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Anticancer activity of acetone and methanol extracts of Terminalia chebula Retz and *Withania somnifera* (Linn.) Dunal on HeLa cell line

Mary Grace Jinukuti and Archana Giri

Centre for Biotechnology, Institute of Science and Technology,
Jawaharlal Nehru Technological University Hyderabad, Kukatpally, Hyderabad-50085, Telangana, India

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Abstract

Terminalia chebula Retz (Combretaceae) and *Withania somnifera* (Linn.) Dunal (Solanaceae), which are native to India, possess immense therapeutic and pharmacological potential. Acetone and methanol extracts of *T.chebula* fruit and *W. somnifera* root were used to determine their anticancer activity towards HeLa cell line. The viability of cells was determined by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay, which is based on the reduction of MTT by mitochondrial dehydrogenase of intact cells to a purple formazan product. Acetone extract of *T. chebula* (IC_{50} at 0.113 mg/ml) and methanol extract of *W. somnifera* (IC_{50} at 0.138 mg/ml) showed effective anticancer activity compared to that of cisplatin as control. The presence of polyphenolics in acetone extract of *T. chebula* and methanol extract of *W. somnifera* were determined using HPLC method. HPLC analysis indicated the presence of catechin, vanillic acid, quercetin and sinapic acid in acetone extract of *T. chebula* and vanillic acid, quercetin and sinapic acid in methanol extract of *W. somnifera*, which are wide spread in plants and their products play an important role in human diet.

Key words: Anticancer, *Terminalia chebula* Retz, *Withania somnifera* (Linn.) Dunal, HeLa cell line, MTT assay, HPLC analysis

1. Introduction

Cancer is a class of diseases in which cells undergo uncontrolled growth. It is a dynamic process that triggers many intricate factors (Hanahan and Weinberg, 2000). Carcinogenesis occur in three stages (initiation, promotion and progression) which represents the transformation of normal cells to malignant cells and regarded as the accumulation of gradual genetic and biochemical changes. Initiation is a mutagenic event that results from the exposure and interaction with carcinogens with cellular constituents such as DNA. Promotion is carried out by replication of abnormal cells that results in the growth of definable mass of preneoplastic cells. Progression which is the final stage of cancer development changes the preneoplastic cells into metastatic cell population (Pitot and Dragan, 1994). Chemoprevention is a strategy to inhibit, delay or reverse carcinogenesis in humans, using natural or synthetic chemical agents. Medicinal plant and its derivatives act as anticancer agents against different type of cancers (Picheswararao *et al*., 2015). Two-third of the human cancers can be eliminated by lifestyle modifications including dietary changes. Consumption of foods rich in natural phytochemicals can reduce the risk of several types of cancers. Despite the significant amount of research, a clear understanding of the individual component in the plants that can prevent cancer is not comprehensive. Polyphenols are the largest group of phytochemicals that play multiple roles in human health and act as antioxidants, antiallergic, anti-inflammatory, anticancer, antihypertensive and antimicrobial agents (Mary *et al*., 2012). Use of phytomedicines is an effort to combat diseases (Biradar, 2015). Development of such phytomedicines based on ethno medical leads is relatively more easy when compared to chemically synthesized drug (Subramoniam, 2014). Several *in vitro* and *in vivo* studies resulted in the discovery of many phytochemicals with cancer preventing properties such as catechins from green tea, curcuminoids from turmeric, isoflavones from soybean, resveratrol from grape seeds, chebulic acid and gallic acid from *T.chebula* Retz and withaferins from ashwagandha (Surh, 2003; Greenwald, 1996; Block, 1991). Dietary polyphenols may exert their anticancer effects *via* a variety of mechanisms such as removal of carcinogenic agents, modulation of cancer cell signaling and antioxidant enzymatic activities, and induction of apoptosis and cell cycle arrest (Vauzour *et al*., 2010). Compared to essential vitamins, dietary polyphenols are considered to have a superior amount of antioxidant and anticancer property (Ramos, 2008).

T. chebula Retz belonging to the family Combretaceae is called “King of Medicines” in Tibet and was listed first in Ayurvedic medicines due to its extraordinary medicinal properties. *T. chebula*, contains several phytoconstituents like tannins, sterols, phenolics, flavonoids, amino acids, essential oils and resin, *etc*. The chief components of tannin are chebulinic acid, chebulic acid, chebulagic acid, corilagin, ellagic acid and gallic acid. Tannins of *T. chebula* are hydrolysable (pyrogallol) type. About 14 types of hydrolysable tannins such as chebulic acid, chebulanin, neochebulinic acid, chebulegic acid, 1,2,3,4,6-penta-O-galloyl-b-D-glucose, 3,4,6-tri-O-galloyl-D-glucose, gallic acid, punicalagin, corilagin, ellagic acid, casuarinin and terchebulin are isolated from *T. chebula* fruits (Juang *et al*., 2004). Phytochemicals such as ethaedioic acid, 4,2,4 chebulyl-d-glucopyranose, terpinenols, anthraquinones, sennoside and

Author for correspondence: Professor Archana Giri
Centre for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological University Hyderabad, Kukatpally, Hyderabad-50085, Telangana, India
E-mail: archanagirii@yahoo.co.in
Tel.: +91-09849028367, 040-23156129

terpinenes are reported to be present in *T. chebula* (Srivastava *et al*., 2010). From the stem bark of *T. chebula* triterpenoids and their glycosides are isolated (Kundu and Mahato, 1993). According to the recent studies, *T. chebula* contain more phenolic compounds than any other plant (Saleem *et al*., 2002). *T. chebula* is known for its beneficial effects on human health due to its extraordinary power of healing (Nair *et al*., 2010). The fruits of *T. chebula* are reported to possess various medicinal properties such as antidiabetic, retinoprotective, antioxidant, chemopreventive, cardioprotective and hepatoprotective activities.

W. somnifera (Linn.) Dunal belonging to the family Solanaceae is a well known Indian medicinal plant, used widely in the treatment of various diseases. This plant consists of valuable phytochemicals, used for treating neurological disorders. The roots of *W. somnifera* are rich in phytochemicals such as alkaloids, starch, volatile oil, glycosides, reducing sugars and steroids. *W. somnifera* consists of bioactive compounds called withanolides which are naturally occurring C28 steroidal lactones. In this compound, C-22 and C-26 are oxidized to form a six membered lactone ring. Alkaloids exhibit potent anticancer activity against various cancers (Karthik *et al*., 2012). The roots of *W. somnifera* are reported to contain different alkaloids such as somniferinine, withamine, withanmine, somniferine, somnine, pseudowithamine, withanaminine (Majumdar, 1955) and also possess acylsteryl-glucoside, glycowithanolide and alkaloids such as sitoindosides VIII, sitoindosides VII , sitoindosides IX, sitoindosides X (Ghosal *et al*., 1998), withananine and withanine. The leaves are rich in steroidal lactones such as withaferin A,D,E (Kirson *et al*.,1977), withanolide Z,B, 27-deoxywithaferin A, trienolide, *etc*. The seeds of *W. somnifera* are rich in aliphatic esters and ketones such as withanolide-WS 2 and withanolide -WS 1 (Bilal *et al*., 2012). The roots of *W. somnifera* are reported to possess medicinal properties such as anti-inflamatory, antistress, antimicrobial (Mary and Archana, 2013) and rejuvenating effect, *etc*. *T. chebula* and *W. somnifera* are rich in compounds which are responsible for antioxidant, antimicrobial, anticancer, antihyperglycemic and protective effects on various organs such as heart, nerves, kidney and liver. Traditionally, these plants are used to treat a variety of human diseases. Therefore, there is an urgent need to determine the biological activity of its phytoconstituents for development of safe and cost effective remedies.

2. Materials and Methods

2.1 Plant materials and preparation of extracts

T. chebula Retz (fruits) and *W. somnifera* (Linn.) Dunal (roots) were purchased from Farm Wealth Biotech, Hyderabad and authenticated by Professor Rana Kausar, Department of Botany, Osmania University, Hyderabad. The roots and the fruits were dried and milled to fine powder. The powdered material was extracted three times with solvents such as acetone and methanol at room temperature for 24 h and filtered. The filtered extracts obtained were concentrated using a rota-vapour (IKA, Germany) at 45^0C. The extracts were stored at 4°C for further use.

2.2 HPLC-DAD Analysis

Polyphenolics in acetone extract of *T. chebula* and methanol extract of *W.somnifera* were analyzed by Shimadzu LC10 HPLC system which was equipped with diode array detector (DAD). Using a Luna C18 column (250 mm×4.6 mm i.d.; particle size, 5 ìm) with a C18 guard column, separation of polyphenolics was carried out. 6% acetic acid in 2 mM sodium acetate (solvent A) and acetonitrile (solvent B) was used as mobile phase. Varying the proportion of solvent A to solvent B, a solvent gradient was maintained in the following manner: a linear gradient of 0-15% of B for 45 min, 15-30% B for 15 min, 30-50% B for 5 min, and 50-100% B for 5 min. Chromatograms and spectra were recorded at set time of 70 min, whereas the total running time was 80 min which includes 10 min post run for equilibration of column at initial conditions. The column temperature was 30°C. The injection volume was 20 µl and flow rate was 1.0 ml/min. At 280 nm, the detector was set for catechin, vanillic acid, protocatechuic acid, syringic acid, and o-coumaric acid; 320 nm for ferulic acid and sinapic acid and 360 nm for quercetin and myricetin. The standard polyphenolics selected for the identification of compounds in *T.chebula* and *W.somnifera* were catechin, vanillic acid, protocatechuic acid, o-coumaric acid, syringic acid, sinapic acid, ferulic acid, quercetin and myricetin. Using the linear regression equations which were derived from authentic standards, polyphenolics in the *T.chebula* and *W.somnifera* extracts were quantified. The polyphenolic compounds were identified by comparing their retention time and spectral matching with that of authentic standards. By coelution, the identity of the compound was further confirmed.

2.3 Cell viability assay

The cells viability was determined by MTT (3, 4, 5-dimethylthiazol -2-yl)-2-5-diphenyltetrazolium bromide) assay. In this assay, mitochondrial dehydrogenase of intact cells reduces MTT to a purple formazan product (Mosmann, 1983). Cells were plated in wells of 96-well plates. The inoculum volume of HeLa cells was 200 µL which was around 3 x 10^3 cells/well concentration and were incubated at 37°C for 24 h in CO_2 incubator to allow adherence, prior to exposure to *T. chebula* and *W. somnifera* extracts, appropriate culture media was added. Briefly, cells were treated with known concentrations (0 - 0.2 mg/ml) in triplicate and incubated for 48 h in CO_2 incubator at 37°C. After the treatment, media containing extract was carefully removed by aspiration. To each well, 200 µl of 0.4 mg/ml MTT in phosphate-buffered saline (PBS) was added and incubated in dark for 4 h. After incubation period, formazan crystals which were formed, were solubilized by adding 200 µl of dimethyl sulfoxide (DMSO) to each well and incubated for 4 h. Amount of formazan was determined by measuring absorbance at 620 nm, using an ELISA plate reader. To determine the sensitivity of cancer cell lines, anticancer drug cisplatin (0 - 0.2 µg/ml) was used as positive reference compound. Cells grown in media containing equivalent concentration of DMSO was used as negative control. The IC_{50} values were calculated from the plotted absorbance data for the dose-response curves.

2.4 Statistical analysis

Results were calculated from triplicate data and expressed as Means ± standard deviations. The data were compared through least significant difference test, using statistical analysis system (Ver.9.1). Graphing, curve fitting and IC_{50} were performed using Graph Pad Prism (ver.6.0).

3. Results and Discussion

Effect of fruit extracts of *T. chebula* Retz. and root extracts of *W. somnifera* (Linn.) Dunal on cell viability was estimated by MTT assay. HeLa cell lines were used for investigation of anticancer

activity of fruit extracts of *T. chebula* and root extracts of *W. somnifera in vitro*. The MTT assay is extensively used for measuring cell survival and proliferation by cleavage of the soluble yellow tetrazolium salt MTT [3 (4,5 dimethyl-thiazole 2 yl) 2, 5 diphenyl tetrazolium bromide] into a blue colored formazan by the mitochondrial enzyme succinate dehydrogenase that is used for assaying cell survival and proliferation. There is a direct proportion between the formazan produced and the number of viable cells. However, it depends on the cell type, cellular metabolism and incubation time with MTT. In this study, acetone (IC_{50} at 0.113 mg/ml) extract of *T.chebula* and methanol (IC_{50} at 0.138 mg/ml) extract of *W.somnifera* showed effective anticancer activity which is comparatively less than that of cisplastin (IC_{50} at 0.042 mg/ml) which is used as control. The results of MTT assay of acetone and methanol extracts of *T.chebula* and *W.somnifera* were represented in Table 1 and Figures 1 and 2, respectively. *T.chebula* (fruit) and *W. somnifera* (root) are rich in unique phytochemicals, which act as effective anticancer agents. Chebulinic acid, one of the major constituent of *T. chebula* was reported to show significant anticancer activity against HT-29 cancer cell lines at various concentrations (Meenavangalapati *et al.,* 2013). In a study, carried out by Saleem *et al.* (2002), 70 % methanolic extract of *T. chebula* fruits decreased the cell viability, inhibited cell proliferation and induced cell death in a dose dependent manner in several human malignant cell lines and in lower concentrations, apoptosis was induced, but at higher concentrations, necrosis was the major mechanism of the cell death. Dolly *et al.* (2014) studied cytotoxic activity of acetone, methanol, ethyl acetate and aqueous extracts of *T.chebula* fruit on human fibroblasts (L929) and keratinocytes cell lines using MTT assay. Among all four extracts, acetone extract showed highest activity which can be comparable with the present study on HeLa cells. Increased concentration of acetone extracts did not affect the metabolic process of cells, indicating biocompatibility of phytoconstituents present in these extracts. In a study by Yadav *et al.* (2010), 50% ethanol extract of root, stem and leaves of *W. somnifera* could exhibit their cytotoxicity against five human cancer cell lines of four different tissues, *i.e.*, PC-3, DU-145 (prostrate), HCT-15 (colon), A-549 (lung) and IMR-32 (neuroblastoma), which determines the anticancer property of this plant. Withaferin A, a major chemical constituent of *W. somnifera* showed anticancer activity against pancreatic cancer cells by exhibiting protein degradation (Yu *et al*., 2010). The root extracts of *W. somnifera* were reported to exhibit effective cytotoxic properties against lung, colon, central nervous system, and breast cancer cell lines (Jayaprakasam *et al*., 2003), which determines the anticancer activity of *W.somnifera* root.

HPLC analysis revealed the presence of polyphenolics such as catechin, vanillic acid, quercetin, sinapic acid in acetone extract of *T. chebula* and vanillic acid, quercetin, sinapic acid in methanol extract of *W. somnifera*, when compared to that of authentic standard. The results of HPLC assay of acetone extract of *T.chebula*, methanol extract of *W.somnifera* and authentic standard were represented in Figures 3a, 3b and 3c, respectively. Catechin, which was identified in the acetone extract of *T. chebula* by HPLC analysis, was reported to show effective anticancer activity against human colorectal cancer cells (Guang *et al*., 2012). Urmila *et al.* (2011) reported the anticancer activity of quercetin against breast cancer (MCF-7), hepatic cancer (HepG2), prostate cancer (PC-3) and colon carcinoma (HCT-15) cell lines. Anticancer activity of vanillic acid against prostate cancer (PC-3) and breast cancer (MCF-7) cells are reported by Eliana *et al.* (2014). Neda and Helena (2014) reported the antiproliferative action of sinapic acid against human breast cancer T-47D cell line. These polyphenols which play an important role in our diet, may act as a potent anticancer agent.

Table 1: Cytotoxic ability of *T. chebula* (Fruit), *W. somnifera* (Root) and cisplatin (control) on HeLa cells as determined by their IC_{50}, expressed as mg/ml

***T. chebula* (Fruit) (mg/ml)**	
Cisplatin	0.042±0.001
Acetone	0.113±0.01 [a]
Methanol	0.149±0.05
***W. somnifera* (Root) (mg/ml)**	
Acetone	0.148±0.05
Methanol	0.138±0.01

[a]All data were average (±SD) of three replicates

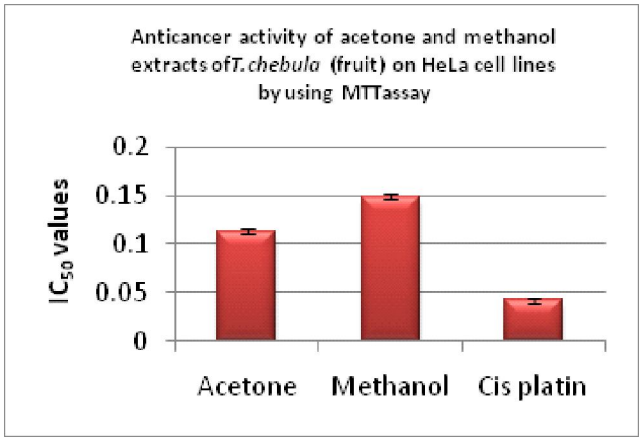

Figure 1: Anticancer activity of acetone and methanol extracts of *T.chebula* (Fruit) on HeLa cell lines by using MTT assay

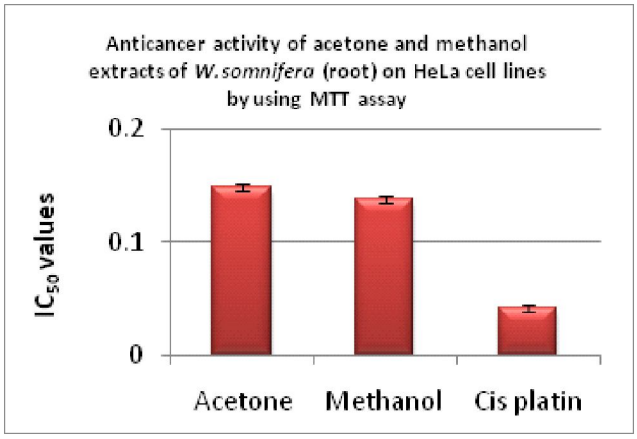

Figure 2: Anticancer activity of acetone and methanol extracts of *W. somnifera* (Root) on HeLa cell lines by using MTT assay

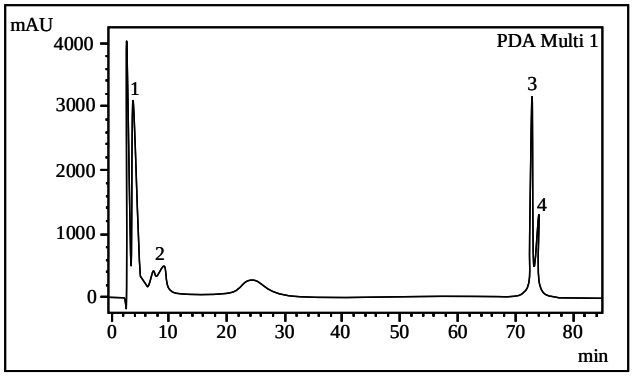

Figure 3(a): HPLC chromatogram of acetone extract of *T. chebula* (Fruit)

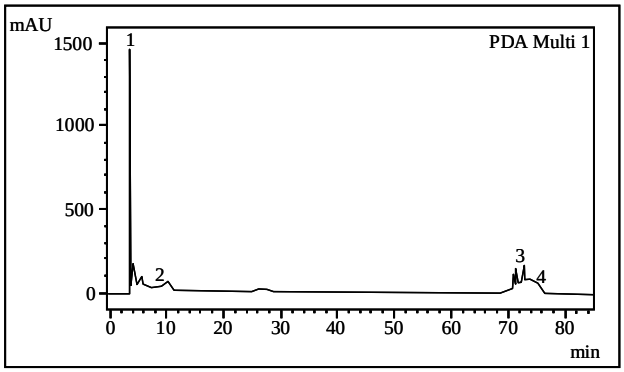

Figure 3(b): HPLC chromatogram of methanol extract of *W. somnifera* (Root)

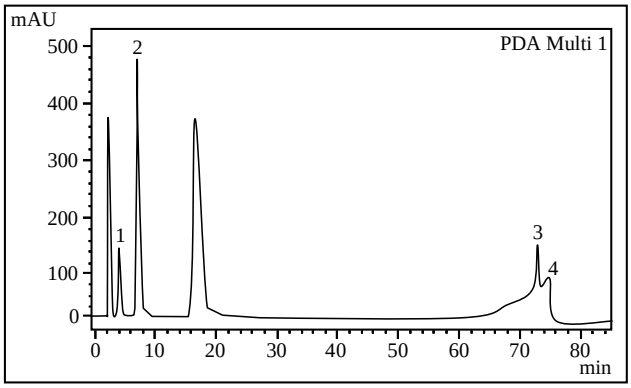

Figure 3(c): HPLC chromatogram of a mixture of standard polyphenolics. (1) Catechin; (2) Vanillic acid; (3) Quercetin; (4) Sinapic acid

4. Conclusion

Comparison of antiproliferative activity of *T.chebula* fruit and *W.somnifera* root extract with standard anticancer drug (cisplatin) showed that these extracts, have growth arresting activity, significantly due to its high polyphenolics content, thus, demonstrating its effectiveness as a cancer preventing agent. Isolation of bioactive components can exhibit the exact potential of these medicinal plants in the development of herbal formulations in future. However, further studies are needed to prove the protective effects observed *in vitro* do indeed translate *in vivo*.

Conflict of interest

We declare that we have no conflict of interest.

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