

In Vitro Cytotoxic Activity Toward Anticancer and Antimicrobial of *Azadirachta Indica*, *Aegle Marmelos*, *Ocimum Sanctum* and *Withania Somnifera* Extracts

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Abstract: *Background:* Resistance to treatment represents the ‘big’ problem and the considerable improvement in survival rates still remains a researcher’s dream. Thus, continued research efforts are required to make treatments more personalized, to minimize side effects and improve overall survival and to also have an insight in toxico-genomics. Indeed proof of facts about medicinal plants worldwide and rich Indian medicinal flora, *Objective:* in the present investigation attempts to evaluate antimicrobials and anticancer therapeutic properties of isolated constituents from *Withania somnifera* (ASHWANGANDHA) Part Used Leaves, *Aegle marmelos* (BEL) Part Used Leaves, *Azadirachta indica* (NEEM) Part Used Leaves, *Ocimum sanctum* (TULASI) Part Used Leaves, *Method:* Hydro alcoholic (1:1) Extracts were evaluated against cancer cell lines i.e. A549 (Lung carcinoma), PA-1 (Ovarian cancer) and MCF-7 (Breast cancer), with standard as Doxorubicin. Moreover the antimicrobial activity on *Staphylococcus aureus*, *Bacillus coagulans* and one Gram-negative—*Escherichia coli*, human pathogenic bacteria; and three fungal strains—*Aspergillus niger*, *Trichoderma viride* and *Fusarium oxysporum*. *Result:* The results showed significant association of phytochemicals on inhibition of test bacteria and fungi with significant p value $p < 0.05$, except For *Trichoderma viride* No Association Was Found $P > 0.05$.

Keywords: In Vitro Anticancer Antibacterial, Antifungal, Aegle Marmeleos, Azadirachta Indica, Ocimum Sanctum and Withania Somnifera

1. Introduction

The term “Cancer” is derived from the Greek word “Karkinos” coined by Hippocrates, a non-communicable disease (NCD) characterized by uncontrolled growth of cells [1, 2]. It is the second leading cause of death after cardiovascular diseases amongst non-communicable disease and neither incidence, morbidity or mortality rate have declined much over the years [2]. Due to the serious side effects and resistance against cancerous cells with highly definite approved anticancer drugs, there is an urgent need to develop alternative approach toward the cancer anticancer drugs for the same context here we are approaching plant sources [3]. A lot of drawbacks are associated with conventional treatments like inter-patient heterogeneity, inter-

tumor and intra-tumor heterogeneity, multidrug resistance (MDR) [4], cytotoxicity to nearby cells. As the effects of treatment are not targeted, they prove to be toxic to non-target tissues and organs of the body, reducing the overall effectiveness of the treatment [5]. Because of the medicinal plants having an excellent work against cancerous cells, with high efficacy, some successful plants ingredients has been approved by FDA [6] moreover Recent research has suggested that phytochemicals target a number of pathways related to breast, lung and ovary cancer and mitigate the adverse effects of conventional treatment providing a positive feedback against malignancies and can also play a key role in preventing cancer [6–9]. Some of attempts are underway to work out the therapeutic and anti-neoplastic properties of medicinal plants [10, 11]. Consequently, herbal medicines

have received much attention as substitute anticancer drugs [12]. Many reasons have been given on why people use medicinal plants as therapy. Another big challenge in the treatment of breast cancer is the development of multiple drug resistance “MDR” in tumors via a number of complex mechanisms including modification of the drug efflux membrane transporters, alterations in beta-tubulin, and multidrug resistance protein (MRP) [13]. A lot of drugs which have been proved successful in the past can be resistant such as- anthracyclines (doxorubicin, daunorubicin, epirubicin, and mitoxantrone), taxanes (paclitaxel, docetaxel) and capecitabine [14, 15]. On the basis of all these observations, it becomes a necessity to find out an alternative approach for cancer management that can minimize the risk and reduce the after-effects of the conventional treatment approaches. A lot of drugs derived from plants showing selective toxicity have led to clinical trials for therapeutic development [16].

2. Materials and Methods

Fresh Plants material *Withania somnifera* (ASHWANGANDHA) Part Used Leaves, *Aegle marmeleos* (BEL) Part Used Leaves, *Azadirachta indica* (NEEM) Part Used Leaves, *Ocimum sanctum* (TULASI) Part Used Leaves, collected during the month of October from *Sanjivani Ayurvedic Nursery Bhopal*, were collected from the collected during the month of October 2018 from *Sanjivani Ayurvedic Nursery Bhopal*. Cell Culture & Freezing Lung cancer cell line A549, MCF-7 and PA-1 were procured from National Center for Cell Science (NCCS) Pune. Cells were routinely cultured in 25 cm² flasks in Dulbecco's modified Eagle's Medium (DMEM) with high glucose (Lonza) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine (Genetix Biotechnology) and penicillin (100 units/ml) streptomycin (100 µg/ml) (Invitrogen) at 37 °C in a CO₂ incubator (Eppendorf).

2.1. Extraction, Fractionation and Isolation

Shade dried powdered material was extracted in Hydro alcoholic (1:1) by using soxhlet apparatus for *Withania somnifera*, *Aegle marmeleos*, *Azadirachta indica*, and *Ocimum sanctum*. The weight of the powder was 200 gm in 150 ml in solvent at 60°C for 96 hours for each sample. Then solution was filtered and concentrated under reduced pressure by rotator evaporator till constant mass is obtained at 40°C. Dark green/brown coloured semisolid crude was obtained. The herbal extract was dissolved in water and sterile filtered through 0.22 µm [17]. For all experiments, the sterile herbal Hydro alcoholic (1:1) extract was diluted with DMSO to the final concentrations are 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml and 100 µg/ml [18].

2.2. Phytochemical Analysis

Withania somnifera, *Ocimum sanctum*, *Azadirachta indica* and *Aegle marmeleos* were subjected to

phytochemical tests for the identification of various constituents, such as alkaloid, flavonoids, phenolic compound and tannis, carbohydrate, steroid, glycosides, proteins, inorganic acid, oxalic acid, amino acid [19]. After phytochemical analysis bioactive compounds present in extract was separated out by column chromatography in a proper solvent system. Column chromatography was performed on a classic 20 cm long × 2 cm diameter glass column packed with 50 g Silica gel of 60-120 mesh size as stationary phase and crude drug were further subjected to column chromatography [CC] and eluted with specific solvent to obtain pure compounds. Silica gel for column chromatography was used as stationary phase [20]. The flow rate used was 5 ml/min. Three and four elutes for each solvent were taken. Further analysis were performed based on: TPC, TFC, antioxidant assay [21].

2.3. Total Phenolic Content (TPC)

The total phenolic content was determined by using calibration curve (5 to 10µg/ml). Four readings were taken for each solution for checking the reproducibility and to get accurate result. The intensity of the solution is proportional to the amount of tannins and can be estimated against standard tannic acid, the total phenolic content, expressed as mg tannic acid equivalents per 100 g dry weight of sample. The total phenolic content was measured by Folin-Ciocalteu reagent assay [22].

2.4. Total Flavonoid Content (TFC)

Total flavonoid contents were measured by Aluminum chloride colorimetric assay. Hydroalcoholic extracts that has been adjusted to come under the linearity range and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 3ml of water. To the above mixture, 0.1ml of 5% C₄H₄O₆KNa₄H₂O (Potassium Sodium L-(+) - Tartrate Tetrahydrate) was added. After 5 minutes, 0.1ml of 10% AlCl₃ was added and the total volume was made up to 3 ml with distilled water. It was left at room temperature for 30 min after which the absorbance of the reaction mixture was measured at 430nm [23].

2.5. Free Radical Scavenging Activity

DPPH (2,2 – Diphenyl 1- Picryl Hydrazyl)- The free radical scavenging activity of aqueous and ethanolic extracts and the standard L-Ascorbic Acid (Vitamin C) was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. Here, 0.1mM solution of DPPH in alcohol was prepared and was protected from light influence by maintaining the dark condition and was kept folded in aluminum foil and 3ml of this solution was added to 1ml various conc.(10 µg/ml) of extracts or standard solution of (10 µg/ml). Absorbance was taken after 30min at 550nm. The percentage inhibition activity was calculated from [(A0-A1)/A0] x 100, where A0 is the absorbance of the control and A1 is the absorbance of extract/standard taken as Ascorbic acid [24].

This is the most reported method for screening of antioxidant activity of many plants. DPPH assay method is based on the reduction of alcoholic solution of colored free radical DPPH by free radical scavenger. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 516nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. The activity is expressed as effective IC₅₀ [24].

2.6. Crude Drug Treatment

In Vitro Assay for Cytotoxic Activity A stock solution of each plant extracts was prepared by dissolving 30 mg of extract in 95 µl of dimethyl-sulfoxide (DMSO, Merck, Germany) and 2000 µl of cell culture medium to a final stock concentration of 10mg/ml and then diluted with complete culture medium to reach the desired concentrations [25].

2.7. Anti-microbial Susceptibility Test

2.7.1. McFarland Standard

McFarland standards are suspensions of either barium sulfate or latex particles that allow visual comparison of bacterial density. A 0.5ml McFarland standard is equivalent to a bacterial suspension containing between 1 x 10⁸ and 2 x 10⁸ CFU/ml of E. coli [26].

2.7.2. Disc-Diffusion Method for Antimicrobial Activity

Test Microorganisms and Growth Media The following microorganisms *Staphylococcus aureus* (MTCC 3160), *Bacillus coagulans* (MTCC 5856) and one Gram-negative—*Escherichia coli* (MTCC 443), human pathogenic bacteria; and three fungal strains—*Aspergillus niger* (MTCC), *Trichoderma viride* (MTCC 800) and *Fusarium oxysporum* (MTCC 284) were chosen based on their clinical and pharmacological importance.[28] The bacterial strains obtained from Institute of Microbial Technology, Chandigarh, were used for evaluating antimicrobial activity. In order to detect potential antimicrobial activity in the plant extracts, paper discs (diameter 12 mm) were soaked in an extract solution containing different concentration (40, 60, 80, and 100%). All plates were then incubated at 37°C for 24 hr and the zones of inhibition were subsequently measured in mm [17-18].

3. Results

3.1. Phytochemical Analysis

The present work has undertaken phytochemical analysis which would show definite action value (IC₅₀) according to growth curve. Some of the parameters which were considered for the study of phytochemical analysis included Saponins, Flavonoids, Steroids, Terpenoids, Alkaloids and Glycoside. Table 1. Phytochemical screening of 4 selected medicinal plants.

Table 1. Phytochemical Analysis.

S. No.	Test	<i>Ocimum sanctum</i> Hydro alcoholic (1:1)	<i>Withania somnifera</i> Hydro alcoholic (1:1)	<i>Aegle marmeleos</i> Hydro alcoholic (1:1)	<i>Azadirachta indica</i> Hydro alcoholic (1:1)
1.	Test for carbohydrate				
	mollish test	++ve	+ve	+ve	+ve
	Tannic acid test	+ve	+ve	+ve	+ve
2.	Test for protein				
	Millons reagent	+ve	+ve	+ve	+ve
	Xantoprotein test	-ve	-ve	-ve	-ve
3.	Test for Amino acid				
	Test for cystien	-ve	+ve	-ve	+ve
4.	Test for steroid				
	Salkowski test	+ve	++ve	+ve	+ve
5.	Test for glycosides				
	For deoxysugar (keller)	+ve	+ve	+ve	+ve
6.	Test for flavonoids	+ve	+ve	+ve	+ve
	Test for alkaloids				
7.	Mayer's test	+ve	+ve	+ve	+ve
	Wagner test	+ve	+ve	+ve	+ve
	Test for tannic and phenolic compound				
8.	5% fecl ₃	-ve	-ve	-ve	-ve
	Lead acetate	+ve	+++ve	+++ve	+++ve
	Dil. Potassium per magnet	+ve	+ve	+ve	+ve
9.	Test for organic acid				
	Conf. Test for oxalic acid	++ve	+ve	+ve	+ve
10.	Test for inorganic acid				
	Test for sulphate	+ve	+ve	+ve	+ve
11.	Test for chloride	+ve	+ve	+ve	+ve

3.2. Qualitative Analysis

By, TLC Rf values were identified for all selected plants which are 0.43, 56, 0.63 and 0.6. *Withania somnifera*, *Aegle marmeleos*,

Azadirachta indica, and *Ocimum sanctum* respectively.

3.3. FT-IR Analysis

FT-IR analysis of the extracts was done for the purpose of

detection of functional groups associated. The FT-IR spectrum of the plants extracts recorded the number of peaks lying between 3100-3400 respectively Figure 2. FTIR analysis was done by BRUKAR FTIR Spectroscopy.

3.4. Total Polyphenols Content (TPC)

Total polyphenols content (TPC) was determined by to the spectrophotometer method with Folin-Ciocalteu's reagent. Gallic acid was used as calibration standard and results were expressed as gallic acid equivalents μg per g dry weight. Total polyphenols content of the extracts was found: 200 $\mu\text{g/g}$, 120 $\mu\text{g/g}$, 66 $\mu\text{g/g}$ and 36 $\mu\text{g/g}$ *Withania somnifera*, *Azadirachta indica*, *Aegle marmeleos*, and *Ocimum sanctum* respectively. Absorbance was measured at 725 nm using a UV-Visible spectrophotometer.

3.5. Total Flavonoid Contents (TFC)

Total flavonoid contents (TFC) were measured by Aluminum chloride colorimetric assay. Hydro-alcoholic extracts that were adjusted to fall under the linearity range and different dilution of standard solution of Quercetin (10-

100 $\mu\text{g/ml}$). Total flavonoid content of the extracts was found : 4.78 $\mu\text{g/ml}$, 5.41 $\mu\text{g/ml}$, 4.77 $\mu\text{g/ml}$ and 5.62 $\mu\text{g/ml}$. *Withania somnifera*, *Aegle marmeleos*, *Azadirachta indica*, and *Ocimum sanctum* respectively. Equivalents per dry weight of sample. Absorbance of the reaction mixture was measured at 430 nm with a single beam spectrophotometer (Systronic).

3.6. DPPH Radical Scavenging Assay

In the present work, Hydro alcoholic extracts was evaluated for antioxidant property by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and Nitric oxide free radical scavenging activities. From the percentage inhibition calculated the Hydro alcoholic extracts possesses antioxidant properties. The significant antioxidant property showed by IC_{50} values extracts is due to the presence of phenolic constituents. Hence it was used in the preparation of herbal formulation. DPPH Antioxidant IC_{50} values of the extracts was found : 51.57 $\mu\text{g/ml}$, 40.0 $\mu\text{g/ml}$, 74.54 $\mu\text{g/ml}$ and 91.52 $\mu\text{g/ml}$ *Withania somnifera*, *Ocimum sanctum*, *Azadirachta indica* and *Aegle marmeleos* respectively.

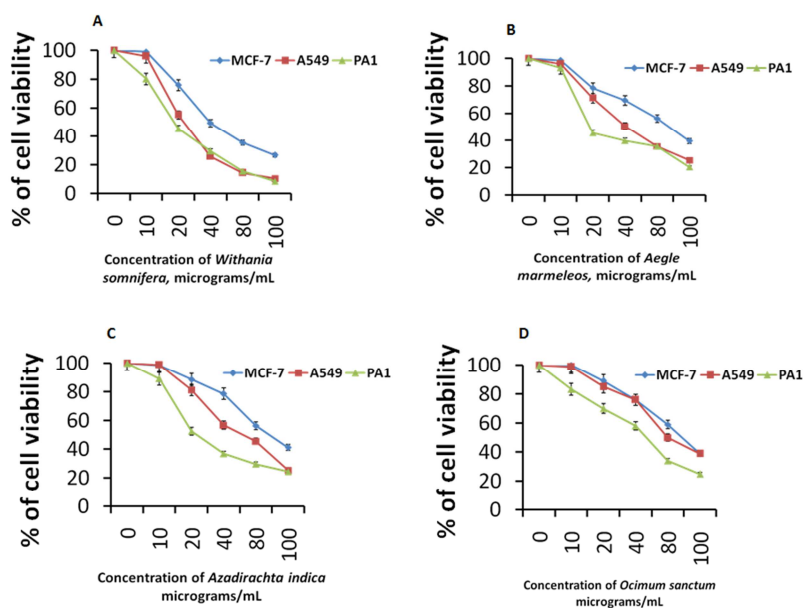


Figure 1. Cytotoxicity of (A) *Withania somnifera* (ASHWANGANDHA) Part Used Leaves, (B) *Aegle marmeleos* (BEL) Part Used Leaves, (C) *Azadirachta indica* (NEEM) Part Used Leaves, (D) *Ocimum sanctum* (TULASI) Part Used Leaves, Hydro alcoholic (1:1) Extracts MTT assay was performed on cells after 24 h treatment with increasing concentrations of flowing Plant extracts. Results are presented as percentage of cell viability from duplicate assays.

3.7. Antimicrobial Assay

The antimicrobial activity was determined in the extracts using agar disc diffusion method. The antibacterial and antifungal activities of extracts (40, 60, 80, 100%) of *Cassia fistula* were tested against two Gram-positive *Staphylococcus aureus*, *Bacillus coagulans* and one Gram-negative—*Escherichia coli*, human pathogenic bacteria; and three fungal strains—*Aspergillus niger*, *Trichoderma viride* and *Fusarium oxysporum*. Zone of inhibition of extracts were compared to that of different standards like Streptomycin for antibacterial activity and

Griseofulvin for antifungal activity. The results showed that the remarkable inhibition of the bacterial growth was shown against the tested organisms. Tables 2 and 3.

3.8. Calculation of Cell Viability or IC_{50} Values

For selected plant active molecules, (column chromatography extracts) IC_{50} value were calculated and separately Mentioned (Figure 1) with special reference to MCF-7 (human breast cancer) cell line which are *Withania somnifera*, *Aegle marmeleos*, *Ocimum sanctum* and *Azadirachta indica* and IC_{50} value wear calculated is 30 ± 1.35 ,

84±3.21, 90±3.2 and 88±2.55 µg/ml respectively. For selected plant active molecules (column chromatography extracts) IC₅₀ value were calculated with special reference to PA-1 (human ovary cancer) cell line which are *Withania somnifera*, *Aegle marmeleos*, *Ocimum sanctum*, *Azadirachta indica* and IC₅₀ value wear calculated is 12±1.03, 15±2.0, 38±1.35 and 18±1.23µg/ml respectively. IC₅₀ value were

calculated with special reference to A-549 (human lung cancer) cell line which are *Withania somnifera*, *Aegle marmeleos*, *Ocimum sanctum*, *Azadirachta indica* and IC₅₀ value wear calculated is 10±2.1, 34±2.3, 65±2.35 and 65±3.36 µg/ml respectively. Standard drug was used for IC₅₀ of Doxorubicin are MCF-7 500nm, A549- 550nm, PA-1- 570nm as mentioned in Figure 1.

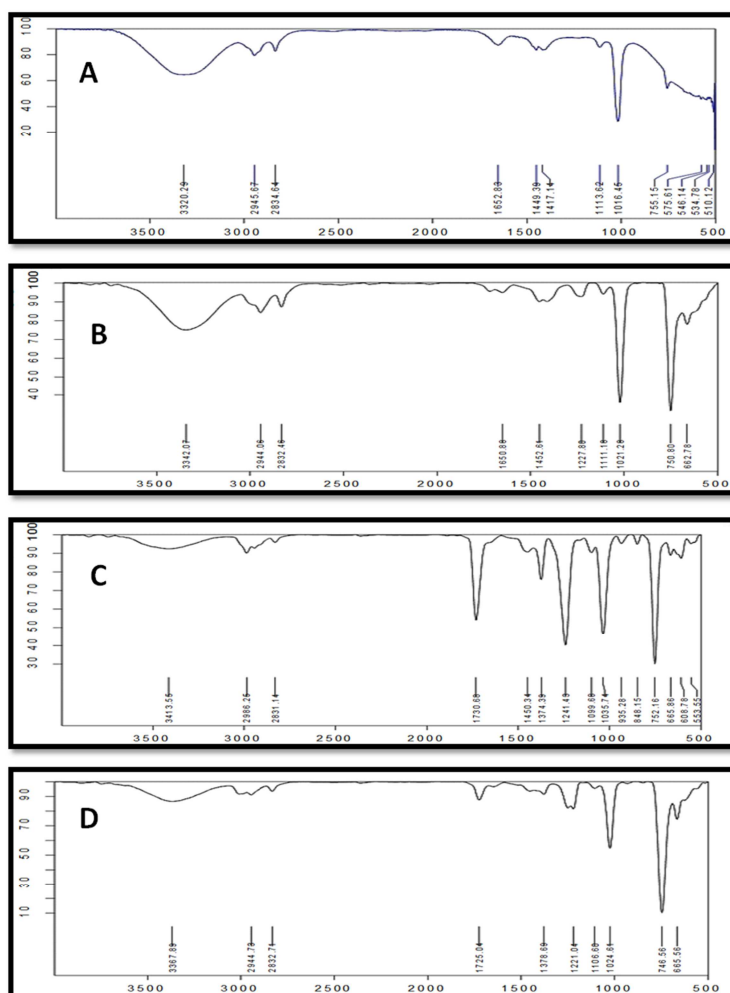


Figure 2. The FT-IR analysis of the samples were done and the functional groups associated were determined The FT-IR spectrum of the sample gave effective peaks. the FT-IR spectrum of the *Withania somnifera* 2A, *Aegle marmeleos* 2B, *Ocimum sanctum* 2C, *Azadirachta indica* 2D samples recorded a number of peaks lying between 3367.89 cm⁻¹, 2944.79 cm⁻¹, 2832.74 cm⁻¹, 1725.04 cm⁻¹, 1378.69 cm⁻¹, 1221.04 cm⁻¹, 1106.60 cm⁻¹, 1024.64 cm⁻¹, 746.56 cm⁻¹, 665.56 cm⁻¹ respectively.

Table 2. Antibacterial Activity of Compounds.

Sample name	Diameter of Zone of inhibition (mm)* against														
	(+) <i>Bacillus coagulans</i>					(+) <i>Staphylococcus aureus</i>					(-) <i>Escherichia coli</i>				
	Concentration of compound					Concentration of compound					Concentration of compound				
	100%	80%	60%	40%	SD	100%	80%	60%	40%	SD	100%	80%	60%	40%	SD
A. I	18.2	15.3	14.9	12.7	2.26	18.0	16.5	11.5	10.7	3.61	14.5	12.0	9.7	9.3	2.39
A. M	18.2	16.2	13.6	9.5	3.75	19.3	16.6	12.0	9.5	4.42	14.5	11.3	9.5	6.2	3.46
O. S	20.0	18.5	16.2	14.5	2.43	17.3	14.2	13.0	10.5	2.82	16.5	13.5	10.5	8.3	3.57
W. S	16.2	11.3	10.5	7.5	3.6	18.3	14.5	11.5	8.5	4.19	17.5	13.0	11.5	7.2	4.24
**Std	21.2	20.0	19.5	18.0	1.32	20.0	19.1	18.3	17.9	0.92	18.3	17.0	15.6	14.0	1.84

*The zone of inhibition (mm) taken as average of four determination in four different direction.

**Streptomycin used as standard antibacterial agent.

Table 3. Antifungal Activity of Compounds.

	Diameter of Zone of inhibition (mm)* against														
	Aspergillus niger					Tricoderma viride					Fusarium oxysporum				
	Concentration of compound					Concentration of compound					Concentration of compound				
	100%	80%	60%	40%	SD	100%	80%	60%	40%	SD	100%	80%	60%	40%	SD
A. I	15.6	13.9	11.0	9.9	2.61	18.6	16.5	14.0	10.5	3.48	16.0	15.9	11.6	10.8	2.76
A. M	11.5	10.2	6.2	5.0	3.11	3.5	13.0	8.8	6.5	4	13.4	12.5	10.6	7.3	2.7
O. S	14.5	12.5	10.6	8.5	2.56	16.5	13.5	10.8	8.5	3.45	17.5	15.2	16.5	13.3	1.81
W. S	15.5	11.8	7.2	6.0	4.36	13.5	10.0	7.6	5.5	3.43	17.4	15.3	12.1	8.0	4.0
**Std.	16.8	15.2	14.0	12.8	1.7	23.4	22.0	20.9	10.5	5.88	20.1	18.7	17.0	15.3	2.0

*The zone of inhibition (mm) taken as average of four determination in four different direction.

**Griseofulvin used as standard antifungal agent.

4. Statistical Analysis

The results of the inhibition percentage of growth of the three bacteria and three fungi as affected by the four concentrations (40%, 60%, 80%, 100%) of the *Azadirachta indica*, *Aegle marmeleos*, *Ocimum sanctum* and *Withania somnifera* extracts were statistically analyzed using One way analysis of variance (ANOVA) using Graph-Pad. The results showed significant association of phytochemicals on inhibition of test bacteria and fungi with significant p value $p < 0.05$, except For *Tricoderma viride* No Association Was Found $P > 0.05$. The MTT assay indicated that Hydro alcoholic (1:1) through CC extracts of the plant exhibited significant cytotoxic effects on MCF-7, A549 and PA-1 cancer cell line (breast, lung and ovary respectively), This finding suggests that the reduction observed in the viable cells following treatment with *Azadirachta indica*, *Aegle marmeleos*, *Ocimum sanctum* and *Withania somnifera*. The further studies on the active components for proper assessment of their chemotherapeutic properties as well as their possible development as promising anticancer drugs in addition more research will be useful to investigate the unknown and unexplored potential of above mentioned plant and drug design analysis.

5. Discussion

In Plant chemistry considerable Contributions have been made by [1, 2, 6, 11–14, 18, 20]. They worked out the cytotoxic activity of medicinal plants and studied their antiproliferative activity against cancer. In this study we demonstrated that *Withania somnifera*, *Aegle marmeleos*, *Azadirachta indica*, and *Ocimum sanctum* might be a source of antibacterial, antifungal and antiproliferative substances/compounds. Optimization of culture conditions may accelerate their growth and stimulate the production of biologically active compounds. It may also limit the need to harvest *Withania somnifera*, *Aegle marmeleos*, *Azadirachta indica*, and *Ocimum sanctum* in the field. It is especially important for *Withania somnifera*. A high variety of biologically active substances present in *Withania somnifera*, extracts may provide new antibacterial and/or anticancer agents. As an urge to study the effect of the extracts of *Withania somnifera*

as a novel therapeutic agent, they were characterized for their cytotoxic effects against all three study cell lines. To conclude, this article induces a concentration-dependent inhibition of cells. Based on these results, further studies could be carried out as a search for *Withania somnifera* compounds to develop alternative therapeutic measures against diseases.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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