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CHEMICAL CHARACTERIZATION OF *Cinnamomum verum*. HYDRODISTILLATION BY-PRODUCTS, EVALUATING THEIR ANTIOXIDANT, AND ANTICANCER ACTIVITIES

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ABSTRACT: Essential oils derived from *Cinnamomum verum* are widely used; nevertheless, the hydrodistillation waste produced with these oils are relatively unexplored and underutilized. With only partial data available in the literature, we analysed the chemical composition of by-products from hydrodistilled cinnamon. Ethanolic solid residue of cinnamon by-product (ECE) was extracted using 80% ethanol. The extract was tested for antioxidant activity and demonstrated a high effect. The overall phenolic content was 85.25 mg GAE/g, whereas the flavonoid content was 23.14 mg QE/g. The inhibitory concentration that inhibits 50% of the cancer cell population (IC_{50}) for the cytotoxic action of ECE extract against HCT 116 (human colon) and PC3 (human prostate) cancer cell lines at varied concentrations (31.25-1000 µg/mL) was 31.4 µg/mL for HCT 116 and 33.4 µg/mL for PC3.We conclude that the solid residue of cinnamon by-product exhibits significant potential in the development of phyto-medicines with anticancer properties. Drugs derived from Ethanolic cinnamon solid by-product (ECE) could serve as an alternative medicinal source due to their anticancer activity. Furthermore, this study suggests that the ethanol extract of this by-product possesses the greatest potential for anticancer activity against (HCT 116) Human colon and human Prostate (PC3) cancer cell lines.

Key words: Cinnamon hydrodistillation, bioactive compounds, antioxidant, anticancer activities.

INTRODUCTION

Herbs and spices have been used since ancient times for nutritional and traditional medicine applications as they are rich in natural bioactive substances eliciting, for example, antioxidant and anti-inflammatory responses. Among these, cinnamon is widely used both as a bark extract and powder. There are over 300 species of cinnamon, but the most diffused as food/nutraceuticals are *Cinnamomum verum J. Presl, Cinnamomum aromaticum* or *Cinnamomum cassia* (L.) J. *Presl*, *Cinnamomum burmanni* (*Nees and Th. Nees*) *Nees ex Blume, 2012* and *Cinnamomum loureiroi Nees*. These species differ in morphology and chemical composition. Specifically, at the phytochemical level, significant differences in the content of polyphenols and volatile phenols have been observed among various genotypes **(Nabavi *et al.*, 2015)**.

Moreover, the secondary metabolites profile also depends on plant growth conditions and the associated environmental pressures **(Avula *et al.*, 2015; Ford *et al.*, 2019)**. Among the most interesting compounds of bioactive Cinnamomum accessions, there are vanillic acid, caffeic acid, gallic acid, *p* coumaric acid, ferulic acid, proanthocyanidins A and B, kaempferol, cinnamic acid and cinnamaldehyde, which exhibit several human beneficial effects, such as neuroprotective, hepatoprotective, cardioprotective and gastroprotective **(Khasnavis and Pahan, 2012; Nabavi *et al.*, 2015)**. Most of these compounds are related to the antioxidant activity, enhancing the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) (**Hemmati *et al.*,**

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2018; Liao *et al.*, 2012). Another important activity of cinnamon phyto-complexes is the anti-inflammatory one, which has been demonstrated in various cell and animal models and diseases, such as colitis, arthritis and diabetes **(Hagenlocher *et al.*, 2017; Hariri and Ghiasvand, 2016; Kim and Kim, 2017; Kim and Kim, 2019)**.

Because of these healthful properties, the cinnamon extract and/or specific interesting compounds have been used as a bioactive ingredient in food products and supplements.

However, it should be considered that the use of only isolated molecules is quite limited both because, in many cases, the beneficial effect is performed by a combination of molecules that act in synergy, and due to their poor systemic distribution and relative bioavailability **(Quero *et al.*, 2020**). For this reason, in recent years, the use of total cinnamon phytoextracts obtained by aqueous or hydroalcoholic extractions capable of optimizing the content of various bioactive molecules has become increasingly widespread. The extracts are often used to produce functional foods, such as yoghurt, creams and ice creams, rather than confectionery products up to food supplements and dietetic foods **(Liu *et al.*, 2022)**.

The solid residues that remain after hydrodistillation can also be used as secondary raw material to obtain different bioactives using conventional extraction techniques, such as solvent extraction in an orbital shaker and Soxhlet apparatus, or innovative techniques, such as ultrasound bath, resulting in considerable concentrations of phenols and flavonoids in the final extracts (**Boulila *et al.*, 2015; Sánchez-Vioque *et al.*, 2015; Santana-Méridas *et al.*, 2014; Veličković *et al.*, 2008; Wollinger *et al.*, 2016)**. When comparing different extraction procedures used to process solid hydrodistillation residues, ultrasound application is considered gentler compared to other extraction procedures, working at a lower temperature and for a shorter time, making it more suitable for the preservation of polyphenols from thermal degradation **(Bashi *et al.*, 2012; Da Porto *et al.*, 2013; Foo *et al.*, 2017).**

In this study, we assessed the antioxidant, total flavonoid, total phenolic, and anticancer properties of ethanolic cinnamon by-product extract's solid residue

MATERIALS AND METHODS

Materials and Chemicals

Cinnamon has been obtained from a local market (Zagazig, Egypt). All solvents used throughout the present work were obtained from different companies. 1, 1-Diphenyl-2, picrylhydrazyl (DPPH), β-carotene, quercetin, gallic acid and Tert-butyl hydroquinone (TBHQ), were purchased from Sigma (St. Louis, MO, USA).

HCT 116 cell (human colon cancer cell line), and PC3 cells (prostate carcinoma cell) were obtained from VACSERA Tissue Culture Unit (Giza, Egypt).

Preparation of Cinnamon Extracts (ECE)

Cinnamon was dried in a vacuum oven (Thermo Fisher Scintific Inc., Japan) at 45°C for 72 h and grounded to a fine powder in a mill (Retsch, Model ZM 1000, Haan, Germany).

Approximately 100 g of dried plant material underwent steam distillation for about 4 hours in a pilot-scale apparatus to extract essential oil. Ethanolic residual solid cinnamon by-product (ECE) post-distillation was initially sun-dried for Forty-eight hours to achieve a moisture content below 10%, then milled in a laboratory grinder to a size smaller than 0.5 mm. The ECE was preserved at 4°C for subsequent analysis.

Ethanolic solid residue of cinnamon by-product (ECE) was extracted using the method described in reference (**Kadifkova Panovska *et al.*, 2005**). A quantity of 100 g of ethanolic solid residue of cinnamon by-product was extracted with 1000 mL of ethanol 80% at room temperature for Twenty-four h. The extract was then centrifuged at 5500 rpm for ten minutes. The remaining residue was re-extracted twice under identical conditions and filtered through filter paper. The ethanol 80% extract was subsequently concentrated under reduced pressure using rotary evaporator and lyophilized to yield a powder.

Phytochemical Analysis of ECE

Determination of total phenolic compounds

Total phenolic compounds of solid residue of cinnamon by-product were determined according to the method described by **(Škerget *et al.*, 2005)**.

Determination of total flavonoids

The total flavonoid compounds of solid residue of cinnamon by-product were determined according to the method described by **Ordonez *et al.* (2006**).

Antioxidant Activity Determination of ECE

DPPH radical-scavenging activity

The electron donation ability of the obtained extracts was measured according to **(Hatano *et al.*, 1988)** by bleaching of the purple-colored solution of DPPH. One hundred µL of each extract (10 mg extract/10 mL solvent) was added to 3.9 mL of 0.1 Mm DPPH dissolved in methanol. After 120 min, the absorbance at 517 nm was measured against control (Gülçin *et al.*, 2004). Percentage of antioxidant activity of free-radical DPPH was determined as follows:

Antioxidant activity (Inhibition) percentage = $[(A_{control} - A_{ECE})/A\ control] \times 100$.

A control is the absorbance of the control reaction, while A_{ECE} is the absorbance in the presence of ECE. TBHQ and gallic acid were utilized as positive controls.

β-Carotene/linoleic acid bleaching

Extracts and synthetic antioxidants were investigated for their ability to prevent β-carotene bleaching (**Dastmalchi *et al.*, 2007**).

A control with no extract was also tested. The antioxidant activity was calculated using the following procedure:

Antioxidant activity (%) = $[1-(Abs^0_{ECE} - Abs^{120}_{ECE})/ (Abs^0_{control} - Abs^{120}_{control})] \times 100$

Abs^0_{ECE} represents the initial absorbance of ECE at 0 time, Abs^{120}_{ECE} designates the absorbance of ECE after 120 minutes, $Abs^0_{control}$ represents the initial absorbance of the control at 0 time, and $Abs^{120}_{control}$ represents the absorbance of the control after 120 minutes.

Antitumor Activity determination of ECE

Sample cytotoxicity on cells is determined using the MTT technique

The effect of ECE concentrations ranging from 31.25-1000 µg/mL on human cell line viability was tested in vitro using MTT-assay. Normal cells (Vero cells) and cancer cells (HCT 116 and PC3) were obtained from the VACSERA Tissue Culture Unit in Giza, Egypt. A 96-well tissue culture plate was filled with 1 X 10^5 cells/mL (100 µL/well) and cultured at 37°C for 24 h to generate a full monolayer. The growing media was then withdrawn from the wells. The confluent cell monolayer was washed twice with washing solution. Two-fold dilutions of the test sample were produced in RPMI medium with 2% serum. 0.1 mL of each dilution was applied to separate wells, with three wells set aside as controls with only maintenance medium. The plate was incubated at 37°C for evaluation. The cells were tested for toxicity signs such as partial or complete monolayer loss, cell rounding, shrinkage, or granulation. An MTT solution (5mg/mL in PBS) supplied by BIO BASIC CANADA INC was made. Each well received 20 µL of this solution. The plate was shaken at 150 rpm for 5 minutes to completely mix the MTT and medium. It was then incubated at 37°C with 5% CO2 for 1-5 h to allow the MTT to metabolize. The media was discarded, and the plate was dried with paper towels as needed. Formazan, a metabolic product of MTT, was resuspended in 200 µL DMSO. The plate was then shaken at 150 rpm for 5 minutes to completely dissolve the formazan. The optical density was measured at 560nm after eliminating the background at 620nm. The optical density of cells is directly proportional to their amount **(Van de Loosdrecht *et al.*, 1994)**.

The cell viability and cytotoxicity percentages were estimated using the following formulas:

Cell viability (%) = (Abs_{ECE} /Abs control) x 100

The following formula was used to determine the tested substance's cytotoxic activity (%):

Cytotoxic activity (%) =100 %- cell viability (%)

The ECE concentration producing 50% growth inhibition is termed IC_{50}.

Statistical Analysis

Experiments were repeated three times and findings were provided as mean ± standard error. The ANOVA variance analysis was performed using the general linear models

(GLM) approach in the Statistical Analysis System software (SAS version 9.1, SAS Institute, 2003). A p-value of < 0.05 indicated statistical significance (**Rutherford, 2012)**.

RESULTS AND DISCUSSION

Total Active Components in ECE

The study found a phenolic content of 85.25 mg GAE g−1 ECE and a flavonoid content of 23.12 mg QE g−1 ECE. Phenolic chemicals are known to inhibit the cyclooxygenase and lipoxygenase pathways **(Ferrandiz *et al.*, 1991; Ferrandiz *et al.*, 1990; MJ, 1991).** Flavonoids are found to inhibit Ornithine decarboxylase enzyme, it is rate-limiting enzyme in polyamine biosynthesis, which has been correlated with the rate of DNA synthesis and cell proliferation in several tissues, hence inhibiting cell proliferation (**Tanaka *et al.*, 1997a; Tanaka *et al.*, 1997b; Makita *et al.*, 1996**). The flavonoids and phenols found in this plant may tell more about the therapeutic effects of cinnamon byproducts.

The Antioxidant Activity of ECE

The antioxidant activity of the extract was assessed using two methods: DPPH and β-Carotene/linoleic acid bleaching assays. These methods provide a comprehensive evaluation of the extract's ability to scavenge electrons or free radicals. Commonly utilized to determine the antioxidant potential of complex samples, these assays are based on different principles **(Munteanu and Apetrei, 2021)**.

The ECE extract demonstrated potent antioxidant properties, as evidenced by the DPPH scavenging activities shown in Fig. 1, compared to standard substances TBHQ and gallic acid. A concentration-dependent increase in antioxidant activity was observed in all samples tested over time, indicating an enhanced ability to scavenge free radicals.

As depicted in Fig. 2, ECE inhibits β-carotene bleaching by neutralizing radicals originating from linoleic acid. The scavenging of radicals derived from linoleates resulted in a higher retention of β-carotene (63.01) in comparison to TBHQ (31.3) and gallic acid (18.1). Phenolic compounds and flavonoids are linked to antioxidative effects in biological systems, functioning as scavengers of both singlet oxygen and free radicals (**Sharma *et al.*, 2015**). There is a strong correlation between the presence of phenolic substances and antioxidant activity (**Shahwar *et al.*, 2010**). Within biological systems, the most significant free radicals are reactive oxygen species (ROS), which include hydroxyl radicals, hydrogen peroxides, and superoxide anions (**Pryor *et al.*, 2006**).

The Cytotoxicity Effect of Ethanolic 80% Solid Residue of Cinnamon By-Product Extracts on (HCT 116) Human Colon and Human Prostate (PC3) Cancer

The MTT assay was used to assess the cytotoxic activity of ECE extract against (HCT 116) human colon and human prostate (PC3) cancer cell lines at different doses (31.25-1000 µg/mL), as stated in Tables 1 and 2. The plant extract's antiproliferative efficacy against cancer cell lines was quantified as an IC_{50} value. IC_{50} is the inhibitory concentration required to reduce cancer cell population by 50%.

The extracts induced an inhibitory variance in cell growth according to the sort of extract and kind of cell line. The phenolic compounds in medicinal plants are bioactive and have an important role in cancer prevention. They have a complementary and overlapping mode of action, which includes antioxidant activity, free radical scavenging, and modulation of carcinogen metabolism, all of which alter important cellular and molecular mechanisms related to carcinogenesis, a multistep process involving tumor cell transformation, survival, proliferation, angiogenesis, and metastases, as discovered by **Vurusaner *et al.* (2012)**.

Ethanolic cinnamon extract inhibit vascular endothelial growth factor subtype 2 (VEGFR2) kinase activity, thereby inhibiting the angiogenesis involved in cancer. The results of the study revealed that cinnamon could potentially be used in cancer prevention (**Lu *et al.*, 2010**). Cinnamaldehydes have been synthesized and tested as inhibitors against angiogenesis **(Kwon *et al.*, 1997)**.

Jeong *et al.* (2003) reported that CB403, a chemical that can be synthesized from 2 -hydroxycinnamaldehyde derived from cinnamaldehyde, can inhibit tumor growth.

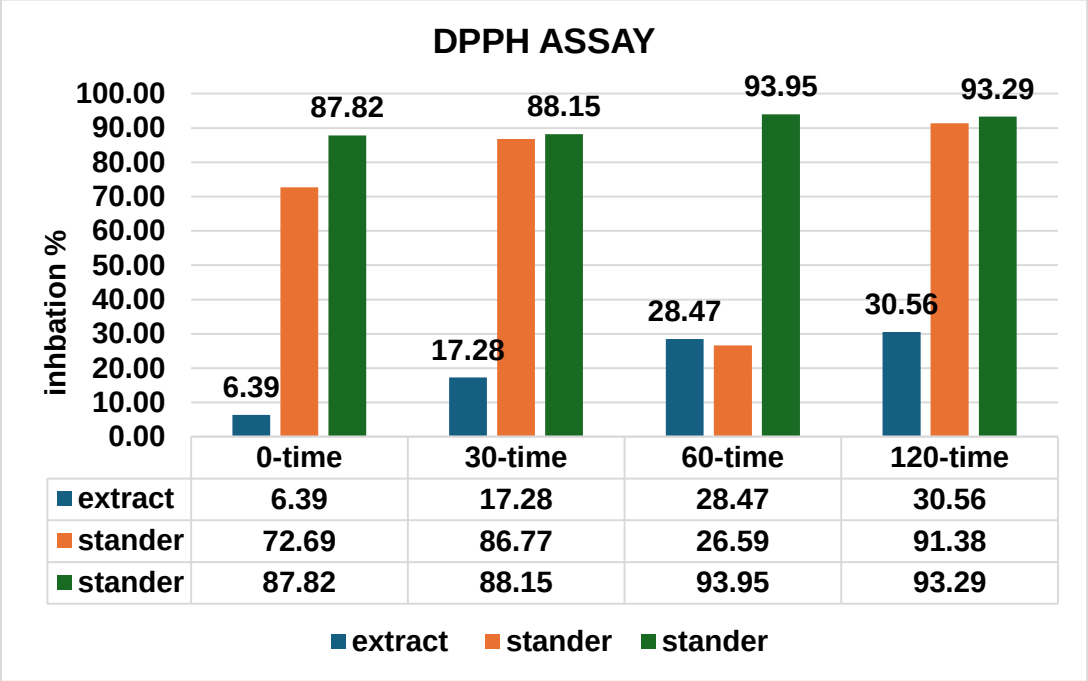

Fig. 1. Antioxidant activity of ECE against DPPH• as compared with TBHQ and gallic acid

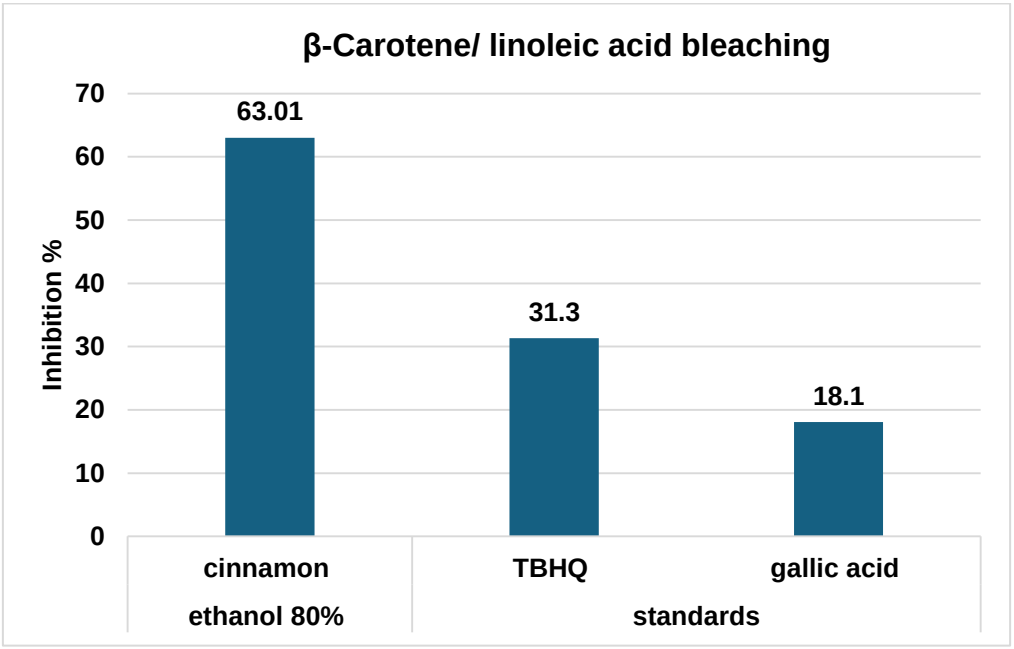

Fig. 2. Inhibition of ECE in β-carotene-linoleic acid emulsion as compared with TBHQ and gallic acid

Table 1. Percent cell viability of ethanolic 80% solid residue of cinnamon by-product extracts of HCT 116 cell line

ID	Conc. µg /mL	Viability %	Toxicity %	IC_{50} (µg/mL)
HCT 116	--------	100	0	
ECE	1000	4.968383017	95.03161698	49.44
	500	4.33604336	95.66395664	
	250	5.239385727	94.76061427	
	125	13.64046974	86.35953026	
	62.5	40.19873532	59.80126468	
	31.25	61.24661247	38.75338753	

Table 2. Percent cell viability of ethanolic 80% solid residue of cinnamon by-product extracts of PC3 cell line

ID	Conc. µg /mL	Viability %	Toxicity %	IC_{50} µg /mL
PC3	--------	100	0	
ECE	1000	4.935064935	95.06493506	54.97
	500	6.32034632	93.67965368	
	250	13.16017316	86.83982684	
	125	19.30735931	80.69264069	
	62.5	34.11255411	65.88744589	
	31.25	69.87012987	30.12987013	

Overall, the antitumor and growthinhibitory properties of CB403 in animal-based studies as well as in cell culture-based studies indicate the potential of cinnamon to be used as an anticancer agent **(Jeong *et al.*, 2003**).

Cabello *et al.* (2009) reported that cinnamic aldehyde inhibits the activity of NF-κ B and the production of tumor necrosis factor alpha (TNFα -) induced interleukin-8 (IL-8) in A375 cells. This inhibition provides additional support to the existing unrecognized role of cinnamic acid as a potential anticancer agent **(Cabello *et al.*, 2009).**

Fang and others reported the anticancer effect of trans-cinnamaldehyde from cinnamon, finding that trans-cinnamaldehyde showed potential effects in restraining tumor cell growth and in enhancing tumor cell apoptosis **(Fang *et al.*, 2004).**

Treatments with the extracts of cinnamon and cardamom augment the activities of the detoxifying and antioxidant enzyme glutathione-stransferase (GST) with a concomitant reduction in lipid peroxidation levels in animals with colon cancer compared to controls (**Bhattacharjee *et al.*, 2007)**.

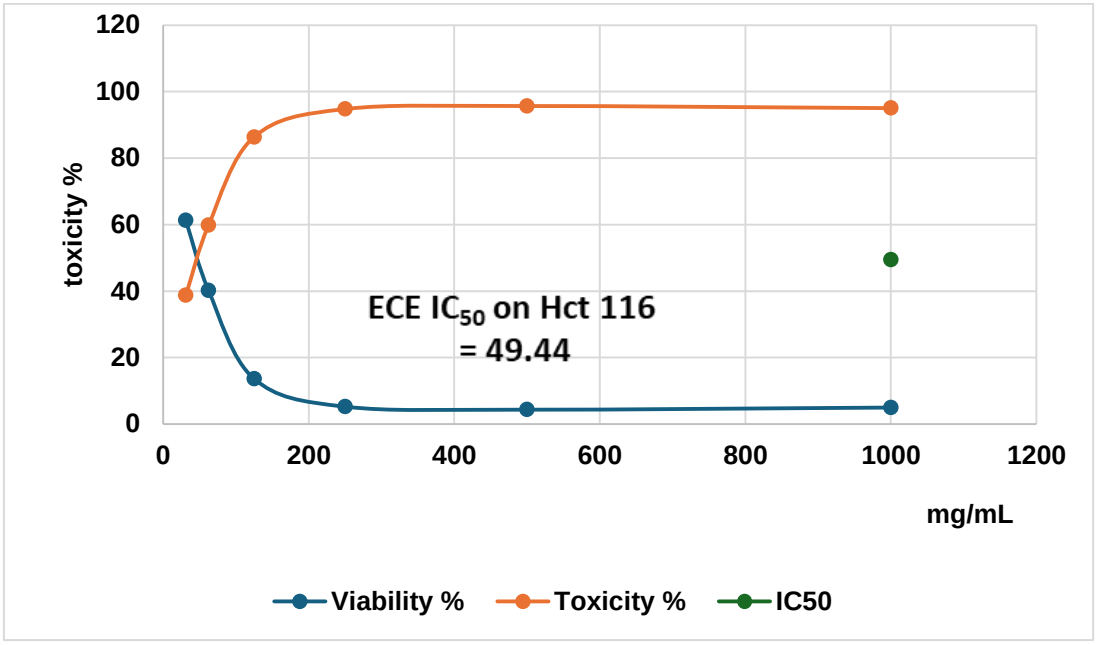

Fig. 3. Effect of ethanolic 80% solid residue of cinnamon by-product extracs on human colon cancer cell line (HCT 116).

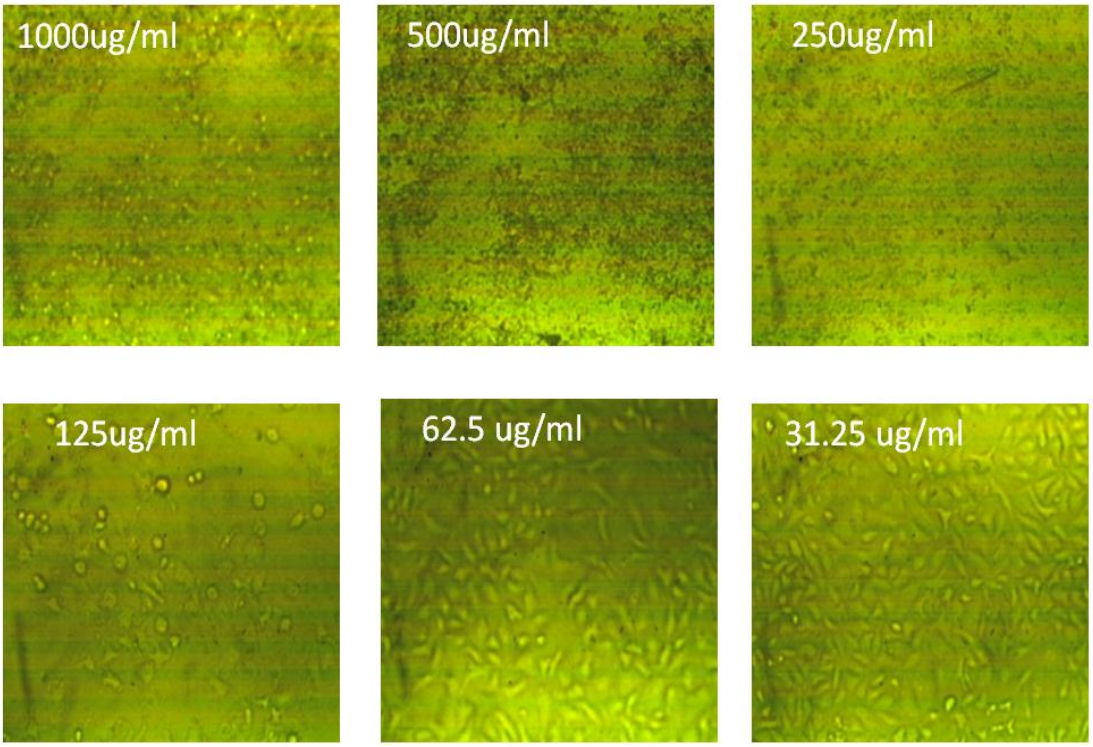

Fig. 4. Percent cell viability and toxicity of ethanolic 80% solid residue of cinnamon by-product extracts of HCT 116 cell line

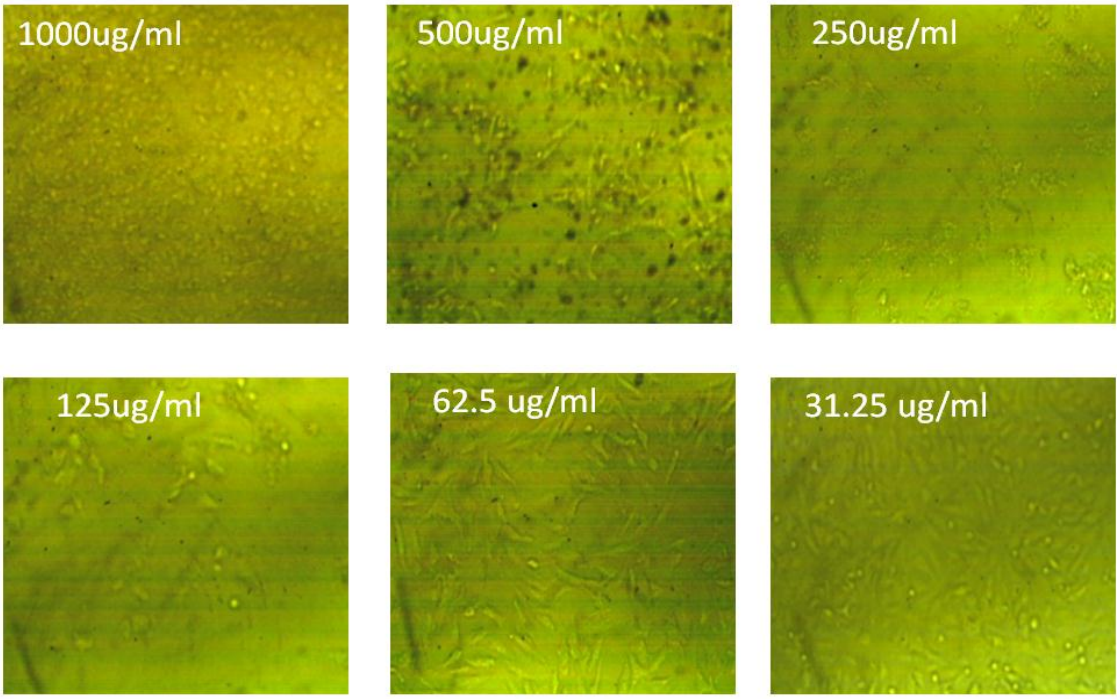

Fig. 5. Effect of ethanolic 80% solid residue of cinnamon by-product extracts on human prostate cancer cell line (PC 3)

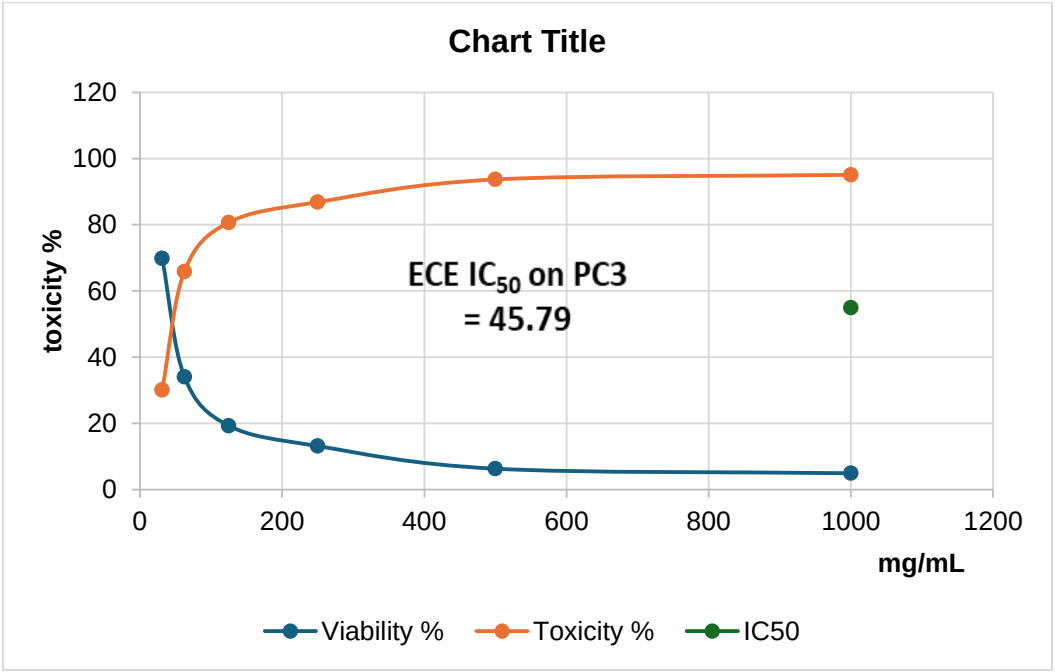

Fig. 6. Percent cell viability and toxicity of ethanolic 80% solid residue of cinnamon by-product extarcts of PC3 cell line

Conclusion

The solid residue of ethanolic cinnamon by-product (ECE) contained phenolic components ECE contains antioxidants as well as anti-cancer properties.

The solid residue of ethanolic cinnamon by-product leaves shows great promise in the creation of phytomedicines with anticancer characteristics. Because of their anticancer properties, drugs generated from ethanolic cinnamon solid by-product (ECE) may serve as an alternative therapeutic source. Furthermore, this investigation indicates that the ethanol extract of this by-product has the strongest anticancer activity against (HCT 116) human colon and human prostate (PC3) cancer cell lines.

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التوصيف الكيميائي لبقايا منتجات التقطير المائي لنبات القرفة، وتقييم أنشطتها المضادة للأكسدة والسرطان

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تُستخدم الزيوت العطرية المشتقة من نبات القرفه على نطاق واسع؛ ومع ذلك، فإن بقايا عمليات التقطير المائي الناتجة عن هذه العمليات غير مستغلة بشكل كافٍ. في هذا العمل، تم استخراج البقايا الصلبة من منتج القرفة الثانوي وتم عمل مستخلص ايثانولي 80%. تم اختبار المستخلص للنشاط المضاد للأكسدة وأظهر تأثيرًا كبيرًا. وقد اظهرت النتائج ان المحتوى الفينولي الإجمالي 85.25 مجم / GAE جم، بينما كان محتوى الفلافونيدات 23.14 مجم/ QE جم. كان التركيز المثبط الذي يثبط 50% من تعداد خلايا السرطان (IC50) باستخدام المستخلص ECE ضد خلايا سرطان القولون البشري (HCT 116) و PC3 (البروستات البشرية) عند تركيزات مختلفة (31.25-1000 ميكروجرام / مل) 31.4 ميكروجرام/ مل لـ HCT 116 و 33.4 ميكروجرام/مل لـ .PC3 نستنتج أن البقايا الصلبة من منتج القرفة الثانوي تُظهر إمكانات كبيرة في تطوير الأدوية النباتية ذات الخصائص المضادة للسرطان. يمكن أن تكون الأدوية المشتقة من المنتج الثانوي الصلب للقرفة (ECE) بمثابة مصدر طبي بديل بسبب نشاطها المضاد للسرطان. علاوة على ذلك، تشير هذه الدراسة إلى أن المستخلص الإيثانولي لهذا المنتج الثانوي يمتلك أكبر إمكانات للنشاط المضاد للسرطان ضد خلايا سرطان القولون البشري (HCT 116) والبروستات البشرية (PC3).

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