprint.

Keywords: *Casuarina equisetifolia* Bark- Phenolics- Antioxidant Capacity- Cytotoxic Activity- Metabolomics

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Introduction

Herbal medicine attracted the attention and gained the acceptance from the public and scientific professionals (Panda and Naik, 2009). The plant extracts exhibit vital role as antioxidants in reducing the tissue injuries induced by attack of the reactive oxygen species (ROS) (Erdemoglu et al., 2006). In recent years, many studies aimed to reveal efficiency of plant extracts against the tissue injuries induced by oxidative stress. It was found that the black tea (Khan et al., 2005), *Paronychia argentea* (Zama et al., 2007), curcumin (Canales-Aguirre et al., 2012), root of green tea (Mehri et al., 2016), different plant oils (Al-Attar et al., 2017) and Ginkgo biloba extracts are rich in various bioactive compounds that exhibited efficiency against the oxidative stress and the peroxidation reaction through restoring the antioxidant system (Hajirezaee et al., 2019).

Casuarina equisetifolia bark contained various active phytoconstituents including alkaloids, steroids, triterpenoids, proteins, saponins, phenolics, glycosides, tannins and flavonoids in addition to reducing sugars (Kishore and Rumana, 2014; Al-Snafi, 2015). It is rich in condensed tannins consisting predominantly of procyanidin. The epicatechin was the main extension unit in polymer varying from trimers to tridecamer in chain length (Gumgumjee and Hajar, 2012). Presence of these active constituents enhanced the pharmacological activities of the *C. equisetifolia* bark extract. It showed antimicrobial (Moazzem Hossen et al., 2014), gastroprotective (Shalini and Kumar, 2011), nephroprotective (El-Tantawy et al., 2013), hepatoprotective (Sriram, 2011), antidiabetic and antihyperlipidemic activity (Kantheti et al., 2014). This was in addition to the scavenging activity against the deleterious effect induced by ROS (Gumgumjee and Hajar, 2012). Most of the active phytoconstituents have

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limited ability to cross the cellular membrane due to their high molecular weights. This consequently leads to decreasing their bioavailability and efficacy. Absorption of these biologically active components was accelerated by integrating the traditional medicinal plants with nanotechnology to reduce their toxicity and enhance their bioavailability (Mamillapalli et al., 2016).

The practical applications of plant extracts increased due to increasing their valuable properties as a result of incorporating metal nanoparticles (M-NPs) into their polymeric matrices and this is considered as the most promising solution for suppressing their inherent stability problem (Rozenberg and Tenne, 2008). Moreover, inclusion of metal nanoparticles showed a higher antioxidant activity at lower concentrations compared to plant extract itself (Johnson et al., 2014 ; Abdel-Aziz et al., 2014). Gold nanoparticles (Au-NPs) are characterized by their unique surface plasmon resonance (SPR) that increases their biomedical applications. Therefore, they have been widely studied. It is interesting for materials scientists, chemists and biologists to find cost-effective and eco-friendly procedures for Au-NPs synthesis especially in light of the efforts to find greener methods of inorganic material synthesis (Spadavecchia et al., 2016; Melani et al., 2017). The oxidized citric acid becomes stabilizing agent during Au-NPs synthesis by mean of green nanotechnology through the Turkevich method (Turkevich, 1985). Similarly, tannic and ascorbic acids (Sau and Murphy, 2004; Ahmad, 2014) and different polysaccharides (Park et al., 2011) can be used during Au-NPs synthesis as capping or reducing agent to reduce Au (III) ions. From this point of view, the present study was designed to appraise the in vitro biological activity for selecting the most effective *C. equisetifolia* bark extract to be utilized for Au-NPs synthesis and hence to compare the biological activities of the extract before and after incorporating Au-NPs.

Materials and Methods

Preparation of plant extracts

Fresh bark of *C. equisetifolia* Linn (*Casuarinaceae*) was collected during Sept. 2020 from the Research and Production Station Unit in El-Nubaria, Egypt and thoroughly washed by double deionized water to remove surface impurities, then subjected to air dryness for 3 weeks. The dried plant material was weighed, chopped and crushed in an electric blender into powder. The air-dried powdered plant bark (1.9 Kg) was soaked in methanol (70%) until complete exhaustion then filtered through a *Whatman No. 1* filter paper. The filtrate was concentrated under reduced pressure at 55°C till complete dryness (208g). The dried methanolic residue (45g) was suspended in least amount of distilled water (200 mL) and extracted successively with petroleum ether, dichloromethane, ethyl acetate and followed by n-butanol. The extracting solvents were evaporated under vacuum.

Phytochemical screening test

The air-dried bark powder was subjected to phytochemical screening in order to detect classes of the chemical constituents responsible for the main biological activities of the plant. Carbohydrates and /or glycosides (Molisch, 1886), flavonoids (free and combined) (Seikel, 1962), coumarins, saponins, alkaloids and/ or nitrogenous compounds, sterols and /or triterpenes (Wagner et al., 1984), tannins (Smith, 1960) and anthraquinones (free and combined) (Wallis, 1967) were determined in the methanolic extract and its successive polar and non-polar fractions.

Quantitative determination of major phytoconstituents
Total polyphenolic content

Concentration of the total polyphenols was quantified in the different *C. equisetifolia* bark extracts as mg gallic acid/100 gm using Folin Ciocalteu reagent based on the method suggested by Singleton and Rossi (1965).

Total tannin content

Content of total tannin was assayed in the total methanolic extract and its polar fractions (ethyl acetate and butanol) using tannic acid as a reference compound according to the method described by Broadhurst and Jones (1978).

In vitro biological activities

All biological activities were assayed in total methanolic *C. equisetifolia* bark extract and its successive fractions to select the most effective one to be incorporated with gold nanoparticles (Au-NPs). All analyses were carried out in three replicates.

Total antioxidant capacity

Total antioxidant capacity (TAC) was determined by evaluating the green phosphate/Mo^{5+} complex at wavelength 695 nm according to the method suggested by Prieto et al., (1999). It was expressed as mg gallic acid equivalent per gram dry weight.

Iron reducing power

Total iron reducing power was assessed as µg/mL using ascorbic acid as standard by the method described by Oyaizu (1986). A high absorbance of the reaction mixture at 700nm indicates a higher reducing power.

Free radical scavenging activities

The scavenging activities of total methanolic *C. equisetifolia* bark extract and its successive fractions were assessed by measuring ability of each fraction to scavenge the free radicals using spectroscopic method. The 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity was assayed by calculating percent of the DPPH free radical inhibition (%) and the median inhibitory concentration (IC_{50}) according to the method suggested by Rahman et al. ,(2015). The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was carried out to determine the ABTS scavenging activity using ascorbic acid as standard according to the modified method described by Arnao et al., (2001).

Alpha-amylase inhibitory assay

The α-amylase inhibitory assay of total methanolic

C. equisetifolia bark extract and its successive fractions was carried out using acarbose as standard drug for calculating percentage of α-amylase inhibition (%) using the 3.5-dinitrosalicylic acid (DNSA) method (Wickramaratne et al., 2016).

Cytotoxic activity

Cytotoxic activity tests (In vitro bioassay on human hepatocellular (HEPG-2) and colon carcinoma (CACO-2) cell lines) were conducted and determined based on the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay suggested by Vichai and Kirtikara (2006). The optical density (OD) was determined at 570 nm by a microplate ELISA reader. Percent of cell-growth inhibition (%) and the IC_{50} were calculated using IC_{50} calculation software.

Synthesis of gold nanoparticles (Au-NPs)

Based on total polyphenolic and tannin contents with the in vitro antioxidant activity (total antioxidant capacity, iron reducing power and scavenging activities against free radicals) and the cytotoxic activity that were assayed in total methanolic *C. equisetifolia* bark extract and its successive fractions, it was found that the total methanolic extract was the most active. Therefore, it was selected to be incorporated by Au-NPs.

Preparation of gold nanoparticles (Au-NPs)

The Au-NPs were fabricated via the chemical reduction method according to the method suggested by Zhao et al., (2013) through two main parts. The first part was concerned with reduction of Au^{+3} ($HAuCl_4$) to Au^0 by the reaction with trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) in an aqueous solution. The second part was the stabilization using cetyltrimethylammonium bromide (CTAB) in order to avoid aggregation of the particles.

Preparation of gold C. equisetifolia nano-extract

Nanoemulsion was prepared using crude plant extracts, non-ionic surfactant Tween 20 (HLB-16.7), cellulose nanocrystal (CNC) and water via spontaneous emulsification method. It was carried out in two steps. In the first step, organic phase was fabricated through mixing plant crude sample with the chosen surfactant (Tween 20) at the ratio 1:5 then 3 gm of CNC was added and the mixture was sonicated for 30 min. In the second step, organic phase (plant extract, Tween 20 and CNC) was added drop by drop (20 mL/min) to water using separating funnel and stirring the system magnetically at 800 rpm (60°C) for 5 hrs. Then, the prepared Au-NPs were added to the prepared nanoemulsion at the ratio 1%. The mixture was sonicated at 50 °C for another 30 min.

Characterization of biosynthesized nanoparticles

The crystalline nature and grain size of the synthesized Au-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) at a temperature of 25-28 ºC. The analysis scans were carried out during run in 2θ range of 5 to 80º with step time of 1s and step size of 0.02. Shape and size of the synthesized M-NPs was 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 $600X10^3$ and a resolution until 0.2 nm. The Au-NPs spectra were assayed by Shimadzu UV-VIS recording spectrophotometer UV-240 at λ 200 - 800 nm after diluting the samples (10-fold) with deionised water. The average hydrodynamic size of the synthesized Au-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) at room temperature with a detection angle of 90° after diluting the samples with double distilled water prior to analysis.

Identification of active metabolites in total methanolic extract before and after incorporating gold nanoparticles (Au-NPs)

Fourier Transform Infrared Spectroscopy (FT-IR) analysis

FT-IR analysis was performed using FT-IR technique manufactured by Bruker based on the method documented by Weigel et al. (2004). The absorbance and transmittance were estimated against blank then the transmittance percent (Trans.%) and relative intensities (Int.%) were calculated.

Metabolite profiling via High Performance Liquid Chromatography/Mass Chromatography (HPLC/MS) analysis

Sample preparation

Sample was prepared by adding 1 mL the reconstitution solvent (DI-Water: Methanol: Acetonitrile - 50: 25: 25) to 50 mg of weighed sample and mixed by vortex for 2 min and by ultra-sonication (10 min) then centrifuged at 10,000 rpm (10 min). Making injected concentration 1µg/µL, 50 µL of the stock was diluted to 1000 µL with reconstitution solvent. Sample (10 µL) was injected on negative mode and the reconstitution solvent (10 µL) was injected as a blank sample.

LC/QTOF-MS-MS (SCIEX)

LC separation was achieved utilizing SCIEX HPLC system that is made up of a precolumn (In-line filter disks) (Phenomenenx 0.5 µm x 3.0 mm) and column (X select HSS T3) (Waters, 2.5 µm, 2.1x150 mm) coupled to Mass Spectrometric detection that performed with a quadrupole-TOF-MS and worked with the negative mode with a Duo Spray ion source. The acquisition software (Analyst TF 1.7.1, AB SCIEX) was used during the detection process, using 35 V Collision Energy (CE) and ± 15 V Collision Energy Spread (CES). Each sample was injected three times randomly using negative polarity. A solvent system involves solvent A (5 mM ammonium formate buffer pH 8 containing 1% methanol) and solvent B (100 % acetonitrile) and full run with flow rate 0.3 mL/min for 28 min was carried. Samples (10 µL) were injected and set at 40°C.

Processing and statistical analysis of data

An HPLC-QTOF full MS record was processed (data

mining, alignment and normalization) using MasterView software (Version 1.2.1, AB SCIEX). Furthermore, the high-resolution MS and MS/MS mass spectra obtained within HPLC-MS analyses were evaluated to estimate elemental formulae of pre-selected marker compounds. Featuring peak extraction from total ion chromatogram (TIC) was performed using MasterView (Version 1.0 with Formula Finder plugin version 1.0, AB SCIEX) based on Non-targeted analysis (Signal-to-Noise ration greater than 5) and that intensities of sample-to-blank ratio should be more than 3.

MS DIAL for data analysis

Both MS-DIAL (Version 3.52) and ReSpect Negative data base were utilized, and free latest versions of these programs were available on website (RIKEN PRIMe) with the identification score cut off 70%.

Determination of the median lethal dose (LD_{50})

The LD_{50} of total methanolic *C. equisetifolia* bark extract (the most effective) before and after incorporating Au-NPs was evaluated based on the methods suggested by Paget and Barnes (1964). Seventy-two adult albino mice (weight 20-25 g) were divided into 6 groups (6 mice in each group) for calculating the LD_{50} of total methanolic extract and 6 groups for that of gold nano-extract. The groups were treated orally by stomach tube with increasing the doses (2000, 4000, 6000, 8000, 10,000 and 12,000 mg/Kg). Number of dead mice was recorded after 24 hrs of extract administration.

Results

Percentage yields, physical and chemical investigations of total methanolic C. equisetifolia bark extract and its successive fractions

All extracts were evaporated to dryness under vacuum at 40 oC before undergoing the phytochemical screening. The dried residue yields with their percentages, physical and chemical properties were presented in Supplementary Table 1. It was found that the total methanolic extract and its polar fractions (ethyl acetate and butanol) are rich in carbohydrates, saponins, tannins and flavonoids. Sterols and/or triterpenes were noticed highly in the non-polar fractions (P. ether and dichloromethane). Coumarins were noticed only in dichloromethane fraction. The total methanolic extract and its successive (polar and non-polar) fractions contain no alkaloids, anthraquinones, cardiac glycosides or volatile constituents.

Quantitative determination of major phytoconstituents

Preliminary phytochemical screening revealed that polyphenolic compounds especially tannins are the major phytoconstituents in *C. equisetifolia* bark. As a result, total phenolic and tannin contents were quantified. Data depicted in Table 1 showed total polyphenolic and tannin contents of total methanolic extract of *C. equisetifolia* bark and its successive fractions and it was found that total methanolic extract contains the highest concentration of total polyphenols (131.39 ± 0.24 mg gallic acid/100 gm) and total tannin (13.10 ± 0.01 µg/mL).

In vitro biological activities of C. equisetifolia bark

The previous literature reported that *C. equisetifolia* bark is a good source of phenolic compounds which were well known by their antioxidant activity (Chen et al., 2007), antidiabetic (Kifle et al., 2020) and cytotoxic activity (Bruyne et al., 1999). The biological activities of total methanolic *C. equisetifolia* bark extract and its successive fractions were measured by assaying total antioxidant capacity and iron reducing power in addition to DPPH and ABTS radicals scavenging activity using ascorbic acid as standard. Data compiled in Table 1 showed the antioxidant activity of total methanolic extract of *C. equisetifolia* bark and its successive fractions and it was found that total methanolic extract possessed the highest total antioxidant capacity (275.64 ± 0.37 mg gallic acid/gm) and iron reducing power (240.31 ± 0.66 µg/mL) as compared to the other successive fractions.

The antioxidant activity of total alcoholic extract and successive fractions of *C. equisetifolia* bark was carried out by assaying their DPPH and ABTS radicals scavenging activities that are expressed as IC_{50} values. Strong antioxidant activity was indicated by low IC_{50} value (Table 2). Among the extractives, total alcoholic extract, ethyl acetate and butanol fractions possessed the highest activity. The phenolic compounds are characterized by their redox properties that enable them to act as hydrogen donors and hence to be used as reducing agents. Presence of these active constituents in plant extracts increased their scavenging activities against both Reactive Oxygen (ROS) and Nitrogen Species (RNS) (Kalim and Nikalje, 2017 ; Abdel-Aziz et al., 2014). At equal concentration (100 µg/mL), the scavenging activities of total methanolic, P. ether, dichloromethane, ethyl acetate and butanol were 82, 73.6, 81.2, 87.7 and 82 %, respectively, whereas at the same concentration, the standard ascorbic acid was 94.7 %. The IC_{50} values of total methanolic, P. ether, dichloromethane, ethyl acetate and butanol were 4.7, 71, 42, 4.8 and 5.3 µg/mL, respectively. The IC_{50} of the standard ascorbic acid was 3.9 µg/mL. Total methanolic extract exhibited the strongest antioxidant activity followed by the polar fractions (ethyl acetate and butanol). They showed over 50% scavenging effect against DPPH at concentrations of 4.7, 4.8 and 5.3 µg/mL, respectively. On the other hand, non-polar fractions (P. ether and dichloromethane) rendered the weakest hydrogen-donating capacity.

As regard to the scavenging activity against ABTS radical, it was found that total methanolic extract possessed the highest scavenging activity (23.97 ± 0.14 %) as compared to successive fractions followed by butanol (17.92 ± 0.10%) at equal concentrations of total methanolic extract and its successive fractions. Dichloromethane fraction showed low scavenging activity (9.06 ± 0.10%), whereas at the same concentration, the standard acorbic acid was 35.07 ± 0.06% (Table 3).

Herbal plants are well known by the presence of a variety of active biomolecules that serve as antioxidants. Flavonoids, tannins and coumarins belong to phenolic antioxidants that scavenge radicals dose-dependently, thus they attracted the attention to be used as promising therapeutic drugs against the pathological lesions

Table 1. Major Phyto-constituents and Antioxidant Activity of Total Methanolic *C. equisetifolia* Bark Extract and Its Successive Fractions.

Extract	Total Polyphenols (mg gallic acid/100 gm)	Total Tannins (µg/mL)	Total Antioxidant Capacity (mg gallic acid/gm)	Iron Reducing Power (µg/mL)
Total methanolic	131.39 ± 0.24*	13.10 ± 0.01*	275.64 ± 0.37*	240.31 ± 0.66*
Petroleum ether	41.06 ± 0.15	-	42.57 ± 0.28	50.06 ± 0.93
Dichloromethane	46.17 ± 0.16	-	72.95 ± 0.07	86.45 ± 1.73
Ethyl Acetate	76.52 ± 0.41	4.69 ± 0.01	253.27 ± 1.29	96.69 ± 0.48
Butanol	107.30 ± 1.23	8.17 ± 0.01	257.48 ± 0.79	217.05 ± 0.62

Table 2. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity and the Median Inhibitory Concentration (IC_{50}) of Total Methanolic *C. equisetifolia* Bark Extract and Its Successive Fractions

	Conc. (µg/mL)								IC_{50} (µg/mL)
	100	50	25	12.5	6.25	3.125	1.56	0.78	
Total methanolic	82.0	81.2	80.9	78.6	64.5	33.9	16.2	7.7	4.7
Petroleum ether	73.6	32.1	17.7	8.9	-	-	-	-	71
Dichloromethane	81.2	58.3	33.0	18.9	-	-	-	-	42
Ethyl acetate	87.7	86.0	83.0	78.3	63.4	32.8	17.5	6	4.8
Butanol	82.0	80.9	79.0	78.3	56.0	34.7	26.5	8.5	5.3
Ascorbic acid (Standard)	94.7	94.3	94.0	93.8	64.1	45.1	32	24.6	3.9

induced by oxidative stress and free radicals attack (Chen et al., 2007). Moreover, Zhang et al., (2010) discussed the antioxidant effect of the *C. equisetifolia* bark which proved good ferric reducing power and scavenging activity against DPPH radical due to presence of condensed tannins from bark of this plant and this proposed that this extract could be an excellent nutrient antioxidant source in food and nutraceutical products. As suggested by Huang et al., (2005), the phenolic antioxidants exhibit their effect on DPPH due to their ability to donate hydrogen. Radical scavenging activities play an effective role in preventing different diseases (i.e. diabetes and cancer) that occurred as a result of attack of free radicals. The scavenging activity against DPPH radical is the most suitable mechanism for screening and predicting the antioxidant activity of different plant extracts because relatively short time is required for undergoing this assay. The results obtained during this study revealed that the total alcoholic extract and polar fractions from *C. equisetifolia* bark showed good radical scavenging activity. The free radical scavenging and antioxidant activities are highly correlated to the total polyphenolic content. This is clear in our study where total methanol extract is a good source of these biomolecules with high antioxidant capacity making it an excellent candidate for green synthesis of metallic nanoparticles.

Phenolic compounds with high antioxidant capacity exhibit potential antidiabetic activity since oxidative stress affect β- cells of pancreas. The α-amylase inhibitory assay is considered as a measurement for antidiabetic activity (Sekhon-Loodu and Rupasinghe, 2019). In our study, α-amylase inhibitory assay was carried out using

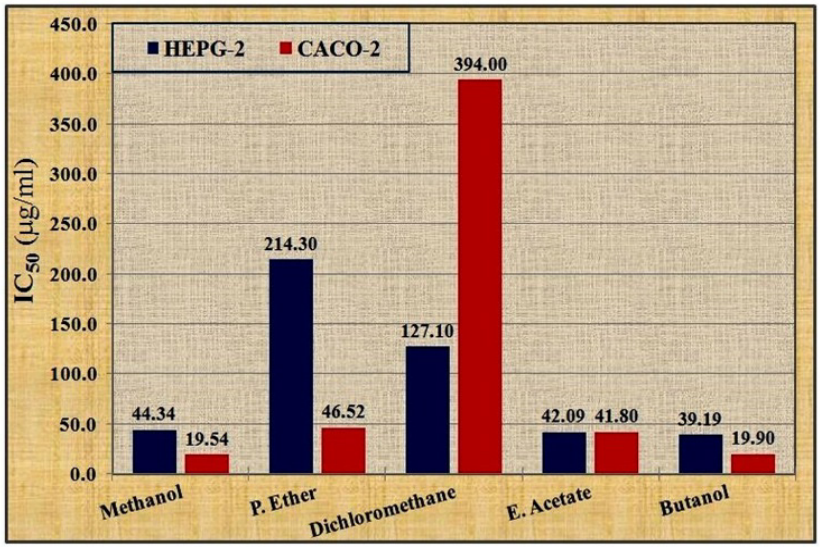

Figure 1. Cytotoxic Activity of Total Methanolic *C. equisetifolia* bark Extract and Its Successive Fractions against Human Liver (HEPG-2) and Colon Cancer (CACO-2) Cells.

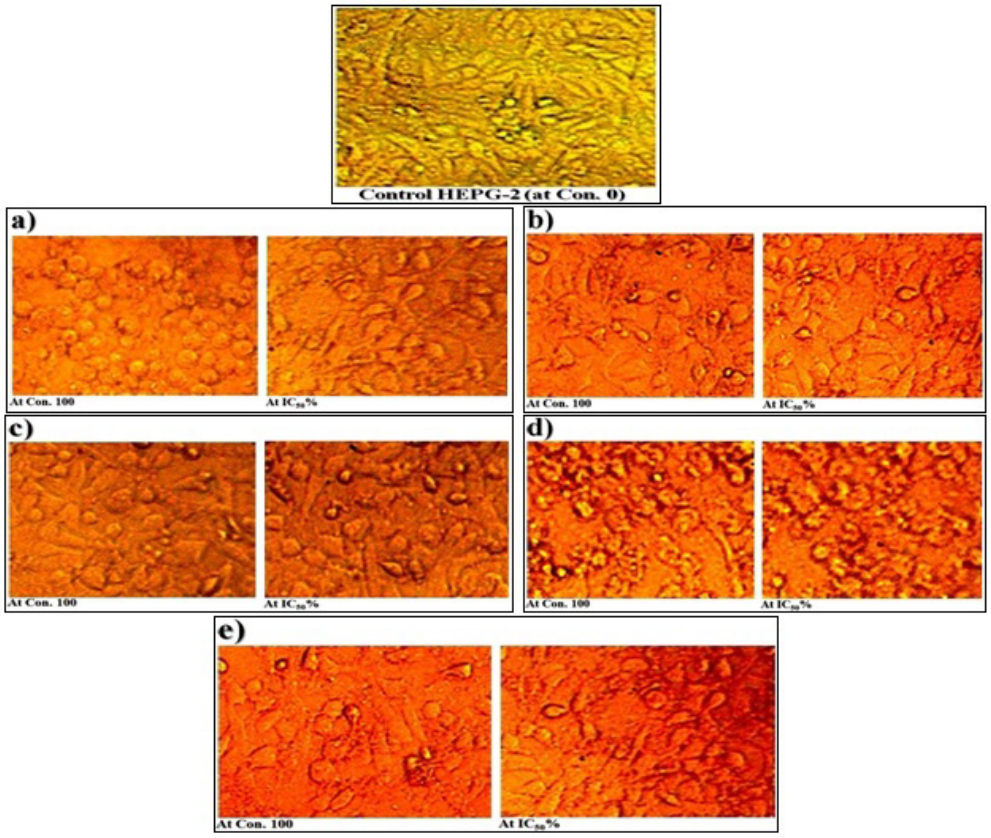

Figure 2. Cytotoxic Activity against Human Liver Cancer (HEPG-2) Showing the Maximum Concentration (100 µg/mL) and Median Inhibitory Concentration (IC_{50}) of a) Total Methanolic Extract, b) Petroleum Ether, c) Dichloromethane, d) Ethyl Acetate and e) Butanol Fractions of *C. equisetifolia* Bark Compared to Control HEPG-2 Cells..

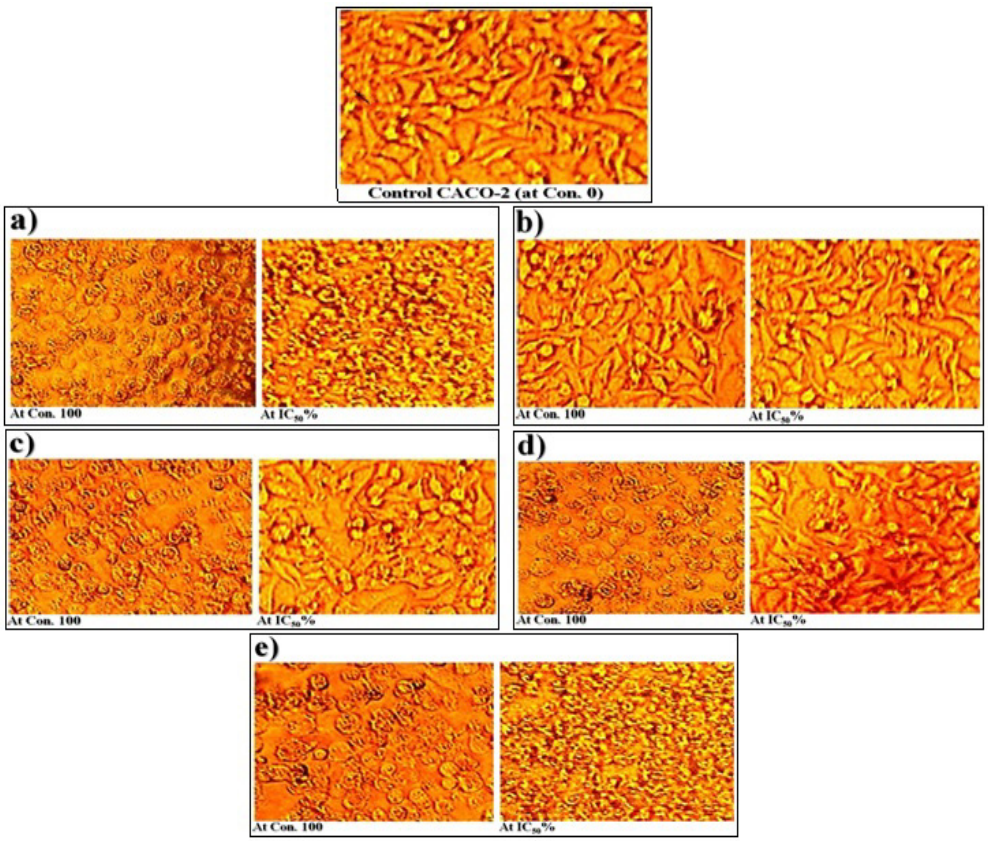

Figure 3. Cytotoxic Activity against Human Colon Cancer (CACO-2) Showing the Maximum Concentration (100 µg/mL) and Median Inhibitory Concentration (IC_{50}) of a) Total Methanolic Extract, b) Petroleum Ether, c) Dichloromethane, d) Ethyl Acetate and e) Butanol Fractions of *C. equisetifolia* Bark Compared to Control CACO-2 Cells.

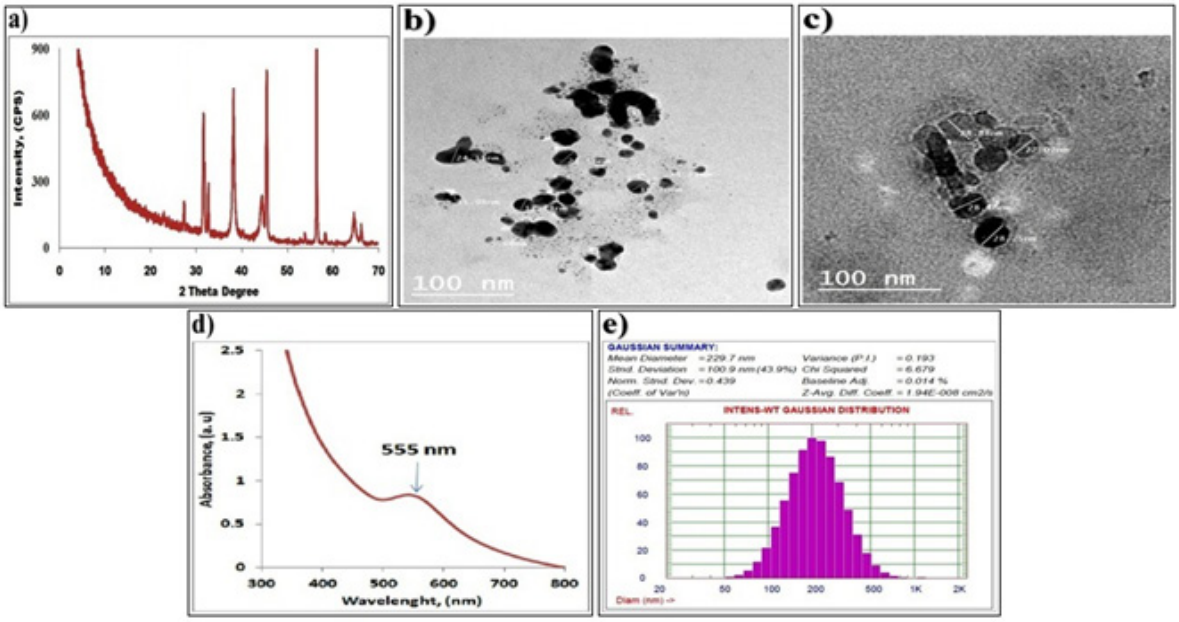

Figure 4. Characterization of the Synthesized Gold Nanoparticles (Au-NPs) Showing a) X-Ray Diffraction (XRD) Spectrum, b) Transmission Electron Microscope (TEM) Image of Au-NPs, c) TEM Image of Au-NPs Incorporated into Total Methanolic *C. equisetifolia* Bark Extract, d) Ultraviolet-visible (UV-VIS) Spectroscopy and e) Dynamic Light Scattering (DLS)

acarbose as standard drug. At equal concentrations of total methanolic extract and its successive fractions, the total methanolic extract exhibited the highest inhibitory effect on α-amylase activity (53.00 ± 0.58%) as compared to the other successive fractions and followed by butanol (polar fraction) (46.33 ± 0.67%). The dichloromethane fraction showed the lowest inhibitory effect (16.67 ± 0.88%), whereas at the same concentration, the standard acarbose was 65.00 ± 0.58% (Table 4). This agreed with Kifle et al., (2020) who emphasized that α amylase inhibitory activity is most likely to be due to the presence of phenolic compounds. Phenolic acids, tannins and flavonoids belong to the polyphenolic compounds that possess inhibitory effect on α-amylase (Kim et al., 2000). During this study, the phytochemical analysis revealed that total methanolic extract and its polar fractions are rich in polyphenolic compounds that belong to the bioactive metabolites and exist in all plant extracts at different concentrations exhibiting an inhibitory effect against α-amylase. Therefore, total methanolic extract exhibited the highest inhibitory effect against α-amylase due to presence of the highest concentrations of total polyphenols and total tannins.

Table 3. The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity of Total Methanolic *C. equisetifolia* Bark Extract and Its Successive Fractions

Extract	Inhibition of ABTS Radicals (%)
Total methanolic	23.97 ± 0.14*
Petroleum ether	11.09 ± 0.10
Dichloromethane	9.06 ± 0.10
Ethyl Acetate	10.91 ± 0.11
Butanol	17.92 ± 0.10
Ascorbic acid (Standard)	35.07 ± 0.06

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

Discussion

In vitro cytotoxic activity of total methanolic extract and its successive fractions against HEPG-2 showed that the polar fractions (ethyl acetate and butanol) exhibited the highest activity against HEPG-2 with the lowest IC_{50} values 42.09 and 39.19 µg/mL, respectively and followed by total methanolic extract (IC_{50} 44.34 µg/mL) (Figure 1 and Supplementary Table 2). Figure 2 showed the maximum concentration (100 µg/mL) and IC_{50} of total methanolic *C. equisetifolia* bark extract and its polar and non-polar fractions compared to control HEPG-2.

As regard to cytotoxic activity against CACO-2, the Figure 3 revealed the maximum concentration (100 µg/mL) and IC_{50} of total methanolic extract of *C. equisetifolia* bark and its polar and non-polar fractions compared to control CACO-2. It was found that total methanolic extract and its butanol fraction exhibited approximately equal anticancer activity (IC_{50} 19.54 and

Table 4. Percent of the Inhibitory Effect of Total Methanolic *C. equisetifolia* Bark Extract and Its Successive Fractions on α-amylase

Extract	Inhibition of α-amylase (%)
Total methanolic	53.00 ± 0.58*
Petroleum ether	23.67 ± 0.33
Dichloromethane	16.67 ± 0.88
Ethyl Acetate	27.00 ± 0.58
Butanol	46.33 ± 0.67
Acarbose (Standard)	65.00 ± 0.58

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

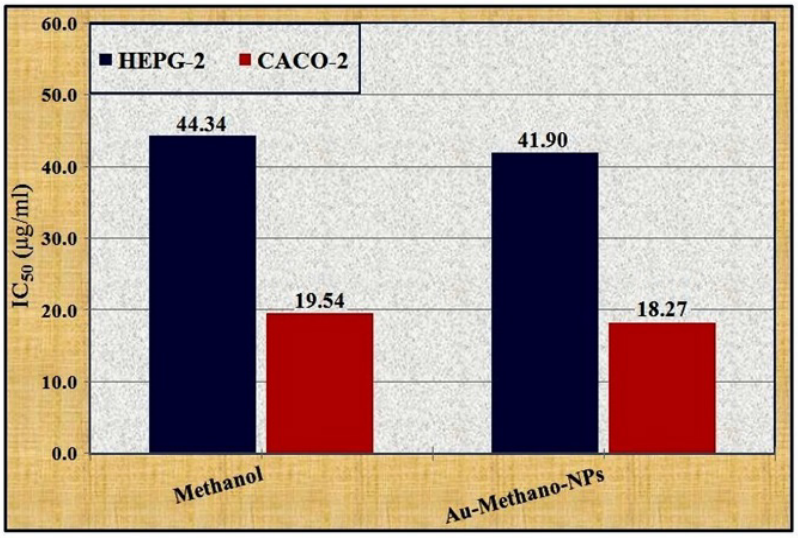

Figure 5. Cytotoxic Activity of Total Methanolic *C. equisetifolia* Bark Extract against Human Liver (HEPG-2) and Colon Cancer (CACO-2) before and after Incorporating Au-NPs.

19.90 µg/mL, respectively) followed by ethyl acetate fraction (IC_{50} 41.80 µg/mL) then dichloromethane fraction (IC_{50} 46.52 µg/mL) (Figure 1 and Supplementary Table 3). Findings of these cytotoxic activities supported that total methanolic extract was the most suitable extract to be utilized during Au-NPs. These findings were supported our antioxidant results mentioned previously.

Studying the structural properties of the synthesized Au-NPs

Total methanolic extract of *C. equisetifolia* bark is considered a good choice for green synthesis of Au-NPs due to the presence of polyphenolic biomolecules exerting strong antioxidant capacity facilitating reduction of gold ions Au+3 to Au0. The XRD and TEM are the most suitable techniques used for studying structural properties of the materials synthesized at nano-scale. The prepared Au-NPs were examined through the XRD diffraction pattern. As illustrated in Figure 4a, the XRD data revealed that the fabricated Au-NPs achieved in presence of AuCl4- analogous diffraction peaks are allocated to metallic Au phase with the greatest significant representative peaks which seem at 38°, 43.8°, and 65° attributed to the crystallographic planes (1 1 1), (2 0 0), and (2 2 0), respectively. The peak width of Au-NPs from crystalline plane (1 1 1), sizes of the Au crystallite were found to be approximately 15 nm for Au-NPs (Youssef et al., 2014). Furthermore, morphology of the prepared Au-NPs was examined by the TEM, which determine shape and size of Au-NPs. The size of Au-NPs has been examined by determining the diameter of whole particles on TEM images. Average Au width was in the range of 15 nm with very little particles of higher and lower size distribution. In addition, the TEM data displayed that Au-NPs are round or spherical in shape as shown in Figure 4b &c.

Also, UV-Vis spectroscopy is easy, fast, simple and discerning for various NPs types, requests simply a short period time for measurement. As illustrated in Figure 4d, the prepared Au-NPs showed a sharp peak at 555 nm that reveals the formation of Au-NPs. Consequently, DLS is mostly used to determine particle size as well as size distributions in aqueous solutions. The size attained from DLS is frequently larger than that particle size obtained from TEM, which might be because of the effect of Brownian motion. DLS is categorized as a nondestructive technique and it is used to acquire the average diameter of the nanoparticles dispersed in aqueous solutions. As presented in Figure 4e, it was found that the

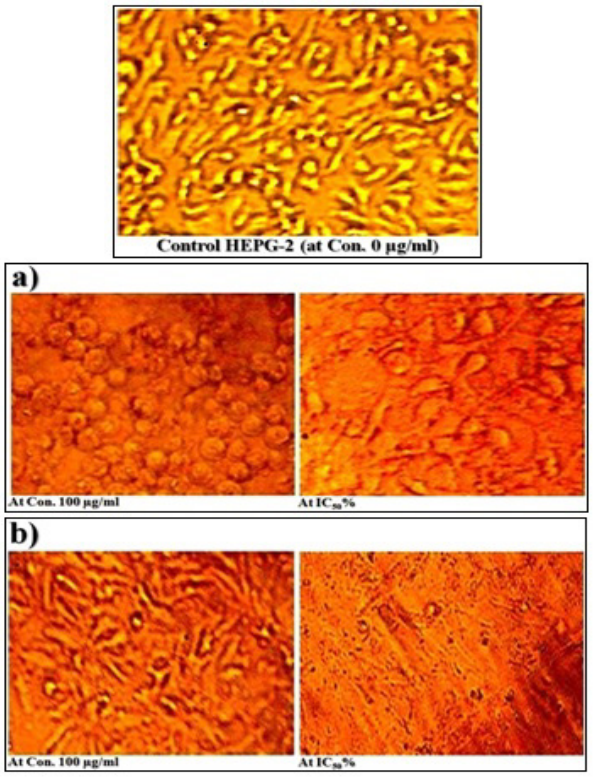

Figure 6. Cytotoxic Activity against Human Liver Cancer (HEPG-2) Showing the Maximum (Conc. 100 µg/mL) and Median Inhibitory Concentration (IC_{50}) of a) Total Methanolic Extract before Incorporating Au-NPs and b) Total Methanolic Extract after Incorporating Au-NPs Compared to Control HEPG-2 Cells.

Table 5. Major Phyto-constituents and Antioxidant Activity of Total Methanolic *C. equisetifolia* Bark Extract before and after Incorporating Au-NPs.

Extract	Total Polyphenols	Total Tannins	Total Antioxidant Capacity	Iron Reducing Power
	(mg gallic acid/100 gm)	(µg/ml)	(mg gallic acid/gm)	(µg/mL)
Total methanolic	131.39 ± 0.24	13.10 ± 0.01*	275.64 ± 0.37	240.31 ± 0.66
Au-methanolic nano-extract	712.79 ± 0.87*	5.08 ± 0.01	454.41 ± 1.32*	661.65 ± 0.48*

Table 6. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity and the Median Inhibitory Concentration (IC_{50}) of Total Methanolic *C. equisetifolia* Bark Extract before and after Incorporating Au-NPs

	Conc. (µg/ml)								IC_{50} (µg/ml)
	100	50	25	12.5	6.25	3.125	1.56	0.78	
Total methanolic	82	81.2	80.9	78.6	64.5	33.9	16.2	7.7	4.7
Au-methanolic nano-extract	95	94.1	93.6	92	62.1	45.9	21.1	9.8	5.4
Ascorbic acid (Standard)	94.7	94.3	94	93.8	64.1	45.1	32	24.6	3.9

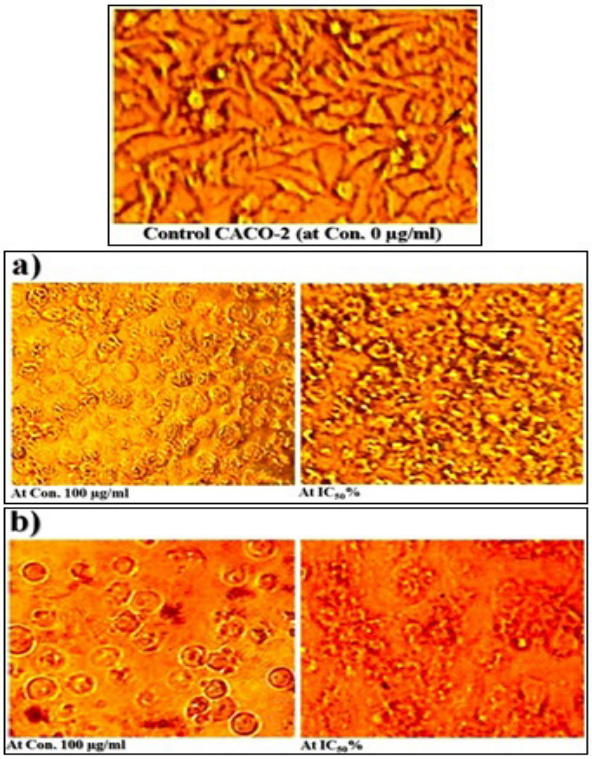

Figure 7. Cytotoxic Activity against Human Colon Cancer (CACO-2) Showing the Maximum (Conc. 100 µg/mL) and Median Inhibitory Concentration (IC_{50}) of a) Total Methanolic Extract before Incorporating Au-NPs and b) Total Methanolic Extract after Incorporating Au-NPs Compared to Control CACO-2 Cells.

Table 7. The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Radical Scavenging Activity of Total Methanolic *C. equisetifolia* Bark Extract before and after Incorporating Au-NPs

Extract	Inhibition of ABTS Radicals (%)
Total methanolic	23.97 ± 0.14
Au-methanolic nano-extract	36.06 ± 0.10*
Ascorbic acid (Standard)	35.07 ± 0.06

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

Table 8. Percent of the Inhibitory Effect of Total Methanolic *C. equisetifolia* Bark Extract before and after Incorporating Au-NPs on α-amylase

Extract	Inhibition of α-amylase (%)
Total methanolic	53.00 ± 0.58
Au-methanolic nano-extract	60.67 ± 0.88*
Acarbose (Standard)	65.00 ± 0.58

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

particle size distribution of the fabricated Au-NPs has main diameter around 200 nm.

Phytochemical and biological assessment of gold total methanolic nano-extract

The gold total methanolic *C. equisetifolia* bark nano-extract is characterized by high contents of tannins together with the presence of saponins, flavonoids, carbohydrates and/or glycosides. Sterols, triterpenes, alkaloids, anthraquinones, cardiac glycosides and volatile constituents are not detected.

It was noticed that concentration of total polyphenols (712.79 ± 0.87 mg gallic acid/100 gm) increased after incorporation of gold nano particles (Table 5), this may be due to interference of metal ions used in synthesis of nano particles with polyphenolic measurements (Mystrioti et al. 2016). While concentration of the total condensed tannins decreased to 5.08 ± 0.01 µg/mL in total methanolic extract after incorporating Au-NPs because they were consumed for reducing Au (III) ions to produce Au-NPs (Sau and Murphy, 2004 ; Ahmad, 2014).

Regarding antioxidant capacity, gold nano extract showed elevation in antioxidant capacity (454.41 ± 1.32 mg gallic acid/gm) and iron reducing power (661.65 ± 0.48 µg/mL) compared to the native total methanolic extract. Incorporation of the Au-NPs into total methanolic extract decreased its DPPH radicals scavenging activity slightly (IC_{50} 5.40 µg/mL) (Table 6). However, it enhanced the ABTS free radicals scavenging activity (36.06 ± 0.10%) as compared to the extract itself before incorporating Au-NPs (23.97± 0.14%) (Table 7). Consistent with our findings, recent studies revealed

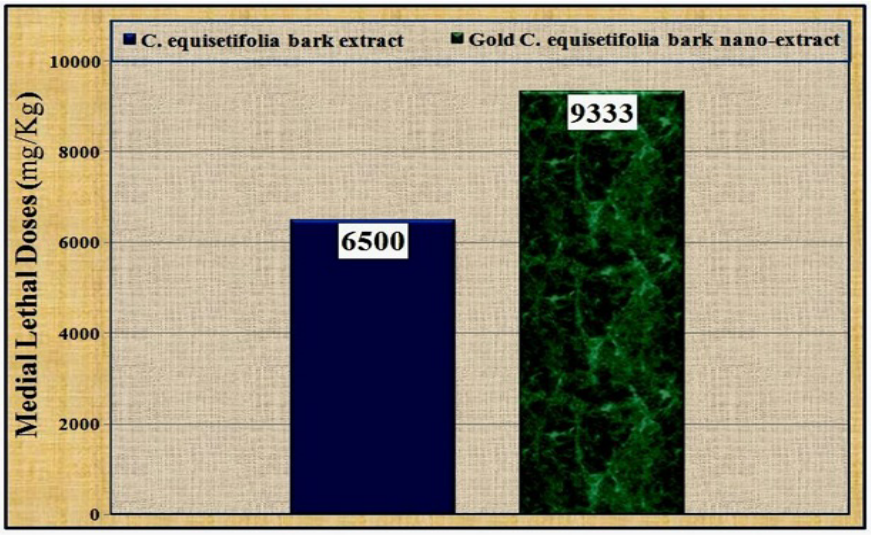

Figure 8. The Median Lethal Doses (LD_{50}) of Total Methanolic *C. equisetifolia* Bark Extract before and after Au-NPs Incorporation

that inclusion of M-NPs into plant extract increased the bioavailability leading consequently to elevating its total antioxidant ability, iron reducing power, and hence increasing the free radicals scavenging ability compared to native plant extract itself (Abdelhady and Badr, 2016; Aboulthana et al., 2019). Also, gold nano-extract enhanced the inhibitory effect on α-amylase activity (60.67 ± 0.88%) as compared to the extract itself (53.00 ± 0.58%) before incorporating Au-NPs (Table 8).

Moreover, inclusion of Au-NPs into total methanolic extract showed obvious elevation in its cytotoxic activity against HEPG-2 (IC_{50} 41.90 µg/mL) (Figure 5 and Supplementary Table 4). Figure 6 showed the maximum concentration (100 µg/mL) and IC_{50} of total methanolic extract against HEPG-2 before and after incorporating Au-NPs compared to control HEPG-2. Figure 7 showed the maximum concentration (100 µg/mL) and IC_{50} of methanolic extract against CACO-2 before and after incorporating Au-NPs compared to control CACO-2. The cytotoxic activity increased against CACO-2 slightly after incorporating Au-NPs (IC_{50} 18.27 µg/mL) as compared to the extract itself before incorporating Au-NPs (IC_{50} 19.54 µg/mL) (Figure 5 and Supplementary Table 5). This might be attributable to presence of the active phyto-constituents that have the ability to prevent development of the invasive cancer effectively (Suganya Devi et al., 2012). Incorporation of Au-NPs into plant extracts increased the cytotoxic activity against growth of human cancer cells compared to the crude extract itself. This was manifested by their reduced IC_{50} for different used cell lines. This was in accordance with Aboulthana et al. (2019) and recently supported by Abdel-Halim et al. (2020) who documented that enhancement of the anticancer activity by lowering growth of cancer cells might refer to increasing the total antioxidant capacity, iron reducing power and free radicals scavenging activity.

Identification of active metabolites in total methanolic extract before and after incorporating Au-NPs

Fourier Transform Infrared Spectroscopy (FT-IR) analysis

FT-IR technique was used to identify functional groups existing in the plant extract that are responsible for reduction of metal ions and stabilization of biosynthesized nanoparticles (Shousha et al., 2019). During the present study, FT-IR was employed for identification of the possible biomolecules' functional groups in both total methanolic *C. equisetifolia* extract and its gold nano-extract (Supplementary Figures 1 and 2) and Supplementary Table 4). Both FT-IR spectra showed broad signal at about 3,200-3,400 cm^{-1} attributed to stretching vibrations of hydroxyl group of phenolic compounds (Alegria et al., 2018) besides a strong band at about 1,600-1,610 cm^{-1} corresponding to the presence of carbonyl group or C=C of aromatic compounds (Firoozi et al., 2016). Peaks at 1,000-1,500 cm^{-1} corresponds to C-O and C-C in aromatic ring stretching vibrations. Shift in the position and intensity of these peaks were observed in the spectrum of the gold nano-extract which is due to involvement of these functional groups in reduction of gold ions and capping of Au-NPs surface. Also, the appearance of new absorption bands proved the formation of covalent bonds in metal nanoparticles (Alamdari et al., 2020). FT-IR results confirmed the abundancy of phenolic and flavonoid bioactive metabolites in total methanolic extract of *C. equisetifolia* bark before and after incorporating gold nanoparticles.

HPLC/QTOF MS-MS metabolite profiling

A comprehensive non-targeted metabolites profiling was carried out for total methanolic *C. equisetifolia* bark extract using HPLC/QTOF-MS-MS technique before and after incorporating Au-NPs. A total of 33 and 17 chromatographic peaks were tentatively annotated before and after Au-NPs inclusion, respectively. Metabolite assignments were carried out by comparing the retention time and mass data (accurate mass and fragmentation pattern in NI mode) with reference literature. HPLC-MS total ion chromatograms of total methanolic *C. equisetifolia* extract in NI mode before and after incorporating Au-NPs are displayed in Supplementary Figure 3 and a list of the annotated metabolites along their spectroscopic and chromatographic data is presented in

Supplementary Table 7. Condensed tannins, flavonoids, phenolic acids and their derivatives represented the most abundant classes of metabolites in total methanolic *C. equisetifolia* bark extract before and after incorporating Au-NPs, whereas fatty acids, organic acids and sugar were also noticed. The identification details for each metabolite class are provided as follows:

Condensed tannins

Condensed tannin, known as proanthocyanidin, is a class of secondary metabolites with pronounced biological activities especially antioxidant activity against free radical-mediated diseases (Unusan, 2020). Condensed tannins are formed of flavan-3-ol units i.e. catechin, gallocatechin and their enantiomer isomer. In B-type proanthocyanidins, flavanol units are linked by C_4–C_6 or C_4–C_8 interflavan-linkage, while in A-type, an additional C_2–C_5 or C_2–C_7 interflavan ether-linkage occurs.

Proanthocyanidin mass fragmentation showed prominent diagnostic ions produced from Retro-Diels-Alder (RDA) fragmentation, heterocyclic ring fission (HRF), benzofuran formation (BFF) and quinone methide (QM) fragmentation processes. RDA fragmentation and HRF define the linkage between monomeric units and hydroxylation of B-ring, while QM fragmentation provides information about the monomeric units, specially the base one (Singha et al., 2018).

HPLC-MS analysis of total methanolic *C. equisetifolia* extract showed two parent ions at *m/z* 289 (peaks 14 & 16) with different retention time showing intense fragment ion at *m/z* 245 due to the loss of 44 amu (CH_2=CH–OH). Other fragment ions at *m/z* 125 [M − H-165]− representing pholoroglucinol moiety, resulted from HRF fission alongside *m/z* 137 resulted from RDA of ring C and *m/z* 179 [M − H-110]− was due to the loss of dihydroxy benzene moiety which was detected by the presence of product ion at *m/z* 109 [M − H-179]− (Said et al., 2017; Yuzuak et al., 2018). Based on MS fragmentation analysis and the order of hydrophobicity in reversed phase HPLC, peak (14) and peak (16) are tentatively identified as diastereoisomers catechin and epicatechin, respectively (Singha et al., 2018). Similarly, Peak (8) and Peak (11) with *m/z* 305.0661 [$C_{15}H_{13}O_7$] – are assigned as gallocatechin and epigallocatechin respectively (Said et al., 2017).

Six flavan-3-ol derivatives were identified based on their characteristic fragmentation pattern. Peak (13) at Rt 4.521 [*m/z* 577.1353 $C_{30}H_{25}O_{12}$-] showed products ions at m/z 451 [M-H-126] − resulted from the loss of a phloroglucinol moiety in HRF process (Lv et al., 2015), beside an intense fragment ion at *m/z* 425 with neutral loss of 152 amu (^{1,3} B group) through RDA process indicating presence of (epi) catechin unit. Product ion at *m/z* 289 [M-H-288]− resulted from QM cleavage of the inter-flavanoid bond identifying upper and terminal units to be (epi) catechin. Peak (13) was tentatively identified as procyanidin dimer B type [(epi)catechin-(epi)catechin]. Peak (17) at Rt 5.846 [m/z 865.1941 $C_{45}H_{37}O_{18}$-] showed the same fragmentation pattern as peak (13) with an intense fragment ion at m/z 577 resulting from QM fragmentation of upper (epi)catechin unit, assigned as procyanidin trimer B type (procyanidin C). The fragmentation behavior of peak (13) could be extrapolated to peak (20) [*m/z* 575.1167 $C_{30}H_{23}O_{12}$-] with a mass that is 2 amu less than that of the corresponding B-type procyanidin dimer indicating the presence of (C2—7) interflavanoid linkage of A-type procyanidin (Li and Deinzer, 2007). Peak (20) was tentatively identified as Procyanidin dimer A type. Likewise, peak (19) [*m/z* 863.1807 $C_{45}H_{35}O_{18}$-] was assigned as Procyanidin trimer A type with only one A-type connection between the upper and the middle units, that was evident by fragment ions at *m/z* 575 and 289 generated from QM cleavage (da Silvaa et al., 2017). Peak (10) at Rt 3.404 and *m/z* 593.1286 showed intense fragment ions at *m/z* 425 [M−H−168]− and 407 [M−H−168-H_2O]− through RDA reaction and successive loss of water molecule, indicating that the B ring of the upper unit has a pyrogalloyl group as the RDA fragmentation on the top unit produces more stable ion (Tala et al., 2013 ; Singha et al., 2018). Fragment ion at *m/z* 289 resulted from QM fragmentation confirming (epi) catechin as base unit. Therefore, peak (10) was assigned as dimeric (epi) gallocatechin-(epi) catechin with B-type linkage, known as Prodelphinidin B3. Similarly, peak (7) at Rt 2.386 and *m/z* 609.1211 was identified as dimeric (epi) gallocatechin-(epi) gallocatechin, known as Prodelphinidin B with characteristic fragment ion at *m/z* 305 resulted from QM fragmentation between two (epi) gallocatechin units.

Regarding HPLC-MS analysis of total methanolic *C. equisetifolia* extract after incorporating Au-NPs, three flavan-3-ol compounds i.e. catechin, epicatechin and gallocatechin were detected together with procyanidin dimer A type, procyanidin dimer and trimer B type.

Flavonoids

Identification of flavonoids aglycones, with the characteristic nucleus of 2-phenyl chromone, was based on specific fragmentation patterns that are informative for structural elucidation. In detail, most of flavonoids undergo neutral losses of 18 amu (H_2O), 28 amu (CO) of the C ring and 44 amu (COO-), besides the characteristic RDA cleavage between different chemical bonds of the C ring (Chen et al., 2016). Based on comparisons of the proposed fragmentation pathways with published data, HPLC-MS of total methanolic *C. equisetifolia* extract showed peak (26) at *m/z* 271.0616, with fragment ions at *m/z* 177, 151 and 119, was identified as naringenin flavanone (Brito et al., 2014 ; Chen et al., 2016). Likewise, peak (27) [*m/z* 301.0713 $C_{16}H_{13}O_6$-] was assigned as hesperetin with extra 4' methoxy group. Peak (24) *m/z* 285.0397, peak (25) *m/z* 301.0365, peak (28) *m/z* 269.0448 and peak (31) *m/z* 315.2533 were identified as luteolin, quercetin, apigenin and isorhamnetin, respectively (Brito et al., 2014). Peak (18) at Rt 6.462 [*m/z* 433.1137] showed characteristic fragment ion at m/z 271 due to loss of 162 amu of hexose, assigned as naringenin O- hexoside. HPLC-MS analysis of total methanolic *C. equisetifolia* extract after incorporating Au-NPs showed three of the aforementioned flavonoids namely, isorhamnetin, apigenin and naringenin.

Phenolic acid derivatives

Negative ion MS revealed the presence of 8 phenolic acids in total methanolic *C. equisetifolia* extract belonging to hydroxybenzoates and hydroxycinnamates. Generally, phenolic acids and their derivatives presented a loss of 18 amu (H_2O) and 44 amu (COO-) in MS/MS (Ammar et al., 2015). Peaks 3, 4, 9 and 21 were assigned as tri-, di-, mono- and methoxy hydroxy benzoic acids, respectively, by comparison of their fragmentation pattern with previously published data in addition to peak 12 annotated as ellagic acid (Mena et al., 2012; Wang et al., 2015). On the other hand, total methanolic *C. equisetifolia* extract after incorporating Au-NPs showed only methyl gallate and hydroxy methoxy benzoic acid.

Chalcones

HPLC-MS analysis of total methanolic *C. equisetifolia* extract before and after incorporating Au-NPs revealed the presence of two chalcones. Peak (22) at *m/z* 435.1300 [$C_{21}H_{23}O_{10}$-] showing product ions at *m/z* 345 and 315 due to loss of 90 and 120 amu, respectively in addition to presence of the characteristic fragment ion at *m/z* 273 of phloretin aglycone. Peak (22) was identified as phloretin c-glucoside (Martini et al., 2018). Peak (29) at *m/z* 273.0753 ($C_{15}H_{13}O_5$-) showed fragment ions at *m/z* 167, 151 and 125 typical for phloretin when compared with literature (Stefova et al., 2019).

Acids

It was noticed that quinic acid (m/z 191.0548) and malic acid (*m/z* 133.014) were identified in negative ionization MS of total methanolic *C. equisetifolia* extract before and after Au-NPs inclusion by comparison with published data (Fernández-Fernández et al., 2010 ; Steimer et al., 2017). In addition, three fatty acids were identified in total methanolic extract i.e. dihydroxy octadecenoic acid (*m/z* $C_{18}H_{33}O_4$-), hexadecanoic acid (m/z $C_{16}H_{31}O_2$-) and heptadecanoic acid (m/z $C_{17}H_{33}O_2$-) showing characteristic loss of 44 amu (COO-). However, only dihydroxy octadecenoic acid (*m/z* $C_{18}H_{33}O_4$-) was found after Au-NPs inclusion.

The net result of metabolites profiling via HPLC-MS showed that metabolites identified in total methanolic extract before and after incorporating Au-NPs mainly belong to polyphenolic compounds i.e. flavonoids, tannins, phenolic acids and chalcones. It was reported that polyphenolic compounds exhibited strong in-vitro antioxidant activity (Yu et al., 2021). Earlier studies revealed that catecholic B-ring in most flavonoids (i.e. quercetin and luteolin) and condensed tannins is responsible for this activity. Antioxidant activity is increased by polymerization of flavan-3-ols into larger condensed tannins. Flavonoids and condensed tannins can act as antioxidants through single-electron or hydrogen atom transfer mechanisms (Gourlay and Constabel, 2018). Other study observed that B-type procyanidins showed stronger antioxidant activity than ascorbic acid and α-tocopherol and it was proved that dimeric procyanidins can trap eight peroxyl radicals more than ascorbic acid and thus help in restoration of the oxidative balance by removal of those ROS (Bruyne et al., 1999). Lately, interests have been withdrawn upon the effects of antioxidants on the inhibition of several degenerative diseases as cancer (Mutalib et al., 2016). Consistent with our findings, previous studies reported that plant polyphenols are responsible for cytotoxic activity. Previous studies proved that compounds with galloyl and quinic acid cores act as cytotoxic agents. Moreover, presence of flavan units and ether linkage in A-type proanthocyanidins also enhance cytotoxic activity. Additionally, the inhibitory effect of epigallocatechins on the promotional stage of oncogenic transformation induced by radiation was demonstrated. Epigallocatechins and catechins proved promising effects in the inhibition of gastrointestinal carcinogenesis, breast carcinoma, colon carcinoma, lung carcinoma and melanoma (Bruyne et al., 1999).

The median lethal dose (LD_{50})

Total methanolic extract incorporated with Au-NPs was safer than the extract itself when administrated orally in the experimental animals (mice) by stomach tube. The gold methanolic nano-extract appeared safer as compared to the methanolic extract itself. This was supported by our findings that emphasized that the LD_{50} of the methanolic extract before incorporating Au-NPs was about 6,500 mg/Kg and the therapeutic dose was about 650 mg/Kg. After incorporating Au-NPs, the LD_{50} was about 9,333.33 mg/Kg and hence the therapeutic dose was 933.33 mg/Kg (Figure 8). This was in accordance with Abdel-Halim et al., (2020) who reported that incorporation of Au-NPs increased safety of plant extract and this was supported by their findings that revealed the LD_{50} values of the Bauhinia variegata extract and its gold nano-extract were found to be 36.50 and 51.5 ml/kg and the doses used for the therapeutic investigation (1/20 LD_{50}) for the extract and nano-extract were about 1.83 and 2.58 ml/ kg, respectively. This was in agreement with the concept explained by Aboulthana et al., (2019) who showed that green route synthesis of M-NPs found with less toxicity. This was supported recently by Aboulthana et al., (2022) who postulated that safety of the nano-extract might refer to the renal clearance that is considered as a multifaceted process and desirable pathway for M-NPs elimination with low degradation rate in the body to avoid the undesirable side effects.

In conclusion, this study attempted to justify that the studied biological activities of *C. equisetifolia* bark are phytochemically related to its secondary metabolite profile. The total methanolic extract has been chosen for Au-NPs biosynthesis as it showed the highest level of polyphenolic and tannin contents as compared to its successive fractions. Although incorporation of Au-NPs into total methanolic extract increased the total polyphenols leading to elevating antioxidant capacity and iron reducing power, it decreased the total tannins. HPLC-MS fingerprint showed 33 and 17 metabolites in total methanolic extract before and after incorporating Au-NPs, respectively. Incorporation of Au-NPs into total methanolic extract kept the major active metabolites such as procyanidins, flavonoids, chalcones and phenolic acids. Total methanolic extract incorporated with Au-NPs

showed higher ABTS radicals scavenging activity in addition to elevating the inhibitory effect against α-amylase activity. Also, it increased the cytotoxic activity against HEPG-2 and CACO-2 slightly as compared to the extract itself. Furthermore, it was safer than the extract itself when administrated orally in mice.

Author Contribution Statement

Wael M. Aboulthana: Writing – original draft, Writing – review & editing, Visualization, Supervision and Project administration. Esraa Refaat: Resources, Data curation, Writing – review & editing. Sally E. Khaled: Methodology, Validation, 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

The experimental design that was concerned with determining the safe doses of the extracts administrated orally in animals (mice) was carried out based on a protocol approved by the Institutional Animal Ethical Committee of National Research Centre, Dokki, Giza, Egypt, and according to guidelines reported in "Guide for the care and use of laboratory animals".

Conflict of interest

The authors who are responsible for the manuscript practically and theoretically declare that they have no conflicts of interest (financial or non-financial) among them.

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