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Some Microbiological Studies on Aqueous and Methanolic Extracts of Cinnamon (Cinnamomumzeylanicum).

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ABSTRACT

This study aimed to studying the gaining of the cinnamon extracts by two methods using water or 95 % methanol and modifying this extracts chemically by sulfation to gain four extracts; native aqueous, sulfated aqueous, native methanolic and sulfated methanolic extracts. From the results, all extracts had antimicrobial activity against pathogenicStaph. aureus, E. coli and Candida albicanswhile the native and sulfated methanolic extracts are more effective than native or sulfated aqueous extracts, the highest inhibition zone (IZ) diameter value recorded are; 31.2, 22.5 and 28.0 mm for Staph. aureus, E. coli and Candida albicans, respectively for methanolic extract while the highest (IZ) diameter value for water extract are: 25.0, 20.4 and 20.0 mm for Staph. aureus, E. coli and Candida albicans, respectively. All extracts show antimicrobial activitiesbut at different degree with different Minimum inhibitory concentration (MIC), all extracts had high antitumor effect against cancer colon cells(Caco-2 cells) ranged from 28.73 % to 71.81 % of cytotoxicity and the sulfation modification did not increase or decrease the biological activities of the extracts.

KEYWORDS Cinnamon, aqueous extract, methanolic extract, sulfation, antimicrobial activity.

Introduction :

The natural products are found to be more effective with least side effects as compared to commercialantibiotics so that reason they are used an alternated remedy for treatment of various infections. (Tepe et al.,2004). Spices are defined as plant substances used to enhance flavor, Cinnamon is one of the oldest spices known. It was used in ancient Egypt not only as a beverage flavoring and medicine, but also as an embalming agent. It was so highly treasured that it was considered more precious than gold. Around this time, cinnamon also received much attention in China, which is reflected in its mention in one of the earliest books on Chinese botanical medicine, dated around 2,700 B.C. Cinnamon's popularity continued throughout history. It became one of the most relied upon spices in Medieval Europe. Due to its demand, cinnamon became one of the first commodities traded regularly between the Near East and Europe. Cinnamon can be used as spice because of its sweet flavoring and spicy characteristics and it also plays an important role in pharmacological effects such as: anti- inflammation, antimicrobial, antioxidant ,antidiabetes type 2, antispasmodic, anti- ulcer and cytotoxic properties, also Many medicinal plants produce antioxidant andantimicrobial properties which protect the host from cellular oxidation reactions and other pathogens highlighting the importance of search for natural antimicrobial drugs (Mothan and Lindequist, 2005).Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Kordali et al., 2005).Some oils have been used in cancer treatment (Sylvester et al.,2006). The aim of this study is to evaluate the chemical structure, antimicrobial activity, minimum inhibitory concentration (MIC) and antitumor activity of Cinnamon extracts.

Materials and Methods:

Materials:

Collection of plant materials:

Cinnamon barks obtained from local Egyptian shops, then were grounded using a grinder into a fine powder, then they were kept in dark bottles until the day of use.

Bacterial and Yeast strains:

Three pathogenic organisms isolated and identified by the clinical laboratory of Faculty of Medicine, Ain Shams University (El-Demerdash hospital), cairo, egypt. One Gm -ve bacterium(*E-coli*), the second is Gm +ve bacterium (*Staph.aureus*) and the third is *Candida albicans* yeast.

Bacteria and Yeast media:

The bacterial media used in this study for isolation andmaintenance of the studied pathogenic bacteria are: Nutrientagar and Nutrient broth,while in case of *Candida albicans*, Potato Dextrose Agar (PDA) is used. All media purchased from DIFCO Company.

Methods :

Analytical Methods:

For each studied samples, chemical properties of moisture, ash, crude proteins, total carbohydrates and crude fats were performed according to (AOAC, 2000).

Preparation of Aqueous and Methanolic Extracts

The spice cinnamon was purchased from local market. The spice was washed with distilled water thoroughly. Dry spice (100 gm) was crushed and sieved through mesh cloth to get the fine powder. Powdered spices were soaked in 200ml of distilled water and were kept at room temperature for 24 hours, then were filtered using Whatman no.1 filter paper. The filtrate

was heated at 40-50°C using water bath until thick paste is formed. The thick paste was considered as 100% concentration of extract. These extracts were stored at 4°C in refrigerator. Extracts of this spice were further diluted to make different concentrations such as 10, 20, 40,60, 80and 100%by mixing with appropriate volumes of distilled water. The methanolic extract was prepared following same procedure with the exception of solvent which was 95% methanol instead of sterilized distilled water(Sana Mukhtar&IfraGhori., 2012).

Preparation of Sulfated Extracts:
This was performed by the modification of the method reported by Yang et al. 2005with some modification. The sulfation of the extract was performed as follows: 0.1g of each extract was suspended in 0.5 ml dry formamide and the mixture was stirred at room temperature for 24h. in order to disperse it into the solvent. The sulfating agent was prepared by dropping 1ml of $HClSO_3$in 4.0 ml of formamide under cooling in an ice-water bath and then added to the extract. The reaction was cooled in ice, neutralized by 30% NaOH solution and dialyzed against running water for 48h and then lyophilized.

Maintenance of bacterial & fungal cultures and inoculum preparation
Pure bacterial cultures were refreshed and maintained on nutrient agar slants and plates on regular basis. The bacterial cultureswere streaked on sterile nutrient agar plates and kept in incubator for 24 hours at 37°C and stored at 4 °C, while the yeast strain grown on PDA. Bacterial & yeast cultures were refreshed after every 3 to 4 days to avoid contamination. Inoculum was prepared bygrowing the pure microbial cultures in appropriate broth media over night at 37°C.

Antimicrobial activity test:
The extracts were examined for its antimicrobial activity against Gm -ve bacteria, Gm +ve bacteria and *Candida albicans*by well diffusion method (Mitscher, et al. 1972 &Pepeljnjak, et al. 2005). The experiment wasperformed using a culture at 37 °C for 24 h. on 20 ml ofnutrient agar for bacteria or PDA for yeast were poured into sterile petri-dishes and allowed for solidification. Wells weremade in agar plates using sterile cork pore of 4 mm diameter. The cultures were adjusted to approximately 10^6 CFU/ml with sterile saline solution. One hundred andfifty microliters of the suspensions were spread over theagar plates using a sterile glass spreader. Each tested sample was dissolved in 1 ml of dist. water and sterilized by filtrationthrough a 0.22 µm membrane filter (by using Millioporemembrane filter apparatus). 150 µl of each sample wereadded separately to the appropriate wells in the Petridishes.

Determination of the minimum inhibitory concentration (MIC) of cinnamon extracts on indicator pathogens:
cinnamon extracts were tested to determine the minimal inhibitory concentration (MIC) for each bacteria or yeast tested in the present study were grown on nutrient broth media for bacteria or PDA for yeast for 24h . After then 150µl of 10^6 cell/ml were spotted on each plate supplement withvarying concentrations (10. 20. 40, 60. 80 and 100 %) ofthe extracts. The plates were incubatedat 37° C for 24 h.The MICs were determinedas the lowest concentration of extract inhibitingvisible growth of each organism on the agarplates(Perez et al.1990).

Antitumor activity :
Antitumor activity of aqueous and methanolic cinnamon extracts:
Quantitative assay of cytotoxicity:
Quantification of fixated monolayer cells was performed spectrophotometrically with crystal violet as a DNA stain. Staining cell nuclei of fixed cells with crystal violet allows for rapid, accurate and reproducible quantification of cell number in cultures grown in 96-well plates (Kueng et al. (1986).Crystal violet assay were used to measure the total count of cancer mammalian cells which well reflect the cytotoxic effects of the aqueous and methanolic extracts of cinnamon by using human colon carcinoma cell line, Caco-2, in 96 well plates. Experiments were performed in basal tissue culture medium (BTC medium) buffered with 1 M HEPES (pH 7.4), 2.20 g of $NaHCO_3$, and 10ml of fetal bovine serum per liter maintained by incubation in 5% CO_2. Briefly, the cell culture type (Caco-2) was subcultured as follows: The growth medium was discarded and the cell sheet was washed with sterile 0.15M PBS, pH 7.5. Cell dispersing solution (Trypsin) was added for 1 min and then discarded. Cells were incubated at 37ºC. After cell separation and roundation was observed microscopically, 50 ml of fresh medium (BTC medium) was added to the flask. Suspended Caco-2 cells were counted and diluted to about 10^5cell/ml. Cell suspension (100 µl) was distributed in 96 tissue culture plate, then incubated in humid 5% CO_2 incubator at 37ºC for 24 h to allow for attachment. After incubation, The medium was discarded and cells were exposed to a dilution series ranging from (0.02, to 0.005 mg/ml) aliquots suspension of each sample of water and methanolic cinnamon extracts (suspended in fresh Basal Tissue Culture (BTC) medium) were inoculated in each 8 well and incubated for 48 h in 5% CO_2 incubator at 37ºC. including controls (contains only BTC medium) After 48 hours of exposure, the medium was discarded and cells were fixed with 100 µl of gluteraldehyde of 1% for about 30 minutes. The excess gluteraldehyed solution was removed then washed with tap water. Crystal violet solution (0.1%), 100 µl was added for about 30 minutes. wells were immersed in very slow rate of running tap water for 15 minutes. The plates were dried then the stained wells were eluted with 100 µl of 10% acetic acid solution. The color intensity in each well was measured using microplate reader at 590 nm. Eight replicates were used foreach isolate, and all tests were performed in triplets. The absorption reading of different aliquots of cinnamon extracts (average reading of 8 wells) divided by the control reading (average reading of 8 wells containing 100 µl of sterile BTC) multiplied by 10^4 (the total number of Caco-2, cell per well). This provides the number of viable Caco-2, cells. Subsequently, the previous number was subtracted from 10^4 to obtain the number of dead Caco-2, cells.

Statistical analysis
Data were statistically analyzed using SPSS, version 10for windows SPSS (Inc., Chicago, IL, USA). Data was presented as mean ± standard deviation.

Results:
1. Chemical composition of extracts:
Data of the chemical composition of investigated cinnamon extracts was shown in Table (1). The results showed a wide range of variation in the yield of the investigated cinnamon extracts. The highest yield was recorded in water extract of (12.0 %) and lowest in methanolic extract (8.0%). Also the data showed a variation in the proportions of this constituent. The highest value of total carbohydrates was observed in methanolic extract of (33.2%) and lowest in water extract (30.2%).

Another a little variation was also observed in the soluble-protein and moisture contents of the studied cinnamon extract. Thus, the percentages of protein varied from (12.1%) in water extract to (12.2%) in methanolic extract and moisture varied from 30.1 % to 33.1 % for water and methanolic extracts, respectively. On the other hand, the ash and crude fibers contents were recorded the same for the two studied extracts 12.0 and 14.0, respectively.

Table (1): Yield, ash,moisure,crude fiber,total carbohydrates and total proteins of aqueous and methanolic extracts of Cinnamon (g/100g).

Extract	Yield (gm/100gm)	Ash %	Moisture %	Crude fiber %	Total carbohydrates %	Total Proteins %
aqueous	12.0	12.0± 0.5	31± 1.0	14.0 ± 0.2	30.2± 0.2	12.1 ± 0.2
methanolic	8.0	12.0± 0.5	30± 0.5	14.0± 0.1	33.2± 0.4	12.2 ± 0.4

2- Sulfation of the aqueous and methanolic extracts:
The results show that the two extracts (aqueous and methanolic extract) had been sulfated and giving rise to 7.7 and 8.0 mg sulfate/ 100 gm of the Cinnamon powder for aqueous and methanolic extract, respectively.

3-Antimicrobial activity of cinnamon extracts:
The spice cinnamon was tested against *E. coli*&*Staph. aureus* as pathogenic bacteria and *candida albicans* as pathogenic yeast. From Table 2, all extracts of the cinnamon are effective against all pathogens but in different degree according to the mean diameter of inhibition zone (IZ) forming a mximum(IZ)of 31.2 mm in case of native methanolic extract against pathogenic *Staph. aureus*and the minor (IZ) was in case of *Candida albicans* (19.9 mm) at sulfated aqueous extract.In all cases, the methanolic extract is more effective to all pathogens than aqueous extract.

Table 2: Antimicrobial activity of cinnamon extracts

Extracts	Mean diameter of inhibition zone (mm)		
	Staph. aureus	E.coli	Candida albicans
Native aqueousext.	25.0 ± 0.5	20.4 ± 0.2	20.0 ± 0.2
Sulfated aqueous ext	25.0 ± 0.5	20.2 ± 0.2	19.9± 0.2
Native Methanolicext.	31.2 ± 0.4	22.5 ± 0.5	28.0 ± 0.2
Sulfated Methanolic ext.	31.1 ± 0.4	22.5 ± 0.5	28.0 ± 0.2
control	27.0	25.0	26.0

Control :Imepenim 10 µg for bacteria and Griseofulvin 10 µg for yeast.

4- The minimum inhibitory concentration (MIC) of the Cinnamon extracts:
Tables (3-6) show the using of the different concentrations of the studied extracts (10,20,40,60,80 and 100 %) to record the minimum inhibitory concentrations (MIC) of the studied extracts. From all the results, the concentration 10 % is the lowest one and the (IZ) increases by increasing the concentration until reach to concentration 100 %, which is the best concentration gives the best (IZ) for all extracts with all pathogens (27 mm, 24 mm and 26 mm for*Staph.aureus, E.coli*and *Candida albicans*, respectively). Also there are not any differences in the (IZ) results of the sulfated extracts resulted from the sulfation modification process of the native extracts.

Table 3 : Antibacterial activity of cinnamon extracts against*Staph. aureus*

Mean diameter of inhibition zone (mm)				
Cinnamon concentration	Native aqueous extract	Sulfated aqueous extract	Native methanolic extract	Sulfated methanolic extract
10 %	0	0	0	0
20 %	0	0	11.5 ± 0.4	11.5 ± 0.4
40 %	11.0 ± 0.5	11.0 ± 0.5	25.8 ± 0.4	25.8 ± 0.4
60 %	20.0 ± 0.5	19.9 ± 0.5	27.8 ± 0.4	27.8 ± 0.4
80 %	24.6 ± 0.5	24.4 ± 0.5	28.6 ± 0.4	28.1 ± 0.4
100 %	25.0 ± 0.5	25.0 ± 0.2	31.2 ± 0.3	31.2 ± 0.2
Control) imepenim 10 µg)	27.0 ± 0.1			

From previous results of table (3), the MICs of the extracts concentrations against the pathogenic *Staph. aureus* are 20 % concentration of the native methanolic extract and its sulfated one which give rise to (IZ) = 11.5 mm diameter, while the concentration 40 % of the native aqueous extract and its sulfated one is the MIC to it due to its (IZ) diameter is 11.0 mm on other hand, table (4) show that 20 % concentrations is the MIC for all extracts against E. coli (IZ ranged from 6.0 to 6.5 mm).

Table 4: Antibacterial activity of cinnamon extracts against *E.coli*

Mean diameter of inhibition zone (mm)				
Cinnamon concentration	Native aqueous extract	Sulfated aqueous extract	Native methanolic extract	Sulfated methanolic extract
10 %	4.5 ± 0.5	4.5 ± 0.5	4.6 ± 0.5	4.5 ± 0.5
20 %	6.0 ± 0.25	6.0 ± 0.1	6.5 ± 0.4	6.5 ± 0.1
40 %	13.0 ± 0.5	13.0 ± 0.2	14.8 ± 0.4	14.8 ± 0.2
60 %	15.0 ± 0.4	15.1 ± 0.1	15.7 ± 0.6	15.7 ± 0.1
80 %	16.2 ± 0.5	16.2 ± 0.5	19.3 ± 0.6	19.3 ± 0.2
100 %	20.4 ± 0.2	20.4 ± 0.2	22.5 ± 0.2	22.5 ± 0.2
Control (imepenim 10 µg)	24.0 ± 0.1			

Table (5) show that concentration 40 % for all extracts is the MIC for all extracts and the IZ ranged from 9.0 to 10.8 mm diameter. only 100 % concentration is the best concentration which give the highest IZ for all extracts to all studied pathogenic organisms.due to it gave the best IZ for all studied pathogens.

Table 5 : Antibacterial activity of cinnamon extracts against *Candida albicans.*

Mean diameter of inhibition zone (mm)				
Cinnamon concentration	Native aqueous extract	Sulfated aqueous extract	Native methanolic extract	Sulfated methanolic extract
10 %	0	0	0	0
20 %	0	0	0	0
40 %	9.0 ± 0.5	9.0 ± 0.3	10.8 ± 0.4	10.0 ± 0.3
60 %	11.0 ± 0.5	11.0 ± 0.3	15.8 ± 0.4	15.6 ± 0.3
80 %	14.6 ± 0.5	14.6 ± 0.4	18.6 ± 0.4	18.5 ± 0.1
100 %	20.0 ± 0.2	20.0 ± 0.2	28.0 ± 0.2	28.0 ± 0.2
Control (griseofulvin 10 µg)	26.0 ± 0.2			

Antitumor activity of cinnamon extracts:
Table 6 clearly illustrate the cinnamon extracts effect on tumor colon cells *in vitro*. The data of the Table 6 indicate that all the tested concentrations (varied from 0.005 to 0.02 mg/ml) of the cinnamon extracts gives positive results with the tumor cells and the cytotoxicity of the tumor cells ranged from 2819 to 7127of dead cellscompared with the control.

Table 6: Antitumor effect of cinnamon extracts:

Extract concentration	Type of the extract	OD	No of dead cells	No of viable cells	% of cytotoxicity
0.02 mg/ml	aqueous	0.361	7173	2827	71.73
	Sulfated aqueous	0.360	7181	2819	71.81
	methanolic	0.360	7181	2819	71.81
	Sulfated methanolic	0.360	7181	2819	71.81
0.01 mg/ml	aqueous	0.790	3833	6167	38.33
	Sulfated aqueous	0.780	3911	6089	39.11
	methanolic	0.795	3794	6206	37.94
	Sulfated methanolic	0.785	3872	6128	38.72
0.005 mg/ml	aqueous	0.912	2874	7126	28.74
	Sulfated aqueous	0.910	2896	7103	28.96
	methanolic	0.913	2873	7127	28.73
	Sulfated methanolic	0.912	2874	7126	28.74
control		1.281	zero	10000	zero

Discussion :

From all results from Table 2 to Table 5, the Antimicrobial activities of the cinnamon extracts are effective against all the studied pathogens by different degrees of effects, the most sensitive pathogen is *Staph. Aureus* bacteria followed by *Candida albicans* yeast and finally *E.coli*, this may be due to that the structural differences in the cell membrane and cell wall structure, Gram negative has outer membrane as well which further block the penetration of antibiotics including the extracts of spices making them slightly resistant to it, in case of *candida albicans*, cell wall structure is almost resemble the cell wall structure of the Gram positive bacteria and stained deep blue by Gram stain so it behave slightly like Gram +ve bacteria. It is agree with Indu et al. (2006) whom reported that the cinnamon extract posses effective antibacterial properties against *E.coli* and *B. subtilis.*

The antimicrobial activity of aqueous extracts could be due to anionic components such as thiocyanate, nitrate, chlorides and sulphates in addition to many other compounds naturally present in plants. (Darout , 2000). The methanolic extracts showed better results as compared to aqueousextracts as being organic dissolves more organic compounds resulting in the release of greater amount of active antimicrobial components (Cowan, 1999).So methanolic extracts of cinnamon are more efficient in its antimicrobial activity as compared to the aqueous extracts. The antimicrobial activity ofcinnamon might be due to the presence of cinnamaldehyde compound which inhibits the amino acid decarboxylation activity in the cell which leads to energy deprivation and microbial cell death (**Wendakoon andSakaguchi, 1995).**

The MICs were determined as the lowest concentration of oil inhibiting visible growth of each organism on the agar plate (Perez et al., 1990).

From previous results of table (3), the results of MIC of the studied extracts were in close agreement with (Saraf et al.,2011) who showed that the cinnamon essential oil inhibit the the growth of *Staph. aureus,* mm on other hand, table (4) show that 20 % concentrations is the MIC for all extracts against *E. coli* (IZ ranged from 6.0 to 6.5 mm) and it agree with Friedman et al. 2002, who found that the cinnamon oil was effective against the pathogenic *E. coli.* While table 5 show that concentaration 40 % for all extracts is the MIC for all and the IZ ranged from 9.0 to 10.8 mm diameter. only 100 % concentration is the best concentration which give the highest IZ for all extracts to all studied pathogenic organisms. due to it gave the best IZ for all studied pathogens.these findings are also quite similar with the results of Chao et al. 2000, who reported that the cinnamon bark oil fully inhibited the growth of some Gram positive and Gram negative bacteria, fungi and yeasts. Therefore, the high antimicrobial activity of cinnamon oil is due to the presence of the high amount ofcinnamaldehyde (Bin Jantan et al., 2008).

The positive effect of the all extracts against colon cancer cells which gave higher cytotoxicity ranger from 2819 to 7149 of dead cells compared with the control, with the extracts concentrations ranged from 0.005 to 0.02 mg/ml. this may be due to that the cinnamon extracts posses high content of carbohydrate and it agree with Hassan et al.(2009) whom stated that there is a positive co relation between antitumor activity and carbohydrate contcentration.

Conclusion :

From above results, the methanolic extracts of cinnamon are more effective than the aquoues extracts, but in all cases the four cinnamon extracts had possesantmicrobial and antitumor activities due to their structure content. On other hand, sulfation modification of the native aquoues and methanolic extracts had no positive or negative effect on all biological evaluations.Finally,this results made cinnamon plant very important spice which can improve the human health , so it considered as important medicinal plant.

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