

Estimation and Action Mechanisms of Cinnamon Bark via Oxidative Enzymes and Ultrastructures as Antimicrobial, Anti-biofilm, Antioxidant, Anti-diabetic, and Anticancer Agents

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Cinnamon is a plant with significant medicinal value that is used extensively as a spice, flavoring, and fragrance ingredient. Phytochemical characterization via high-performance liquid chromatography, antimicrobial activity against various microorganisms, anti-diabetic properties by α -glucosidase and α -amylase assessments, antioxidant activity via 2,2-diphenyl-1-picrylhydrazyl-hydrate, and total antioxidant capacities of cinnamon bark were evaluated. Moreover, antitumor potential against human breast cancer (MCF-7) and human fetal lung fibroblast (WI-38) cells of the bark extract were detected. A promising antimicrobial action towards *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Candida albicans*, and *Mucor circinelloides* where the inhibition areas were 25, 23, 18, 19, 29, and 16 mm, respectively, was recorded. Cinnamon bark has IC_{50} of 5.01 and 2.58 $\mu\text{g/mL}$ compared to standard acrobose with their IC_{50} values 4.32 and 1.99 $\mu\text{g/mL}$, respectively for α -amylase and α -glucosidase inhibitions. Cinnamon bark extract has IC_{50} of 77.39 ± 0.84 and 162.67 ± 0.28 $\mu\text{g/mL}$ toward cell lines of MCF-7 and WI-38, respectively. The protective effect of cinnamon in accelerating the apoptosis of MCF-7 cells has been verified by flow cytometric evaluation employing Annexin-V and cell cycle kits, as well as the increasing levels of malondialdehyde, hydrogen peroxide, and nitric oxide with decreasing glutathione and catalase levels.

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INTRODUCTION

The perennial tropical tree recognized as cinnamon (*Cinnamomum*) is native to Sri Lanka. This plant is a member of Lauraceae, and an evergreen tree. Many species of *Cinnamomum* are used in medicine. The term was borrowed into English at the last decade of the 14th century through the historical French word “cinnamon,” which itself originated in Latin through the Greek term “kinnamomon” and is thought to have Semitic roots (*cf.* Hebrew “qinamon”). The term was originally printed in a print work in 1430, in “Fall of Princes” (Rao and Gan 2014; Al-Rajhi *et al.* 2023a). Manganese, calcium, and dietary fiber are all found in cinnamon. It contains additional substances that have properties related to inflammatory treatments, inhibition of microbes, and cancer-fighting treatments. Cinnamon’s various benefits have been documented in several studies on the bark and bark powder. Cinnamon’s phenolic compounds along with its essential oils are good for people’s health. Recent study has demonstrated the useful functions of cinnamon in the regulation of arteriosclerosis, rheumatism, and dementia (Hariri and Ghiasvand 2016). Regarding daily consumption of cinnamon, a sufficient amount (0.11 mg/L kg of body mass) of coumarin can be considered safe without risking adverse consequences (Abraham *et al.* 2010). Based on accessible research findings, risk evaluation should be directed towards the potentially hazardous ingredients of cinnamon extract, particularly the toxic ones such as safrole and styrene (Kowalska *et al.* 2021).

The emergence and spread of pathogenic bacteria have caused increased bacterial infections, which has raised concerns about the creation of new antimicrobial medications. Moreover, controlling infection and healthcare faces a significant global challenge due to the formation of microbial biofilms, owing to their resistance to several drugs. In the field of food science, as mentioned by Lu *et al.* (2022), biofilm formation is a significant physiological indicator that can cause food safety issues and control the buildup of various bio-products. Plant extracts, oils, and the substances derived from them have been utilized for centuries to combat infections caused by microbes because they are known to be effective towards an extensive number of microorganisms (Al-Rajhi *et al.* 2022; Qanash *et al.* 2022; Al-Rajhi *et al.* 2023b). Cinnamon has been shown in many investigations to have antibacterial properties towards various bacteria (Trinh *et al.* 2015; Utchariyakiat *et al.* 2022). Many investigators have directed their attention towards the antimicrobial characteristics of conventional medicinal compounds (Freires *et al.* 2015; Abdelghany *et al.* 2021; Al-Rajhi and Abdelghany 2023; Bakri *et al.* 2024).

In addition to bacterial infections, researchers are investigating treatments for diabetes and cancer. *Diabetes mellitus* is a long-term metabolic condition related to a higher risk of retinopathy, cardiovascular disorder, and periodontal disease. Regarding cancer, statistics from 2020 show that there are roughly 20 million individuals with cancer worldwide and nearly 11 million mortalities due to cancer (Bakhtiari *et al.* 2019). Carcinogenesis, which has four stages—tumor initiation, tumor outreach, cancerous switching, and tumor progression—is the gradual change of healthy cells into malignant cells. Tumor initiation, the initial stage of carcinogenesis, is characterized by modifications in DNA (deoxyribonucleic acid) resulting from exposure to biological or chemical carcinogenic stimulus (Subhashini and Geetha 2015).

Solvent selection is one of the steps of planning for the separation as well as extraction processes. Solvent selectivity to extract the target ingredients from the biomass

of plants is correlated to polarity of the elected solvent; ethanol is considered less toxic in comparison to other solvents except water for human, as well as animal in the extraction of natural contents for both natural medicinal and food purposes. Aqueous and absolute ethanol has been employed to extract the active phenolic, flavonoids, and their derivatives from natural materials with good outcomes (Al-Garadi *et al.* 2023). This work investigated antimicrobial beside anti-biofilm, antioxidant, and anti-diabetic properties, as well as certain antitumor effects and mechanisms of ethanolic cinnamon bark extract.

EXPERIMENTAL

Materials

Cinnamon (*Cinnamomum zeylanicum*) bark powder was bought from a local store at Jeddah, Saudi Arabia. At 25 °C (temperature of room), the powder was stored in a plastic sack. The used chemicals in the investigation were bought from Sigma Co., Ltd. in city of Heidelberg at Germany. *Enterococcus faecalis* (ATCC10541), *Klebsiella pneumonia* (ATCC70063), *Salmonella typhi* (ATCC6539), *Candida albicans* (ATC10221), and *Mucor circinelloides* (AUMMC11656) were graciously supplied by Prof. Tarek M. Abdelghany at University of Al-Azhar, Egypt.

Extraction of Cinnamon

A stoppered container filled with 50 g of ground cinnamon bark powder was placed in absolute ethanol (0.5 L) for 72 h at 22 °C. Then, the extract was sonicated for 50 min at 50 °C. The crude cinnamon bark extract was assembled by filtration, which was followed through concentration of these extracts in a rotatory evaporator under vacuum at 50 °C (Kallel *et al.* 2019).

Detection of the Total Phenolic and Flavonoid Contents

A content of 2.50 mL of Folin-Ciocalteu solution and 2.50 mL (7.5 g/100 mL) sodium carbonate were fused, and then they were mixed with aliquots (500 µL extracts). After 10 s, the tubes were vortexed, followed by keeping them for 2 h at 28 °C. Using a MiltonRoy 310 spectrophotometer (Tokyo, Japan), the wavelength was recorded at 760 nm to find total phenol (De Giani *et al.* 2022). In a 10-mL flask, 1.0 mL of cinnamon bark extract and 2.0 mL of methyl alcohol were combined. Using water in a flask of 25 mL capacity, solutions of 5% NaNO₃, and 5% NaOH, beside 7% AlCl₃ were made. Just about 200 µL of tested extract was taken in a vial of glass and mixed with 75.0 µL of 5.0% NaNO₃, and then at 28 °C the blend was kept for ten min. Following sonication and keeping for 10 min at 28 °C, 1.25 mL and 0.5 mL of AlCl₃ (10 %) and NaOH (1 M) were added, respectively to the container. Following incubation, a Milton Roy 310 spectrophotometer (Tokyo, Japan) was employed to evaluate the wavelength of reaction solutions besides the standard after blanking with methanol at 520 nm to determine total flavonoid content (De Giani *et al.* 2022).

Assessment of Phenols and Flavonoids Employing High Performance Liquid Chromatography (HPLC)

Approximately 10 µL of the cinnamon bark extract was inoculated into the HPLC (Agilent Technologies, CA, USA). The C18 column was used for analysis at 50 °C. The mobile phase contained 0.05% acetic acid in acetonitrile (B) and H₂O (A) flowing at a level of 0.9 mL/min. The programming sequence for the mobile stage was as follows: 0 min (80% A); 0 to 1 min (80% A); 1 to 11 min (75% A); 11 to 18 min (60% A); 18 to 20 min (80% A); and 22 to 24 min (82% A). The detector was adjusted at 290 nm. Five µL of tested sample were injected in the column of HPLC with constant temperature (40 °C). The ultraviolet (UV) detector uses a single wavelength at 280 (Icon Scientific Inc., MD, USA) (Mizzi *et al.* 2020).

Assessment of Antimicrobial Potential and of Minimal Inhibitory (MIC), Besides Bactericidal Concentrations (MBC)

The antimicrobial ability of cinnamon bark extract was measured employing the well agar diffusion procedure. On malt extract agar for fungi and nutrient agar plates for bacteria, a hole of 6.0 to 8.0 mm was drilled with a sterilized cork borer tip before an antibacterial substance or extract solution was added to the well. Dimethyl sulfoxide was employed as a control group. The inhibition zone was detected after the bacteria were left for 24 h at 36 °C and 28 °C for 7 days for fungi (Abdelghany *et al.* 2023; Alghonaim *et al.* 2023). Using nutritional broth for bacteria and malt extract broth for fungi, the micro-dilution broth method can be operated to calculate the MIC of the samples under investigation. The samples were diluted twice to determine their final concentrations, which varied between 0.98 and 1000 µg/mL. The 96-well microtiter plates were planned by putting 200 µL of the dilutions of the examined extract in liquid medium into each well. After the inoculum was generated with new microbial cultures according to the turbidity of 1.0 McFarland standard, 2 µL of sterile 0.85% NaCl was added to the wells to reach a final density of 3.0×10^6 CFU/mL after incubating the bacteria for 72 h at 35 °C and the cells for fungi at 28 °C. The extract concentrations at which the growth of the standard strain was totally prevented were measured through visuals to calculate the MICs. There was a negative control (tested extracts without an inoculum) and a positive control (inoculum without the examined specimens) on each microplate (Nemattalab *et al.* 2022). Through subculturing 100 µL of the bacterial culture onto nutrient agar plates in each well that have complete growth repression, from the final positive, and from the development control, the MBC was found. After incubating the plates at 35 °C for 72 h, the MBC was identified as the smallest level of extracts that did not cause microbial growth.

Anti-biofilm Assay and Bacterial Ultrastructure

The effect of the specimens on the formation of bacterial films was determined in 96-well polystyrene flat bottom plates. A final level of 10⁶ CFU/mL was determined by placing 280 µL of newly amended by microorganisms inoculated trypticase soy yeast broth (TSY) into each well of a microplate. The microplate was then cultured in an amount of MBC at previously determined sub-lethal doses (75%, 50%, and 25%). Wells filled by medium and those containing just ethanol and no samples were employed as controls. Plates were left at 36 °C for 48 h. Then, the supernatant was discarded, and sterile distilled water was applied to thoroughly clean any free-floating cells from each well. After letting

the plates air dry for 30 min, the biofilm that had formed was dyed for 15 min at ambient temperature with an aqueous solution of stain (0.1% crystal violet). After 15 min, the excess stain was detached by washing using sterile distilled H₂O. Following the pouring of 250 µL of ethanol (95%) to wells to finally liquefy the dye connected to the cells, wavelength was detected at 570 nm by a microplate reader after 15 min of incubation (Alsolami *et al.* 2023). The highly affected *Klebsiella pneumonia* via biofilm study was subjected to transmission electron microscope (TEM) (JOEL, Tokyo, Japan) to evaluate the changes in the cell ultrastructure.

Anti-diabetic Testing

α-Amylase investigation

The experiment was carried out using the 3,5-dinitrosalicylic acid (DNSA) procedure. The cinnamon bark extract was added to buffer (consisted of 0.03 M NaH₂PO₄/NaH₂PO₄ besides 0.007 M of NaCl at pH 7.0) to dissolve it further, yielding levels (1.9 to 1000 µg/mL), after first being dissolved in 10% dimethyl sulfoxide (DMSO). About 200 µL of the tested extract with 2 units/mL solution of the α -amylase were combined, and the blend was then left at 32 °C for 9.0 min. After that, tubes were filled with 200 µL of the 1% liquefied starch (w/v) solution, and it was left for 3 min. After the reaction was ended with 200 µL of DNSA (12.0 g of sodium potassium tartrate tetra-hydrate in 8.0 mL of 2 M sodium hydroxide and 20 mL of 96 mM of 3,5-dinitrosalicylic acid solution), it was heated in a water bath for 10 min at 95 °C. The mixture was allowed to cool to 20 °C before being diluted with 5.0 mL of distilled H₂O. The wavelength at 540 nm was then detected by a UV-Visible MiltonRoy310 (Tokyo, Japan) spectrophotometer (Nawaz *et al.* 2023).

α-Glucosidase investigation

α -Glucosidase activity of the samples under examination was to be measured. The α -glucosidase enzyme solution (1.0 U/mL) and 0.1 M phosphate buffer (pH 6.8) were left for 20 min at 35 °C with 10 µL of each specimen containing a range of doses, as mentioned in α -amylase investigation. After 20 min, 20 µL of 1 M pNPG (4-nitrophenyl β -D-glucopyranoside as chromogenic substrate) was injected to start the reaction. All contents of the mixture were left for 35 min. Addition of 50 µL of 0.1 N Na₂CO₃ was required to finish the reaction, and the wavelength at 405 nm was detected using the MiltonRoy, (Tokyo, Japan) 310 plus spectrophotometer (Ortiz-Andrade *et al.* 2007).

Antioxidant Testing

DPPH assay

To assess the antioxidant impact of the tested sample *in vitro*, the ethanol solution containing 0.1 mM DPPH was utilized. A total of 1 mL of this solution was split into three and mixed with samples that had different ethanol levels (3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL). All contents of mixture were well shaken and then approved to remain for 30 min at the ambient temperature. The MiltonRoy (Tokyo, Japan) 310 plus was then utilized to detect the wavelength at 520 nm (Hussain *et al.* 2021).

Total antioxidant capacity (TAC)

Through the phosphomolybdenum method, the following components, 0.6 M sulphuric acid, 28.0 mM NaH₂PO₄, 4 mM ammonium molybdate, and 1.0 mL of extract were mixed. Three mL of the prepared solution were left at 90 °C for 160 min. Following the mixture's ambient cooling, absorbance was detected at 630 nm employing a microtiter plate reader (Holzel ELX800; Holzel Diagnostics, GmbH, Hohenzollernring, Germany). The values were presented as ascorbic acid equal (AAE) µg/mg of extracts (Shahid *et al.* 2018). The blank solution contained just 4 mL of the reagent solution.

Ferric reducing antioxidant power (FRAP) assess

Ten mM TPTZ solution in 40 mM HCl, 300 mM acetate buffer (pH 4.0), and 20 mM FeCl₃.6H₂O solution were combined in a 10.0: 1.0: 1.0 ratio to form ferric reducing antioxidant power (FRAP). Cinnamon bark extract solution (39 to 20,000 µg/mL) were prepared, with solution of ascorbyl glucoside (3.9 to 2000 µg/mL) serving as the guideline solution. Samples (20 µL) were added to 96-well plates and allowed to react for 32 min away from light at 37 °C with 180 µL solution of FRAP. Ferrous tripyridyl triazine complex wavelength was recorded at 595 nm for both the extract and standard. The FRAP level in the extract was given in µM AAE (II) equivalent. The FRAP content was estimated by building the standard curve with a ferrous sulphate solution that ranged from 9.8 to 5000 µM (Rachid *et al.* 2022).

Antiproliferative Assay

The ability of cell lines to transfer soluble yellow of tetrazolium [3-(4, 5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide] (MTT) to (E, Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenyl (formazan) (insoluble form), which follows its dissolution with DMSO, is the basis for the cytotoxic effect of cinnamon bark extract (from 500 to 15.63 µg/mL) on MCF-7 (human breast cancer cells) and WI-38 cells (human fetal lung fibroblast cells), as determined by MTT analysis. Using standard dilutions, the result is a purple color, whose level is correlated with the number of non-dead cells. The wavelength at 570 nm was recorded *via* microplate reader (Holzel ELX800; Holzel Diagnostics, GmbH, Hohenzollernring, Germany).. For imaging the treated cells, as mentioned in this assay, the tested cells were treated with cinnamon bark extract (from 500 to 15.63 µg/mL), and then incubated for an additional 24 h at 37 °C. Once the new medium was added, MTT solution (100 µL) was placed in and allowed to sit for 4 h at 37 °C. The cells were inspected with a CCD camera connected to Olympus (1205, Tokyo, Japan) (Gahtori *et al.* 2023).

Detection of Annexin V/PI Apoptosis

Cell death developments were observed *via* measuring kit of Annexin V-FITC apoptosis (Sigma-Aldrich, Darmstadt, Germany) in compliance with the supplier's steps. First, MCF-7 cells (1.0×10^6 cells/well) were placed into 6-well plates, and for a full day they were incubated. Following that, they received treatment with the identified IC₅₀ of cinnamon bark extract for a further 24 h. Following treatment, three PBS washes were carried out on the treated and untreated cells (negative control), followed by trypsinization. The dead cells were subsequently floated in PBS, followed by the addition of 10.0 µL of Propidium iodide (PI) solution and 5.0 µL of Annexin V-FITC to the floated cells. The

floated cells were stored without light for 15 min and at 25 °C. In less than 60 min, a flow cytometer (BD Biosciences, CA, USA) was employed to detect cell death using (BD FAC-Caliber software, USA) (Chittasupho *et al.* 2023).

Cell Cycle Assay

Flow cytometry was employed for this assay. The influence of cinnamon bark extract on the MCF-7 cells' cycle pattern was investigated. MCF-7 cells were cultivated in 6-well plates (1.0×10^6 cells/well) for 24 h before being exposed to the IC_{50} of the extract. Following trypsinization, the cells were rinsed with PBS buffer and fixed at -20 °C using ice-cold 70% ethanol. After that, the cells were treated with PI solution (BD Biosciences, CA, USA) for 35 min at 5 °C. BD FAC-Caliber software, USA, was utilized to analyze the phases of cell cycle (Schoene *et al.* 2005).

Oxidative Enzymes Assessment

To detect the impact of cinnamon bark extract on specific indicators of oxidative stress, measurements were made of the levels of malondialdehyde (MDA), a byproduct of lipid peroxidation, nitric oxide (NO), and hydrogen peroxide (H_2O_2), as well as the actions of superoxide dismutase (SOD), and catalase (CAT), besides glutathione (GSH). The intended enzymes were identified using the reported method of Popovici *et al.* (2021).

Statistical Analysis

Every test was conducted thrice, and the results are displayed as mean \pm . After a one-way analysis of variance (one-way ANOVA) was performed *via* Graph Pad Prism V5 (San Diego, CA, USA) software to evaluate the data.

RESULTS

Assessment of Phytochemicals in Cinnamon Bark Extract

The phytochemicals in the cinnamon bark extract comprising flavonoids and total phenolic content were assessed. The flavonoids had the level of 53.68 ± 0.07 mg(QuE)/mL, while total level of phenolic content was 66.31 ± 0.03 mg(GAE)/mL (Table 1). HPLC was applied to quantitatively investigate the various levels of flavonoids and phenolic compounds in cinnamon bark extract. About 11 various compounds were recorded in the cinnamon bark extract (Fig. 1 and Table 2), where hesperetin and gallic acid were the major compounds. While daidzein, chlorogenic acid, and cinnamic acid were present in moderate levels. Lastly, ferulic acid, ellagic acid, quercetin, syringic acid, syringic acid, and methyl gallate were present in minimal values.

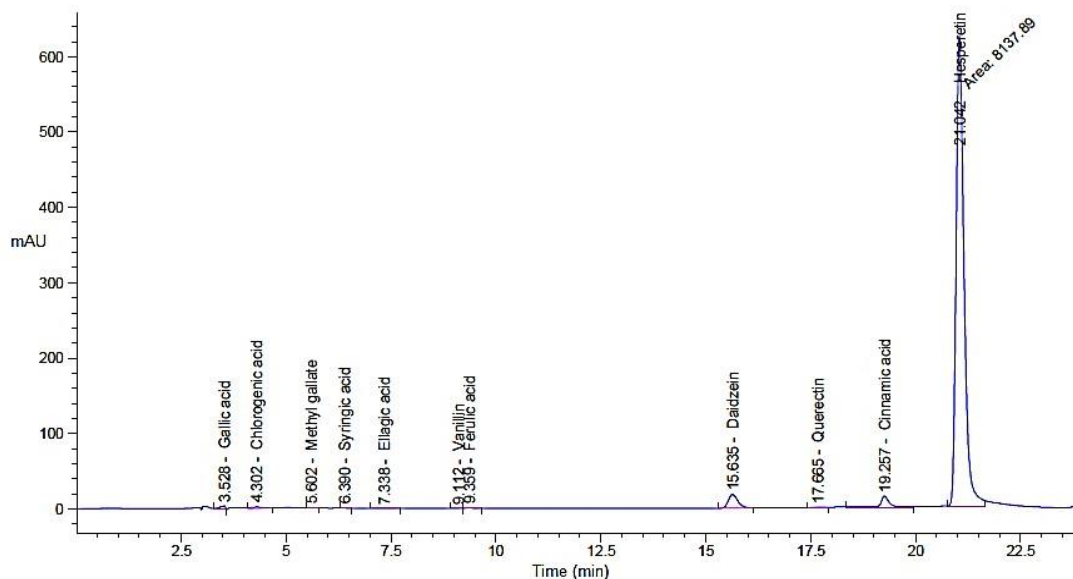


Fig. 1. Peaks of phenol and flavonoid compounds in cinnamon bark extract using HPLC

Table 1. Total Flavonoid and Total Phenolic Content in Extract of Cinnamon Bark

Flavonoids (mg (QuE)/mL)	Phenolic (mg (GAE)/mL)
53.68 ± 0.07	66.31 ± 0.03

Table 2. Various Detected Compounds of Phenolic and Flavonoid in Extract of Cinnamon Bark Utilizing HPLC

Compound	Area	Concentration	
		µg/mL	µg/g
Gallic acid	51.82	229.18	11459.13
Chlorogenic acid	35.40	4.59	229.70
Methyl gallate	1.40	0.07	3.52
Syringic acid	1.26	0.09	4.60
Ellagic acid	3.17	0.32	15.85
Vanillin	1.99	0.07	3.70
Ferulic acid	5.83	0.34	16.94
Daidzein	279.11	15.65	782.67
Quercetin	4.78	0.65	32.27
Cinnamic acid	244.18	4.37	218.64
Hesperetin	8137.89	400.10	20005.03

Antimicrobial Action of Cinnamon Bark Extract, MIC, and MBC

The antimicrobial action of the ethanolic extract of cinnamon bark *versus* *K. pneumoniae*, *S. typhi*, *E. faecalis*, *S. aureus*, *C. albicans*, and *M. circinelloides* were recorded (Fig. 2 and Table 3). The cinnamon bark extract produced inhibition zones *versus* all the tested microorganisms extracted for fungi, which were approximately in the same level for the applied positive control in some tested microorganisms. The inhibition zone *versus* *E. faecalis* was 25 ± 0.1 mm, while MIC and MBC were 15.62 $\mu\text{g/mL}$. The inhibition zone, MIC, and MBC for *K. pneumoniae* were 18 ± 0.2 mm, 125 and 250 $\mu\text{g/mL}$, correspondingly. While the inhibition zone was 23 ± 0.1 mm, MIC and MBC were 15.62 $\mu\text{g/mL}$ *versus* *S. aureus*. Moreover, the inhibition zone against *S. typhi* was 19 ± 0.1 mm while MIC and MBC were 62.5 $\mu\text{g/mL}$. Furthermore, the inhibition zone was 24 ± 0.1 mm, MIC and MBC were 15.62 $\mu\text{g/mL}$ *versus* *S. aureus*. Additionally, the inhibition zone *versus* *C. albicans* was 29 ± 0.1 mm. Besides, 7.8 $\mu\text{g/mL}$ was attributed to MIC and MBC of *C. albicans* (Table 3). Moreover, *M. circinelloides* was inhibited also with inhibition of 16 ± 0.2 mm.

Biofilm formation *versus* *E. faecalis*, *S. aureus*, *K. pneumoniae*, and *S. typhi* upon using various levels (75%, 50%, and 25%) of cinnamon extract were tested. It was noticed that at 75% of MBC for the tested bacteria, the highest reduction in biofilm formation was observed. Meanwhile, 25% of MBC had the lowest reduction in biofilm. For instance, the anti-biofilm activity was found at 84.95%, 77.80%, 89.27%, and 82.79% at 25% of MBC, while it was 94.18%, 89.56%, 95.26%, and 90.88% at 75% of MBC *versus* *E. faecalis*, *S. aureus*, *K. pneumoniae*, and *S. typhi*, in that order. Furthermore, *K. pneumoniae* was the most influenced bacteria compared to other tested bacterial biofilms against cinnamon bark extract (Fig. 3). *K. pneumoniae* ultrastructure was examined *via* SEM, which reflected changes in structures of cell including cell wall, cell membrane, and internal organelles. The cell wall was damaged and separated from the cell membrane (Fig. 4).

Table 3. Antimicrobial Actions, MIC and MBC Levels of Cinnamon Bark Extract *Versus* Tested Microorganisms

Microorganism	Inhibition Zone Diameter (mm)		MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
	Extract of Cinnamon Bark	Positive Control		
<i>E. faecalis</i>	25 ± 0.1	25 ± 0.1	15.62	15.62
<i>S. aureus</i>	23 ± 0.1	24 ± 0.1	15.62	15.62
<i>K. pneumoniae</i>	18 ± 0.2	21 ± 0.2	125	250
<i>S. typhi</i>	19 ± 0.1	20 ± 0.2	62.5	62.5
<i>C. albicans</i>	29 ± 0.1	28 ± 0.3	7.8	7.8
<i>M. circinelloides</i>	16 ± 0.3	18 ± 0.1	-	-

Data are represented as means \pm SD

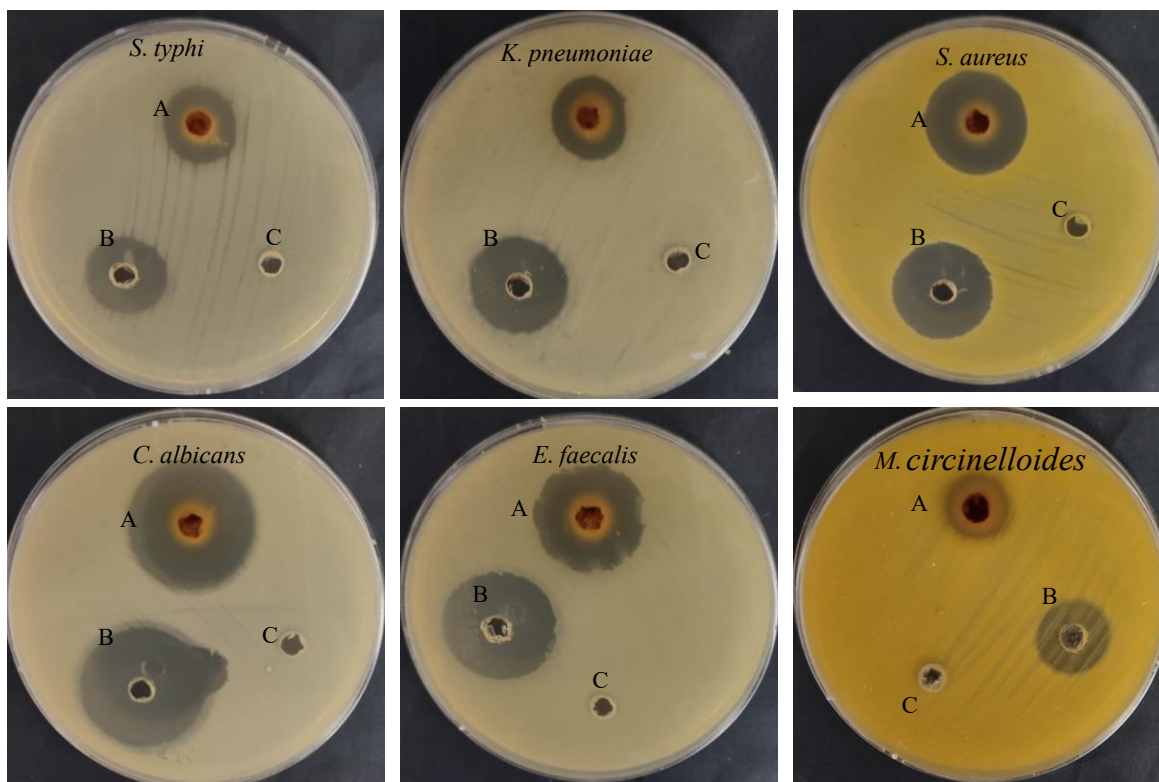


Fig. 2. Different inhibition zones upon testing with cinnamon bark extract *versus* microorganisms in different plates upon using (A) Positive control cinnamon bark extract; (B) Positive control antibiotic/antifungal; (C) Negative control. (Each sample was tested three times on each plate and a negative control (DMSO) could well be seen in each plate)

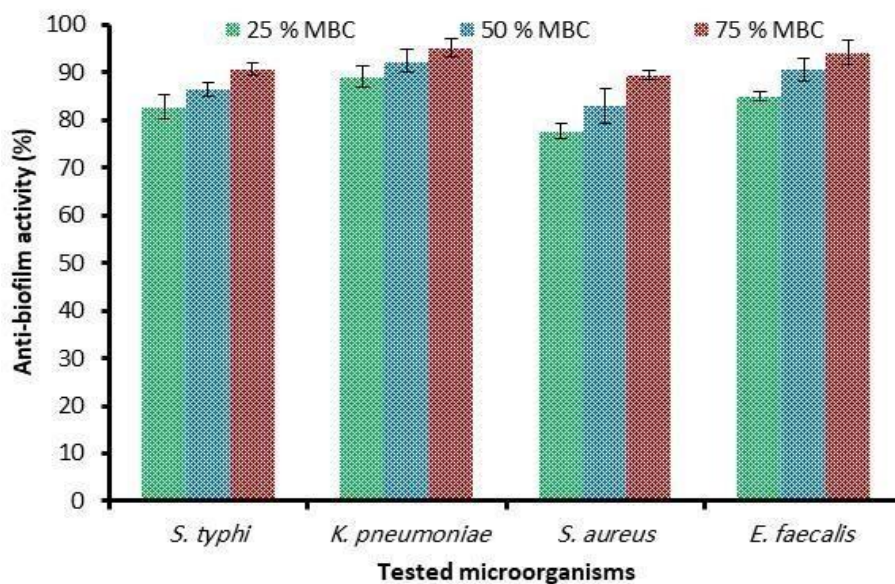


Fig. 3. Anti-biofilm activity of 25%, 50%, and 75% MBC dose of ethanol extract of cinnamon bark against *E. faecalis*, *S. aureus*, *K. pneumoniae*, and *S. typhi* (Statistics are signified as means \pm SD)

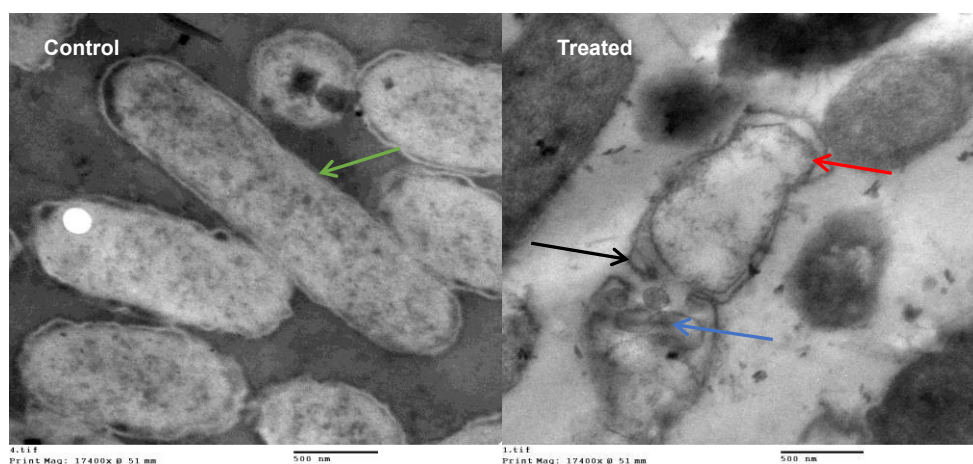


Fig. 4. Ultrastructure of untreated (control) and treated *K. pneumoniae* by cinnamon bark extract. Control cells with normal cells and identical cell walls (Green arrow). The presence of periplasmic, demonstrating the split-up of cell wall from the cytoplasmic membrane (Red arrow), is shown. Rupture of cell wall (Black arrow), and disordered internal structures (Blue arrow) appeared due to a breach of the cell membrane. Direct magnification 40000x.

Anti-diabetic role of cinnamon bark extract via inhibition effects of α -amylase and α -glucosidase

Inhibitions of both actions of α -amylase and α -glucosidase increased as cinnamon bark extract increased from 1.95 $\mu\text{g/mL}$ to 1000 $\mu\text{g/mL}$ (Figs. 5, 6). Excellent results were obtained for prevention of both α -glucosidase and α -amylase with 2.58 $\mu\text{g/mL}$ and 5.01 $\mu\text{g/mL}$ of IC_{50} values, respectively compared to standard acarbose, where the quantities of IC_{50} were 1.99 and 4.32 $\mu\text{g/mL}$, respectively.

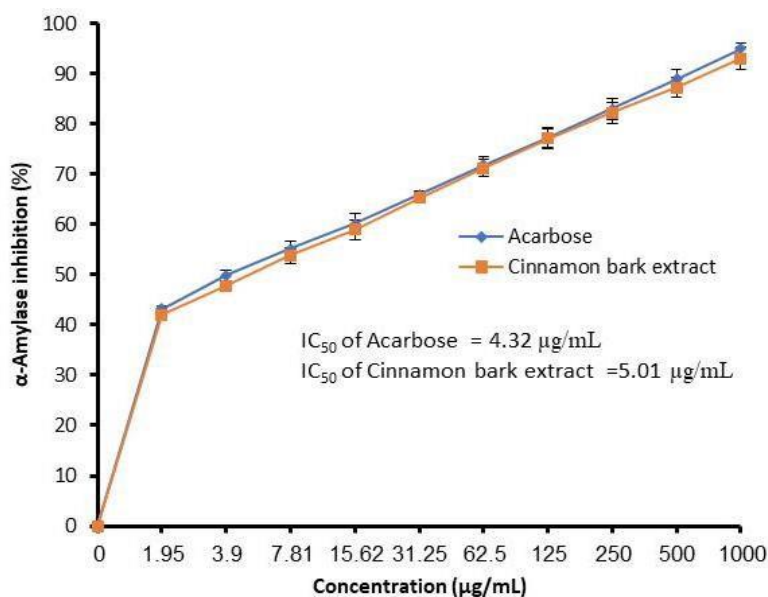


Fig. 5. α -Amylase inhibition of cinnamon bark extract versus acarbose standard (Results are illustrated as means \pm SD)

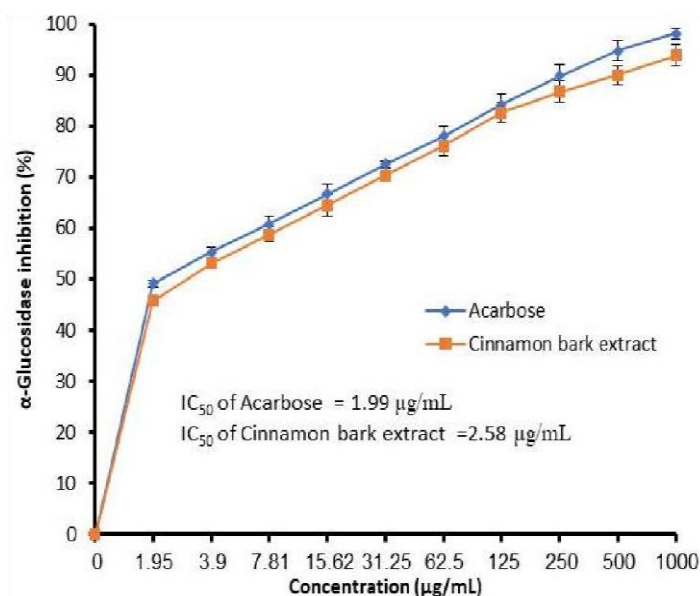


Fig. 6. α -Glucosidase inhibition of cinnamon bark extract *versus* acarbose standard (Data are illustrated as means \pm SD)

Antioxidant Impact

Antioxidant activity by DPPH

Using DPPH, cinnamon bark extract showed a promising antioxidant action, particularly with the increasing dose that accompanied with rising DPPH scavenging percentage. Slight differences between the antioxidant activity of extract and standard compound ascorbic acid at all tested concentrations (Fig. 7) were observed. Cinnamon bark extract possess IC_{50} quantity of 4.57 μ g/mL, whereas ascorbic acid presented a value of IC_{50} to be 2.79 μ g/mL.

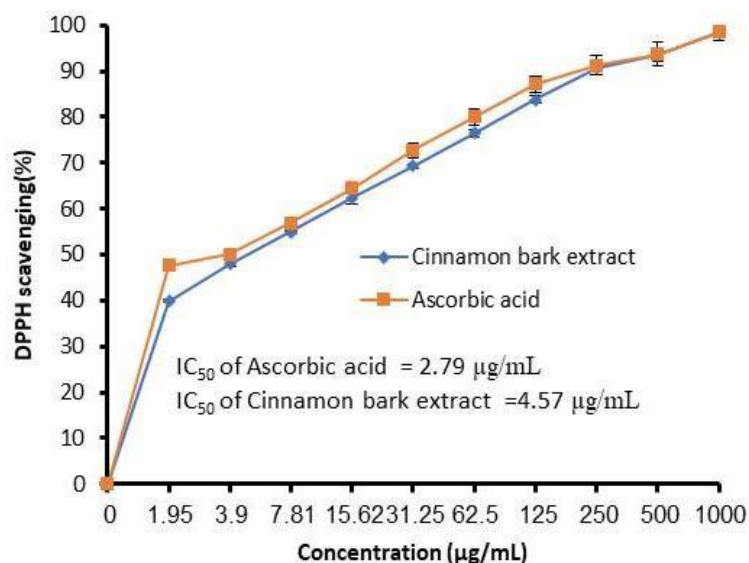


Fig. 7. Antioxidant influence using DPPH assay of cinnamon bark extract and ascorbic acid (At each concentration, the data are illustrated by results mean \pm SD)

Testing antioxidant impact by TAC and FRAP methods

Ethanollic extracts of cinnamon bark were tested by total antioxidant capacity (TAC) protocol resulting in a rate of 816.95 ± 0.050 equiv. AAE $\mu\text{g}/\text{mg}$ of sample. Furthermore, the cinnamon bark extract had a value of 844.65 ± 0.41 equiv. AAE $\mu\text{g}/\text{mg}$ of cinnamon bark extract upon testing employing FRAP assay, as shown in Table (4).

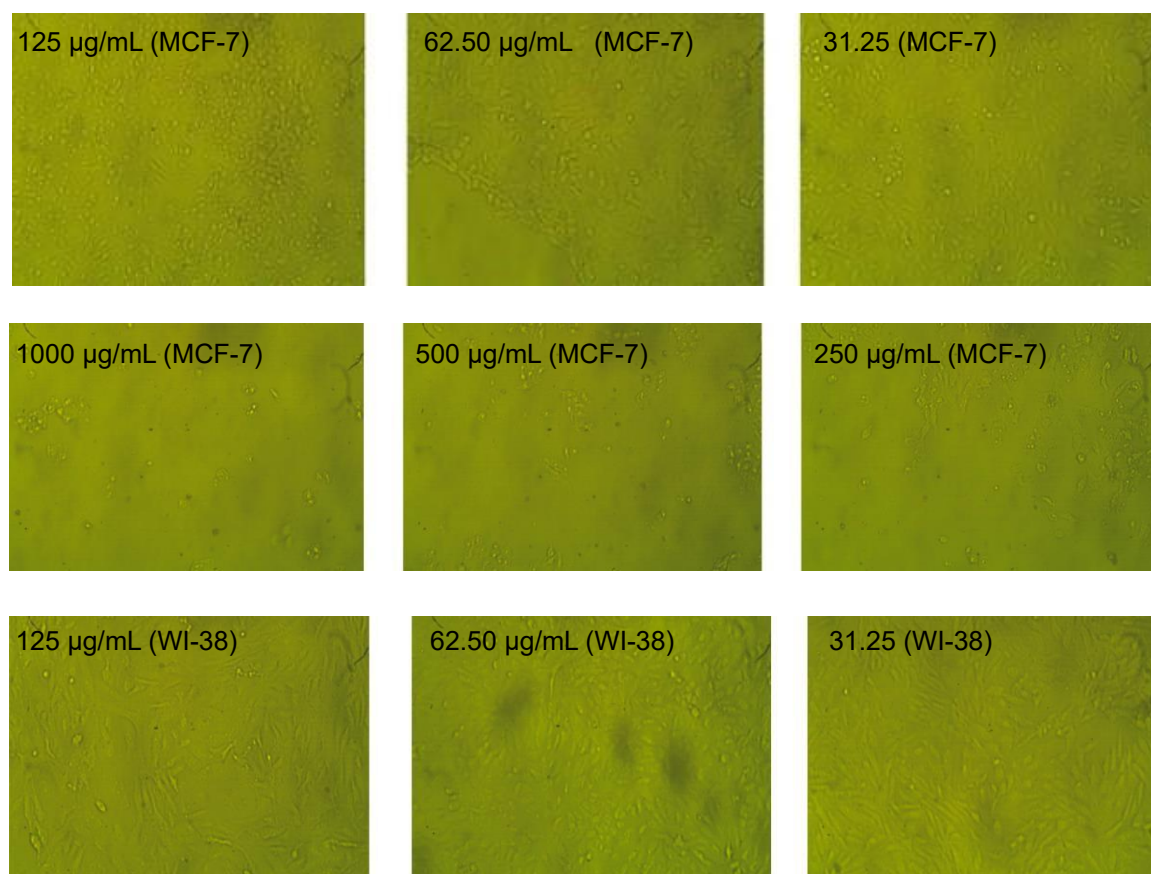
Table 4. Antioxidant Potential of Cinnamon Bark Ethanollic Extract Using TAC and FRAP

TAC Equivalent (AAE)	FRAP Equivalent (AAE)
$816.95 \pm 0.050 \mu\text{g}/\text{mg}$	$844.65 \pm 0.41 \mu\text{g}/\text{mg}$

(Data are represented as means \pm SD)

Anticancer impact of cinnamon bark extract

Cinnamon bark extract displayed a quantity-dependent development inhibitory influence on MCF7 and WI-38 cell lines at quantities from 31.25 to 1000 $\mu\text{g}/\text{mL}$, for one day (Fig. 8). The cinnamon bark extract revealed the lowest IC_{50} quantities towards MCF-7 (cells line of breast cancer) in contrast to WI-38 cells. The extract of cinnamon bark presented the greatest effectiveness with IC_{50} quantities of 77.39 ± 0.84 and $162.67 \pm 0.28 \mu\text{g}/\text{mL}$ on proliferation of MCF-7 and WI-38 cell lines, correspondingly (Fig. 8).



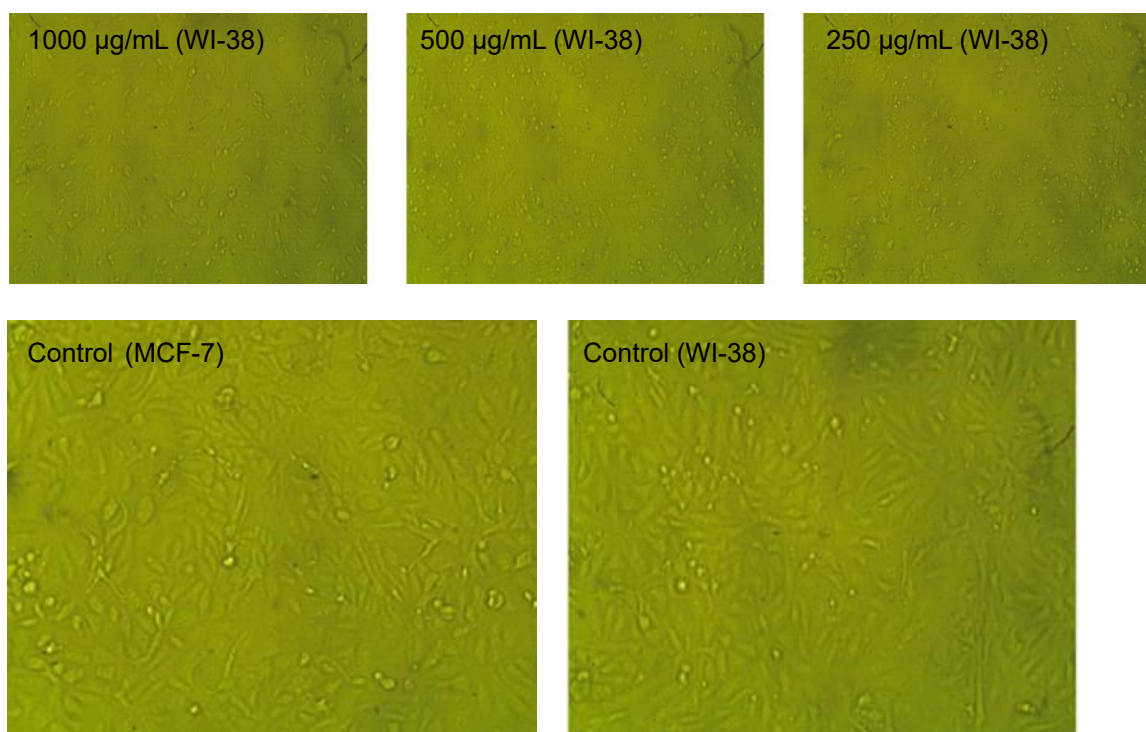


Fig. 8. Anticancer impact of cinnamon bark extract (different concentrations) *versus* MCF-7 and WI-38 cells. Pictures were captured next 24 h incubation using microscope (inverted) (Magnification at 40x)

Annexin V-FITC Apoptotic Study

The impact of cinnamon bark extract on MCF-7 was tested by flow cytometry using Annexin V-FITC staining test, to evaluate either non-specific necrosis or apoptosis that were used to kill the cells. It could be noticed that cinnamon extract dramatically elevated the cells percentage of MCF-7 that were Annexin V-FITC positive throughout both the initial and final steps of apoptosis. The effect was about 14 times what was observed for the untreated cells ($p < 0.05$). Additionally, few cells caused non-specific necrosis cell death (Fig. 9).

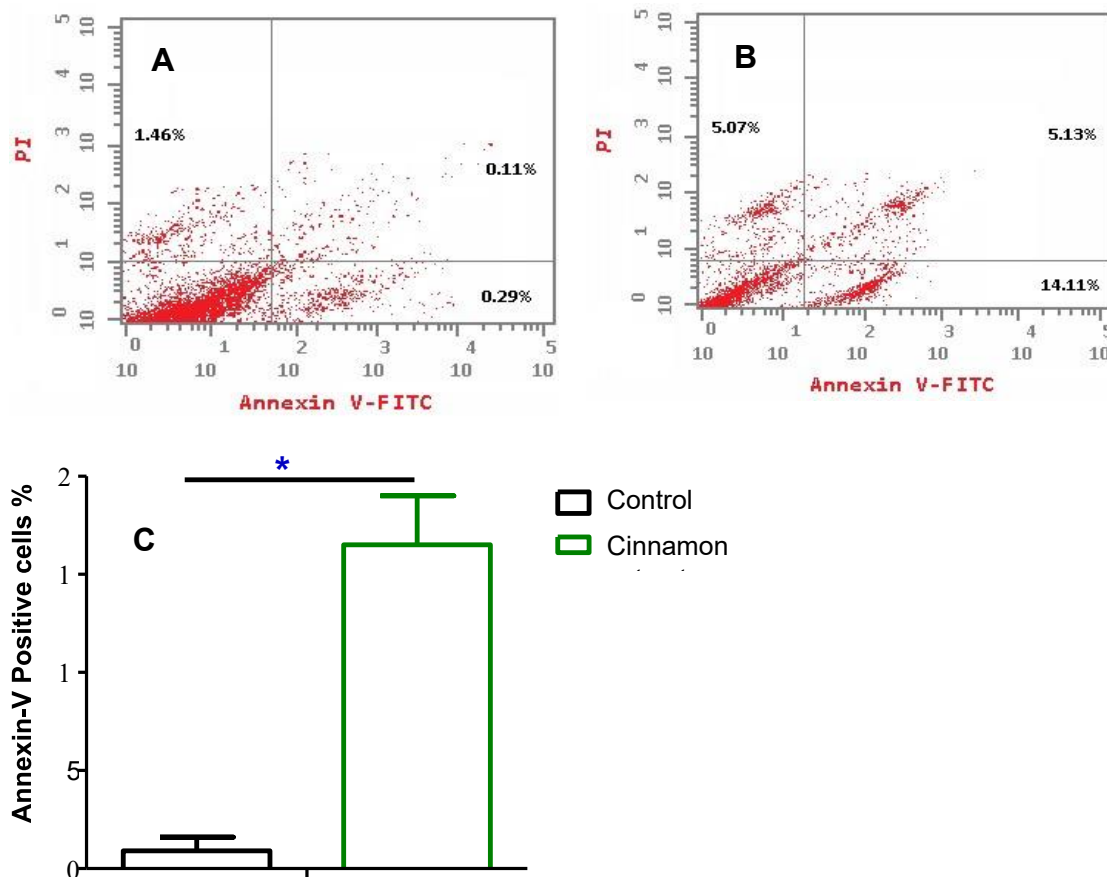


Fig. 9. Annexin V-FITC dual staining in treated MCF-7 cells by $77.39 \pm 0.84 \mu\text{g/mL}$ of cinnamon bark extract for 24 h (A), and control (B); Exposed cells to cinnamon bark extract having melodramatic apoptosis induction (C). Statistics showing difference among cells (untreated and treated) ($P < 0.05$)

Analysis of cell cycle

Distinct cells may be seen at numerous stages of the cell cycle once cinnamon bark extract was applied to MCF-7 cells at its IC_{50} . Comparing the treated MCF-7 control cells (Fig. 10A) to untreated MCF-7 cells (Fig. 10B), cinnamon bark extract revealed a dramatic rise in the cell number at the G2/M stage. Additionally, the outcomes demonstrate that cinnamon bark decreases MCF-7 cell proliferation in the G0/G1 stage to exert its lethal impact (Fig. 10C).

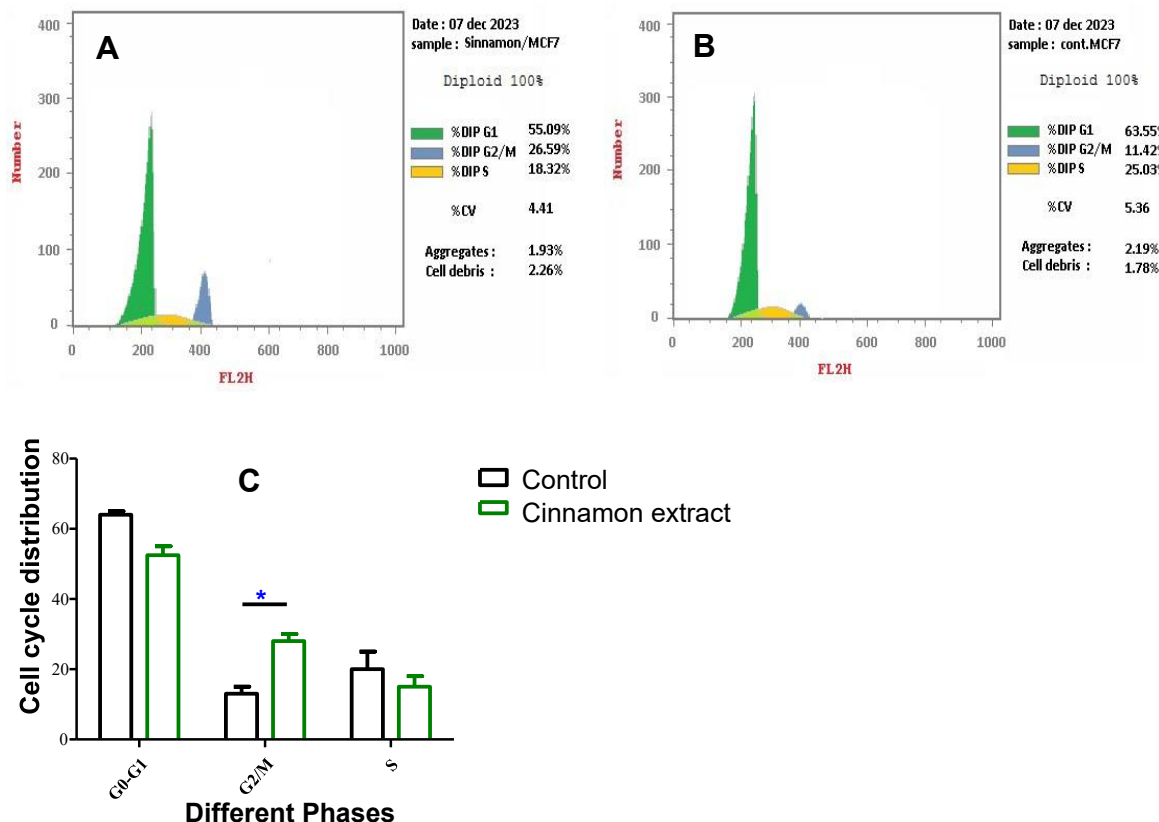


Fig. 10. The cell cycle testing for MCF-7 cells after administration of $77.39 \pm 0.84 \mu\text{g/mL}$ of cinnamon bark extract for 24 h demonstrating various stages of cell cycle namely G1, S, and G2/M (a) Flow cytometry histogram of normal MCF-7 cells; (b) Histogram of flow cytometry of MCF-7 cells exposed to cinnamon bark extract; (c) Statistical analysis between stages of cell cycle (Results are symbolized as averages \pm SD since $p \leq 0.05$ was respected as significant.)

Oxidative enzymes assessment

A reduction in GSH and catalase enzymes levels with a significant ($p \leq 0.05$) p-value was noticed in treated MCF-7 cells using cinnamon bark extract *versus* untreated MCF-7 cells. There was a dramatic elevation in MDA ($p \leq 0.05$) and NO levels in treated MCF-7 cells using cinnamon bark extract *versus* untreated MCF-7 cells. Furthermore, there was a high elevation of H_2O_2 level ($p \leq 0.001$) in treated MCF-7 cells using cinnamon bark extract against untreated MCF-7 cells. Further, a notable elevation in SOD level in treated MCF-7 cells was seen using the extract *versus* untreated MCF-7 cells (Table 5).

Table 5. Testing Levels of Oxidative Enzymes Exuded in Unexposed and exposed MCF-7 Cells to Extract of Cinnamon Bark

Cells	GSH (mg/g Protein)	SOD		MDA (nmol/g Protein)	Catalase (U/g Protein)	NO ($\mu\text{mol/g}$ Protein)	H_2O_2 (nmol/g)
		Inhibition (%)	Activity (U/g Protein)				
Unexposed	8.91	60.8	226.8	7.2	62.4	15.2	0.93
Exposed	0.68*	84.0	313.3	25.3*	32.6*	36.5*	5.23**

* $p \leq 0.5$ and ** $p \leq 0.01$

DISCUSSION

Herbs that contain many bioactive molecules are globally utilized natural substances in health care because of their potent medicinal qualities (Alsolami *et al.* 2023; Alghonaim *et al.* 2024). The aim of the present paper was to examine the potential of cinnamon bark extract against microbial development, biofilm formation, beside its potential as anti-diabetic, anti-tumor and antioxidant, as an extensive herbal remedy.

Absolute ethanol in the present experiments has been used to extract cinnamon bark as the best solvent to obtain all bioactive products from cinnamon, as reported recently by another research group (Alawlaqi *et al.* 2023). To identify the phytochemical components in the cinnamon ethanol extract that may have biological effects, phytochemical testing was conducted in tandem with this investigation. The phytochemical assessments verified the occurrence of medicinal components, such as flavonoids and phenols substances, in the cinnamon ethanolic extract (Al-Garadi *et al.* 2023).

In this study hesperetin, daidzein, chlorogenic acid, gallic acid, and cinnamic acid were identified with notable levels, as reported by other investigators (Khalisyaseen and Mohammed 2021). The primary constituents of cinnamon essential oil are eugenols and cinnamaldehyde, which account for the potent antimicrobial properties of the extract. The phenolic ingredients in the extract are the highest contributors for management of inflammatory, oxidative stress, diabetic, and tumor. Cinnamaldehyde is an electronegative molecule that is involved in biological mechanisms (Vivas *et al.* 2019; Iwata 2022). This study illustrated the successive antimicrobial role of cinnamon bark extract *versus E. faecalis*, *S. aureus*, *K. pneumonia*, *S. typhi*, and *C. albicans*. The biological process behind the antimicrobial activity of cinnamon bioactive molecules on cell walls has been shown by other studies (Shabani *et al.* 2016).

The current investigation illustrated the effective anti-biofilm formation of cinnamon bark extract *versus E. faecalis*, *S. aureus*, *K. pneumonia*, and *S. typhi*, which is comparable to the report of Waty and Suryanto (2018). They examined effects of an ethanolic cinnamon extract at various concentrations and found that alkaloids and flavonoids slowed the development of six distinct Gram-positive bacterial species (Gulcin *et al.* 2016). Flavonoids have antibacterial properties because they can combine with extracellular proteins to generate complex chemicals that damage bacterial cell membrane integrity (Qanash *et al.* 2023a). Phenolic compounds have antibacterial properties because of their alkaline content and ability to alter osmotic tension, which can harm bacteria's cell walls and surrounding tissues. The osmotic pressure that exists among bacteria and their surroundings is impacted by the alkaline properties of phenolic compounds (Qanash *et al.* 2023b). It is believed that a number of variables, comprising the occurrence of extracellular polymeric molecules around cells of bacteria, contribute to biofilm sensitivity to antibacterial chemicals. From the obtained results, extract of cinnamon bark exhibits good anti-biofilm properties. The extract inhibits the bacterial biofilm through penetration or preventing the formation of extracellular polymeric molecules. Since this herb is used as a food additive, it can be applied in the form of a paste or cream to fight periodontal pathogens or pathogenic microbes in diabetic foot, as well as in the form of tablets to fight stomach microbes.

Many plant extracts have been subjected to free radical scavenging assays, with DPPH, TAC, and FRAP being commonly employed assays. The present results showed

that cinnamon bark extract has a promising antioxidant impact. At a dose of 0.01 mg/mL, the extracts demonstrated strong free radical scavenging action, according to an investigation conducted recently (Madushika Wariyapperuma *et al.* 2021). Furthermore, the examined assays to test anti-diabetic impact of cinnamon bark extract showed its efficient anti-diabetic outcome. Agreeing to the research managed by Wickramasinghe *et al.* (2018), the *C. zeylanicum* methanolic extract exhibited 80% α -amylase inhibitory action. Moreover, according to Nawaz *et al.* (2023), the enzyme activity of α -glucosidase (36.0 ± 8.0 $\mu\text{g/mL}$) and α -amylase (57.0 ± 8.0 $\mu\text{g/mL}$) was decreased by cinnamon extracts. Moreover, Hayward *et al.* (2019) showed that cinnamon extract was more beneficial for diabetes individuals and exhibited anti-hyperglycemic qualities. As mentioned previously, the present enzymes (α -amylase and α -glucosidase) perform an essential role in the controlling of blood glucose degrees besides hyperglycemia (Al-Rajhi *et al.* 2023a, Almehayawi *et al.* 2024). In this context, polysaccharides are broken down into simple sugars such as glucose and fructose during metabolic processes which regulated in some cases by these enzymes.

In this study the extract was found to be useful in decreasing the viability of MCF-7 cells with a notable role in accelerating apoptotic rate of MCF-7 cells. In accordance with the previously published work, several mechanisms have been recommended to clarify the anti-proliferative activity of cinnamaldehyde on certain tumor cell lines. These processes include apoptosis induction, cell cycle arrest, and disruption of cellular structures (Banerjee and Banerjee 2023). Nevertheless, Roth-Walter *et al.* (2014) found that cinnamaldehyde therapy resulted in apoptosis and the prevention of viability and proliferation of cell in primary and immortalized immune cells. This shows unequivocally that, even though cinnamaldehyde has anti-carcinogenic properties, administering it to cancer patients may not be appropriate because of its potential for preventing immune system stimulation.

Antioxidant enzymes have been developed by mammalian cells as a defense against oxidative damage. One specific indicator of how cells react to oxidative damage is a shift in the rate of activity of antioxidant enzymes (Nordberg and Arnér 2001). Thus, in the present investigation, the curative influence of processed cinnamon extract in MCF-7 cells was approved by the outcome of the tested enzymes. Cinnamon bark extract contains phenolic compounds such as *trans*-cinnamic acid and hesperetin that may contribute to antioxidant function in several ways: by eliminating free radicals, attaching ions of metals, blocking the enzymatic processes that generate free radical forms, raising the levels of physiologically significant natural antioxidants, and stimulating the development of numerous genes that generate the enzymes that lower oxidative damage (Primiano *et al.* 1997).

CONCLUSIONS

1. The ethanol extract of cinnamon bark contained phenolic and flavonoid active components.
2. The investigated bacterial and fungal pathogens were all susceptible to the antimicrobial action of the cinnamon ethanol extract, where *Enterococcus faecalis* and *Candida albicans* were the most impacted microbes by the extract. Additionally, it has an anti-biofilm effect that is strongest against *Klebsiella pneumoniae*.

3. Tests conducted *in vitro* demonstrated the probable benefits of cinnamon bark extract as an antioxidant and anti-diabetic agent.
4. The ethanol extract from cinnamon bark was attained to be efficient *versus* breast cancer in this investigation. Tests on cells indicate that the extract can interact with cell mechanisms and regulate oxidative enzymes and apoptosis.
5. The ultrastructure changes in *K. pneumoniae* as well as antioxidant enzymes, morphological changes, and apoptosis induction indicated the action mechanisms of cinnamon bark extract against bacteria and cancer cells.

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