African J. Biol. Sci., 18 (1): 53-69 (2022)
ISSN 1687-4870 **e- ISSN 2314-5501 (online)**
www.ajbs.journals.ekb.eg **E.mail: aasdjournal@yahoo.com**

Anti-cancer effect of some Egyptian natural plants extraction against prostate cancer cells

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Received: May 12, 2022; Accepted: June 1, 2022; Available online: June 10, 2022

ABSTRACT

Prostate cancer (PCa) is a malignant tumor that usually caused by many epigenetic alteration that lead to uncontrolled proliferation, differentiation and invasion to nearby tissues. Different types of treatment for patients of PCa have been applied including surgery, radiation, hormone and chemo-therapy. This work presented an experimental study for using low-cost and ecologically friendly Egypt plants' extracts (garlic, cinnamon and liquorice) that have been dissolved in DEMSO for the treatment of PCa. To evaluate the potential effect of the selected extracts on, PC3 cells, MTT assay, qRT-PCR and ELISA techniques were studied. The results showed that the garlic extract exhibited a significant cytotoxic effect against PC3. It was significantly increased the production of relative LDH in the treated cells rather than the extracts of cinnamon and liquorice. Also, it was found that garlic components effects on proapoptotic signaling including P53, Caspase-3 and modulates the Raf-1 signaling pathway, and down-regulating the RAF/MEK/ERK pathway in PC3 cells compared with the effects of cinnamon and liquorice extractions. ELISA results indicated that the mean concentrations of IL-1α and IL-6 highly increased after 24h. In conclusion, the garlic extract was found to have an ability to adjust the secretion of pro-inflammatory cytokines. Meanwhile, garlic based herbal can be used as effective and harmless anti-cancer agent for PCa treatment.

Keywords: Garlic, cinnamon, liquorice, P53, Raf-1, IL-6, IL-1α.

INTRODUCTION

Now a day, prostate cancer (PCa) is documented as the second most common worldwide cancer and the fifth cause of cancer death in males (Torre *et al*., 2015; Dunn and Kazer, 2011). Initially, the traditional treatment for PCa is prostatectomy or radiation that aims to remove or destroy the malignant cells that have not metastasized (Feldman and Feldman, 2001, Kotb and Elabbady, 2011). PCa is a malignant tumor that usually begins in the outer part of the prostate caused by many epigenetic alteration that lead to un-controlled proliferation, differentiation and invasion to nearby tissues (Lawrence *et al*., 2010). Different types of treatment for patients of PCa have been occurred such as surgery, radiation, hormone and chemo-therapy, the increase in some adverse actions confines its use in some patients include negatively effect on the healthy tissues or organs as well as dependent on prostate cancer stage. However, various patients can't be recovered totally by these types of treatments, and may be followed by cancer recurrence or metastasis (Feldman and Feldman, 2001; Kotb and Elabbady, 2011).

The majority of PCa growth is androgen dependent. Androgen deprivation therapy (ADT) for instance surgery or

gonadotropin-releasing hormone (GnRH) analog treatment is that the main therapeutic and dramatically operative intervention for the treatment of androgen dependent PCa (ADPC) in placing patients with tumors in retardation (Huggins, 1978). Nevertheless, after this medical care, most of these PCa patients step by step become androgen independent, in progress, metastasize and resist to ADT within about 13–24 months accompanied with the increase in prostate-specific antigen (PSA) level. Siegel *et al*. (2017) mentioned that the failure of ADT treatment is responsible for the ~27,000 metastatic PCa deaths in the United States of America annually.

In recent years, many natural products and extracts have been scientifically investigated in vitro or in vivo and used as potential anti-prostate cancer agents (diet and nutrition) who have been considered as an active preventive strategy for cancer. A group of dietary natural products have been also shown a potential role in prevention and treatment of cancers (Zheng *et al*., 2016; Zhou *et al.*, 2016; Zhang *et al.*, 2016 & 2015;Li *et al*., 2013). There is a tendency to use natural products acted as treatment for PCa because of their safety, low toxicity and anti-oxidant properties (Georgios *et al.*, 2016). Moreover, fruits usually enriched with polyphenols giving them advantages to have great antioxidant activity which may help in reducing the risk of cancer (Li *et al.*, 2013 & 2016; Fu *et al*., 2011 & 2010; Xia *et al*., 2010).

Egyptians over 3500 years ago used garlic (*Allium sativum*) as a treatment solution for some tumors (Block, 1985). Furthermore, it has been used in folk medicine to treat a variety of diseases (Yun *et al*., 2014). Some garlic ingredients such as saponins and phenols have positive antioxidant effects. The garlic compounds can hinder tumor proliferation by controlling DNA repair and cell cycle regulation (Doll, 1992). Liquorice is a popular sweetener and a dryness quencher in many food products particularly in Middle East. It possesses effective pharmacological activities such as anti- cancer (Kuramoto *et al*., 2017). Its root contains variety of oils, alkaloids, poly saccharides, phenolic acid and flavonoids. Also, it is a source of proteins, amino acids, mineral salts such as calcium, sodium (Wang *et al*., 2015; Zhixin *et al*., 2021;Yang *et al*., 2016) .

A scientific outline and data of future prospects are necessary to facilitate additional chemical and medicine studies for anti-prostate cancer agents. Consequently, the present work aims to study the using of dietary natural products and their bioactive compounds in reducing the deterioration caused by PCa in cell lines. In addition, herbal based materials could be used as effective and safe anti-cancer agent. Also, garlic, liquorice, and cinnamon extracts were investigated the ability to adjust the secretion of pro-inflammatory cytokines.

MATERIALS AND METHODS

1. Preparation of herbal extracts:

The used materials were smooth powder of garlic, liquorice, and cinnamon. To prepare the plants extraction, 10 mg of the raw materials were carefully crouched and sterilized with ethanol 75% and was kept drying at room temperature. Each sterilized extraction was dissolved in 1 mL dimethyl sulfoxide (DMSO) to get the final concentration of 100 µg/µl. The final extraction was incubated at 4°C until used.

To plant extracts for GCMS and HPLC analysis was obtained from UGC Pharma Co. (Badr city, Cairo governorate, Egypt). The preparation procedures were: 1kg of used plants was macerated for 48 h in a mixture of water and ethanol (100% con.) with a ratio of 20: 80. After that, the prepared mixture was heated in thermal

stainless-steel tank at about 70°C for 6-7 h until reaching the concentration of 5% measured by refractometer concentration tester. The obtained liquid was cooled at room temperature, filtered, and preserved materials (2g from sodium methylparaben and 1g from sodium propyl paraben) were added.

2. Tests and instruments:

The fine chemical compositions of garlic, cinnamon and liquorice ethanol extracts were examined.

2.1 Gas chromatography mass spectrometry (GC-MS) analysis:

The chemical composition of the prepared liquids of garlic, cinnamon and liquorice were performed using Trace GC-TSQ Mass Spectrometer (Thermo Scientific, Austin, Tx, USA) equipped with a direct capillary column TG-5MS. The constituents were identified by comparison of their mass spectra with those of WILEY 09 and NIST 14 mass spectral database.

2.2 High performance liquid chromatograph (HPLC) analysis:

The content of garlic, cinnamon and liquorice liquids was quantitatively measured by high performance liquid chromatography (HPLC) (**El-Fadl *et al.*, 2021)**. The used HPLC (Agilent 1100) is composed of a quaternary pump and a UV/V as a detector. Phenolic and flavonoid compounds were determined.

3. Cell lines:

The used prostate cancer cells (PC3 cell line) were provided from National Cancer Institute (NCIE), Cairo, Egypt. The cells were grown in RPMI media supplemented with 4 mM L-glutamine, 4 mM sodium pyruvate, and 5% of heat-treated bovine serum albumin (BSA). The normal hepatocytes were grown in RPMI media that contains 4 mM L-glutamine and 10% BSA. All cell lines were incubated at 37°C with 5% CO_2 condition (Khalil *et al.*, 2017; Abd El Maksoud *et al.*, 2019). The imaging of cultured cells was determined by using inverted microscopy with a Zeiss A-Plan 10X.

4. Cytotoxic concentration 50% (CC_{50}):

The prepared extracts were tested for their cytotoxic effect and the potential CC_{50} in PC3 cells line was calculated. Therefore, the cells were cultured in 96-well plates in a density of $10x10^3$cells/well and were incubated in CO_2 incubator at 37°C. The cells were treated with different concentrations of each of the investigated plant extract (0.1-10 mg/mL) followed by overnight incubation. The cell viability rate and cytotoxic concentration were monitored by using colorimetric assay MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell growth kit (Sigma-Aldrich, Germany) based on the amount of formazan dye which has been determined by measuring absorbance at 570 nm.

5. Lactate dehydrogenase (LDH) production:

LDH assay kit (Abc-65393) was used to measure LDH production in the fluid media that collected from cultured-treated cells. It was determined according to the manufacture procedures; 100µl of lysed cells was incubated with 100µl LDH reaction mix for 30 min at room temperature. LDH activity was measured by a plate reader at OD450 nm. The relative LDH production was calculated by dividing the mean values of treated cells on the mock values and indicated by fold change (Khalil, 2012; Khalil *et al.*, 2019).

6. Enzyme-linked immunosorbent assay (ELISA):

ELISA assay was used for the quantification analysis of the released interleukin-6 (IL-6) and IL-1α using human ELISA kits (Abcam 46042 and Abcam 46028, respectively). PC3 cells cultured in 96-well plates were overnight incubated. Then the cells were treated with either 500 µg/mL of each indicated extract followed by incubation periods of 0, 6, 12, 24, 36 and 48h. At each time point, the cells were lysed using 1X cell lysis buffer (Invitrogen, USA) then 100 µl of the lysed cells were transferred into the ELISA plate reader and were incubated for 2 h at room temperature (RT) with 100 µl control solution and 50µl 1X biotinylated antibody. Then after, 100 µl of 1X streptavidin-HRP solution was added to each well of samples and incubated for 30 min in the dark place. 100 µl of the chromogen TMB substrate solution was added to each samples well and incubated for 15 min at RT away from the light. Finally, 100 µL stop solution was added to each well of samples to stop the reaction. The absorbance of each well was measured at 450 nm (Khalil *et al.*, 2017; Khalil *et al.*, 2020).

7. Quantitative real time PCR (qRT-PCR):

The genes expression quantification was analyzed by using qRT-PCR in which the cellular total RNA was obtained using TriZol (Invitrogen, USA) and was purified by using RNA purification kit (Invitrogen, USA). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using M-MLV reverse transcriptase (Promega, USA). The mRNA expression of Raf-1, P53, and Casp3 were analyzed by using QuantiTect-SYBR-Green PCR Kit (Qiagen, USA). The specific primers of the used genes are listed in Table (1).

The housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression level was used for normalization in the real-time PCR data analysis. The PCR reaction system conatined 10 µl SYBR green, 0.25 µl RNase inhibitor (25 U/µl), 0.2 µM of each primer, 2 µL of synthesized cDNA, and nuclease-free water up to a final volume of 25 µL. The PCR was used at conditions of 94°C for 5 min, 35 cycles (94°C for 30 sec, 60°C for 15 sec, 72°C for 30 sec) (Khalil *et al*., 2019; El-Fadl *et al*., 2021).

Table (1): Oligonucleotides primer sequences used for mRNA quantification of the studied genes.

Description	Primer sequences (5'-3')
Raf-1-sense	TTTCCTGGATCATGTTCCCCT
Raf-1 antisense	ACTTTGGTGCTACAGTGCTCA
P53-sense	GCGAGCACTGCCCAACAACA
P53-antisense	GGTCACCGTCTTGTTGTCCT
Casp3-sense	GGACAGCAGTTACAAAATGGATTA
Casp3-antisense	CGGCAGGCCTGAATGATGAAG
GAPDH-sense	TGGCATTGTGGAAGGGCTCA
GAPDH-antisense	TGGATGCAGGGATGATGTTCT

8. Data analysis:

All the manuscript enclsed histogrames and charts were illustereted by the Microsoft Excel. Delta-Delta Ct analysis was used in the quantification analysis of mRNA delivered from qRT-PCR assay

based on the following equations: (1) delta-Ct = Ct value for gene- Ct value for GAPDH, (2) (delta–delta Ct) = delta Ct value for experimental –delta Ct for control), (3) quantification fold change = ($2^{-\text{delta-delta ct}}$) (Rao *et al.*, 2013; Khalil *et al.*, 2017). The student's two-tailed t-test was used for statistical analysis. P-value ≤ 0.05 was considered statistically significant.

RESULTS

1. Quantitative analysis of garlic, cinnamon and liquorice by analytical HPLC:

Tables (2, 3 and 4) show the changes in phenolic and flavonoid compounds in the ethanol extract for garlic, cinnamon and liquorice. The phenolic compounds found in all extracts were chlorogenic, p-coumaric. But other specific compounds were found in two extract such as syringic, protocatechulic and isoferulic (garlic and liquorice), vanillic (garlic and cinnamon), Pyrogallol (cinnamon and liquorice). Only, gallic and catechol compounds were found in garlic. While, p-OH benzoic and ferulic were found in cinnamon only.

From the results, it was observed that the highest concentrations were for cholorogenic in garlic (11.26 µg/mL), catechin and p-coumaric in cinnamon (35.16 and 17.36 µg/mL, respectively), isoferulic and syringic in liquorice (18.76 and15.3 µg/mL, respectively). The major compounds found in the study were agreed with those given by Kayiran *et al.* (2019) as a result of using LC-MS/MS analysis. Also, it is worthy to record that the quinic acid can be obtained by the hydrolysis of chlorogenic acid (11.26 µg/mL) that found in thepresent study.

The flavonoid compounds found in the studied extracts which have relatively the same retention times (RT) such as rutin, naringin, querestin, kampferol, apigenin, catechin. Other compounds are found in specific extracts such as chrysin (liquorice), myrecetin and rosmarinic (liquorice and garlic). Also, it was found that the highest concentrations were for querestin in cinnamon and garlic (15.23 and 14.36 µg/mL, respectively) and myrecetin in liquorice (12.36µg/mL).

Table (2): Result of HPLC test for phenolic and flavonoid compounds in garlic extraction according to specific retention time (RT).

Group	RT (min)	Compound	Concentration (µg/mL)	Group	RT (min)	Compound	Concentration (µg/mL)
Phenols	3.0	Syringic	3.55	Flavenoid	3.0	Myrecetin	5.21
	4.0	Gallic	2.14		4.6	Rutin	4.88
	6.3	Protocatechulic	6.88		5.2	Naringine	3.48
	8.1	Chlorogenic	11.26		7.0	Querestin	14.36
	9.3	Catechal	6.19		8.0	Kampferol	1.79
	11.0	Isoferulic	5.69		9.1	Luteolin	2.41
	13.0	P-counaric	4.36		10.0	Apigenin	1.98
	15.6	Vanillic	5.99		11.0	Hyperoside	1.69
					12.0	Catechin	3.14
					16.0	Coumaric	5.69
					18.0	Epicatechin	9.73

Table (3): Result of HPLC test for phenolic and flavonoid compounds in cinnamon extraction.

Group	RT (min)	Compound	Concentration (µg/mL)	Group	RT (min)	Compound	Concentration (µg/mL)
Phenols	5.0	Pyrogallol	10.33	Flavenoid	4.4	Rutin	9.14
	7.0	Catechin	35.16		5.2	Naringine	8.16
	8.0	Chlorogenic	6.47		7.0	Querestin	15.23
	9.8	p-PH benzoic	8.16		7.9	Kampferol	6.17
	12.0	Ferulic	9.12		9.1	Luteolin	7.46
	13.0	P-coumaric	17.36		10.0	Apigenin	14.56
	15.6	Vanillic	8.66		12.0	Catechin	9.52

Table (4): **Result of HPLC test for phenolic and flavonoid compounds in liquorice extraction.**

Group	RT (min)	Compound	Concentration (µg/mL)	Group	RT (min)	Compound	Concentration (µg/mL)
Phenols	3.0	Syringic	15.3	Flavenoid	2.0	Chrysin	2.66
	5.0	Pyrogallol	7.25		3.0	Myrecetin	12.36
	6.0	Protocatechulic	6.98		4.1	Rutin	1.99
	7.1	Cinnamic	5.36		5.3	Naringine	6.44
	8.5	Chlorogenic	8.66		6.2	Rosmarinic	5.14
	11.0	Isoferulic	18.76		7.2	Querestin	4.26
	11.5	Caffeic	2.36		8.1	Kampferol	4.55
	13.0	P-coumaric	4.18		10.1	Apigenin	5.69
					12.0	Catechin	2.11

2. Gas chromatography-mass spectrometry analysis:

The physiologically active components of ethanol garlic, cinnamon and liquorice extracts were determined using GC-MS analysis (Tables 5-7). The results of garlic extracts showed that diallyl disulphide, trisulfide, di-2-propenyl, 1-isopropenyltricyclo hexane and ethyl aminomethyl formimidate compounds have the highest peak areas (%) (32.19, 20.17, 8.62 and 5.94, respectively). Other compounds were found in less than 5% at different retention times (RT). Where in cinnamon extracts, it was found that the compounds with the highest peak area (%) in decreasing order were alfa.-copaene (42.00), naphthalene (16.55), cinnamaldehyde, (E) (13.37), à-muurolene (10.74) and cinnamaldehyde dimethylacetal (8.31) and other compounds were found in less than 4% at different RT. Furthermore, in liquorice the highest peak area (%) were found for benzene1-methoxy-4-(2-propenyl) (19.55), 5(E)- [(4-acetylphenyl) methyl) liden] tetrahydrofuran-2-one (16.19), and benzene, 6-isopropenyl-3-methoxymethoxy-3-methyl-cyclohexene, ethanol,2-butoxy (app. 7). Other compounds were found in less than 6% at different RT.

Table (5): Phyto-components identified by GC-MS analysis of garlic extraction

Retention time (min)	Compound name	Area %	Molecular formula	Molecular weight	Cas #
4.26	10-Heptadecen-8-ynoic acid, methyl ester, (E)-	3.39	$C_{18}H_{30}O_2$	278	16714-85-5
4.44	(S)-(-)-2-Amino-3-phenyl-1-propanol	1.61	$C_9H_{13}NO$	151	3182-95-4
4.54	Ethyl aminomethylformimidate	5.94	$C_4H_{10}N_2O$	102	NA
4.71	1-ISOPROPENYLTRICYCLO [3.1.0.0(2,6)]HEXANE	8.62	C_9H_{12}	120	126978-74-3
4.96	CYCLOHEXANE, 1,2,4-TRIS(METHYLENE)-	0.31	C_9H_{12}	120	14296-81-2
5.44	1-PROPANONE,2-DIAZO-1-PHENYL-	4.98	$C_9H_8N_2O$	160	25451-15-4
6.11	10,12-Octadecadiynoic acid	0.52	$C_{18}H_{28}O_2$	276	7333-25-7
7.50	Diallyl disulphide	32.19	$C_6H_{10}S_2$	146	2179-57-9
8.88	Trisulfide, methyl 2-propenyl	2.50	$C_4H_8S_3$	152	34135-85-8
9.47	Benzoic acid, 4-(1,1-dimethylethoxy)-	0.56	$C_{11}H_{14}O_3$	194	13205-47-5
10.35	BENZENE, 1-METHOXY-4-(2-ROPENYL)-	2.39	$C_{10}H_{12}O$	148	140-67-0
10.76	ALLICIN	1.38	$C_6H_{10}OS_2$	162	539-86-6
12.57	Trisulfide, di-2-propenyl	20.17	$C_6H_{10}S_3$	178	2050-87-5
14.43	2-AMINOETHANETHIOL HYDROGEN SULFATE (ESTER)	0.47	$C_2H_7NO_3S_2$	157	2937-53-3
16.37	2-AMINOETHANETHIOL HYDROGEN SULFATE (ESTER)	0.28	$C_2H_7NO_3S_2$	157	2937-53-3
18.76	2-Methyl-E,E-3,13-octadecadien-1-ol	1.08	$C_{19}H_{36}O$	280	NA
19.52	9-OCTADECENOIC ACID (Z)-	0.40	$C_{18}H_{34}O_2$	282	112-80-1
20.20	9-OCTADECENOIC ACID (Z)-	1.33	$C_{18}H_{34}O_2$	282	112-80-1
21.47	2-Methyl-E,E-3,13-octadecadien-1-ol	3.95	$C_{19}H_{36}O$	280	NA
24.48	9-OCTADECENOIC ACID	1.46	$C_{18}H_{34}O_2$	282	NA
26.08	9-OCTADECENOIC ACID	1.26	$C_{18}H_{34}O_2$	282	NA
27.28	9-OCTADECENOIC ACID (Z)-	1.39	$C_{18}H_{34}O_2$	282	112-80-1
27.68	9-OCTADECENOIC ACID (Z)-	0.13	$C_{18}H_{34}O_2$	282	112-80-1
28.73	9-OCTADECENOIC ACID (Z)-	3.66	$C_{18}H_{34}O_2$	282	112-80-1

Table (6): Phyto-components identified by GC-MS analysis of cinnamon extraction.

Retention time (min) RT	Compound Name	Area %	Molecular Formula	Molecular Weight	Cas #
4.58	Ethanol, 2-butoxy-	1.16	C6H14O2	118	111-76-2
5.08	Benzene, 1-ethyl-3-methyl-	0.68	C9H12	120	620-14-4
12.42	Cinnamaldehyde, (E)-	13.37	C9H8O	132	14371-10-9
13.79	à-ylangene	0.40	C15H24	204	NA
14.00	.alfa.-Copaene	42.00	C15H24	204	NA
14.44	á-Longipinene	1.55	C15H24	204	41432-70-6
14.82	CINNAMALDEHYDE DIMETHYL ACETAL	8.31	C11H14O2	178	NA
15.75	á-Longipinene	0.74	C15H24	204	41432-70-6
16.19	ç-Muurolene	3.27	C15H24	204	30021-74-0
16.69	à-Muurolene	10.74	C15H24	204	31983-22-9
17.16	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	16.55	C15H24	204	483-76-1
17.43	.alfa.-Copaene	0.51	C15H24	204	NA
19.85	à-Cadino	0.70	C15H26O	222	481-34-5

Table (7): **Phyto-components identified by GC-MS analysis of liquorice extraction**

Retention time (min) RT	Compound Name	Area %	MF	Molecular Formula	Molecular Weight
4.63	Ethanol, 2-butoxy-	7.59		C6H14O2	118
5.00	Benzene, 1-ethyl-3-methyl	2.10		C9H12	120
5.66	Benzene, 1-ethyl-3-methyl	3.95		C9H12	120
6.21	6-Isopropenyl-3-methoxymethoxy-3-m ethyl-cyclohexene	7.57		C12H20O2	196
7.62	2,6-OCTADIENAL, 3,7-DIMETHYL-	4.02		C10H16O	152
10.40	BENZENE, 1-METHOXY-4-(2-PROPENYL)-	19.55		C10H12O	148
11.73	BENZENE, 1-METHOXY-4-(2-PROPENYL)-	1.85		C10H12O	148
12.60	Benzene, 1-methoxy-4-(1-propenyl)-, (Z)-	7.13		C10H12O	148
14.53	Methyl 9,11-octadecadiynoate	1.77		C19H30O2	290
15.53	9-OCTADECENOIC ACID (Z)-	1.06		C18H34O2	282
16.37	Methyl 6,8-octadecadiynoate	3.28		C19H30O2	290
18.75	Bicyclo[4.4.0]dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl)-	1.37		C15H24O2	236
19.03	Methyl 9,11-octadecadiynoate	3.51		C19H30O2	290
19.54	9-OCTADECENOIC ACID	1.89		C18H34O2	282
20.21	5(E)-[(4-ACETYLPHENYL)METHY LIDENE]TETRAHYDROFURAN-2 -ONE	16.19		C13H12O3	216
24.49	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, cis-	1.73		C19H36O3	312
26.19	9-OCTADECENOIC ACID (Z)-	4.42		C18H34O2	282
27.30	9-OCTADECENOIC ACID (Z)-	4.59		C18H34O2	282
28.78	9,12-Octadecadienoic acid (Z,Z)-	6.45		C18H32O2	280

3. Cytotoxic effect of plants extract on PC3 cells:

The PC-3 cell line was treated with the studied plants extracts (garlic, cinnamon and liquorice) using different concentrations of each extract (0 - 20 mg/mL) compared with the DEMSO pre-treated cells. To investigate the cytotoxic effect of the indicated plants extract, the cytotoxic concentration 50% (CC50) was monitored in PC3 cells that were treated overnight. The cell viability rate and cytotoxicity were determined using the MTT assay. Interestingly, the treated cells by cinnamon and liquorice extracts showed weak cytotoxic effects as shown in Figure (1A). In contrary, the garlic obviously decreased the cell viability percentage of the treated cancer cells compared to the DEMSO and other extracts as appeared in microscopic images (Fig.1B).

As indicated in Figure (2), the cytotoxic effect of garlic extraction decreased with the concentration increasing. The garlic exhibited a significant cytotoxic effect against PC3 almost in concentration 1.25 mg/mL as CC_{50} (concentration that inhibits 50% of cells). The results were statistically analyzed using the T-Test hypothesis with significance set at *P* value of < 0.05 as shown in Table (8).

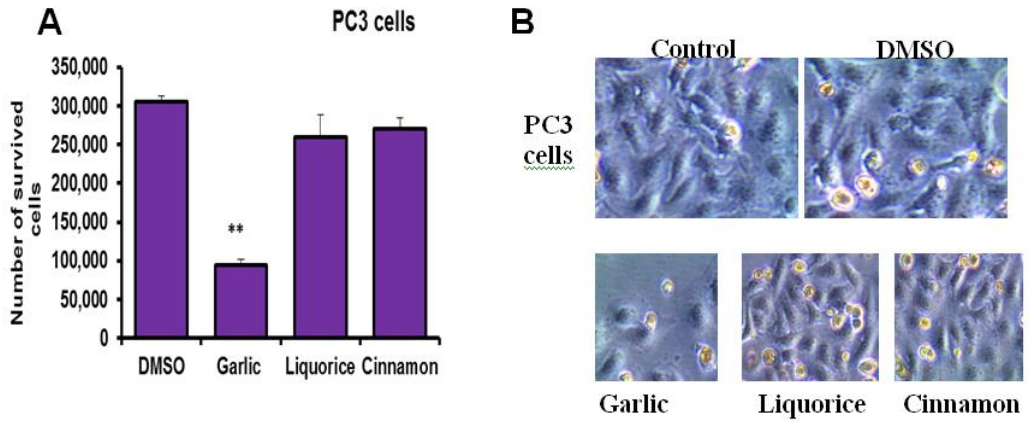

Fig. (1): The cytotoxic effect of indicated plants extract in PC3 cells. (A) The number of survived cells treated with garlic, liquorice and cinnamon extractions. Error bars indicate the SD of four independent experiments. A Student's two-tailed t-test was used for the significance analysis of represented values. *p ≤ 0.05 and **p ≤ 0.01. (B) Representative inverted microscopy images of cells morphology two days upon treatment with garlic, liquorice and cinnamon extract in comparison with DMSO-treated cells and untreated cells (NT).

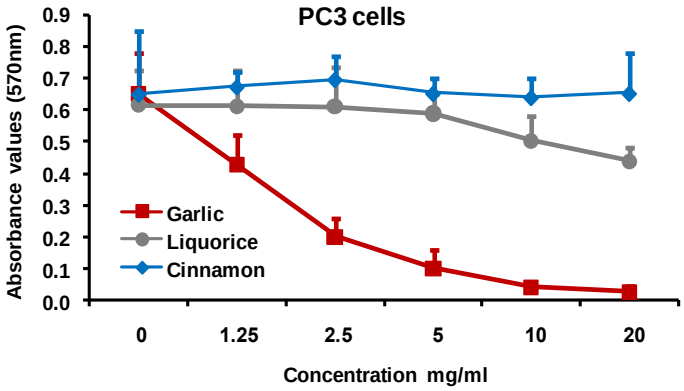

Fig. (2): The cytotoxic effect of plants extraction

Table (8): Results of statistical parameters of cell viability rate of treated cells.

Statistical measurements	Control	Plants extract		
	DMSO	Garlic	Liquorice	Cinnamon
Mean absorbance	0.63	0.43	0.61	0.67
STD	0.14	0.10	0.11	0.05
P values	-	0.032*	0.975	0.832

*Significant effect at *p* value < 0.05

4 Production of LDH in treated cells:

LDH is a soluble cytosolic enzyme that is released into the culture medium following the loss of membrane integrity, therefore the measurement of LDH serve a general means to cell viability which is associated with necrotic of these cells. Interestingly, the relative LDH production was significantly increased up to 5 fold in the treated cells with garlic rather than that were treated with cinnamon and liquorice extracts (1.58 and 1.24, respectively) in comparison with the non-treated cells (NT) as shown in Figure (3).

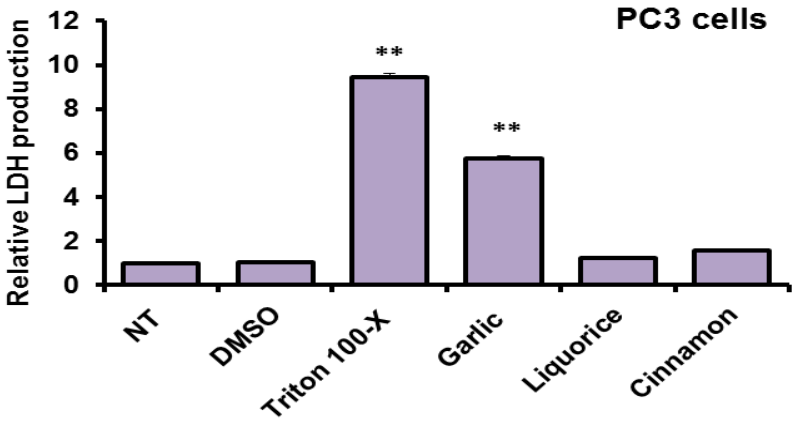

Fig. (3): Relative LDH production from treated cells compared with Triton 100-X treated cells. Error bars indicate standard deviation (SD) of four different replicates. Student two-tailed test was used to determine P values and significance of LDH production level.

5. Determination of gene expression using Real-Time qRT-PCR:

To highlight the biological activity of natural plants extraction on cellular signaling, the m-RNA of RAF-1, P53 and Caspase-3 in treated PC3 cells using qRT-PCR was quantified. The PC3 cells were treated with garlic, cinnamon and liquorice, then isolated total RNA and c-DNA. Fig. (4A-C) demonstrated that the using of garlic in concentration of 0.25mg/mL has a dramatic inhibition of Raf-1 with highly significant (P = 0.00396), associated with the relative expression level of p53 that was up regulated more than 3 fold (P = 0.01652).

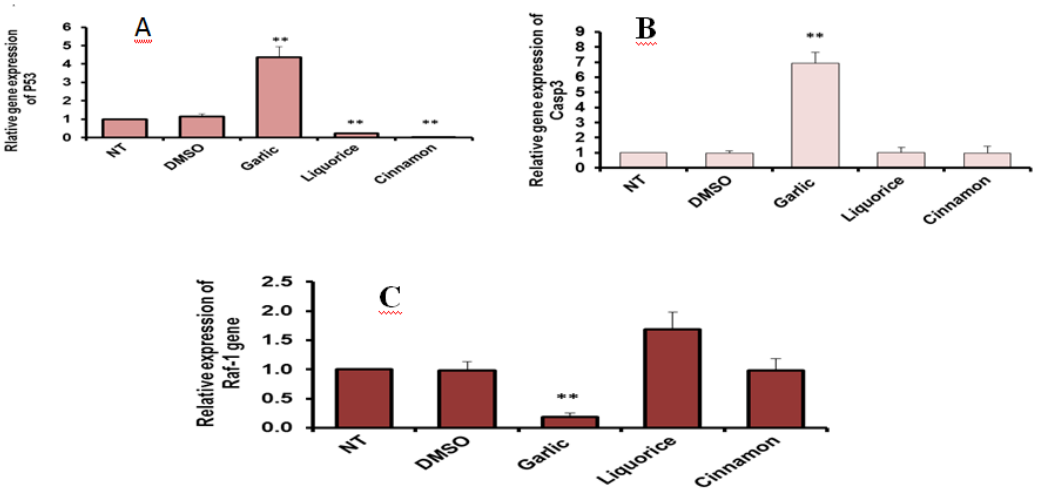

Fig. (4): Relative expression of P53, Caspase and Raf-1 in treated PC3 cells. (A) Steady-state mRNA of P53 gene indicated by fold change in PC3- treated cells compared with DMSO-treated cells. (B) Steady-state mRNA of Caspase-3 gene indicated by fold change in PC3 cells in comparison with DMSO-treated cells. (C) Steady-state mRNA of Raf-1 gene indicated by fold change in PC3 cells in comparison with DMSO-treated cells. Levels of GAPDH-mRNA were used as an internal control. Error bars indicate the SD of three independent experiments. Student two-tailed t-test was used for significance analysis of cycle threshold (Ct) values. *p ≤ 0.05 and **p ≤ 0.01.

6. Quantitative of IL-6 and IL-1 α secretion by ELISA:

A number of human tumor cells express cytokines such as interleukins, to measure the released pro inflammatory cytokines from the treated cells with garlic, cinnamon and licorice. The mean concentrations of IL-1α (Fig. 5A) and IL-6 (Fig. 5B) increased dramatically after 24h compared with untreated cells or DEMSO treated cell. The findings demonstrated the ability of garlic extract to regulate the secretion of pro inflammatory cytokines.

Fig. (5): Levels of pro-inflammatory cytokines IL-1α and IL-6 in treated-cells. (A) The concentration of produced IL-1α (pm/mL) in the fluids media of PC3 cells that were subjected at the indicated time points compared with DMSO-treated cells. (B) Concentration of produced IL-6 from infected cells that treated with the plants extract indicated by pm/mL.

DISCUSSION

In comparison between the results obtained from the quantitative analysis of garlic, cinnamon and liquorice by analytical HPLC as well as GC-MS and the results of Takim *et al.* (2021) it was found that the present test results displayed for the garlic species generally have the same photochemical compounds at different amounts according to the species used. In addition, the detected major compounds of garlic can be different according to the climatic environments of the area were the garlic grows.

Results obtained from the cytotoxic effect of plants extract on PC3 cells are confirmed with the result of Bagul *et al.* (2015) which reported that the inhibition of cell proliferation of 80-90% was observed for Hep-G2, PC-3 and other cancer cells after the treatment by crude garlic extract (CGE). The methanol extracts of Allium tuncelianum (MAT) have strongest cytotoxic effect on endometrial carcinoma (ECC-1) and PC3 cells (Takim *et al.,* 2021). Based on these studies, the cytotoxic effects are attributed to the major phenolics and sulphuric compounds in the garlic extract. Furthermore, the CC50 for MAT Turkish garlic (Takim *et al.,* 2021) achieved high cytotoxic effect at low concentration (0.105 mg/mL) in PC3 cells than was found in the present study (1.25mg/mL) and this may be due to the garlic species used in the experimental work.

Results of LDH production in treated cells by the used of plants extraction and as a comparison with triton 100-x, it was found that the garlic extract has a superior effect on PCa cells rather than other extracts. So, LDH leakage is considered as a marker of cytoxicity.

Furthermore, the morphological modifications can be used as indicators for the cell death induced by garlic extractions; the cell death was induced mostly by necrosis, correlating the results with the LDH assay. The test results are matched with other results that stated that the Allium sativum (garlic) and its bioactive sulfur compounds have a cytotoxic effect depending on the dose that was added as a treatment (Țigu *et al.,* 2021).

The result of determination of gene expression using qRT-PCR can be attributed to the quinic acid found which can be used as effective drug candidate against PCa, due its pharmacokinetic properties (Inbathamizh and Padmini, 2013). On the other hand, a research investigated the effect of liquorice roots extractions on hepatocellular carcinoma cells and they found that an initiation of apoptosis and cell cycle arrest through activating AMPK and p53 and stressed on its unfavorable effects (Abdel-Wahab *et al.,* 2020). Hence, the using of liquorice for prevention and therapy should be used with care. Flavonoids are the main active component isolated from roots inhibited cancer cells through blocking cell cycle and regulate multiple signaling pathways ex. MAPK and P13K/AKT pathways (Zhixin *et al.,* 2021). One of phenolic constituent of licorice is licochalcone E (LicE) which increased the expression of pro-apoptotic factors (Bax and BCl-2) associated against of cell death (Bad) and tumor suppressor gene P53 (Yang *et al.,* 2016). Cinnamon is one of the most widespread and oldest plant spices that have wide range of pharmacological functions including anti-cancer, anti-microbial and anti-oxidant activities (Sahand *et al.,* 2019). Researchers found that cinnamon and its components can induce death in cancer cell mechanisms such as inhibition of Ap-1 or change in mitochondrial membrane potential (Koppikar *et al.,* 2010).

It is worthy to mention that, the proteins in caspase family play critical roles

in the apoptotic process. Thus, the expression levels of the caspase -3 was measured in treated cells and the results revealed that the garlic is highly significantly regulation with more than 6 fold (p= 0.00712). This result agreed with Bagul *et al.* (2015) which demonstrated that the crude garlic extract (CGE) encouraged cell cycle arrest and had a four-fold increasing in caspase activity (apoptosis) found in PC-3 cells when treated at a dose of 0.5 or 1 µg/mL. These findings indicated that the gene expression levels of Raf-1, P53 and Caspase-3 by qRT-PCR displayed that the PCa status could be relived as advance warning of the disease.

Furthermore, the stimulatory effects of plants extraction on P53, Caspase-3 and modulate the Raf-1 pro-apoptotic signaling pathway. Where, the garlic extraction induces apoptosis by activating the mitochondria-dependent pathway executed by caspase-3, increasing p53 expression level, and down-regulating the RAF/MEK/ERK pathway in PC3 cells compared with cinnamon and liquorice extractions. Cinnamaldehyde, (E) (13.37) is one of cinnamon compounds that has the highest peak area (%) reduced the anti-apoptotic protein Bcl-XL level and raised the level of proapoptotic protein Bax together with p53 in a time-dependent manner in liver cancer cell (HepG2) at 30 µM concentration (Dutta and Chakraborty, 2018).

Kaempferol, one of glycine flavonoids found in garlic, inhibits PCa cells proliferation by up-regulating the expression of caspase-8,-9,-3 (Halimah *et al.,* 2015). The ability of querestin, the highest flavonoid compound in garlic to induce intrinsic mitochondrial apoptotic was verified by Chou *et al* (2010). It mediates P53 apoptotic pathway (Batra and Sharma, 2013). It was found that the MAT increased caspase-3, 9 gene expressions in PC3 (Takim *et al.,* 2021) and this result is in the same line with the present study.

The effect of polyphenols results are confirmed with the study that had investigated the effect on proteins coded by tumor suppressor genes, such as p53 (apigenin, curcumin, EGCG), p21 (apigenin, EGCG), p27 (apigenin) and checkpoint proteins CHK1, 2 (gallic acid). Also, the RAF1 gene that was found in the living cells affords orders to make a protein that is part of a signaling pathway called the Ras/MAPK pathway. It helps to control the growth, division, differentiation, migration and apoptosis (David and Deborah, 2018).

The work result of quantitative of IL-6 and IL-1 α secretion by ELISA is approved with the study of Lee *et al.* (2018) which found that black garlic extracts produced reduction in IL-6, IL-8 expression level. However, targeting Raf-1 signaling pathway increases the production levels of the pro-inflammatory cytokines including IL-6 and IL-8 in targeted cells. Furthermore, the productions of IL-6 and IL-8 were subsequently maintained cell growth and survival, inflammatory events, and cancer development (El-Fadi *et al.,* 2021). In addition, after the oncogenic event, interleukin signaling in cancer cells can become a pathological mechanism of tumor growth, metastatic and cancer progression (Bruni *et al.,* 2020).

Conclusion

This research was focused on the possibility of using some Egyptian plants (garlic, cinnamon and liquorice) extraction dissolved in DEMSO as anticancer therapy. The results from the analyses and experimental studies showed that the garlic exhibited a significant cytotoxic effect

against PC3 almost in concentration 1.25 mg/mL. Interestingly, the relative LDH production was significantly increased up to 5 fold in the treated cells with garlic rather than that were treated with cinnamon and liquorice extracts (1.58 and 1.24, respectively) which means that the garlic extract has a superior effect on PCa cells. Garlic components effects on pro-apoptotic signaling including P53, Caspase-3 and modulate the Raf-1 signaling pathway.

Also, garlic extraction has an advantage to induce apoptosis by activating the mitochondria-dependent pathway executed by caspase-3, increasing p53 expression level, and down-regulating the RAF/MEK/ERK pathway in PC3 PCa cells rather than cinnamon and liquorice extractions. By using ELISA at the indicated time points, the mean concentrations of IL-1α and IL-6 increased dramatically after 24h compared with untreated cells or DEMSO treated cell. The findings demonstrated that the ability of garlic extract to adjust the secretion of pro inflammatory cytokines.

Finally, garlic based herbal is encouraged for using as operative and harmless anti-cancer agent for PCa treatment which may attract public health authorities, pharmaceutical industries. Garlic derived dietary supplements are essential by nutritionists, physicians, food technologists and food chemists.

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تأثير بعض مستخلصات النباتات الطبيعية المصرية ضد خلايا سرطان البروستاتا

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المستخلص

سرطان البروستاتا (PCa) لدى الرجال هو ورم خبيث ينتج عادة عن العديد من التغيرات الجينية التي تؤدي إلى الانتشار غير المنضبط والغزو للأنسجة المجاورة. ويوجد أنواع مختلفة من العلاج لمرضى PCa مثل الجراحة والإشعاع والعلاج الهرموني والعلاج الكيميائي. لذا قدم هذا العمل دراسة تجريبية لاستخدام مستخلصات لنباتات مصرية منخفضة التكلفة وصديقة للبيئة (الثوم والقرفة وعرق السوس) حيث تم إذابتها في مذيب DEMSO. وخلال البحث تم الاستعانة بعدد من الطرق والتقنيات التحليلية المختلفة مثل HPLC و GC-MS.لتقييم التأثير المحتمل للمستخلصات المختارة. وقد تمت دراسة الخلايا السرطانية للبروستاتا (PC-3) عن طريق استخدام تقنيات qRT-PCR وELISA وMTT. وأظهرت النتائج أن الثوم له تأثير سام للخلايا ضد PC-3 ومن المثير للاهتمام ، أن استخدام الثوم أدى الى زيادة إنتاج LDH النسبي في الخلايا المعالجة بدلاً من تلك المعالجة بمستخلصات القرفة وعرق السوس. أيضًا ، كما توصلت الدراسة الى أن مكونات الثوم تؤثر بشكل ملحوظ على الإشارات التي ترسلها الجينات والتي تؤدي الى موت الخلايا المبرمج مثلP53 ، Caspase-3 وتعديل مسار إشارات Raf-1 ، والتنظيم المنخفض لمسار RAF / MEK / ERK في خلايا سرطان البروستاتا مقارنةً بمستخلص القرفة وعرق السوس . وقد أشارت نتائج ELISA إلى أن متوسط تركيزإفراز السيتوكينات المؤيدة للالتهابات IL-6 IL-1α قد ازدادت بشكل كبير بعد 24 ساعة. وبناء عليه فإنه يوصى بأمكانية استخدام نبات الثوم كعامل فعال وغير ضار كمضاد لسرطان البروستاتا .