JSRMBS Journal of Scientific Research in Medical and Biological Sciences
ISSN 2709-0159(print) and ISSN 2709-1511 (online)
Volume 3, Issue 3 Article 2
DOI: https://doi.org/10.47631/jsrmbs.v3i3.530

EVALUATION OF ANTICANCER ACTIVITY *OF AZADIRACHTA INDICA* AND *WITHANIA SOMNIFERA* CRUDE EXTRACTS ON HUMAN PROSTATE CANCER CELL LINES PC3 AND DU145

Chhavi[1], Manali Kaintura[2],Tikkam Singh[3], Ajeet Singh[4]

[1]Zakir Husain Delhi College, University of Delhi, Delhi, India
[2]Hemvati Nandan Bahuguna Garhwal University, Srinagar Garhwal, Uttarakhand, India
[3]Research Scientist, R & D Unit, Helix Biogenesis Pvt. Ltd. First Floor, Sector 2, Noida Uttar Pradesh-201308, India.
[4]R & D Unit, Helix Biogenesis Pvt. Ltd. First Floor, Sector 2, Noida Uttar Pradesh-201308

ARTICLE INFO

Recieved: 26 July 2022
Revised: 22 Sept 2022
Accepted: 28 Sept 2022

Keywords:
Prostrate cancer,
PC3,
DU145,
Azadirachta indica,
Withania somnifera

Corresponding Author:

Dr. Tikkam Singh

Email:

tikkam.singh35@gmail.com

ABSTRACT

Purpose: *Recent years have witnessed a shift from chemically processed drugs* ***to*** *more therapeutic natural domains. Several plant parts like root, stem, leaves, etc. are utilized to develop 'natural drugs' for treatment of deadly diseases, one such being cancer. A plethora of plant secondary metabolites such as alkaloids, flavonoids, terpenes, steroids, phenolics have displayed viable roles in the human diseases like diabetes, cancer and have been known to show anti-inflammatory, analgesics and antipyretic properties. The present investigation has mainly focused on the evaluation of crude extracts of Azadirachta indica A. Juss. (Neem), Withania somnifera (L.) Dunal (Ashwagandha) against human prostate cancer cell lines PC3 and DU145 by performing cell viability tests.*

Methods: *The crude extracts of plants were prepared in organic solvents such as ethanol and methanol and their anticancer activity were evaluated by employing resazurin and DNA fragmentation assays.*

Results: *The crude extracts of A. indica and W. somnifera have showed inhibition on growth of PC3 and DU145 cells. In PC3, ethanolic extract of W. somnifera (WSEE) at 80 µg/mL was found to be optimum as cancer cells growth inhibitor and showed 45.03% inhibition whereas, 42.63% inhibition in DU145 cells was observed in 100 µg/mL of methanolic extract of A. indica (AIME). Fragmented or damaged DNA was observed in the extract treated PC3 and DU145 cancer cells assessed by DNA fragmentation assay.*

Conclusion: *The secondary metabolites present in the crude extracts showed moderate anticancer activity against prostate cancer cell lines such as PC3 and DU145. These natural agents stand out as chemopreventive substances and are have been used more in practice with allopathic substitutes, however investigation with the aid of advance technology and extrapolating the potential benefits of various other herbs can be vital in this regard.*

INTRODUCTION

Amongst the deadliest incurable disease is cancer, which took more than 9 million lives worldwide, in 2018. Prostate cancer is the second most common cancer in men that is the

leading cause of cancer death after lung cancer in many countries. Prostate cancer is the cancer of prostate gland which usually occurs in males after the age of 50. The cancer begins when cells start dividing very rapidly. These cells accumulate and form a tumor, which grows to elude other nearby tissues. Symptoms of advanced prostate cancer are trouble in urinating, bone pain, blood in urine, blood in semen, etc. Several studies have associated the cause of cancer with gonorrhea, diet high in red meat, milk products. Raised blood pressure also increases the risk of prostate cancer (Chitta et al., 2013). However, individuals having BRCA mutations are in high risk of getting prostate cancer (Lee et al., 2017). Prostate-Specific Antigen (PSA) test is used as a firstline screening test for prostate cancer. This test is used for early detection of prostate cancer which gives hope to patients for better and appropriate treatment (Catalona, 2018). Prostate cancer like the malignancies known otherwise have been linked to the widening genetic susceptibility, and the environmental influences, in most cases both. Statistics reveal that men with the age group above 65 years are more prone to the risk of prostate cancer and is more common in the Scandinavian countries than in Asia. (Crawford, 2003) The inception of prostate cancer is by the activation of the domain coded by CAG nucleotide repeat. In a healthy man, the sequence is usually 11-31 repeat, reduction in the projected sequence can however be projected with the increase risk of prostate cancer lines (Crawford, 2003). Dietary supplements that contain excess of fat, emulsified and processed food, smoking- overconsumption of alcohol, coupled with a sedentary lifestyle is the welcome mat for many ailments, cancer being one. A study shows a group of Japanese men to U.S.A (with low rate of cancer incidents in their native) had inflicting local rates in prostate cancer, this was correlated with the soyabean intake by the Japanese, the isoflavones present in which are known to inhibit proteins that are required for the proliferation of cell, thus limiting the uncontrolled propagation of the cells (Crawford, 2003).

Prostate cancer is in parallel with various other categories of cancer that disseminate in the living body through EMT. The tumor cells via the circulating fluids transmit throughout the body to accumulate in the endosteal bone surface (Wang et al., 2018). The accommodation of prostate tumor cells within the bone marrow increases its susceptibility to be transmitted. Progressive spread of prostate cancer is also induced by the levels of androgenic hormones, high levels of which bear catastrophic implications. Therefore, the hormone therapy that aims at the reduction of androgens levels by introducing anti-androgens, induction of female sex hormones, or suppressing the adrenal glands have proven to be an effective strategy in the treatment of prostate cancer (Taplin and Balk, 2004). Understanding the etiological mechanism of any cancer, diagnosing the synergistic effects of various agents and herbal drugs, and devising the treatment with combined approach has been the key to management of this ailment.

Since the time immemorial, plants have been considered as immense source of various pharmaceutical bioactive compounds. These bioactive compounds have been utilized all over the world for millions of years as natural drugs having pharmacologic and therapeutic potential for the treatment of different diseases. Medicinal plants are known to synthesize a plethora of important bioactive compounds in response to biotic or abiotic stress for their biological functions. In recent times, these bioactive compounds acquired utmost interest to pharmaceutical industries for drug designing and many other therapeutic uses. However, the synthesis of these therapeutic bioactive molecules is site–specific for a particular plant part, depends on the growth and developmental stages, environmental conditions and availability of micro– and macronutrients. *Azadirachta indica* A. Juss (family name: Meliaceae, commonly known as Neem) and *Withania somnifera* (family name: Solanaceae, commonly known as Ashwagandha) are traditional medicinal plants that have been used from a very long time. *A. indica* has various potent biological activities such as antibacterial, antifungal, antiviral,

antidiabetic, antimalarial, anti-inflammatory, antiseptic, anticancer, etc. (Omale and Okafor, 2008; Demiray et al., 2009; Ismail et al., 2010). About more than 135 novel compounds with various chemical structures have already been identified from different parts of this medicinally important plant (Hayat et al., 2010). Similarly, *W. somnifera* has also possesses antioxidant, anxiolytic, adaptogen, memory enhancing, antiparkinsonian, antivenom, anti-inflammatory, antitumor properties (Gupta and Rana, 2007). Ayurveda uses these herbs for various diseases and disorders due to the presence of bioactive compounds such as phenols, flavonoids, terpenoids, alkaloids, etc. Therefore, present investigation is focused on the evaluation of anticancer activity of plant extract prepared in ethanol and methanol.

METHODOLOGY

Cell Lines and Culture

PC3 (Caucasian prostate adenocarcinoma) and DU145 (prostate cancer cell line) were procured and authenticated from National Centre for Cell Sciences, India. These were grown in Roswell Park Memorial Institute (RPMI) media (Sigma, USA) supplemented with 10% fetal calf serum (Gibco, USA) and 1% antibiotic-antimycotic solution (Hi-Media, India). The cells (70-80% confluency) less than five passages were used for experiments. The cells were maintained in a humidified CO_2 (5%) incubator (ThermoForma.Series II water jacketed CO_2 Incubator) at 37 °C.

Preparation of plant extract

Dried plant material of *A. indica* and *W. somnifera* were taken and ground into course powder using mixer grinder. The course powder of 10g each plant was added in 100 ml of ethanol and methanol separately in glass jars. Further, these glass jars were kept in an incubator shaker at 25 ± 3°C temperature for 48 h. The prepared solutions were filtered through Whatman Paper No. 1 and the obtained extracts were evaporated to dryness, stored at 4 °C for further experiments. Extracts of *A. indica* prepared in ethanol and methanol named as AIEE and AIME, respectively. Similarly, extracts of *W. somnifera* prepared in ethanol and methanol represented as WSEE and WSME, respectively.

Resazurin assay

Resazurin assay was performed to evaluate the viability of the PC3 and DU145 prostate cancer cells when being treated with the choice of drugs: *A. indica* ethanolic extract (AIEE), *A. indica* methanolic extract (AIME), *W. somnifera* ethanolic extract (WSEE) and *W. somnifera* methanolic extract (WSME). This redox dye indicates the presence of metabolically active and viable cells based on the change in the coloration of solution mixture from violet to pink. This colour change is due the conversion of resazurin to resorufin by the activity of mitochondrial enzymes (McMillian et al., 2002). The cell suspension of both PC3 and DU145 cell lines were prepared using the modified protocol of Vicentini et al. (2003). The cell suspension of PC3 and DU145 cells (500 µl each), complete RPMI media was added to obtain a of 2000 µl volume each in ELISA plate. In each well 200 µl cell suspension was added and incubated for 24 hours at 37°C in a CO_2 incubator. After incubation, the cell suspension of PC3 and DU145 were treated with plant extract prepared in different solvents such as ethanol and methanol. After 24 h of seeding in a 5% humidified CO2 incubator at 37 ^{o}C, a range of concentrations of the extracts 20, 40, 60, 80 and 100 µg/mL were added in triplicate. The other wells were of vehicle control (0.1% DMSO in the incomplete RPMI media) and control (all in triplicates). These plates were incubated at 37 °C for 24 h in a CO_2 (5%) incubator. After the required incubation, 20 µL of resazurin dye (Sigma, USA) reagent (5 mg/mL in PBS) was added to it, followed by incubation for 4 h, subsequently all wells were cleared off their constituents and 150 µL of

DMSO was added to each well. The plates were then read in an ELISA plate reader (Tecan, Austria) at 540 nm. Percent (%) inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of extract treatment}}{\text{Absorbance of control}} \times 100$$

DNA fragmentation assay

For DNA fragmentation, $0.1x10^6$ cells were seeded in 40 mm petri-dishes for 24 h. These cells were treated with different concentration of AIME and WSEE (20-100 µg/mL) for 24 h. The Cell suspension was centrifuged at 2000 rpm for 5 minutes and then 1 mL of PBS solution was added without disturbing the pellet and centrifuged again for 5 minutes. PBS solution was then discarded and supplied with 500 µl of Lysis buffer. The cell suspension was incubated at 60 °C in a water bath for 30 minutes. Equal volume of phenol-chloroform and isoamyl alcohol were added and centrifuged at 8000 rpm for 15 minutes. The obtained aqueous layer was transferred to another fresh microcentrifuge tube in which ethanol and 3M sodium acetate were added followed by an incubation period of 30 minutes at -20 °C. It was further centrifuged at 8000rpm for 15 minutes. The supernatant was discarded and added 70% ethanol followed by centrifugation. The tubes were air dried until ethanol completely evaporated. Obtained DNA was dissolved in 50 µl of nuclease free water and stored at 4 °C. Isolated DNA sample from each treatment ran on 1.2% agarose gel at 90 V for 30 min and visualized under UV Transilluminator.

RESULTS AND DISCUSSION

Resazurin Assay

In order to evaluate anticancer activity of WSEE, WSME, AIEE and AIME against PC3 and DU145 cell lines, cell viability was assessed by using resazurin assay after 24 h of treatment. PC3 cells when treated with different concentrations (20, 40, 60, 80 and 100 µg/mL) of WSEE showed dose dependent inhibition in growth of cancer cells. Maximum percent growth inhibition i.e., 45.03 ± 1.21% was observed in PC3 cells at 80 µg/mL of WSEE (Figure 1a) whereas, only 32.78 ± 1.4% growth inhibition was seen in PC3 cells when treated with 100 µg/mL WSME. In case of DU145 cells, treatment was given with different concentrations (20, 40, 60, 80 and 100 µg/mL) of AIEE and AIME, which were effective in inhibiting the growth of DU145 cells. However, higher concentrations of AIME (60-100 µg/ mL) were capable of inhibition. Optimum percent growth inhibition of 42.63 ± 1.48% was reported at 100 µg/mL AIME in DU145 prostate cancer cells (Figure 1b). Contrary, AIEE at 100 µg/mL was found to inhibit only 29.31 ± 1.33% growth of DU145 cells. The probable reason for difference in inhibiting activity of ethanolic and methanolic extract is the polarity of solvent against *A. indica* and *W. somnifera*. Secondary metabolites present in plant showed enhanced activity in more polar solvent. The major biological compounds of *W. somnifera* which possess anti-carcinogenic activity are Withanolide D, Withalongolide A (Dutta et al., 2019).

The ethanolic extract of Selenium-enriched green tea has also been inhibited the growth of A549 and HepG2 lung cancer cells in a concentration-dependent manner having IC50 values of 278.6 µg/ml and 431.6 µg/ml, respectively (Li et al., 2008). The cytotoxicity of ethanolic leaves extract of *Peristrophe bivalvis* was found to be 175.25 ± 12.34 µg/mL in EAC cell lines (Tanavade et al., 2012). The methanolic extract of *Stephania elegans* has been showed potent cytotoxic activity towards MCF-7 cells with IC50 158.7 ± 0.13 µg/mL (Sharma et al., 2017). Kandil et al. (2019), have reported the cytotoxic effect of methanolic extract of *Cichorium intybus* on MCF-7 cell line achieved at 500 µg m/L. Interestingly, methanolic extract of aerial parts of *A. indica* and *M. azaderach* have been reported to inhibit the MCF

cell lines having their respective IC50 values of 165.5629 and 280.8989 µg/mL, wherein 200 µg/ml of the methanolic extract of *A. indica* revealed highest percent inhibition of 65.5% (Malar et al., 2020). Our report of inhibitory activity of ethanolic as well as methanolic extracts of the *A. indica* and *W. somnifera* against prostate cancer cell lines PC3 and DU145 is in accordance of above studies.

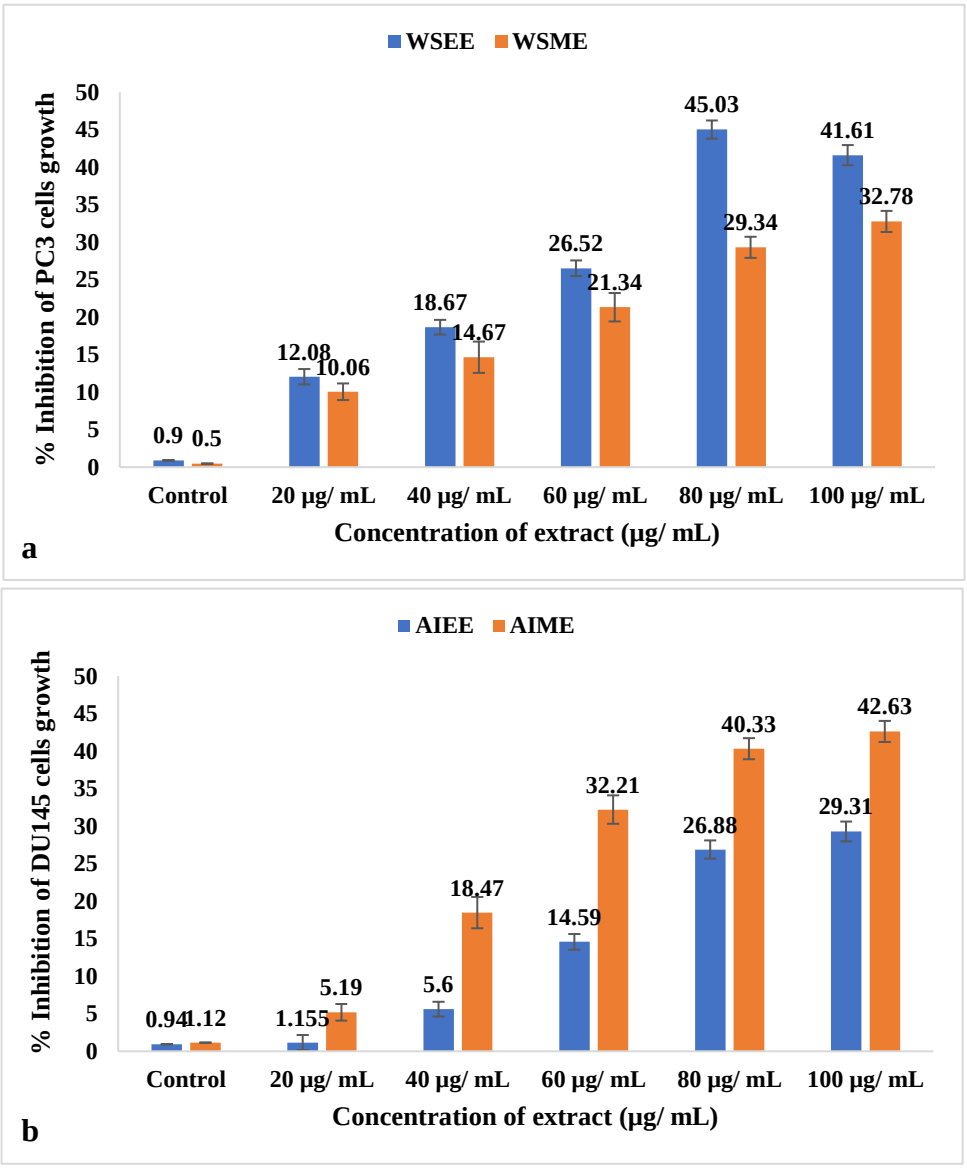

Figure1. Graph representing % inhibition on ***(a)*** *PC3 cells treated with different concentrations of WSEE and WSME,* ***(b)*** *DU145 cells treated with different concentrations of AIEE and AIME.*

DNA fragmentation

DNA fragmentation was carried out to detect DNA damage in PC3 and DU145 cells after 24 h of treatment with AIEE, AIME, WSEE and WSME. Interestingly, ladder like patterns were observed in both PC3 and DU145 cells when treated with different concentrations (20, 40, 60, 80 and 100 µg/mL) of above mentioned extracts. This is due to apoptosis in cancer cells induced by the *A. indica* extract. The major secondary metabolites that induce apoptosis, inhibit cell viability in *A. indica* are flavonoids, triterpenoids, limonoids (Dutta et al., 2019). Nimbolide is a major limonoid constituent of *A. indica* and studies have already been reported

that it has anti-tumor activity. It inhibits the expression of p-STAT3, important signaling molecule in regulation and growth of tumour (Biswas et al., 2002). Herbal products have opened up new vistas in management of cancer as has been proved in several existing products (Baker et al., 2007). The probable reason is attributed to their synergistic action and multi-targeted approach with minimum side effects (Srivastava et al., 2005).

CONCLUSION

Plant based drugs/ extracts have been proving to be of greatest importance due to their multi-targeted healing approach. The present investigation revealed that both ethanolic and methanolic extracts of *A. indica* and *W. somnifera* moderately inhibited the growth of PC3 and DU145 prostate cancer cells. The results suggest that these medicinally important plants could be effective in management of prostate cancer at a certain extend. However, further and advanced studies are required pertaining to the anticancer activity and mechanism of secondary metabolites of *A. indica* and *W. somnifera* involved in the inhibition of growth of prostate cancer cells.

ACKNOWLEDGMENTS

The study and experiment associated with the "Evaluation of anticancer activity of *Azadirachta indica* and *Withania somnifera* crude extracts on human prostate cancer cell lines PC3 and DU145" was done in the laboratory of Helix Biogenesis, Pvt. Ltd., Noida, Uttar Pradesh, with supreme precision and accuracy. The project is carried out under the direct supervision of Dr. Tikkam Singh.

AUTHOR'S CONTRIBUTIONS

Tikkam Singh and Ajeet Singh have designed experiments, monitored, assisted in writing and edited the manuscript. Chhavi and Manali Kaintura both have equal contribution in performing all the experiments and also in the writing the manuscript.

CONFLICT OF INTEREST

The authors do not have any conflict of interest to declare.

FUNDING

None was received for this research.

REFERENCES

Baker, D. D., Chu, M., Oza, U., & Rajgarhia, V. (2007). The value of natural products to future pharmaceutical discovery. *Natural Product Reports*, 24(6), 1225-1244. https://doi.org/10.1039/b602241n

Biswas, K., Chattopadhyay, I., Banerjee, R. K., & Bandyopadhyay, U. (2002). Biological activities and medicinal properties of neem (Azadirachta indica). *Current Science*, *82*(11), 1336–1345. http://www.jstor.org/stable/24106000

Catalona, W. J. (2018). Prostate cancer screening. *Medical Clinics*, 102(2), 199-214. https://doi.org/10.1016/j.mcna.2017.11.001

Chitta, K. S., Khan, A. N., Ersing, N., Swaika, A., Masood, A., Paulus, A., Qadeer, A., Advani, P., Sher, T., Miller, K. C., Lee, K., & Chanan-Khan, A. A. (2013). Neem leaf extract induces cell death by apoptosis and autophagy in B-chronic lymphocytic leukemia cells. *Leukemia & Lymphoma*, 55(3), 652-661. https://doi.org/10.3109/10428194.2013.807927

Crawford, E. D. (2003). Epidemiology of prostate cancer. Urology, 62(6 SUPPL. 1), 3–12.

Demiray, S., Pintado, M. E., & Castro, P. M. L. (2009). Evaluation of phenolic profiles and antioxidant activities of Turkish medicinal plants: *Tilia argentea, Crataegi folium* leaves and *Polygonum bistorta* roots. *World Academy of Science, Engineering and Technology*, 54, 312-317. https://doi.org/10.5281/zenodo.1075066

Dutta, R., Khalil, R., Green, R., Mohapatra, S. S., & Mohapatra, S. (2019). *Withania somnifera* (Ashwagandha) and withaferin A: Potential in integrative oncology. *International journal of molecular sciences*, *20*(21), 5310. https://doi.org/10.3390/ijms20215310

Hayat, K., Zhang, X., Farooq, U., Abbas, S., Xia, S., Jia, C., ... & Zhang, J. (2010). Effect of microwave treatment on phenolic content and antioxidant activity of *Citrus mandarin* pomace. *Food chemistry*, 123(2), 423-429. https://doi.org/10.1016/j.foodchem.2010.04.060

Ismail, H. I., Chan, K. W., Mariod, A. A., & Ismail, M. (2010). Phenolic content and antioxidant activity of cantaloupe (*Cucumis melo*) methanolic extracts. *Food chemistry*, 119(2), 643-647. https://doi.org/10.1016/j.foodchem.2009.07.023

Kandil, A. S., Abou-Elella, F., & El Shemy, H. A. (2019). Cytotoxic profile activities of ethanolic and methanolic extracts of chicory plant (*Cichorium intybus* L.). *Journal of Radiation Research and Applied Sciences*, 12(1), 106-111. https://doi.org/10.1080/16878507.2019.1594136

Lee, M. V., Katabathina, V. S., Bowerson, M. L., Mityul, M. I., Shetty, A. S., Elsayes, K. M., Balachandran, A., Bhosale, P. R., McCullough, A. E., & Menias, C. O. (2017). BRCA- associated cancers: role of imaging in screening, diagnosis, and management. *Radiographics*, *37* (4), 1005-1023. https://doi.org/10.1148/rg.2017160144

Li, F., Wang, F., Yu, F., Fang, Y., Xin, Z., Yang, F., ... & Hu, Q. (2008). In vitro antioxidant and anticancer activities of ethanolic extract of selenium-enriched green tea. *Food Chemistry*, 111(1), 165-170. https://doi.org/10.1016/j.foodchem.2008.03.057

Malar, T. J., Antonyswamy, J., Vijayaraghavan, P., Kim, Y. O., Al-Ghamdi, A. A., Elshikh, M. S., ... & Kim, H. J. (2020). In-vitro phytochemical and pharmacological bio-efficacy studies on *Azadirachta indica* A. Juss and *Melia azedarach* Linn for anticancer activity. *Saudi Journal of Biological Sciences*, 27(2), 682-688. https://doi.org/10.1016/j.sjbs.2019.11.024

McMillian, M. K., Li, L., Parker, J. B., Patel, L., Zhong, Z., Gunnett, J. W., ... & Johnson, M. D. (2002). An improved resazurin-based cytotoxicity assay for hepatic cells. Cell biology and toxicology, 18(3), 157-173. https://doi.org/10.1023/a:1015559603643

Omale, J., & Okafor, P. N. (2008). Comparative antioxidant capacity, membrane stabilization, polyphenol composition and cytotoxicity of the leaf and stem of *Cissus multistriata*. *African Journal of Biotechnology*, 7(17). http://dx.doi.org/10.4314/ajb.v7i17.59240

Sharma, R., Chandan, G., Chahal, A., & Saini, R. V. (2017). Antioxidant and anticancer activity of methanolic extract from *Stephania elegans*. International Journal of Pharmacy and Pharmaceutical Sciences, 9(2), 245-9. https://doi.org/10.22159/ijpps.2017v9i2.16204

Srivastava, V., Negi, A. S., Kumar, J. K., Gupta, M. M., & Khanuja, S. P. (2005). Plant-based anticancer molecules: a chemical and biological profile of some important leads. *Bioorganic & Medicinal Chemistry*, 13(21), 5892-5908. https://doi.org/10.1016/j.bmc.2005.05.066

Tanavade, S. S., Naikwade, N., & Chougule, D. D. (2012). In vitro anticancer activity of Ethanolic and Aqueous Extracts of *Peristrophe bivalvis* Merrill. *Research Journal of Pharmacy and Technology*, 5(10), 1324-1327.

Vicentini, C., Festuccia, C., Gravina, G. L., Angelucci, A., Marronaro, A., & Bologna, M. (2003). Prostate cancer cell proliferation is strongly reduced by the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 in vitro on human cell lines and primary cultures. *Journal Of Cancer Research And Clinical Oncology*, 129(3), 165-174. https://doi.org/10.1007/s00432-003-0420-3

Wang, G., Zhao, D., Spring, D. J., & DePinho, R. A. (2018). Genetics and biology of prostate cancer. *Genes & development*, *32*(17-18), 1105-1140. https://pubmed.ncbi.nlm.nih.gov/30181359

Zhang, J., Ahn, K. S., Kim, C., Shanmugam, M. K., Siveen, K. S., Arfuso, F., Samym, R. P., Deivasigamanim, A., Lim, L. H. K., Wang, L., Goh, B. C., Kumar, A. P., Hui, K. M., Sethi, G. (2016). Nimbolide-induced oxidative stress abrogates STAT3 signaling cascade and inhibits tumor growth in transgenic adenocarcinoma of mouse prostate model. *Antioxidants & Redox Signaling*, *24* (11), 575-589. https://doi.org/10.1089/ars.2015.6418