**Original Research Article**

# Anticancer activity of aqueous myrrh extract alone and in combination with cisplatin in HeLa cells

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## Abstract

**Purpose:** To study the impact of an aqueous myrrh extract on the proliferation of cervical cancer cells in vitro.

**Methods:** First, 100 g of ground myrrh resin was boiled in 1000 mL of distilled water for 30 min. Different components of the decoction were identified using gas chromatography–mass spectrometry and high-performance liquid chromatography. The effects of high (20 µg/mL) and low (10 µg/mL) concentrations of cisplatin, serial concentrations of myrrh (20, 40, 60, and 80 µg/mL), and a combination of both were evaluated using cell proliferation assay, DNA fragmentation, and electron microscopy.

**Results:** All four myrrh concentrations decreased the viability of HeLa cells (16.25 % $p < 0.01$). A significant further decrease occurred when myrrh was combined with 10 µg/mL cisplatin (11.42 % $p < 0.01$). This was confirmed by quantifying DNA fragmentation. Ultrastructurally, HeLa cells showed typical apoptosis after treatment with 10 µg/mL cisplatin or a high dose of myrrh. The use of 20 µg/mL cisplatin or the combined therapy resulted in cell necrosis with ruptured cell membranes and autophagosomes. The effect of combined therapy was more lethal than the effect of either of them alone.

**Conclusion:** Myrrh induces apoptosis and autophagy and enhances the activity of cisplatin in vitro. It is potentially a basis for further studies of other types of cancer cell. The clinical use of myrrh in the palliative therapy of human cervical carcinoma might be justified.

**Keywords:** Cervical cancer, Cell viability, Chemotherapy, DNA fragmentation, Myrrh, Cisplatin, Cell ultrastructure, HeLa cells

## INTRODUCTION

Platinating agents, including cisplatin (CDDP), are therapeutic agents used in many types of cancer affecting the gonads, female genital system, and head and neck [1]. CDDP is often administered after surgery or radiation [2]. Patients receiving platinating agents can experience dose-dependent side effects resulting in permanent toxicity and morbidity [3]. Cancer cells can also develop resistance through decreased drug vitality, which requires metabolic induction, or due to changes in apoptosis-related proteins [4,5].

Studies have examined the development of CDDP analogs to minimize its toxicity or resistance, which is crucial for the success of cancer chemotherapy [6]. Products extracted from species in the genus *Commiphora* have significant antibacterial, anti-inflammatory, and antitumor properties [7], in addition to their anti-proliferative effects in lung, pancreas, breast, and prostate cancers [8]. Therefore, this work evaluated the cytotoxic, apoptotic, autophagic, and chemosensitizing potential of the inexpensive medicinal plant, myrrh, alone and in combination with CDDP, on human cervical cancer cells *in vitro* using a cell proliferation assay, the quantification of DNA fragmentation, and electron microscopy.

## EXPERIMENTAL

### Materials

HeLa cells (CCL-81) were purchased from ATCC (USA) and Dulbecco's modified Eagle's medium (DMEM) from Biochrom (Germany). CDDP was provided by the Gynecology Oncology Unit in King Abdulaziz University Hospital (KAUH). CDDP (Ebewe Pharma, Austria, 50 mg/100 mL) was prepared as a 3.3 mM stock solution [9]. Myrrh resin was purchased from a local merchant, cleaned, and ground. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] was from Sigma-Aldrich.

### Preparation of aqueous extract of myrrh

First, 100 g of clean ground myrrh resin was added to 1000 mL of distilled water. The mixture was heated to boiling at 100 °C for 30 min. The decoction was centrifuged, filtered, frozen, and then lyophilized [10]. Then, 20 µg of the powder was dissolved in DMEM with 10 % fetal calf serum (FCS) before it was added to a monolayer of HeLa cells for 24 h.

### Gas chromatography–mass spectrometry (GC-MS)

The analysis was performed using a GCMS-QP2010 plus (Japan) and a DB-5 column (30 m × 0.25 mm i.d.; film thickness 1.00 µm) (USA). A 1.0-µL sample of the aqueous extract was injected using splitless mode. Details of the procedure have been reported elsewhere [11].

### High-performance liquid chromatography (HPLC)

The aqueous extract was partitioned with diethyl ether using a separating funnel. The diethyl ether layer was evaporated under reduced pressure until dryness. The combined diethyl ether layers were evaporated and the resulting residue was dissolved in HPLC-grade methanol. Further technical details were as described previously [12].

### HeLa cell culture

First, 10 % fetal bovine serum (FBS) and 100 µg/mL penicillin/streptomycin were added to the DMEM. For the culture and cytotoxicity experiments, $5 \times 10^4$ HeLa cells/well were added to 96-well plates in 5 % $CO_2$–95 % air at 37 °C. Triplicate wells were prepared.

### Cell proliferation assay

To evaluate the proliferation of HeLa cells, the MTT assay was used. MTT is reduced to purple formazan by mitochondrial dehydrogenase, indicating normally functioning mitochondria [13]. HeLa cells were incubated with increasing doses of CDDP (2.5, 5, 10, 20, and 40 µg/mL) for 24 h, and cell viability was assayed. Three independent experiments were performed in quintuplicate. CDDP had a dose-dependent anti-proliferative effect on cell viability. The $IC_{50}$ of CDDP was 20 µg/mL after 24 h (Figure 1).
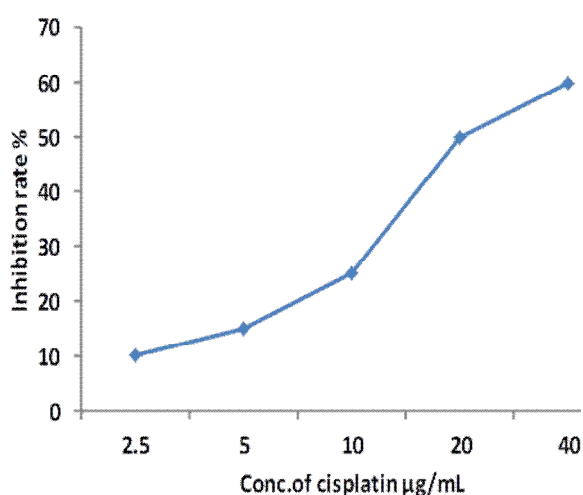


**Figure 1:** Dose- and time-dependent curve of the inhibition rate (%) of cisplatin (µg/mL) on HeLa cells using MTT assay. *Data are presented as mean ± SD of three independent experiments performed in quintuplicate. CDDP revealed a dose-dependent anti-proliferative effect on cell viability. The IC50 of CDDP was 20 µg/ml after 24 hr*

The medium was removed and replaced with high- (20 µg/mL) or low-dose (10 µg/mL) cisplatin solutions. Monolayer cells were also incubated with 20, 40, 60, and 80 µg/mL of the aqueous myrrh extract. Another group was incubated with 10 µg/mL cisplatin and the serial doses of myrrh simultaneously. Cultured cells not subjected to any treatment acted as a control group. All wells were incubated for 24 h at 37 °C in 5 % $CO_2$–95 % air. The absorption of the culture plates was measured at 570 nm after 24 h in MTT using an ELISA reader (Bio-Rad) and compared with the control cultures. All experiments were performed three times. The viability of the cultured cells was calculated as in Eq 1.

Viability (%) = {(At – Ac)/Ac}100 …………….. (1)

where At and Ac are the absorbance of drug-treated and control samples, respectively.

## Quantitation of DNA fragmentation

DNA fragmentation in cultured cells was tested using the diphenylamine technique [14]. Cells (13 × 10$^6$) were incubated for 18 h with or without CH-11 monoclonal antibody (100 ng/mL), collected, and washed with PBS. Total DNA was separated with 0.5 mL of lysis buffer {5 mM Tris-HCl, 2 mM ethylenediaminetetraacetate (EDTA), 0.5 % Triton X-100, pH 8.0}, and the lysate was centrifuged to separate high- from low-molecular-weight DNA. The cleaved DNA in the supernatant was precipitated with 5 % trichloroacetic acid and the DNA fragments were quantified using spectrophotometry at 570 nm after adding diphenylamine.

DNA fragmentation (%) was taken as the ratio of the amount of DNA in the supernatant to the total DNA in the supernatant and pellet, expressed as a percentage.

## Assessment of ultrastructural features

The cell ultrastructure was studied using transmission electron microscopy, following the protocol of the King Fahd Medical Research Center at KAUH. When the cultured HeLa cells reached confluence, they were treated with trypsin diluted in phosphate-buffered saline (PBS) at a density of 200,000 cells per mL, and fixed in 2.5 % glutaraldehyde at room temperature for at least 2 h at 4 ºC. The cells were centrifuged three times until a pellet formed. The pellet was processed as described previously [15]. Grids were examined and photographed with a transmission electron microscope (Philips CM 100) at 60 kV.

## Statistical analysis

The data were analyzed using SPSS version 22 (IBM-USA). One-way analysis of variance (ANOVA) was used to determine the significance of differences. When equal variance was assumed, the least significant difference (LSD) $t$-test was applied. The data are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

# RESULTS

The different components of the decoction of myrrh obtained using GC-MS and HPLC are listed in Table 1.

**Table 1:** Relative concentrations (%) of different chemical components of myrrh decoction

| Chemical category | Composition (%) |
|---|---|
| A-Cadinene | *3.6* |
| Cuminaldehyde | 1.8 |
| 2-Methoxyfuranodiene | *3.2* |
| 3-Epi-Alpha-Amyrin | 1.8 |
| Hydrocarbons | 0.4 |
| Phenols | 0.1 |

## Cell viability

There was a significant decrease in the viability of HeLa cells at all concen-trations of the aqueous extract of myrrh tested. Viability decreased by 16.25% on increasing the concentration from 20 to 80 µg/mL. In addition, cisplatin at both 20 and 10 µg/mL caused significant decreases in cell viability (50% and 36%, respectively). The simultaneous combi-nation of 10 µg/mL cisplatin and the serial concentrations of myrrh revealed a dose-dependent decrease in cell viability. The greatest decrease was revealed upon combination with 80 µg/mL myrrh (11.42 %) and was 35.6 % greater than that produced by the high concentration of cisplatin alone (Figure 2).

## DNA fragmentation

To detect apoptosis, the DNA fragmentation was quantified. The use of myrrh alone increased the percentage of DNA fragmentation in a dose-dependent manner. Cisplatin at 20 and 10 µg/mL induced 40.61 and 26.34 % DNA fragmentation, respectively. Moreover, the combined therapy of cisplatin and aqueous extract of myrrh enhanced DNA fragmentation and appeared to be more effective than cisplatin alone (Figure 3).

## Ultrastructural morphology

The ultrastructure of HeLa cells was studied using transmission electron microscopy. The untreated HeLa cells had intact plasma membranes with many microvilli; the nucleus had prominent nucleoli; and viable mitochondria were observed in the cytoplasm (Figure 4a).

HeLa cells treated with 10 μg/mL Cisplatinwere typical apoptotic pyknotic cells with shrunken cytoplasm and condensed nuclear chromatin (Figure 4b). On increasing the dose of cisplatin to 20 μg/mL, fragmentation of the nucleus, leakage of chromatin into the cytoplasm, development of autophago-somes, and rupture of the cell membrane were noted (Figure 4c). HeLa cells treated with the aqueous extract of myrrh contained clumps of nuclear chromatin and there was dilatation of the rough endoplasmic reticulum. Typical nuclear fragmentation was evident with higher doses (Figure 4d to Figure 4g). Combining 10 μg/mL cisplatin and increasing serial doses of myrrh resulted in progressive condensation and leakage of chromatin, loss of integrity of the cytoplasmic organelles, and the presence of autophagosomes (Figure 4h Figure 4j). In addition, the combined therapy resulted in rupture of the plasma membrane of HeLa cells (Figure 4k).
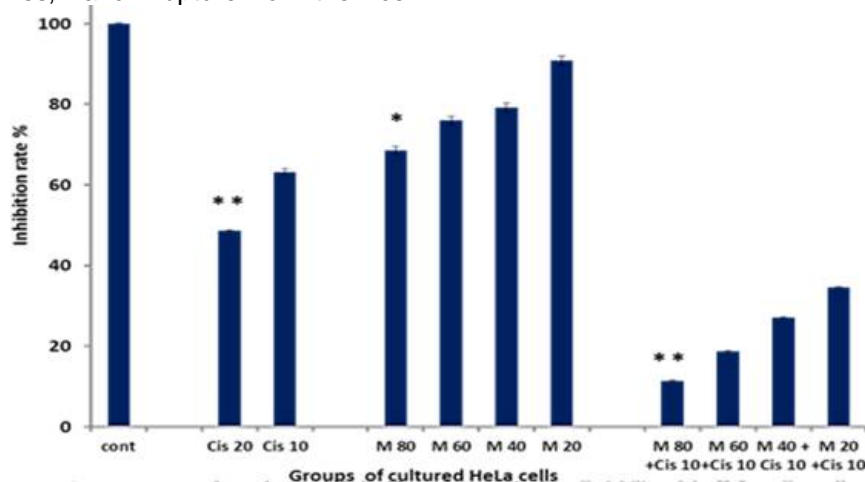


**Figure 2:** Cytotoxic effect of cisplatin and aqueous extract of myrrh on HeLa cells. *The aqueous extract of myrrh caused a significant decrease in the viability of Hela cells at all concentrations. Simultaneous combination of 10 μg/mL cisplatin (Cis) and the serial concentrations of myrrh caused a dose-dependent decrease in cell viability. Untreated cells were used as a control (Cont). The results were analysed using one-way ANOVA followed by LSD post hoc test. Values are expressed as the mean ± SD*
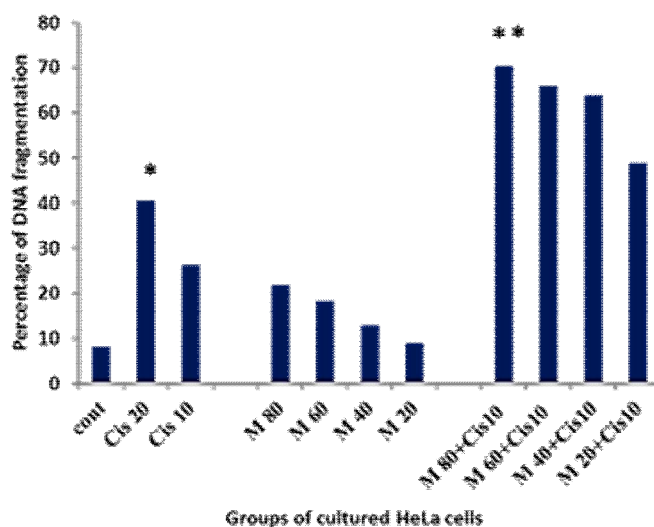


**Figure 3:** Percentage of DNA fragmentation of hela cells using cisplatin (Cis) and aqueous myrrh (m) extract separately and in combination. *There was high DNA fragmentation with combination therapy with 80 μg/ml myrrh (m) and 10 μg/ml Cis. Untreated cells were used as a control. The results were analysed using one-way ANOVA followed by LSD post hoc test. Values are expressed as the mean ± SD*
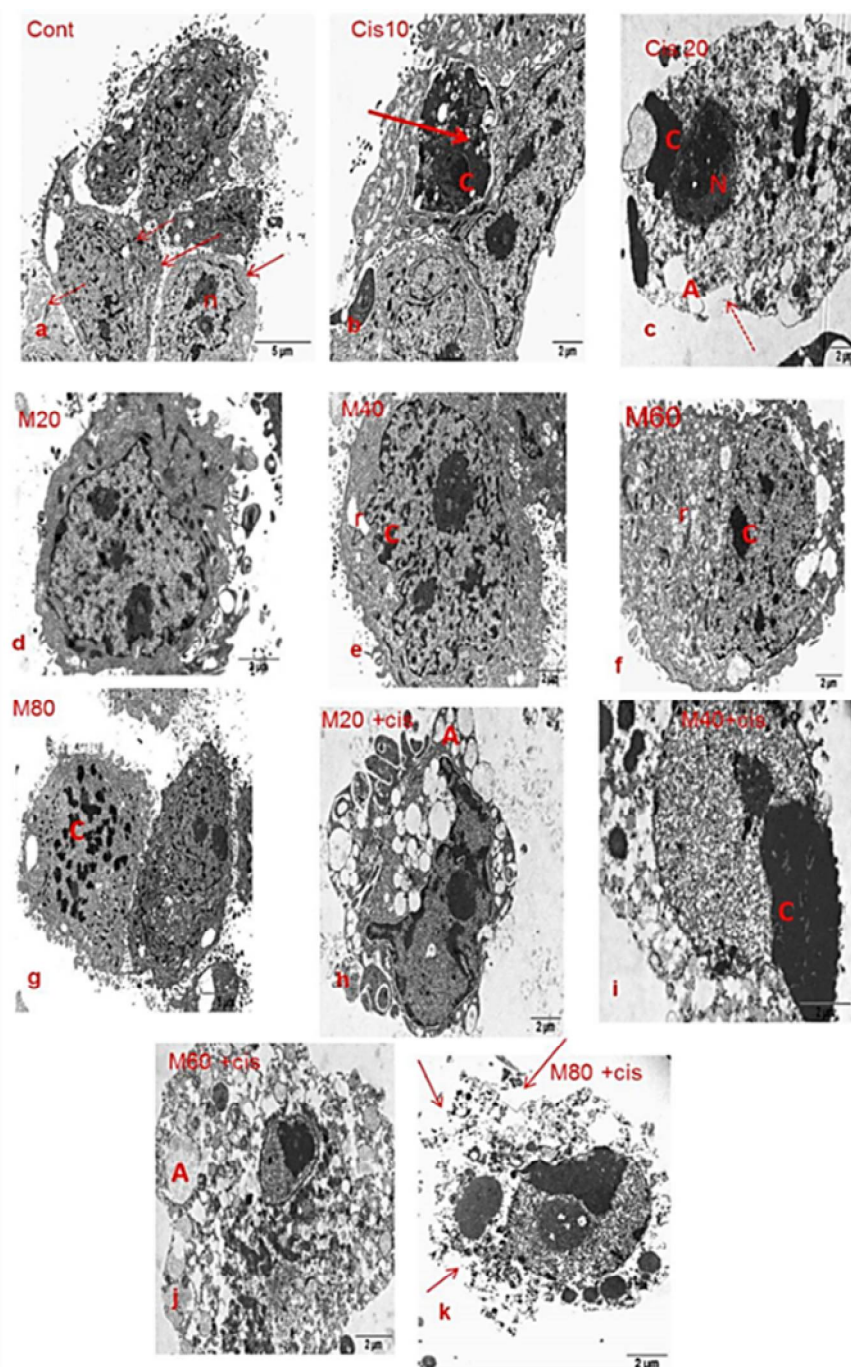
**Figure 4:** An electron micrograph showing the ultrastructural features of HeLa cells: (a) Untreated control group; (b, c) cells treated with 10 and 20 µg/mL cisplatin, respectively; (d–g) increasing doses of myrrh; and (h–k) both 10 µg/mL cisplatin and increasing doses of myrrh. *The untreated Hela cells had intact plasma membranes and numerous microvilli (thin arrows). Nuclear membrane was intact and there were prominent nucleoli (n). The cytoplasm contained many viable mitochondria (dashed arrows) (a). (b, c). Treatment with 10 and 20 µg/mL cisplatin produced typical apoptotic pyknotic cells with shrunken cytoplasm and condensed nuclear chromatin (N) (b), while in (c) there was fragmentation of the nucleus, leakage of chromatin into the cytoplasm, and cell membrane rupture. (d-g) Treatment with increasing doses of myrrh resulted in clumps of nuclear chromatin and dilatation of the rough endoplasmic reticulum. (h-k) Treatment with 10 µg/mL Cisplatin and increasing serial doses of myrrh resulted in progressive condensation and leakage of chromatin ©, loss of integrity of the cytoplasmic organelles, and the presence of autophagosomes (A); (k) shows rupture of the plasma membrane*

## DISCUSSION

This study used HeLa cells as a model to evaluate the therapeutic mechanism of myrrh in human cervical cancer by investigating its cytotoxic, apoptotic, and autophagic potential in comparison with CDDP.

Myrrh alone caused a concentration-dependent decrease in the viability of HeLa cells. In addition, the concentration of CDDP had a profound effect, decreasing the cell viability to 11.42 %. Apoptotic effects were confirmed by the DNA fragmentation assay.

A previous study found that the majority of patients with cancer use complementary and alternative medicine (CAM); however, CAM is usually not effectively integrated with conventional oncology treatment (COT) [16].

The constituents of the decoction of myrrh included sesquiterpenes, such as α-cadinene, 2-methoxyfuranodiene, cuminaldehyde, and 3-epi-α-amyrin. In trials to induce cancer cell apoptosis, sesquiterpenes arrested the cell cycle at the $G_0/G_1$ phase in prostate cancer cells. Both the antiproliferative and the immune/inflamma-tory-modulating effects of myrrh were attributed to sesquiterpenes [17].

Physicochemical assays *in vitro* characterized these components as antioxidants. Nevertheless, they were described as cytotoxic. This was explained based on the cell membrane damage and permeability of its different layers, in addition to a decrease in the mitochondrial membrane potential leading to membrane depolarization [18] and a reduction in the pH gradient resulting in the seepage of cytochrome C, calcium ions, and proteins [19].

The ultrastructural morphology of HeLa cells revealed signs of apoptosis using either (10 μg/mL) cisplatin or myrrh, while features of necrotic cell death were obvious with the higher dose of cisplatin or the combined treatment. In accordance with these results, it was reported that cisplatin can induce either apoptosis or necrosis depending on the dose [20] and the metabolic and energy status of the cells [21].

Autophagosomes were also seen with the combined treatment. Autophagy may act as a therapeutic mechanism to suppress cancer or as an adaptive mechanism to protect cells from chemotherapy. The interaction between the microenvironment and autophagy is a complicated process [22]. The interactive link between apoptosis and autophagy has been documented and it has been reported that the same factors induce autophagy and apoptosis [23]. p53, a powerful motivator of apoptosis, can also drive autophagy. Autophagy increases DRAM, a direct p53 target gene [24]. In addition, activation of the PI3 kinase/Akt pathway inhibits both apoptosis and autophagy [25]. Various biochemotherapeutic combinations have been developed to combine the cytotoxic action of cancer chemotherapy with substances that modulate the antitumor biological response. This is in addition to counteracting the suppressive effect of cancer chemotherapy on host immune/biological responses, which has a dominant effect in tumor progression [26]. Natural molecules isolated from myrrh have both immunostimulatory activity and anti-proliferative and antioxidant effects [27]. This could improve the efficacy and tolerability of standard anticancer therapies.

**CONCLUSION**

The findings of this study indicate that the use of myrrh alone and in combination with CDDP leads to apoptosis and autophagy. This is probably the basis for further studies of the effects on the survival of HeLa cells. The clinical use of myrrh in the palliative therapy of human cervical carcinoma may be justified.

# DECLARATIONS

## Conflict of Interest

No conflict of interest associated with this work.

## Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

## Open Access

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