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EVALUATION OF CYTOTOXIC EFFECT OF MORINGA OLEIFERA LEAF EXTRACT ON HEAD AND NECK SQUAMOUS CELL CARCINOMA CELL LINE: AN IN VITRO STUDY

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ABSTRACT

Despite new advances in better diagnosing and treating HNSCC, yet, survival rates still remain unchanged relatively. Nowadays, natural resources as plants and trees are used to cure cancer. Moringa oleifera (MO), a plant used in clinical pharmacy, grows mainly in South Africa and Asia. It is useful in the treatment of various conditions including cancer. The present study was designed to evaluate the cytotoxic and apoptotic effect of moringa oleifera leaf extract (MOLE) on head and neck squamous cell carcinoma cell line. Hep-2 cells were cultured in various concentrations (half IC50, IC50 and double IC50) for 24 hours. Cell viability was evaluated by MTT assay. Caspase 3 expression was measured using ELISA assay. In addition, Microscopic slides were prepared for each drug concentration and were photomicrographed and analyzed for the estimation of nuclear area factor. Results were compared to those of cisplatin, a well known chemotherapeutic agent. Both MOLE and cisplatin showed a concentration-dependent inhibition of cell proliferation of Hep-2 cells. The antiproliferative effect of MOLE and cisplatin was also associated with increase in caspase 3 expression as well as apoptotic morphological changes together with a decrease in the mean values of NAF which further indicates the presence of apoptosis in treated Hep-2 cells. **So,** MOLE had strong antiproliferative and apoptotic induction capability. M. oleifera leaf extracts may be used as a substitute of cancer chemoprevention.

KEYWORDS: Moringa Oleifera Leaf Extract, Head and neck squamous cell carcinoma, Cytotoxicity, Apoptosis, Cisplatin.

INTRODUCTION

Squamous cell carcinoma of the head and neck region (HNSCC) arises from the the upper aerodigestive tract mucosa and represents more than 90% of all H & N malignancies. It is the sixth most prevalent cancer worldwide. Surgery, radiation, and chemotherapy, either single or in combinations, are classically used as remedied options for patients suffering from HNSCC (Hodge et al., 2009). Chemotherapy is one of the most important modalities used to alleviatelife in advanced HNSCC. When used alone, chemotherapy can't be a sole cure method for solid tumors of HNSCC. Its role is confined to pre- or post-surgical protocols. Although clinicians work continuously to improve surgical techniques, radiation delivery and chemotherapy and although this supportive care has improved the quality of life for patients with HNSCC, unfortunately, survival as a whole has not been markedly improved (Rothenberg and Ellisen, 2012).

Generally, chemotherapeutic drug agents are sub-divided into two large families based upon their mode of action on the malignant cell cycle. Drugs non-specific for cell cycle, as alkylating agents and organic products, they act by killing tumor cells both in the resting and in the cycling phases, and drugs specific for the cell-cycle. They are most active against tumor cells specially rapidly proliferating ones that are in cycles rather than cells in resting G0 phase (Dy and Adjei, 2006).

Cisplatin is a platinum based chemotherapeutic agent. It is one of the most recently and effectively agents used in cancer treatment (Florea and Büsselberg, 2011). Cisplatin used in high dose remains the gold standard chemotherapy protocol applied in the remedy of HNSCC (Marur and Forastiere, 2016).

Despite the positive effects of platinum compounds on cancerous cells, they have poisonous impact on normal tissue. Patients treated by such drugs suffer from several harmful side effects and this limits the dose which can be taken (Florea and Büsselberg, 2011).

Phytochemicals are biologically active non-nutrient compounds that are widely distributed in plants. They have the ability to reduce the risk of occurrence of many diseases. Recent pharmaceutical researches accomplished in advanced countries have significantly ameliorate the quality and efficiency of the herbal extracts used in the cure of cancer. Part of these extracts act by protecting the body from cancer by increasing detoxification capability of the body. Others can decrease the harm of radiotherapy and chemotherapy. Researchers all over the world focus on the herbal medicines to boost immune cells against cancer (Manju et al., 2017).

Moringa oleifera L (Genus: Moringa) (Family: Moringaceae), perennial plant, (Al-Asmari et al., 2015) has been given the name of "miracle tree", or "gift of nature" or "mother's best friend", because of its elevated nutritional contents as B-carotene, fats, iron, proteins, potassium, and vitamin C added to other nutrients. Its leaves are in general the most commonly part used for extraction in medicine because it contains the highest vitamins, iron, amino acids, minerals and proteins. The antidiabetic and anticancer properties are due to the presence of flavonoids such as isothiocyanates and quercitin which are known for their anti-proliferative, anticancer power(Leone et al., 2015).

Many research papers are assessing the anti-proliferative effect of moringa and its relation to induction of reactive oxygen species (ROS) in cancer cells. Scientists proved that the ROS generated in malignant cells lead to apoptosis. This is also ascertained by the up regulation of caspase 3 and caspase 9, which are part of the apoptotic pathway (Gopalakrishnan et al., 2016).

In The present study, the effect of Moringa leaf extract on Hep-2 cells regarding the proliferation and apoptotic profile with reference to cisplatin was assessed.

MATERIALS

- Primary culture of laryngeal squamous cell carcinoma (Cancer research institute, Cairo, Egypt).
- DMEM routine culture media.
- MTT assay kit, capase-3 Elisa kit, (Sigma Aldrich Chemical co., St.Louis, U.S.A.)

METHODOLOGY

Propagation and maintenance of HNSCC cell line: -

All the procedures were carried out in the stem cells laboratory at the excellence center, ARC, Dokki, Egypt. All steps were performed in the laminar flow hood under complete aseptic conditions. Hep-2 cell line was used for cytotoxicity determination. The cells were grown in RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum in T-25 tissue culture flasks at 37°C in 5% CO2 in a CO2 incubator. Media in flasks were changed every other day. Cells were examined under inverted light microscope till reaching 70-80% confluency. Then the cells were subcutured two times a week.

2) IC50 calculation Of Moringa and Cisplatin

IC50 preparation and calculation were carried out in VACSERA labs, Cairo, Egypt. Stock drug solutions (Moringa and Cisplatin) were prepared in 100% dimethylsulphoxide (DMSO at 10 mg/ml) and sonicated to dissolve the sample. After use, the stocks were kept at –20°C. For the assays, the drugs were further diluted to the appropriate concentration using complete medium. The DMSO concentration in the wells with the highest drug concentration does not exceed 1%. Assays were performed in 96-well microtiter plates, each well receiving 100 µl of culture medium with 4 x 10^4 cells. The plates were read using an excitation wave length of 630 nm. IC50 values were determined using the microplate reader software ROBONIK P2000 EIA Reader. The absorbance data were transferred to an excel sheet and the average absorbance (mean) was calculated. To calculate cell viability, the absorbance measured in each compound was divided by the absorbance of the control cells (negative control) X 100. The compound concentrations (X axis) versus average % cell death (Y axis) were plotted. The IC50 was calculated.

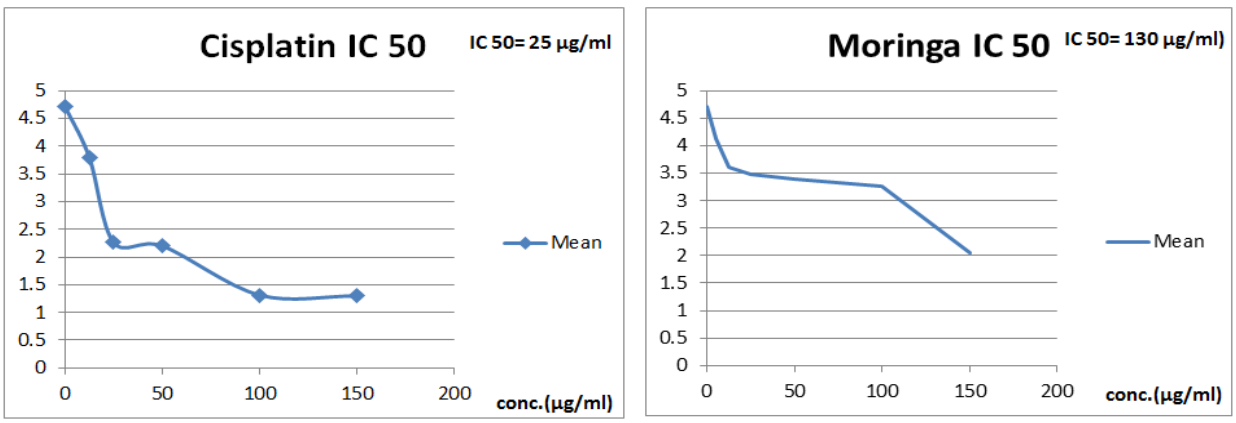

Fig. 1:- IC50 Calculation Chart.

3) Grouping

Group I: Hep-2 cells treated with Moringa Leaf Extract for 24 hours.

Group II: Hep-2 cells treated with cisplatin for 24 hours.

Group III: Control Group.

In all groups, the effect of Moringa or Cisplatin was estimated using different concentrations representing: half IC50, IC50 and twice IC50 concentrations.

4) Assessment of the effect of Moringa and Cisplatin on Hep-2 cell line

I- Cytotoxicity assay (MTT)

MTT assay is a colorimetric test used to measure the metabolic activity of cells. Color intensity is measured at 500-600 nanometers using a microplate reader. The darker the color produced, the greater the number of actively, viable cells (Bernas and Dobrucki, 2002). Before the MTT assay, one ml of cells (50,000 to 100,000 cells/ml) was placed into each well of the 96-well culture plates. Plates were then incubated for 24 hours in a CO2 incubator. Media were then removed and cells were washed with PBS. Different concentrations of cisplatin and moringa were then added and kept for 24 h. 50 µL of serum-free media and 50 µL of MTT solution were added into each well. The plates were incubated at 37°C for 3 hours. After incubation, 150 µL of MTT solvent were added into each well. Absorbance was read at OD=590 nm within 1 hour.

II- ELISA for caspase 3 apoptotic marker: *Caspase 3 Cell-Based ELISA Kit* is a convenient, lysate-free, high throughput and sensitive assay kit that can monitor caspase 3 protein expression profile in cells. The kit can be used for measuring the relative amounts of caspase 3 in cultured cells as well as screening for the effects that various treatments have on caspase 3.

III- Estimation of Nuclear Area factor assay

i. Slides preparation

After the period of treatment has elapsed, cells were detached from flasks using trypsin as previously described. Cells were collected and centrifuged to form a pellet. 50 µl were dispended on the glass slide, dried and fixed using methanol. Fixed slides were rehydrated in descending concentrations of alcohol (100%, 90%, 75% and 50%). Slides were washed in distilled water for 5 minutes. Then the slides were stained with hematoxylin and eosin stain. For each group, two slides were prepared.

ii. Photomicrography and nuclear morphometric analysis

For each slide (each concentration at each duration), ten microscopic fields were photomicrographed at the power of 1000X (oil immersion) using a digital video camera (EOS 650D, Canon, Japan) that was built in a light microscope (BX60, Olympus, Japan). Images were transferred to the computer software for further analysis. Fields were chosen having the greater number of apoptotic cells. The cells were assessed for the criteria of apoptosis. Image analysis software (Image J, 1.27 z, NIH, USA) was used for analysis. Images were corrected for brightness and contrast automatically. Images were converted into 8-bit gray scale type. Phase contrast coding of the desired areas was done automatically. The surface area and circularity of the nuclei were automatically measured. Nuclear area factor were calculated using the following equation:

Nuclear area factor= Circularity x Object area

- The data were then tabulated in Microsoft Excel sheet (Microsoft Office 2010).

iii. Statistical analysis

Data were presented as median, range, mean and standard deviation (SD) values. Kruskal-Wallis test was used to compare between different groups as well as different concentrations. Dunn's test was used for pair-wise comparisons when Kruskal-Wallis test is significant. Spearman's correlation coefficient was used to determine the correlations between different outcomes. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.

RESULTS

I. MTT assay after 24 hours

As regards Moringa group; there was a statistically significant difference between the concentrations (*P*-value <0.001, Effect size = 0.546). Control group showed the statistically significantly highest median value. Half IC50 and IC50 concentrations; both showed statistically significantly lower median values. Double IC50 showed the statistically significantly lowest median value. While for Cisplatin group; there was a statistically significant difference between the concentrations (*P*-value <0.001, Effect size = 0.613). Control group showed the statistically significantly highest median value. Half IC50 concentration showed lower median value followed by IC50. Double IC50 showed the lowest median value. With half IC50 as well as IC50 and double IC50 there was a statistically significant difference between the groups. Control group showed the statistically significantly highest median value. Cisplatin group showed statistically significantly lower median value. Moringa group showed the statistically significantly lowest median value.

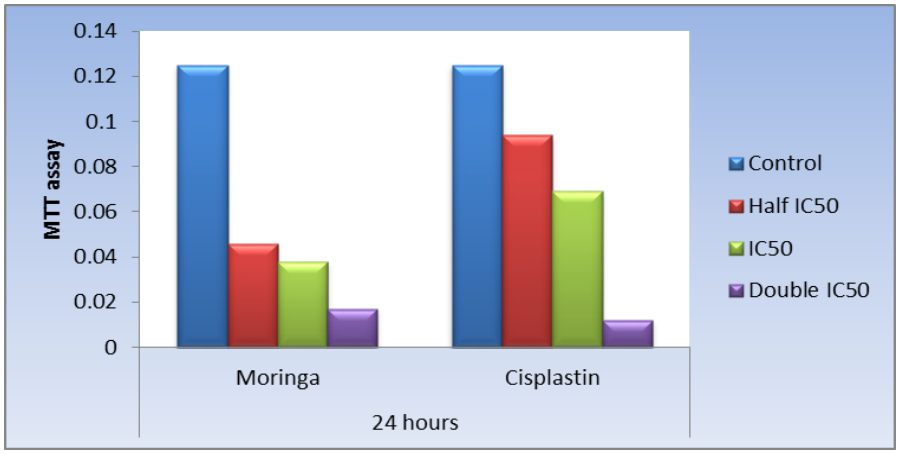

Fig. 2: Bar chart representing median values for MTT assay with different concentrations.

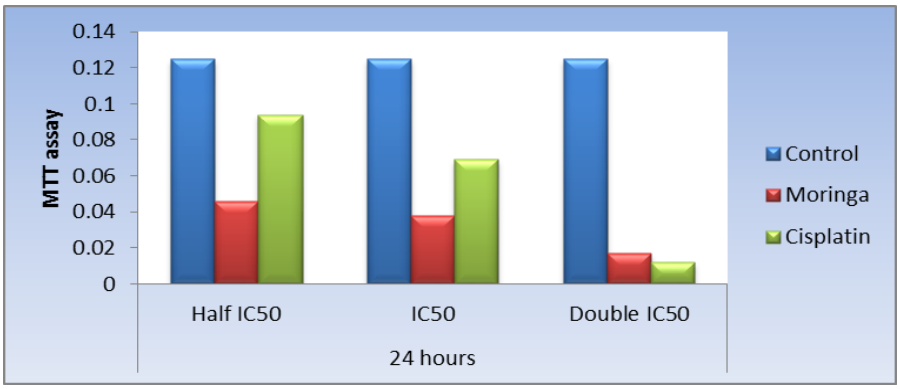

Fig. 3: Bar chart representing median values for MTT assay in the different groups.

II. ELISA after 24 hours

As regards Moringa group; there was a statistically significant difference between the concentrations (*P*-value <0.001, Effect size = 0.579). Double IC50 concentration showed the statistically significantly highest median value. IC50 concentration showed lower median value followed by half IC50. Control group showed the statistically significantly lowest median value.

While for Cisplatin group; there was a statistically significant difference between the concentrations (*P*-value <0.001, Effect size = 0.452). No statistically significant difference between double IC50 and IC50 concentrations; both showed the highest median values. Half IC50 concentration showed statistically significantly lower median value. Control group showed lowest median value. With half IC50 as well as IC50 concentrations and double IC50 there was a statistically significant difference between the groups. Pair-wise comparisons between the groups revealed that Cisplatin group showed the statistically significantly highest median value. Moringa group showed statistically significantly lower median value. Control group showed the statistically significantly lowest median value.

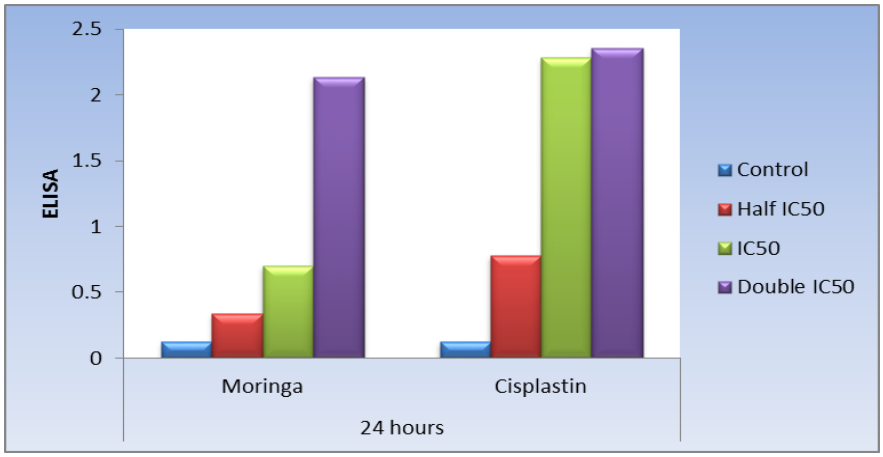

Fig. 4: Bar chart representing median values for ELISA in the different groups.

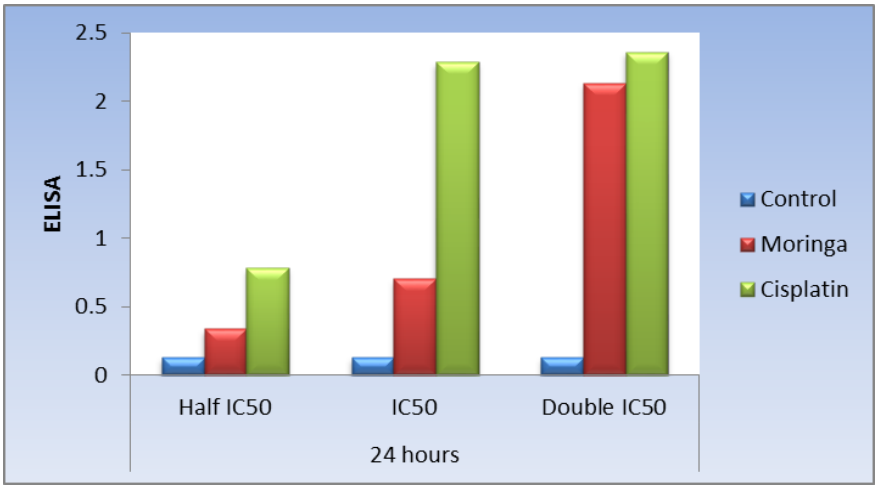

Fig. 5: Bar chart representing median values for ELISA with different concentrations.

III. Nuclear Area Factor Assay (NAF) after 24 hours

As regards Moringa group and cisplatin group there was a statistically significant difference between the concentrations. Half IC50 concentration showed lower median value followed by IC50. Double IC50 concentration showed the statistically significantly lowest median value.

With half IC50, IC50 as well as double IC50 concentrations; there was a statistically significant difference between the groups (*P*-value <0.001, Effect size = 0.528), (*P*-value <0.001, Effect size = 0.613) and (*P*-value <0.001, Effect size = 0.653), respectively. Control group showed the statistically significantly highest median value. Moringa group showed statistically significantly lower median value. Cisplatin group showed the statistically significantly lowest median value.

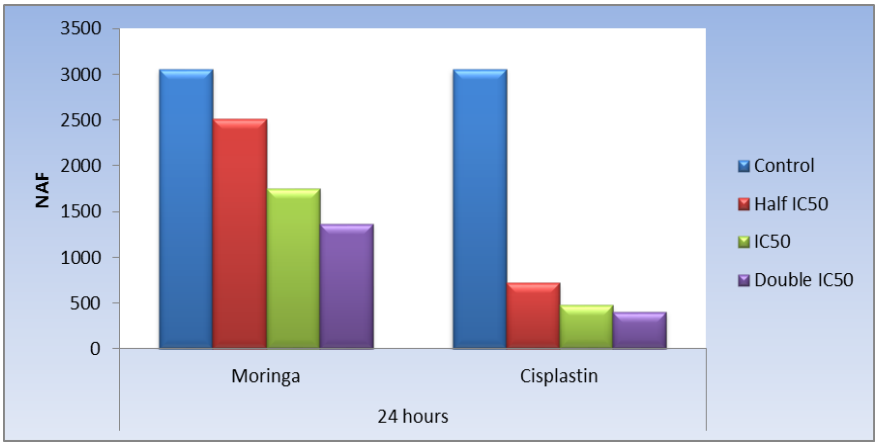

Fig. 7: Bar chart representing median values for NAF with different concentrations.

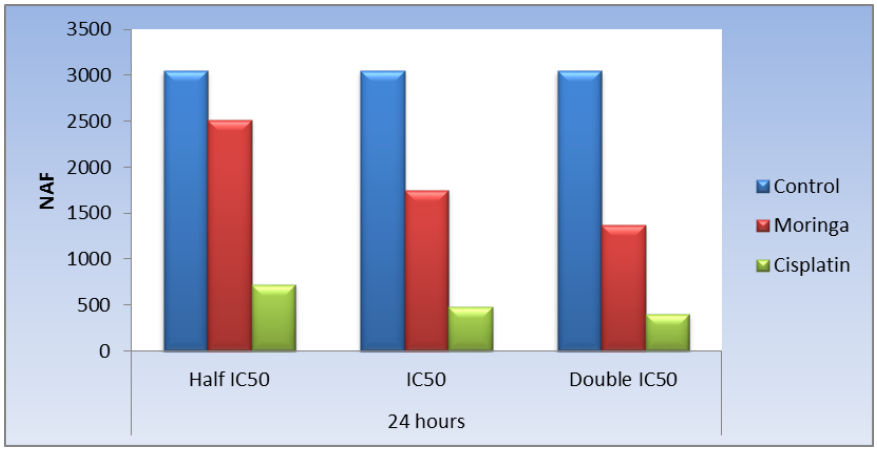

Fig. 6: Bar chart representing median values for NAF in the different groups.

IV. Correlation between different outcomes after 24 hours

There was a statistically significant inverse correlation between MTT assay and ELISA.

There was a statistically significant direct correlation between MTT assay and NAF.

There was a statistically significant inverse correlation between ELISA and NAF.

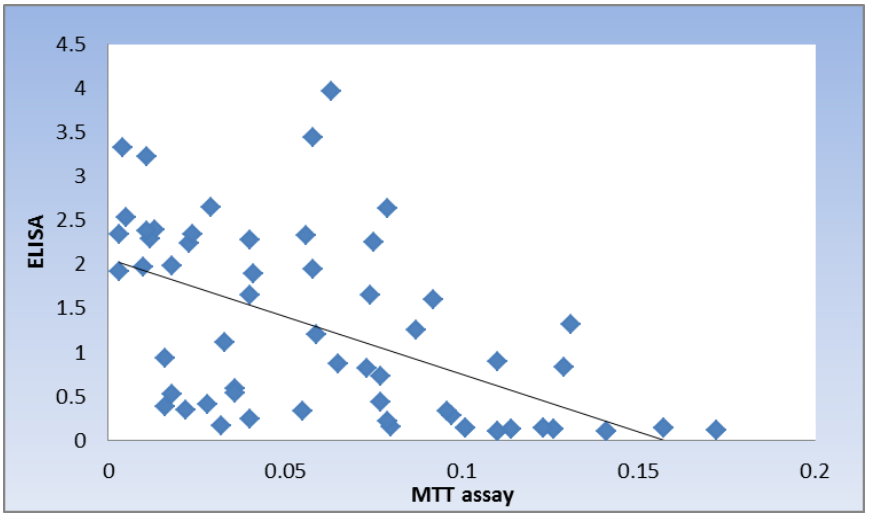

Fig. 8: Scatter diagram representing inverse correlation between MTT assay and ELISA after 24 hours.

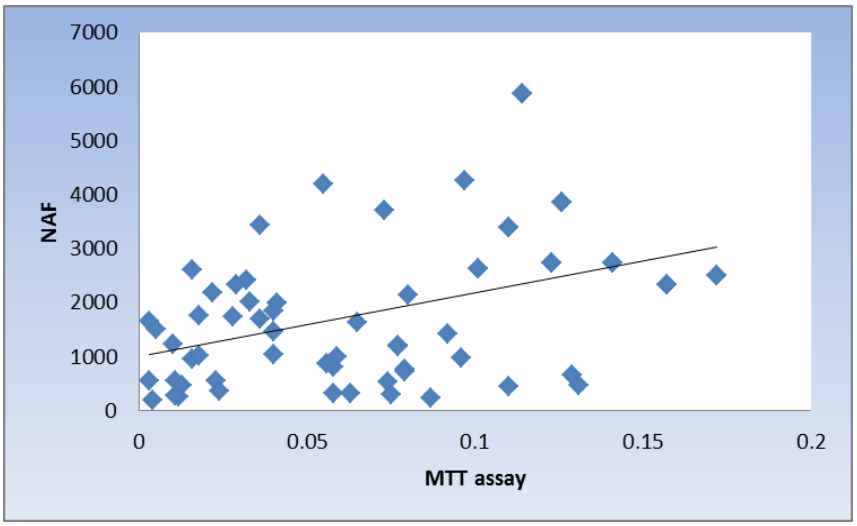

Fig. 9: Scatter diagram representing direct correlation between MTT assay and NAF after 24 hours.

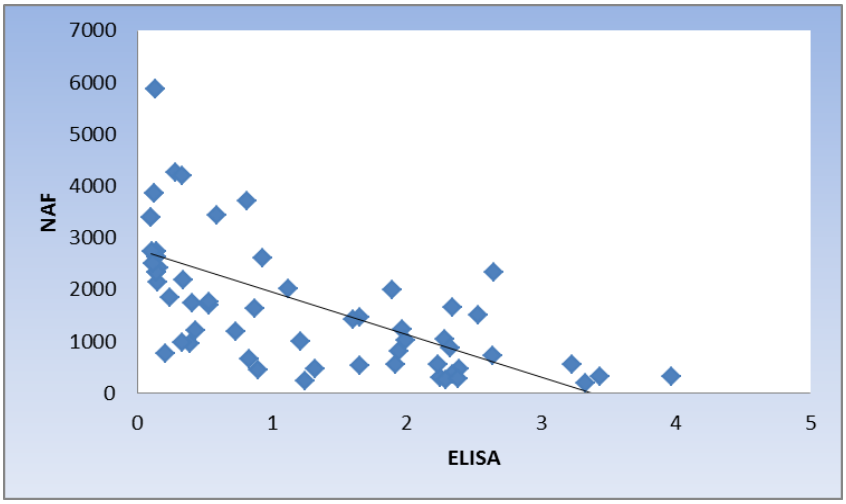

Fig. 10: Scatter diagram representing inverse correlation between ELISA and NAF after 24 hours.

V. Cytological evaluation

1. **Control cells:** most of control Hep-2 cells showed almost regular cellular outline without evidence of folding in cellular membrane. Nuclear and cellular pleomorphisms were detected among most of these cancer cells. Only few showed the early morphological criteria of apoptosis confined to peripheral condensation of chromatin against nuclear membrane (fig.11).
2. **Moringa treated Hep-2 cells and Cisplatin treated Hep-2 cells:** unlike control cells, most moringa and cisplatin treated HEp-2 cells showed morphological criteria of apoptosis in its different stages. These criteria included peripheral condensation of chromatin against nuclear membrane, irregularities in cellular membrane (fig.13, 16), cellular and nuclear shrinkage, nuclear fragmentation (fig.12) and apoptotic bodies (fig.12, 13, 14, 15, 16). In addition to these apoptotic cells, some cells still showed proliferation and pleomorphism similar to control cells (fig.12, 15). Morphological changes of necrosis were only seen in cisplatin in the form of necrotic debris (fig.17).

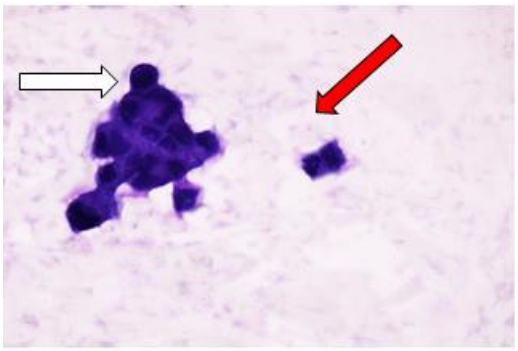

Fig. 11: Photomicrograph of control cells after 24 hours incubation showing proliferating (white arrow) and pleomorphic HEp-2 cells (red arrow) (H&Ex1000oil).

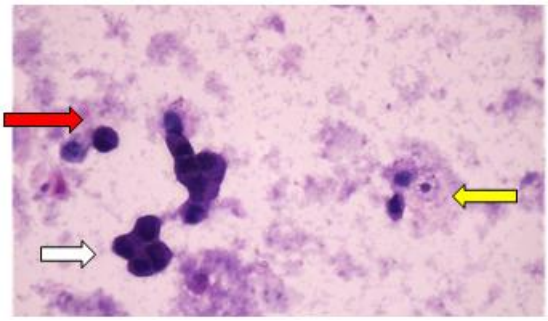

Fig. 12: Photomicrograph of HEp-2 cells 24 hours post treatment with half IC50 of moringa showing proliferating and pleomorphic HEp-2 cells (white arrow), nuclear fragmentation (red arrow), apoptotic body (yellow arrow) (H&Ex1000oil).

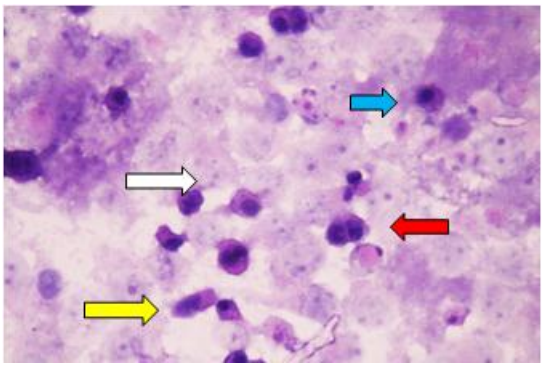

Fig. 13: Photomicrograph of HEp-2 cells 24 hours post treatment with IC50 of moringa showing irregular cellular outline (white arrow), cellular and nuclear shrinkage (red arrow), apoptotic body (yellow arrow), peripheral condensation of chromatin (blue arrow) (H&Ex1000oil).

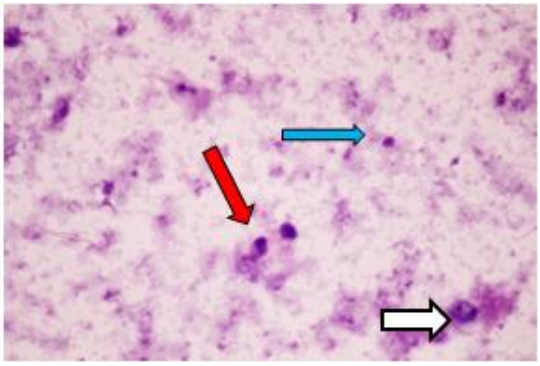

Fig. 14: Photomicrograph of HEp-2 cells 24 hours post treatment with double IC50 of moringa showing peripheral condensation of chromatin (white arrow), cellular and nuclear shrinkage (red arrow) apoptotic body (blue arrow) (H&Ex1000oil).

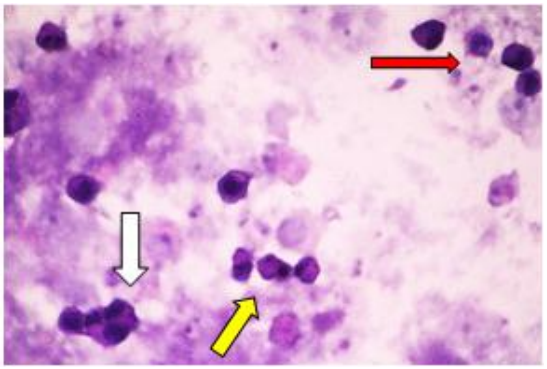

Fig. 15: Photomicrograph of HEp-2 cells 24 hours post treatment with half IC50 of cisplatin showing proliferating and pleomorphic HEp-2 cells (white arrow), apoptotic bodies (red arrow), cellular and nuclear shrinkage (yellow arrow) (H&Ex1000oil).

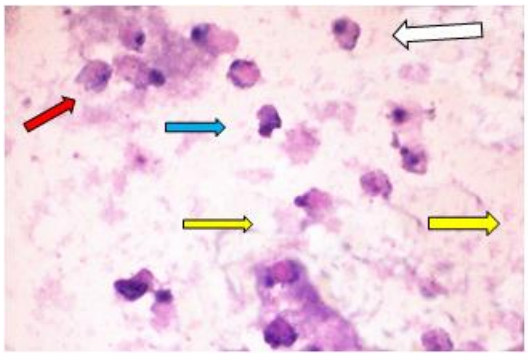

Fig. 16: Photomicrograph of HEp-2 cells 24 hours post treatment with IC50 of cisplatin showing irregular cellular membrane (white arrow), apoptotic body (red arrow), cellular and nuclear shrinkage (yellow arrow) peripheral condensation of chromatin (blue arrow) (H&Ex1000oil).

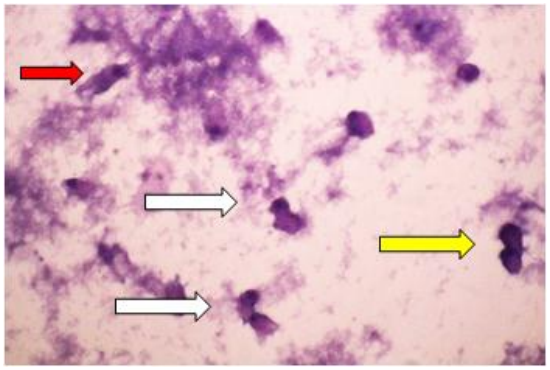

Fig. 17: Photomicrograph of HEp-2 cells 24 hours post treatment with double IC50 of cisplatin showing peripheral condensation of chromatin (white arrow), necrotic debris (red arrow), cellular and nuclear shrinkage (yellow arrow) (H&Ex1000oil).

DISCUSSION

Although recent advances in cancer management and diversity of treatment protocols, the survival rate of HNSCC patients is still below expectations (Rothenberg and Ellisen, 2012). More than 1500 anticancer agents are actively developed and more than 500 are in clinical trials. Yet we still need more efficient and less toxic protocols for managing cancer (Mehrotra and Yadav, 2006). The main regimen used to treat HNSCC is chemotherapy especially in advanced OSCC when tumors are inaccessible for surgical removal or when masses are too large to be completely excised.

In the present study, the assessment of cytotoxicity of moringa and cisplatin on Hep-2 cells revealed that both moringa and cisplatin had a clear cytotoxic effect on Hep-2 cells when compared to untreated control cells. There cytotoxic effect showed a concentration dependent manner. Double IC50 concentrations of both moringa and cisplatin showed highest cytotoxicity.
These results could be explained by several studies that found that moringa IC50 was attained in 24 h and this caused cellular death in more than 50% of cultured cells in 24h as Nair and Varalakshmi, 2011 and Tiloke et al., 2013. In contrast, Apirakramwong et al., 2018 stated that the anti-proliferative effect of moringa on cancer cells after 24 h was more than 30% inhibition and after 48 h more than 60% inhibition. In the same way, Pamok et al., 2012 proved that moringa extract was found to be toxic against colon cancer cells as the concentration and time increased (24 and 48h).

By comparing the viability of cells cultured with moringa to those cultured with cisplatin, it was found that moringa was more cytotoxic compared to cisplatin.

These results were in agreement with the results of other studies such as those of Tiloke et al., 2019, Apirakramwong et al., 2018, Gaffar et al., 2019 and Sreelatha et al., 2011 who stated that moringa cytotoxicity was dose dependant. Their researches were performed on hepatocellular carcinoma, breast and colorectal cell lines, colorectal carcinoma and cervical adenocarcinoma cell lines, breast cancer cell line and KB cancer cell line respectively.

The present study proved that MO enhanced caspase-3 mediated apoptosis compared to untreated cells. Tiloke et al showed that MO leaf extract mediates its cytotoxic effect in malignant cells via affecting viability of mitochondria and via inducing apoptosis in a ROS-dependent manner. MOE significantly causes high ROS levels. The elevated levels of ROS have a negative impact on proteins, DNA and lipids leading to damage. In response to damage od DNA, biochemical cascades occur to determine whether the cellular fate will be cell-cycle arrest, repair or apoptosis (Tiloke et al., 2019). MOE also caused an increase in Bax pro-apoptotic protein and a significant decrease in p-Bcl2 anti apoptotic protein. ATP levels are reduced in cells treated by MOE. This may be explained by the fact that ATP may have been sequestered in the form of an apoptosome, which would then activate caspase-9 via cleavage, an initiator for the intrinsic apoptotic pathway. These findings suggest that MOE might activate cell death mediated via apoptosis through the intrinsic pathway (Tiloke et al., 2019).

MOE induced apoptosis is most probably attributed to its phytochemical constituents. MOE is composed of a wide range of compounds including glucosinolates, isothiocyanates, niazimicin, quercetin and kaempferol. They all possess anticancer potential as proved by their antiproliferative effect on in Hep-2 cancer cells in the present study. (Tiloke et al., 2013), (Tiloke et al., 2019), (Al-Asmari et al., 2015).

Different viability percentage between studies may be attributed to different cell lines used with subsequent variations in cellular uptake of a same drug (Al-Asmari et al., 2015).

In the present study, ELISA assessment of caspase-3 expression in Hep-2 cells treated with moringa and cisplatin revealed that both moringa and cisplatin treated Hep-2 cells showed increased levels of caspase 3 expression in a concentration dependent manner when compared to untreated control cells. Double IC50 concentrations of both moringa and cisplatin showed highest expression of caspase 3. This was in agreement with the results of studies of Tiloke et al., 2019, Jung et al., 2015, Tiloke et al., 2013, Madi et al., 2016 and Dany et al., 2012 who showed that moringa mediates apoptosis by upregulating caspase-3 expression in epatocellular cancer liver and lung cell lines respectively.

On comparison of the effect of moringa and cisplatin on caspase-3 expression by ELISA, it was found that cisplatin treated Hep-2cells showed the highest levels of caspase 3 expression in all 3 concentrations (half IC50, IC50 and double IC50) when compared to moringa treated Hep-2 cells. This could be explained by the fact that cisplatin is a purified compound that is highly cytotoxic and has already been used as a commercial chemotherapeutic drug since a long period of time. MOE is still a new compound which may contain many constituents causing antagonistic effect and masking its anticancer impact(Gaffar et al., 2019).

In the present study, treatment of Hep-2 cells with different concentrations of moringa and cisplatin resulted in a dose-dependent decrease in number of proliferating cells and appearance of morphological features of apoptosis. After 24h, half IC50 and IC50 concentration of moringa and cisplatin treated HEp-2 cells still showed some proliferating and pleomorphic HEp-2 cells which was not obvious in double IC50 concentrations, this finding supports the previous tests (MTT and caspase 3), which showed that with increasing the concentration of moringa and cisplatin, there was a decrease in cytotoxicity and an increase in caspase 3 expression. Although half IC50 and IC50 concentration of moringa and cisplatin showed criteria of apoptosis but still they showed higher viability of cells, this could be due to the fact that MTT assay detects late apoptosis and cells at this concentration showed mainly early apoptotic criteria.

In the present study, the data recorded revealed that there is a decrease in NAF values in moringa and cisplatin treated Hep-2 cells when compared to untreated control cells confirming the occurrence of a considerable amount of apoptosis in relation to control cells. Moringa and cisplatin treated Hep-2 cells showed a concentration dependent manner. Double IC50 concentrations of both moringa and cisplatin showed lowest NAF values. In accordance, Helmy and Abdel Azim, 2012 demonstrated that NAF values decreased in cultured Hep-2 cells when cisplatin concentration increased. In case of moringa, no previous studies used NAF to detect apoptotic and necrotic cells in cancer cell lines.

By comparing the effect of moringa and cisplatin on NAF values, it was found that cisplatin treated Hep-2cells showed the lowest NAF values in all three concentrations (half IC50, IC50 and double IC50) when compared to moringa treated Hep-2 cells. These results were found to be in agreement with caspase-3 expression by ELISA test, that found that cisplatin treated Hep-2cells showed the highest levels of caspase 3 expression in all 3 concentrations (half IC50, IC50 and double IC50) when compared to moringa treated Hep-2 cells.

CONCLUSION

Moringa Oleifera has an antiproliferative impact on Hep-2 cells by enhancing apoptosis via upregulation of caspase 3 apoptotic marker.

ACKNOWLEDMENTS

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REFERENCES

1. **Park, B., Chiosea, S., and Grandis, J. (2011).** Molecular changes in the multistage pathogenesis of head and neck cancer. Cancer Biomarkers, 9(1-6): 325-339.
2. **Hodge, C., Khuntia, D., Manon, R., and Harari, P. (2009)** Adjuvant Therapy for Patients with Oral Cavity Cancer. In: Myers, J. Oral cancer metastasis. Springer Science & Business Media; 121-34.
3. **Noguti, J., De Moura, C., De Jesus, G., Da Silva, V., Hossaka, T., Oshima, C., and Ribeiro, D. (2012).** Metastasis from oral cancer: an overview. Cancer Genomics-Proteomics, 9(5): 329-335.
4. **Mehrotra, R., and Yadav, S. (2006).** Oral squamous cell carcinoma: etiology, pathogenesis and prognostic value of genomic alterations. Indian journal of cancer, 43(2): 60-66.
5. **Marur, S., & Forastiere, A. (2016).** Head and neck squamous cell carcinoma: update on epidemiology, diagnosis, and treatment. In Mayo Clinic Proceedings, 91(3): 386-396.
6. **Rothenberg, S., and Ellisen, L. (2012).** The molecular pathogenesis of head and neck squamous cell carcinoma. The Journal of clinical investigation, 122(6): 1951-1957.
7. **Dy, G. and Adjei, A. (2006).** Principles of chemotherapy. In: Chang, A., Ganz, P., Hayes, D., Kinsella, T., Pass, H., Schiller, J., Stone, R. and Strecher, V. Oncology An Evidence-Based Approach 2nd ed. Springer: New York, 14-40.
8. **Florea, A. and Büsselberg, D. (2011).** Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. Cancers, 3(1): 1351-1371.

9. **Specenier, P., and Vermorken, J. (2009).** Current concepts for the management of head and neck cancer: chemotherapy. Oral oncology, 45(4-5): 409-415.
10. **Cooper, J., Pajak, T., Forastiere, A., Jacobs, J., Campbell, B., Saxman, S. and Machtay, M. (2004).** Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. New England Journal of Medicine, 350(19): 1937-1944.
11. **Bernier, J., Domenge, C., Ozsahin, M., Matuszewska, K., Lefèbvre, J. Greiner, R. and Cognetti, F. (2004).** Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. New England Journal of Medicine, 350(19): 1945-1952.
12. **Gibson, M., Li, Y., Murphy, B., Hussain, M., DeConti, R., Ensley, J. and Forastiere, A. (2005).** Randomized phase III evaluation of cisplatin plus fluorouracil versus cisplatin plus paclitaxel in advanced head and neck cancer (E1395): an intergroup trial of the Eastern Cooperative Oncology Group. Journal of Clinical Oncology, 23(15): 3562-3567.
13. **Lam, M. (2003).** Natural medicine. In: Lam, M. Beating cancer with natural Medicine. 1st ed. Bloomington: United States of America. 79-104.
14. **Sharma, P., McClees, S., and Afaq, F. (2017).** Pomegranate for prevention and treatment of cancer: an update. Molecules, 22(1): 177-195.
15. **Calcabrini, C., Catanzaro, E., Bishayee, A., Turrini, E., and Fimognari, C. (2017).** Marine sponge natural products with anticancer potential: An updated review. Marine drugs, 15(10): 310-334.
16. **Demain, A. and Vaishnav, P. (2011).** Natural products for cancer chemotherapy. Microbial biotechnology, 4(6): 687-699.
17. **Manju, K., Jat, R., and Anju, G. (2012).** A review on medicinal plants used as a source of anticancer agents. International Journal of Drug Research and Technology, 2(2): 177-183.
18. **Al-Asmari, A., Albalawi, S., Athar, M., Khan, A., Al-Shahrani, H., and Islam, M. (2015).** Moringa oleifera as an anti-cancer agent against breast and colorectal cancer cell lines. PloS one, 10(8): 1-14
19. **Leone, A., Spada, A., Battezzati, A., Schiraldi, A., Aristil, J., and Bertoli, S. (2015).** Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of Moringa oleifera leaves: an overview. International journal of molecular sciences, 16(6): 12791-12835.
20. **Jung, I., Lee, J., and Kang, S. (2015).** A potential oral anticancer drug candidate, Moringa oleifera leaf extract, induces the apoptosis of human hepatocellular carcinoma cells. Oncology letters, 10(3): 1597-1604.
21. **Gopalakrishnan, L., Doriya, K., and Kumar, D. (2016).** Moringa oleifera: A review on nutritive importance and its medicinal application. Food Science and Human Wellness, 5(2): 49-56.
22. **Sreelatha, S., Jeyachitra, A., and Padma, P. (2011).** Antiproliferation and induction of apoptosis by Moringa oleifera leaf extract on human cancer cells. Food and Chemical Toxicology, 49(6): 1270-1275.
23. **Tiloke, C., Phulukdaree, A., and Chuturgoon, A. (2013).** The antiproliferative effect of Moringa oleifera crude aqueous leaf extract on cancerous human alveolar epithelial cells. BMC complementary and alternative medicine, 13(1): 226-234.
24. **Khalafalla, M., Abdellatef, E., Dafalla, H., Nassrallah, A., Aboul-Enein, K., Lightfoot, D. and El-Shemy, H. (2010).** Active principle from Moringa oleifera Lam leaves effective against two leukemias and a hepatocarcinoma. African Journal of Biotechnology, 9(49): 8467-8471.
25. **Tiloke, C., Phulukdaree, A., Gengan, R., and Chuturgoon, A. (2019).** Moringa oleifera aqueous leaf extract induces cell-cycle arrest and apoptosis in human liver hepatocellular carcinoma cells. Nutrition and cancer, 71(7): 1165-1174.
26. **Afifi, N., Abdel-Hamid, E., Baghdadi, H., and Mohamed, A. (2012).** Nuclear Area Factor as a Novel Estimate for Apoptosis in Oral Squamous Cell Carcinoma-Treated Cell Line: A Comparative in-vitro Study with DNA Fragmentation Assay. Journal of Clinical & Experimental Pathology, 2(2): 1-5.
27. **Apirakramwong, A., Tragulpakseerojn, J., Sithisombut, C., and Pamonsinlapatham, P. (2018).** Anti-proliferation effect of Moringa oleifera Lam.extracts on human cancer cell lines. Thai Journal of Pharmaceutical Sciences, 42: 1-4
28. **Gaffar, S., Apriani, R., and Herlina, T. (2019).** n-Hexane fraction of Moringa oleifera Lam. leaves induces apoptosis and cell cycle arrest on T47D breast cancer cell line. Journal of Pharmacy & Pharmacognosy Research, 7(3): 173-183.
29. **Adebayo, I., Balogun, W., and Arsad, H. (2017).** Moringa oleifera: An apoptosis inducer in cancer cells. Tropical Journal of Pharmaceutical Research, 16(9): 2289-2296.
30. **Dany, M., Madi, N., Nemer, N., Beyrouthy, M., Abdoun, S., and Usta, J. (2012).** Moringa oleifera: Natural leaf extract with potential anti-cancerous effect on A549 lung cancer cells. Lung Cancer, 77: 21-22.
31. **Madi, N., Dany, M., Abdoun, S., and Usta, J. (2016).** Moringa oleifera's nutritious aqueous leaf extract has anticancerous effects by compromising mitochondrial viability in an ROS-dependent manner. Journal of the American College of Nutrition, 35(7): 604-613.
32. **Potestà, M., Minutolo, A., Gismondi, A., Canuti, L., Kenzo, M., Roglia, V. and Montesano, C. (2019).** Cytotoxic and apoptotic effects of different extracts of Moringa oleifera

Lam on lymphoid and monocytoid cells. Experimental and Therapeutic Medicine, 18(1): 5-17.

33. **Nair, S., and Varalakshmi, K. (2011).** Anticancer, cytotoxic potential of Moringa oleifera extracts on HeLa cell line. Journal of Natural Pharmaceuticals, 2(3): 138-142.
34. **Pamok, S., Vinitketkumnuen, S., and Saenphet, K. (2012).** Antiproliferative effect of Moringa oleifera Lam. and Pseuderanthemum palatiferum (Nees) Radlk extracts on the colon cancer cells. Journal of Medicinal Plants Research, 6(1): 139-145.