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Antioxidant and Antitumor Activities of Methanolic Extracts Obtained from Red Delicious and Granny Smith Apples' Seeds

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ABSTRACT
The relationship between oxidative stress and many life-menacing diseases has been documented in the last two decades. This correlation has resulted in extensive investigations to find potential antioxidants for the protection and management of such diseases. In this work, the seeds of Red Delicious and Granny Smith apples were extracted by four solvents including water, methanol, chloroform, and n-hexane. Extraction was operated by the application of three techniques, which are kinetic maceration, ultrasound- and microwave-encouraging extraction methods. With each technique, three patterns were followed involving non-sequential, sequential declingly- and growingly-ordered in polarity. Nine methanolic extracts from each apple seeds' phenotype were evaluated for their in vitro biological properties. This assessment included the antioxidant activity tested versus hydroxyl and DPPH free radicals, and antitumor activity explored via MTT assay versus AMN3, HeLa, MCF-7, and SKG cancer cell lines. The results reported that the obtained methanolic extracts displayed a convenient biological activity versus the test free radicals and cancer cell lines, with notability contributed to those obtained from the seeds of Red Delicious apple. Also, the results documented that the desirable extraction pattern was the sequential declingly-ordered in polarity and the recommended technique was the ultrasound-encouraging extraction. This study inferred that there is a significant relationship between the antitumor activity of these methanolic extracts and their antioxidant activity. This may guide to explore novel antioxidant and/or antitumor agents from the seeds of the applied apple phenotypes.

Keywords: Red Delicious apple, Granny Smith apple, Seeds, Antioxidant, Antitumor.

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INTRODUCTION

In a human body, free radicals are normally generated from the metabolic processes and can act as cellular regulatory mediators as well as protective agents against the pathogens. Also, free radicals may assault the body from exogenous sources such as ultraviolet light, ionizing radiation, and contaminants (1).

Oxidative stress can be resulted from either the overproduction and exposure to free radicals or failure of the biological system to counteract their damaging effects. Currently, it is documented that these effects are correlated to the development of several life-menacing diseases such as cancer, diabetes, and cardiovascular disorders (2).

Plants are still considered as a treasure of natural products which identified by their diversity in the chemical structures and biological activities. Currently, there is an expanding trend to apply the natural products in the protection and management of many diseases especially those linked to oxidative stress (3). Vast number of phytochemicals has been isolated and examined for their antiradical activity and the results reported their effectiveness in buffering the harmful effects of damaging free radicals (4).

Red Delicious and Granny Smith apples are two cultivars which are highly produced and consumed worldwide. Also, they are characterized by their wealthy in the dietary fibers, vitamins, minerals and antioxidants. However, the research papers regarding the beneficial health effects of the apple were focused on the editable parts of this fruit and omitted its waste products particularly the seeds (5,6).

A considerable amount of apple seeds is annually released as a by-product of the worldwide consumption of apples (7). To deal with this waste product, recent studies targeted the identification of phytochemicals found in the apple seeds, isolation and characterization of their biological activities (8). The results indicated the presence of many valuable phytochemicals in these seeds such as flavonoids, coumarins, and essential oils. Also, the results reported the biological activities of these phytochemicals which are characterized by their diversity and efficiency (9).

Based on our literature review conducted in December 2019, the trials to find any informative data concerning the biological activities of the methanolic extracts of Granny Smith and Red Delicious apples' seeds have failed. Accordingly, the aim of the present study is to prepare methanolic extracts from the seeds of aforementioned apple phenotypes and to test their antiradical and antitumor activities. These extracts were prepared via three extraction techniques including kinetic maceration, ultrasound- and microwave-encouraging extraction methods. Concerning each one, the extraction was conducted in three patterns, which are non-sequential, sequential declingly- and growingly-ordered in polarity.

MATERIALS AND METHODS

Solvents, reagents, cancer cell lines and their cultures utilized in this study were acquired from Sigma-Aldrich, Bio-World, and Tokyo Chemical Industry. The two apple phenotypes were purchased from a local market and their botany identified by professionals from the College of Agriculture and Forestry/ University of Mosul, Mosul, Iraq. The instruments employed in the extraction process were shaker water bath (SWBR17 SHEL LAB shaking water bath, USA) for kinetic maceration, ultrasonic water bath (40 kHz, 350 W, Power sonic410, Korea) for ultrasound-encouraging extraction, and home microwave oven (Moulinex - MW

Steam 23L, MW531070, France) for microwave-encouraging extraction .

Preparation of apple seeds' extracts

Each lone apple of the purchased batches was scoured carefully by hand-rubbing with tap water and then with distilled water, and silted into four fractions by a sharp home blade. The secluded seeds were air-dried in dark place at room temperature for 15 days, pulverized by an electric grinder, and sieved to afford a fine powder. The powder was kept in air-tight bottles under cold and dark storage conditions until its extraction by the following solvents: water, methanol, chloroform and n-hexane (10).

The extraction was executed by utilizing three methods, which are kinetic maceration (KM), ultrasound-encouraging (UEE) and microwave-encouraging (MEE) extraction methods. In the first one, the extract mixture was kinetically soaked at 30°C for 72 hr by using a shaker water bath. In the second method, the extract mixture was sonicated by employing an ultrasonic water bath at 30°C for 30 min. In the last method, the extract mixture was beamed with microwaves at 100 W for 5 min utilizing a home microwave oven (11).

For each method, three patterns of extraction were applied including non-sequential, sequential decliningly- and growingly-ordered in polarity. In a non-sequential pattern, the dried seed powder (2 g) was extracted with methanol (20 ml); while in a sequential pattern, the extract mixture was prepared by mixing the powdered seeds (2 g) with 20 ml of the first solvent in the order. As the extraction process completed, the mixture was filtered and the residual solid was extracted with solvent next to the first one in the employed arrangement. These proceedings were also applied for the third and the fourth solvents in the same fashion (12).

Antiradical activity

The antiradical activity of the resultant 18 methanolic extracts was assessed by examining their capacity to neutralize the hydroxyl and DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radicals applying methanol and vitamin C as negative and positive standards respectively. The percentage of antiradical activity acquired by each extract was computed by the following mathematic formula: ARC (%) = $(A_{st} - A_m / A_{st}) \times 100$. Where ARC is the antiradical activity of the methanolic extract, A_{st} is the absorbance of positive standard and A_m is the absorbance of extract (13).

Antiradical activity versus DPPH free radicals

The selected methanolic extract (1.5 ml) was mixed with methanolic DPPH solution (0.5 ml, 0.1 mM). The tested mixture was plated with aluminum foil to preserve it from light, and then brooded at room temperature for 30 min. The capacity of methanolic extract to decolorize the DPPH violet color was followed spectroscopically at 517 nm versus a negative standard solution composed from DPPH (0.5 ml, 0.1 mM) and MeOH (1.5 ml). The positive control is a methanolic solution of vitamin C at the following concentrations: 25, 12.5, and 6.25 μM (13).

Antiradical activity versus hydroxyl free radicals

The tested solution was attained by the following order of additions: the selected methanolic extract (1.5 ml), potassium phosphate buffer (2.4 ml, 200 mM, pH 7.8), ferric chloride (60 μl, 1 mM), orthophenanthroline (90 μl, 1 mM), and hydrogen peroxide solution (150 μl, 170 mM). This mixture was brooded for 5 min at room temperature and the absorbance was followed spectroscopically at 560 nm versus a negative standard tested solution in which the extract was replaced by methanol. The positive control is a methanolic solution of vitamin C at the following concentrations: 25, 12.5, and 6.25 μM (13).

Antitumor activity

For each well of a 96-well plate, the specific cancer cells (10000 cells) were applied and independently treated after 24 hr with the selected methanolic extract. Cell viability test was carried out in the next 72 hr of treatment by evicting the medium, furnishing the MTT dye (27 μl, 3.28 mM), and subsequently brooded the treated cells for 1.5 hr at 37°C . The absorbances of the treated well (W_t) and untreated well (W_u) were detected via a microplate reader adapted at 492 nm. The % growth inhibition of three independent trials was calculated by applying the following formula: Growth inhibition (%) = $(W_u - W_t)/ W_u \times 100$. The positive standard solution was a methanolic solution of 5-Flurouracil (25, 12.5, 6.25 μM) while a negative standard was methanol (14).

RESULTS AND DISCUSSION

The feasible and practical safety of a vast number of natural products have encouraged their utilization via many antique and neoteric communities to take the advantages of their biological activities (15). The methanolic extracts acquired from various parts of many plants are widely accepted as a component of conventional home remedies (16–19). Since now, this is the original report regarding the antiradical and antitumor activities of methanolic extracts captured from the seeds of two apple phenotypes, Red Delicious and Granny Smith.

Antioxidant activity

Oxidative stress is regarded as a considerable risk factor which directly involved in pathogenesis of many age-contributed diseases such as atherosclerosis, osteoarthritis, diabetes, cancer, and cardiovascular disorders (20,21). Accordingly, the management of this risk factor becomes a global medical request and requires an extensive attempt to explore effective antiradical agents (22). Part of this attempt is directed toward those agents extracted from the plants. Since now, there are many crude methanolic extracts from many plants and their parts exhibited a significant in vitro antiradical activity (16,23,24). This can be attributed to the presence of specific phytochemicals with a potential activity (25).

The results documented in Table 1 and displayed in Figures 1, 2 indicated that the antiradical activities of crude methanolic extracts obtained from the UEE are superior to those acquired from the other applied extraction methods. This revealed that the physical, chemical and mechanical changes induced by the applied ultrasound energy resulted in a better dislocation of the phytochemicals into methanol

than those changes achieved by the other utilized extraction methods (26).

Also, the results indicated that the pattern of sequential decliningly-ordered in polarity was generally afforded the best outcomes than those of the other patterns. This may be attributed to the moderate polarity of the phytochemicals which may exhibit the antiradical activity. Commonly, most of the phytochemicals with this activity are belonged to phenolic and polyphenolic compounds which preferably dissolved in methanol and chloroform (27).

Additionally, the crude methanolic extracts obtained from the seeds of Red Delicious apple exhibited a better antiradical activity than those obtained from Granny Smith apple. This may indicate that the phytochemicals found in the extracts of the first phenotype are more potent as antiradicals than those found in the extracts of the second phenotype (9).

Table 1: Results acquired from testing the in vitro antiradical activity of the reference compound and resultant methanolic extracts.

Compound Name	ARC% ± SD (n=3) Versus DPPH free radicals	ARC% ± SD (n=3) Versus hydroxyl free radicals	Compound Name	ARC% ± SD (n=3) Versus DPPH free radicals	ARC% ± SD (n=3) Versus hydroxyl free radicals
R1	37.91 ± 1.20	36.83 ± 1.00	R1	37.91 ± 1.20	36.83 ± 1.00
R2	25.88 ± 1.00	22.18 ± 0.90	R2	25.88 ± 1.00	22.18 ± 0.90
R3	12.56 ± 1.35	5.84 ± 1.15	R3	12.56 ± 1.35	5.84 ± 1.15
GS1	8.57 ± 1.15	6.42 ± 1.00	RD1	11.12 ± 1.20	8.95 ± 1.05
GS2	10.58 ± 1.00	7.62 ± 1.20	RD2	11.94 ± 0.90	9.86 ± 1.05
GS3	10.47 ± 1.20	7.13 ± 1.05	RD3	11.23 ± 1.15	9.39 ± 0.95
GS4	14.09 ± 0.90	10.12 ± 1.15	RD4	15.37 ± 0.95	13.11 ± 1.05
GS5	16.22 ± 1.05	12.09 ± 0.95	RD5	18.43 ± 1.00	14.17 ± 0.75
GS6	14.37 ± 0.95	10.46 ± 1.12	RD6	17.02 ± 1.15	13.78 ± 1.00
GS7	12.10 ± 1.20	8.55 ± 1.10	RD7	12.65 ± 1.00	11.03 ± 0.90
GS8	12.58 ± 1.15	10.03 ± 1.25	RD8	14.68 ± 1.20	12.39 ± 1.05
GS9	12.10 ± 1.30	8.93 ± 0.95	RD9	13.14 ± 1.00	12.35 ± 1.15

R1: Vitamin C at the concentration of 25 µM, R2: Vitamin C at the concentration of 12.5 µM, R3: Vitamin C at the concentration of 6.25 µM, GS: Granny Smith apple, GS1: Methanolic extract obtained from KM set on non-sequential pattern, GS2: Methanolic extract obtained from UEE set on non-sequential pattern, GS3: Methanolic extract obtained from MEE set on non-sequential pattern, GS4: Methanolic extract obtained from KM set on sequential decliningly-ordered in polarity, GS5: Methanolic extract obtained from UEE set on sequential decliningly-ordered in polarity, GS6: Methanolic extract obtained from MEE set on sequential decliningly-ordered in polarity. GS7: Methanolic extract obtained from KM set on sequential growingly-ordered in polarity, GS8: Methanolic extract obtained from UEE set on sequential growingly-ordered in polarity, GS9: Methanolic extract obtained from MEE set on sequential growingly-ordered in polarity, RD: Red Delicious apple, SD: Standard deviation of three independent trials (n).

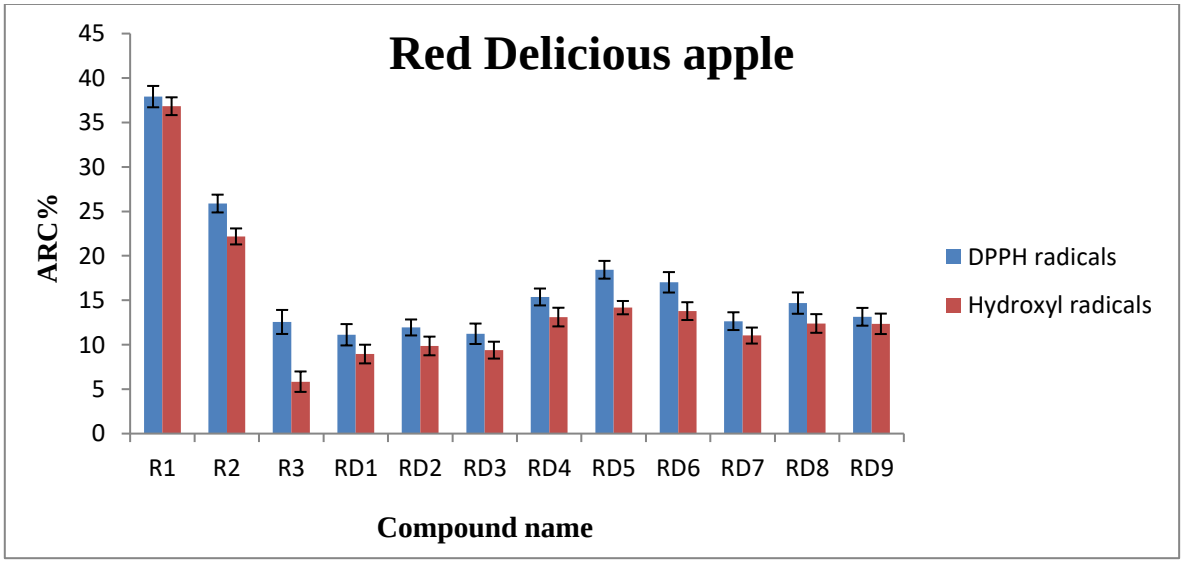

Figure 1: Diagram represented the results acquired from testing the antiradical activity of methanolic extracts obtained from Red Delicious apple' seeds.

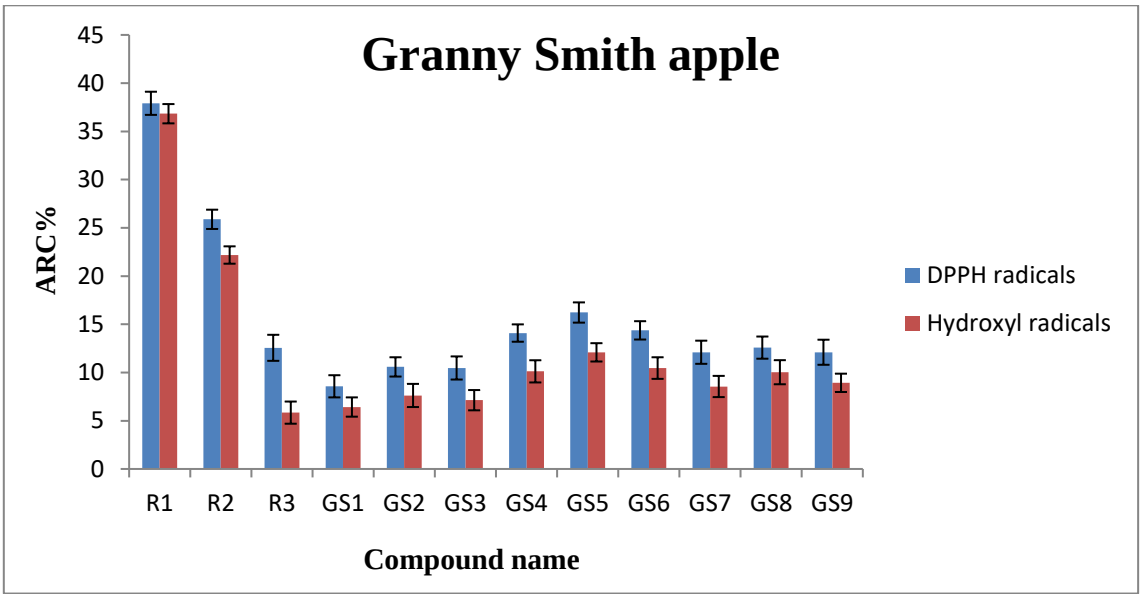

Figure 2: Diagram represented the results acquired from testing the antiradical activity of methanolic extracts obtained from Granny Smith apple' seeds.

Antitumor activity

The prepared 18 methanolic extracts were examined for their antitumor activity versus four cancer cell lines, which are AMN3 (murine mammary adenocarcinoma), HeLa (cervix), MCF-7 (breast), and SKG (esophageal). This examination was performed via MTT test using three concentrations of 5-Fluorouracil as positive controls, and methanol as a negative control.

The results documented in Tables 2, 3 and displayed in Figures 3, 4 indicated that the prepared methanolic extracts have a promising antitumor activity versus the test cancer cell lines. Also, the same findings afforded from studying the antiradical activity of the prepared extracts were reported in the testing of their antitumor activity . Additionally, the extracts obtained from Red Delicious apple' seeds showed a more potent antitumor activity versus the following test cancer cell lines: HeLa, MCF-7, and SKG than those obtained from Granny Smith apple' seeds. For AMN3 cancer cell line, the extracts from Granny Smith apple' seeds exhibited a better antitumor activity than those acquired from Red Delicious apple' seeds.

There is a good correlation between the antiradical activity and antitumor activity of each of the tested extracts. Generally, the results acquired from testing the antitumor and antiradical activities of the prepared extracts were paralleled to each other. This may lead to conclude that the possible antitumor mechanism of these extracts be contributed to their antioxidant activity (28–30).

Table 2: Results acquired from examining the in vitro antitumor activity of the reference compound and methanolic extracts obtained from Red Delicious apple' seeds.

Compound Name	Growth inhibition % ± SD (n=3)			
	AMN3	HeLa	MCF-7	SKG
R1	44.34 ± 0.80	54.71 ± 0.76	56.76 ± 1.20	42.45 ± 0.85
R2	36.10 ± 1.10	47.67 ± 0.90	50.17 ± 1.00	37.24 ± 0.90
R3	30.16 ± 1.00	41.06 ± 0.90	38.82 ± 1.15	31.81 ± 1.05
RD1	8.48 ± 0.90	7.21 ± 1.10	9.02 ± 1.15	8.19 ± 1.00
RD2	9.23 ± 1.10	7.44 ± 1.05	10.39 ± 0.95	10.42 ± 0.90
RD3	9.01 ± 1.05	7.20 ± 1.25	9.38 ± 0.90	9.49 ± 1.10
RD4	11.12 ± 1.25	9.23 ± 1.20	13.58 ± 1.10	11.49 ± 1.05
RD5	11.46 ± 1.05	9.29 ± 1.35	14.45 ± 1.25	12.56 ± 1.05
RD6	11.23 ± 1.15	9.31 ± 1.00	13.87 ± 1.10	12.05 ± 1.00
RD7	9.45 ± 0.95	8.11 ± 0.95	11.67 ± 1.10	10.42 ± 1.20
RD8	10.34 ± 0.90	8.68 ± 0.90	13.06 ± 1.15	11.21 ± 1.05
RD9	10.05 ± 1.00	8.46 ± 1.10	11.93 ± 1.25	11.18 ± 1.20

R1: 5-Fluorouracil at the concentration of 25 μM, R2: 5-Fluorouracil at the concentration of 12.5 μM, R3: 5-Fluorouracil at the concentration of 6.25 μM, SD: Standard deviation of three independent trials (n).

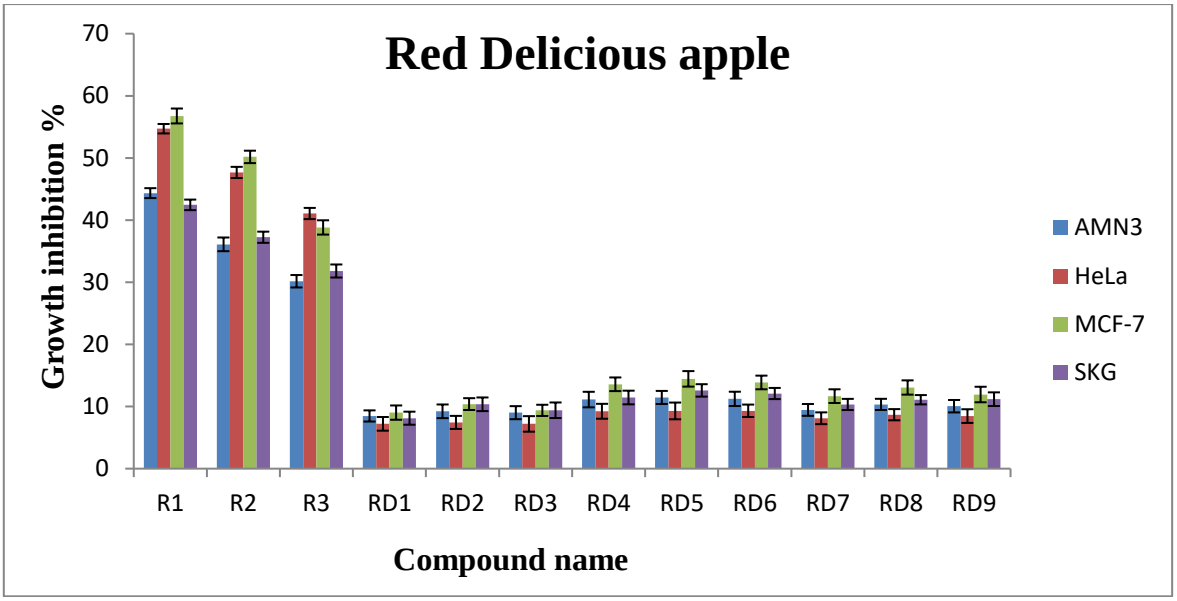

Figure 3: Diagram represented the results acquired from testing the antitumor activity of methanolic extracts obtained from Red Delicious apple' seeds.

Table 3: Results acquired from examining the in vitro antitumor activity of the reference compound and methanolic extracts obtained from Granny Smith apple' seeds.

Compound Name	Growth inhibition % ± SD (n=3)			
	AMN3	HeLa	MCF-7	SKG
R1	44.34 ± 0.80	54.71 ± 0.76	56.76 ± 1.20	42.45 ± 0.85
R2	36.10 ± 1.10	47.67 ± 0.90	50.17 ± 1.00	37.24 ± 0.90
R3	30.16 ± 1.00	41.06 ± 0.90	38.82 ± 1.15	31.81 ± 1.05
GS1	11.51 ± 0.95	7.13 ± 1.15	8.90 ± 1.00	8.12 ± 1.05
GS2	12.26 ± 1.00	7.36 ± 1.10	10.27 ± 1.00	10.36 ± 1.10
GS3	12.09 ± 1.15	7.12 ± 1.00	9.24 ± 1.05	9.40 ± 1.25
GS4	14.36 ± 1.05	9.14 ± 1.05	13.44 ± 1.15	11.45 ± 1.10
GS5	14.53 ± 1.00	9.20 ± 1.00	14.30 ± 1.00	12.59 ± 1.00
GS6	14.32 ± 1.05	9.23 ± 0.90	13.71 ± 1.25	12.09 ± 0.90
GS7	12.56 ± 1.15	8.00 ± 1.05	11.55 ± 1.05	10.33 ± 0.90
GS8	13.42 ± 0.95	8.57 ± 0.95	13.07 ± 1.00	11.09 ± 0.75
GS9	13.01 ± 1.15	8.39 ± 1.00	11.82 ± 0.95	11.10 ± 1.10

R1: 5-Fluorouracil at the concentration of 25 µM, R2: 5-Fluorouracil at the concentration of 12.5 µM, R3: 5-Fluorouracil at the concentration of 6.25 µM, SD: Standard deviation of three independent trials (n).

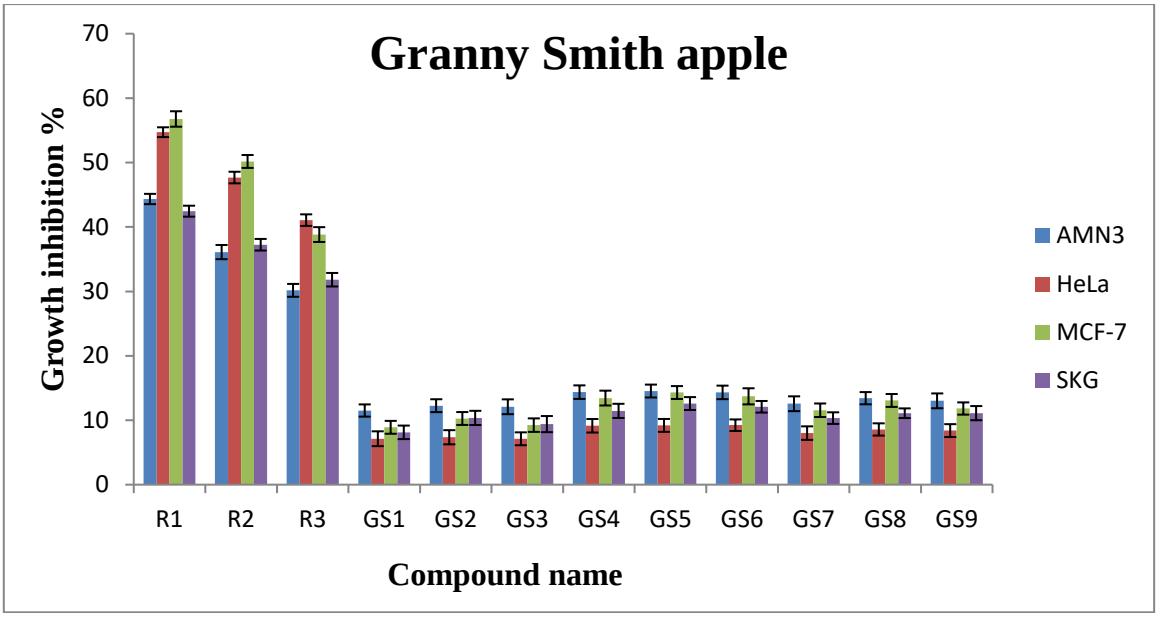

Figure 4: Diagram represented the results acquired from testing the antitumor activity of methanolic extracts obtained from Granny Smith apple' seeds.

CONCLUSION

This study demonstrated the success in the preparation of 18 methanolic extracts from the seeds of Red Delicious and Granny Smith apples' seeds. This preparation was established by utilizing three extraction methods, each one was operated in three patterns. The tested biological studies revealed the following conclusions: the prepared methanolic extracts were generally exhibited reasonable antiradical and antitumor activities versus the test radicals and cancer cell lines. The extracts from Red Delicious apple' seeds provided a better antiradical activity than those from Granny Smith apple' seeds. The extracts from Red Delicious apple' seeds exhibited a better antitumor activity versus the following cancer cell lines: HeLa, MCF-7, and SKG than those obtained from Granny Smith apple' seeds. For AMN3 cancer cell line, the extracts from Granny Smith apple' seeds exhibited a better antitumor activity than those obtained from Red Delicious apple' seeds. Finally, the best results were acquired from the utilization of UEE operated in the pattern of sequential decliningly-ordered in polarity.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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