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Phenolic composition and biological activities of methanolic extract of *carica- papaya*

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ABSTRACT

Our study aimed to evaluate antimicrobial, antioxidant and antitumor activities and currently phenolic of polar extract of *carica papaya* leaves. Nine phenolic compounds were isolated from *carica papaya* leaves and identified as flavonol, flavonol glycosides, and phenolic acids. The antioxidant activity was measured by 2, 2' - Diphenyl Picrylhydrazyl (DPPH) radical scavenging method. The methanolic extract completely inhibited DPPH at three different concentrations 19, 38, 77 µl which showed very high antioxidant capacity, which was close to ascorbic acid standard use. Antimicrobial activities of plant extracts were studied against five bacterial strains and five fungal species, 0.1 ml of plant extract (10 mg / 1 ml) had inhibitory effect for most bacterial spp. and some for all fungal spp., and clearly inhibitory effect against all of the tested strains at 0.3 ml (10 mg /1 ml) of the extract, the inhibitory effect increase with increasing the concentration of the phenolic extract.

KEYWORDS

Carica papaya leaves;
Flavonol;
Flavonol glycosides;
Phenolic acids;
UV;
MS;
NMR;
Antimicrobial;
Antioxidant;
Antitumor activities.

INTRODUCTION

Carica papaya (papaya) is a tree-like herbaceous plant, a member of the small family *Caricaceae* and widely cultivated for its edible fruits. It originates in the lowlands of eastern Central America, from Mexico to Panama, and can be found in all tropical countries and many subtropical regions of the world. Parts of the plant are used in tropical diets as a fruit or vegetable; it is sometime used as therapeutic remedy for several of its medicinal properties. *Papaya* fruit is thought to contain some immuno-stimulating and anti-oxidant agents[6,15]. Brazil is the world's greatest producer of *papaya* fruit, and contributed to 25% of the worldwide production in 2002. Brazil, Mexico, and Malaysia are the main exporting countries of this fruit mainly to the European market[11]. Very little has been published on the chemical composition or biochemistry of *Carica papaya*. Chemical characterization of the metabolites extracted from the plant has shown the presence of active compounds in the plant tissues, such as cysteine endopeptidases, a class-II and class-III chitinase, and glutaminyl cyclase in the latex[8]. The leaves are used to relieve the symptoms of asthma and as a vermifuge, in the treatment of gastric problems, fever and amoebic dysentery. Methanolic leaf extracts demonstrated vasodilatatory and anti-oxidant effects, both implicated in the reduction of cardiovascular risks[16].

Papaya contains 108 mg ascorbic acid per 100 g of fresh fruit, which is higher than oranges 67 mg/100

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g[14]. The study screened for anthelmintic and/or antitumour bioactive compounds from Thai indigenous plants and evaluated effectiveness against three different worm species and two cancer cell lines[8]. Ethanolic extracts of 20 selected plant species used by Yemeni traditional healers to treat infectious diseases were screened for their antibacterial activity against both Gram-positive and Gram-negative bacteria, as well as for cytotoxic activity. Fourteen of the ethanolic extracts showed variable degrees of antibacterial activity[5]. The quercetin glycosides rutin and manghaslin were isolated from the fruits of the mountain *papaya* Vasconcellea pubescens A. DC. Grown in Chile by selective fractionation using the bleaching of the free radical scavenger 1,1-diphenyl-2-picrylhydrazyl (DPPH) as the guiding assay. The structures were characterized by spectroscopic methods. Furthermore, 19 phenolic compounds were identified for the first time in the edible fruits by HPLC with UV and ESI–MS–MS detection. Ten of the compounds detected in the fruits and active fractions were tentatively characterized as hydroxyl cinnamic acid glycosides and nine as quercetin glycoside derivatives. The results provide relevant information on the low molecular weight constituents of this important fruit crop[19]. Food levels of vitamin C and flavonoids not only vary greatly depending on species and variety, growing location, harvesting time, storage, processing, and other conditions, but also with respect to methodological differences. For accurate dietary exposure determination and in support of future studies on the effects of dietary vitamin C and flavonoids, it was determined ascorbic acid, and the major dietary flavones (apigenin,luteolin), flavonols (k.ampferol, quercetin, myricetin), flavanones (hesperetin, naringenin and their glycosides), and anthocyanidins (pelargonidin, cyanidin, delphinidin) in fruits and vegetables commonly consumed in Hawaii[1]. The effect of ripening on the chemical composition of papaya (*Carica papaya L.*), especially regarding volatile components, was investigated in four ripening stages. Ripening was characterized sensorily, as well as through physical and chemical analyses. Volatile compounds were isolated by a simultaneous distillation / solvent extraction method. α-terpineol showed maximum concentrations in the third maturation stage, in correspondence with fruit ripeness. Other ripeness indicators could be hardness and soluble solids[2].

The aim of the present study was to identify the different phenolic classes of the polar extract of leaves of the Egyptian *carica-papaya* and to investigate the antimicrobial, antioxidant and anti-tumor activities of polar extracts from leaves of *carica-papaya*, for which a limited data have been previously published.

MATERIAL AND METHODS

Plant materials

Fresh leaves of *carica papaya* was collected on December (2007) from Sharkiaa, Egypt. And identified by Botany Department, Faculty of Science, Zagazig University.

Test oraganisms

The bacterial and fungal strains were personally obtained from the microbiology Lab., Botany Department, Faculty of Science, Zagazig University. Bacterial species tested were *Psudomonas areuginosa, Kelbseilla, Salmonella typhi, Staphyllococcus aureus* and *E. coli*. and fungal species were *Fusarium oxysporum, Aspergillus flavus, Aspergillus niger, Cladosporium* sp. *and penicillium* sp.

Materials for cytotoxic activity

Human tumor cell lines: HEPG2 (liver carcinoma cell line), MCF7 (breast carcinoma cell line) and HCT116 (colon carcinoma cell line).

Chromatographic materials

Paper chromatography

Sheets of Whatman paper No 1 or 3 MM were used for two- dimensional, comparative or preparative paper chromatography.

Column chromatography

The separation of the phenolic and flavonoid components was performed by column over Polyamide powder, polyamide 6-S for CC, Riedel-De Haen AG, seelze- Hannover, Germany.

Physical tests

Ultra-Violet spectrophotometric analysis

Chromatographically, pure materials dissolved in

analytically pure methanol were subjected to UV spectrophotometric investigation in 4 ml capacity quartz cells Zeiss spectrometer PMQ -II. In case of flavonoids, AlCl3, AlCl3/HCl, fused NaOAc/H3BO3 and NaOMe reagents were separately added to methanolic solution of the investigated material and UV measurements were then carried out[12].

Nuclear magnetic resonance spectroscopic analysis

NMR spectra were measured on Jeol ECA 500 MHz NMR Spectrometer at National Research Center, Dokki, Cairo, Egypt. 1H chemical shifts (δ) were measured in ppm, relative to dmso-d6 and converted to TMS scale

Mass spectrometric analysis

The isolated pure compounds were subjected, in most cases to Fast Atom Bombardment (positive and negative) mass spectroscopic analysis (FAB-MS) on MM 7070 E spectrometer (VG analytical). Some other compounds were subjected to electron spry ionization mass spectroscopic analysis (ESI – MS) a Varian Mat112-ET Spectrometer.All measurements were carried out at Institute Fur Chemie, Humboldt Universität zu Berlin, Germany.

Methods

Extraction

Two kilograms of air dried leaves thoroughly crushed and exhaustively extracted under reflux over a water bath with 5 liters of a methanol / bidistilled water (3: 1) mixture for 3 hours. The solvent was removed under reduced pressure at about 45°C. The residual finally yielded 150 g of a sticky dark brown material.

For the primary detection of the phenolic content in methanolic extract, the following investigations were carried out

Two dimensional paper chromatography of the extract on Whatman paper No 1, irrigated in the solvent system (acetic acid – water 6%) (HOAc – 6), followed by butanol: acetic: water (4:1:5) revealed the presence of mainly seventeen phenolic compounds, corresponding spots gave positive response towards $FeCl_3$ spray reagent, some of which appeared under UV light as dark purple spots which turned orange or lemon yellow or reddish orange when fumed with ammonia vapor or when sprayed with Naturstuff spray reagent[13].

A sample of 33 gm of the extract dissolved in 50 ml aqueous methanol (3:1) was fractionated over 300 gm polyamide 6S, Riedel-De Haen AG, Seelze – Hannover, Germany. Column (150 X 3.5 cm) and elution with methanol / bidistilled water mixtures of decreasing polarities for gradient elution led to the desorption of seven individual fractions (I-VII) which were dried, individually, in vacuum, and then subjected to rechromatography for several times to have the pure phenolic compounds.

Antioxidant assay

Antioxidant scavenger activities were spectrophotometrically determined at 517 nm according to method adopted by[9], with some modification[10]. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

Where A_B is the absorbance of blank sample and A_A is the absorbance of tested extract.

Antimicrobial activities

Pretreatment of extract: Dissolving the extract in dimethylforamide (DMF) for antimicrobial investigation at the final concentration of (10 mg / 1 ml).

Antibacterial activity

Antibacterial activities of extract were tested using pour plate technique on nutrient agar medium. Culturing and incubated of different bacterial species were carried out at 27 °C for 24 hours. Extract was tested at two concentrations 0.1 ml and 0.3 ml (10 mg / 1 ml). After the elapse of incubation periods, the diameter of inhibition zones was measured (mm). Mean of 3 replicated was calculated. The inhibition zone formed by the extracts against the particular test bacterial strain determined as the antibacterial activities of the extract[17].

Antifungal activity

Czepak Dox media used for cultivation of fungal species. The medium was seeded with different fungal species. After solidification of media on plates, make pores in agar with cup porer (15 mm) diameter. Two concentrations 0.1 ml and 0.3 ml (10 mg / 1 ml) of the extract were transfered into the well. Dimethyl foramide (DMF) was used only as a control. The plates were incubated for 7 days at 30 °C. The inhibition zone (mm)

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TABLE 1 : Chromatographic and spectral analysis of the phenolic compounds from methanolic extract of *carica papaya* leaves.

Compd No.	R_f values (x 100)	UV λ_{max} (nm) MeOH	MS	^{1}H- NMR Spectral Data (DMSO-d_6)δ (ppm)
1	57 (H2O), 59 (HOAC-6%), 81 (BAW)	260, 295.	153	6.89 (d, J=8 Hz, H-5), 7.46 (dd, J=8, 2.5Hz, H-6), 7.52 (d, J=2.5 Hz, H-2).
2	67 (H2O), 65 (HOAC-6%), 59 (BAW)	220, 245, 300, 330.	-	Caffeic acid moiety :7.42 (*d*, J= 16Hz, β-H), 705 7.05(*d*, J= 2Hz, 6-H), 6.96 (dd, J=7.5 Hz and J=2 Hz, 2-H), 6.79 (*d*, J= 7.5Hz, 3-H),6.19 (*d*, J= 16Hz, α-H). Quinic acid moiety: 5(m, 1'-H),1.88(m, 2'-H and 6'-H), 3.85(m, 3'-H and 5'-H), 3.5 (m, 4'-H).
3	22(H2O), 48 (HOAC-6%), 42 (BAW)	359, 299*, 266*, 259	-	Quercetin moiety:- 6.18 (d, J=2.5Hz, H-6), 6.37 (d, J-2.5 Hz, H-8), 7.55 (d, J=2.5 Hz, H-2'), 6.85 (d, J=8 Hz, H-5'), 7.56 (dd, J=2.5 & 8 Hz, H-6'). Glucose moiety:- 5.32 (d, J=8 Hz, H-1"), 3-3.75 (m, Hz, H-2"-6"), 4.35 (broad s, $\Delta\nu_{1/2}$ = 4). Rhamnose moiety:- 3-3.75 (m, H-1''', 2''', 5'''), 0.97 (d, J=6 Hz, CH_3-rhamnose).
4	17 (H_2O), 34 (HOAC), 45 (BAW)	256, 356	463	6.21 (d, J=2.5 Hz, H-6), 6.43 (d, J=2.5Hz, H-8), 7.56 (d, J=2.5 Hz, H-2'), 6.89 (d, J=8 Hz, H-5'), 7.56 (dd, J=2.5 & 8 Hz, H-6'), 5.5 (d, J=8 Hz, H-1"), 3.2-3.8 (m, H-2''-H-6'')
5	22 (H_2O), 48 (HOAC), 68 (BAW)	259, 297 sh., 348	448	Quercetin moiety: 6.17 (*d*, J=2.5 Hz,H-6),6.36 (*d*, J=2.5 Hz, H-8), 7.256 (*d*, J=2.5, H-2'), 6.82 (*d*, J=8 Hz,H-5'), 7.251 (dd, J=2.5 and 8 Hz, H-6') Rhamnose moiety: 5.20 ($\Delta\nu_{1/2}$ = 4 Hz,H-1"), 3.1 – 3.9 (*m*, overlapped with water proton resonances, H-2"-H-6").
6	25 (H_2O), 45 (HOAC), 81 (BAW).	218, 245, 298, 325.	180	6.2 (*d*, J= 16Hz, β-H), 6.76(J=7.5 Hz,5-H),6.88 (dd, J=7.5 Hz and J=2.5 Hz, 6-H), 6.98(*d*, J= 2.5Hz, 2-H), 7.48 (*d*, J= 16Hz, α-H).
7	33 (H_2O), 54 (HOAC), 60 (BAW).	278	390	4.51 (*d*, J= 7.3, H-2), 3.84 (*m*, H-3), 2.38 (ax, *dd* J=16.0, 7.9, H-4) 2.68 (eq, *dd*, J= 16.0, 5.3, H-4) 5.90 (*d*, J= 2.2, H-6), 5.72 (*d*, J=2.2, H-8),6.74 (*d*, J= 1.9, H-2'), 6.7 (*d*, J= 8, H-5'),6.61(*dd*, J= 8, 1.9, H-6')
8	0 (H_2O), 7 (HOAC), 75 (BAW).	255, 268, 370	302	6.19 (*d*, *J* =2.5, H-6), 6.4 (*d*, *J* =2.5, H-8), 7.64 (*d*, *J* =2.5, H-2'), 6.88 (*d*, *J* =8.5, H-5'), 7.53 (*dd*, *J* =2.5 &8.5, H-6')
9	0 (H_2O), 10 (HOAC), 85 (BAW)	268, 369	286	6.4 (*d*, *J*=2.5, H-8), 6.18 (*d*, *J*=2.5, H-6) 8.14 (*d.*, *J*=8, H-2' and H-6'), 6.89 (*d*, *J*=8, H-3' and H-5')

formed by the extract against the particular test fungal strain determined as the antifungal activities of the extract.

Measurements of potential cytotoxicity by SRB assay

Potential cytotoxicity of the extract was tested using the method of[18]. Cells were plated in 96-multiwell plate (104 cells / well) for 24 hrs. Before treatment with the extract allow attachment of cell to the wall of the plate. Different concentrations of the extract under test (50, 100, 125, 250 and 500 µg /ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the extract for 48 hrs. at 37 °C and atmosphere of 5% CO_2. After 48 hrs. cells were fixed, washed and stained with Sulforhodamine B strain. Excess strain was washed with acetic acid and attached strain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and extract concentration is plotted to get the survival curve of each tumor cell line after the specified compound.

RESULTS AND DISCUSSION

Identification of phenolic constituents in methanolic extract

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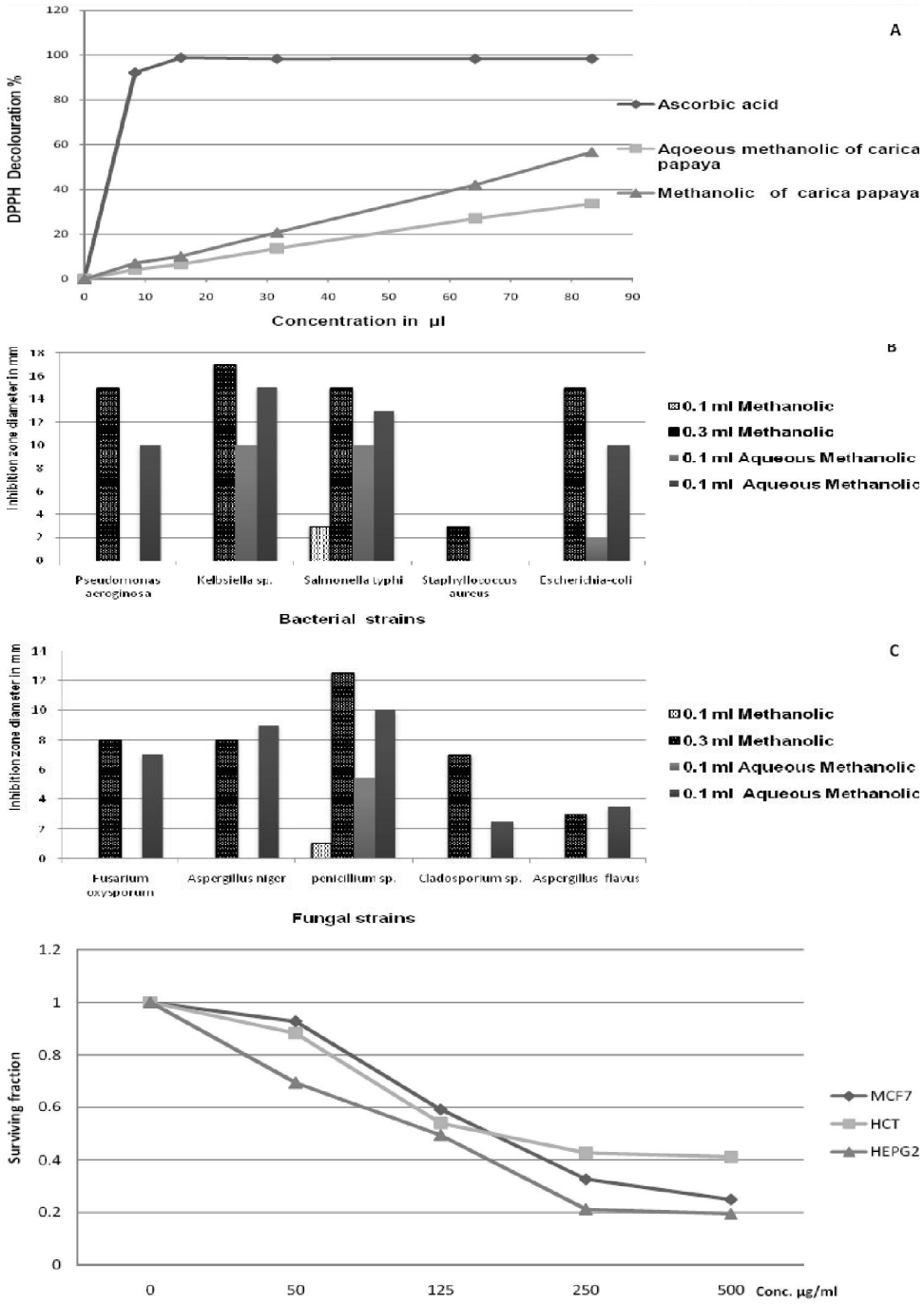

Figure 1 : A: Antioxidant activity curve of the extracts of *carica papaya.* B: Antibacterial activity of the methanolic and aqueous methanolic extract of *carica papaya.* C: Antifungal activity of the methanolic and aqueous methanolic extract of *carica papaya* leaves. D: % of survival fraction of colon, breast and liver carcinoma cell lines against concentration (µg/ml) of methanolicextract of *carica papaya* leaves.

Investigation of the phenolic compounds was done by fractionation of the extract, over polyamide column and elution with methanol/bidistilled water mixtures of decreasing polarities for gradient elution led to the desorption of seven individual fractions (I-V) which were dried, individually, in vacuum, and then subjected to rechromatography for several times led to the separation of nine pure phenolic compounds. Fraction I; was found to contain two compounds 1) protocatechuic acid and 2) chlorogenic acid, fraction II; contain 3) Quercetin-3-O-rutinoside, fraction III; contain 4) Quercetin - 3-*O*- glucopyranuronide and 5) Quercetin 3-*O*-α-$^{1}C_{4}$-

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TABLE 2 : Antioxidant activity of methanolic and aqueous methanolic of *carica papaya leaves*.

Methanolic solution (µl) of the substance added to 3ml DPPH	%Inhibition		
	Ascorbic acid	Aqoeous methanolic of *carica papaya*	Methanolic of *carica papaya*
0	0	0	0
10	92.15	4.21	7.06
19	98.8	6.61	10.07
38	98.27	13.68	20.75
77	98.35	27.06	41.95
100	98.37	33.68	56.69

rhamnopyranoside, fraction IV; contain 6) Caffeic acid and 7) ρ-coumaric acid, fraction V; 8) Quercetin, and 9) Kaempferol. The structure of these compounds were confirmed by chromatographic, UV, MS, H- NMR spectral data and the results were listed in TABLE 1.

Biological activities of *carica papaya* leave extracts

Assay for antioxidant capacity

The methanolic extract of *carica papaya* completely inhibited DPPH absorbance at three different concentrations used, (19, 38 and 77µl), (Figure 1: A) and (TABLE 2). The percentages obtained can be considered as a full absorbance inhibition of DPPH, because after completing the reaction, the final solution always possesses some yellowish color and therefore its absorbance inhibition compared to colorless methanol solution can not reach 100%. Permanent residual absorbance results in up to 7% of the total absorbance inhibition.

Antibacterial activity

Methanolic extract of *carica papaya* leaves showed inhibitory activity against *Salmonella typhi* at 0.1 ml concentration, but at 0.3 ml concentration showed inhibitory activity against all species. Aqueous methanolic extracts of *carica papaya* leaves showed inhibitory activity against *Salmonella typhi*, *Kelbseilla* sp. and *Escherichia coli* at 0.1 ml concentration, but at 0.3ml concentration the aqueous methanolic showed inhibitory activity against all species except for Staphyllococcus aureus (Figure 1: B) and (TABLE 3). These results agreement with that obtained by Ayoola *et al.*[7] and Ayad[6]. Ayoola *et al.*[7] reported that, the alcoholic extracts of clove, ginger, peppermint spearmint and thyme were the most effective than aqueous extracts against *E.coli* isolated.

TABLE 3 : Antibacterial activity methanolic and aqueous methanolic of *carica papaya* leaves.

Bacterial strains	Inhibition zone diameter in mm			
	Methanolic		Aqueous methanolic	
	0.1 ml	0.3 ml	0.1 ml	0.3 ml
Pseudomonas aeroginosa	_	15	_	10
Kelbsiella sp.	_	17	10	15
Salmonella typhi	3	15	10	13
Staphyllococcus aureus	_	3	_	_
Escherichia-coli	_	15	2	10

TABLE 4 : Antifungal activity of methanolic and aqueous methanolic of *carica papaya* leaves.

Fungal strains	Inhibition zone diameter			
	Methanolic		Aqueous methanolic	
	0.1 ml	0.3 ml	0.1 ml	0.3 ml
Fusarium oxysporum	_	8	_	7
Aspergillus niger	_	8	_	9
penicillium sp.	1	12.5	5.5	10
Cladosporium sp.	_	7	_	2.5
Aspergillus flavus	_	3	_	3.5

TABLE 5 : Cytotoxic activity of the methanolic extract of *carica- papaya* leaves against colon, breast and liver carcinoma cell lines.

Conc. µg/ml	HCT	MCF7	HEPG2
0.0	1.000000	1.000000	1.000000
50.0	0.717484	0.763665	0.738469
125.0	0.527279	0.365105	0.666767
250.0	0.375569	0.301902	0.197970
500.0	0.370140	0.275331	0.168325

Antifungal activity

Methanolic and aqueous methanolic extracts of *carica papaya* leaves showed inhibitory activity against only *penicillium* sp. at 0.1 ml concentration, but at 0.3

ml concentration showed inhibitory activity against all species (Figure 1: C) and (TABLE 4).

Results of anti tumor activity

The methanolic extract of *strawberry* leaves were tested against three human cell lines [HEPG2 (liver carcinoma cell line), MCF7 (breast carcinoma cell line) and HCT116 (colon carcinoma cell line)]. The results showed that the extract has activity against all cell lines tested (TABLE 5) and (Figure 1: D). IC50 of HEPG2= 168 µg / ml, MCF7=98.3 µg / ml and HCT= 144 µg / ml.

CONCLUSION

The results of this study suggest the possibility of using these extracts as natural food preservatives and potential sources of antibacterial, antifungal and antioxidant ingredients for the food and pharmaceutical industry.

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