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RESEARCH ARTICLE

In Vitro Experimental Evaluation of Antiviral and Anticancer Potentials of Both Orange Albedo and Grape Seed Extracts

Eman A. Ismail[1], Mohamed AF[1*] and Ramadan EM[2]

[1]Holding Company for Production of Vaccines, Sera and Drugs, Egypt
[2]Department of Microbiology, Faculty of Agriculture, Ain Shams University, Egypt

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Correspondence:
Dr. Mohamed AF,
Holding company for production of vaccines, Sera and Drugs, Egypt, Tel: +201222477069;
Email: fahmy.aly@gmail.com

ABSTRACT

Nowadays, the recovery of health benefit bioactive compounds from fruit wastes is a research trend not only to help minimize the waste burden, but also to meet the intensive demand from the public for phenolic compounds which are believed to have protective effects against chronic diseases. The present work aimed to evaluate the antiviral and anticancer potentials of reusable Orange Albedo Extract (OAlbE) and Grape Seed Extract (GSE) wastes. Results revealed that the IC_{50} values of OAlb M/E and GSE M/E extracts in MCF-7 cell line were 184439, 48108, 344, 11.68 µgm / ml and was 6016 µgm / ml for SGSE 24 hrs post treatment respectively, while the IC_{50} values of the extracts in Caco-2 cell line were 23708, 14107, 1158, 15607 µgm / ml and was 430 µgm / ml for SGSE 24 hrs post treatment, respectively. The SGSE was of a higher potential toxicity than experimental extracted one. Antiviral activity of E/M OAlbE and GSE showed the same antiviral potential; 0.51 log (10) / 0.1 ml (12.9%) respectively. While the antiviral potential against HAV revealed that OAlbE/M and GSME showed low potential against HAV recording 0.26 log (10) /0.1 ml. On the contrary both O-AlbE and GSME showed no antiviral potential compared with standard IFN (5 IU/ml) recorded a depletion of viral infectivity titers recording 3.25 log (10) / 0.1 ml (41.93%) and 3 log (10) /0.1 ml (40%) for RVFV and HAV respectively. Also, anticancer potentials was proved as there was a remarkable BCL-2 , P53 and Bax genes expression compared with control respectively. It can be concluded that both E/M OAlb and GSE are promising antiviral and anticancer agents. More intensified investigations must be conducted to maximize the biological potentials of both extracts. A higher level of characterization of extract contents must be achieved and evaluated for targeting the most promising fraction has both bioactivities considered.

Introduction

Fruits and vegetables wastes and by-products, which are formed in great amounts during industrial processing, represented a serious problem, as they exert an influence on environment and need to be managed and/or utilized. On the other hand, they are very rich in bioactive components, which are considered to have a beneficial effect on health. Using the agro wastes therapeutically are new ideas which are slowly gaining popularity. They are

high value products and their recovery will be economically attractive. These are novel, natural, eco friendly and economic sources of antimicrobics, which can be used in the prevention of diseases caused by pathogenic microbes and also reduce pollution [1]. Citrus fruit are consumed as fresh or utilized for processed citrus products and citrus-by-products. The peel of citrus fruit is a rich source of flavanones and many polymethoxylated flavones, which are very rare in other plants. In vitro, flavonoids display anti-proliferative effect on various human neoplasic cell lines as observed in myeloid and lymphoid leukemia cells [2] gastric, ovarian, prostrate cancer cells [3], and squamous cell carcinoma [4]. Another working group proved that the antioxidant and antimicrobial properties of methanol (100% and 80% aqueous) extracts pummelo fruit's Albedo [5], contain by-products responsible components were purified, and the isolated compounds were tested for antioxidant and antimicrobial potential [5]. Vitis vinifera (grape) is a rich source of several biologically active compounds including anthocyanins, proanthocyanidins, and stilbenes [6]. Grape Seed Extract (GSE), a mixture containing about 95% standardized proanthocyanidins, is a popular dietary supplement due to its anti-cancer and anti-inflammatory properties [7]. In vitro studies showed that GSE has significant growth inhibitory action on a variety of colon cancer cells in a dose- and time-dependent manner [8]. Studies have found that grape seed extract may enhance the growth of breast, stomach, colon, prostate and lung cancer cells in test tubes, however there is no clear evidence yet whether it works in human. Antioxidants such as those found in grape seed extract are thought to reduce the risk of developing cancer. Grape seed extract may also help prevent damage to human liver cells caused by chemotherapy medication [9-11]. The aim of the present study was to evaluate the antiviral and anticancer potentials of reusable orange and grape seed ethanolic and methanolic extracts.

Materials and Methods

1. Preparation of grape seed and orange albedo extracts

Grape seed (from Meloky grape) and orange albedo (from Common balady orange) 100 gm from each were washed with distilled water to remove unwanted materials. Grape seeds were grinded in a sterile mortar to the finest size as possible. Orange Albedo was dried in hot oven at 40□C for 48 hrs till dryness, followed by grinding as previous. Powder of both was divided into two separate groups for differential extraction using both ethanol and methanol according to [12]. The prepared powders were soaked in both methanol and ethanol for 7 days. Extracts were cold centrifuged and dried using rotary evaporator [13,14]. Dry extracts were retracted in both solvents for 3 days. Extracts were sterile filtrated using 0.22 µm Millipore disposable stericup filters

2. Cell cultures and treatments

Breast cancer (MCF7) and colon cancer (CaCo-2) cell lines were supplied from the VACSERA cell culture laboratory. Cells were grown in RPMI -1640 growth medium supplemented with 10% fetal bovine serum and 1% penicillin streptomycin solution (10,000 units of penicillin and 10 mg of streptomycin in 0.9% NaCl) in a humidified atmosphere of 5% CO_2, 95% air at 37□C. The cells were cultured in 75 cm2 cell culture flasks. For experimental purposes, cells were cultured in 96- well plates (0.1 ml of cell solution/well 2x 105 / ml). Cells were allowed to attach for 24 h before treatment with ethanolic and methanolic orange albedo and grape seed extracts. Growth medium was decanted 24 hrs post cell culturing .Cells were treated with 2 fold serially diluted extracts starting from 100 mg for orange Albedo and grape seed extracts and 10 mg for grape standard seed extract till 0.0017 mg / ml while 0.00015mg / ml for standard grape seed according to [15].

3. Cytotoxicity:

Cytotoxic effect of extracts was determined against CaCo-2 and MCF7 cell lines. Where test extracts were 2 fold serially diluted in RPMI-1640 medium supplemented with 2% Foetal Bovine Serum (FBS). Cell treatment was conducted for 24 hrs post removal of growth medium, [16].

4. MTT assay

The (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)MTT assay is based on the protocol described for the first time by [17]. With little modification the assay was optimized for the cell lines used in the experiments. Briefly, for the purposes of the experiments at the end of the incubation time, cells were incubated for 4 h with 0.5 mg/ml of MTT, dissolved in Phosphate Buffer Saline (PBS). Treated plates were washed with PBS (0.2 ml) twice followed by the addition of DMSO (0.05 ml), gentle shaking in dark for 10 min for complete dissolution of developed crystals. The resulting solutions were transferred in 96-well plates and absorbance was recorded at 570 nm using the micro plate spectrophotometer system (Biotek LX-800 device). Results were analyzed with the Masterplex - 2010 software. Viability % was blotted against concentration of test materials.

***5. Invitro* anticancer activity**

RNA was extracted from 24 hrs methanolic and ethanolic extracts treated breast and colon cancer cell lines. Untreated control cells were included. Test was performed according to manufacturer's protocol using SV Total RNA Isolation System (Promega-USA). Extracted RNA was reverse transcripted to cDNA using Revert Aid first strand cDNA synthesis Kit (Fermantas-Lithuania). The expression of pro-apoptotic genes (P53and Bax) and anti-apoptotic gene (Bcl-2) was carried out using the newly synthesized cDNA as template for PCR. Semi-quantitative RT-PCR was carried out in triplicates according to [18] followed by densitometric analysis of band intensities

6. Antiviral:

Antiviral potentials of both O-Alb. and GSE were monitored where precultured Vero cells were treated with OAlb and GS extracts for 24hrs. Treatment media was decanted and test virus was prepared according to Abed El gaied, et al., [19], where virus was 10 fold serially diluted. Viruses dilutions were dispensed to the treated and non-treated cells. Virus infectivity titer was evaluated according to Reed and Muench [20] and the antiviral activity was determined by subtracting the virus infectivity titer of non treated cells and virus infectivity titer of treated cells.

Results

1. Cytotoxicity

Evaluation of the cytotoxicity of test extracts to both MCF7 and CaCo-2 cells showed that both extracts (methanolic or ethanolic) were cytotoxic to cell lines. Data showed that standard grape extract was more toxic than the rest of test experimental extracts in a significant way ($P<0.05$) and the IC_{50} concentration of test extracts was listed in (Table 1).

Table 1: The IC_{50} concentration of test extracts with MCF7 and CaCo-2 cells.

Cell lines	GSEE	GSME	SGSE	OAlbEE	OAlbME
MCF7	11.68 µgm	344µgm	6016 µgm	48108 µgm	184439 µgm
CaCo-2	15607µgm	1158 µgm	430 µgm	14107 µgm	23708 µgm

GSEE: Grape Seed Ethanol Extract; GSME: Grape Seed Methanol Extract; SGSE: Standard Grape Seed Extract; OAlbEE: Orange Albedo Ethanol Extract; OAlbME: Orange Albedo Methanol Extract Also, it was noticed that GSE was significantly toxic than OAlbE in MCF7 and CaCo2 cells ($P<0.05$), and GSE was significantly toxic to MCF7 than CaCO2 ($P<0.05$) and OAlbE was toxic significantly to CaCo2 than MCF7 ($P<0.05$).

2. Antiviral activity

Regarding the antiviral activity (Figures 1,2) it was recorded that, OAlbME and OAlbEE extracts could reduce the viral infectivity titer in the order of, 0.51 log (10) / 0.1ml (6.58%), 1 log (10) / 0.l (12.9%) for RVFV, respectively. in the mean times GSME and GSEE could reduce the RFV infectivity titer in the order of 0.51 log (10) /0.1 ml (6.58%), 1 log (10) / 0.l (12.9%) for RVFV, concurrently SGSE showed the same antiviral potential of GSME compared with the antiviral activity of IFN as a positive control (3.25 log (10) / 0.1 ml) (41.93%) depletion rate in virus infectivity titer. In the same context using of HAV as a DNA virus model, data showed that methanolic albedo extract and grape seed ethanolic extract showed weak antiviral potential recording 0.26 log(10) /0.1 ml (3.46%) depletion rate, while albedo ethanolic and methanolic standard grape extract showed no effect on virus infectivity titer. Data recorded revealed that the extracts were potentially weak antiviral compared to the standard IFN that could reduced the infectivity titer of RVFV and HAV in the

order of 3 log (10) / 0.1 ml (40%) for HAV (Figures 1,2).

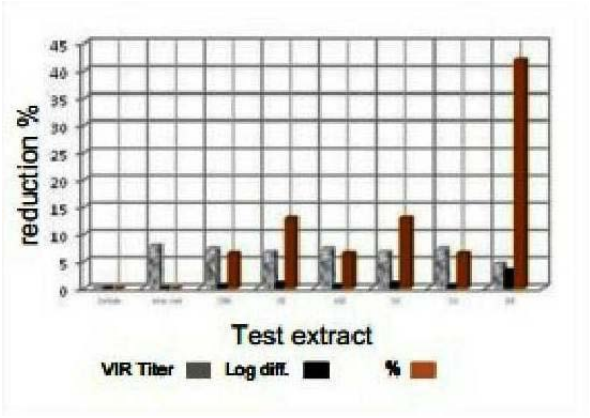

Figure 1: Evaluation of antiviral activity of Orange ALbedo and Grape seed extracts against Rift Vallkey Fever virus using Cell culture.

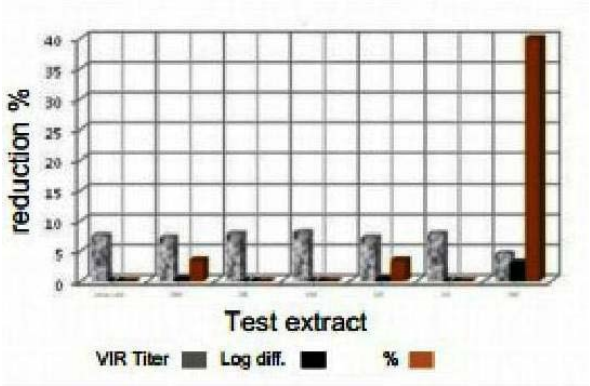

Figure 2: Evaluation of antiviral activity of Orange ALbedo and Grape seed extracts against Hepatitis A virus as a DNA virus Model using Cell culture.

3. Anticancer

Regard to in vitro anticancer potential, both ethanolic and methanolic extracts showed promising anticancer effect which detected via the up regulation of pre-apoptotic genes namely P53. Bax and anti apoptotic gene; Bcl-2 in a non significant way ($P>0.05$) compared with non treated cells (negative control) when extracts tested against breast and colon cancer cell lines (Table 2 & Figure 3).

Discussion

The present work aimed to evaluate the antiviral and anticancer potentials of reusable fruit wastes that used to produce huge amounts of fruits juices.

Regarding the antiviral activities of OAlb E, it was found that methanolic Albedo extract microbial activity against both RVFV and HAV showed a variable potentials, that attributed to phenolics and phlavinoids contents those contain aromatic ring to which the activity attributed .Also, present data are in agreement with Cowan, [21] and Emami et al., [22] who recording that most of the organic chemical constituents reported are known to possess aromatic phenolics compounds, which known for their wide spectra of antimicrobial activity as to be synthesized by plants in response to microbial infection. In addition, it was recorded that Albedo had the highest antioxidant activity reflecting its higher flavonoid and total phenolics content and results showed that Albedo was the main source of glycosylated flavanones and flavedo of 299 metoxylated flavones [23,24]. Vitamin C as OAlb E content proved to exert virucidal activity as was proved by Abed el gaied, et al., [19] and Abd el-Razek, et al., [25] recording that Vitamin C (Ascorbic acid could inactivate both Polio and Rift valley fever viruses when used as 1.5 and 3 mM within 24 hrs respectively compared with BPL and Formalin as a current inactivants. The viricidal activity was proved by Madhusudana, et al., [26] and Rawal, et al., [27], who mentioned that Rabies and HIV viruses could be completely inactivated with ascorbic acid. Regarding the antiviral activity, present data recoded considered the use of HAV and RVF as viral models was in agreement with Su, & D'Souza, [28], despite our use of safe concentrations for both OAlbE and GSE were effective to reduce the infectivity titers of HAV in a dose-dependent manner. Others recorded that GSE at 1 mg/ml after 2 h of incubation at 37□C decreased infectivity titer of viruses (~7 log10 PFU/ml) by 3.20 log10 PFU/ml for HAV. [29-33]. Also, Matias, et al., [34] evaluated the effect of extract obtained from winemaking by-products (composed of both grape skin and seeds) on adenovirus type 5 (Adeno-5) infection and found that the extract at a concentration of 0.8 mg/ml caused a 5-log10 reduction and also has strong antiviral activity against herpes simplex virus type 1 [35,36], polyomavirus Berardi, et al., [37], and varicella-zoster virus [38]. Therefore, we could compare our results with the antiviral effects of proanthocyanidins

(PAC) obtained from other sources. Cheng, et al., [39] studied the effect of proanthocyanidin A-1 from Vaccinium vitis-idaea (lingonberry) against herpes simplex virus type 2 (HSV-2) and showed that 63 μM PAC-A1 decreased HSV-2 titers by 1 log10 PFU/ml post 1 h of incubation at 37□C. The antiviral mechanism of GSE against food-borne viruses has not been established. Nair et al., [40] studied the antiviral mechanisms of GSE against HIV and showed that GSE significantly down regulated the expression of HIV entry co receptors and thus that GSE can interfere with the binding of the virus to the cell receptor and prevent HIV entry into the normal lymphocyte. Again, one must be aware that these are only speculations, and therefore, further studies on the mechanism of action of GSE are needed. Since grape seeds are waste or by-products from the wine and grape juice industry, they are readily available and inexpensive to acquire. In addition, grape seed extract has been proven to have considerable health benefits and antimicrobial activity. These combined associated health benefits and chemo preventive properties provide great advantages for the use of GSE in the food industry. Recently, applications of GSE in the food industry have been explored. [41-43]. The report of Crowell [44] is agreement with our reported data that OAblE showed a clear anticancer potentials as there was a significant up regulation and down regulation of both pro-apoptotic and anti apoptotic genes namely P53, Bax and Bcl-2 respectively in both cancer cell lines that may be attributed to its contents as vitamins, especially vitamin C, phytochemical compounds like liminoids, synephrine, hesperidin flavonoid, polyphenols, pectin, Quercetin and etc. It is clear that antioxidant content of which the flavonoids those are excellent radical-scavengers of the hydroxyl radical as reported by [45-50]. In the present study, the cytotoxic effect of red Grape Seeds Effect (GSE) was in alignment with reports of Hussien et al., [51] recording that GSE procured from XIAMEN against human colon cancer HCT-116 and normal epithelial WISH cell lines. It was clear that GSE enhanced the growth of the normal human epithelial cells WISH at low concentration and then it inhibited their proliferation at higher concentration with an IC_{50} at 2000μg/ml. However, it was estimated that GSE decreased the proliferation of HCT-116 cells in concentration-dependent manner with an IC_{50} at 80μg/ml. This indicates that GSE preferentially target cancer cells while sparing their normal counterparts. Also, recorded that the exact reason for this was not clear but could be due to differential metabolism of bioactive compounds in normal and cancer cells according to [52,53]. Also, our data presented was in agreement with Ye et al., [54], recording that grape seed proanthocyanidin extract exhibited cytotoxicity towards some cancer cells (MCF-7; breast cancer, A-427; lung cancer and gastric adenocarcinoma cells) in a concentration- and time-dependent manner. Regarding the anticancer potentials of grape seed our data concerned the pre - apoptotic; P53 and Bax genes in addition to the antiapoptotic gene namely Bcl-2, they were significantly expressed in treated cancer cell lines compared with the non-treated control cells, these data was in agreement with several reports those suggested the pro-apoptotic Bax protein to act as a tumor suppressor in human malignancies [55] playing a key role in mediating the apoptotic programme in response to genotoxic stress [56]. Also, Hussien, et al., [51] demonstrated a reduction in BCL-2 mRNA expression and increase in Bax and p53 mRNAs expression that had been treated with GSE. It was clear that the effect of GSE in down-regulation of Bcl-2 and up-regulation of Bax and P53 mRNAs expression appeared at 72-96 hrs post treatment at different doses (25, 50 and 100μg/ml). Also, recent articles have pointed out the importance of the Bax, Bcl-2 and ROS production in tumor cells [57]. Also, Hussien et al., [51] indicated that anticancer potency of GSE obtained from XIAMEN exhibits some differences from other previous reported studies (using different GSE cultivars), In addition, Dinicola et al., [58] reported that HCT-8 colon cancer cells are differently sensitive to the anticancer effects triggered by GSE than Caco2 colon cancer cells.

Table 2: Evaluation of Anticancer Activity Relative to Normalization % in breast (MCF-7) and colon (CaCO2) cancer cell lines treated with test grape seed and orange albedo methanolic and ethanolic extracts.

Cell lines	Gene / Normalization %	Extracts					Control
		M-OAlbE	E-OAlbE	M-GSE	E-GSE	SGS	
MCF-7	P53	198	238	268	248	278	158
	N %	1.25	1.5	1.7	1.6	1.75	
CACO-2	P53	233	254	289	268	275	189
	N %	1.23	1.34	1.52	1.41	1.45	
MCF-7	Bax	102	132	175	148	133	89
	N %	1.14	1.48	1.96	1.66	1.5	
CACO-2	Bax	99	122	168	154	176	78
	N %	1.26	1.56	2.15	1.97	2.2	
MCF-7	Bcl-2	83	7	88	72	69	153
	N %	0.54	0.31	0.37	0.42	0.45	
CACO-2	Bcl-2	99	72	82	115	92	168
	N %	0.6	0.43	0.48	2.68	0.54	

$$N\% = \left(\frac{\bar{x}\ OD\ of\ Exp\ Gene}{\bar{x}\ OD\ of\ cell\ cont}\right)$$

P53/ Bax (Pre apoptotic genes) and Bcl-2 (Anti Apoptotic gene)
Normalization % = Increase of Gene expression rate compared to its rate in cell control.

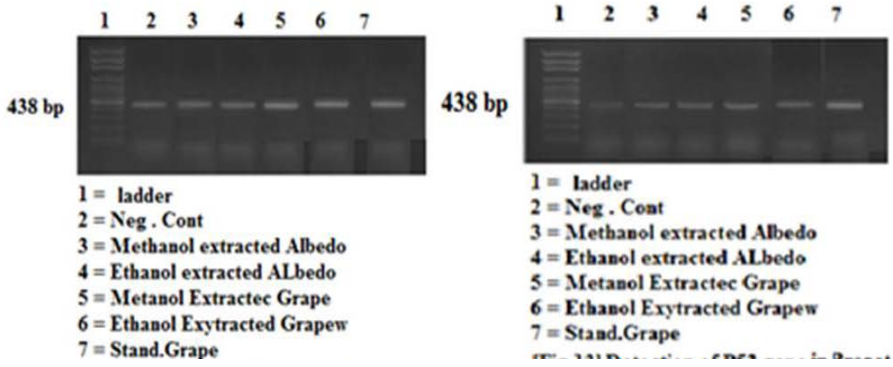

Figure 3(a): Detection of P53 gene in Colon cancer cell line (CaCo2) under the effect of Test Extracts using PCR.

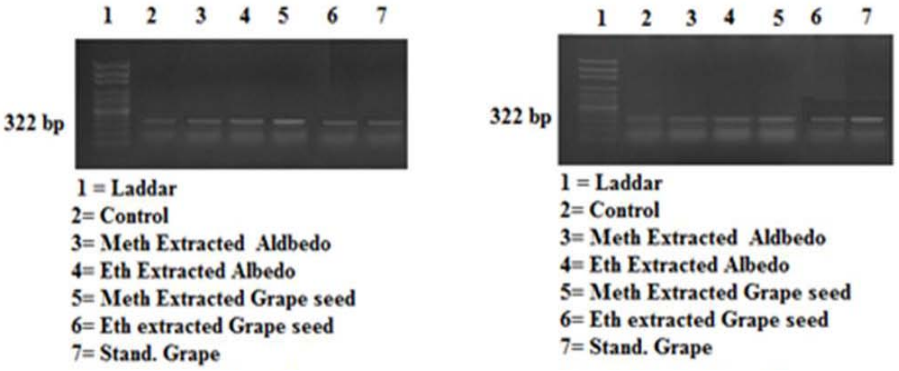

Figure 3(b): Evaluation of BAX gene expression rate in Breast cancer cell line (MCF7) using PCR under the effect of Test extracts.

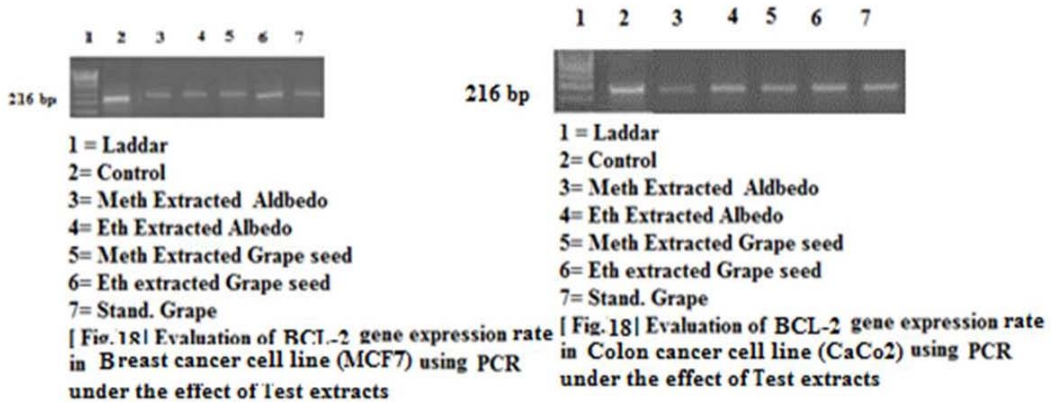

Figure 3(c): Evaluation of Bcl-2 gene expression potential under the effect of Test Extracts on Colon cancer cell line (CaCo2) cells using PCR.

Conclusion

Finally it can be concluded that both OAlbE and GSE are promising source of antiviral and anticancer derivatives can be prepared from fruits and vegetable wastes to be environmental friends.

Recommendations

More intensified investigation for the grape seed and orange Albedo extracts studied for detection more extractable materials subjected to evaluation. Also, chemical characterization of extracted materials for more detailed active groups to which the bioactivity is attributed finally more types of viral models and cancer origins to be tested for host range effect of our extracted materials.

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