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Extraction, Purification and Biological Properties of Polyphenols and Flavonoids from Lebanese *Punica granatum* Peels

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ABSTRACT

Pomegranate peels were used to extract crude polyphenols and flavonoids. The polyphenol crude extract and the flavonoid crude extract were both evaluated for TPC giving 33.5 mg/g of dry powder and TFC giving 41.8 mg/g of dry powder respectively. Also, these crude extracts were evaluated for their antioxidant, antiproliferative and antibacterial activities. The antioxidant activity of crude polyphenols and flavonoids showed very satisfactory results (90% and 88% at 100µg/mL respectively) that were close to those of ascorbic acid (90.58% at 100µg/mL). The antiproliferative activity was tested on two different cancer cell lines (HT-29 and HCT-116 cells) where the extracted polyphenols showed their best inhibitory effect after 72 hours from application, at 25 µg/mL with 46.07% inhibition for HCT-116 cells and 81.31% at 5µg/mL for HT-29 cells. Therefore, the extracted flavonoids showed their best inhibitory effect after 72 hours from application to HCT-116 cells at 200µg/mL with 62.72% inhibition and 58.53% at 5µg/mL for HT-29 cells after 24 hours from application. Finally, the polyphenol extract showed a MIC of 1.05 and 0.525mg/L for *S. epidermidis* and *E. coli* respectively, and a MBC of 2.1 and 1.05mg/L for the respective strains. However, the flavonoid extract showed a MIC of 1.575 and 0.7875mg/L for *S. epidermidis* and *E. coli* respectively, and a MBC of 3.15 and 1.575mg/L for the respective strains. All of these obtained results demonstrated that Lebanese pomegranate peels are rich in bioactive compounds and could be used for treating diseases related to oxidative stress.

INTRODUCTION

The advancement in drug discovery technology, diversification of the health sector and reduced funding for natural product-based drug discovery, couldn't replace the herbs and herbal treatment systems. The natural products from plants and biological sources still remain an unlimited and uncondensed source of new phytochemicals and nutraceuticals (Rizwan *et al.,* 2017). Natural phytochemicals are involved in various sectors starting with the medicinal one, moving to food technology and plant pathology sectors. In recent years, attention has been focused on the natural substances for biological control of plant pathogens such as agro-industrial wastes where the pomegranate peel is considered as a good example for this type of agro-industrial byproducts. From this, Arabs countries and more particularly Lebanon are distinguished by a great wealth of plant species especially with medicinal properties (Abou-Chaar, 2004; Deeb *et al.,* 2013). One of the most famous plants known in Lebanon and in the world is pomegranate. It is known for their beneficial rich components that can be used for a variety of their chemical properties, such as their antioxidant scavenging activity and antifungal activity (Sabbah *et al.,* 2017). Besides the pomegranate juice, pomegranate peels are also important for their containment of many phytochemicals including polyphenols and flavonoids (Sabbah *et al.,* 2017).

Hence shows the increasing need for a constant search of new natural phytochemicals which can be used as a substitute for synthetic chemicals in many different sectors. So the objective of our research work was **first**, to extract crude polyphenols and flavonoids from the dried peels of the Lebanese *Punica granatum* and **second**, to (1) evaluate their antioxidant power using an *in vitro* method, the DPPH test, (2) study their antiproliferative capacity on two cancer cell lines, the epithelial cell HT-29 and the human colorectal cell HCT-116 using the MTT method, and (3) determine their antibacterial property on two bacteria strains, one Gram positive bacteria (*Staphylococcus epidermidis* CIP 444) and one Gram-negative strains (*Escherichia coli* ATCC 35218).

MATERIAL AND METHODS

Preparation of sample

Pomegranates were washed and cleaned with water after their collection from south of Lebanon at 375 m of altitude. The peels were dried at room temperature in shade, away from sunlight, with continual turning over for homogeneous drying. After that, the dried peels were

grinded to obtain a powder and preserved in a container away from light, heat and moisture for later use.

Extraction and purification of polyphenols (Chart 1)

The extraction and purification of polyphenols have been determined according to the method of Bharadwaz and Bhattacharjee (2012). Briefly, peels powder (100g) were first dissolved in 750 mL of methanol (70%) in a water bath (40°C) for 30 minutes. The solution was then filtrated and the methanol was completely eliminated by a rotary evaporator at 50 °C. After the elimination of the methanol, 0.3g of ascorbic acid was added to the solution to prevent oxidation and the solution was allowed to separate in a separatory funnel after the addition of 500 mL of ethyl acetate. The solution was divided into two parts during separation, the upper yellow part (containing polyphenols and ethyl acetate), and the dark brown part (containing water and other components). The upper yellow part was taken and the ethyl acetate was totally eliminated under reduced pressure in the rotary evaporator to collect the powder of polyphenols remaining in the flask and analyze it.

Extraction of flavonoids (Chart 2)

Flavonoids were extracted according to the method of Zhang *et al.* (2011). Briefly, 20g of pomegranate peels powder were dissolved in 250 mL of ethanol (70%) using the ultrasound machine for 1 hour. Then, the solution was filtrated and the ethanol was completely eliminated using a rotary evaporator. After that, the solution was frozen at -20 °C and then placed in the lyophilisator to dry the extract to be used later in the analysis.

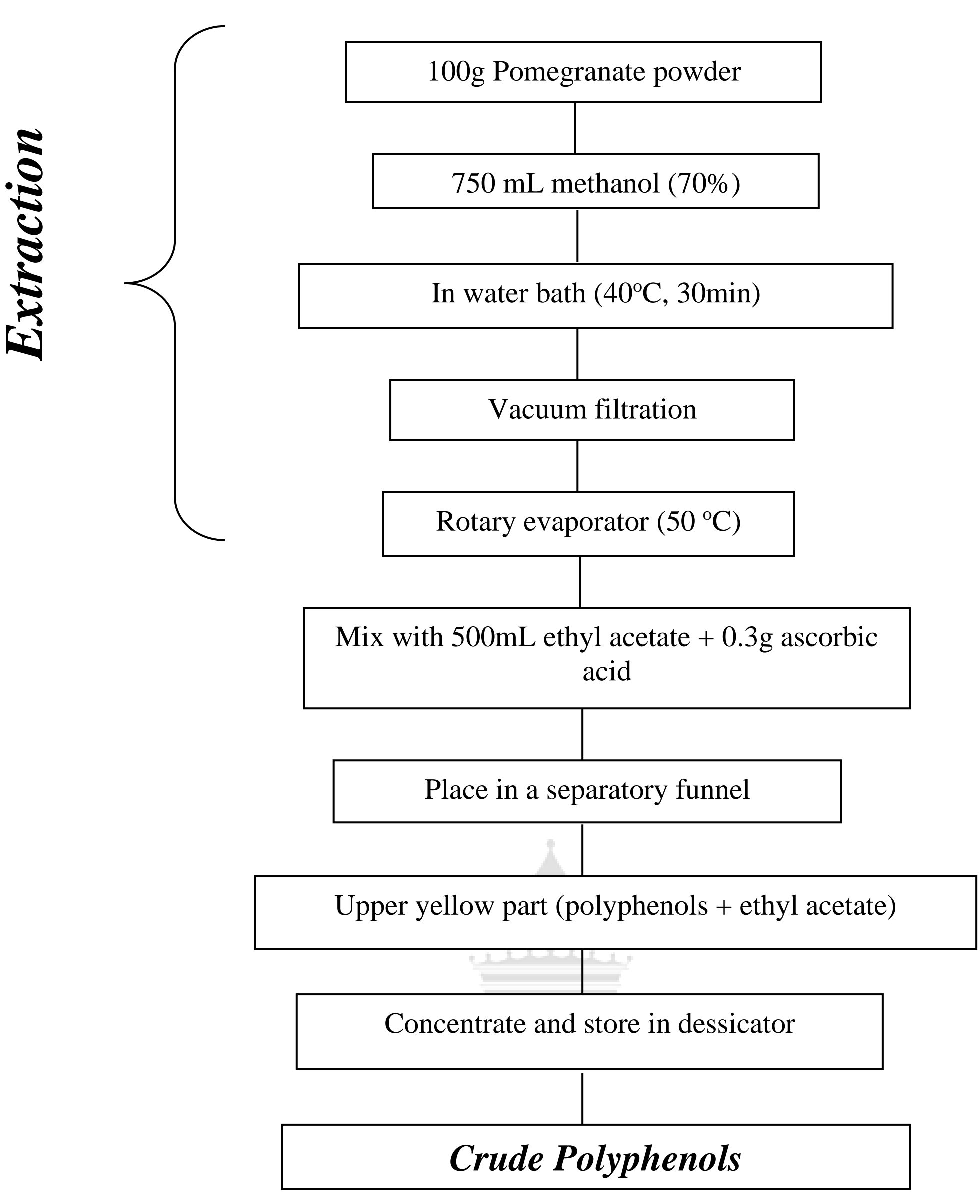

Chart 1: Polyphenols extraction and purification

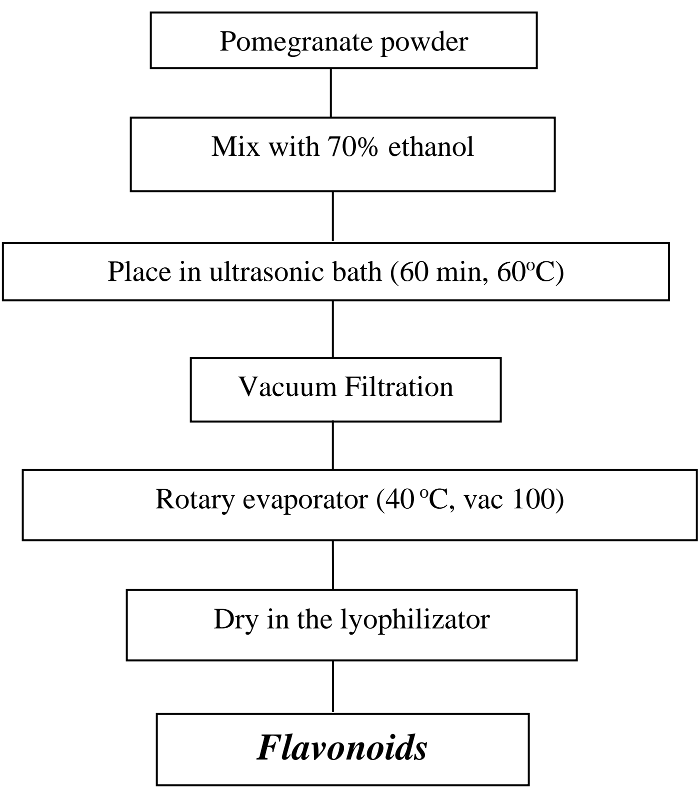

Chart 2: Flavonoids extraction

Total phenolic content (TPC)

The Folin–Ciocalteau reagent method has been used for the estimation of TPC Farhan *et al.* (2012). Briefly, 100 µL of each extract was taken and mixed with 1 mL of Folin–Ciocalteau reagent (1/10 dilution in water). After 5 minutes, 1 mL of Na_2CO_3 (7%) has been added. The blend was incubated in the dark at room temperature for 30 minutes. The absorbance of blue-colored solution of all samples was measured at 765 nm using a Gene-Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg of gallic acid equivalent (GAE) per g of dry weight of plant powders.

Total phenol content = GAE x V x D /m,

Where **GAE** is the gallic acid equivalence (mg/mL); **V** is the volume extract (mL), **D** is dilution factor and **m** is the weight (g) of the crude plant extract.

Total flavonoids content (TFC)

The aluminum chloride method was used according to Quettier-deleu *et al.* (2000) for the determination of TFC. 1 mL of various concentrations of the extract was mixed with 1 mL of 2 % methanolic aluminum chloride solution. After an incubation period at room temperature in the dark for 30 minutes, the absorbance of all samples was determined at 415 nm using a

Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg per g of rutin equivalent (RE) and methanol was used as blank.

$$TFC = RE \times V \times D/W$$

Where **RE** is Rutin equivalent (µg/ml), **V** is the total volume of sample (ml), D is dilution factor, W is the sample weight (g).

Biological activities

Antioxidant activity with 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The method of Rammal *et al.* (2013) has been used for the scavenging ability of DPPH antioxidant test. 1 mL of different concentrations (100, 50, 25 and 5 µg/mL) of polyphenol and flavonoids was added to 1 mL of DPPH (0.15 mM in methanol) and at the same time, a control consisting of 1 mL DPPH with 1 mL methanol was prepared. The reaction mixtures were mixed very well by hand and then incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 517 nm by a Gene Quant 1300 UV-Vis spectrophotometer. The ascorbic acid was used as a positive control and the methanol was used as blank. The DPPH scavenging ability of plant extracts was calculated using the following equation:

$$\%\ Scavenging\ \text{activity} = [(\text{Abs control} - \text{Abs sample})]/\ (\text{Abs control})] \times 100$$

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The Abs control is the absorbance of DPPH + alcohol; Abs sample is the absorbance of DPPH radical + sample. Also, three controls have been prepared.

Antibacterial activity assay

Bacterial strains: The strains used in this study were one Gram positive bacteria (*Staphylococcus epidermidis* CIP 444) and one Gram-negative strain (*Escherichia coli* ATCC 35218). The Gram-positive CIP 444 strain is a clinical strain that is isolated from an infected implanted device of a patient who is hospitalized in the Mignot Hospital of Versailles, France Chokr *et al.* (2006). The other strain is ATCC. The latter were stored in glycerol stocks at -80°C and used as required. Brain heart infusion (BHI), Brain heart agar (BHA), and Mueller–Hinton broth (MHB) were purchased from HIMEDIA (Mumbai, India), in which they were prepared and then autoclaved as indicated by the manufacturer before their use.

MIC and MBC assays: extracts of plant were tested for their corresponding Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) by broth microdilution assay, as recommended by the Clinical Laboratory and Standard Institute

(CLSI). A concentration of 4.2mg/mL of polyphenol crude extract and 6.3mg/mL of flavonoid extract were prepared. In a 96-well plate (200 µL/well) (Greiner Bio-One, Essen, Germany), serial two-fold dilutions in MHB of the different extracts were done. The wells were inoculated with 5×10^5 bacteria/ml. After incubating the plates at 37 °C for 24 hours, the MIC (which is defined as the lowest concentration that yielded no growth) was determined. In addition, the wells with no visible growth were placed on BHA in order to determine the MBC (which is defined as the lowest concentration which killed ≥99.9% of the initial inoculum). The Petri plates were incubated overnight at 37°C, and the MBC was determined.

Antiproliferative activity:

To study the antiproliferative activity of powder's extracts from the studied plant, cell culture was performed using the epithelial cells HT-29 and HCT-116 cells of the human colon. Then the measure of inhibition of cell proliferation was applied using the yellow tetrazolium MTT technique.

Cell culture was performed in 96-well plates, each containing 100 µL DMEM at 10.000 cells for HT-29 and 15.000 cells for HCT-116. The powder's extracts were diluted with the DMEM culture medium in decreasing concentrations (200, 100, 50, 25 and 5 µg/mL) and were then added to the wells after pre-incubation for 24 h. The plates were then incubated under 5% CO_2 and at a temperature of 37°C during 24, 48 and 72 hours respectively.

After incubation, 10 µL of MTT solution were added per well and incubated for 3 hours at 37°C. Then a 100 µL solubilization solution was added to each well. Finally, the absorbance was measured with a spectrophotometer at 570 nm. This quantity is directly proportional to the number of cells with an intact membrane.

RESULTS AND DISCUSSION

TPC and TFC

The TPC and TFC were estimated using the gallic acid and the rutin standard curves. Our results indicated that TPC is 33.5 mg/g of dry powder and the TFC is 41.8 mg/g of dry powder.

These phenolic compounds (polyphenols and flavonoids) are very important fruit constituents which can be used as an important indicator of several functional properties like antioxidant

or antibacterial capacities. Additionally, they can be used as a preliminary screen for any product when intended as a natural source of antibacterial compounds.

Antioxidant activity

The antioxidant activity of the crude polyphenols is illustrated in Figure 1. The polyphenols showed higher percentage of scavenging activity than that of ascorbic acid at the concentration 5µg/mL. This percentage reaches the 90 % at the concentration 100 µg/mL for both extracted polyphenols and ascorbic acid.

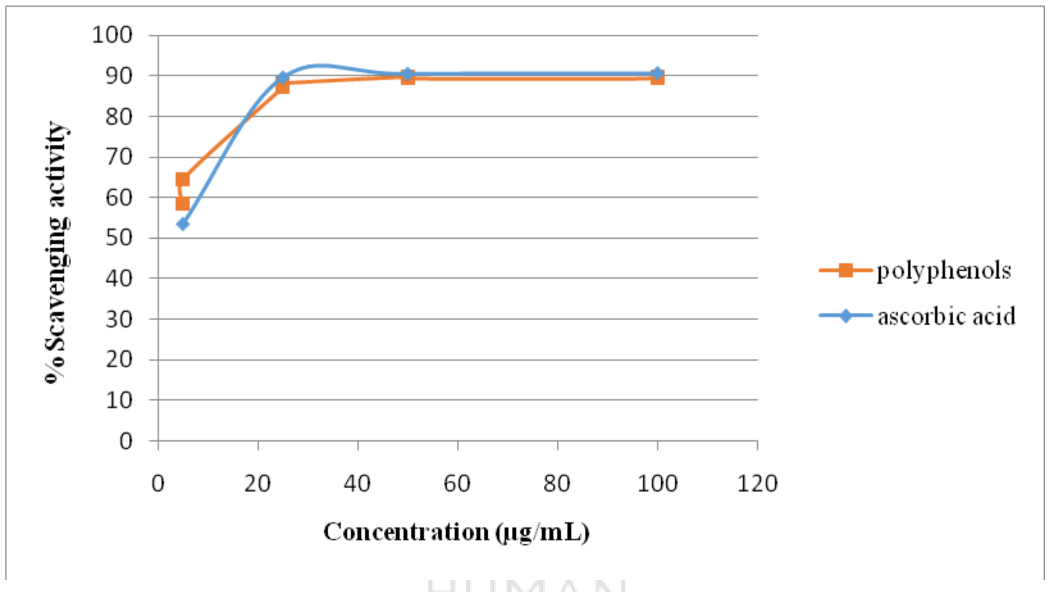

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Figure No. 1: % of scavenging activity of both polyphenols and ascorbic acid

The antioxidant activity of flavonoids is illustrated in Figure 2. The extracted flavonoids have exerted an antioxidant power reaching the 88 % at the concentration 100 µg/mL, a percentage closed to that showed by the ascorbic acid.

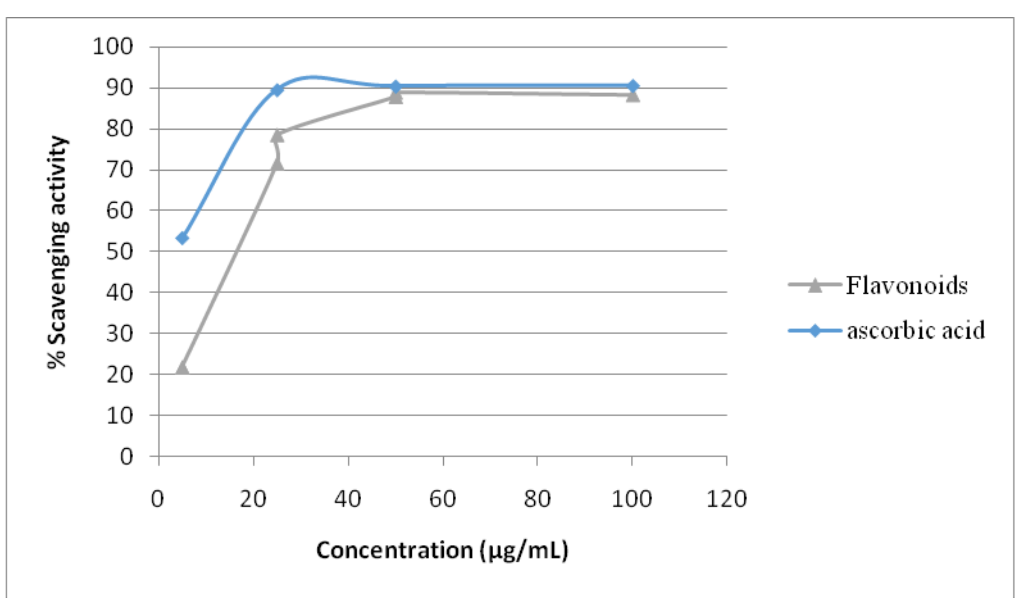

Figure No. 2: % of scavenging activity of both flavonoids and ascorbic acid

Oxidative stress is defined as an imbalance between the production and removal of free radicals that lead to potential oxidative damage (Poljsak and Milisav, 2012). Free radicals can be produced during aerobic metabolism and can trigger a series of oxidative damage to biomacromolecules, including lipids, proteins, and DNA. Also, they have been implicated in the pathogenesis of a wide variety of diseases (Davies, 1995). Factors, such as environmental pollution, drug intake, a high fat diet, moldy food, or oxidized oil intake, may cause oxidative stress. In addition to trying to avoid these factors, antioxidant introduction is generally believed to be an effective approach to prevent oxidative stress (Yu-qing *et al.,* 2017). Antioxidants are the compounds which terminate the attack of free radicals and thus reduce the risk of these disorders (Meena *et al.,* 2012).

The presence of antioxidant compounds such as polyphenols and flavonoids in pomegranate peels leads to the disappearance of radical chromogens, and the activity in doing is calculated from the disappearance of color or absorbance readings generated from a specific UV spectrum. Our findings suggest that multiple polyphenol and flavonoid compounds in pomegranate peels may exert very similar or even equal antioxidant activity than single purified ascorbic acid in scavenging free radicals *in vitro*.

Antiproliferative activity

In this study, the effect of extracted polyphenols and flavonoids was studied on two cell lines (HCT-116 and HT-29 cells) for three days, and the results were recorded and analyzed.

Polyphenols

The effect of different concentrations applied of extracted polyphenols varied against HCT-116 cells (Figure 3). After **24 hours** of the addition of polyphenols to these cells, none of the concentrations showed inhibitory effect except for 200 µg/mL and 25 µg/mL with 14.45 % and 16.45 % inhibition respectively.

After **48 hours** from the introduction of the different concentrations, there was an increase in the inhibitory activity for all concentrations except for 25 µg/mL that showed 0 % inhibition at this period. The percentage of inhibition increased from 27.47 % to 41.9 % as the concentration decreased from 200 µg/mL to 100 µg/mL respectively. However, it decreased sharply at 50 µg/mL to 4.09 % and then it increased again to be 19.39 % at 5 µg/mL.

After **72 hours** from the introduction of polyphenols to the cells, there was a notable inhibition activity above 29% at all concentrations. The highest effect was for 25 µg/mL with an inhibitory effect of 46.07% inhibition. Noticeable results were recorded for the 5 µg/mL concentration showing 40.42% inhibition. On the other hand, 50 µg/mL and 100 µg/mL concentrations showed close inhibitory effect with 37.32% and 35.64% respectively, whereas 200 µg/mL showed the lowest inhibitory effect with 29.35%.

After **24 hours** from the introduction of polyphenols to the HCT-116 cells, only the doses 25 µg/mL and 200 µg/mL showed inhibitory effect with 16.45% and 14.45% inhibition respectively. However, after **48 hours**, the % inhibition increased to 41.09% for a higher dose (100 µg/mL) and 27.47% for 200µg/mL. This result continued to increase to reach 46.07% after **72 hours** being affected by the dose 25 µg/mL.

It is clear that polyphenols have satisfactory results on HCT-116 cells. This effect is noticed after 48 hours from the beginning of the experiment with results of 41.09% for 100 µg/mL. However, the best results were recorded after 72 hours by the 25 µg/mL and 5 µg/mL doses with 46.07% and 40.42% respectively. This shows that polyphenols have the best effect on HCT-116 cells after 72 hours of being in contact. As for the appropriate concentration, we find that 5 µg/mL is the most suitable one since it shows one of the highest effects (40.42%) with respect its low concentration.

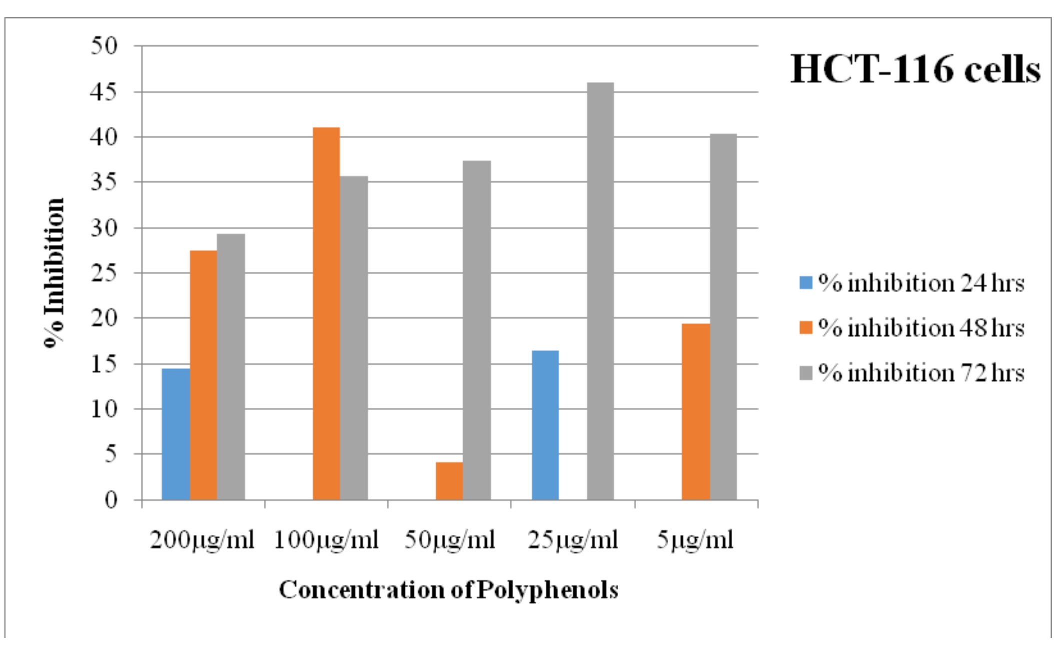

Figure No. 3: The inhibitory effect of different doses of polyphenols against HCT-116 cells

The effect of the different doses of polyphenols applied to HT-29 cells was different than that of HCT-116 cells (Figure 4).

After **24 hours**, the % inhibition increased from 10.09% to 19.03% as the dose decreased from 200µg/mL to 100µg/mL, and then it decreased slightly to 17.31% at 50µg/mL to be totally absent at lower doses (5µg/mL & 25µg/mL).

However, after **48 hours** from being in contact, the % inhibition took an opposite slope where it increased with the increase of doses from 7.51% at 5µg/mL to 67% at 200µg/mL.

After **72 hours**, there was no effect of any of the doses on inhibition but at 50µg/mL where it recorded 19.56% inhibition and at 5µg/mL where it recorded a surprising % that reached 81.31%.

The effect on the HT-29 cells was very low at day1 where it recorded % inhibition of 19.03% at 100µg/mL. This % increased in the 2nd day at a higher dose (200µg/mL) to reach 67.84%. However, the best results were recorded at the lowest dose (5µg/mL) which was 81.31% after 72 hours from applying the polyphenols.

These sums up that polyphenols have remarkable effect at HT-29 cells starting from day 2 proportional to the doses; however, abrupt results were recorded after 72 hours for all the doses. No or slight results were recorded for all the doses except for the 5µg/mL which showed a sharp inhibitory effect with 81.31% inhibition. This shows that the high doses have

an immediate but temporary effect on HT-29 cells. These results might be explained that these cells although they are affected by polyphenols in the beginning, but then they show resistance against these phytochemicals. Besides, it is shown that the most appropriate routine to inhibit the proliferation of these cells is by applying low dose (5µg/mL) where the result will not be immediate, however effective after 72 hours. The reason behind the high effect of such low dose could be related to the fact that the effect of low concentrations is revealed after a certain period of time.

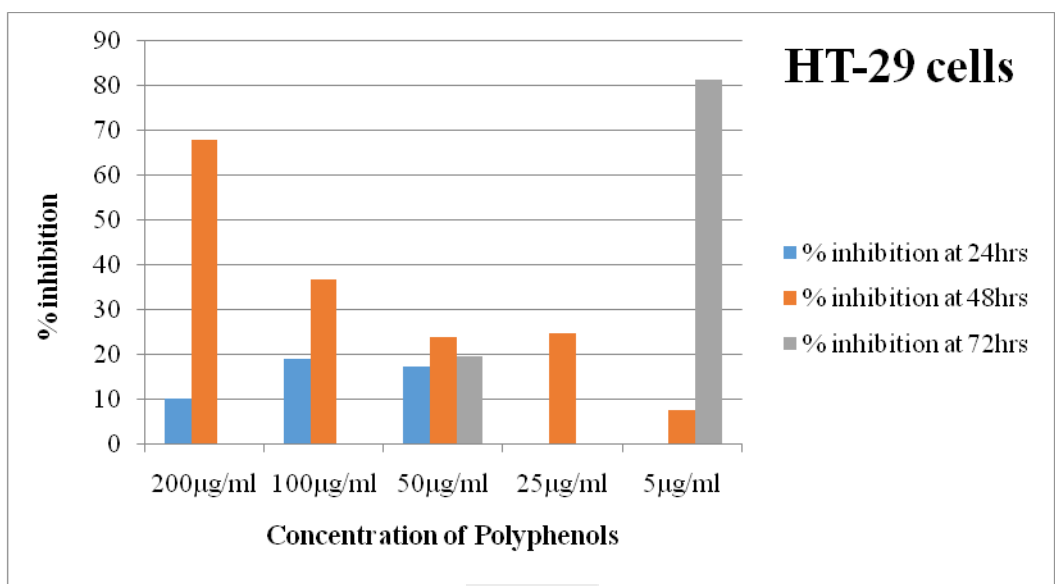

Figure No. 4: Inhibitory effect of different doses of polyphenols against HT-29 cells

Flavonoids:

The effect of flavonoids against HCT-116 cells was also studied (Figure 5). The results recorded after **24 hours** showed high inhibitory effect at the highest dose (200µg/mL) which was 33.97%. However, this % decreased enormously with the decrease of dose to be totally absent at 50µg/mL. However, a slight inhibitory effect was recorded for the doses 25µg/mL to 5µg/mL with 4.49% to 12.75% inhibition respectively.

After **48 hours**, the % inhibition was also the best at the highest dose (200µg/mL) which recorded 58.75%. Then this % was sharply decreased to be totally absent at doses 50 and 25µg/mL, and then it increased again at the lowest dose 5µg/mL to 5.19%.

After **72 hours**, the inhibitory effect started to appear at 50µg/mL by 7.4% inhibition and increased with the increased of the dose to reach 62.72% at 200µg/mL.

At the first day of introduction of flavonoids to the cells, the highest effect was recorded at the highest dose 200µg/mL achieving 33.97% inhibition, which is lower than that recorded in

the second day at the same dose that was 58.75% inhibition. However, this result continued to increase to reach at the third day 62.72% inhibition at the same dose.

Reasonable effects were found of flavonoids on HCT-116 cells. Negligible effects were recorded for low doses, while acceptable results were recorded for the 200µg/ml dose starting from day1, and the effect reached its maximum after 72 hours. This shows that flavonoids are effective only at high doses (200µg/mL).

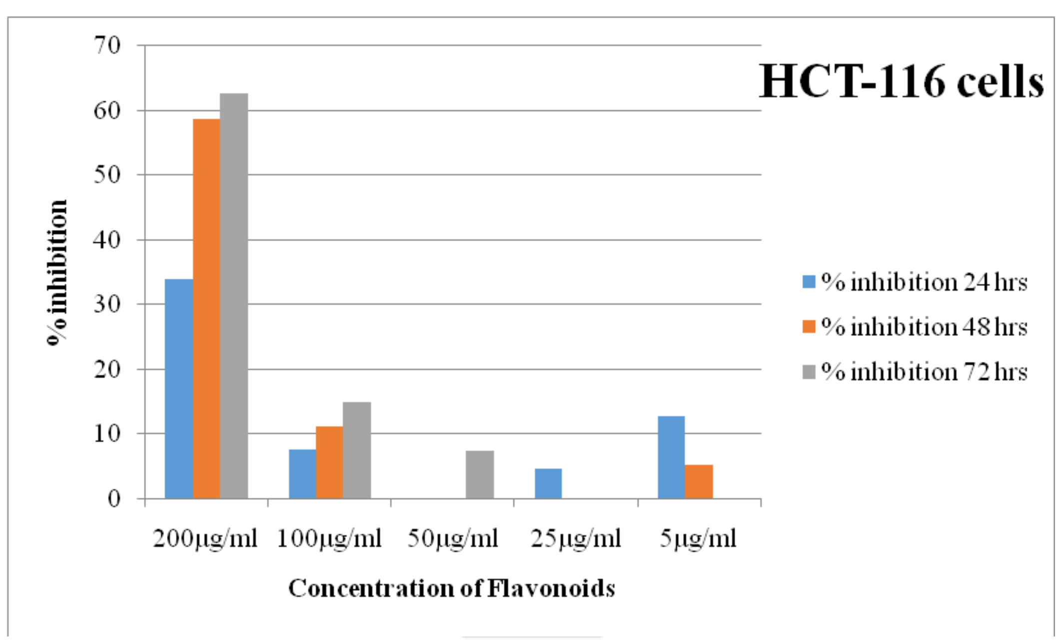

Figure No. 5: Inhibitory effect of different doses of flavonoids against HCT-116 cells

HT-29 cells were also affected by different doses of flavonoids (Figure 6). After **24 hours** of the introduction of flavonoids to the cells, the inhibition was only accomplished by the lowest dose 5µg/mL and recorded a notable inhibitory effect of 58.53% inhibition.

After **48 hours**, the effect was negligible at doses 25µg/mL and 100µg/mL. However, the % inhibition increased from 3.63% to 34.14% as the dose increased from 5µg/mL to 200µg/mL.

After **72 hours**, there was no inhibitory effect at the lowest dose. However, it was recorded that the effect increased with the increase of dose from 5.57% to 40.23% at doses 25µg/mL and 200µg/mL respectively.

The inhibitory effect of flavonoids on the HT-29 cells was the highest at the first day where the lowest dose 5µg/mL recorded 58.53% inhibition. This result decreased at the second day to reach 34.14% inhibition that was recorded by the highest dose 200µg/mL, which is in turn lower than that of day 3 that recorded 40.23% inhibition at 200µg/mL.

Regarding HT-29 cells, there were also reasonable effects for flavonoids. The % inhibition effect increased with the increase of doses having best results at 200µg/mL and starting from the second day. This shows that flavonoids have effect on these cells at high doses. On the other hand, confusing results were recorded for the lowest dose (5µg/mL) after 24 hours with a % inhibition of 58.53%. This high level of inhibition decreased sharply after 48 hours to become 3.63% and totally disappeared after 72 hours. The decrease in effect could be related to the fact that the effect of flavonoids diminishes with time at low doses. More experiments need to be performed in order to better explain these results.

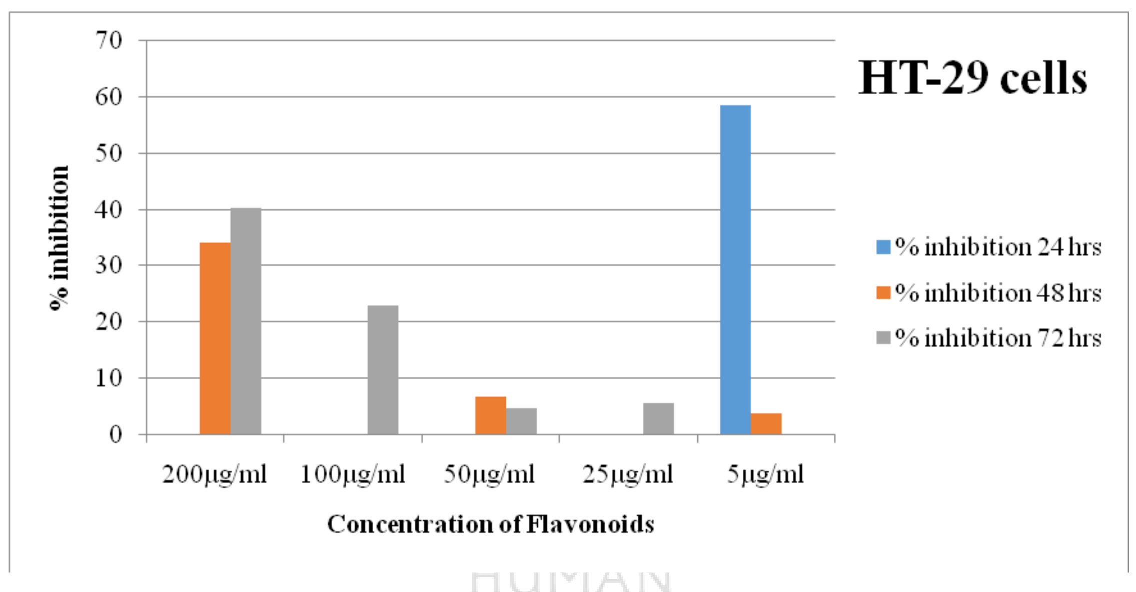

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Figure No. 6: The inhibitory effect of different doses of flavonoids against HT 29 cells

Antibacterial Activity

The development of drug resistance in human pathogens against commonly used antibiotics necessitated a search for new antimicrobials of mainly plant origin. The antibacterial screening of the two extracts of *P. granatum* peels was conducted against two bacterial strains, one Gram positive (*S. epidermidis*) and one Gram negative (*E. coli*). These microorganisms were chosen due to they are commonly associated with spoilage of refrigerated foods and considering a future use of the pomegranate peel flour as a possible food ingredient.

For polyphenol extract, the MIC for the two bacterial strains *S. epidermidis* and *E. coli* was 1.05 and 0.525mg/L respectively, whereas the MBC for the respective strains was 2.1 and 1.05mg/L.

For flavonoid extract, the MIC for the two bacterial strains *S. epidermidis* and *E. coli* was 1.575 and 0.7875mg/L respectively, whereas the MBC for the respective strains was 3.15 and 1.575mg/L.

The antibacterial activity of peels of *P. granatum* may be indicative of presence of metabolic toxins or broad spectrum antimicrobial compounds that act against both grams positive and gram negative bacteria. However, the polyphenol extract showed better bactericidal action against *S. epidermidis* than that of the flavonoids extract. On the other hand, the bactericidal action against *E. coli* of both extracts was similar with a slight difference (0.52mg/L).

Antibacterial effects of pomegranate peel flour may be attributed to the combined action of various bioactive compounds which can provoke the bacterial death following diverse action mechanisms. So in order to have better idea of these bioactive compounds, we have extracted crude polyphenols and flavonoids from the peels of pomegranates and had confirmed the hypothesis that attributes the antibacterial activity to polyphenolic compounds.

Therefore, several mechanisms, acting on specific targets simultaneously, have been proposed to explain the antimicrobial action of fruit extracts. The inhibitory mechanisms of polyphenolic compounds, including hydrolysable polyphenols the main components of pomegranate peel, are believed to be associated with precipitation of bacterial cell membrane proteins by the reaction of peel phenolics entails bacterial cell lysis (Akhtar *et al.,* 2015), the inhibition of microbial enzymes through reaction with sulfhydryl groups or by nonspecific interactions with the proteins (Cowan, 1999). Likewise, phenolic compounds may react with protein sulfydryl groups and make them unavailable for microbial growth thereby generating phenolic toxicity (Haslam, 1996). However, it was reported that the antibacterial activity of phenolic acids and flavonoids may be attributable to the cytoplasmic membrane damage caused by perforation and/or a reduction in membrane fluidity (Akhtar *et al.,* 2015).

CONCLUSION

In this study, we have confirmed that pomegranate peel powder has considerable levels of polyphenols and flavonoids and their chemical characteristics induce in general strong biological activities such as antioxidant activity that may be exploited for use against anti-oxidative stress for humans or animals; as well as bactericidal activity against *E. coli* and *S. epidermidis*, which are some of the most common sources of food-borne diseases. Also, an anticancer activity against two cell lines (HCT-116 and HT-29) that belong to colon cancer cells which opens the door for a wide field of studies for cancer treatment and prevention.

Due to the low cost of fruit co-products, which otherwise would be discharged as waste in the environment, they should be regarded as potential ingredients for food industry or nutraceutic resources, capable of offering significant low-cost as well as nutritional dietary supplements for low-income communities.

In a future study, the chemical profile of these extracted polyphenol and flavonoids will be determined using a HPLC/MS or another analytical technique.

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