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Original article

The antioxidant and anticancer activity of *Quercus coccifera* plant leaves extracts

Saif Aldeen Jaber

Faculty of Pharmacy, Middle East University, Amman, Jordan
Applied Science Research Center, Applied Science Private University, Amman, Jordan

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ABSTRACT

Quercus species are one of the medicinal plants that commonly used in the treatment of different diseases. *Quercus coccifera* (*Q. coccifera*) is part of the *Quercus* species which grow in Jordan and used in traditional folklore medicine. The aim of this study is to confirm the ability of (*Q. coccifera*) leaves extracts to exert anticancer activity.

In this study, an extraction method of the dried-leaves using different polarity solvents was used. Extracts were pre-evaluated for antioxidant and anticancer activities while active extracts were used to measure half maximal effective concentration (EC_{50}) against 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and Half-maximal inhibitory concentration (IC_{50}) against cancer cells.

Methanol, boiled and microwaved water extracts had greater than 80 % antioxidant activity, and the strongest activity, of more than 99 %, was boiled water extract. Similarly, the pre-evaluation treatments of cancer cell lines indicated a strong biological activity of more than 70 % from the previously mentioned extracts, and the highest activity, of greater than 90 %, was from boiled water extracts against all cancer cell lines. The highest EC_{50} against DPPH was obtained by using 0.009 mg/ml boiled water extracts, which was lower than positive control quercetin. In the same manner, lung, breast, and prostate cancer cell lines were highly affected by boiled water extracts with IC_{50} of 14.1, 7.2, and 25.1 μg/ml, respectively, and a selectivity index (SI) of greater than 4.71.

Q. coccifera leaves extracts show promising ability to be a source of a new anticancer therapeutics.

1. Introduction

Quercus species (*Fagaceae*) are group of plants refers to Oak trees with the ability to grow in high temperate and tropical places (Jaber, 2023b). Four hundred and fifty species are listed in this genus within the flowering and fruiting dynamics (Tejerina et al., 2011). *Quercus coccifera* (*Q. coccifera*) is one of the major species growing in Jordan, and its extracts are widely used by Jordanian population in traditional medicine (Genç et al., 2012). It was found that the decoction of the plant parts produces different biological activity such as reducing hemorrhage, treating diarrhea, healing wounds and burns, and recently bactericidal activity (Söhretoglu et al., 2014, Anlas et al., 2019, Jaber, 2023a, Jaber, 2023b).

Cancer is one of the widely spread diseases, and one of the leading causes of death which is continually increasing (Deo et al., 2022, Ming et al., 2022). The statistical analysis and mortality rate indicated that one out of six, and one out of five of women and men, respectively were found to have deathly tumors in their life (Anand et al., 2008, Dutta et al., 2021). The reason of the high deaths rate of cancer patients are either the lack of the drug selectivity which target tumors rather than normal tissues, or the drug induced resistance (Hamilton and Mack, 2003, Timin et al., 2022). Natural products, especially the extracts or compounds which have antioxidant activity, were found to be a successful alternative in decreasing the tumor cell mass, or preventing tumors from being formed at early stages (Elbouzidi et al., 2022). Many phytochemical classes of compounds were found to exert this antioxidant activity including flavonoids, polyphenols, alkaloids, saponins, terpenes and other compounds. *Quercus coccifera* was found to have a high concentration of the mentioned classes which have an antioxidant activity (Tanase et al., 2022, Jaber, 2023b). In this research, different extracts of *Quercus coccifera* plant leaves extracts were used to evaluate the antioxidant activity of their chemical profile using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay. DPPH assay is an assay used as for the pre-evaluation of the possible antioxidant activity which is used as an indicator for the anticancer activity In addition, the active antioxidant extracts were tested, using *in vitro* techniques, to evaluate their activity

E-mail address: Sjaber@meu.edu.jo.

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against breast, lung, and prostate cancer cell lines. The extracts were also added to a normal fibroblast to evaluate their toxicity to human normal cells. Finally, active extracts were applied to caspases-3 and 5 enzymes that initiate cell apoptosis were tested as another suggested mechanism of action against cancer cell lines.

2. Materials and methods

2.1. Materials

Plant leaves extracts were collected from Amman, Jordan dried and stored in suitable bags, and identified as mentioned in a previously published research (Jaber, 2023b). All reagents and chemical solvents including DPPH, n-hexane, chloroform, methanol were obtained from Sigma-Aldrich, USA. Purified water was obtained through using Barnstead Water Purification System/Type 1, obtained from Thermo Fisher Scientific, UK. Different cancer cell lines like breast cancer cells (BT-20), lung cancer cell lines (NCI-H2126), and prostate cancer cell lines (DU-145) were obtained from Cell Lines Service (CLC), Berlin, Germany. In addition, Dulbecco's Modified Eagle Medium (DMEM) selected for the growth of the cells was purchased from CLC, Berlin, Germany. Finally, normal human cell lines (A2780) were purchased from Sigma-Aldrich, USA. While AlamarBlue® solution was obtained from Thermo Fisher, USA. While Varioskan LUX Multimode Microplate Reader was used for absorbance reading obtained from Thermo Fisher Scientific, USA. Sinoped freeze drying machine, were obtained from China. ELISA reader used for Caspase enzymes activity ELX800 was obtained from Promega, US.

2.2. Methods

2.2.1. Botanical identification

The botanical identification of the plant leaves was done by Professor Jameel Allaham, Faculty of science, Al Yarmouk University, Irbid, Jordan.

2.2.2. Plant extracts preparations

For each extract, 300 g were soaked with the selected solvents (n-hexane, chloroform, methanol, boiled, and microwaved water). Soaking took a place under a fume hood, for 24 h. n-hexane, chloroform, and methanolic containing metabolites were dried using a rotary evaporator. Whereas, water extracts were dried through lyophilization using a Freeze-Drying machine. All produced extracts were stored in fridge at temperature between 2 and 8 °C to avoid the degradation of any heat-sensitive compounds.

2.2.3. Phytochemical screening

Different phytochemical screening tests were done to the extracts using Mayer's, Dragendorff's tests for alkaloid detection as per (Kognou et al., 2016). While other tests were used for the detection of tannins, glycosides, and terpenoids as per (Uddin and Rauf, 2012, Elezabeth and Subramanian, 2013).

2.2.4. DPPH inhibitory activity assay

DPPH inhibitory activity for all extracts (n-hexane, chloroform, methanol, boiled and microwaved water) was performed using 10 mg/ml in dimethyl sulfoxide (DMSO) with each extract. 100 µl of the prepared extracts solutions were transferred to 96-well plates with the following concentrations (1, 0.5, 0.25, 0.125, 0.05, and 0.03 mg/ml), followed by the addition of 50 µl of 0.6 mM DPPH ethanol solution. An equal concentration of DMSO was prepared as a negative control. The plates were left to react for 30 min, and absorbance was measured at wavelength of 518 nm. The blank solution was prepared by mixing of 50 µl ethanol with 100 µl of 10 mg/ml of each extract as all extracts are colored and can affect absorbance values. While 150 µl of 0.6 mM of DPPH solution were used as a negative control. Antioxidant activity (AA %) of plant leaves extracts were measured according to the equation below. EC_{50} of each extract was calculated using linear regression by Prism 8 program. Through the experiment the concentration of DMSO didn't exceed 5 %. All samples were prepared in a triplicate.

$$AA\% = 100 - \{[(Abs(Sample) - Abs(Blank))*100]/Abs(Control)]\}$$

2.2.5. Anticancer biological activity assay

Normal fibroblast derived from human cells (A2780), lung cancer cells NCI-H2126, breast cancer cells BT-20, and prostate cancer cells DU-145 were cultivated in DMEM which contains (10 %v/v fetal bovine serum, 2 mM glutamine, and 50 µg/ml penicillin/streptomycin solution), and incubated in a humidified incubator at normal cells temperature (37 °C) with 5 % CO_2. Human normal fibroblast was added to 96-well plates with a concentration of 7500cell/well, while the three types of cancer cell lines were added to the 96-well plates with a concentration of 3500cell/well, and incubated overnight. Stock solutions of the plant extracts were prepared as 10 mg/ml concentration, with serial dilutions by concentration ranges between 1 mg/ml and 0.0025 mg/ml. Each extract concentration used for the first evaluation of the anticancer activity were 30 µg/ml. These extracts concentrations were added to the plates containing cells (normal and cancer cell lines) followed by incubation for 42 h. Cells viability were measured by adding AlamarBlue® followed by an incubation for 6 h. The absorbance of the 96-well plates were measured using florescence mode, with excitation and emission wavelengths of 560 and 590 nm, respectively. For IC_{50}, a serial dilution of each extract was prepared with the following concentrations (1, 0.5, 0.25, 0.125, 0.0625, 0.0315, and 0.025 mg/ml) using 2.5 %v/v DMSO, following the same protocol mentioned above. Selectivity Index (SI) was calculated by dividing the IC50 of active extracts treated with normal human fibroblast over the IC50 results on cancer cell lines. Through the assay the concentration of DMSO didn't exceed 5 %. All samples were prepared in a triplicate.

2.2.6. Caspase-3 and −9 activity

Caspase enzymes (3 and 9) were determined by using Radioimmunoprecipitation Assay (RIPA) reagent from MDA-MB-231 cells after being treated for 48 h with 0.025, 0.0315, 0.0625, 0.125, 0.5, and 1 mg/ml of active *Q. coccifera* extracts. KeyGen Biotechnology, Nanjing, China kit was used for testing the activity, while ELISA reader at a wavelength of 405 nm was used for absorbance measurements. All samples were prepared in a triplicate.

2.3. Statistical analysis

All graphs, mean, SD, and statistical analysis were created and measured using Graph Pad Prism 8®.

3. Results

3.1. Plant extract preparation

An enough yield of each extract was produced to be used in the biological activity testing, with the highest yield of 26.1 g from methanolic extracts, see Table 1.

Table 1
Yields (g) and % Yield of the produced extracts.

Plant Extract	Yield (% Yield)	Plant Extract	Yield (% Yield)
n-hexane	2.7 (0.9)	Chloroform	3.8 (1.3)
Methanol	26.1 (8.7)	Boiled water	22.6 (7.5)
Microwaved water	14.8 (4.9)		

Table 2
Phytochemical screening of plant leaves crude extracts.

Extract type	Type of compounds			
	Alkaloid	Tannins	Glycosides	Terpenoid
n-hexane	–	–	–	–
Chloroform	+	+	–	+ +
Methanol	+ + +	+ + +	++	+ +
Boiled water	+ +	+ +	++	+
Microwaved water	+	–	+	–

+: indicates the presence of compound after 10–15 min, + +: indicates the presence of compounds after 5–10 min, + + +: the presence of the compounds before 5 min, and -: the absence of the compounds after 1 h.

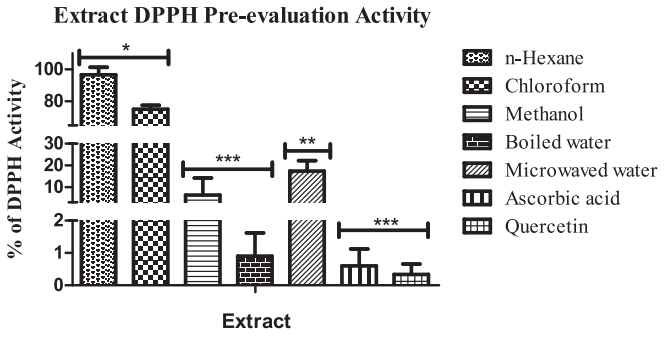

Fig. 1. DPPH pre-evaluation activity of *Q. coccifera* plant leaves extracts. Legend: *: Inactive samples, **: Moderately active samples, and ***: Highly active sample.

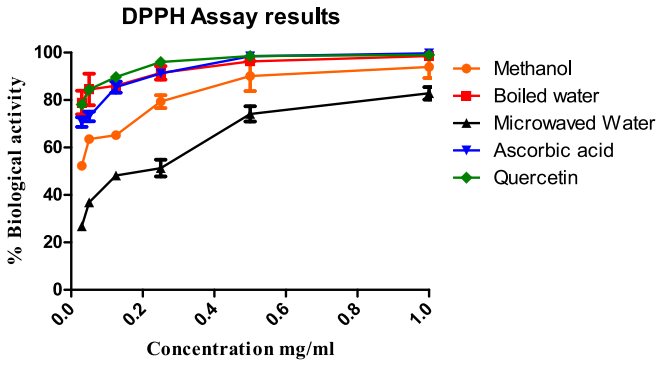

Fig. 2. DPPH inhibitory activity for selected active extracts.

3.2. Phytochemical screening

According to different phytochemical testings for the identification for the presence of alkaloids, tannins, glycosides, and terpenoids, methanol and boiled water extracts were found to exert all tested compound while n-hexan extract was found to exert negative results. On the other hand, microwaved water were found to exert a positive results with alkaloids and glycosides tests while chloroform exert a positive results with every test except with glycosides test. Phytochemical screening results are presented in Table 2.

3.3. DPPH inhibitory activity assay

According to the pre-evaluation of the DPPH inhibitory activity of the produced extracts, presented in Fig. 1, n-hexane and chloroform extracts were not able to inhibit DPPH with an activity of less than 25 %. On the other hand, the rest of the extracts (methanol, boiled and microwaved water) were with an activity greater than 80 %. Among all the extracts, boiled water extract was found to exert a similar biological activity to positive controls (ascorbic acid, and quercetin) with an activity greater than 99 %. While the lowest biological activity was produced by n-hexane extract with an activity less than 5 %.

Table 3
Quercus coccifera plant leaves active extracts EC_{50} for DPPH inhibitory activity.

Extract	EC_{50} (mg/ml)	Extract	EC_{50} (mg/ml)
Methanol	0.027	Boiled water	0.009
Microwaved water	0.170	Ascorbic acid	0.011
Quercetin	0.010		

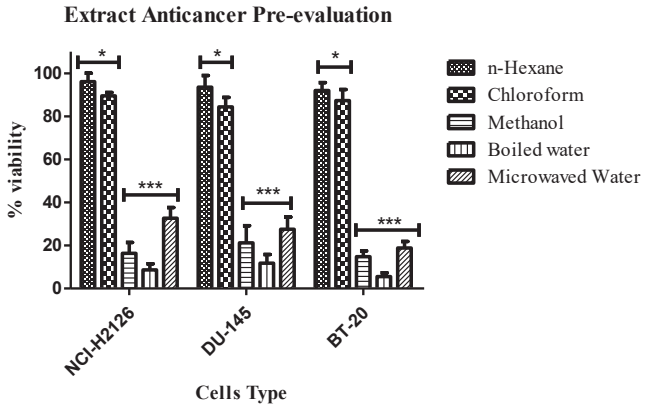

Fig. 3. The pre-evaluation activity of *Q. coccifera* plant leaves extracts. NCI-H2126: lung cancer, BT-20: breast cancer, DU-145: prostate cancer. Legend: *: Inactive samples, ***: Highly active sample.

According to the pre-evaluation of the extracts, methanolic extract and both boiled and microwaved extracts were selected for the measurements of EC_{50} against DPPH activity. Again, boiled water extract was found to exert a comparable EC_{50} as the positive controls (quercetin, and ascorbic acid) with a concentration of 0.009 mg/ml. Methanolic extract was found to exert a very promising activity with EC_{50} of 0.027 mg/ml. The lowest EC_{50} between the selected extracts, for further DPPH inhibitory activity, was microwaved water extract with a concentration of 0.17 mg/ml. DPPH inhibitory activity and EC_{50} results are presented in Fig. 2 and Table 3, respectively using GraphPad Prism 8.

3.4. Anticancer biological activity

All extracts were subjected to pre-evaluation for their possible anticancer activity as presented in Fig. 3. Methanolic, and both boiled and microwaved water extracts were found to exert a promising anticancer activity against breast, lung and prostate cancer cell lines. A highest pre-evaluation anticancer activity was found to be exerted after cells treatment with boiled water extract with a concentration of 1 mg/ml. The cells viability for cells treated with boiled water extracts were found to be less than 20 % against all cells. On the other hand, n-hexane, and chloroform extracts failed to be active against cancer cells with a cell viability greater than 85 %.

For $IC_{50,}$ both water and methanolic extracts were selected for the measurements of IC_{50}, due to their biological activity in the pre-evaluation anticancer testing. While toxicity assay was performed for same extracts in the presence of normal cells fibroblast. All three extracts were found to be safe with no significant toxicity on the normal cells fibroblast. Similar to the pre-evaluation anticancer activity, boiled water extract was found to have the lowest IC_{50} of 14.1, 7.2, and 25.1 μg/ml against lung, breast and prostate cancers cell line, respectively, followed by methanolic extracts. Whereas the lowest anticancer activity with IC_{50} of 117.8, 39.6, and 57.3 μg/ml was found in cells treated with microwaved water extract. All three extracts were found to exert the strongest activity against breast cancer cells. While the lowest activity for both

Table 4
IC_{50} of active extracts obtained from *Quercus coccifera* against cancer cell lines.

Active extract	NCI-H2126 IC_{50} (µg/ml)	BT-20 IC_{50} (µg/ml)	BU-145 IC_{50} (µg/ml)	Cell toxicity	SI
Methanolic	53.2	27.4	72.5	No toxicity	> 4.25*
Boiled water	14.1	7.2	25.1	No toxicity	> 4.71*
Microwaved water	117.8	39.6	57.3	No toxicity	> 4.01*

SI: Selectivity Index, *: Safe.
NCI-H2126: lung cancer, BT-20: breast cancer, DU-145: prostate cancer.

boiled water and methanolic extracts were found to be against prostate cancer cells and the lowest activity for microwaved water against lung cancer cell lines. All IC_{50} and anticancer activity are presented in Table 4 and Fig. 4.

3.5. Caspase-3 and −9 activity

According to Fig. 5, and Table 5, all active extracts were found to exert an agonistic effect on Caspase-3, and −9. The highest agonistic activity for both enzymes was found after being treated with boiled water extract with an EC_{50} lower than 25 µg/ml. According to the results both water extracts have activity on both Caspase-3, and −9, while methanolic extract was found to have a poor agonistic activity against Caspase-9 even at a high concentration (1 mg/ml).

Table 5
EC50 for caspases enzymes treated with active extracts.

	EC50 (µg/ml)	
Extracts	**Caspase-3**	**Caspase-9**
Methanol	127.1	<1000
Boiled water	19.1	21.2
Microwaved water	99.3	139.5

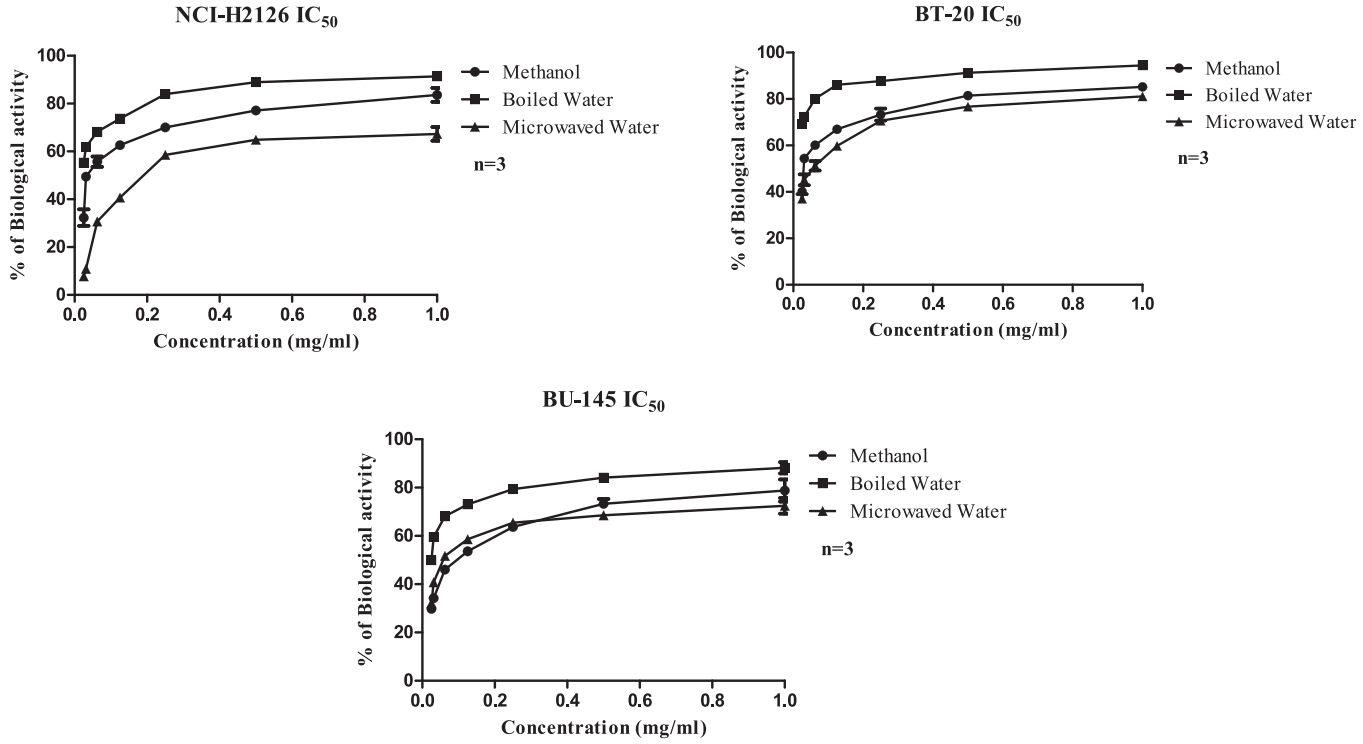

Fig. 4. Anticancer activity of methanolic, boiled water and microwaved water extracts against lung, breast, and prostate cancer cell lines. NCI-H2126: lung cancer, BT-20: breast cancer, DU-145: prostate cancer.

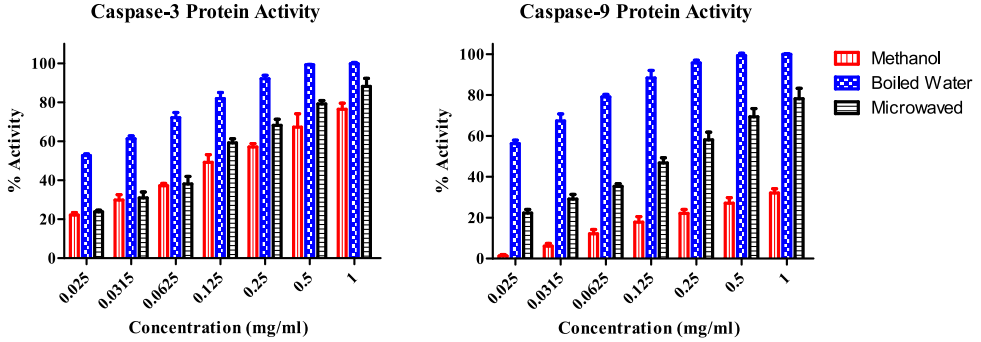

Fig. 5. Caspase-3 and –9 enzyme activity after treating with different concentrations of *Q. coccifera* active extracts.

4. Discussion

The current investigation was performed to evaluate the antioxidant and anticancer activity of *Q. coccifera* plant leaves extracts using different *in vitro* assays namely: DPPH and anti-cancer assays. The reason of performing such assays is the presence of highly diverse chemical classes such as alkaloids, terpenoids, tannins, glycosides, and flavonoids (Fersi et al., 2023, Jaber, 2023b). All the mentioned chemical classes are with antioxidant potential activity, and were proven before by different research groups (Luo et al., 2022b, Plazas et al., 2022, Shen et al., 2022, Zhang et al., 2023). In addition, other *Quercus* species were found to exert an antioxidant and an anticancer activity like *Quercus cerris*, and *Quercus rubra* (Nisca et al., 2022, Tanase et al., 2022).

Upon performing a pre-evaluation antioxidant activity for the extracts in the presence of ascorbic acid and quercetin, boiled water and methanolic extracts showed a comparable and a close antioxidant activity, respectively against DPPH. While microwaved water extract showed a promising antioxidant activity against DPPH. Quercetin and ascorbic acid are considered to have a potent antioxidant activity, and are used by different antioxidant assays protocols as positive controls (Peng and Shahidi, 2022, Sixtus and Pillai, 2022). Thus, a promising EC_{50} was measured from the aforementioned extracts against DPPH. When EC_{50} for the DPPH inhibitory activity of the active extract was performed, very promising results were obtained. The boiled water extract was found to be with a lower EC_{50} than positive controls quercetin and ascorbic acid which had EC_{50} values which indicates the strong antioxidant activity. On the other hand, methanolic and microwaved water extracts were found to have a higher EC_{50} which indicates a moderate biological activity. Those higher inhibitory activity of DPPH indicates a promising anticancer activity (Grigalius and Petrikaite, 2017).

Cancer cells are characterized with its oxidative stress which resulted in an imbalance between the reactive oxygen species (ROS) and antioxidants in human body (Luo et al., 2022a). Thus, a metabolic disturbance and signaling aberrations can exacerbate the carcinogenesis and malignancy (Luo et al., 2022a). Antioxidant, as a therapeutic agents (non-enzymatic antioxidant), can mask the effect of ROS which lead either to the restoration of the metabolic disturbance and/or signaling aberration (Luo et al., 2022a). According to the anticancer activity assay presented in Fig. 4 and Table 4, a highest biological activity with the lowest IC_{50} was found with cells treated with boiled water extract. This could be as a result of the presence of the highest concentration of antioxidant between all extracts. It was found before that water extracts produced from natural source contains high concentration of antioxidant like the water extract of *Moringa oleifera* and *Bambusa chungii* (Cao et al., 2022, Kirindage et al., 2022). While methanolic and microwaved water extracts were found to exert a lower biological activity with a higher IC_{50}. It was found that the difference in the biological activity against DPPH, and against cancer cells between microwaved water and boiled water is obvious. This could be due to the microwaving effect on the peptides and polyphenolic compounds. It was suggested before that microwaving can lead to the degradation of natural compounds, and this can lead to the loss of their biological activity (Kurtulbaş et al., 2022).

Another suggested mechanism for active extracts is the ability of extracts to activate Caspases enzymes especially (3, and 9). Caspase-3, and −9 are important mediators for cells apoptosis which is considered to be a dose-dependent triggered enzyme (Asadi et al., 2022, Anvarbatcha et al., 2023). The unique chemical profile of *Q. coccifera* extracts suggests an ideal ability to interact with DNA and proteins to enhance cells selectivity in treatment. The strongest agonistic activity was obtained after being treated with boiled water extract, which explains the lowest IC_{50}. While a promising activity for both methanolic and microwaved water extracts is due to the activity of microwaved water extracts on Caspase-3 and −9, and for methanolic extract strong activity on Caspase-3 only. As a result of the high selectivity, a minimization of normal cells damaged was found to be exerted after the treatment of the normal fibroblast with active extracts. The polar extracts had the highest antioxidant, anticancer, and agonistic activity due to the presence of different functional groups that can for a strong interaction with different targets which lead to higher activity (Castro-Varela et al., 2023, Jaber, 2023a).

5. Conclusion

In conclusion the suggested antioxidant and anticancer activity of *Q. coccifera* plant leaves extracts have been proved for the future use in drug discovery. It was found that extracts extracted through solvents with intermediate polarity or higher (methanol, and both boiled water, and microwaved water extracts) contain compounds with antioxidant and anticancer activity at a low concentration (below 100 μg/ml). The highest antioxidant and anticancer activity were found to be exerted with DPPH and different cancer cell lines treated with boiled water extract. In addition, it was found that boiled water extract was either comparable or with better biological activity than positive controls used in the antioxidant activity. While, both methanolic and microwaved water extracts were found to be resulted in a promising biological activity. All extracts found to be active with a high Selectivity Index which mean that *Q. coccifera* leaves extracts are safe to be used by people having prostate, lung, or breasts cancers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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