



Jordanian *Ducrosia flabellifolia* Inhibits Proliferation of Breast Cancer Cells by Inducing Apoptosis

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Authors' contributions

This work was carried out in collaboration between all authors. Author WHT designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors RAI and FK managed the phytochemical analyses of the study. Author AMM contributed to the study design, the analysis of the results, and the technical support. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: to evaluate the potential apoptosis inducing effect of *Ducrosia flabellifolia* extracts against different cancer cell lines.

Methodology: The antiproliferative activity of *Ducrosia flabellifolia* extracts was tested against three cell lines using MTT assay. The apoptosis induction ability of ethanol extract was determined using TUNEL colorimetric assay while agarose gel electrophoresis was used to detect DNA fragmentation. Morphological changes associated with apoptosis were observed using scanning electron microscopy. LC/MS-MS analysis was used to determine the main flavonoids present in the plant extract.

Results: *Ducrosia flabellifolia* ethanol extract showed selective antiproliferative activity against different cell lines. The highest activity was against MCF-7 cell line with IC₅₀ value of 25.34 µg/mL, followed by Hep-2 cell line with IC₅₀ value of 98.01 µg/mL. While the lowest activity was against Vero cell line with IC₅₀ value of 98.01 µg/mL. The

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antiproliferative effect was exerted by inducing apoptosis as indicated by the presence of DNA fragmentation, nuclear condensation, and formation of apoptotic bodies in treated cancer cells. LC/MS-MS analysis revealed the presence of five flavonoids (quercetin, fisetin, kaempferol, luteolin, and apigenin) and their derivatives in the extract.

Conclusion: The apoptosis inducing ability of *Ducrosia flabellifolia* ethanol extract validate the use of this plant in traditional medicine to treat cancer. The anticancer synergistic effect of *Ducrosia flabellifolia* compounds has broad implication for understanding the anticancer potential of plant natural products *in vivo*, where different compounds may act in concert to reduce tumor burden.

Keywords: *Flavonoids; anticancer; antiproliferative; plant extract; quercetin.*

1. INTRODUCTION

Cancer is the second cause of death after cardiovascular diseases [1]. The majority of cancer deaths (above 70%) occur in countries with low and middle income [2]. Jordan is among these countries and recent estimates in Jordan reported 5000 cancer cases per year [3]. Commercially available anticancer agents are either synthetic compounds or natural products originating from different sources including plants.

Synthetic chemistry is dominating the field of new drug discovery. However, the potential of bioactive plants to provide new and novel products for disease treatment and prevention is still enormous [4]. The antitumor area has the greatest impact of plant derived drugs, where drugs like vinblastine, vincristine, taxol, and camptothecin have improved the chemotherapy of some cancers [5].

Ducrosia species are normal flora in different countries including Jordan, Iraq, Iran, Afghanistan, Pakistan and in the region along the Arabic Gulf [6]. *Ducrosia* belong to Apiaceae family which characterized by having different phytochemicals especially coumarins [7].

In traditional medicine, different *Ducrosia* species were used as analgesic, pain reliever and cold treatment [8]. Antimicrobial, antimycobacterial, antifungal, central nervous system depressant, and antianxiety effects were reported for different *Ducrosia* species including *D. anethifolia* and *D. ismaelis* [6,9-10].

Ducrosia flabellifolia is a wild plant growing in the eastern desert of Jordan and considered as a rare plant [11]. No reports are available about the biological activities of *Ducrosia flabellifolia*.

Taking into account the use of *Ducrosia flabellifolia* in traditional medicine, and the lack of studies about its biological activities, this study was conducted to evaluate the antiproliferative and apoptosis inducing effects of *Ducrosia flabellifolia* against different cancer cell lines. For a better understanding of the chemical composition of this plant, the major constituents of the plant extract was identified using LC/MS-MS.

2. METHODOLOGY

2.1 Plant Material and Extraction Procedure

The plant was collected from Wadi Hassan in the eastern desert of Jordan. The taxonomic identity of the plant was authenticated by Prof. Dawud EL-Eisawi (Department of Biological Sciences, University of Jordan, Amman, Jordan). Voucher specimens were deposited in the Department of Biological Sciences, University of Jordan, Amman, Jordan. The air dried areal parts of *Ducrosia flabellifolia* were finely ground. Suitable amounts of the powdered plant materials were soaked in 95% ethanol (1L per 100g) for two weeks. The rotary evaporator (Buchi R-215, Switzerland) was used to evaporate ethanol at 40°C to obtain the crude ethanol extract. The ethanol extract was further subjected to solvent-solvent partitioning between chloroform and water. All solvents were evaporated to dryness under reduced pressure to produce the crude extracts which were collected and stored at -20°C for further testing [12].

2.2 Cell Lines and Culture Conditions

Hep-2 (larynx carcinoma), MCF-7 (breast epithelial adenocarcinoma), and Vero (African green monkey kidney) cell lines were kindly provided by the Department of Biological Sciences, University of Jordan. Cells were grown in Minimum Essential Medium Eagle (Gibco, UK) supplemented with 10% heat inactivated fetal bovine serum (Gibco, UK), 29 µg/mL L-glutamine, and 40 µg/mL Gentamicin. Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

2.3 Antiproliferative Activity Assay

The antiproliferative activity of *Ducrosia flabellifolia* extracts was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Promega, Madison, WI, USA). Exponentially growing cells were seeded at 17,000 cells/well (for Hep-2 and Vero cell line) and 11,000 cells/ well (for MCF-7 cell line) in 96 well microplates (Nunc, Roskilde, Denmark). After 24 h incubation, a partial monolayer was formed then the media was removed and 200 µL of the medium containing the plant extract (initially dissolved in DMSO) were added and re-incubated for 48 h. Then 100 µL of the medium were aspirated and 15 µL of the MTT solution were added to the remaining medium (100 µL) in each well. After 4 h contact with the MTT solution, blue crystals were formed. One hundred µL of the stop solution were added and incubated further for 1 h. Reduced MTT was assayed at 550 nm using a microplate reader (Biotek, Winooski, VT, USA). The plant extracts was tested at the following concentration 200, 150, 100, 50, 25, 15, 10, and 5 µg/ml. Control groups received the same amount of DMSO (0.1%). Untreated cells were used as a negative control while, cells treated with vincristine sulfate were used as a positive control. IC₅₀ values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line. IC₅₀ values were reported as the average of three replicates. The antiproliferative effect of the tested extracts was determined by comparing the optical density of the treated cells against the optical density of the control (cells treated with media containing 0.1% DMSO).

2.4 Assessments of Apoptosis in Cell Culture

Apoptosis was detected using terminal deoxynucleotidyl transferase (TdT) mediated-16-deoxyuridine triphosphate (dUTP) Nick-End Labelling (TUNEL) system (Promega, Madison,

WI, USA). MCF-7 cell line was selected because it is the only cell line that showed sensitivity toward *Ducrosia flabellifolia* ethanol extract. MCF-7 cells cultured in 24 well plates were treated with 30 µg/mL *Ducrosia flabellifolia* ethanol extract for 28 h. The assay was conducted according to the manufacturer's instructions. Briefly, treated cells were fixed using 10% formalin followed by washing with phosphate buffer saline (PBS). Cells were then permeabilized using 0.2% triton X-100.

Biotinylated dUTP in rTdT reaction mixture was added to label the fragmented DNA at 37 °C for one hour, followed by blocking endogenous peroxidases using 0.3% hydrogen peroxide. Streptavidin HRP (1:500 in PBS) was added and incubated at room temperature for 30 min. Finally, hydrogen peroxide and chromagen diaminobenzidine were used to visualize nuclei with fragmented DNAs under the light microscope (Novex, Arnhem, Holland). Cells treated with 40 nM vincristine sulfate were used as a positive control while untreated cells were used as a negative control.

2.5 Scanning Electron Microscopy

MCF-7 cells were cultured in complete DMEM containing 30 µg/mL of *Ducrosia flabellifolia* ethanol extract and incubated for 48 h in humidified CO₂ incubator. Negative control cells were incubated with complete DMEM and positive control cells received complete DMEM containing 40 nM vincristin sulfate. Treated cells were fixed with 3% glutaraldehyde for 1.5 h followed by osmium tetroxide (2% in PBS) for 1 h. After fixation, cells were washed in PBS and sequentially dehydrated using 30%, 70%, and 100% ethanol [13]. Fixed cells were attached to a metal stubs and sputter coated with platinum using Emitech K550X coating unit (Qourum Technology, West Sussex, UK). The coated specimens were viewed using Inspect F50/FEG scanning electron microscope (FEI, Eindhoven, The Netherlands) at accelerating voltage of 2–5 kV.

2.6 Detection of DNA Fragmentation

Agarose gel electrophoresis was used to analyze DNA fragmentation. MCF-7 cells (11 X 10⁵) were incubated with different concentrations of *Ducrosia flabellifolia* ethanol extract for 48h followed by cell detachment using trypsin-EDTA and washing using PBS. DNA purification kit (Promega, USA) was used for DNA extraction. The cell pellets were incubated with 600 µl nuclei lysis solution until no visible clumps remain followed by 20 seconds incubation with 200 µl protein precipitation solution. After centrifugation, the supernatant containing the DNA was separated and mixed with 600 µl isopropanol followed by centrifugation and mixing pellets with 600 µl 70% ethanol followed by centrifugation. The pellets were incubated with 100 µl of DNA rehydration solution at 65 °C for 1 hour. DNA bands were separated using 2.0% agarose gel electrophoresis containing ethidium bromide and visualized using UV transilluminator.

2.7 Qualitative Phytochemical Screening

Thin layer chromatography (TLC) was used for qualitative phytochemical screening of the ethanol extract of *Ducrosia flabellifolia*. Aliquots (50–75 µl) of the extract were applied 1cm from the base of the TLC plates (0.25 mm, Macherey-Nagel, Germany). Serial mixtures of chloroform and methanol (from 0–100%) were used as eluents. Development of the chromatograms was performed in a closed tank in which the atmosphere had been saturated with the eluent vapor by lining the tank with filter paper wetted with the eluent. For

flavonoids and terpenoids detection, plates were sprayed with p-anisaldehyde/sulfuric acid reagent and carefully heated at 105°C for color development [14]. For alkaloids detection, plates were sprayed with iodoplatinate acid and placed in the fume hood for drying.

2.8 LC/MS-MS Triple Quadrapole

Liquid chromatography with MS/MS triple quadrapole was used to identify flavonoids content of *Ducrosia flabellifolia* ethanol extract. For chromatographic separation and mass spectral analysis a Shimadzu LC-8030 MS system (degasser, binary gradient pump, autosampler, and mixer) was used coupled with Shimadzu 8030 triple quadrapole system equipped with ESI ion source interface. Separation was conducted on Shimpack ODS C18 column (250mm X 4.7 mm, 5µm) at 30 OC. Linear gradient system was applied as solvent A (water), solvent B (methanol) and solvent C (acetonitrile with 0.2 acetic acid).

The mobile phase was eluted for the first 5 minutes as 88% (v/v%) solvent A, 8% (v/v%) solvent B, and 4% (v/v%) solvent C. for the last 40 minutes the mobile phase was eluted as 70% (v/v%) solvent A, 16% (v/v%) solvent B, and 14% (v/v%) solvent C.

2.9 Statistical Analyses

The results of the antiproliferative part are presented as means ± SEM of three independent experiments. Statistical differences among fractions were determined by one way ANOVA using Graph Pad Prism5 (GraphPad Software Inc., San Diego, USA). Differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

Screening of plants and their products for their potential to induce apoptosis have become the major strategy in the search for new anticancer agents. The present study was conducted to evaluate the apoptosis induction ability of *Ducrosia flabellifolia* against three cell lines including MCF-7 (breast cancer) cell line.

MTT dye was used in different studies to determine cell viability for many herbals and phytochemicals [15]. In the present study, the antiproliferative activity of three (ethanol, aqueous, and chloroform) *Ducrosia flabellifolia* extracts was tested on MCF-7, Hep-2, and Vero cell lines using the MTT reduction assay.

The ethanol extract exhibited the highest antiproliferative activity with IC_{50} values of 25.34, 98.01, and 87.50 µg/ml against MCF-7, Hep-2, and Vero cell lines, respectively (Table 1).

Both chloroform and aqueous extracts showed limited antiproliferative activity against Hep-2 and Vero cell lines with IC_{50} values above 144 µg/ml. On the other hand, the aqueous extract was more active than chloroform extract against MCF-7 cell line with IC_{50} values of 33.04 and 47.25 µg/ml, respectively. These results may indicate that the polar active principles are more responsible for the antiproliferative activity. Our results agree with previous studies that reported the antiproliferative activity of polar principles in plants like *Trametes robiniophila*, *Alocasia macrorrhiza*, and several Thai medicinal plants [16-18]. All extracts were more active against MCF-7 cell line (Table 1). This selectivity could be the result of the sensitivity of the cell line to the compounds in the extract or to tissue specific response [19].

Table 1. Percentage yield and IC₅₀ determination of *Ducrosia flabellifolia* solvent extracts

Extract	Yield (w:w %)	IC ₅₀ value (µg/ml) ± SEM		
		MCF-7	Hep-2	Vero
Ethanol	6.56	25.34 ± 0.68	98.01 ± 1.80	87.50 ± 1.65
Chloroform	13.50	47.25 ± 1.75	144.35 ± 0.97	152.33 ± 1.98
Aqueous	4.57	33.04 ± 1.55	159.33 ± 1.36	164.06 ± 0.88
Vincristin sulfate	-	11.02 ± 0.05	> 90	> 90

Apoptosis (programmed cell death) is the main mechanism of cell death that is essential for diverse processes ranging from cell development to stress response [15]. Apoptosis induction seems to be an attractive goal to kill cancer cells since many cancers inactivate apoptosis to survive [20]. The main features of apoptosis include chromatin condensation, cell shrinkage, DNA fragmentation, and formation of apoptotic bodies [21].

The ability of the ethanol extract of *Ducrosia flabellifolia* to induce apoptosis in MCF-7 cells was evaluated using a TUNEL colorimetric assay which detects DNA fragmentation during programmed cell death. Cell shrinkage and DNA fragmentation were clearly observed in cells treated with 30 µg/mL *Ducrosia flabellifolia* ethanol extract (Fig. 1).

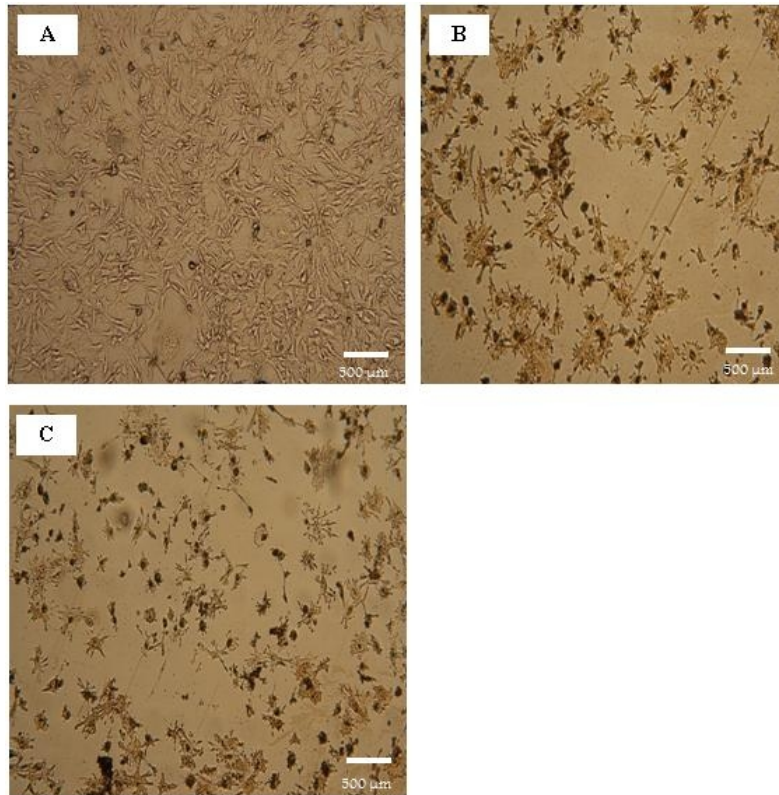


Fig. 1. MCF-7 cells assayed by DeadEnd™ colorimetric TUNEL system to indicate cell apoptosis. (A) Negative control; (B) Cells treated with 30 µg/ml *Ducrosia flabellifolia* ethanol extract; (C) Positive control

Systemic cleavage of DNA to produce nucleosomal fragments of 200 bp (or its multiples) is considered as a clear characteristic of apoptosis [22]. For more confirmation of our results, fragmented DNA molecules were detected using agarose gel electrophoresis. Clear DNA fragmentation was observed in cells treated with 30, 50, and 100 $\mu\text{g}/\text{mL}$ *Ducrosia flabellifolia* ethanol extract, whereas untreated cells showed no evident DNA fragmentation (Fig. 2). Previous studies have reported that large fragment of 50-300 kb can be detected in early stages of apoptosis while further fragmentation to 200 bp or its multiples was observed in late stages of apoptosis [23].

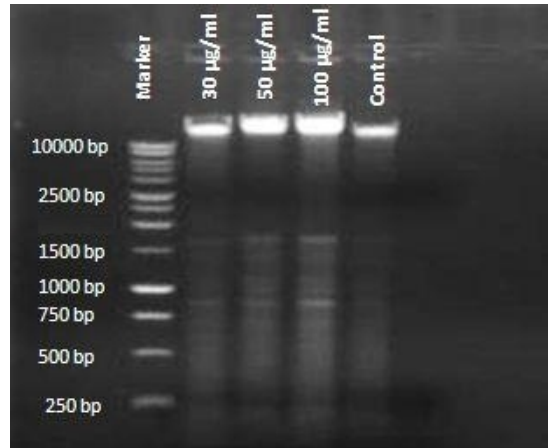


Fig. 2. Effect of different concentrations of *Ducrosia flabellifolia* ethanol extract on MCF-7 cells detected by agarose gel electrophoresis

Morphological alterations during apoptosis include chromatin condensation, nuclear remodeling and membrane blebbing [24]. In order to gain more insight into programmed cell death, morphological changes associated with apoptosis were detected using scanning electron microscopy. Cytomorphological changes corresponding to a typical morphology of apoptosis were detected in MCF-7 cells treated with 30 $\mu\text{g}/\text{mL}$ *Ducrosia flabellifolia* ethanol extract. These changes include cell shrinkage, membrane blebbing, loss of contact with neighboring cells, and formation of apoptotic bodies (Fig. 3). The presence of small degraded apoptotic bodies around cells was also observed (Fig. 3). On the contrary, untreated cells showed normal morphology while cells treated with vincristine (positive control) showed morphological changes similar to those observed in cells treated with the plant extract.

Phytochemical screening is essential to identify the chemical nature of the active components that may involve in the apoptosis induction ability. Qualitative thin layer chromatography revealed the presence of flavonoids and terpenoids in *Ducrosia flabellifolia* ethanol extract. The link between flavonoids and reduced cancer risk was documented in previous studies [25-26]. Comprehensive analysis of the flavonoids present in the extract was achieved using HPLC- MS/MS (Fig. 4). The flavonoid profile of *Ducrosia flabellifolia* ethanol extract consisted of quercetin, fisetin, kaempferol, luteolin, and apigenin in addition to their derivatives (Table 2). This is the first study to report the antiproliferative activity and detailed phytochemical screening of *Ducrosia flabellifolia* flavonoids.

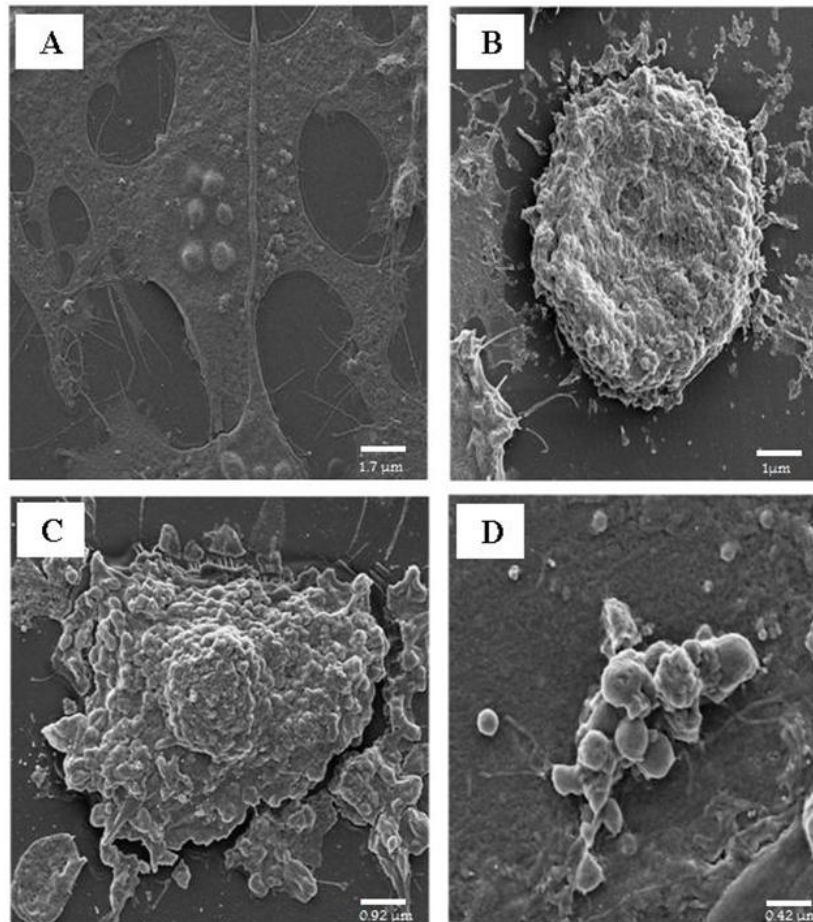


Fig. 3. Scanning electron micrographs of MCF-7 cells. (A) Untreated cells; (B) Cells treated with 40 nM vincristin sulfate; (C) Cells treated with 30 μ g/mL of *Ducrosia flabellifolia* ethanol extract; (D) formation of apoptotic bodies in cells treated with 30 μ g/mL of *Ducrosia flabellifolia* ethanol extract

Quercetin is one of the flavonoids that are widely distributed in different plants [27]. Many biological effects of quercetin have been reported, including anticancer, anti-inflammatory, antibacterial and muscle relaxation [28]. The anticancer and antimicrobial activity of quercetin derivatives were also documented [29]. It seems that the presence of quercetin and some of its derivatives in *Ducrosia flabellifolia* ethanol extract participate directly in the antiproliferative activity of this plant. On the other hand, kaempferol is not as widely distributed as quercetin, but is present in some plants that have potential anticancer activity like broccoli and endive [30]. Previous studies showed the ability of kaempferol to induce apoptosis in different cancer cell lines including osteosarcoma [31] and glioblastoma cell lines [32]. The synergistic antiproliferative effect of kaempferol and quercetin against human gut and breast cancer cells was also reported [30]. For other flavonoids detected in *Ducrosia flabellifolia*, previous studies reported antiproliferative activity for apigenin [33-35], fisetin [36-37], and luteolin [38-39]. Furthermore, the synergistic antiproliferative activity of luteolin with quercetin [40] and luteolin with diosmetin [41] was also documented.

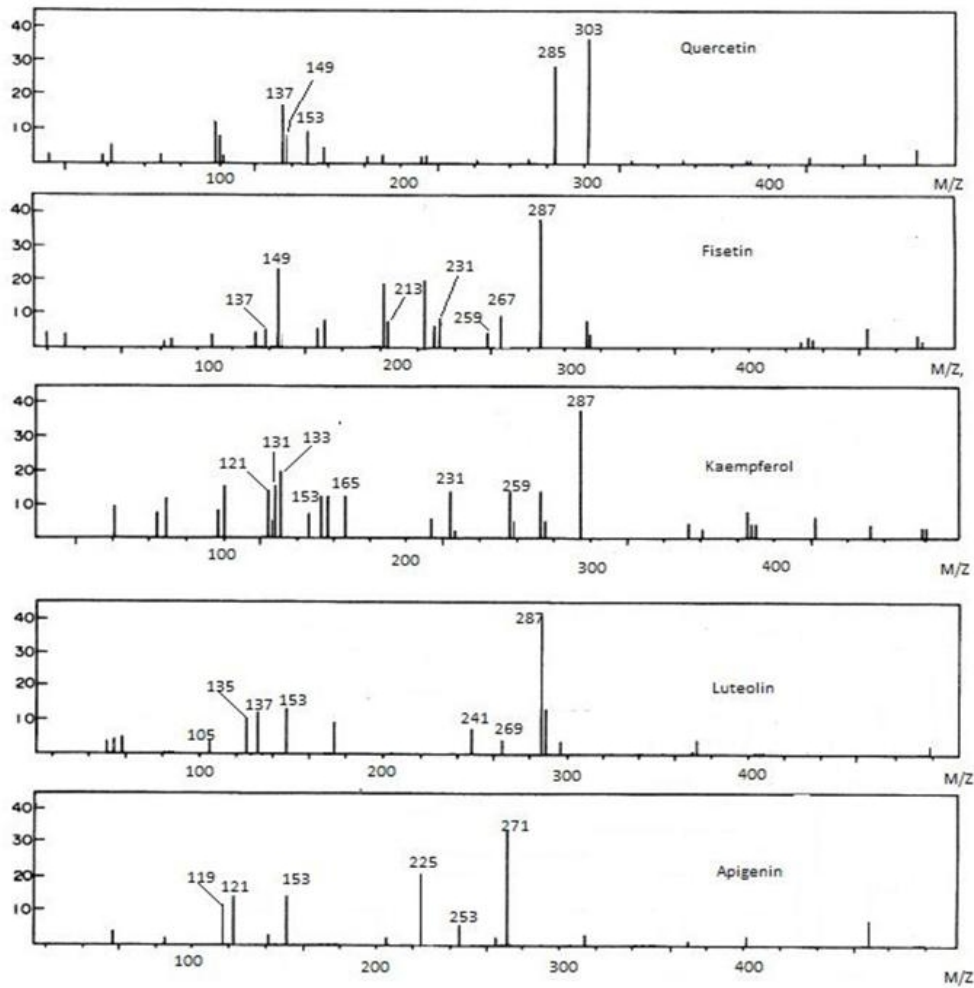


Fig. 4. The MS₂ spectra of flavonoids of *Ducrosia flabellifolia* ethanol extract.

Table 2. The [M+H]⁺ molecules of *Ducrosia flabellifolia* flavonoids, as well as the defining structure fragments produced by MS₂

Assessed fragments	Flavonols			Flavones	
	Quercetin (1)	Fisetin (2)	Kaempferol (3)	Luteolin (5)	Apigenin (6)
[M+H] ⁺	303	287	287	287	271
Defining subgroup fragments					
[M+H-H ₂ O] ⁺	285	269	269	269	253
[M+H-H ₂ O-CO] ⁺	257	241	241	241	225
[M+H-H ₂ O-2CO] ⁺	229	213	213	—	—
[M+H-CO] ⁺	275	259	259	—	—
[M+H-2CO] ⁺	247	231	231	—	—
[M+H-CH ₂ CO] ⁺	—	—	—	—	—

4. CONCLUSION

Our study is the first to evaluate the antiproliferative effect of *Ducrosia flabellifolia* ethanol extract and the results necessitate the protection of this rare plant. It is unlikely that the apoptosis induction ability of *Ducrosia flabellifolia* ethanol extract is due to the action of a single agent; it is more likely to be due to one or several synergistic effects of a combination of active flavonoids present in this plant. Such synergistic effect has broad implication for understanding the anticancer potential of plant natural products *in vivo*, where different compounds may act in concert to reduce tumor burden. Further studies are necessary to confirm the apoptosis inducing potential of the pure compounds isolated from *Ducrosia flabellifolia*.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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