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[Abdullah A. Shaito](#) ^{*} , Islam Omairi , [Najlaa Al-Thani](#) , Fatiha Seglab , Esraa Ad-darwish , [Firas H. Kobeissy](#) ^{*} , [Salam Nasreddine](#)

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Article

***Medicago orbicularis* Has Antioxidant, Anti-hemolytic, and Anti-Cancerous Activities and Augments Cisplatin-Induced Cytotoxicity in A549 Lung Cancer Cells**

Abdullah Shaito ^{1,2,*}, Islam Omairi ³, Najlaa Al-Thani ⁴, Fatiha Seglab ¹, Esraa Ad-darwish ⁵, Firas Kobeissy ^{6,*} and Salam Nasreddine ^{3,7,*}

¹ Biomedical Research Center, Qatar University, P.O. Box 2713, Doha, Qatar. abdshaito@qu.edu.qa (A.S.) and fseglab@qu.edu.qa (F.S.)

² College of Medicine, and Department of Biomedical Sciences at College of Health Sciences, Qatar University, P.O. Box 2713, Doha, Qatar. abdshaito@qu.edu.qa (A.S.)

³ Faculty of Sciences-Section I, Groupe Anti-Cancer Therapeutic Approaches (ATAC), Rammal Rammal Laboratory, Lebanese University, Beirut, Lebanon. isslamomairi96@gmail.com (I.O)

⁴ Research and Development Department, Barzan Holdings, 7178 Doha, Qatar; nalthani@barzanholdings.com (N.A.)

⁵ Biotechnology in Forensic Science Program, Faculty of Health Sciences, American University of Science and Technology, Beirut, Lebanon. darwishesraa1997@gmail.com

⁶ Department of Neurobiology, Center for Neurotrauma, Multiomics & Biomarkers (CNMB), Morehouse School of Medicine, 720 Westview Dr. SW, Atlanta, GA 30310 Atlanta, GA, United States; firasko@gmail.com (F.K.)

⁷ Doctoral School of Science and Technology, Research Platform for Environmental Science (PRASE), Lebanese University, Beirut, Lebanon; salam.nasreddine@ul.edu.lb (S.N.)

* Correspondence: abdshaito@qu.edu.qa or abdshaito@gmail.com (A.A.S.); firasko@gmail.com (F.K.); salam.nasreddine@ul.edu.lb (S.N.)

Abstract: Cancer is second leading cause of death, worldwide. Lung cancer is the leading cause of cancer-related mortality. Plant-based therapeutics and herbal medicine have played a vital role in the development of several anti-cancerous agents, and has been used to reduce the severe side effects of chemotherapy as well. Since the anti-lung cancer properties of the plant *Medicago. orbicularis* are not explored yet, we identified its phytochemical composition and investigated the anti-oxidant, anti-hemolytic, and anti-cancerous properties of extracts of this plant in A549 human lung adenocarcinoma cells. Results show that all parts of *M. orbicularis* (stems, leaves, and fruits) exhibit remarkable anti-oxidant and hemolytic activities. In addition, all extracts showed a dose-dependent anti-cancerous cytotoxic activity against A549 cells; with fruit extracts being the most potent. This cytotoxic effect could be related, at least partly, to the induction of apoptosis, where *M. orbicularis* fruit extracts activated Caspase-3 and PARPP-1, and reduced the ratio of anti-apoptotic BCL-2/ pro-apoptotic BAX, thereby promoting cellular death. Furthermore, the use of *M. orbicularis*, in combination with a conventional chemotherapeutic agent, cisplatin, was assessed. Indeed, combination of cisplatin and *M. orbicularis* fruit extracts was more cytotoxic and induced more aggregation of A549 cells than either treatment alone. GC-MS analysis and total polyphenol and flavonoid content determination indicated that *M. orbicularis* is rich in compounds that have anti-cancerous effects. *M. orbicularis* may be a potential source of anti-cancerous agents to manage progression of lung cancer and its resistance to therapy.

Keywords: *Medicago orbicularis*; lung cancer; herbal medicine; antioxidants; hemolytic; cisplatin; A549 cells

I. Introduction

Cancer remains the second leading cause of disability and death worldwide where around 1 in 6 deaths are related to cancer. In 2020, around 19 million new cancer cases and almost 10 million cancer-related deaths were recorded globally [1].

Lung cancer is a major public health concern, with significant impact on individuals and communities worldwide. Lung cancer has a high incidence rate; it was the second most commonly diagnosed cancer in 2020. Lung cancer is the leading cause of cancer-related deaths (around 1.8 million deaths or 18% of global cancer-related deaths in 2020) [1]. With a 21% 5-year survival rate, lung cancer has one of the most poor prognoses [2].

Depending on its progression, or the presence of metastasis, treatment of lung cancer will differ. Surgical resection, combination chemotherapy, and stereotactic body radiation are considered as the main modes of treatment. A significant obstacle in lung cancer therapy is the resistance that develops to chemo- and radio-therapy. Following therapy, resistance can lead to the relapse of lung cancer around 6 months post-therapy, in many patients [3,4]. Cisplatin is a cytotoxic chemotherapeutic agent used to treat several cancers including lung cancer [5]. The standard of treatment for limited-stage lung cancer remains chemotherapy using four to six cycles of cisplatin and etoposide and concurrent radiation therapy. However, 60-70% of patients are at extensive-stage lung cancer and require a different treatment regimen; also involving cisplatin [4,6]. Nevertheless, these treatments can become refractory in patients. When relapse takes place lung cancer is usually refractory to treatment and has poor prognosis due to the limited availability of therapeutic options [3,4]. As a result, there is an urgent demand for new treatment options and alternative therapeutic modalities that can treat lung cancer and circumvent its chemoresistance. Herbal medicine represents a promising alternative in this regard.

Herbal medicine and phytotherapy, medicinal treatment based on plants or herbs and their extracts, have played a central role in the development of several anti-cancer agents such as Paclitaxel, Camptothecin, and Vincristine [7–11]. Herbal medicine can be utilized as an assistant therapeutic modality as well. It can enhance the response rate to chemotherapy as well as radiotherapy, help overcome resistance to therapy, decrease the severity of side-effects caused by chemotherapy and radiotherapy, and enhance the quality of life and survival of cancer patients [12–15]. Relatedly, herbal preparations have been used as well to reduce the severe side effects of chemotherapy, including cancer-related fatigue [7]. Herbal preparations and herbal active compounds act as anti-cancerous and anti-metastatic agents through several mechanisms, including scavenging of reactive oxygen species (ROS), modulation of epithelial to mesenchymal transition (EMT), impairment of angiogenesis, modification of the expression and activity of matrix metalloproteinases (MMPs), among others [16,17].

The Fabaceae family of plants includes a large number of domesticated species which are harvested as crops for human and animal consumption as well as oils, fuel, fertilizers, and medicinal and agricultural varieties [18]. *Medicago* is a genus of the Fabaceae family and it comprises more than 83-87 different species of flowering plants [18–20]. Twenty of these species are herbaceous perennials, 63 are herbaceous annuals and only 2 are shrubs [20]. *Medicago orbicularis* L. Bartal. (common names: black disk, button medick, or button clover) is a species of the *Medicago* genus indigenous to Eurasia and North Africa [19]. The plant is distributed throughout the Mediterranean basin mainly in Palestine, Lebanon, Syria, Algeria, Spain, France, Italy and Greece, in Middle East countries such as Iraq and Iran, and several other countries [18]. *M. orbicularis* is a winter annual plant which flowers in Spring and early Summer. Its stems are leafy, stipules are fimbriate, leaflets are oval non-hairy with toothed margins, flowers are orange-yellow, seed pods are flat, coiled and lack spines [19].

M. orbicularis phytochemical composition has not been investigated. But, other *Medicago* species have been reported to be rich in phytochemicals, including flavonoids, carotenoids, saponins, phenolic acids, and phytoestrogens [21–23]. These phytochemicals have been demonstrated to exhibit anticancer activities through several mechanisms including apoptosis, antiproliferative, immunomodulatory, and anti-inflammatory activities, and the capacity to modulate oxidative stress

[24–28]. It is well recognized that antioxidant activity is one mechanism through which medicinal plants may exert a therapeutic effect [27–32]. Close-by species such as *M. polymorpha*, *M. sativa*, *M. arabica*, or *M. truncatula* were shown to possess a robust antioxidant activity [22,23]; an indication to the potential anti-oxidant and therapeutic value of *M. orbicularis*. Güleç and Kültür reported a traditional therapeutic use of *M. orbicularis* in Turkey for heart diseases [33]. However, there are no studies currently on the antioxidant potential, therapeutic applications or anti-cancerous properties of *M. orbicularis*. Therefore, we assessed the antioxidant capacity, hemolytic properties, phytochemical composition and the effects of *M. Orbicularis* on cell proliferation, cell aggregation, and cell migratory potential against A549 human lung cancer cells. Additionally, the combinatorial effect of a treatment using both cisplatin and *M. orbicularis* on A549 cells was tested.

II. Materials and Methods

Cell Culture and Reagents

Human lung adenocarcinoma cell line A549 and primary normal neonatal fibroblast (HDFn) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained in a humidified chamber at 37 °C and 5% CO₂ in DMEM (Dulbecco's Modified Eagle's Medium; cat# D0819 Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (cat# F9665, Sigma-Aldrich) and 100U/ml penicillin and 0.1 mg/ml streptomycin (cat# P4333, Sigma-Aldrich). Cells were passaged by trypsinization when they reached 90% confluence.

Plant Collection and Extraction

Fresh *Medicago orbicularis* L. *Bartal* was collected from Almat, Byblos, Lebanon between May-June 2021. A voucher specimen was stored in the herbarium of the Faculty of Pharmacy, Lebanese University, Beirut, Lebanon and was authenticated by Professor George Tohme, an experienced taxonomist and herbalist. *Medicago orbicularis* L. *Bartal*: Kingdom Plantae; Phylum Tracheophyta Class Magnoliopsida; Order Fabales; Family Fabaceae; Genus *Medicago* L. (Index Kewensis; <https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:506322-1>).

Extracts were obtained from leaves, stems and fruits of *M. orbicularis*. In brief, the different plant parts were cleaned, air-dried in the shade at room temperature, ground to powder, and kept in plastic containers, away from light, heat and moisture. Then, 10 grams of the powder were mixed with 100 ml of 70% aqueous ethanol, and the mixture was placed in a reciprocating shaker and continuously agitated at 150rpm for 3 to 4 days. The solutions were filtered and concentrated by a rotary evaporator at 40°C and then lyophilized using a lyophilizer. The lyophilized extracts were stored at -20°C until their use. At the time of use, the extracts were dissolved in 70% ethanol.

DPPH (α , α -diphenyl- β -picrylhydrazyl) Antioxidant Activity

The antioxidant free radical scavenging ability of the ethanolic extracts of the different parts of *M. orbicularis* (leaves, fruits and stems) was measured using the DPPH radical scavenging assay which was performed as described [34]. DPPH changes color from intense purple to pale-yellow due to the donation of a hydrogen atom, if the plant extract has radical scavenging activity. For *M. orbicularis* ethanolic extracts, 1 ml of different concentrations of the ethanolic plant extracts (50, 100, 150, 200, 300 or 500 μ g/ml) was mixed with an equal volume of DPPH (cat# D9312, Sigma-Aldrich Co.) solution (0.15 mM in ethanol). The blank consisted of 1 ml of DPPH solution and 1 ml of 70% ethanol. Mixed samples were then kept in the dark for 30 min and the OD was measured at a wavelength of 515 nm using a spectrophotometer. Ascorbic acid, a potent antioxidant, was employed as a positive control and reference. The DPPH scavenging activity of each concentration of the extracts was calculated using the formula:

$$\% \text{ Radical scavenging activity} = \left[\frac{\text{OD}_{\text{blank}} - \text{OD}_{\text{plant extract at each concentration}}}{\text{OD}_{\text{blank}}} \right] \times 100$$

In vitro Cytotoxicity Assay

In vitro cytotoxicity of *M. orbicularis* extracts was assessed using MTT assay which is a reduction assay that measures cellular metabolic activity and is reflective of cell viability. A549 and neonatal fibroblast cells were seeded in a 96-well tissue culture plate at a density of 0.7×10^4 cells per well in DMEM culture medium containing 10% FBS and penicillin/ streptomycin. After an overnight incubation, cells were treated with different concentrations (50, 100, 150, 200, 300 or 500 $\mu\text{g}/\text{ml}$) of *M. orbicularis* extracts for 24, 48 and 72 h. As a control, cells were treated with a concentration of ethanol (vehicle-control cells) equal to the concentration of ethanol in the extract-treated cells. Following the treatment, MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide; cat# M5655, Sigma-Aldrich Co.) was added for 3 hours until a purple formazan precipitate was formed. Media were removed gently, and an isopropanol-HCl solvent was added to dissolve the formazan precipitates. The Optical Density (OD) was measured by an ELISA plate reader at a wavelength of 595 nm. The percentage of cell viability of the treated cells was calculated using the following formula:

$$\% \text{ Cell Viability} = \left(\frac{OD_{\text{treated cells}}}{OD_{\text{vehicle-control cells}}} \right) \times 100$$

The IC₅₀ (half-maximal inhibitory concentration) value, was determined by extrapolation from the cell killing curve to determine the concentration of the extract that induced 50% cell death.

Cisplatin (Sigma-Aldrich Co.), dissolved in DMSO, was used as a positive control of the cytotoxic effect of *M. orbicularis* parts extracts on A549 cells. A549 cells were treated for 24, 48 and 72 hours with increasing concentrations of cisplatin (2.5, 5, 10 and 20 $\mu\text{g}/\text{ml}$) and compared to the DMSO treated vehicle-control cells. IC₅₀ values of cisplatin in A549 cells were determined at the three time points. Data are displayed as the mean \pm SEM of three independent experiments.

Cell Aggregation Assay

Seeded A549 cells were detached using 2mM EDTA PBS (calcium- and magnesium-free). Cells were pelleted and washed with PBS, and then resuspended in 1ml PBS including or not 100 or 150 $\mu\text{g}/\text{ml}$ of *M. orbicularis* fruit extract alone, 5 $\mu\text{g}/\text{ml}$ cisplatin alone, or a combination of both and kept shaking on a rocker for 60 minutes at 37°C in a cell culture incubator. Cells were fixed with 1ml of 1% formaldehyde and imaged using an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). Percentage of aggregation was calculated using the formula: $\% \text{ Aggregation} = (1 - \frac{N_t}{N_c}) \times 100$; where N_t is the number of single cells in treated wells and N_c is number of single cells in the vehicle control cells.

Assay of Hemolysis and Anti-hemolytic activity

Hemolytic activity assay was performed as previously described [35]. Erythrocytes were obtained after separation from plasma by centrifugation at 2500 rpm for 10 min at 4°C starting from fresh sheep blood. Briefly, RBCs were washed three times with 1X PBS then pelleted and resuspended in PBS to obtain a 5% suspension of RBCs. Then, 50 μL of different concentrations (10, 20, 40, 50 and 100 $\mu\text{g}/\text{ml}$) of ethanolic extract of fruits of *M. orbicularis* were added to 1 ml of the RBC suspension. The mixture was incubated at 37°C for 1.5 hours, then the suspension was centrifuged at 2,500 rpm for 10 min at 4°C and the OD measured at 540 nm using a spectrophotometer. Ethanol concentrations equal to those present in the fruit extracts were added to blood and used as a negative control, 1% SDS as a positive control of hemolysis of RBCs, and 1X PBS as the blank. Hemolytic levels were calculated as follows:

$$\% \text{ Hemolysis} = \left(\frac{OD_{\text{extract}}}{OD_{\text{vehicle-control}}} \right) \times 100$$

Anti-hemolytic activity assessment was performed as previously published [36]. Briefly, the same steps were performed as in the hemolysis assay except that H₂O₂ was added to induce hemolysis. After adding *M. orbicularis* extracts to the RBC suspension for 20 min, RBCs were incubated with 350 μL of 30% H₂O₂ at 37°C for 1.5 h. The suspension was centrifuged at 2500 rpm for 10 min at

4°C and OD at 540 nm was determined. Thirty % H₂O₂ was used as a positive control, and 1X PBS as the blank. Anti-hemolytic levels were calculated according to the following equation:

$$\% \text{ inhibition of hemolysis} = \left(\frac{OD_{\text{vehicle-control}} - OD_{\text{extract}}}{OD_{\text{vehicle-control}}} \right) \times 100$$

Scratch/ Wound-Healing Assay

A549 cells were grown in 12-well tissue culture plates until confluence. A scrape was made through the confluent monolayer using a sterile yellow 200 µL pipette tip. The culture medium was then removed, the cells were washed twice with PBS (Sigma-Aldrich, St. Louis, MO, USA) to remove cellular debris, and incubated at 37°C in fresh medium in the presence or absence of the indicated concentrations of *M. orbicularis* extracts. Photomicrographs of the scratch were obtained at baseline (0 h) and for the next 4 to 12 h using an inverted microscope (objective 10×). The width of the wound was expressed as the average ±SEM of the difference between the measurements taken at time zero and the corresponding time points. Ethanol was used as a vehicle control.

Western Blotting Analysis

A549 cells were seeded in a 6-well plate at a density of 2.8x10⁵ cells per well and cultured for 24 h. The cells were treated with extracts of *M. orbicularis* for 48 h and then the cells were washed twice with PBS and lysed using a lysis buffer containing 2% SDS, 60 mM Tris lysis buffer (pH 6.8), and protease inhibitors and centrifuged at 10,000 g for 10 min. The protein concentration of the supernatants was determined using the Bradford protein assay kit (Biorad, Hercules, CA, USA) and 25 µg of protein lysates were resolved on 10% SDS-PAGE before being transferred to a polyvinylidene difluoride membrane (Immobilon PVDF; Biorad, Hercules, CA, USA). The membranes were then blocked for 1 h at room temperature with 5% non-fat dry milk in TBST (TBS and 0.05% Tween 20). Immunodetection was performed by incubating the membrane overnight with specific primary antibodies at 4°C. Primary antibodies included rabbit IgG anti-full length human poly-adenosine diphosphate (ADP) ribose polymerase-1 (PARP-1) (Cell Signaling Technologies, CST 9542; dilution 1/1,000), rabbit anti-human proCaspase 3 (Santa Cruz, sc-7148; dilution 1/1,000), rabbit anti-human B-cell lymphoma 2 (BCL-2) (Abcam, ab32124; dilution 1/5000), rabbit anti-Bcl-2 associated X protein (BAX) (Abcam, ab32503; dilution 1/2,000), and rabbit anti-GAPDH (Abcam, ab181602; dilution 1/10,000). Membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (Abcam, ab6721; dilution 1/5,000) for 1 h followed by washing in TBST. Immunoreactive bands were detected using ECL substrate kit (Thermo Scientific, Rockford, IL, USA), and membranes were scanned using the Chemidoc imaging system (Bio-Rad). The intensity of the obtained bands was quantified using ImageJ software (NIH, MD, USA). All bands were normalized to GAPDH, which was used as a loading control.

Spectroscopic determination of total phenolic content

Total phenolic compounds content (TPC) was determined using the Folin-Ciocalteu colorimetric oxidation/reduction-based reaction as previously described [37], with slight modifications. Briefly, 100µl of diluted fruit extracts of *M. orbicularis* were added to 500µl of Folin-Ciocalteu reagent, followed by the addition of 400µl of saturated sodium carbonate solution (2%). The oxidation products of the reaction show a blue color with a broad light with an absorption maximum of 765 nm. OD 765 of the different mixtures was measured, against a blank, using a spectrophotometer. A calibration curve was prepared using a strong antioxidant, gallic acid, and the results were expressed as gallic acid equivalents (GAE) in milligrams per gram of the extract.

Spectroscopic determination of total flavonoids content

The total flavonoids content (TFC) of *M. orbicularis* fruit extracts was determined by the aluminium chloride colorimetric method as previously detailed [38]. AlCl₃ forms a flavonoid-aluminium complex that has a maximum OD at 510 nm. Briefly, a 100µl aliquot of appropriately

diluted fruit extracts of *M. orbicularis* were added to 400 μ l of distilled water. Then, 400 μ l of 5% NaNO₂ were added, followed by 300 μ l of 10% AlCl₃. After 6 min of incubation 200 μ l of 1M NaOH were added to the mixture followed by 240 μ l of distilled water. The absorbance of the resulting pink solution was determined at 510 nm versus a blank (water). A calibration curve was prepared using a known flavonoid, quercetin. The curve was used to calculate the amount of flavonoids in the plant extracts which was expressed as quercetin equivalents in mg/g dry weight of the plant part.

Gas Chromatography/ Mass Spectrometry (GC/MS)

The phytochemical compounds of *M. Orbicularis* fruit extracts were analyzed using GC/MS analysis. A Perkin Elmer Clarus 680 (Perkin Elmer, USA) system attached to a triple quadrupole mass spectrometer was used. Chromatography was conducted on a hydrophobic capillary column RTxi-5 Sil MS column (30 m \times 0.25 mm ID \times 0.25 μ m) using an injection volume of 10 μ l, a flow rate of 1.5 mL/min, a pressure of 23.1 KPa, and an average velocity of 0.2 sec. The temperatures of the source and the interface were 200 °C and 280 °C, respectively. The initial temperature was set at 80 °C for 2 min, increased to 250 °C at 15 °C/min, and raised to 280 °C at 15 °C/min (held for 12 min). Identification of phytochemicals in extracts was carried out by comparing the obtained retention indices with those of chemical compounds in the database of the National Institute of Standards and Technology (NIST).

Statistical Analysis

Results were evaluated for statistical difference using One-way ANOVA followed by Bonferroni test to calculate *P* values. Data are presented as mean \pm standard error of the mean (SEM) and a *p*-value of *P* <0.05 was considered as statistically significant. Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA).

III. Results

M. orbicularis plant ethanolic extracts exhibit remarkable antioxidant properties

The study commenced by testing the anti-oxidant capacity of *M. orbicularis*, as this was not addressed in previous studies. The antioxidant activity of the different parts of *M. orbicularis* was measured using the DPPH radical scavenging assay. Ethanolic extracts from all parts of *M. orbicularis* exhibited significant free radical scavenging activity which was concentration dependent (Figure 1). *M. orbicularis* leaves ethanolic extracts had the highest antioxidant potential (17% free radical scavenging activity at 200 μ g/ml), followed by *M. orbicularis* fruits ethanolic extracts while *M. orbicularis* stems ethanolic extracts showed the lowest antioxidant activity (Figure 1).

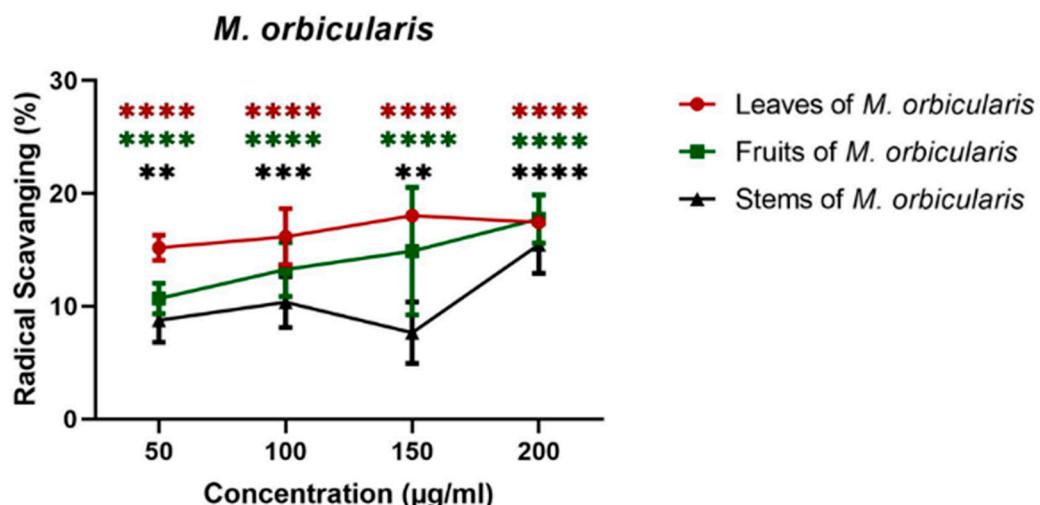


Figure 1. Ethanolic extracts of *M. orbicularis* exhibit antioxidant properties. The percentages of radical scavenging activity of different concentrations (50, 100, 150, 300 and 500 $\mu\text{g}/\text{ml}$) of leaves, fruits, and stems of *M. orbicularis* ethanolic extracts were measured by DPPH radical scavenging assay. Data are presented as the mean \pm SEM of three independent experiments ($n=3$). ** denotes $P<0.01$, *** denotes $P<0.001$, and **** denotes $P<0.0001$.

M. orbicularis plant parts ethanolic extracts reduce the viability of A549 lung adenocarcinoma cells

Antioxidant ability usually correlates with the antiproliferative effects of plant extracts [34,39,40]. Therefore, the antiproliferative effects of extracts from different aerial parts of *M. orbicularis* were tested against A549 human lung adenocarcinoma cells. A549 cells were treated with ethanolic extracts (50, 100, 150, 200 $\mu\text{g}/\text{ml}$) of *M. orbicularis* plant parts (leaves, fruits and stems) for 24, 48, and 72 h, and their antiproliferative effects were determined using the metabolism-dye-based MTT assay (Figure 2). All plant extracts reduced the number of metabolically active A549 cells in a concentration and time dependent manner (Figure 2). *M. orbicularis* leaves extracts at the concentration of 100 $\mu\text{g}/\text{ml}$ caused a significant ($P<0.05$) decrease in the number of A549 cells starting at 48 hours, compared to control vehicle-treated cells (Figure 2A). *M. orbicularis* fruits extract on the other hand significantly ($P<0.0001$) reduced A549 cell number at the same starting concentration of 100 $\mu\text{g}/\text{ml}$, but at the earlier time point of 24 hours (Figure 2B). Figure 2C, shows that *M. orbicularis* stems ethanolic extracts required a higher concentration (150 $\mu\text{g}/\text{ml}$) to cause a significant ($P<0.0001$) decrease in A549 cell number, at the later time point of 72 hours. These data show that all *M. orbicularis* parts ethanolic extracts can reduce the viability of A549 cells. Furthermore, *M. orbicularis* fruits ethanolic extracts seem to be the most potent at decreasing the viability of A549 cells.

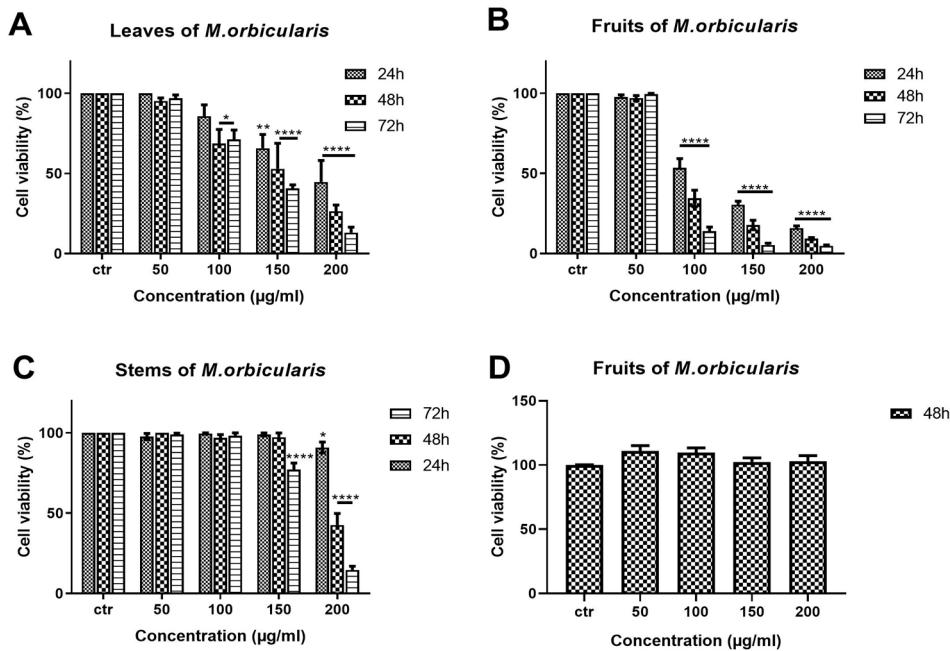


Figure 2. *M. orbicularis* plant parts ethanolic extracts reduce the viability of A549 cells. MTT assay was used to measure cell viability of A549 cells treated for 24, 48 and 72 h with increasing concentrations (50, 100, 150, 200 $\mu\text{g}/\text{ml}$) of *M. orbicularis* plant parts ethanolic extracts. A, B and C show the results of MTT cell cytotoxicity assay of A549 cells treated with the indicated concentrations of ethanolic plant extracts of *M. orbicularis* leaves, fruits and stems, respectively. D shows the cell viability of neonatal fibroblast cells treated with the indicated concentrations of *M. orbicularis* fruits ethanolic extract. Viability of treated cells was compared to the control (ctr) vehicle-treated cells. Data are displayed as the mean \pm SEM of three independent experiments ($n=3$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

Calculation of IC50 values of the different plant extracts confirmed the time- and concentration-dependent cytotoxic effect of *M. orbicularis* extracts. **Table 1** reveals that indeed *M. orbicularis* fruits ethanolic extracts have the lowest IC50 value of A549 cell viability (116.5 ± 2.06 $\mu\text{g}/\text{ml}$ at 24 hours, 91.86 ± 1.96 $\mu\text{g}/\text{ml}$ at 48 hours, and 86.18 ± 1.93 $\mu\text{g}/\text{ml}$ at 72 hours). Additionally, A549 cells were treated with the chemotherapeutic agent, cisplatin, which potently decreases A549 cell viability (**Table 1**). *M. orbicularis* fruits ethanolic extracts show a decent inhibitory effect when compared with cisplatin (around 10 times higher IC50 at 48 hours). Given that *M. orbicularis* fruits ethanolic extracts were the most effective at reducing lung cancer cell viability, we decided to perform the next experiments using *M. orbicularis* ethanolic fruit extracts.

Table 1. IC50 values (in $\mu\text{g}/\text{ml}$) of ethanolic extracts of parts of *M. orbicularis* used to treat A549 cells for 24, 48 and 72 hours. The IC50 values of cisplatin in A549 cells are also listed for comparison.

	24 hrs	48 hrs	72 hrs
A549			
Leaves of <i>M. orbicularis</i>	146.8 ± 2.16	144.6 ± 2.16	130 ± 2.11
Fruits of <i>M. orbicularis</i>	116.5 ± 2.06	91.86 ± 1.96	86.18 ± 1.93
Stems of <i>M. orbicularis</i>	310 ± 2.49	194 ± 2.29	177 ± 2.25
Cisplatin	24.73 ± 1.46	9.56 ± 1.07	4.71 ± 0.88

The antiproliferative effects of *M. orbicularis* ethanolic extracts appear to be specific to cancer cells. *M. orbicularis* fruits ethanolic extracts did not decrease the number of normal human neonatal fibroblast cells, even at the highest tested concentration of 200 $\mu\text{g}/\text{ml}$ (**Figure 2D**). In comparison, the same concentration of the fruits extracts caused more than 90% decrease in the number of A549 cells at 48 h, compared to control vehicle-treated cells (**Figure 2B**).

M. orbicularis fruit ethanolic extracts induce apoptosis of A549 lung adenocarcinoma cells

Herbal extracts have been reported to act by inducing apoptosis in cancer cells [16]. To assess whether *M. orbicularis* reduced the viability of A549 cells by inducing apoptosis, we treated A549 cells with *M. orbicularis* fruit extracts for 48 h and used Western blotting to evaluate the protein levels of apoptosis effector proteins PARP-1 and proCaspase 3, the anti-apoptotic protein BCL-2 and the pro-apoptotic protein BAX, hallmark proteins of apoptosis. Activation of Caspase 3 is a central irreversible event in the process of apoptosis. Once activated Caspase 3 will drive apoptosis by proteolytically digesting its substrates including DNA and proteins like PARP-1 [41]. Initiation of apoptosis involves an increase in protein levels of the pro-apoptotic protein BAX, which acts by forming channels in the mitochondrial membrane, leading to the release of cytochrome C, initiating apoptosis. On the other hand, BCL-2 is an anti-apoptotic protein which prevents the release of cytochrome C from the mitochondrial membrane, thereby inhibiting apoptosis [42]. **Figure 3** shows a significant decrease ($P < 0.01$) in the protein levels of full length PARP-1 and proCaspase 3 in A549 cells treated with *M. orbicularis* fruit extracts. This decrease seems to be dose dependent at 100 and 150 $\mu\text{g}/\text{ml}$ concentrations of *M. orbicularis* fruit extracts. In addition, the protein levels of BAX have decreased while BCL-2 protein levels have increased leading to a decrease in the protein ratio of BCL-2/BAX, a ratio that determines the balance between anti- *versus* pro-apoptotic events. Indeed, the ratio of BCL-2/ BAX was dose dependently and significantly ($P < 0.05$), decreased by treatment of A549 cells with 150 $\mu\text{g}/\text{ml}$ of *M. orbicularis* fruit extracts. These results indicated that *M. orbicularis* fruit extracts dose dependently induced apoptosis in A549 cells and that apoptosis may be responsible, at least in part, for *M. orbicularis*-induced cell death of A549 cells.

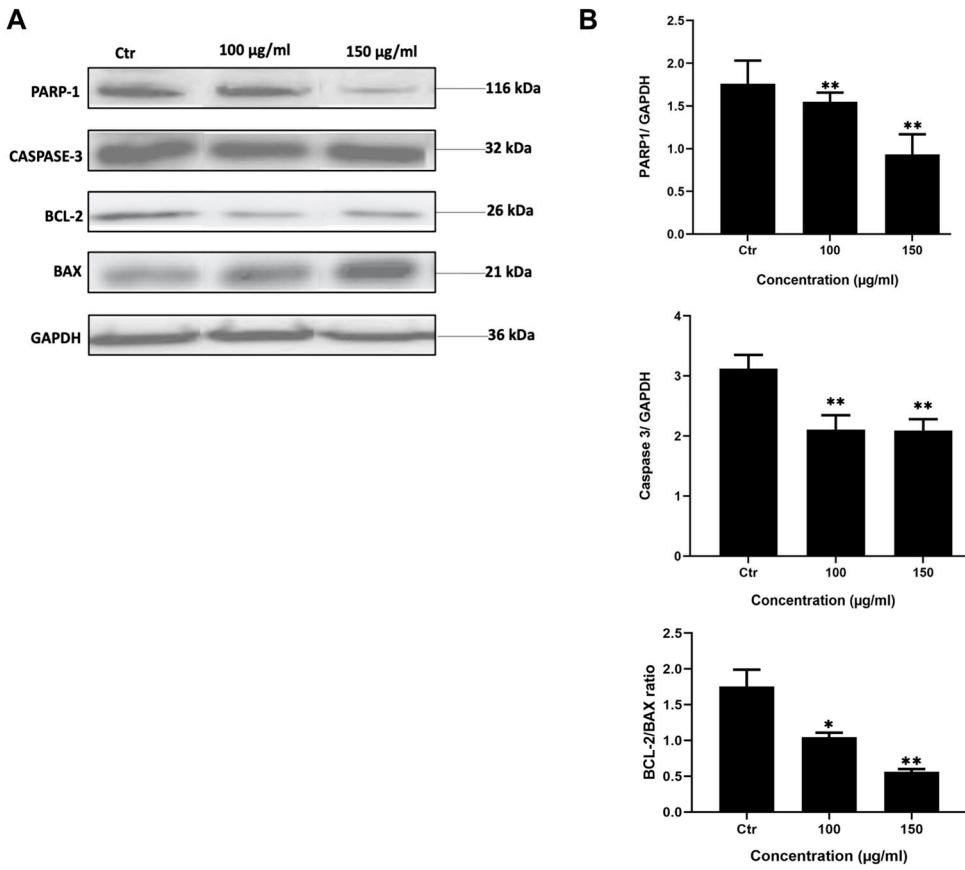


Figure 3. *M. orbicularis* fruit ethanolic extracts induce apoptosis of A549 cells. (A) Representative western blot of protein lysates from A549 cells treated 100 and 150 µg/ml of *M. orbicularis* fruit extracts for 48 h. Levels of proteins were normalized to GAPDH protein levels. (B) Quantification of images in (A). Intensity of protein bands were quantified by ImageJ software and normalized to intensity of bands of GAPDH protein. The ratio is expressed in arbitrary units. Data are presented as the mean ± SEM of 3 independent experiments. .

Ethanolic extracts of fruits of M. orbicularis reduce the migration of A549 lung adenocarcinoma cells

Cell migration is critical for proper development as well as physiological processes in adult tissues. Cell migration is also elevated in certain pathological states such as cancer. Increased cell migration may lead to enhanced cancer metastasis contributing to a malignant cancer phenotype. Non-small cell lung cancer cells (NSCLC) such as A549 cells are notorious for their highly invasive and migration potentials. We tested the ability of the ethanolic extracts of *M. orbicularis* fruits to inhibit A549 cell migration using a wound healing scratch assay. **Figure 4** shows the outcome of the wound healing scratch assay where *M. orbicularis* fruits inhibited migration of A549 cells. A549 cells treated with 100 and 150 µg/ml have traveled less distance to cover the scratched area than control cells, 12 h following scratching. **Figure 4B** shows a significant anti-migration activity ($P < 0.05$) due to treatment of A549 cells with 150 µg/ml of *M. orbicularis* fruit extracts. This shows that *M. orbicularis* may attenuate metastatic properties of A549 cells by inhibiting cell migration, a hallmark of metastasis.

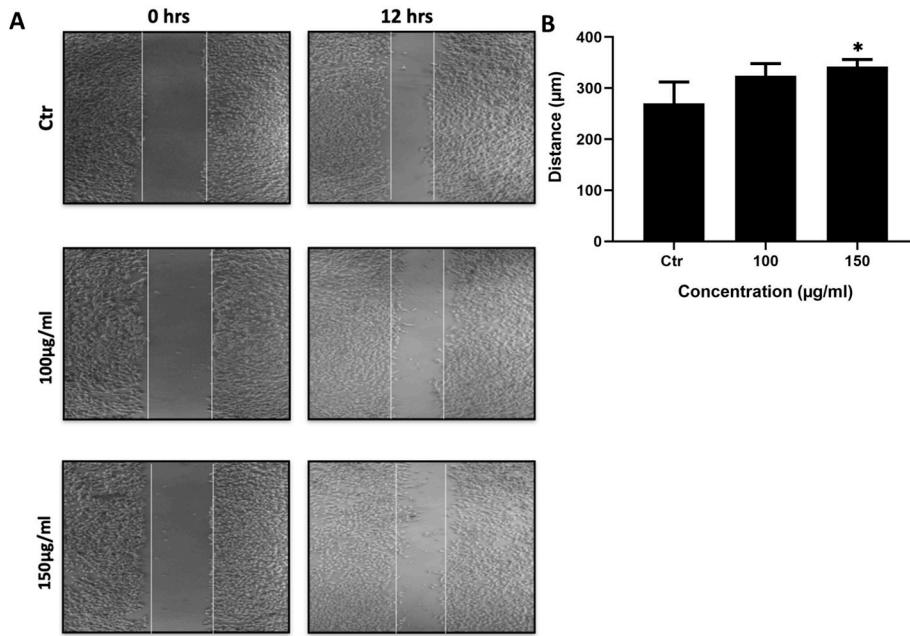


Figure 4. *M. orbicularis* fruits ethanolic extracts inhibit migration of A549 cells. (A) A confluent monolayer of A549 cells was wounded by scratching with a pipette tip. The cells were then incubated with the indicated concentrations of *M. orbicularis* fruits extracts. After 12 h, the scratch was photographed using an inverted microscope and then measured and analyzed. Values represent the fold change in migration compared to the control vehicle-treated cells. (B) Quantification of the micrographs in (A). Bar graphs represent the distance (μm) migrated by the cells after 12 h of treatment. Values represent the average of three independent experiments ($n=3$) and are represented as mean \pm SEM. * $P < 0.05$.

Ethanolic extracts of M. orbicularis fruits exhibit potent antihemolytic properties

One reason behind preference of herbal preparations and plant extracts over synthetic drugs is the general public conception that they are of natural source and therefore “safe” [30]. A drug safety profile includes the ability to safely administer a drug or herbal preparation by systemic infusion into a patient through the blood. Thus, it is empirical to test if extracts of *M. orbicularis* can impact the hemolysis of red blood cells (RBCs). A hemolysis assay was performed to test if *M. orbicularis* fruits ethanolic extracts can induce hemolysis of RBCs. Ethanolic extracts of *M. orbicularis* fruits did not cause hemolysis of RBCs (data not shown).

This result prompted us to test the ability of the ethanolic extracts from the fruits of *M. orbicularis* to protect RBCs against hemolysis which usually results from the use of chemotherapeutic agents in cancer therapy. To this end, an anti-hemolysis assay was performed. Indeed, pretreatment of RBCs with different concentrations (40, 50, or 100 $\mu\text{g}/\text{ml}$) of the ethanolic extracts from the fruits of *M. orbicularis* protected RBCs from H_2O_2 -induced hemolysis (Figure 5). Figure 5 shows that 40, 50, or 100 $\mu\text{g}/\text{ml}$ of ethanolic extracts of *M. orbicularis* fruits significantly inhibited hemolysis of RBCs. For example, at 100 $\mu\text{g}/\text{ml}$ fruit extracts of *M. orbicularis* caused a significant 75.65 % inhibition of RBCs hemolysis. This result sheds the light on the safety of fruit extracts of *M. orbicularis* and underscores the potential use of these extracts in combination therapy with classical therapeutic agents, known to induce RBCs hemolysis.

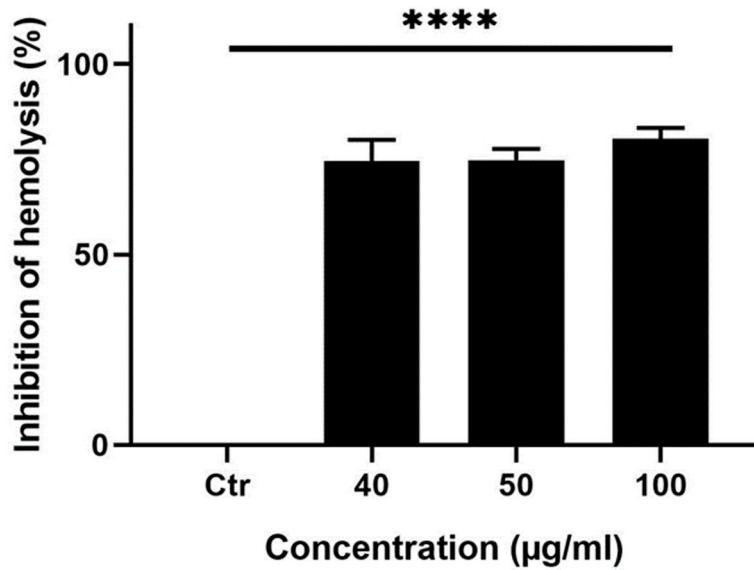


Figure 5. *M. orbicularis* fruits ethanolic extracts show potent anti-hemolytic activity. Erythrocytes were pretreated with different (40, 50 and 100 µg/ml) concentrations of ethanolic extracts from the fruits of *M. orbicularis*. H₂O₂ was then added to induce hemolysis. Data represent % inhibition of hemolysis compared to control (Ctr) vehicle-treated cells. Data are displayed as the mean ± SEM of three independent experiments; **** denotes $P<0.0001$.

Ethanolic extracts of fruits of M. orbicularis enhance cisplatin-induced cytotoxicity in A549 lung cancer cells

Cisplatin has potent anti-proliferative effects against A549 cells [43], and the anti-proliferative effect of cisplatin were confirmed in this study. The IC₅₀ values of cisplatin-induced reduction of viability of A549 cells were 24.73, 9.56 and 4.72 µg/ml at 24, 48 and 72 hours, respectively (Table 1 and Figure 6A). These values are comparable to the values previously reported in the literature [43,44].

Many types of cancer including lung cancer develop resistance to chemotherapy and as a result they usually relapse following chemotherapy with cisplatin or other chemotherapeutic agents [45]. Combination therapy can help to overcome the chemoresistance that develops against chemotherapeutic agents. When combined with chemotherapy, plant extracts with anti-cancerous properties may offer a way of overcoming chemoresistance and cancer relapse. This is because plant preparations usually have a different mechanism of action than chemotherapeutic agents [16]. Therefore, we assessed the effect of a combination treatment using ethanolic extracts of *M. orbicularis* fruits on cisplatin-induced killing of A549 cells. A549 cells were treated for 48 h with a low-dose of cisplatin (5 µg/ml) in combination with 100 µg/ml of *M. orbicularis* fruits extracts. Cell viability assays showed that at these concentrations, cisplatin alone and *M. orbicularis* fruits extracts alone have moderate cytotoxicity levels (Table 1, Figure 2B, and Figure 6A). Hence, any enhanced cytotoxic effects can be noticed using these chosen concentrations. For example, the cell viability of A549 decreased significantly ($P<0.0001$) from 85.9 % in A549 cells treated with cisplatin alone to 25.8% in cells treated with the combination of cisplatin and *M. orbicularis* fruits extracts (Figure 6B). In addition, the combination treatment enhanced the cytotoxic effect of *M. orbicularis* fruits extracts where the A549 cell viability decreased from 34.4% in cells treated with *M. orbicularis* fruits extracts alone to 25.8% in the combination treatment (Figure 6B). These results showed that *M. orbicularis* fruits ethanolic extracts sensitized A549 cells to the cytotoxic effects of cisplatin.

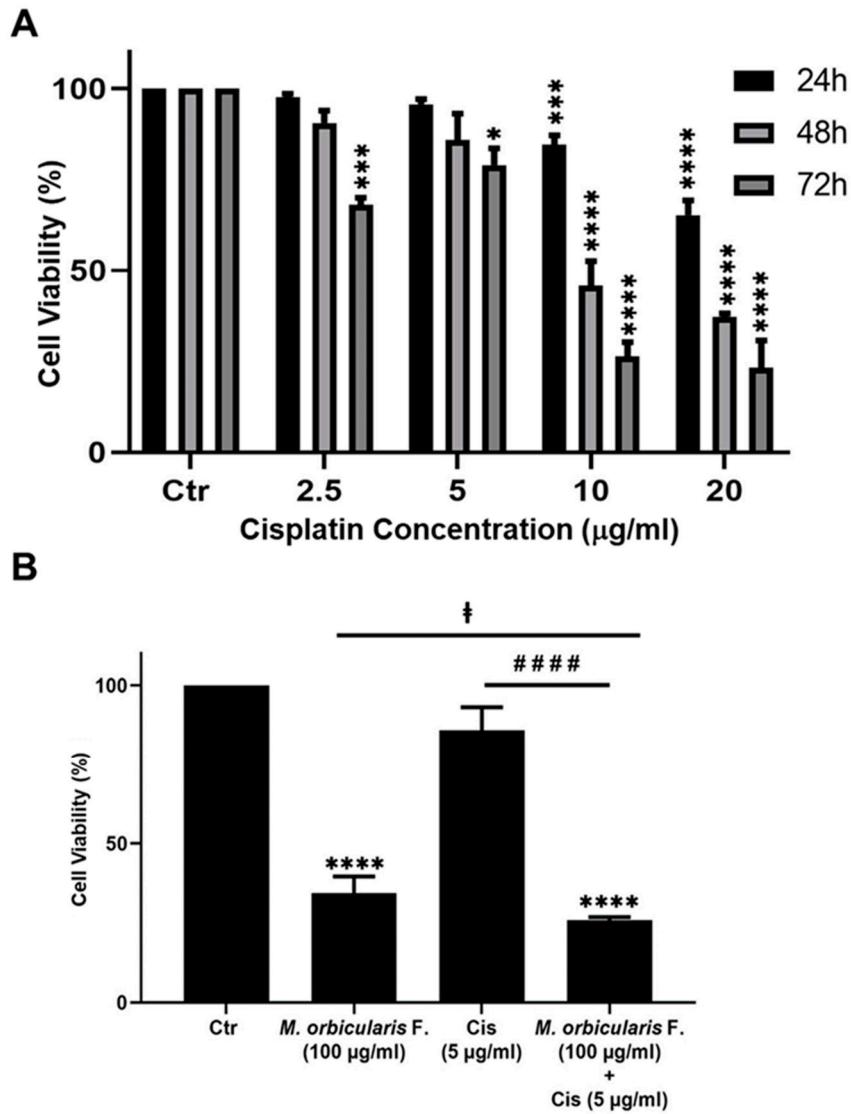


Figure 6. *M. orbicularis* enhance cisplatin-induced cytotoxicity in A549 lung cancer cells. (A) Cell viability of A549 cells treated with the indicated concentrations of cisplatin was measured using MTT assay. Data are displayed as % cell viability relative to control (Ctr) cells. (B) A549 cells were treated with fruit extracts of *M. orbicularis* (100 $\mu\text{g/ml}$) alone or combined with 5 $\mu\text{g/ml}$ cisplatin (Cis) for 48 h, and then assayed for cell viability using MTT assay. Bar graphs represent % cell viability relative to control (Ctr) cells. Data are presented as the mean \pm SEM of three independent experiments (n=3). Significant difference from control: **** $p < 0.0001$; significant difference from cisplatin treatment alone: ##### denotes $P < 0.0001$; and significant difference from *M. orbicularis* alone: † denotes $P < 0.05$. *M. orbicularis* F. denotes *M. orbicularis* fruits extracts.

Ethanol extracts of fruits of *M. orbicularis* augment cisplatin-induced aggregation of A549 lung cancer cells

Cisplatin is a standard chemotherapeutic drug used to manage different types of cancers [5], including lung cancer [46]. When cancer cells undergo metastasis, they lose their ability to adhere together in order to invade other organs [47]. When a cancer is treated with chemotherapeutic drugs, cell-cell adhesion usually increases in order to reduce metastasis [48]. Chemoresistance of cancer cells to drugs such as cisplatin results in altered cell adhesion patterns, permitting cancer cells to metastasize even under treatment [49]. Therefore, we next sought to examine the effect of *M. orbicularis* fruit extracts on the cell-cell adhesion of A549 cells. Toward this, a cell aggregation assay was performed, where A549 cells were incubated, while shaking in a non-adherent cell culture plastic

plate, in the absence or presence of the extract alone, cisplatin alone, or the extract combined with cisplatin. **Figure 7** shows that after 1 h of treatment of A549 cells with 100 and 150 μ g/ml of *M. orbicularis* fruit extracts, the percentages of cell aggregation significantly ($P<0.001$) increased by 47 % and 65 %, respectively, compared to control cells (Figure 6 A, B). However, the percentage of cell aggregation of A549 is 44 % after treatment with cisplatin (5 μ g/ml) alone and significantly increased to 71.5 % after the co-treatment with cisplatin (5 μ g/ml) and *M. orbicularis* (150 μ g/ml) (Figure 5A, B). These results showed that the *M. orbicularis* extract significantly augmented cisplatin-induced aggregation of A549 lung cancer cells aggregation.

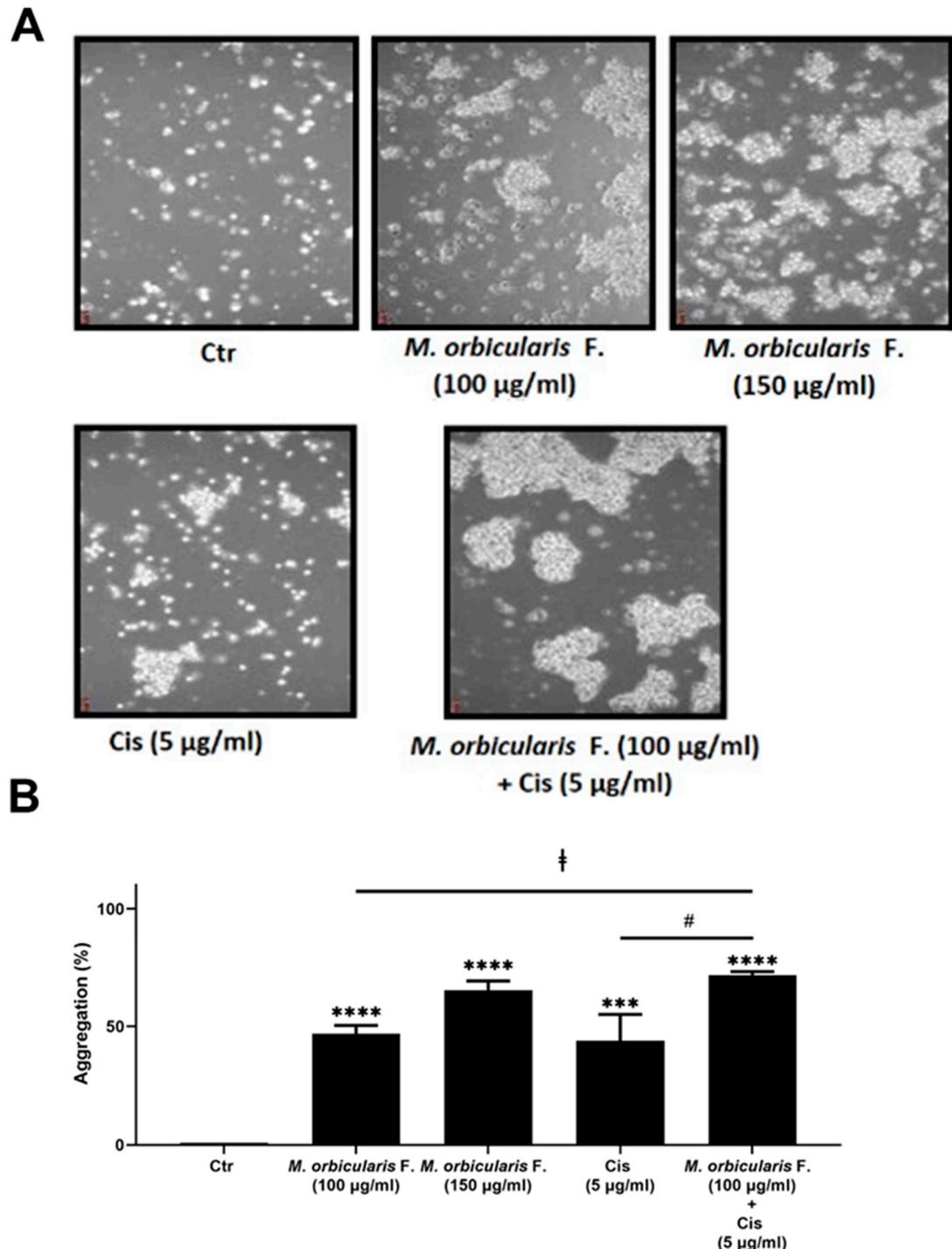


Figure 7. *M. orbicularis* augments cisplatin-induced aggregation of A549 lung cancer cells. (A) A549 cells were incubated with *M. orbicularis* alone (100 or 150 μ g/ml) or combined with 5 μ g/ml cisplatin (Cis), and then subjected to cell aggregation assay as described in the materials and methods. (B)

Quantification of the data in (A). Micrographs of cells were taken after 1h of treatment and the percentage of cell-cell aggregations was measured using the following equation: % aggregation= $(1 - \frac{N_t}{N_c}) \times 100$, where N_t is the number of single cells in the control and N_c is the number of single cells in the treated sample. Data represent the mean \pm SEM of three independent experiments ($n=3$). Significant difference from control: *** $P < 0.0001$; significant difference from Cisplatin treatment alone: # denotes $P < 0.005$; and significant difference from *M. orbicularis* extracts alone: † denotes $P < 0.05$. *M. orbicularis* F. denotes *M. orbicularis* fruits extracts.

Total polyphenols and flavonoids contents of *M. orbicularis* fruits ethanolic extracts

The anticancer properties of plant extracts are frequently attributed to their rich phytochemical content [29,50]. Results of this study have indicated that *M. orbicularis* fruits ethanolic extracts have strong antioxidant effects. These effects could be mediated by polyphenol and flavonoids [26,51,52] of *M. orbicularis* fruits extracts among other phytochemicals. Furthermore, the phytochemicals of *M. orbicularis* have not been previously identified. In order to characterize the phytochemical contents of ethanolic extract of *M. orbicularis* fruits, we measured the total phenolics and flavonoids contents of *M. orbicularis* fruits extracts. Indeed, the total phenolics content was 1.1583 ± 0.00005 mg GAE/g of dry matter of *Medicago orbicularis* fruits. Total flavonoids content was 0.0246 ± 0.00003 mg quercetin equivalents/g of dry matter of *Medicago orbicularis* fruits. These results show that *Medicago orbicularis* fruits are rich in phenolics and flavonoids and may explain the anticancer properties of *Medicago orbicularis* fruits against A549 cells.

Identification of phytochemical composition of *M. orbicularis* fruits ethanolic extracts by GC/MS

To further characterize the phytochemical contents of *M. orbicularis* fruits, GC/MS analysis was used (Figure 8). Table 2 lists 18 of the major compounds of *M. orbicularis* fruits as identified by GC/MS, by comparing their mass spectral fragmentation patterns to those of known compounds listed in the NIST library.

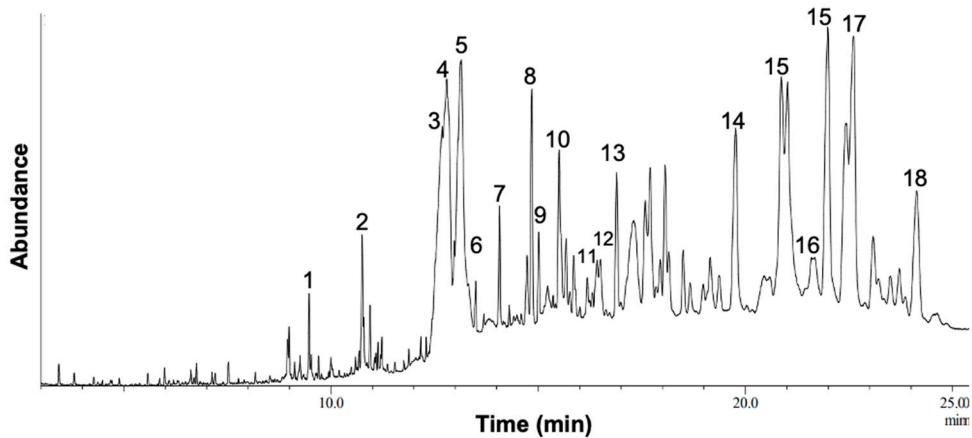


Figure 8. GC/MS chromatogram of *M. orbicularis* fruit ethanolic extract displaying the elution profile of phytochemicals listed in Table 2. The detected phytochemicals are arranged according to their retention time, compound name, and molecular weight.

Table 2. Results of GC-MS analysis of *M. orbicularis* fruits extracts. The Table presents the name of the identified compounds, their chemical nature and retention time RT(min), and their molecular formula and molecular weight (MW). The listed compounds have highest similarity indices to candidates in the NIST database.

S.N ^o	Name of the Compound	Compound Nature	RT(min)	Molecular Formula	MW
1	7-Hexadecenal, (Z)-	Unsaturated aldehyde	9.530	C16H30O	238
2	Octadecanamide, N-(2-hydroxyethyl)-	Fatty acid	10.95	C20H41NO2	327
3	Lup-20(29)-en-3-ol, acetate, (3.beta.)	Triterpenes	12.690	C32H52O2	468
4	6-methoxy-2-methyltetrahydroquinoline	Quinolines	12.88	C32H50N6O2	550
5	12-Oleanen-3-yl acetate, (3.alpha.) -	Triterpenoids	13.19	C32H52O2	468
6	Trismethoxyresveratrol, trans-	Polyphenol resveratrol	13.49	C17H18O3	270
7	Linoleic acid ethyl ester	Fatty acid	14.03	C20H36O2	308
8	Lup-20(29)-en-3-one	Triterpenoid	14.86	C30H48O	424
9	4-Nitrophenyl laurate	4-Nitrophenyl esters	15.15	C18H27NO4	321
10	Lupeol, trifluoroacetate	Triterpene alcohol	15.69	C32H49F3O2	522
11	N-Methyl-pseudotomatidine diacetate	Alkaloids	16.74	C32H51NO4	513
12	Pyridine, 1-acetyl-1,2,3,4-tetrahydro-5-(2-piperidinyl)-	Piperidine alkaloid.	16.91	C12H20N2O	208
13	Meristic acid	Fatty acid	17.19	C14H28O2	228
14	Oxirane	Rrganic compound	19.74	C18H36O	268
15	gamma-Sitosterol	Sterol	20.91	C29H50O	414
16	beta-Amyrone	Sesquiterpene	21.62	C30H48O	424
17	Lupeol	Triterpene alcohol	22.89	C30H50O	426
18	Dihydroniloticin diacetate	Flavonoids	24.35	C34H54O5	542

IV. Discussion

Cancer is a complex and devastating disease which affects millions of lives worldwide. Lung cancer, particularly non-small cell lung cancer, has the highest mortality rate among malignant tumors across the globe [43]. Current treatments for lung cancer such as chemotherapy and radiotherapy are associated with considerable toxicity and other side effects. In addition, lung cancer patients develop resistance to therapy and suffer from lung cancer recurrence as early as 6 months following therapy [3,4]. In this regard, there is a revived research interest in using herbal- and plant-based therapies as sources of anti-cancer bioactives. Several herbal remedies have been shown to have efficacy and minimal toxicity and side effects for disease treatment [12,13]. In addition, plants and their phytochemicals are being used as starting scaffolds for developing more potent anticancer agents [15,30]. Historically, plants or herbs and their extracts were used for the development of several important anti-cancer agents such as Paclitaxel, Camptothecin, and Vincristine [7–11,30]. Furthermore, several intricate biochemical pathways are simultaneously dysregulated in cancer [6,15], and herbal-based or herbal-derived remedies can target several molecular pathways and can become an alternative or complementary treatment to conventional cancer therapies [7,12–15,53,54]. Relatedly, multiple recent *in vitro* and *in vivo* studies demonstrated that concurrent use of natural products with conventional treatments (chemo- and radio-therapy) can synergistically sensitize tumors to therapy, enhancing therapeutic efficacy and reducing toxicities [15]. Many studies have documented the promising use of herbal plant extracts against cancer cell lines *in vitro* and *in vivo* in animal models of cancer, and are now being tested in clinical trials. These studies have shown that herbal-based remedies have antioxidant, antihemolytic, and apoptosis-inducing effects. They can also impact cell proliferation, aggregation and adhesion, migration, and metastasis [16,17,55].

Medicago orbicularis L. Bartal is an understudied plant species as far as its therapeutic effects are concerned. Except for a study reporting a traditional use of *M. orbicularis* for heart diseases management in Turkey [33], there are no reports on the therapeutic applications or phytochemical composition of *M. orbicularis*. Notably, *M. orbicularis* anti-cancerous activities have not been explored yet [18]. It was therefore pertinent to study phytochemical composition, antioxidant capacity, antihemolytic properties, and the effects of *M. orbicularis* on cell proliferation and apoptosis, cell aggregation, and cell migratory potential against human lung adenocarcinoma A549 cells, in addition to the combinatorial effect of a treatment of both the chemotherapeutic agent cisplatin and *M. orbicularis* on A549 cells.

There are no studies currently on the anti-oxidant potential of *M. orbicularis*, but other species of the genus *Medicago* such as *M. polymorpha*, *M. sativa*, *M. arabica*, and *M. truncatula* were shown to have potent anti-oxidant activities and to be rich in phytochemicals [22,23]. Since anti-oxidant activity is often times accompanied by therapeutic effects [27–32], this study was initiated with an evaluation of the antioxidant activity of the ethanolic extract of different plant parts of *M. orbicularis* including its leaves, fruits and stems. All plant parts of *M. orbicularis* showed anti-oxidant potential and the leaves showed the highest antioxidant activity.

To test if the anti-oxidant capacity of *M. orbicularis* is accompanied by an anti-cancerous effect, we tested the cytotoxic effect of *M. orbicularis* (leaves, fruits and stems) on A549 human lung adenocarcinoma cells. All *M. orbicularis* plant parts inhibited proliferation of A549 cells, but *M. orbicularis* fruits exhibited the highest reduction of viability of A549 cells and were chosen to perform the rest of the experiments of the study. The National Cancer Institute (NCI, USA) considers an IC₅₀ of 30 µg/mL to indicate a strong cytotoxic activity and a promising candidate for the further purification of a crude extract, and an IC₅₀ of 31–200 µg/ml to indicated moderate cytotoxicity [56]. The IC₅₀ of the fruit extracts at 72 h of treatment was 86.18± 1.93 µg/mL, indicating that this crude extract has moderate cytotoxicity. To date, no other study has reported the cytotoxicity levels of *M. orbicularis* against cancerous cells. Importantly, the cytotoxic effects of the fruit extracts were showed selectivity to A549 cancerous cells and did not affect human neonatal fibroblast cells even at high concentrations. This indicated that *M. orbicularis* fruits extracts may not have side effects when used *in vivo*.

Plant bioactives can decrease viability of cancer cells through several mechanism including induction of apoptosis [7,12–15,53,54]. Cytotoxic agents can cause an increase in the expression of the pro-apoptotic protein BAX that makes pores in the mitochondrial membrane leading to the release of cytochrome C [57]. Release of cytochrome C initiates the execution phase of apoptosis and activates Caspase 9 which cleave pro-Caspase 3 into active Caspase 3, the main executer effector caspase [58,59]. Active Caspase 3 can cleave many protein substrates [60], such as caspase activated DNase that fragments genomic DNA [61] and PARP-1, to promote apoptosis [59]. PARP-1 is required for DNA repair where it adds poly (ADP ribose) polymers to acceptor proteins to promote repair of DNA damage. PARP-1 cleavage by Caspase 3 inactivates PARP-1 leading to reduction of DNA repair and promotion of apoptosis [58,59,62,63]. These events are recognized as the intrinsic apoptotic pathway. The anti-apoptotic protein BCL-2 can inhibit the release of cytochrome C into the cytoplasm, thereby attenuating the intrinsic apoptotic pathway [59,64]. Western blotting analysis of lysates of A549 cells treated with 100 and 150 g/ml *M. orbicularis* fruit extract indicated that *M. orbicularis* fruit extracts activated Caspase-3, inactivated PARP-1, and lowered the expression of BCL-2 and increased the expression of BAX, suggesting the activation of the intrinsic apoptotic pathway. The results showed a significant decrease in the protein levels of full-length PARP-1 and proCaspase 3 in A549 cells in a dose-dependent manner. Moreover, there was a dose dependent decrease in the BCL-2/BAX ratio. This is the first report showing that *M. orbicularis*-induced death of A549 cells is mediated, at least partly, by the intrinsic apoptotic machinery. Other mechanisms of cell death, such as autophagy, necroptosis, or pyroptosis, may also be induced by *M. orbicularis*, and need to be tested in future studies.

As part of the safety profile of a cancer drug, its systemic infusion through the blood of a patient should not cause any hemolysis of red blood cells [65]. *M. orbicularis* fruit extracts did not cause any hemolysis of RBCs. Even more, the fruits extracts protected RBCs against hemolysis. This attested to the possible safety of these extracts in future *in vivo* or clinical studies.

Metastasis is the major culprit behind cancer-associated mortality [66–68]. Cell migration is a key requirement for the metastatic process. Although cell migration is essential in many physiological processes such as wound repair, tissue formation, and proper immune response, its deregulation is required for the initial steps of cancer metastasis as cells spread away from the primary tumor site. Cell migration involves the downregulation of cell junction and adhesion proteins and proteolysis of ECM proteins by metalloproteinases (MMPs) [67,69]. We assessed the effect of the fruit extract on migration of A549 using a wound healing assay. Non-cytotoxic concentrations of the fruit extracts of *M. orbicularis* inhibited migration of A549 cells in a concentration-dependent manner. This indicated that *M. orbicularis* fruits could attenuate the malignant phenotype of A549 cells by inhibiting cell migration and consequently metastasis. This result invites future investigation of the ability of *M. orbicularis* to inhibit the invasive potential of A549 cells through its action on MMPs, and proteins of the process of epithelial-to-mesenchymal transition (EMT), a hall mark of carcinogenesis and metastasis [27,28,67,68,70].

In order for cancer cells to migrate and metastasize, they should lose their adhesion to neighboring cells allowing them to migrate and invade at secondary tumor sites and organs [27,28,67,68,70]. Cancer therapeutics can act by strengthening cell adhesion and aggregation to prevent cell migration and metastasis [71]. In this study, an aggregation assay showed that *M. orbicularis* fruits significantly enhanced cell aggregation, signifying that the extract enhanced adhesion of A549 cells. This result further attests to the ability of *M. orbicularis* to attenuate the malignant phenotype of A549 cells. Future, studies should focus on elucidating the molecular mechanisms underlying this finding, include examination of cell adhesion proteins, connexins gap junction proteins, and EMT markers such as E-cadherin, N-cadherin, vimentin, Snail, among others [72].

Apart from their direct suppressive effects on carcinogenesis and cancer metastasis, herbal remedies and plant extracts have been used to complement conventional therapies since they can target several molecular pathways other than those targeted by conventional therapy, and therefore may overcome refractoriness that develops against cancer therapeutics [7,12–15,53,54]. When

combined with chemotherapy, plant-based therapies can enhance response to chemotherapy, help overcome resistance to therapy, decrease the severity of side-effects of chemotherapy including cancer-related fatigue [7,12–15]. Chemotherapeutic agents, including cisplatin can become refractory in lung cancer patients, and as result the cancer may relapse with poor prognosis [3,4]. Combination of cisplatin with a multitargeted herbal remedy may circumvent this problem. In this study, the cytotoxic effect of cisplatin alone on A549 cells was confirmed. The IC₅₀ of cisplatin with A549 that we obtained are similar to those reported in the literature [43]. In order to determine the impact of a combination treatment on cisplatin-induced death of A549 cells, we applied a co-treatment of cisplatin and the ethanolic extract of *M. orbicularis* fruits. The results showed that the combination treatment significantly augmented cisplatin-induced decrease in cell viability of A549 cells, when compared to either the extract alone, or cisplatin alone. These results show that *M. orbicularis* may be a source of a complementary therapy for lung cancer, in combination with chemotherapy. The combination treatment showed a similar result when tested on aggregation of A549 cells. Co-treatment of *M. orbicularis* fruits extracts and cisplatin showed that the combination treatment significantly augmented the increase in aggregation of A549 cells, when compared to either the extract alone, or cisplatin alone. Taken together, these results support *M. orbicularis* as a source for development of anti-lung cancer drug candidates that can complement chemotherapy.

M. orbicularis phytochemical composition has not been defined yet. But, other *Medicago* species have been reported to be rich in phytochemicals [21–23], which have anticancer activities [24–28]. Natural polyphenols are plant secondary metabolites which have two or more phenol rings. Polyphenols health benefits include being antioxidant, antidiabetic, cardioprotective, neuroprotective. Relatedly, phenolics have been reported to have strong anticancer effects through various mechanisms including removal of cancer cells, inhibition of cell cycle, and induction of apoptosis, inhibition of metastasis among others [52]. Flavonoids are natural polyphenols with documented anticancer and antioxidant properties. Flavonoids have been reported to inhibit carcinogenesis by suppressing oxidative stress through their antioxidant activities [26,51]. Our results indicated that *M. orbicularis* fruits ethanolic extracts have strong antioxidant and cytotoxic effects against A549 cells. These effects could be mediated by polyphenols and flavonoids of *M. orbicularis* fruits extracts among other phytochemicals. In this study, *Medicago orbicularis* fruits were shown to be rich in phenolics and flavonoids, which may explain the anti-oxidant and anticancer properties of *Medicago orbicularis* fruits against A549 cells.

To further evaluate the phytochemical composition and establish a more comprehensive profile of the phytochemical constituents of *Medicago orbicularis* fruits, GC-MS analysis was conducted. We identified 18 prominent peaks that correspond to bioactive compounds of *Medicago orbicularis* fruits. *Medicago orbicularis* ethanol extract was found to be a complex mixture of various classes of phytochemicals, including flavonoids, alkaloids, diterpenes, triterpenes, sesquiterpenes, sterols, alcohols, aldehydes, fatty acids, and hydrocarbons. Among the diverse array of phytochemicals present in the extract, several compounds were correlated with therapeutic properties, including anti-cancerous activities. For example, lupeol and its derivatives, which were the most frequently identified compounds, have been recently demonstrated to possess a diverse array of pharmacological activities [73]. These activities included anticancer, antimicrobial, and antidiabetic effects, with certain lupeol derivatives exhibiting greater potency than lupeol [74]. Future investigation should focus on more comprehensive assessment of the plant extracts of *M. orbicularis*, using more advanced techniques such as preparative HPLC followed by LC-MS/MS or NMR, to identify the bioactives responsible for the therapeutic effects of crude extracts from this plant.

In conclusion, this study shows for the first time that the hydroalcoholic extract of *M. orbicularis* has anti-oxidant, anti-hemolytic, and cytotoxic properties against A549 lung cancer cells. The decrease in cell viability could be at least partly mediated by the intrinsic pathway of apoptosis, involving PARP-1, Caspase 3, and BCL-2/BAX. *M. orbicularis* was also able to suppress cell migration and enhance cell aggregation of A549 cells. These activities could be attributed to the phytochemicals present in *M. orbicularis* such as polyphenols and falvonoids, and the other compounds that were

identified by GC/MS. These properties place *M. orbicularis* as potential source of new candidate effective natural agents for the treatment of the lung cancer.

Author Contributions: Conceptualization: SN, Experimental design: SN, FK, and AS; Formal analysis: AS, FK, and SN did analysis and interpretation of data; Resources: SN, FK, and AS; Performed experiments: IO, NA, and FS; Writing: original draft preparation, AS, IO, FS; Writing: review and editing, all authors; Supervision: SN, FK, and AS; Funding acquisition, SN, FK, and AS. All authors read and approved the final manuscript.

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Conflict of Interest statement: The authors declare that there are no conflicts of interest.

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