Research Article

In Vitro Cytotoxic Potential of Plant Terpenes and Selected Medicinal Plants of Pakistan against NIH 3T3 Cell Line

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Abstract

Medicinal plants are used in anticancer therapies from ages. The very first time we have not only screened anticancer medicinal plants from Pakistan but also identified anticancer plant terpenes. This study will definitely pave the way towards anticancer drug development after animal trials.

Methods: This study was based on ethanolic extraction of fruits/seed of 25 plants. The cytotoxic potential of extracts and plant terpenes was evaluated against mouse fibroblasts (NIH 3T3) cell line by using MTT (3- [4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay and compared with cyclohexamide.

Results: Ethanolic extracts were nontoxic against NIH3T3 cell line. On the basis of the % inhibition plants were graded as B.lycium, 48% > T.bellerica, 47% > C.verum, 45% > Z.tenuior, 39% > H. adenophyllum, 38% > C. carandas, 31% > C.behen, 29% > P. juliflora, 28% & P.granatum, 28% W.somnifera, 27% > E.cheiri, 26% > S. potatorum, 24% & M.incana, 24% > D. peregrina, 21% & B.lanzan, 21% > L. maldivica, 20% > F. lyrata, 18% > F.arabica, 16% & R. indica, 16% > C.absus, 13% & C.paniculatus, 13%. > C. diurnum, 12% > J.mimosifolia, 11% > D.malabarica, 8% > C. speciosa, 3% as compared to the reference drug Cyclohexamide, 70%. Whereas the maximum % inhibition was exhibited by n-Hexadecanoic acid (88%), followed by Hexadecanoic acid, methyl ester (16%) and Methyl decanoate displayed 11%. IC50 of n-Hexadecanoic acid and cyclohexamide was 3.1±0.2 and 0.8±0.2 respectively.

Conclusion: Plant extracts and terpenes was found to be inactive against 3T3 cell line.

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Keywords | *Cytotoxic Effects, NIH 3T3 Cell Line, Anticancer Activity, MTT Assay, IC50.*

Introduction

Natural and plant based medicines have been used to treat 87% of human diseases covering microbial infection, cancer and other immunological disorders.[1] Cancer is the supreme evident reasons of human deaths worldwide, resulted 8.8 million deaths in 2015. Middle-income countries especially Pakistan and India, unfortunately 70% of deaths results from cancer.[2] In recent years, an increase in cases of breast, oral cavity, lung and liver cancers including hepatitis and Human Papilloma Virus (HPV), are detected in Pakistan.[3] Costly and painful treatments, inaccessible diagnosis and late-stage presentation are the major limitations in treating cancer especially in Pakistan. The overall economic impact of cancer is increasing especially in Pakistan. Total annual economic cost of cancer in 2010 estimated at approximately US$ 1.16 trillion.[3]

Every cancer type involves specific treatment procedures, surgeries, radiotherapy, and chemotherapy. But these treatments may cause side effects and problems affecting healthy tissues or organs.[4,5] These side effects include anemia, loss of appetite, bleeding, edema, fatigue, hair loss, infection and memory or concentration problems hence affecting overall quality of life (QOL) of patients.[6,7] Various complementary and alternative methods (CAM) are used especially traditional medicinal plants have been used to treat various ailments and serious diseases.[8] Medicinal plants contain bioactive compounds that play vital role being structural and functional parts of enzymes, proteins and hormones. Scientists and nutritionists have been using plant extracts to cure and improve human life style. Plant based secondary metabolites such as tannins, terpenoid, flavonoids and alkaloids possess a lot of potential to be used for possible curative and medicinal use.[9]

Pakistan has a rich and prestigious heritage of medicinal plants. However, majority of the plants have not been yet explored for their therapeutic and medicinal properties. Inspired by the teachings and traditional herbal practices of a Pakistani scholar of international repute and traditional healer, Khawaja Shamsuddin Azeemi, who prescribed T. patula petals for all types of cancer patients with extra-ordinary results.[10] Hence, in this study we decided to determine cytotoxic effect of 25 traditional medicinal plants of Pakistan against NIH/3T3 cell lines. The plants are: Prosopis juliflora, Carissa carandas, Ceiba speciosa, Heterophragm aadenophyllum, Cestrum Diurnum, Jacaranda mimosifolia, Diospyros malabarica, Terminalia bellerica, Terminalia bellerica, Ficus lyrata, Cinnamomum verum, Erysimum cheiri, Buchanania lanzan, Withania somnifera, Fagonia arabica, Berberis lycium, Strychnos potatorum, Matthiola incana, Ziziphora tenuior, Centaurea behen, Rosa indica, Punica granatum, Cassia absus, Celastrus paniculatus are collected from different cities of Pakistan. Whereas fruit of Lodoicea maldivica was purchased from local herbal market. These plants or their parts are used in traditional medicines for the treatment of inflammation and infectious diseases.[11] But very limited research has been done in the area of their anticancer and cytotoxic potential.

Plant terpenoids are used widely for their aromatic qualities and have played a role in traditional herbal preparations in India and China.[12] These compounds exhibit cytotoxicity against tumor cells, and have been tested for anticancer efficacy in preclinical animal models.[13] Epidemiological, experimental and research studies suggest that Terpenoids are helpful in prevention and therapy of cancers and carcinomas.[14] To our knowledge, so far in Pakistan no one has used natural terpenes to see their cytotoxic effects. Here, we report for the first time on the cytotoxic activity of ethanolic extracts of 21 Pakistani medicinal plants and compared them with natural terpenes against normal mouse fibroblasts (NIH3T3) cells.

The purpose of this research work was to explore the cytotoxic effects of these medicinal plants and to compare them with natural plant based Terpenes, so that they can be isolated, characterized and used as potential anticancer drugs in future.

Methods

The experimental study is based on the comparative evaluation of cytotoxic potential of medicinal plants of Pakistan, plant Terpenes and standard medicine. Fresh fruits of 25 selected plants were collected from different cities of Pakistan. The voucher specimens were deposited in Prem Madam the herbarium of Lahore College for Women University, Lahoreas : P. juliflora (Sw.) DC. (LCWU-15-128), C. carandas L. (LCWU-15-129), C. speciosa (A. St.-Hil., A. Juss. & Cambess.) P. Ravenna (LCWU-15-130), H. adenophyllum Wall. ex G.Do Seem. ex Benth. & Hook. Fil. (LCWU-15-131), C. Diurnum L. (LCWU-15-132), J. mimosifolia D.Don (LCWU-15-133), D. malabarica (Desr.) Kostel. (LCWU-15-134), T. bellerica (Gaertn.) Roxb. (LCWU-15-135), F. lyrata Warb. (LCWU-15-117), D. peregrina (Gaertn.) Gurke (LCWU-15-136), C. verum J. S. Presl (LCWU-15-137), E. cheiri (L.) Crantz (LCWU-15-138), B. lanzan Spreng. (LCWU-15-139), W. somnifera (L.) Dunal (LCWU-15-89), F. arabica L. (LCWU-15-140), B. lycieum Royle. (LCWU-15-141), S. potatorum L. fil. (LCWU-15-142), M. incana (L.) W.T.Aiton. (LCWU-15-143), Z. tenuior L. (LCWU-15-144), C. behen L. (LCWU-15-145), R. indica L. (LCWU-15-122), P. granatum L. (LCWU-15-146), L. maldivica (J.F. Gmel.) Pers. (LCWU-15-147), C. absus L. (LCWU-15-148), C. paniculatus Willd. (LCWU-15-149). The collected samples were

washed properly, shade dried at room temperature (20°C-30°C) and pulverized into fine powder for the further use.

Sample powdered material of all selected plants was extracted with ethanol by maceration for successive 3 days. 2.0 g of each sample was extracted and 50 mL of ethanol was added with gentle stirring for 72 h. The extracts were filtered, collected in glass vials and were concentrated by using a rotary evaporator.

Standard methods of phytochemical analyses were performed to detect the phyto-constituents, viz. terpenoids, flavonoids, alkaloids, phenolics and glycosides[15] in plant extracts. Whereas, the qualitative and quantitative estimation of terpenoids[16] and their role as antioxidants was also performed by us in previous study.[17]

Normal mouse fibroblast cells (NIH/3T3, ATCC #: CRL-1658 (lot. No. 59049195) were used for in vitro cytotoxic comparison of plant extracts. Cell lines was cultured in advanced Dulbecco's Modified Eagle Medium (DMEM) augmented with 10% inactivated NBCS (newborn calf serum) and 5mM l-glutamine, and maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Cytotoxic activity of ethanolic extracts as well as selected three plant terpenes (Methyl decanoate, Hexadecanoic acid, methyl ester, n-Hexadecanoic acid) were assessed in 96-well format substrate for high throughput screening (HTS) by MTT colorimetric assay [(18)]. Mouse fibroblasts (3T3) cells were cultured in DMEM medium containing 5% FBS (fetal bovine serum), penicillin (100 IU/mL) and streptomycin (100 µg/mL). The flasks 75 cm^2 were incubated in 5% CO_2 incubator at 37°C. Repeatedly growing cells were picked carefully under aseptic condition and counted by haemocytometer. Cellular concentration (5×104cells/mL) was prepared and poured into 96-well plate and left for overnight. Medium was removed after overnight incubation and 200 µL of fresh medium added along with different concentrations (1-30µM). After 48 hours MTT substrate was prepared (0.5 mg/mL) in a physiologically balanced solution, 200 µL was added into the medium and placed in incubator for further 4 hours. Afterwards, 100µL of DMSO was mixed to each well. Viable cells with active metabolism reduced MTT to formazan product (purple colour), that was monitored and calculated by absorbance at 540 nm using a Micro Plate Reader (Spectra Max plus, Molecular Devices, CA, USA). Extracts showing cytotoxic activity were further tested and calculated the IC50 values. IC50 of the compound for the cytotoxicity noted at concentration caused 50% cellular inhibition of 3T3 cells.

Following formula was used for the determination of cytotoxic inhibition (%)

Inhibition (%) = 100-((mean of values of test compound – mean of values of negative control)/ (mean of values of positive control – mean of values of negative control) x100).

The results were analyzed by using Soft- Max Pro software (Molecular Device, USA). Each experiment was performed in triplicate. The IC50 values were calculated with Probit analysis software (LdP Line software, USA).

Results

Qualitative phytochemical analysis of fruit/seed extracts indicated terpenoids, flavonoids, phenols, quinine, saponins, glycosides and alkaloids. The results of our previous study revealed that these plants had high terpenoid contents,[16] In another study, we purposed that these terpenoid contents may be the major contributing factors for the highest antioxidant potential of the fruit extracts by DPPH free radical scavenging activity.[17]

A total of 25 plant ethanolic extracts and three selected terpenes were selected for their cytotoxic activity and photographed (Figures 1 & 2, Text Figures 1 & 2). The % inhibition of the extracts of the plants as well as terpenes are also summarized in Text Figure 1 & 2 along with their IC50 values (Tables 1 & 2). The results showed that all plant extracts were found to beless toxic against 3T3 cell lines as compared to n-Hexadecanoic acid and cyclohexamide. Whereas the selected terpenes; methyl decanoate exhibited 11%, Hexadecanoic acid, methyl ester (16%) were also inactive against cellular activity except n Hexadecanoic acid (88%), and standard cyclohexamide 70% were found to be toxic against 3T3 cell line with IC50 (cyclohexamide 0.8±0.2).

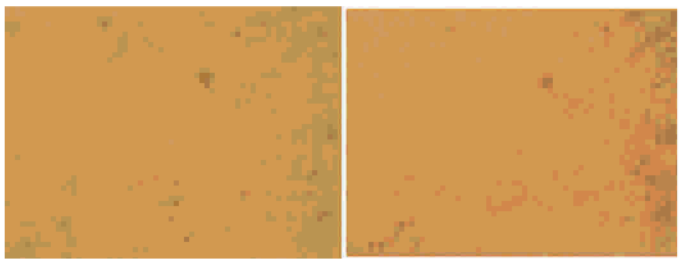

Figure 1 & 2: *Representing cytotoxic effects of terpenes against selected normal mouse fibroblasts (NIH 3T3) cells*

a: % inhibition of Hexadecanoic acid, methyl ester (Uniphat A60) against 3T3 cells

Table 1: *Cytotoxic Potential of Ethanolic Extracts of Plants in Comparison with Cyclohexamide Against 3T3 Cell Line.*

Sr. No.	Ethanolic Extracts of Selected Plants	Conc. (µg/mL)	% Inhibation/ Stimulation	IC_{50}±SD
1	*P. juliflora*	30	28	inactive
2	*C. carandas*	30	31	inactive
3	*C. speciosa*	30	3	inactive
4	*H. adenophyllum*	30	38	inactive
5	*C. diurnum*	30	12	inactive
6	*J. mimosifolia*	30	11	inactive
7	*D. malabarica*	30	8	inactive
8	*T. bellerica*	30	47	inactive
9	*F. lyrata*	30	18	inactive
10	*D. peregrina*	30	21	inactive
11	*C. verum*	30	45	inactive
12	*E. cheiri*	30	26	inactive
13	*B. lanzan*	30	21	inactive
14	*W. somnifera*	30	27	inactive
15	*F. arabica*	30	16	inactive
16	*B. lycium*	30	48	inactive
17	*S. potatorum*	30	24	inactive
18	*M. incana*	30	24	inactive
19	*Z. tenuior*	30	39	inactive
20	*C.behen*	30	29	inactive
21	*R. indica*	30	16	inactive
22	*P. granatum*	30	28	inactive
23	*L. maldivica*	30	20	inactive
24	*C. absus*	30	13	inactive
25	*C. paniculatus*	30	13	inactive
26	Std: Cyclohexamide	30µM	70	0.8±0.2

±SD = Standard Deviation

Table 2: *Cytotoxic Potential of Identified Terpenes in Comparison with Cyclohexamide against 3T3 Cell Line.*

Sr. No.	Identified plant terpenes	Conc. (µM)	% Inhibation/ Stimulation	IC_{50}±SD
1	Methyl decanoate (Uniphat A30)	30	11	inactive
2	Hexadecanoic acid, methyl ester (Uniphat A60)	30	16	inactive
3	n-Hexadecanoic acid (Glycon P-45)	30	88	3.1±0.2
4	Std: Cyclohexamide	30	70	0.8±0.2

±SD = Standard Deviation

b: % inhibition of Methyl decanoate (Uniphat A30) against 3T3 cells

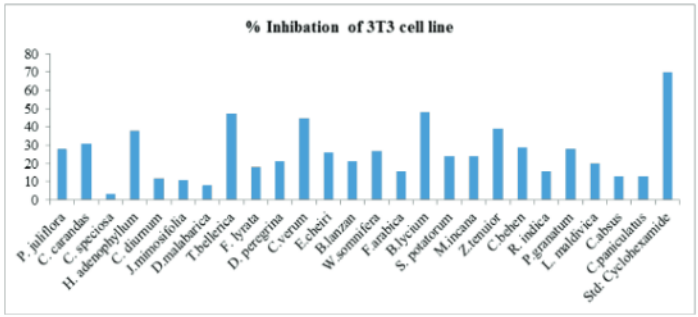

Figure 1: *Cytotoxic inhibition of Ethanolic Extracts of Plants in Comparison with Cyclohexamide Against Normal Mouse Fibroblasts (NIH 3T3).*

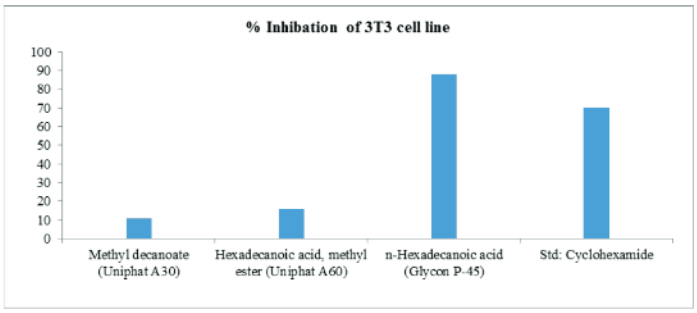

Figure 2: *Cytotoxic Inhibition of Identified Plant Terpenes in Comparison with Cyclohexamide Against Normal Mouse Fibroblasts (NIH 3T3).*

Discussion

Pakistan being an agricultural land is a precious source of medicinal and ornamental plants. The overall environment, humidity level, and several other contributing factors affect overall medicinal and pharmacological properties of the plants.[17] Local herbalists use these medicinal plants to cure local infectious problems.[6,11]

A big population of Pakistan can't afford costly anticancer synthetic medicines, therefore, they have to import them. If we are successful to use these plants as anticancer, then this will not only decrease the burden on economy of Pakistan but also provide local medication that can be more effective for the local people because of their same environment. Moreover, these medicines will have low side effects, cost effective, easily available help to improve quality of life of patients.

Our study describes cytotoxic investigations of 25 medicinal plants so far not studied and screened for

cytotoxic activity against healthy mouse fibroblasts by using MTT assay. All these plant extracts were found to be inactive against 3T3 cells and exhibited various range of inhibition. The fruit/seed extracts of C. speciosa (3%), D. malabarica (8%), J. mimosifolia (11%), C. absus (13%), C. paniculatus (13%), F.arabica (16%), B. lanzan (21%), M. incana (24%), E. cheiri (26%), H.adenophyllum (38%), Z.tenuior (39%), T.patula (39%) and C.verum (45%) were inactive, and methyl decanoate (11%), methyl palmitate (16%) was almost nontoxic. All these plants extracts can be used safely in the cancer treatment without exerting damage to normal healthy cells.

In this study, for the first time we have compared the cytotoxic activity of our selected plants with natural terpenes. In our previous study, we have investigated that these medicinal plants have high Terpenoids.[16] Terpenoids are natural compounds with antimicrobial, antitumor and anticancer properties.[19,20] Some scientists also gave their expert opinion that Terpenoids are the natural products for cancer therapy favoring this experimental work of natural Terpenoids quest.[21,22,23,24,25] Hence, we can say that these Terpenoids may be acting as shield for normal healthy fibroblasts cells and found to be inactive against them as compared to the cancerous cells.

Now, our further plans will be the use of isolation and standardization of potent Terpenoids from these medicinal plants and use them against cancer cell lines. If we are successful in doing this, it will bring a revolution in cancer treatment with minimum side effects and low cost. Moreover, local medicine may lead to personalized medicine in future too. We also welcome the researchers, pharmacists, healthcare professionals and drug analysts for future experimentation, leading to natural compounds purification, in vitro anticancer activity, in vivo administration (animal models) before clinical trials of these medicinal plants. In this way, they may help towards the anticancer drug development after investigating in vitro and in vivo administration and finally on clinical trials leading to drug standardization.

Conclusion

This study confirms that the selected medicinal plants are rich in anticancer, chemo-preventive and medicinally important Terpenoids and proved to be inactive against healthy mouse fibroblasts (NIH 3T3) cells. Whereas, the further steps requiring anticancer potential and preclinical trials on cancer animal model will be vital steps to use them on commercial scales in future.

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References

1. Abdul-Hafeez, E, Y., Karamova, N. S. and Ilinskaya, O. N. Antioxidant activity and total phenolic compound content of certain medicinal plants. International Journal of Biosciences. 2014:5(9):213 - 222.
2. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10 Lyon. 2010.10
3. Sarwar MR, Saqib A. Cancer prevalence, incidence and mortality rates in Pakistan in 2012. Cogent Medicine. 2017;4(1):1288773.
4. Ayaz, M., Junaid, M., Ullah, F., Sadiq, A., Subhan, F., Khan, M. A., Ahmad, W., Ali, G., Imran, M. and Ahmad, S. Molecularly characterized solvent extracts and saponins from Polygonum hydropiper L. show high anti-angiogenic, anti-tumor, brine shrimp, and fibroblast NIH/3T3 cell line cytotoxicity. Frontiers in pharmacology. 2016:7(74):1 – 13.
5. Sharma, P., McClees, S.F. and Afaq, F. Pomegranate for Prevention and Treatment of Cancer: An Update. Molecules. 2017; 22(1):177 – 195.
6. Plummer M, de Martel C, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. The Lancet

Global Health. 2016;4(9):609-16.

7. Weng, J. R., Yen, M. H. and Lin, W. Y. Cytotoxic constituents from Celastrus paniculatus induce apoptosis and autophagy in breast cancer cells. Phytochemistry. 2013; 94:211 - 219.
8. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Frontiers in pharmacology. 2014; 4:177.
9. Mustafa, G. H., Ahmed, S., Ahmed, N. I. and Jamil, A. M. Phytochemical and antibacterial activity of some unexplored medicinal plants of Cholistan desert. Pakistan Journal of Botany. 2016;48(5):2057 - 2062.
10. Azeemi KS. (1999). Rang-o-Roshni se Ilaj. Karachi: Al-Kitab Publications, 42–3.
11. Hamidpour, R., Hamidpour, M., Hamidpour, S. and Shahlari, M. Cinnamon from the selection of traditional applications to its novel effects on the inhibition of angiogenesis in cancer cells and prevention of Alzheimer's disease, and a series of functions such as antioxidant, anticholesterol, antidiabetes, antibacterial, antifungal, nematicidal, acaracidal, and repellent activities. Journal of Traditional and Complementary Medicine. 2015; 5(2):66 -70.
12. Ahmed, S. I., Hayat, M. Q., Tahir, M., Mansoor, Q., Ismail, M., Keck, K. and Bates, R. B. 2016. Pharmacologically active flavonoids from the anti-cancer, antioxidant and antimicrobial extracts of Cassia angustifolia Vahl. BMC Complementary and Alternative Medicine. 2016;16(1):460–469.
13. Grassmann A, Gioberge S, Moeller S, Brown G. ESRD patients in 2004: global overview of patient numbers, treatment modalities and associated trends. Nephrology Dialysis Transplantation. 2005; 20(12): 2587-93.
14. Weng, J. R., Yen, M. H. and Lin, W. Y. Cytotoxic constituents from Celastrus paniculatus induce apoptosis and autophagy in breast cancer cells. Phytochemistry. 2013;94:211-219.
15. Harborne AJ. Phytochemical methods a guide to modern techniques of plant analysis. springer science & business media; 1998.pp.49-188.
16. Malik SK, Ahmad M, Khan F. Qualitative and quantitative estimation of terpenoid contents in some important plants of punjab, pakistan. Pakistan Journal of Science. 2017;69(2).
17. Malik SK, Ahmad M, Khan F. Estimation of radical scavenging potential of selected medicinal plants of lahore, Pakistan. Pakistan Journal of Science. 2017;69: 246-250.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983;65(1-2):55-63.
19. Ercioglu, E., Velioglu, H. M. and Boyaci, I. H. 2018. Determination of terpenoid contents of aromatic plants using NIRS. Talanta. 2018;178:716-721.
20. Jain PK, Soni A, Jain P, Bhawsar J. Journal of Chemical and Pharmaceutical Research, 2016, 8 (2): 1-6. Journal of Chemical and Pharmaceutical Research. 2016;8(2):1-6.
21. Huang M, Lu JJ, Huang MQ, Bao JL, Chen XP, Wang YT. Terpenoids: natural products for cancer therapy. Expert opinion on investigational drugs. 2012; 21(12): 1801-18.
22. Araniti, F., Sánchez-Moreiras, A. M., Graña, E., Reigosa, M. J. and Abenavoli, M. R. Terpenoid trans-caryophyllene inhibits weed germination and induces plant water status alteration and oxidative damage in adult Arabidopsis. Plant Biology. 2017;19(1):79-89.
23. Di Carro, M., Ianni, C. and Magi, E. Determination of Terpenoids in Plant Leaves by GC-MS: Development of the Method and Application to Ocimum basilicum and Nicotiana langsdorffii. Analytical Letters. 2013; 46(4):630-639.
24. Zhang, C. and Liu, Y. Targeting cancer with sesterterpenoids: the new potential antitumor drugs. Journal of natural medicines. 2015;69(3):255-266.
25. Asif, H. M., Sultana, S., Akhtar, N., Rehman, J. U. and Rehman, R. U. 2014. Prevalence, risk factors and disease knowledge of breast cancer in Pakistan. Asian Pacific Journal of Cancer Prevention. 2014;15(11):4411-4416.