

A COMPARATIVE STUDY OF THE EFFICACY OF DIFFERENT *ARTEMISIA CINA* EXTRACTS AND THEIR NANOPARTICULATED FORMS AGAINST A549 LUNG CANCER CELL LINE

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The present study aimed to investigate the possible antiproliferative effects of the plant extracts of *Artemisia cina* on lung cancer, and whether the nanoencapsulation of the plant extracts in the form of nanoemulsion would potentiate their therapeutic efficacy. Soxhlet extraction of the freshly collected shade-dried plant using *n*-hexane and methanol was performed, and the two extracts were loaded within nanoemulsion using the water dilution method. The nanoemulsion was characterized for its particle size, zeta potential, polydispersity, physicochemical stability under refrigeration conditions, and was morphologically visualized using transmission electron microscopy. The antiproliferative activity of the extracts and their nanoforms was tested in A549 lung cancer cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results revealed the successful preparation of the plant extract-loaded nanoemulsions with suitable particle size from 15–16 nm, a homogenous dispersion with polydispersity index (PDI) ranging from 0.23–0.31, and neutral surface charge. The nanoemulsions displayed insignificant changes in their physicochemical properties after storage for 3 months, and their spherical morphology was confirmed using transmission electron microscopy. Both *n*-hexane and methanol extracts displayed antiproliferative activity against A549 cells, with IC₅₀ values of 35.96 ± 1.7 and 41.6 ± 2.8 μg/ml, respectively. On the other hand, their respective nanoparticulated forms displayed superior antiproliferative activity, with IC₅₀ values of 12.59 ± 0.7 and 5.6 ± 0.4 μg/ml, respectively. Therefore, it can be concluded that nanoencapsulation of plant extracts would significantly potentiate their antiproliferative activity, which paves the way towards a more effective anticancer therapy.

Keywords: nanoemulsion; extract; *Artemisia cina*; A549 cells; lung cancer.

1. INTRODUCTION

Lung cancer (both small-cell and non-small-cell) represents the leading cause of death in cancer patients worldwide which might be considered as the second most common cancer type in both men and women coming after prostate and breast cancers, respectively [1–3]. Yearly, the number of deaths caused by lung cancer exceeds that resulting from colon, breast and prostate cancers altogether [4]. Survival rate of lung cancer differs according to the stage of diagnosis

with a five-year survival rate ranging from 92 to 0% for the earliest and the latest stages, respectively [5]. Despite the several advances achieved in early detection and treatment, lung cancer is still featuring poor prognosis and hence, its prevention and treatment are still unfulfilled demands [2–5].

In view of the poor prognosis obtained for the standard treatment of lung cancer, there is a continuous need for searching and finding out new drugs, particularly those obtained from natural sources such as medicinal plants. Among the most commonly used plants in herbal medicine for its wide range of bioactivities is *Artemisia cina*, family Asteraceae. The genus *Artemisia* is mainly spread in Asia, Europa and North America [6]. A plenty of studies reported its diverse bioactivities including antiseptic, antispasmodic, antimalarial and anticancer [7–10].

Recently, the incorporation of plant extracts into nanoparticles have garnered enormous scientific interests for

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being a very useful tool for minimizing the amount used of the bioactive plant extract and optimizing therapeutic outcomes through increasing the bioavailability of the used amount of plant extracts [11, 12]. Moreover, the encapsulation of actives in nanoparticulated forms was reported to particularly potentiate the anticancer effect of those actives [13–16]. Among the different types of nanoparticles, nanoemulsions present a very useful form for encapsulation of therapeutics, owing to the presence of aqueous and oily domains in presence of surfactants and cosurfactants [17, 18]. In our continuing research, a sample of Jordanian wormwood (*Artemisia cina*, family Asteraceae) has been extracted, fractionated and assessed for its *in vitro* antiproliferative activity against lung cancer (A-549) cells as different crude fractions and the corresponding nanoparticulated forms.

2. MATERIALS AND METHODS

2.1. Plant Material and Chemicals

The aerial parts of *Artemisia cina* (Asteraceae) collected in February, 2019 during a scientific excursion at the mountains of Al-Taibah City, Al-Karak Governorate, Jordan. The plant was authenticated by one of our co-authors and a voucher specimen coded ACM-201902 was kept at Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mu'tah University.

Oleic acid, Tween 20, absolute ethanol, disodium hydrogen phosphate, potassium dihydrogen phosphate, dimethylsulfoxide, hexane, methanol, butanol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye were purchased from Sigma Aldrich Co., USA. Fetal bovine serum, Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI-1640), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, L-glutamine, gentamycin and 0.25% trypsin-EDTA were purchased from Lonza, Belgium. A549 cancer cells were obtained from the American type culture collection (ATCC, USA).

2.2. Extraction and Fractionation

Freshly collected aerial parts of *Artemisia cina* plant were left drying in the shade and then ground to yield a final weight of 500 g. Using Soxhlet extraction method, the dry ground sample (100 g) was extracted with 500 mL *n*-hexane for 6 h. The *n*-hexane extract was then separated and evaporated under reduced pressure to yield the first fraction (E₁, 1.2 g). Thereafter, the plant marc obtained was similarly extracted using 500 mL methanol for 6 h, and the methanol extract was separated and concentrated under vacuum affording the second fraction (E₂, 2.1 g).

2.3. Preparation of Nanoparticulated Extracts

A nanoemulsion form of three different plant extracts was prepared using the water titration method [17, 18], in

which 10 mg of extract was dispersed in a mixture composed of 4.1 mL Tween 20, 0.28 mL oleic acid, and 0.32 mL ethanol, and stirred on a magnetic stirrer. The mixture was titrated to a total weight of 10.0 g with water in a dropwise manner for the formation of an oil-in-water nanoemulsion loaded with *A. cina* plant extracts. Formulations of *n*-hexane and methanol extracts will be termed below as N₁ and N₂, respectively.

2.4. Determination of the Particle Size, Polydispersity Index and Zeta Potential of Nanoparticulated Extracts

The particle size, polydispersity index (PDI) and zeta potential of the prepaid nanoparticulated extracts were measured using the Zetasizer device (model ZS3600, Malvern, UK) [19].

2.5. Morphological Examination of Nanoparticulated Extracts Using Transmission Electron Microscopy

The prepared nanoparticulated extracts were visualized using transmission electron microscopy (TEM) without staining, after being dried on a carbon-coated grid (VERSA 3D, USA) [20, 21].

2.6. Assessment of the Stability of Nanoparticulated Extracts

The properties of nanoparticulated (nanoemulsion) formulations (particle size, PDI and zeta potential) were re-measured after 3-month storage at room temperature so as to assess the stability of prepared formulations [22].

2.7. Evaluation of the Cytotoxicity of Plant Extracts Compared to Their Nanoparticulated Forms with Respect to A549 Lung Cancer Cell Line

The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 µg/mL gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and were sub-cultured twice to three times weekly. The cells were suspended in the medium at concentration 5 × 10⁴ cell/well in Corning 96-well tissue culture plates, and then incubated for 24 h. The tested extracts (E₁ and E₂) and their respective nanoparticulated forms (N₁ and N₂) were then added into 96-well plates (three replicates each) to achieve twelve concentrations for each extract. Six vehicle controls with media or 0.5% DMSO were run for each 96-well plate as a control. After incubation for 24 hours, the number of viable cells was determined by the MTT assay [23]. Briefly, the media were removed from the 96 well plates and replaced with 100 µL of fresh RPMI 1640 culture medium, then 10 µL of 12 mM MTT stock solution (5 mg of MTT per 1 mL of PBS) was added to each well including the untreated controls. Then, 96 well plates were incubated at 37°C under 5% CO₂ for 4 h. Aliquots (85 µL) of the media were taken from the wells and 50 µL of DMSO was added to each well, mixed thoroughly with a pipette, and incubated at 37°C for 10 min. Then, the optical density of each sample was measured at 590 nm with the microplate

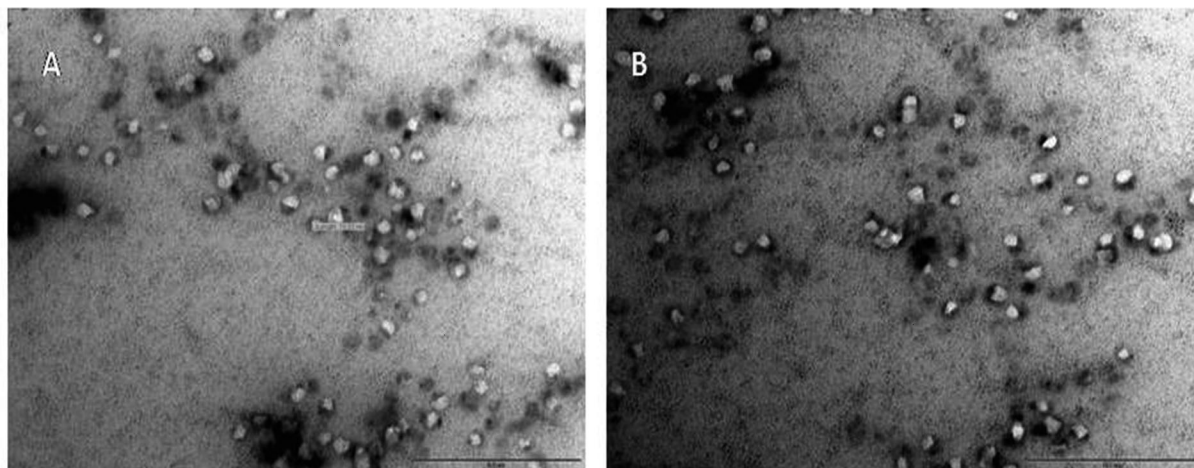


Fig. 1. TEM micrographs of (A) formulation N₁ (B) formulation N₂.

reader (SunRise, TECAN Inc., USA). The relation between surviving cells and drug concentration was plotted to obtain the survival curve after treatment with particular plant extract. The 50% inhibitory concentration (IC₅₀), defined as the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each concentration using Graphpad Prism software (San Diego, CA, USA). Percentage cell viability was calculated using the following formula:

$$\text{Viability}\% = \frac{OD_t}{OD_c} \times 100\%,$$

where OD_t and OD_c are optical densities of the treated and untreated control cells, respectively. The IC₅₀ value (concentration causing 50% cellular death) was calculated for each sample.

2.8. Statistical Data Analysis

Measurements were done in triplicate and represented as mean \pm S. D. The IC₅₀ values were calculated using Graphpad Prism software (San Diego, CA, USA), whereas the T-test at a significance level of $p \leq 0.05$ was performed using Graphpad Instat software.

3. RESULTS AND DISCUSSION

3.1. Particle Size, PDI and Zeta Potential of Nanoparticulated Extracts

The obtained results (Table 1) revealed that the nanoparticulated extracts were characterized by particle sizes ranging within 15 – 16 nm, PDI values ranging from 0.23 to 0.31, and neutral zeta potential values around 1 mV. There was no significant difference in the particle sizes, PDI values or zeta potential values of the three prepared nanoemulsions ($p > 0.05$). The small particle size obtained for these nanoemulsions could be ascribed to their surfactant and co-surfactant content [24]. The low polydispersity of nanoemulsions (less than 0.4) suggests the existence of homogenous nanoparticle formulation and the effective loading of extracts within the nanoemulsion domains. An almost neutral charge on the particles of nanoemulsions could be attributed to the non-ionic nature of the surfactant constituting the majority of the formulation.

3.2. TEM Examination of Nanoparticle Morphology in Extract Nanoemulsions

As can be seen from Fig. 1, the nanoemulsion-based extracts displayed homogenous non-aggregated spherical drop-

TABLE 1. Measurement of the Particle Size, PDI and Zeta Potential of Nanoparticulated *A. cina* Extracts as Freshly Prepared and after Storage for 3 Months at 4 – 8°C

Formulation code	Particle size (nm) freshly prepared (mean \pm S. D.)	Particle size (nm) after storage (mean \pm S. D.)	Zeta potential (mV) freshly prepared (mean \pm S. D.)	Zeta potential (mV) after storage (mean \pm S. D.)	PDI freshly prepared (mean \pm S. D.)	PDI after storage (mean \pm S. D.)
N ₁	15.03 \pm 0.97	16.71 \pm 1.24	0.76 \pm 0.14	0.67 \pm 0.09	0.23 \pm 0.09	0.21 \pm 0.02
N ₂	16.19 \pm 3.40	14.56 \pm 0.92	1.04 \pm 0.23	0.98 \pm 0.38	0.31 \pm 0.01	0.31 \pm 0.07

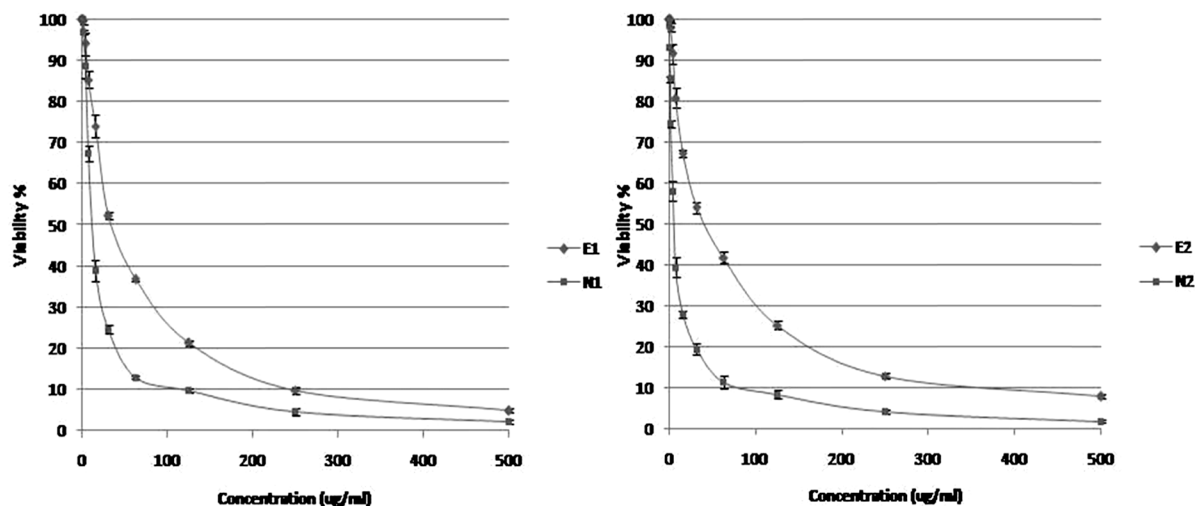


Fig. 2. Percentage viability of A549 cells versus concentration of *A. cina* extracts and their nanoparticulated forms.

lets with particle size values coinciding with the small size obtained from measurements using the Zetasizer device.

3.3. Stability of Nanoparticulated Extracts

The results of stability testing (Table 1) showed no statistically significant changes in the particle size, zeta potential or PDI of the nanoparticulated extracts after storage for 3 months ($p > 0.05$), suggesting the stable nature of the prepared nanoemulsions stored under refrigeration conditions. The stability of nanoparticles despite their neutral charge can probably be attributed to a very small size of the particles, which causes a faster Brownian motion, hence imparting kinetic stability and resistance to aggregation and sedimentation.

3.4. Evaluation of the Cytotoxicity of Plant Extracts Compared to Their Nanoparticulated Form against A549 Lung Cancer Cell Line

The results of cytotoxic activity testing of the nanoparticulated extracts in comparison to the non-encapsulated form are displayed in Fig. 2 with the IC_{50} values tabulated in Table 2. As evident from the results, both extracts of *A. cina* exhibited antiproliferative effects against A549 lung cancer cell lines, with the *n*-hexane extract (E_1) exhibiting significantly lower IC_{50} value compared to the methanol ex-

tract (E_2) ($p < 0.05$). Using solvents of different polarities for the extraction process can influence the chemical classes of natural products in each extract. This can impart differences in the pharmacological activity results. The genus *Artemisia* comprises about 470 species widely spread worldwide and referred to as “wormwood”, “sagebrush” or “tarragon” [25, 26]. Phytochemical exploration of the genus *Artemisia* afforded a plethora of bioactive natural products which can be grossly divided into terpenoids including mono-, sesqui- and triterpenes which were found to exert antiproliferative activity against liver tumor cells [27–30], in addition to polyphenolic secondary metabolites such as flavonoids, flavonoid glycosides, coumarins which were well-proven to possess antioxidant activity [30–32]. These two major chemical classes of different polarities, namely, terpenoids (non-polar) and polyphenolics (polar) explain the superior cytotoxic activity of *n*-hexane extract compared to methanol extract of *A. cina*.

Upon the encapsulation of extracts into nanoemulsions, significant potentiation of their antiproliferative activity occurred, as manifested by 3-fold decrease in IC_{50} value in case of *n*-hexane extract and more than 7-fold decrease in case of methanol extract. This can be attributed to the increased cellular uptake of polar polyphenolic constituents of methanol extract which resulted in the abrupt increase in its cytotoxic activity when nanoparticulated. However, in case of *n*-hexane extract with its non-polar constituents featuring good permeability through lipophilic cell membrane when nanoparticulated only yielded 3-fold decrease in its IC_{50} value compared to the non-encapsulated extract. The superior antiproliferative activity of nanoemulsions could be related to their small size, which allow better cellular uptake of the plant extract, and hence enhanced antiproliferative activity [16, 23].

TABLE 2. IC_{50} Values ($\mu\text{g/mL}$) of *A. cina* Extracts and Their Nanoforms

Cell line	<i>A. cina</i> preparation			
	Extract		Nanoform	
	<i>n</i> -Hexane (E_1)	Methanol (E_2)	<i>n</i> -Hexane (N_1)	Methanol (N_2)
A-549 (lung)	35.96 ± 1.7	41.6 ± 2.8	12.59 ± 0.7	5.60 ± 0.4

It is worthy to note that this study is a proof of the principle that *Artemisia cina* extracts can have potential antiproliferative effect on lung cancer. Future studies will include standardization of these extracts in order to provide more information about their chemical composition and to identify the exact components responsible for the antiproliferative effect.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. R. S. Herbst, J. V. Heymach, and S. M. Lippman, *New Eng. J. Med.*, **359**, 1367 – 1380 (2008).
2. R. D. Neal, F. Sun, J. D. Emery, and M. E. Callister, *BMJ*, **365**, 11725 (2019).
3. J. Ferlay, E. Steliarova-Foucher, J. Lortet-Tieulent, et al., *Eur. J. Cancer*, **49**, 1374 – 1403 (2013).
4. *American Cancer Society: Key Statistics for Lung Cancer 2019*, American Cancer Society. Atlanta, USA. January, 2019. <https://www.cancer.org/cancer/non-small-cell-lung-cancer/about/key-statistics.html>
5. P. Goldstraw, K. Chansky, J. Crowley, et al., *J. Thorac. Oncol.*, **11**, 39 – 51 (2016).
6. K. S. Bora and A. Sharma, *Pharm. Biol.*, **49**, 101 – 109 (2011).
7. R. N. Chopra, S. L. Nayar, and I. C. Chopra, *CSIR New Delhi*, **26**, 2 (1956).
8. A. Narwaria, R. L. Khosa, and S. K. Dhar, *Anc. Sci. Life*, **14**, 10 – 15 (1994).
9. T. Efferth, *Biochem. Pharmacol.*, **139**, 56 – 70 (2017).
10. G. Lian, F. Li, Y. Yin, L. Chen, and J. Yang, *JBUON*, **23**, 73 – 78 (2018).
11. T. Rasheed, M. Bilal, H. M. N. Iqbal, and C. Li, *Colloids Surf. B: Biointerfaces*, **158**, 408 – 415 (2017).
12. B. Mousavi, F. Tafvizi, and S. Z. Bostanabad, *Artif. Cells Nanomed. Biotechnol.*, **46**, 499 – 510 (2018).
13. S. Aldalaen, R. I. El-Gogary, and M. Nasr, *Drug Dev. Ind. Pharm.*, **45**, 55 – 62 (2019).
14. M. Fadel, K. Kassab, D. A. Abd El Fadeel, et al., *Drug Dev. Ind. Pharm.*, **44**, 1809 – 1816 (2018).
15. R. Said-Elbahr, M. Nasr, M. A. Alhnan, et al., *Eur. J. Pharm. Biopharm.*, **103**, 1 – 12 (2016).
16. L. Ramzy, M. Nasr, A. A. Metwally, and G. A. S. Awad, *Eur. J. Pharm. Sci.*, **104**, 273 – 292 (2017).
17. M. Nasr, S. Abdel-Hamid, N. H. Moftah, et al., *Curr. Drug Deliv.*, **14**, 426 – 432 (2017).
18. M. Nasr, and S. Abdel-Hamid, *Drug Dev. Ind. Pharm.*, **42**, 636 – 643 (2016).
19. O. Ashraf, M. Nasr, M. Nebsen, et al., *Int. J. Pharm.*, **539**, 83 – 94 (2018).
20. M. Nasr, S. Mansour, N. D. Mortada, and A. A. Elshamy, *J. Microencapsul.*, **25**, 499 – 512 (2008).
21. M. Nasr, S. Mansour, N. D. Mortada, and A. A. El Shamy, *AAPS PharmSciTech*, **9**, 154 – 162 (2008).
22. M. A. Mouez, M. Nasr, M. Abdel-Mottaleb, et al., *Int. J. Biol. Macromol.*, **93**, 591 – 599 (2016).
23. R. I. El-Gogary, S. A. A. Gaber, and M. Nasr, *Sci. Rep.*, **9**, 11064 (2019).
24. L. Deng, F. Que, H. Wei, et al., *PLoS One*, **10**, e0127291 (2015).
25. D. Obistoiu, R. T. Cristina, I. Schmerold, et al., *Chem. Cent. J.*, **8**, 1 – 11 (2014).
26. G. Tajadod, A. Mazooji, F. Salimpour, et al., *Ann. Biol. Res.*, **3**, 385 – 389 (2012).
27. J. J. Mills, R. S. Chari, J. J. Boyer, et al., *Cancer Res.*, **55**, 979 – 983 (1995).
28. X. Song, X. Wen, J. He, et al., *J. Funct. Foods*, **52**, 648 – 662 (2019).
29. A. Taleghani, S. A. Emami, and Z. Tayarani-Najaran, *Bioorg. Med. Chem.*, **28**, 1 – 22 (2020).
30. B. Koul, P. Taak, A. Kumar, et al., *J. Glyco. Lipid*, **7** (2017).
31. J. Y. Lee, E. J. Chang, H. J. Kim, et al., *Arch. Pharm. Res.*, **25**, 313 – 319 (2002).
32. J. Suresh, K. Mruthunjaya, N. Paramakrishnan, and M. N. Naganandhini, *Int. J. Curr. Chem. Res.*, **3**, 49 – 52 (2011).