Anti-Dysplastic Effect of Nettle Extract and Its Nano-Formulation on 7, 12-Dimethylbenz [A] Anthracene Induced Hamster Buccal Pouch Carcinogenesis

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Abstract:

Objective: Oral squamous cell carcinoma (OSCC) is the most common malignancy in the oral cavity with no definitive cure. In this study, chitosan loaded with nettle was used for the treatment of 7,12-Dimethylbenz[a] anthracene (DMBA)-induced oral squamous cell carcinoma compared to regular chemotherapy with 5-Fluorouracil (5-FU).**Materials and Methods: :** Sixty pathogen-free male Syrian golden hamsters were used. The control group served as a negative control with no intervention, while the remaining animals received DMBA for 16 weeks then they were divided equally into 5 groups as follows; DMBA group received no treatment, while the 5-FU group was treated with 15mg/kg of 5-FU intraperitoneally 3 times per week for 16 weeks. The Nettle group received (15mg/kg) nettle extract, while the Chitosan group was treated with 200µl chitosan only, and the Nano-nettle group received a corresponding dose of chitosan nanoparticles loaded with nettle. All the previous treatment modalities were given orally by oral gavage 3 times a week for 16 weeks. The hamster cheek pouches (HCP) were excised, processed, and stained with haematoxylin and eosin, anti-cyclin D1, and anti-Nkp44**. Results:** Both Nettle and Nano-nettle groups showed a statistically significant reduction of dysplastic changes associated with OSCC induced by DMBA. Both groups showed decreased expression of Cyclin D1 and increased NKp44 expression compared to other experimental groups($P<0.001$). **Conclusions**: Both Nettle and Nano-nettle can be considered nutraceutical therapeutic modalities targeting the cell cycle, increasing immunity, and reducing the dysplastic changes in the hamster model of OSCC.

Introduction:

Oral squamous cell carcinoma (OSCC) represents the most frequent oral neoplasms, with an incidence of almost 90%. OSCCs arise as a result of multiple molecular events that are caused by several factors like genetic predisposition, environmental carcinogens such as tobacco, alcohol, chemical carcinogens, ultraviolet or ionizing radiation, and microorganisms.[1] The genetic material of an individual is damaged due to chronic exposure to carcinogens causing mutations or amplification of oncogenes that control cell survival and proliferation. Mutations in oncogenes like cyclin D1 (CD1, cell-cycle regulators) and inactivation of tumor suppressor genes that regulate inhibition of cell proliferation may cause autonomous growth and development of invasive mechanisms.[2] Natural killer (NK) cells are innate lymphoid cells that boast several complex killing mechanisms involved in preventing tumors and controlling tumor growth without requiring prior sensitization or antigen presentation. NKp44 is a member of cytotoxicity receptor 2 (NCR2) that regulates several NK functions and other innate lymphoid cells.[3] 5-Fluorouracil (5-FU) is an antimetabolite anticancer drug, which has been used for the treatment of several types of cancers including OSCC via influencing the cell cycle and inducing apoptotic death of cancer cells.

Unfortunately, 5-FU has shown various complications such as poor absorption from gastrointestinal tract leading to reduced effectiveness and gastrointestinal toxicity, furthermore non-specific targeting of tumor cells can result in a destructive effect on both cancer and normal cells.[4] Urtica dioica L., also known as stinging nettle (SN) is an annual, wild plant of the family Urticaceae. Extracts from SN (leaves, roots, stem, stalks) have been used for the treatment of several diseases such as gout, eczema, arthritis, and anaemia along with kidney, bladder, and urinary disorders.[5] The biological effects of root extract may be attributed to its chemical composition, including phenol compounds, such as ferulic acid and polyphenols such as naringin, ellagic acid, and myricetin. The exploitation of these antioxidant and anti-inflammatory compounds defined the plant as a valuable tool against mutagenesis and carcinogenesis.[6] During processing conditions, unstable essential oils that are included in plant extracts and volatile compounds evaporate or decompose. Therefore, encapsulation of bioactive ingredients is one of the most recent and effective ways of protecting them against degradation and extending their shelf life. Various techniques have been investigated for Nano encapsulation of bioactive ingredients.[7] Ionotropic gelation is one of the most effective encapsulation methods which leads to increased stability, loading capacity, dispersibility in water, and controlled release of bioactive compounds. Chitosan is a cationic amino polysaccharide, formed by deacetylation of chitin, which is used for nanoparticle production as it is easy to extract, non-toxic, and biodegradable.[8] There is no relevant study concerning the anticancer effect of stinging nettle roots encapsulated in chitosan nanoparticles, this study aimed to evaluate the anticancer effect of nettle and its Nano-formulation in comparison to 5-FU in the OSCC hamster model.

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Materials and methods:

Chitosan and chitosan nanoparticles loaded with nettle formulation and characterization: Nettle was extracted (www.irealherb.com) and loaded on chitosan nanoparticles (Nanogate Company, Cairo, Egypt) via ionotropic gelation. For characterization Transmission electron microscope (TEM) (Nanogate company) was used, and size distribution by the intensity and zeta potential were conducted (Nawah, Almokattam, Cairo, Egypt) using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Triplicates were diluted (1:10) with distilled water for each formulation and expressed as (z- average mean) with the polydispersity index (PDI).[9] **Study design and grouping:** All experimental procedures were approved by the ethical committee of the Faculty of Dentistry, Mansoura University, and complied to ARRIVE reporting guidelines for experimental research. The National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) was followed. Sixty pathogen-free male golden Syrian hamsters were used in this study. The G*power 3.1.9.4 was used to calculate the sample size using priori F-test with an alpha error of 0.05 and a power (1-β) of 0.80 to achieve an actual power of 82.4%. Hamsters were of an average of 6 weeks old and 175 g weight. They were pre-acclimatized for one week and kept in standard temperature, relative humidity, light and dark cycle, and free access to water and a soft diet. The hamsters were coded and randomly allocated into six equal groups (10 animals each) using (the random.org list) and then housed in 10 cages. The control group served as a negative control, while the remaining 5 groups received DMBA for 16 weeks. After confirmation of carcinogenesis the remaining five groups were divided as follows; the DMPA group received no treatment and served as a positive control, 5-FU group was treated with 15mg/kg of 5-fluorouracil intraperitoneally 3 times a week for another 16 weeks (Sigma Aldrich Company, MO, USA), Nettle group received nettle root extract (15mg/kg), Chitosan group received 200µl chitosan only, while Nano nettle group was treated with 200µl chitosan nanoparticles loaded with nettle extract. All treatment the modalities except 5-FU were administrated orally via oral gavage 3 times per week for 16 weeks by the same expert technician.[10,11] **DMBA-induced modal of oral squamous cell carcinoma hamster cheek pouch (HCPs):** Right buccal pouches of hamsters of experimental groups were topically painted 3 times/week with 0.5% DMBA (Sigma Aldrich Company, MO, USA) dissolved in mineral oil (Acros organics, New Jersey, USA) using a camel hair brush.[11] **Euthanasia and Biopsy collection:** Hamsters were anesthetized and euthanized with an overdose of halothane. The HCPs were carefully dissected, washed then fixed for 24 h in 10% buffered formalin and processed for routine haematoxylin and eosin (H & E) stain[11] and Immunohistochemical staining with antibodies against CD1 (CCND1, 0.5-1mg/ml, Sigma Aldrich, USA) and NKp44 (1:2000, Abclonal comp, Woburn, USA).

Image analysis and statistical analysis: Slides were photographed using an Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 20X objective. The result images were analyzed on Intel® Core I7® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for epithelial thickness measurement and color intensity The (thickness) length of the epithelium was measured at 5 different points for each sample then the means were calculated and compared between the groups (10 samples for each group) followed by statistical analysis. For standardization, the measurements were started from the epithelial surface (keratin layer) to the basal layer.

The data were tabulated and tested for normality using the Shapiro-Whilk test and then the parametric data was analyzed using One-Way ANOVA followed by Tukey post-hoc test. The positive Immunohistochemical serial sections for each group were carefully examined to measure the intensity of the reaction in all groups. Data were coded and tabulated using Statistical Package for Social Science software computer program version 25 (SPSS, Inc., Chicago, IL, USA). The data were tested for normality using the Shapiro Wick test, One-way analysis of variance (ANOVA) followed by post-hoc Tukey tests were used. P value less than 0.05 was inferred as significant. The data were decoded for final graph presentation.

Result:

Characterization of Chitosan and Chitosan loaded nettle: Chitosan was provided in white suspension form with 85% degree of DE acetylation, with a spherical shape of average size less than 50nm by TEM. Additionally, the Z-average size for chitosan was 232.4 d.nm, Pdl 0.223 with a zeta potential of 40.4mV, while for chitosan loaded nettle Z-average size was 819.7d.nm, Pi 0.63, and zeta potential of 41.9mV.

Histological results and epithelial thickness measurement: In the control group (group I), HCP's mucosa revealed a very thin keratinized stratified squamous epithelium. The epithelial-mesenchymal interface was flat with an intact basement membrane, underlying sub mucosa with loose delicate connective tissue (CT) and mild inflammatory infiltrate. DMBA group showed severe hyperplasia with broad rete processes, cells with marked basilar hyperplasia, cellular pleomorphism, abnormal mitosis, and hyperchromatism. Also, microinvasion of the basement membrane and keratin pearls were apparent, underlying connective tissue showed invasion by epithelial nests, and marked inflammatory infiltrate. **5**-FU group HCP's mucosa still showed signs of epithelial dysplasia and hyperchromatism with drop-shaped epithelial rete peg.

Basilar hyperplasia, discontinuity of basement membrane, and invasion of underlying mesenchyme by epithelial cells were also observed.

The Nettle and Chitosan groups revealed moderate epithelial dysplasia with broad rete pegs, and moderate inflammatory cellular infiltrates in the connective tissue. The Nano-nettle group showed decreased epithelial dysplasia, intact basement membrane, and mild inflammatory cells infiltrating in underlying connective tissue. As regards epithelial thickness, there was a significant difference between the epithelial thickness between Group II (DMBA) and all other groups including the control group. Regarding variations in thickness, there were no significant differences between the control group, the 5FU, the Nettle, the Chitosan, and the Nano-nettle groups, (Figure1).

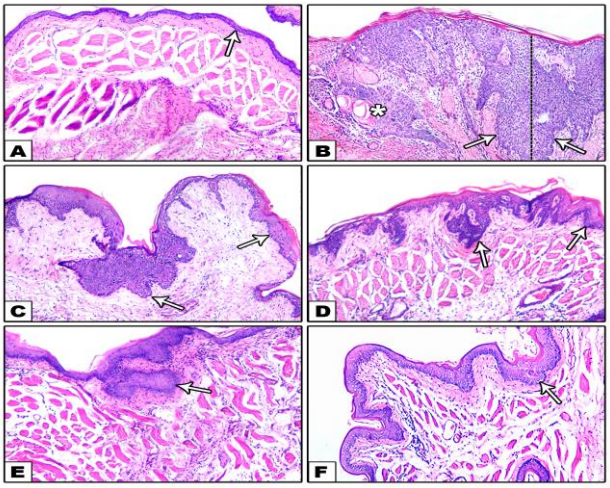

Figure 1: Photomicrograph showing HCP mucosa of group I, showing thin keratinized stratified squamous epithelium with flat epithelial mesenchymal interface with mild inflammatory infiltrate in submucosa (arrow) (A). Sever hyperplasia with broad rete process (dotted line), marked dysplastic epithelial changes with marked basement membrane discontinuity (arrow), and presence of Keratin pearl (*) (B). The epithelium of 5FU, Nettle, and Chitosan groups reveal moderate hyperplasia with drop shaped rete pegs, microinvasion, and moderate inflammatory infiltrate (arrow) (C, D, E). Nano-nettle group showed mucosa with areas of mild epithelial dysplasia. There is basilar hyperplasia, without microinvasion in group (F), (H & E 100X).

Regulatory role of nettle and its nano-formulation on cell cycle and natural killer cells: As regards CD1 results, the expression was confined to basal layers and scattered in the CT in the control group. DMBA group showed a statistically significant higher expression in all hyperplastic and dysplastic epithelial cell layers and with scattered nuclear expression in the underlying connective tissue. The 5-FU group revealed a statistically significant reduction of CD1 (P< 0.001) compared to the DMBA group. While the Nettle, the Chitosan, and the Nano-nettle groups showed a statistically significant reduction in CD1 expression intensity (p=0.000) compared to other groups. There was no significant difference between Nettle and Chitosan groups (Figures 2&4, and Table).As regards NKp44, the control, DMBA, 5-FU groups showed minimal to no expression, while the Nettle, Chitosan, Nano-nettle groups showed a statistically significant increase in the reaction of NKp44 compared with other groups. There was no significant difference between the Nettle and the Chitosan groups. The Nano-nettle group showed the highest (P<0.0001) expression, (Figures 3&4).

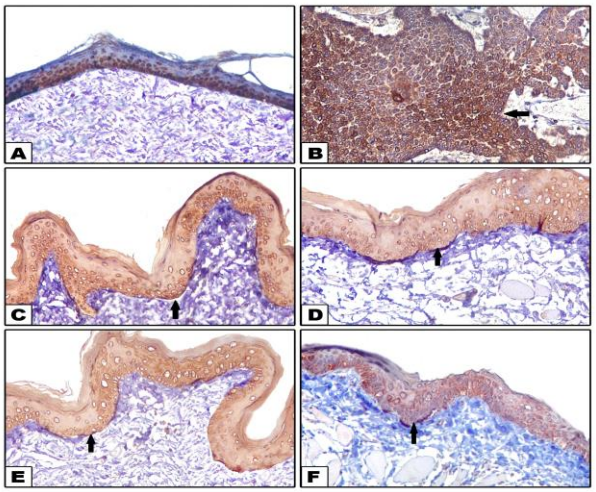

Figure 2: Photomicrograph showing HCP mucosa of different groups with variable expression of CD1. Where group I showed the lowest expression (A), while DMBA group showed highest expression of CD1(arrow)(B). 5FU, Nettle, Chitosan and Nano-nettle groups showed variable moderate to low expression (arrow) (C, D, E, F), (IHC, x200).

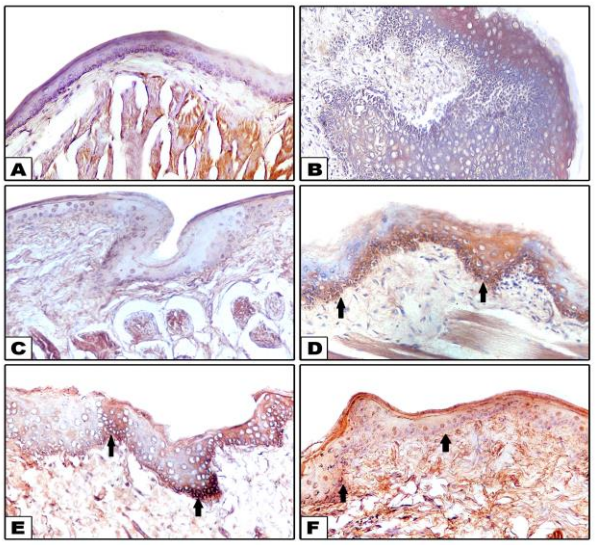

Figure 3: Photomicrograph showing HCP mucosa of different groups with variable expression of NKp44. Where group I showed the almost negative expression (A), while DMBA group showed low expression of NKp44 (B). 5-FU group showed little expression of NKp44 (C), while Nettle, Chitosan and Nano-nettle groups showed variable higher expression (arrow) (D, E, F), (IHC, x200).

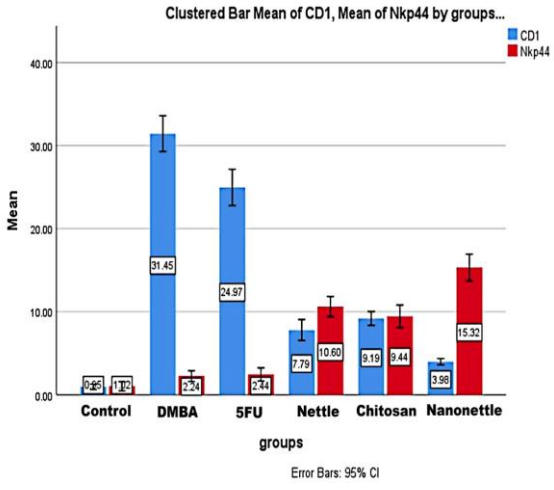

Figure 4: the percentage area of positive expressions of CD1 and NKp44 in experimental groups.

Table: One-way ANOVA followed by Tukey post-hoc test comparing the thickness of the epithelium in the different groups

	Group I control	Group II DMBA	Group III 5FU	Group IV Nano-Nettle	Group V Chitosan	Group VI combination	F value	P value
Mean& SD	13.5[b]±2.4	255.7 [a]±87.9	36.75[b]±17.54	64.49[b]±32.24	54.49[b]±34.91	41.66 [b]±19.73	44.318	0.0001

Superscript dissimilar letters represent significant difference between the groups

Discussion:

In this study, DMBA group results agreed with Zhang et al who found that DMBA application revealed that 100% of the animals with OSCC showed the most severe hyperplasia and dysplasia, due to increased mRNA and protein expression of NF-κB p50 and p65[12].The 5-FU group showed moderate signs of dysplasia compared to the DMBA group, concomitantly, Eloraby et al. reported epithelial hyper keratinization, dysplastic changes in the lower third, basal cell hyperplasia, with drop-shaped rete ridges, and loss of polarity [13]. Current results can be explained by the ability of 5-FU to target cells in the S phase causing blockage of DNA replication and triggering cell death [14].Nettle group, revealed moderate dysplasia, according to Telo et al.; animals treated with SN showed mild ductular proliferation with focal epithelial hyperplasia in chemically induced cancer in mammary tissue of rats, nettle exhibited powerful antioxidant effect via decreasing Superoxide dismutase (SOD), lipid peroxidation, and MDA levels [15]. Nettle also was found to inhibit cell growth without inducing damage to normal control cells by increasing the mRNA expression levels of caspase-3, caspase-9, And decrease in the bcl2 [16].The Chitosan group displayed moderate dysplastic changes as that shown by Wimardhani et al. who reported a cytotoxic effect of chitosan on cancer cells only. Because high positive charged amino groups in chitosan molecules are attracted to the cancer cell membrane, which has a greater negative charge than that of normal cells, chitosan interacts with the tumor cell membrane or extracellularly via a specific receptor, or via endocytosis [17].Nano-nettle group revealed the least dysplastic changes in all groups. This may be attributed to the preservation of volatile components in nettle by encapsulation process, and the improvement Of the antioxidant activity of the bioactive components compared to their free forms as a consequence of protection against unfavorable effects of oxygen and temperature, The antioxidant activity of Nano-nettle was significantly higher than that of free form nettle [18].As regards CD1 results of the control group, Rousseau et al. and Swaminathan et al. reported nuclear expression of CD1 in normal mucosa [19] in the basal and suprabasal region due to the proliferative activity of the basal layer of normal mucosa. On the contrary, Vijayalakshmi and Annamalai reported negative expression in normal samples in hamster model [20]. The DMBA group results showed the highest statistically significant expression of CD1. In OSCC, CD1 is possibly involved in a disturbance in the normal cell cycle control and mitogenic signaling pathways enhancing the cell transformation, and Tumorigenicity not only by increasing cell proliferation but also by suppressing cancer cell apoptosis [21]. In the 5-FU group, the reduction of CD1 expression might be due to the 5-FU ability to inhibit DNA synthesis causing cell death [22]. Concomitantly, Li et al. reported that 5-FU-induce changes in cell cycle regulation of oral cancer cell lines via down-regulation of cyclin D and alteration of other cyclins [23]

Similarly, Nettle, Chitosan and Nano-nettle groups showed a reduction in CD1 expression. Nettle treated group showed a reduction in CD1 expression. A previous study reported that nettle decreased cell viability and induced apoptosis in several cancer cell lines, especially Prostate Carcinoma LNCaP Cells, poorly differentiated and chemo-resistant colon cancer cells. The anti-proliferative effects of nettle extract were equivalent to anti-neoplastic drug as oxaliplatin [6].Additionally, Lichius et al stated that nettle root extract caused 51.4% growth inhibition of benign prostatic hyperplasia in Balb/c mouse [24], as pro-apoptotic and anti-tumor effects of nettle extract may be attributed to its composition of Patuletin, m/p-hydroxybenzoic acid [25]. In the chitosan group the reduction of CD1 expression was explained by Wimardhani et al. as chitosan induces apoptosis and cell cycle arrest in the oral cancer cell line. Consequently, the statistically significant reduction of CD1 in the Nano-nettle group might be due to the synergetic added effect of chitosan to nettle root extract [17].As regards NKp44, both control and DMBA groups showed minimal to no expression. These results agreed with those of Zancope et al. who reported little or no expression of NK and CD8 in patients with tongue OSCC, almost similar to control, suggesting tumorigenesis advancement via evasion of the immune surveillance in hamsters [26].5-FU group showed minimal expression of NKp44. This might be attributed to immunosuppression associated with 5-FU-based chemotherapy. Additionally, Shinko et al. reported a reduction of NK cell sub-population after long-term chemotherapy in patients with colorectal cancer [27].On the other hand, Nettle, Chitosan and Nano-nettle groups showed a statistically significant increase in NKp44 expression. Herrera et al. reported immunomodulatory effects of nettle extracts in rats with severe malnutrition promoting the differentiation of the CD4+ CD8+ T lymphocytes [28]. Urtica dioica extract significantly increased the sensitivity of the

MDA-MB-468 human breast cancer cell line to paclitaxel [15].In agreement with Chitosan group results, li et al. attributed the anti-tumor activity of chitosan to dendritic cell activation, increased NK cell INF-γ production, cytotoxic activity, and cell survival in B16 melanoma mouse model. This may explain the potentiated effect in Nano nettle group by the synergistic effect of both chitosan and nettle root extract [29]. Conversely, Yeh et al. reported non-significant effect on cytotoxic activity of NK cells, and no effect on T- and B-cell proliferation in WEHI-3 cell-generated leukaemia mice [30].

Conclusion:

Nettle extract and its chitosan nano-formulation reduced cyclin D1 and associated cell cycle. Alteration and increased natural killer cells infiltration creating a microenvironment that is unfavorable for tumor growth. Chitosan nanoparticles loaded with nettle are a promising natural product for the reduction of dysplastic changes associated with oral squamous cell carcinoma. The presented results should be considered in a higher animal with different doses to be translated to human trials.

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