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In vitro anticancer activities of *Withania coagulans* against HeLa, MCF-7, RD, RG2, and INS-1 cancer cells and phytochemical analysis

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ABSTRACT

Background: The Pakistani Salt Range has a rich floral diversity including *Withania coagulans* from the Solanaceae family.
Methods: The crude methanolic extracts of the root, leaf, leaf stalk, and fruit of this plant were screened for their cytotoxic activity against human (HeLa, MCF-7, RD) and rat (RG2 and INS-1) cancer cell lines at 20 μg/mL and compared to methotrexate. The IC_{50} values indicated that leaf stalk and fruit extracts exert an 80% or higher cytotoxic activity against all cell lines at 24 hours.
Results: The leaf stalk extract showed the highest cytotoxic efficacy against all tested cell lines, with IC_{50} values ranging from 0.96 ± 0.01 μg/mL to 4.73 ± 0.05 μg/mL followed by the fruit extract with IC_{50} values of 0.69 ± 0.01–6.69 ± 0.06 μg/mL after 48–72 hours incubation. The leaf stalk and seed extracts were analyzed for polyphenols and flavonoids using RP-HPLC. The total flavonoid content (TFC) was calculated for all tested samples, and the highest TFC was recorded for the root extract (394.34 ± 1.26 μg/g). The total phenolic content (TPC) was found in the seed extract (307.86 ± 9.42 μg/g) of *W. coagulans*. The highest contents of myricetin (358.46 ± 2.91 μg/g) were noted in the leaf extract, and highest quercetin was recorded in the seed extract (21.43 ± 0.13 μg/g). The highest gallic acid concentration (83.62 ± 0.71 μg/g) was recorded in leaf stalk extract and *p*-hydroxybenzoic acid in the seed extract (157.46 ± 1.43 μg/g).

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Conclusion: The present study gives a scientific insight and comparative analysis of various plant parts in this medicinally important plant species from the Salt Range of Pakistan against both human and rat cancer cells.

1. Introduction

Cancer caused over 8 million deaths worldwide in 2013; in 1990, it was the 3rd leading cause of death, so it advanced to the 2nd place in 2013. According to an estimate of the American Cancer Society (ACS), about 1,658,370 new cancer cases were diagnosed and 589,430 cancer deaths registered in the United States (US) alone during 2015. For some cancers, effective albeit expensive therapies are available, whereas no effective therapies are available for others.

Plants are an important source of natural products providing the raw material for diverse pharmaceutical and therapeutic applications due to the presence of phytochemicals commonly known as secondary metabolites. A large number of metabolites are utilized against various diseases including cancer and other cellular disorders.[1,2] The medicinal plants and their bioactive constituents have been extensively used as therapeutics against various cancer types, and a vast number of medicinal plants belong to the Solanaceae family.[3,4]

Medicinal plants are important sources for the treatment of noxious diseases like cancer in developing countries like Pakistan. Plants are used due to their cost-effectiveness, availability, and low toxicities.[5] Scientists now need to focus primarily on the scientific validation of the plant species that are used in folk medicines based on ethnobotanical surveys. Once promising plant species are identified, identification of the bioactive substance and their therapeutic effects are subsequent tasks.[5]

Pakistan has a diverse flora of medicinal plants starting from the deserts of Sind, passing through the plane of Punjab to the Hilly areas of northern Pakistan. The salt range of Pakistan is also very rich in its medicinal plants, traditionally used by local herbalists (*Hakeems*) to treat various noxious diseases including cancer.[6] One important plant family of the region is Solanaceae, and *Withania coagulans* is one important regional endemic, edible, and medicinal plant of this family. The plant is ethnobotanically reported in cancer treatment by local practitioners (*Hakeems*) as well as other noxious diseases for centuries; however, scientific authentication of its use has yet not been established. Concerning phytochemical isolation and cytotoxic activity, no study has been reported to date on *W. coagulans* from Pakistan. This plant is commonly known as Indian Rennet, vegetable rennet (English), Paneer Dodi/Jangli Paneer (Hindi & Punjabi), and Ning gu shui qie (Chinese). It has been reported to possess a variety of ethnomedicinal uses, and its extracts have shown potential activities, in particular, anticancer activities, wound healing, immune modulating, as well as antihyperglycemic and hypolipidemic activities.[7]

A plant's secondary metabolites are synthesized in response to stress conditions, and salt stress is one of the inducing factors. The plant phenolics have a major role to combat oxidative stresses to prevent cellular damages which can lead to DNA damages and abnormal cell division. In this context, the plant phenolics can be used as a cancer chemopreventive or cancer chemotherapeutics agents. Keeping in view, the present study was conducted to validate cytotoxicity effects of this plant's extracts and its fractions in comparison to its phenolic contents against MCF-7 (breast), HeLa (cervix), RG2 (brain), RD (rhabdomyosarcoma), and INS-1 (pancreas) cancer cell lines, following the guidelines and recommendation of the United States NCI plant screening program and analysis of their phytochemicals.

2. Methods

2.1. Chemicals

Methanol HPLC grade, 99.9% and n-hexane, 95% were purchased from Sigma–Aldrich, USA; dimethyl sulfoxide (DMSO) was obtained from Fisher BioReagents, Fair Lawn, NJ.

2.2. Collection of plant material

W. coagulans plant material was collected during a field survey in April 2013 from different growing localities in the Salt Range, Punjab, Pakistan (32.2416°N, 72.0237°E, and 867 m elevation). One set of specimens was prepared and identified in the Taxonomy Lab., Department of Botany, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, following the methodology of Qureshi et al.[8] The voucher specimen (Specimen No. MAQ-313(01)-2013) was deposited in the Department Herbarium of Botany, PMAS-Arid Agriculture University Rawalpindi.

2.3. Preparation of crude extract

The plant material (fruit, leaf, leaf stalk, and root) was washed, cut into pieces, dried in the shade, and pulverized using a grinder. The fine plant powder (1 kg of each) was soaked in methanol for 5–10 days to extract soluble compounds with the remaining material removed by filter paper. The first crude methanolic extract (CME) was obtained by concentrating the filtrate under reduced pressure in a rotary evaporator and further dried in a vacuum oven at 40°C. The CME was stored at 4°C in the dark until further use.[9,10]

2.4. Preparation of stock solution of crude extract

The dried plant extracts of fruit, leaf, leaf stalk, and root were weighed using a standard analytical balance (OHAUS, model

A560, S/N 2162-USA) and dissolved in DMSO to a final stock solution concentration of 1 mg/mL.

2.5. Dilution of stock solution

Dilutions of the stock solution were made in cell culture medium, utilizing minimum essential medium MEM alpha for MCF-7, DMEM for RG2, and Roswell Park Memorial Institute medium (RPMI-1640) for HeLa and INS-1 cells. All media contained phenol red and 2% fetal bovine serum (FBS), 1% L-glutamine (2mM), 1% sodium pyruvate (1mM) and 1% antibiotics penicillin (5000 U/mL), and streptomycin (2500 U/mL) (all from Gibco, Invitrogen, CA, USA) as supplements. Logarithmic dilutions of the stock solution between 20 μg/mL and 0.1 μg/mL were used to determine the extract fractions cytotoxicity (IC50 value). Dilutions were kept at 4°C until future use.

The INS-1 cells required additional supplements for their growth medium, comprising 100 mL of Glutamax 1 (100×, 200mM, Gibco), 100 mL of MEM sodium pyruvate (100mM, Gibco) as an energy source during anabolic processes, and 36 μL of β-mercaptoethanol (2.5mM, Sigma) as an antioxidant reducing agent. The pH of this stock solution was adjusted to 7.3 using 5N NaOH, filtered using to 0.2 μm pore size and stored at −20°C until future use. For INS-1 cell medium preparation, a 2% concentration (by volume) of the stock solution was added to the medium.[11]

2.6. Cell culture

MCF-7 breast cancer cells were cultured in minimum essential medium (MEM) alpha, human cervical cancer cells (HeLa), and rat insulin-secreting beta cell line (INS-1) in RPMI 1640 (1×) medium. Rat brain malignant glioma cells (RG2) and human rhabdomyosarcoma cells (RD) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) having phenol red supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (2mM), 1% sodium pyruvate (1mM) and 1% antibiotics penicillin (5000 U/mL), and streptomycin (2500 U/mL), all purchased from Gibco, Invitrogen, CA, USA. Cells were maintained at 37°C in 5% CO_2 humidified incubator. Cells were passaged at 80% confluency, and media was exchanged completely every 2–3 days.[6,12,13]

2.7. In vitro cytotoxicity tests

For cytotoxicity testing, MCF-7, INS-1, RD, RG2, and HeLa cells were seeded into 96 well cell polystyrene culture plates (COSTAR 3596 Corning Incorporated, Invitrogen, CA, USA) at a concentration of 10,000 cells/well in 200 μL of culture media for 24–48 hours before the experiments. Plates were maintained at 37°C and 5% CO_2 in a humidified incubator. When plates reached 80% confluence, the medium was replaced with 2% MEM medium containing different concentrations of a plant extract ranging from 0.1 μg/mL to 20 μg/mL. Experiments were executed in triplicate. After incubation (24 hours, 48 hours & 72 hours), the cytotoxicity was evaluated using the Presto Blue cell viability assay.[14]

2.8. Presto Blue cell viability assay

Cell viability required 10 μL of the Presto Blue cell metabolic assay (Presto Blue, Invitrogen, Frederick, MD, USA) along with 90 μL of 2% Fluorobrite Media supplemented with 2% fetal bovine serum (FBS), 1% L-glutamine (2mM), 1% antibiotics penicillin (5000 U/mL), and streptomycin (2500 U/mL) (all Gibco, Invitrogen, CA, USA). The phenol red media was added to each well of 96-well plates for viability calculation. Plates were incubated at 37°C in an incubator for 60–90 minutes. Only metabolically live cells provide a color change from blue to purple. Fluorescence quantification was provided using a 96-well plate reader Spectra Max M5 (Molecular Devices, Sunnyvale, CA, USA) with $\lambda_{ex} = 560$ nm and $\lambda_{em} = 600$ nm via the bottom of the plate.[13,14] The viability (%) is defined as:

$$\text{Viability} \quad (\%) = \frac{\text{mean fluorescence of treated cells}}{\text{mean fluorescence of control cells}} \times 100$$

2.9. HPLC analysis

The conditions or parameters that were used for the analysis of the samples are described in Table 1. The performance or efficiency of the column was tested according to the certificate of analysis of Discovery HS C18, 5 μm, cat. # 568523-U (Fig. 1). Identification of flavonoids standards (quercetin and myricetin) and phenolic acid standards (gallic acid, syringic acid, vanillic acid, ρ-hydroxybenzoic acid, ρ-coumaric acid, ferulic acid, and protocatechuic acid) were used for identification and quantification. A qualitative analysis in plant extracts was made by comparing their retention times with standards (standards were purchased from Sigma Chemicals Co., St Louis, MO, USA). Quantitative determination was carried out by using calibration curves of the standards. The conditions for the HPLC analysis of samples are provided in Table 1. Quantification of compounds was determined by using the following formula:

$$\text{Concentration of compound} = \left(\frac{\text{injected mass (ng)}}{\text{peak area of standard}}\right) * \frac{\text{peak area of sample} * \text{final volume of sample}}{\text{volume of injected } (\mu\text{L}) * \text{mass of sample (g)}}$$

2.10. Statistical analysis

The statistical package SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL) was used to analyze the data by applying an ANOVA analysis. Multiple comparisons were executed by Duncan's multiple range tests (DMRT) at $p < 0.05$. To determine the IC_{50} value, the logarithmic concentrations of the plant extract were calculated using Graph Pad Prism Version 6.0(b) [Graphpad, La Jolla, CA, USA].

3. Results

The CMEs of the root, leaf, leaf stalk, and fruit of *W. coagulans* were tested at a concentration of 20 μg/mL against HeLa, MCF-7, RD, RG2, and INS-1 for their cytotoxicity potential. Chemo-drug (MTX), cell culturing medium (CCM), and DMSO

Table 1 – HPLC Conditions for Flavonoids and Phenolic Acids Analysis

Sr. No	Analysis	Condition
1	HPLC system	LC-10A, Shimadzu, Japan
2	System controller unit	(SCL-10A), Shimadzu, Japan
3	Column	C18 (250 mm × 4.6 mm, 5 μm), Supelco, USA
4	Mobile phase (phenolics)	88:10:2, water:acetonitrile:acetic acid
5	Mobile phase (flavonoids)	3% TFA (A), ACN:MeOH (80:20) (B), (50:50)
6	Flow rate	1.0 mL/min
7	Column temperature	30°C (CTO-10A)
8	Injection	20.0 μL
9	Wavelength (λ)	280 nm (phenolics), 360 nm (flavonoids)
10	Pressure	145 kgf/cm^2
11	Acquisition software	Class LC-10
12	Detector	UV-Vis (SPD-10A), Shimadzu, Japan
13	Injection loop	20 μL (Rheodyn), USA

were also tested at the same concentration for comparison, acting as a positive control for the first and negative control for the other two ingredients. The latter is confirmed by not observing a significant ($p < 0.05$) effect of DMSO and CCM in either HeLa, MCF-7, RD, RG2, or INS-1 cells even after 72 hours of incubation.

For MCF-7, INS-I, and HeLa cells, the highest activity was recorded using leaf stalk followed by fruit extracts (>80% cell death). Although there is no significant difference in the activity from leaf stalk and fruit extracts, the leaf stalk extract has a slightly higher activity compared to fruit extract. However, in case of RG2 and RD cells, the activity showed a similar pattern for both leaf stalk extract and fruit extract having >80% cell death at 20 μg/mL for 24–72 hours incubation (Fig. 2). This activity was almost twofold to fourfold higher than that of the tested drug in the same concentration. On the basis of initial screening at 20 μg/mL, leaf stalk and fruit extract were selected to calculate IC_{50} value against all five cancer cell lines following the guidelines and recommendation of US NCI plant screening program. According to the US NCI, a plant extract is generally considered to have active cytotoxic effect if it inhibits the growth of 50% of the cells (IC_{50} value) at 20 μg/mL or less, following an incubation time of 48–72 hours.[15–17]

3.1. IC_{50} values of W. coagulans

The results of IC_{50} values of leaf stalk extract and fruit extract of *W. coagulans* at 48–72 hours incubation against all tested cell lines are shown in Table 2. The IC_{50} values were in the range of 0.96 ± 0.01–4.73 ± 0.05 μg/mL for leaf stalk extract and 0.69 ± 0.01–6.69 ± 0.06 μg/mL for fruit extract after 48–72 hours of incubation against all tested cell lines revealing very strong activity for all the tested cell lines. The lowest IC_{50} values after 72 hours incubation were recorded for fruit extract (0.69 ± 0.01 μg/mL) in MCF-7 cells and leaf stalk extract (0.96 ± 0.01 μg/mL) in RD cell (Table 2).

The cytotoxic activities of leaf stalk extract and IC_{50} values of this important medicinal plant species have been reported for the first time, and very promising results were obtained (Table 2).

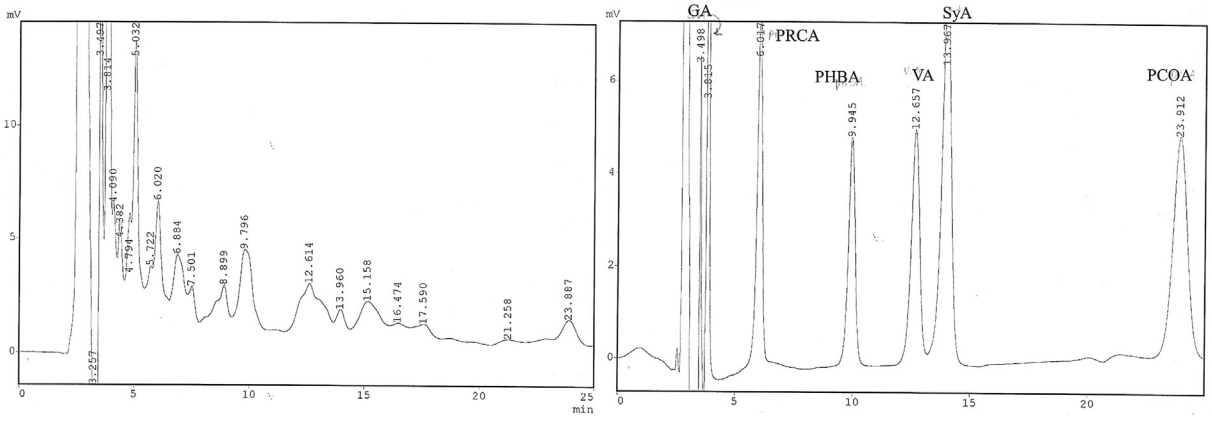

Fig. 1 – HPLC analysis, *left*: chromatogram of crude extract sample analyzed through HPLC. *Right*: chromatogram of phenolic standards (GA = gallic acid, PRCA = protocatechuic acid, PHBA = *p*-hydroxybenzoic acid, VA = banilic acid, SyA = syringic acid, PCOA = *p*-coumaric acid).

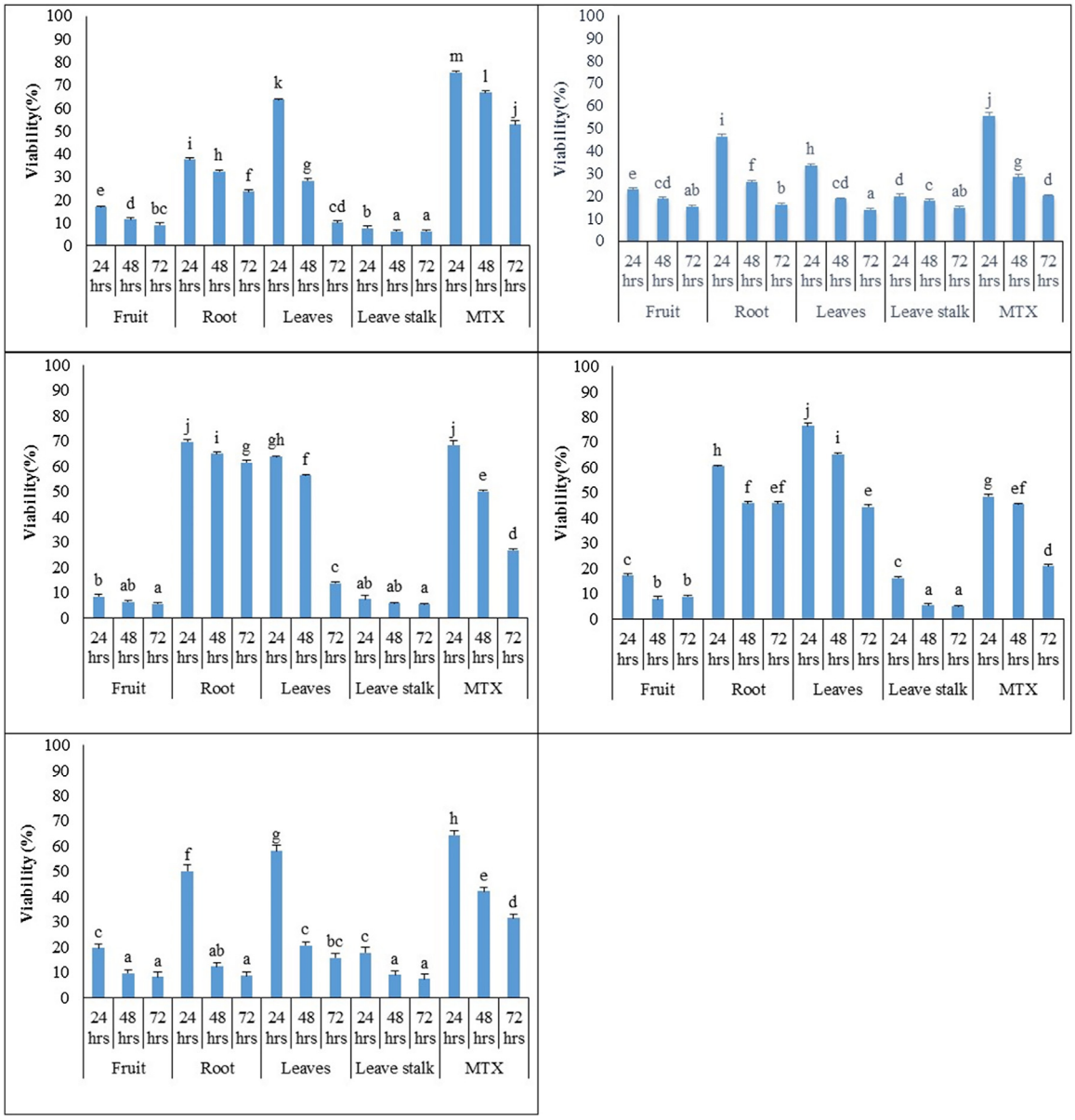

Fig. 2 – Effect of methanolic extract of *W. coagulans* extracts on various cell line following exposure to the NCI recommended a critical concentration of 20 μg/mL for 24 h, 48 h, and 72 h, for *top left* MCF-7, *top right* INS-1, *bottom left* RG2, *bottom center* HeLa, *bottom right* RD. Bars labeled with different letters are significant (Duncan's $p < 0.05$).

3.2. *Phytochemical analysis*

A comparative study on identification and quantification of polyphenols and flavonoids from various plant parts (fruit, leaf, leaf stalk, and root) of *W. coagulans* was achieved by reverse-phase high-performance liquid chromatography (RP-HPLC). Data for the flavonoids (myricetin and quercetin) contents of selected medicinal plant part(s)/organs also indicated that myricetin was the most common and abundant flavonoid present in the tested plants (Table 3). The highest contents of myricetin were observed in the leaves of *W. coagulans* at a concentration of 358.46 ± 2.91 μg/g followed by 321.98 ± 1.76 μg/g in the extract of its fruits, while for other plant parts, the concentrations of myricetin were recorded from 63.92 ± 2.91 μg/g to 292.02 ± 1.10 μg/g.

Quercetin, another important flavonoid, was also quantified in the present study from the selected plant/part(s) and its highest concentration was recorded in the fruit (21.43 ± 0.13 μg/g) followed by the leaf stalk (14.57 ± 0.19 μg/g), while for the plant's root, a concentration of 12.32 ± 0.10 μg/g was recorded, as shown in Table 3.

Total flavonoid content (TFC) was calculated for these tested samples, and the highest TFC was found in the root extract of *W. coagulans* (394.34 ± 1.26 μg/g) followed by its

Table 2 – IC_{50} Values of *Withania coagulans* (Fruit and Leaf Stalk) Against the Selected Cell Lines

Cell line	Incubation time (h)	$IC_{50\ Value}$ (μg/mL)	
		Fruit extract	Leaf stalk extract
MCF-7	48	0.99 ± 0.01	4.73 ± 0.05
	72	0.69 ± 0.01	1.02 ± 0.01
INS-1	48	2.84 ± 0.03	4.56 ± 0.04
	72	1.95 ± 0.02	2.97 ± 0.03
RG2	48	6.69 ± 0.06	2.55 ± 0.03
	72	1.97 ± 0.02	1.68 ± 0.02
HeLa	48	2.79 ± 0.03	1.79 ± 0.02
	72	2.19 ± 0.02	1.68 ± 0.01
RD	48	5.16 ± 0.04	1.74 ± 0.02
	72	2.98 ± 0.03	0.96 ± 0.01

Activity range; >100: not active: weak; 20–100: moderate; 10–20: strong; 5–10: <5: very strong (Wibowo et al, 2011).

Table 3 – The Yield of Flavonoids (μg/g) in Various Plant Parts of *Withania coagulans*

Plant species	Part(s)	Myricetin	Quercitin	Total flavonoid
W. coagulans	Root	292.02 ± 1.10	12.32 ± 0.100	394.34 ± 1.26
	Leaf	358.46 ± 2.91	ND	358.46 ± 2.91
	Leaf stalk	236.27 ± 1.85	14.57 ± 0.19	250.84 ± 1.28
	Fruit	321.98 ± 1.76	21.43 ± 0.13	343.41 ± 1.96

ND; not detected. Results are expressed in values of means ± SD, for analyzed in triplicate.

Table 4 – The Yield of Phenolic Acids (μg/g) in Various Plant Parts of *Withania coagulans*

Plant species	Part(s)	Gallic acid	Protocatechuic acid	*p*-Hydroxybenzoic acid	Vanillic acid	Syringic acid	*p*-Coumaric acid	Ferulic acid	Total phenolics
W. coagulans	Root	28.09 ± 0.69	31.89 ± 0.35	116.46 ± 0.93	19.56 ± 0.15	17.29 ± 0.38	14.79 ± 0.09	19.92 ± 0.10	219.91 ± 4.89
	Leaf	43.12 ± 0.85	9.39 ± 0.76	142.46 ± 1.47	13.27 ± 0.11	22.9 ± 0.49	29.49 ± 0.16	11.29 ± 0.08	228.8 ± 6.40
	Leaf stalk	83.62 ± 0.71	34.09 ± 0.09	123.46 ± 0.97	17.26 ± 0.18	34.95 ± 0.17	12.37 ± 0.17	ND	222.13 ± 5.71
	Fruit	35.28 ± 0.32	39.09 ± 0.18	157.46 ± 1.43	21.27 ± 0.19	14.26 ± 0.10	32.76 ± 0.21	43.02 ± 0.13	307.86 ± 9.42

ND; not detected. Results are expressed in values of means ± SD, for analyzed in triplicate.

leaf (358.46 ± 2.91 μg/g) and fruit (343.41 ± 1.96 μg/g) extracts. The average concentration of TFC in leaf stalk was only 250.84 ± 1.28 μg/g, as detailed in Table 3.

The total phenolic content (TPC) was determined equally, and the highest TPC concentration was recorded for the *W. coagulans* fruit extract (307.86 ± 9.42 μg/g). The total TPC for the remaining plant extracts was comparable with concentrations in the leaf, leaf stalk, and root extracts of 228.8 ± 6.4 μg/g, 222.13 ± 5.71 μg/g, and 219.91 ± 4.89 μg/g, respectively, see Table 4.

Among all, the highest gallic acid (83.62 ± 0.71 μg/g) and syringic acid (34.95 ± 0.17 μg/g) were recorded in the leaf stalk, whereas in fruit extracts, the highest concentrations of protocatechuic acid (39.09 ± 0.18 μg/g), *p*-hydroxybenzoic acid (157.46 ± 1.43 μg/g), vanillic acid (21.27 ± 0.19 μg/g), *p*-coumaric acid (32.76 ± 0.21 μg/g), and ferulic acid (43.02 ± 0.13 μg/g) were recorded, also shown in Table 4. The TPC was highest in the fruit extract of *W. coagulans*.

4. Discussion

Cancer being complex/noxious in nature is very difficult to cure, equally in high-income and middle-income or low-income countries. According to World Cancer Report 2017,[18] the number of cancer cases is expected to rise to 22 million over the next two decades compared to 14 million cases reported for 2012.[19] Scientists are targeting plant extracts to treat these complicated diseases, hypothesizing on their long utilization history by traditional communities.[6,20] According to the WHO report, the drug market for plant-derived compounds was approximately 100 million USD in 2007, and it is expected to reach 5 trillion USD by the year 2050.[5] A remarkable number of anticancer drugs have been made from medicinal plants and are currently in clinical use, based on ethnobotanical as well as ethnopharmacological exploration.[5,21]

Cancer chemotherapy plays a vital role in curing various malignancies, and its primary objective is to kill

the cancer cells but not harms the healthy host cells.[22] Oncologists are predominantly interested in cancer-treating compounds which are selective and induce cellular apoptosis or autophagy, a property satisfied by most secondary plants metabolites. Several studies reported that the mechanism of action of these anticancer drugs leads to the induction of apoptosis.[2]

Plant-based cancer therapeutics are considered a preferable treatment as they are natural, easily available, and can be readily used via a dietary intake. A number of plant-based compounds have very promising potential toward cancer therapeutics, particularly cyanogenic glycosides, taxanes, phenolics (saponins), and lectins. If a plant-based compound is causing cell apoptosis in cancer cells but is safe to the non-cancer cells, then it presents a potential candidate to enter *in vivo* and ultimately clinical trials for its translation into becoming a cancer therapeutic. Plants-based anticancer drugs come in four categories, viz., (a) chemopreventive drugs (antioxidants), (b) inhibitors of histone deacetylase (HDAC), (c) inhibitors of methyltransferases, and (d) mitotic disruptors.[5]

Keeping these four points in view, the present study was designed to explore medicinal plants of the Salt Range Pakistan used ethnobotanically by the traditional herbal practitioner (THP), for their potential use in treating malignancies, due to their cytotoxicity. The bioassay-guided isolation and purification of the CMEs of *W. coagulans* of aerial parts have been reported by Ihsan-ul-haq et al[23] for understanding cancer chemopreventive mechanisms. They isolated three new steroidal lactones, namely, withacoagulin G, withacoagulin H, and withacoagulin I. The withacoagulin G and withacoagulin H were the most active compounds possessing the IC_{50} values of 3.1–1.9 μM and toward inhibiting tumor necrosis factor-α (TNF-α) at 1.60–12.4 μM, respectively. The present study is the first-time report of a leaf stalk extract of *W. coagulans* showing the maximum cytotoxic effect on all tested cell lines with the IC_{50} values of 0.96 ± 0.01–4.73 ± 0.05 μg/mL after 48–72 hours incubation, revealing very strong activity for all tested cell lines (Table 2). The range of IC_{50} values against HeLa cells was lowest (1.68–1.79 μg/mL) compared to MCF-7 (1.02–4.73 ± 0.05 μg/mL) and RD cells (0.96 ± 0.01–1.73 μg/mL). In RG2 cells, IC_{50} values from 1.68 μg/mL to 2.55 μg/mL were recorded, while in INS-1 cells, they were between 2.97 μg/mL and 4.56 μg/mL (Table 2).

Our findings are in agreement with Ihsan-ul-haq et al[23] who reported IC_{50} comparable values for the aerial plant parts of *W. coagulans*. In the present study, the fruit extract of *W. coagulans* had a cytotoxic effect on all tested cell lines with IC_{50} values of 0.69 ± 0.01–6.69 ± 0.06 μg/mL following 48–72 hours incubation. Against HeLa cells, the IC_{50} values were 2.19–2.79 μg/mL, and for MCF-7, the same were calculated as 0.69 ± 0.01–0.99 μg/mL. In the case of RD cells, IC_{50} values were 2.98–5.16 μg/mL. The IC_{50} values of RG2 cells ranged between 1.97 μg/mL and 6.69 ± 0.06 μg/mL, whereas, in the case of INS-1, an IC_{50} of 1.95–2.84 μg/mL was recorded (Table 2). Esmaeili et al[24] reported the anticancer activities in terms of IC_{50} values for leaf extracts of *W. coagulans* collected from South Iran (Sisatan-va-Baluchestan, Bushehr, Hormozgan, and Fars provinces). According to Esmaeili et al,[24] only CME of leaves were tested against five cell lines (HepG-2, MDBK, WEHI-164, and A-549) and the IC_{50} values were >50 μg/mL. The possible reason for higher IC_{50} values reported by Esmaeili et al[24] could be the difference in the geographical distribution of the plants and exposure to the stress. It seems that plants collected from Salt Range of Pakistan have higher anticancer potential compared to the plants of Iranian Regions as reported by Esmaeili et al.[24] Rhizomes CME of *Curcuma zedoaria* was subjected to antiproliferative effect against MCF-7 cells by Hamdi et al,[25] and they have reported an IC_{50} value of 18.4 μg/mL. These findings are in agreement with the present study based on criteria given by Wibowo et al.[26] The present study gives a comparative phytochemical analysis and anticancer activities of various parts (fruit, leaf, leaf stalk, and root) of *W. coagulans* against both human (HeLa, MCF-7, RD) and rat (RG2 and INS-1) cancer cells. It indicates that fruit and leaf stalk extract of this plant are highly significant with IC_{50} values <3 μg/mL for all five cancer cells after 72 hours of incubation.

The results of this study indicate that leaf stalk and fruit extracts of *W. coagulans* can be considered as a putative source for metabolite extraction toward further drug development owing to the very low $IC_{50} < 3$ μg/mL after 72 hours incubations against five cell lines (against HeLa, MCF-7, RD, RG2, and INS-1). The leaf stalk and fruit extract have higher phenolic and flavonoid contents compared to other plant parts. The leaf stalk extract has highest gallic acid and syringic acid contents whereas fruit extract has highest protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, ferulic acid, and TPCs. The highest quercitin contents were also found in fruit extract. The leaf stalk and fruit extract of *W. coagulans* collected from the Salt Range of Pakistan are recommended for isolation of active constituents and identification of the mechanism of action at the molecular level to clarify their role in cytotoxicity leading toward drug development.

Conflict of interest

The authors declare no conflict of interest.

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