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Original Article

Phytochemistry, allelopathy and anticancer potentiality of *Withania somnifera* (L.) Dunal (Solanaceae)

Fitoquímica, alelopatia e potencialidade anticancerígena de *Withania somnifera* (L.) Dunal (Solanaceae)

H. A. Ahmed[a,b*] and S. M. El-Darier[b]

[a]King Faisal University, College of Science, Biological Sciences Department, Al-Ahsa, Saudi Arabia
[b]Alexandria University, Faculty of Science, Botany and Microbiology Department, Alexandria, Egypt

Abstract

Withania somnifera is a wild plant that shows great activity and safety against several human diseases. The current research explored the plant's chemical composition and allelopathic effects on *Rumex dentatus* (recipient plant). Moreover, anticancer activity is also tested against four types of human cancer cell lines. Chemical analysis of *W. somnifera* showed a high percentage of saponins and tannins, while glycosides, alkaloids, and flavonoids occurred in the second order. Results of the allelopathic experiments revealed significant inhibition of the *R. dentatus* plumule and radicle lengths as well as their relative dry weights. In addition, significant reductions in some primary metabolites of *R. dentatus,* like non-reducing and total sugar as well as soluble proteins, were determined. Cytotoxic potentiality of *W. somnifera* was also proved against four different cancer lines, namely; human hepatocellular carcinoma cell line (HepG2), human non-small cell lung cancer cell line (A549), human breast cancer cell line (MCF7), and colon cancer cell line (CaCo2) with IC_{50} value of about 38, 19, 27, and 24 μg/ml, respectively.

Keywords: *Withania somnifera*; *Rumex dentatus*; germination; growth; metabolites; cytotoxic potential.

Resumo

Withania somnifera é uma planta silvestre que apresenta grande atividade e segurança contra diversas doenças humanas. A presente pesquisa explorou a composição química da planta e os efeitos alelopáticos em *Rumex dentatus* (planta receptora). Além disso, a atividade anticancerígena também é testada contra quatro tipos diferentes de linhagens de células cancerígenas humanas. A análise química de *W. somnifera* mostrou alta porcentagem de saponinas e taninos, enquanto glicosídeos, alcaloides, e flavonoides ocorreram na segunda ordem. Os resultados dos experimentos alelopáticos revelaram uma inibição significativa dos comprimentos de plúmula e radícula de *R. dentatus*, bem como seus pesos secos relativos. Além disso, foi determinada redução significativa em alguns metabólitos primários de *R. dentatus* como não redutores e açúcar total, bem como proteínas solúveis. A potencialidade citotóxica de *W. somnifera* também foi comprovada contra quatro diferentes linhas de câncer, a saber: linha celular de carcinoma hepatocelular humano (HepG2), linha celular de câncer de pulmão de células não pequenas humanas (A549), linha celular de câncer de mama humano (MCF7) e linha celular de câncer de cólon (CaCo2) com valor de IC50 de cerca de 38, 19, 27 e 24 μg/ml, respectivamente.

Palavras-chave: *Withania somnifera; Rumex dentatus;* germinação; crescimento; metabólitos; potencial citotóxico.

1. Introduction

Wild plants produce many biochemicals in the environment that may positively or negatively influence other organisms and communities. Allelopathy is a branch of chemical ecology that studies the effects of such biochemical on organisms' germination, productivity, yield, and metabolic changes (Cheng and Cheng, 2015).

Withania somnifera (Ashwagandha) is one of the valuable perennial plant species with wide therapeutic applications in traditional and modern system of medicine (Datta et al., 2011) as well as a friendly corrosion inhibitor of carbon steel in HCl solution (Fouda et al., 2021). The species was distributed mainly in the neglected areas next to buildings and garden flora of cities. Roots contain flavonoids, alkaloids, steroid, polyphenols and many bioactive functional constituents (Kumar et al., 2015). The chemistry of the plant has been extensively studied, leading to the isolation and characterization of several groups of chemical constituents, which are of great biological and pharmacological interests. Aerial parts, roots and berries of this species considered as important

*e-mail: haahmed@kfu.edu.sa
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sources of some secondary metabolites like 12 or more from alkaloids, 40 withanolides and several sitoindosides (Dhar et al., 2015; Mirjalili et al., 2009). Besides these, the plant comprises a number of other secondary metabolites, including flavonols glycosides, sterols, phenolics, glycosides, starch, reducing sugar, and a variety of amino acids including aspartic acid, proline, tyrosine, alanine, glycine, glutamic acid, cysteine, tryptophan and high amount of iron (Gupta and Rana, 2007).

There is always an increase in research interest in identifying bioactive molecules that have a role in treating cancer or protecting against it. In particular, molecules extracted or isolated from plants are most attractive because of their safety, cost-effectiveness and feasibility of oral administration. Nutraceuticals have played an important role in the overall well-being of humans for many years, with or without rigorous evidence backing their health claims (Abid et al., 2024). Traditional medicine systems worldwide have utilized plants for millennia that have medicinal properties, providing an opportunity for modern-day researchers to assess their efficacies against ailments such as cancer (Mendonça et al., 2020; Palliyaguru et al., 2016).

Withania somnifera is a plant that has been demonstrated to have anti-tumorigenic properties in experimental models (Dutta et al., 2019). While scientific research on the plant has exploded in the past decade, much regarding the mode of action and molecular targets involved remains unknown. In the present study, the phytochemistry, allelopathy and anticancer potentiality were performed to evaluate *W. somnifera* as a potent herbicide against *Rumex dentatus* L., (Polygonaceae) and antitumor agent in plants and humans. The two species are invasive and waste-land weeds.

2. Materials and Methods

2.1. Collection and extract preparation

Withania somnifera was collected from Alexandria, Egypt, in August 2020. S.M. El-Darier identified the plant, and a voucher specimen has been deposited in the Herbarium of Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt.

Fresh *W. somnifera* roots of good quality were obtained in a single lot from herbal garden, washed under running water to remove adhering foreign particles, weighed fresh, and dried at low temperature (45-55°C), then ground using an electric grinder. The powder obtained was sieved twice to remove the coarse particles and stored in air-tight containers until further analysis. The yield of the dry matter was calculated as about 69%. Ten grams of root powder were soaked in ethanol in a 1:10 w/v ratio for 24 hours. The extract was filtered and alcohol was evaporated using rotary evaporator apparatus under low temperature. The residue was used later *in vitro* cytotoxic assay.

2.2. Phytochemical screening

The root extract of *W. somnifera* was analyzed for the presence of alkaloids, glycosides, saponins, steroids, flavonoids and phenolic compounds. One gram of dry root sample was weighed and soaked in ethanol (ethanolic extract) and water (aqueous extract). The sample was saturated and allowed to stay overnight for 24 hours. After overnight incubation with continuous shaking the sample extract was filtered with Whatman filter paper then the filtrate was centrifuged at 10,000 to 25,000 rpm for 10 minutes. After that, the supernatant was used for phytochemical screening (Soni et al., 2012). The supernatant was kept in a refrigerator until use.

2.3. Collection and preparation of recipient seeds

Healthy uniform seeds of *R. dentatus* were collected from wheat and broad bean fields in El-Bihara province in June 2019. The seeds were scratched with sandpaper and soaked in distilled water for 1 h, then surface sterilized with 70% ethanol for 2 min and rinsed with double-distilled water several times for complete removal of the sterilant.

2.4. Allelopathy experiment

To perform the germination bioassay test, the surface sterilized seeds were placed evenly in sterilized glass Petri dishes (9 mm). Each Petri dish contained 10 seeds. Then equal volumes (5 ml) of varying concentrations of the aqueous extract were introduced into each Petri dish. Similar volumes of double distilled water were used as control. All the Petri dishes were incubated in the dark at room temperature (24-26°C). The allelopathic behavior was evaluated by recording the number of germinated seeds, plumule (PL) and radicle (RL) lengths using a millimeter ruler and plumule (PDW) and radicle (RDW) dry weights for 7 days after sowing. The vigor Index (VI) was calculated according to Vashisth and Nagarajan (2010) as follows (Equation 1):

$$Vigor\ Index(VI) = Germination\ percentage\ x\ mean\ of\ seedling\ length(plumule + radicle) \quad (1)$$

Germination and inhibition percentages (GP & IP) of radicle growth were calculated by the following formulae (Equation 2):

$$\begin{aligned} &\textbf{Germination percentage} = Number\ of\ germinated\ seeds\ /\ Total\ number\ of\ seeds \times 100 \\ &\textbf{Inhibition}\ (\%)\ \textbf{of radicle growth} = (X - Y)\ /\ X \times 100 \end{aligned} \quad (2)$$

where, X= Control mean radicle length and Y= Treated mean radicle length.

2.5. Determination of primary metabolites

Seven-day-old *R. dentatus* seedlings were used for the extraction and determination of carbohydrates as reducing sugar (Hernández-López et al., 2020), soluble protein (Singh et al., 2015), and total amino acids (Lee et al., 2013).

2.6. Potential cytotoxicity of W. somnifera

Ethanolic extract was tested using the method of (Skehan et al., 1990) by using Sulforhodamine B (SRB) Assay

at the National Cancer Institute, Cairo, Egypt by serial sub culturing. HepG2, A549, MCF7, and CaCo2 were tested.

1- Cells were plated in a 96- multiwall plate (10^4 cells/ well) for 24 hours before treatment with the compound (s) to allow attachment of the cell to the wall of the plate.
2- Different concentrations of the compound under test (0, 1, 2.5, and 10 µg) were added to the cell monolayer triplicate wells and were prepared for each dose.
3- Monolayer cells were incubated with the compound (s) for 48 hours at 37°C and in an atmosphere of 5% CO_2.
4- After 48 hours at 37°C, Cells were fixed, washed, and stained with Sulforhodamine B.
5- The excess stain was washed with acetic acid, and the attached stain was recovered with Tris-EDTA buffer.
6- Color intensity was measured using an ELISA reader.
7- The relation between surviving fraction and drug concentration is plotted to get the survival curve of each cancer cell line after the specified treatment.

Surviving fractions of cells throughout drug exposure were characterized graphically by IC_{50} values (drug concentration that yields 50% fewer cells than the drug-free control). The IC_{50} values were estimated by linear least-squares regression of the growth values versus the logarithm of the extract concentration; only concentrations that yielded growth values between 10 and 90% were used in the calculation. National Cancer Institute (NCI, USA) recommended that 30 µg/ml as the upper IC_{50} limit is considered promising for purification of a crude extract (Mothana et al., 2009).

Predominantly, a bar (P) parallel to the x-axis and intersecting the point 50% on the y-axis was constructed. In the next step, a bar was plotted parallel to the y-axis, starting from the point of intersection of P with the dose-response plot. The IC_{50} could then be directly determined at the point of intersection with the x-axis.

2.7. Growth Inhibition Percentage (GIP)

Growth inhibition percentage (GIP) was calculated according to the general equations of Mosmann (1983) (Equation 3):

$$GIP = \left[100 - \left(\text{Treated survival cells} / \text{control cells}\right) * 100\right] \quad (3)$$

2.8. Statistical analysis

Data concerning the effect of different concentrations of *W. somnifera* aqueous extract on some germination and growth criteria as well as some metabolites were subjected to standard analysis of variance (ANOVA) (Zar, 1984) using the COSTAT 2.00 statistical analysis software manufactured by Cohort software.

3. Results

3.1. Phytochemical screening

The phytochemical screenings of *W. somnifera* are presented in Table 1. Data showed that the plant contains appreciable amounts (+++) of saponins and tannins while glycosides, alkaloids and flavonoids occurred in the second order (++). Fats and fixed oils and volatile oils were not detected in the two types of extracts, and carbohydrates in ethanol extract only. Total phenolics constituents identified by RP-HPLC analysis at λ = 280 nm (Table 2) and at λ = 330 nm of the ethanolic extracts (Table 3) exerted high values of about 8.03% (at λ = 280 nm) and 13.95% (at λ = 330 nm). The results showed the absence of catechin and rutin at λ = 280 and 330 nm respectively.

Table 1. Qualitative screening of the two plant extracts.

Test	Ethanolic extract	Aqueous extract
Alkaloids	++	+
Carbohydrates	-	+
Fats and fixed oil	-	-
Flavonoids	++	+
Glycosides	++	+
Proteins and amino acids	+	+
Saponins	+	+++
Tannins	+	+++
Volatile oil	-	-

(-) The active compound under investigation was not found; (+) Weak to moderate amounts of the active compound under investigation; (++, +++) high amounts of active compound under investigation.

Table 2. Phenolics identified by RP-HPLC* analysis (at λ = 280 nm) of the ethanolic extracts of *Withania somnifera*.

Retention time	Identified constituent	Relative area (%)
6.81	Pyrogallol	0.13
6.92	Gallic acid	0.04
8.235	Protocatechuic	0.15
8.444	Catechin	-
8.593	Chlorogenic acid	0.48
8.950	Catechol	0.13
10.040	Caffeic acid	0.22
11.073	Vanillic acid	0.40
11.620	Ferulic acid	0.36
12.466	Salicylic acid	0.69
12.943	Ellagic acid	1.20
13.127	Benzoic acid	1.04
13.789	Coumaric acid	0.55
14.980	Cinnamic acid	0.84
18.657	Chrysin	1.80
Total identified constituents		8.03

*Reverse-phase high performance liquid chromatography.

3.2. Allelopathic potentiality

Results in Figure 1 showed significant inhibition in seed germination percentages of *R. dentatus* treated with *W. somnifera* extracts at the concentrations inclined from control to 8% concentration level. Similarly, seedling growth of the weed species was highly affected with donor species extracts showing a reduction in the plumule and radicle lengths as well as total seedling length and their relative dry weights. Consequently, the vigor index (VI) attained the same trend and the effect was concentration dependent. Notably, the inhibition percentage increased significantly as the extract concentration increased.

The remarkable reduction in the growth of the tested weed species was accompanied by a significant reduction in reducing, non-reducing and total sugar content as well as soluble proteins. Contrarily, free amino acid content increased significantly as extract concentration increased (Figure 2).

Table 3. Phenolics identified by RP-HPLC analysis (at λ = 330 nm) of the ethanolic extracts of *Withania somnifera*.

Retention time	Identified constituent	Relative area (%)
3.83	Quercetin	0.04
11.78	Rosmarinic acid	2.65
12.06	Hesperidin	1.13
12.43	Rutin	-
13.267	Quercitrin	3.82
14.576	Naringenin	1.00
14.952	Hesperitin	1.20
15.147	Kampferol	1.11
16.167	Apigenin	1.00
Total identified constituents		11.95

3.3. Anti-proliferative activity of W. somnifera ethanolic extract

In vitro cytotoxicity, *W. somnifera* was tested on four human cancer cell lines. Dose response curves constructed for the SRB method between 19-92 µg/ml ranges. Results in Figure 3 showed that the incubation of *W. somnifera* extract with HepG2 and A549 significantly inhibited cell proliferation with IC_{50} values of about 38 and 19 μg/ml, respectively. Likewise, apoptosis in MCF7 was enhanced by *W. somnifera* with an IC_{50} value of 27μg/ml, whereas the potential cytotoxic activity of *W. somnifera* against CaCo2 attained IC_{50}= 24 μg/ml.

3.4. Growth inhibition percentage

The samples of *W. somnifera* root ethanolic extract were verified on four cell lines (HepG2, A549, MCF7 and CACo2). Growth inhibition percentage (GIP) is illustrated and statistically represented in Figure 4. The radar presentation method displayed values relative to the center point. The proliferation of HepG2 was significantly

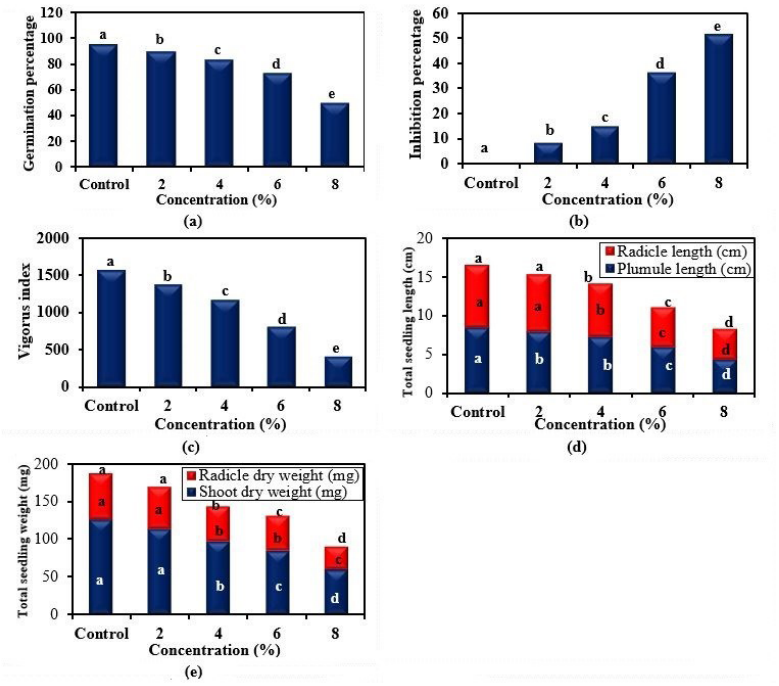

Figure 1. Response of germination and some growth criteria of *Rumex dentatus* seedlings to variation in extract concentration (%) of *Withania somnifera* 7 days after sowing. Different letters on bars indicate a significant difference at P < 0.05 according to one-way ANOVA test.

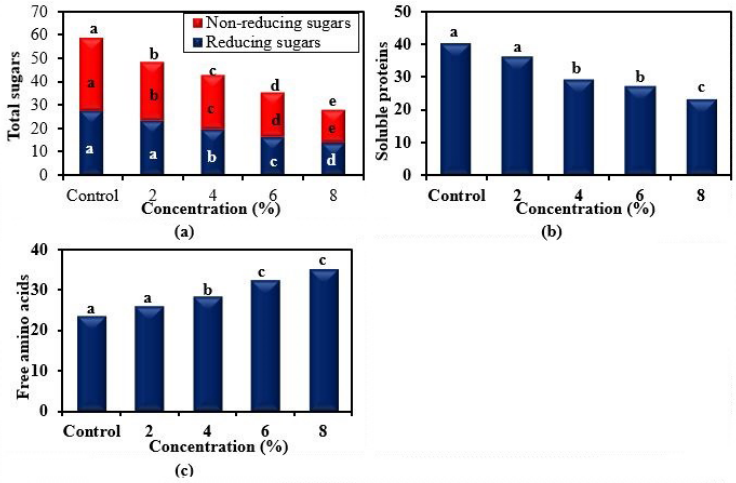

Figure 2. Response of some metabolic products (mg/g.d.w.) in *Rumex dentatus* seedlings to variation in extract concentration (%) of *Withania somnifera* 7 days after sowing. Different letters on bars indicate a significant difference at P < 0.05 according to one-way ANOVA test.

inhibited by 80.2% suppression. Likewise, the proliferation of MCF7 and CACo2 was significantly inhibited by 79 and 68.9%, respectively, while the proliferation of A459 was inhibited by 59.5%.

4. Discussion

Medicinal plants are a rich source of bioactive phytochemicals or bionutrients. A proper understanding of phytochemical screening for plants is essential for drug discovery, developing novel therapeutic agents and for interpreting the allelopathic effects of several plant species (Anwar et al., 2020; Egbuna et al., 2018). Studies carried out during the past 2– 3 decades have shown that phytochemicals have an important role in ecosystem management and in preventing chronic diseases like cancer (Saxena et al., 2013). The chemical analyses infer the effective role that plant extracts play and the mechanism by which they work, whether on the ecological or therapeutic levels (Altemimi et al., 2017; Kamal et al., 2022).

The present study explored that in *W. somnifera* root extract, saponins and tannins attained the first order followed by glycosides, alkaloids and flavonoids. Total phenolics constituents exerted considerable high values. The interrelationship between these constituents and the apparent interaction of some germination and growth factors in the recipient *R. dentatus* plant was significant. Seed germination percentages, and seedling growth (plumule and radicle lengths, total seedling length and their relative dry weights, vigorous index) were reduced, accompanied by a reduction in some metabolites (reducing, non-reducing and total sugar content as well as soluble proteins). In contrarily, free amino acid content increased significantly as extract concentration increased. Li et al. (2010) stated that phenolic allelochemicals had been observed in natural and managed ecosystems, where they cause many ecological and economic problems, such as

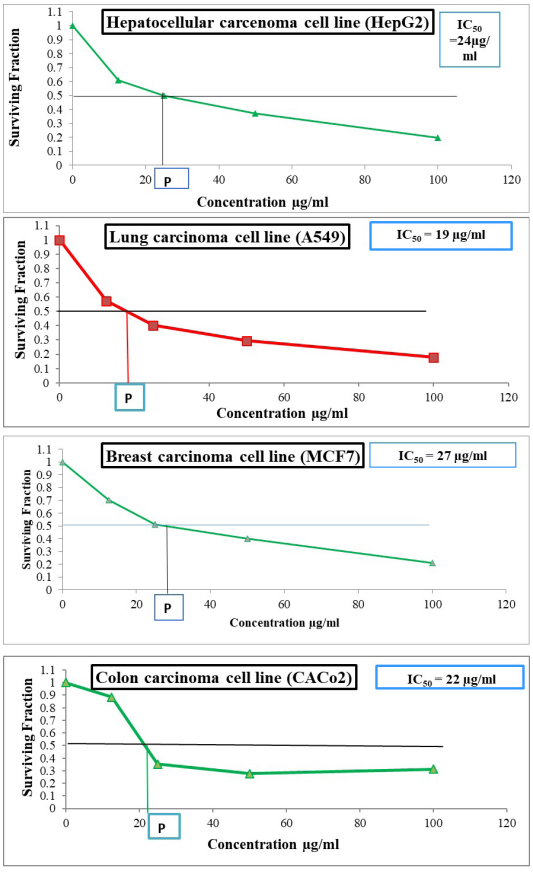

Figure 3. *In vitro* cytotoxicity of *W. somnifera* on four human cancer cell lines.

declines in crop yield due to soil sickness. For example, in agroecosystems, allelochemicals have detrimental effects on the growth of associated and next-season

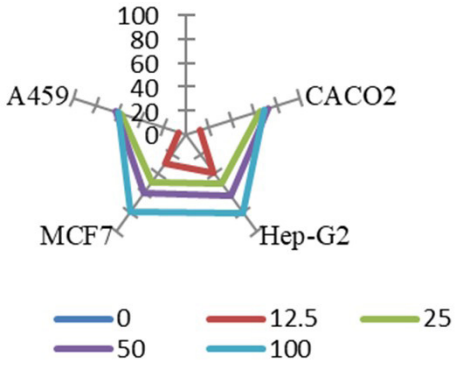

Figure 4. *In vitro* cytotoxicity of *Withania somnifera* ethanolic extracts against four cell lines HepG2: human liver hepatocellular carcinoma cell line, A549: human non-small cell lung carcinoma cell line, MCF7: human breast carcinoma cell line and CACO2: colon cancer cell line in the SRB assay using radar method.

crops (Ridenour and Callaway, 2001). The current study illustrated that *W. somnifera* root aqueous extract (WSRAE) affects the germination percentage (GP) of *R. dentatus* due to phytotoxic allelopathic interactions. Additionally, the obtained data established that inhibition in germination percentage (IP) of *R. dentatus* was significantly affected due to the allelopathic action of the WSRAE. Ordinarily, IP increased with the increase of WSRAE concentration and reached the maximum value of 51.25% at 8% WSRAE concentration. These results agreed with Shteliyana et al. (2012) on seeds of soybean, peas and vetch in response to higher concentrations of the Johnson grass roots extract and with that recorded by Raoof and Siddiqui (2012).

Many therapeutics with low side effects are of natural origin (Newman and Cragg, 2007; Sabir et al., 2021; Santana et al., 2021). In this respect, plants are extensively exploited as a potential source for active components with high antitumor activity. Due to the side effects of chemical drugs, the use of herbs for medicinal purposes is expanding in the clinical field (Yuan et al., 2016). Continuing oxidative damage to lipids, proteins, DNA, and other molecules may contribute to cancer development (Neri et al., 2021; Thanan et al., 2015).

Approximately 60% of all drugs now undergoing clinical trials for the multiplicity of cancers are either natural products or compounds derived from natural products (Réthy, 2007). Earlier studies indicated that only the root extract of *W. Somnifera* is a potential source of new molecules that can curtail cancer growth (Dredge et al., 2003). *W. Somnifera* leaves have also been shown to inhibit the growth of human cancer cell lines comparared to that produced by Adriamycin (Yadav et al., 2010). In this study, growth inhibitory activity of root of *W. somnifera* was investigated against four cell lines representing four different tissues, $HepG_2$, A549, MCF7, and $CaCo_2$. Current results clearly indicated that the cytotoxic effect of *W. somnifera* beyond the four human cancer cell lines resulted in promising starting sources for further investigation. The study supports that *W. somnifera* ethanol extract was highly cytotoxic to the studied human cell lines. In conclusion, this herb is important among various anticancer medicinal plants. It is essential to further screen and investigate different formulations for anticancer therapy in vitro and in vivo in combination with established chemotherapy.

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