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RESEARCH ARTICLE

Exploring Anticancer Potential of Aqueous, Hydroethanolic and Methanolic Extract of an Age-old *Unani* Formulation

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Abstract

Background: Cancer poses a foremost public health challenge worldwide. Several cancer types are preventable and treatable, provided that cancer is diagnosed at an early stage. The derivatives of plant metabolites such as camptothecin, podophyllotoxin, vinca, and others have been a crucial source of numerous clinically effective anti-cancer agents. **Aim:** The present study was conducted to evaluate the cytotoxic potential of a traditional *Unani* formulation against cancer cell lines. **Methods:** A compound Polyherbal *Unani* Formulation (PUF) mentioned in the *Unani* text *Kamil-Us-Sana* to evacuate *Khilt-e-Sawdā'* in the chapter of *Saraṭān* (Cancer) was selected to screen its anti-cancer potential against A-549, HeLa, MCF-7 and B16F10 cells. Aqueous, hydroethanolic (1:1), and methanolic extract of PUF was prepared and tested. **Results:** Total phenolics and flavonoids were detected in significant quantities in PUF. PUF and its ingredients exhibited powerful anti-oxidant potential in DPPH, ABTS, and FRAP assay. Methanolic extract of PUF was found most potent cytotoxic (IC_{50} value of 28.50±1.53 µg/mL) in B16F10 cells, almost equal to positive control doxorubicin (IC_{50} 24.62±0.19 µg/mL). PUF also increased the caspase-3 activity in HeLa and A-549 cells, suggesting apoptosis induction. The cytotoxic activity of PUF may be attributed to the effect of its ingredients in MCF-7 cells and other cancer cell lines. **Conclusion:** Present findings validate the traditional claim mentioned in the *Unani* text and open up a new avenue for further research to explore molecular mechanisms and development of this *Unani* formulation for effective cancer management.

Major Findings: Present study demonstrated the cytotoxic potential of a traditional polyherbal *Unani* formulation with IC_{50} values ranging from 28.50±1.53 µg/mL to 207.86±1.24 µg/mL in HeLa, A-549, MCF-7, and B16F10 cancer cells. The methanolic extract of PUF exhibited greater cytotoxic potential compared to hydro-ethanolic and aqueous extract. The cytotoxic effects of PUF are likely due to its ingredients, which have been reported to have cytotoxic activity in other cancer cell lines.

Keyword: Cancer, Polyherbal, *Saraṭān,* Traditional Medicine, *Unani*

1. Introduction

Cancer stands as a prominent global cause of mortality, contributing to nearly 10 million deaths in the year 2020, representing almost one in six deaths. Approximately one-third of cancer-related fatalities can be attributed to factors such as tobacco use, elevated BMI, alcohol consumption, unhealthy dietary habits, and sedentary lifestyle. Among the most prevalent cancers are those affecting the breast, lungs, colon and rectum, prostate, skin, and stomach. In terms of cancer-specific mortalities in 2020, lung cancer claimed the highest toll with 1.80 million deaths, followed by colon cancer and rectum carcinoma (9,16,000 deaths), liver cancer

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(8,30,000 deaths), stomach (7,69,000 deaths), and breast cancer (6,85,000 deaths) [1].

In the past 50 to 60 years, there has been substantial effort to discover naturally occurring substances as potential new drugs for cancer therapy. In 1950, the American Cancer Institute initiated a widespread screening program of substances and extracts from various origins to find anti-neoplastic agents. Compounds derived from plants have been extremely important in developing several therapeutically useful anti-cancer drugs including *vinca* alkaloid like vincristine and vinblastine, taxol, epipodophyllotoxin, camptotheca derivatives[2]. A comprehensive review found that out of 175 small molecules, discovered since 1940s, in the area of cancer, 131, or 74.8%, were not synthetic drugs. Of these 85, or 48.6%, were either of natural origin or their close derivatives. Moreover, only 20.2% of the anticancer medications approved from 1981 to 2010 were entirely synthetic. In contrast, the remaining 79.8% were either natural products or derived from natural sources[3].

In the philosophy of *Unani* system of medicine, cancer, referred to as *'Saraṭān'* (derived from Arabic, meaning crab), is discussed in the renowned texts. *Saraṭān* is characterized as *Ṣalāba al-Waram* (hardening of swelling) associated with lesions and preponderance of *Mādda-e-Sawdāwī* (abnormal melancholic humour) [4-5]. It is caused by the abnormal accumulation of the "*Sawdā' Ghayr Ṭabī'ī*" (*i.e.*, abnormal black bile)[6-9]. Some ancient *Unani* physicians assumed that *Saraṭān* is having the morphological resemblance to crab because there are various root-like structures in *Saraṭān* and when these structures spread widely into the organ, the organ loses its softness and elasticity and becomes harder[10]. Aslam *et al.*, provided a comprehensive list of such classical texts along with important *Unani* formulation used topically as well as systemically[10]. Khan *et al.*, has written an excellent overview with reference to the treatise *'Kitab ul Havi Al Kabeer Fit Tibb'* written by Abu Bakar Mohammad Bin Zakaria Al Razi (925-865 AD) and presented various types of cancer and their management strategies mentioned in this classical book[11]. Ibn-e-Sina in his famous treatise, *Al-Qanoon*, mentioned that *Saraṭān* mostly occurs in nerves, muscles, tendon and lymph node[6]. The famous physician of *Unani* medicine, Zakaria Al Razi stated in his great treatise *'Kitab-ul-Mansoori'* that *Saraṭān* is a *'Marz-e-Muhlik'* (fatal disease)[12].

According to *Unani* principles, cancer develops with (i) Excess production of *Sawdā'* (black bile), (ii) Initiation of burning of that *Sawdā'* into abnormal *Sawdā'*, (iii) Formation of abnormal *Sawdā'* in result of blood burning (iv) Aberrant *Sawdā'* formation brought on by burning of *'Balgham' (phlegm)*, (v) Production of abnormal *Sawdā'* due to burning of *'Ṣafrā'* (yellow bile)[13]. Therefore, during the treatment of *Saraṭān*, the correction and regaining the balance of *Sawda* is the primary goal; which can be achieved by *Istifrāgh -i-Khilṭ Sawdā'* (evacuation of morbid black bile) with the help of *Munḍij-i-Sawdā' Adwiya* (*i.e.*, black bile concoctive drugs) and *Mushil-i-Sawdā' Adwiya* (*i.e.,* black bile purgative drugs)[12].

Ancient *Unani* scholars have explained the symptoms, signs, prevention, and therapy of *Saraṭān-e-pistan* or *Saraṭān-al-Thady* (breast cancer)[14]. The present study was designed to generate comprehensive scientific data on this *Unani* formulation for cytotoxicity potential against cancer cell lines such as A-549 (human alveolar basal epithelial adenocarcinoma) cell line, HeLa (cervical cancer) cell line, and MCF-7 cells (Michigan Cancer Foundation-7; Breast adenocarcinoma), and B16F10 (Mouse melanoma).

2. Materials and Methods

2.1 Cell Lines, Kits and Chemicals

A-549, HeLa, and MCF-7 cells used in the study were purchased from NCCS, Pune. B16F10 was sourced from NIPER Hyderabad. Ethanol (MB228-500ML), Methanol (MB113-500ML), DMSO (MB058-500ML), Trypan blue (TCL046), FBS Superior (RM10432), PBS (TL1031), DPPH reagent and other chemicals were procured from Himedia. MTT assay kit (Sigma-TOX1-1KT), Caspase-3 assay kit (Sigma-CASP3C; colorimetric), DMEM (D6546), Antibiotic-antimycotic solution (A5955), Trypsin (T4174), Cell Freezing Media, DMSO (C6164), ABTS, Gallic acid, Quercetin were obtained from Sigma.

2.2 Test Drug

The selected formulation (Table 1) is mentioned in the *Unani* textbook *Kamil-Us-Sana* for the purpose of evaluation of *Khilt-e-Sawdā'* (Black Bile) in the chapter *Saraṭān*[8]. Ingredients of formulation were supplied by Good Manufacturing Practices (GMP)- certified *Unani*

Pharmacy of the Institute. The representative specimens were submitted to Botany section of the institute, for the identification and authentication of drugs (Figure 1). Authentication of raw ingredients is of paramount importance for the quality control of any traditional formulation. Botanist verified the identities of all the ingredients of PUF procured from commercial sources. Voucher specimen numbers were assigned to these ingredients SMPU/CRI-Hyd 14369 to 14374. After authentication, the ingredients were coarsely powdered and powder was passed through a sieve (aperture: 355 microns) to obtain uniform powder.

2.3 Extraction Method

Aqueous (Aq), Hydroethanolic (HE; 1:1) and Methanolic (Me) extracts were prepared by soaking

Table 1. Composition of *Unani* formulation

S. No.	Ingredient (*Unani* Name)	Botanical Name	Part	Quantity*
1	*Halela Siyah Hindi*	*Terminalia chebula* Retz.	Fruit pulp	3.5 g
2	*Aftimoon Afriti*	*Cuscuta reflexa* Roxb.	Stem	5.25 g
3	*Bisfayej*	*Polypodium vulgare* L.	Rhizome	5.25 g
4	*Ustukhuddus*	*Lavandula stoechas* L.	Flowers	5.25 g
5	*Kutki Siyah*	*Picrorhiza kurroa* Royle ex Benth.	Root	1.75 g
6	*Ghariqoon*	*Agaricus albus* Schaeff.	Fruiting body	3.5 g
7	*Milh Nafti* (Kala namak)	N/A	--	1.50 g

* Quantities in classical text are given in *Masha*.



Figure 1. Representative specimens of ingredients of PUF.

10g of powder of PUF as well as individual ingredients in 100 mL of the solvent system with manual shaking at ambient temperature. The Aq extract was freeze-dried using lyophilization technique (Southern Scientific Lab Instruments, Chennai, India). The HE and Me extracts were filtered and heated at 60° C. The obtained powdered extracts were stored protected from light and moisture and used for different assays. The dried powder was weighed to determine the percent yield.

2.4 Evaluation of Total Phenolic Content

Total Phenolic Content (TPC) in PUF as well as its ingredient was evaluated by Folin-ciocalteu method as described previously[15]. Total phenolic contents were calculated as µg Gallic Acid Equivalents (GAE) per mg of PUF or its ingredients.

2.5. Estimation of Total Flavonoid Content

Total Flavonoid Content (TFC) in PUF and its ingredients were determined using aluminium chloride colorimetric assay, reported previously[16]. Total flavonoid content was reported as µg Quercetin Equivalents (QE) per mg of PUF or its ingredient.

2.6 DPPH (2,2-DiPhenyl-1-Picryl Hydrazyl) Free Radical Scavenging Activity

DPPH assay was performed as described by Abeysekera *et al*[17]. Gallic acid and Quercetin were used as antioxidant standards[18]. The results are expressed as μg/mL of each extract required to inhibit the DPPH radical signal by 50% (IC_{50}).

$$\%\ \text{DPPH quenched} = 1 - \left(A\text{sample} - \frac{A\text{blank}}{A\text{control}} - A\text{blank} \right) \times 100$$

2.7 ABTS (2, 2′-Azino-bis (3-ethyl Benzothiazoline-6-Sulfonic acid)) Free Radical Scavenging Activity

The ABTS assay was performed according to the method as described earlier[19,20]. Gallic acid and Quercetin were used as standards[18]. The results are expressed as μg/mL of extract, required to reduce ABTS radical signal by 50%.

$$\%\ \text{ABTS quenched} = 1 - \left(A\text{sample} - A\text{blank} / A\text{control} - A\text{blank} \right) \times 100$$

2.8 FRAP (Ferric Reducing Antioxidant Power) Assay

FRAP assay was performed according to the procedure reported in the literature[21,22]. The FRAP value was expressed as (mM $FeSO_4$ equivalent/mg extract). Gallic acid and Quercetin were used as standards.

2.9 Cell Culture: Reviving Cells from Liquid Nitrogen and Cell Counting

Cells were taken out from liquid nitrogen and thawed at 37°C. Cells were suspended in 5 mL Dulbecco's Modified Eagle Medium (DMEM) growth media used for MCF-7 (breast adenocarcinoma), HeLa (human cervical adenocarcinoma), B16F10 (mouse melanoma), and RPMI1640 (Roswell Park Memorial Institute) growth media used for A-549 (human lung carcinoma) cell lines. The pellet obtained after discarding the supernatant was again suspended in 1 mL of media and transferred into T25 flasks with an additional 9 mL of media. The flasks were placed in a CO_2 incubator (5% flow). Flasks were monitored daily for confluence. The media was discarded, washing was performed with PBS 2-3 times, and fresh media was gently added before incubating at 37°C. Passaging occurred at 80% confluence. Cells were washed with PBS, trypsinized with 2 mL of chilled trypsin or EDTA solution at 37°C, and 2 mL of media was added. The cells were transferred to a centrifuge tube and spined at 1000 rpm for 5 min. The pellet obtained after discarding the supernatant was suspended again in 3 mL media and mixed by pipetting and transferred to T75 flask. Cell counting was performed using a hemocytometer (Figure 2). A glass hemocytometer with a cover slip was used; it was cleaned with alcohol before use. A coverslip was dampened with water before placing on the hemocytometer.

2.10 MTT Cytotoxicity Assay

Cytotoxic effect of PUF was evaluated using MTT based *in vitro* toxicology bioassay kit (Sigma-TOX1-1KT) as per the manufacturer's instructions. MTT assay was carried out on total of four cancer cell lines, of which three were human carcinoma lines HeLa, A-549, and MCF-7, and one was mouse cancer cell line B16F10. MCF-7, HeLa and B16F10 cells were maintained in DMEM while A-549 cell lines were grown in RPMI1640;

supplemented with FBS (10%), 0.5% antibiotic and antimycotic solution, 0.5% L-glutamine at 37°C in the cell culture incubator with 5% CO_2. For analysis of cell cytotoxicity, 100µL of cell suspension was seeded in 96-well cell culture plate in triplicates and was incubated for 24-hours in an incubator at 37°C with 5% CO_2 atmosphere. Afterwards, PUF or individual extract was added at different dilutions and plates were incubated for 48 hours. After incubation, media was aspirated and well was rinsed with 100µL of PBS individually. Subsequently, plate was incubated for three hrs after adding 10µL MTT. The resulting formazan crystals were dissolved by adding 100µL of MTT solubilization solution. The solution was gently mixed in a shaker to enhance dissolution or pipetted up and down thrice. Absorbance was measured (570 nm) with a reference wavelength of 690 nm.

2.11 Caspase-3 Activity

The effect of PUF extracts on caspase-3 activity was measured using the caspase-3 assay kit (CASP-3-C, Sigma Aldrich, USA) by following the standard

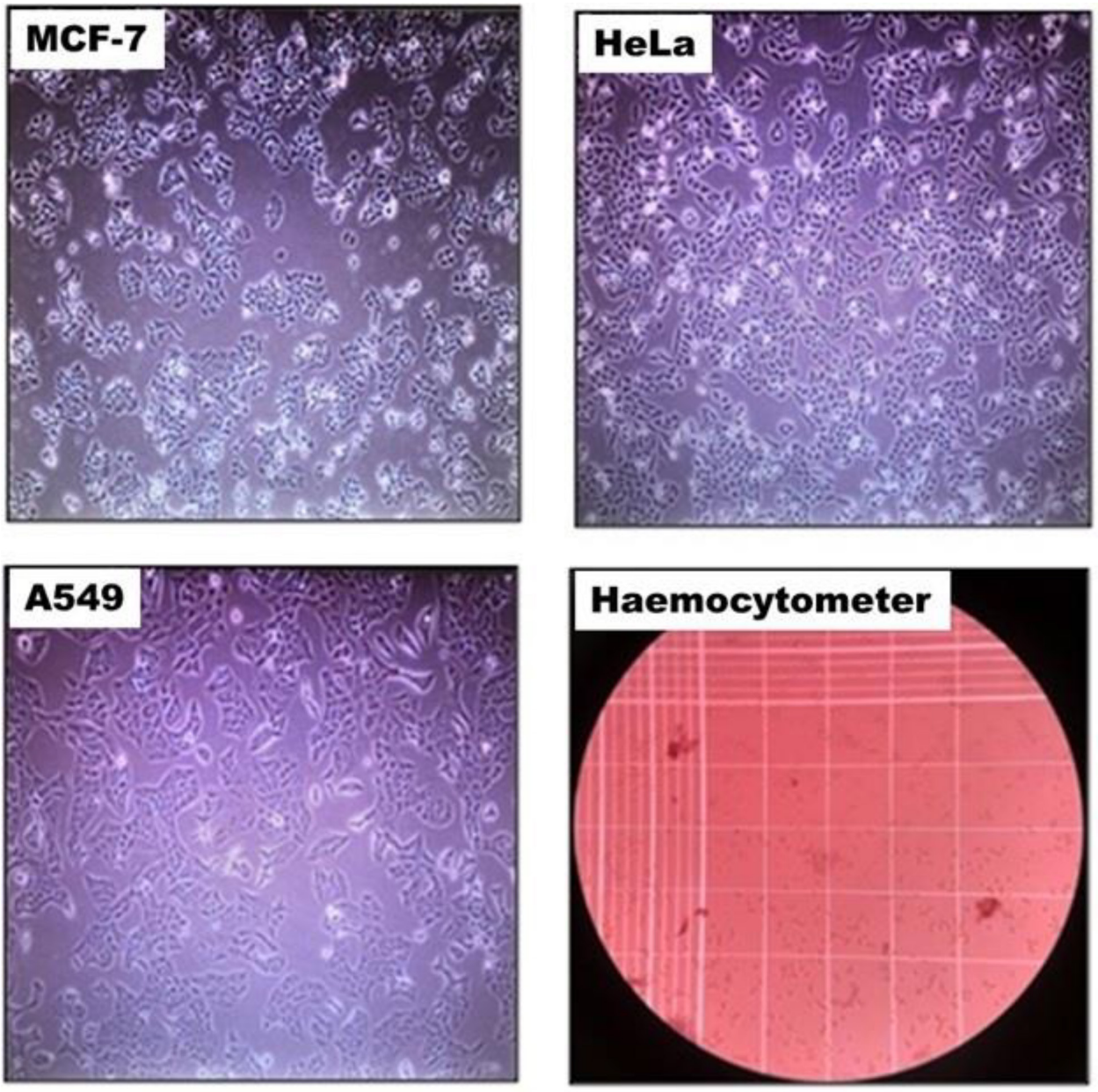


Figure 2. Representative images of different cell lines and view of hemocytometer.

protocol described in the product insert. Caspase-3 activity was determined in µmol of pNA released per minute per mL of cell lysate or positive control based on the kit's formula.

2.12 Statistical Analysis

Experiments were performed in triplicates (n=3). Data presented as mean values and (±) Standard Error of Mean (SEM). IC_{50} values were calculated using Microsoft excel by plotting percentage inhibition on y-axis and concentration on x-axis. One way ANOVA using GraphPad prism software was used for analysis of data of Caspase-3 assay. Values of $P < 0.05$ were considered as statistically significant difference.

3. Results

3.1 Percentage Yield of Extracts of PUF and its Ingredients

Data on the percentage yield of various extracts of PUF and its ingredients is given in Table 2. In aqueous extracts, the yield of Ustukhuddus was lowest (5.33±0.55%), while yield of Halela Siyah was highest (31.86±0.86%). In case of Hydroethanolic extracts, the yield of Ghariqoon was lowest (7.99±1.06%), while Halela Siyah showed highest yield (51.99±0.94%). In methanolic extraction, the yield of Ustukhuddus was lowest (7.92±1.04%), while the yield of Ghariqoon was highest (56.09±3.03%).

3.2 Preliminary Phytochemical Analysis

3.2.1 Qualitative Analysis of PUF

PUF extract confirmed the presence of alkaloids, total phenols, flavonoids, and glycosides in aqueous, hydroethanolic and methanolic extracts. Qualitative measurements are indicated by +, ++, and +++ (low, moderate and high concentration, respectively) (Table 3).

3.2.2 Quantitative Estimation of PUF and its Ingredient

Data from the quantitative analysis of TPC and TFC of PUF and its ingredients is presented in Table 4. Samples were analysed in triplicate. TPC was estimated by gallic acid and expressed as µg gallic acid equivalent per mg of extract. Aq. extract of *T. chebula* showed the highest TPC, *i.e.*, 117.26±1.75 µg of GAE/mg of extract, while Me extract of *A. albus* showed the lowest *i.e.* 17.63±3.80 µg of GAE/mg of extract. Aq. extract of PUF showed the highest TFC, *i.e.,* 123.4±4.06 µg of QE/mg of extract, while Me extract of *A. albus* showed the lowest *i.e.* 1.08±0.67 µg of QE/mg of extract.

3.3 Antioxidant Activity

Data of standards used for anti-oxidant activity (gallic acid and quercetin) is presented in Table 5. DPPH, ABTS, and FRAP data of PUF and its ingredients are shown in Tables 6, 7, and 8 respectively.

Aq. extract of *P. vulgare* showed the best DPPH scavenging activity with IC_{50} value, *i.e.*, 641.31±6.75 µg/mL, while Me extract of PUF showed the lowest *i.e.*, 6.71±0.10 µg/mL. Me extract of *A. albus* showed the highest ABTS radical scavenging activity, IC_{50} value *i.e.,* 712.70±12.26 µg/mL while Me extract of *T. chebula* showed the lowest *i.e.,* 6.71±0.10 µg/mL. HE extract of *T. chebula* showed highest ferric reducing antioxidant power, IC_{50} value *i.e.,* 8.52±0.049 µg/mL while Me extract *A. albus* of showed the lowest *i.e.,* 0.07±0.03 µg/mL.

Table 2. Percentage yield of extracts of PUF and its ingredients

Si. No.	Formulation or Ingredient *Unani* Name or Botanical Name Aqueous Extract		Percentage (%) Yield		
			Hydro Ethanolic Extract	Methanolic Extract	
1	Polyherbal *Unani* Formulation (PUF)		21.76±0.61	31.92±2.78	33.87±3.13
2	*Halela Siyah Hindi*	*T. chebula* Retz.	31.86±0.86	51.99±0.94	52.52±2.91
3	*Aftimoon Afriti*	*C. reflexa* Roxb.	12.03±0.43	15.19±1.67	10.78±0.71
4	*Bisfayej*	*P. vulgare* L.	11.94±0.20	17.28±1.70	12.58±0.18
5	*Ustukhuddus*	*L. stoechas* L.	5.33±0.55	10.10±2.62	7.92±1.04
6	*Kutki Siyah*	*P. kurroa* Royle ex Benth.	28.16±1.20	23.27±1.27	21.26±0.55
7	*Ghariqoon*	*A. albus* Schaeff.	5.64±0.56	7.99±1.06	56.09±3.03

Table 3. Qualitative analysis of PUF extract

Si. No.	Constituents or (Standard)	Test	Observation	Aqueous Extract	Hydro-Ethanolic Extract	Methanolic Extract
1	Alkaloids (Scopolamine)	Dragendorff's test	Orange red precipitate	+	++	++
		Mayer's test	White precipitate	+	++	+++
		Wagner's test	Yellowish orange	+	+	+
2	Total Phenols (Gallic Acid)	Ferric chloride test	Bluish green	+++	+++	+++
3	Flavonoids (Quercetin)	Shinoda test	Pink	+	++	+++
		Alkaline reagent test	Colourless	+++	+++	+++
4	Glycosides (Naringenin)	Liebermann's test	Green	++	+	+
		Salkowski's test	Reddish brown	+	++	++

+ indicates low concentration, ++ indicates moderate concentration, and +++ indicates high concentration, of the phytoconstituent.

Table 4. Total phenolic content and total flavonoid content of PUF and its ingredients

S. No.	PUF or Ingredients	TPC (µg of GAE/mg of Extract)			TFC (µg of QE/mg of Extract)		
		Aq	HE	Me	Aq	HE	Me
1	PUF	106.47±2.09	106.78±5.69	64.90±4.73	14.90±0.55	123.4±4.06	73.81±2.84
2	*T. chebula*	117.26±1.75	87.36±4.22	97.38±6.44	44.57±1.82	57.58±3.03	54.58±1.64
3	*C. reflexa*	52.68±1.02	28.18±3.18	22.60±2.45	12.44±0.18	48.20±3.06	58.18±5.79
4	*P. vulgare*	26.52±0.35	28.93±3.23	20.70±1.56	5.26±0.66	8.16±0.36	28.15±2.06
5	*L. stoechas*	79.97±1.73	64.94±4.07	35.34±1.33	5.61±0.39	29.85±1.35	38.47±4.09
6	*P. kurroa*	55.08±0.44	30.79±3.98	27.86±1.16	8.68±1.92	21.3±1.94	27.61±0.51
7	*A. albus*	64.83±1.22	17.63±3.80	ND	1.08±0.67	7.76±0.49	22.83±4.84
ND: Not Detected							

Table 5. Antioxidant activity of gallic acid and quercetin (standards)

S. No.	Standard(s)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP mM $FeSO_4$ / mg of Standard
1	Gallic acid	4.33±0.18	4.60±0.23	37.14±0.09
2	Quercetin	6.64±0.08	5.83±0.001	27.83±1.32

Table 6. DPPH radical scavenging assay of PUF and its ingredients

S. No.	PUF or Ingredients	DPPH Assay IC_{50} (µg/mL)		
		Aq	HE	Me
1	PUF	36.01±3.05	12.18±0.29	6.71±0.10
2	*T. chebula*	10.43±0.40	21.29±0.35	9.04±0.04
3	*C. reflexa*	376.15±4.57	155.32±13.00	246.18±5.59
4	*P. vulgare*	641.31±6.75	234.84±3.10	133.50±0.57
5	*L. stoechas*	62.44±3.60	78.18±2.51	138.44±0.38
6	*P. kurroa*	276.76±3.16	189.81±13.84	239.40±15.11
7	*A. albus*	102.01±0.65	300.24±9.42	508.16±6.55
Data presented as Mean±SEM (n=3)				

3.4 Cytotoxicity Assay

IC_{50} values of aqueous, hydroethanolic (50:50), and methanolic extract of PUF extracts and doxorubicin on various cancer cell lines are shown in Table 9. Overall, the methanolic extract of PUF showed most potent cytotoxic activity in all tested cell lines, followed by hydroethanolic extract, whereas aqueous extract of PUF showed least cytotoxic potential. When compared to any PUF extract, doxorubicin was far more powerful cytotoxic in all tested cell lines. Methanolic extract of PUF was found to be most potent in term of cytotoxicity, with IC_{50} value of 28.50±1.53 µg/mL in B16F10 cells, which was almost equal to positive control doxorubicin (IC_{50} 24.62±0.19 µg/mL).

IC_{50} values of extracts of individual ingredients of PUF on MCF-7 cancer cells are shown in Table 10.

Table 7. ABTS radical scavenging assay of PUF and its ingredients

S. No.	PUF or Ingredients	ABTS Assay IC_{50} (µg/mL)		
		Aq	HE	Me
1	PUF	40.79±0.52	36.06±0.08	43.97±0.23
2	*T. chebula*	12.83±0.66	12.11±2.42	9.45±0.56
3	*C. reflexa*	181.93±1.64	134.20±1.08	213.77±0.49
4	*P. vulgare*	328.68±2.19	150.20±0.89	115.83±8.45
5	*L. stoechas*	78.20±0.90	56.32±0.35	164.43±1.81
6	*P. kurroa*	196.60±2.11	124.82±1.66	189.62±1.28
7	*A. albus*	123.25±1.13	241.07±6.47	712.70±12.26
Data presented as Mean±SEM (n=3)				

Table 8. Ferric Reducing Antioxidant Power (FRAP) of PUF and its ingredients

S. No.	PUF or Ingredients	FRAP (mM $FeSO_4$ / mg of Extract)		
		Aq	HE	Me
1	PUF	2.98±0.029	2.76±0.104	1.48±0.03
2	*T. chebula*	8.21±0.029	8.52±0.049	2.80±0.27
3	*C. reflexa*	0.47±0.008	0.81±0.010	0.61±0.01
4	*P. vulgare*	0.29±0.002	0.75±0.038	0.59±0.08
5	*L. stoechas*	1.75±0.006	2.63±0.026	0.92±0.06
6	*P. kurroa*	0.79±0.001	0.996±0.031	0.70±0.07
7	*A. albus*	1.24±0.012	0.246±0.001	0.07±0.03
Data presented as Mean±SEM (n=3)				

Table 9. Cytotoxicity data of different PUF extracts and doxorubicin in HeLa, A-549, MCF-7, and B16F10 cell lines

S. No.	Test Substance	IC_{50} (µg/mL)			
		HeLa	A-549	MCF-7	B16F10
1	PUF (Aqueous)	164.44±7.99	207.86±1.24	136.63±3.58	140.60±0.12
2	PUF (Hydroethanolic)	167.39±10.58	177.20±10.74	129.10±1.43	127.55±6.11
3	PUF (Methanolic)	146.41±1.56	103.54±5.20	91.08±8.81	28.50±1.53
4	Doxorubicin	27.03±0.42	29.75±0.79	30.61±0.55	24.62±0.19
Data presented as Mean±SEM (n=3)					

Table 10. Cytotoxicity data of individual ingredients of PUF in MCF-7 cell lines

S. No.	Test Substance	$ICIC_{50}$ (µg/mL) in MCF-7 Cell Lines		
		Aqueous Extract	Hydroethanolic Extract	Methanolic Extract
1	*T. chebula*	133.91±5.10	130.99±4.35	104.76±4.00
2	*C. reflexa*	198.23±7.17	59.49±5.74	34.02±7.92
3	*P. vulgare*	282.12±11.77	94.82±3.67	86.05±4.22
4	*L. stoechas*	100.12±10.33	94.57±1.09	76.66±2.28
5	*P. kurroa*	379.10±7.51	318.73±24.20	241.34±8.25
6	*A. albus*	69.09±1.68	353.47±10.21	407.13±35.30
Data presented as Mean±SEM (n=3)				

C. reflexa methanolic extract was found to be most cytotoxic (IC_{50} 34.02±7.92 µg/mL), while methanolic extract of *A. albus* was least potent cytotoxic (IC_{50} 407.13±35.30 µg/mL).

3.5 Caspase-3 Activity

Caspase-3 activity of aqueous, hydroethanolic, and methanolic extract of PUF and doxorubicin in HeLa cell lines is presented in Figure 3. Doxorubicin showed significantly high caspase-3 activity compared to non-induced control cells ($p<0.001$) in HeLa cell lines. Hydroethanolic and methanolic extracts of PUF also resulted in a significant rise in caspase-3 activity in HeLa cell lines ($p<0.01$ and $p<0.001$ in comparison with non-induced control cells, respectively). Conversely, no statistically significant increase in caspase-3 activity. Effect of methanolic extract was found similar to that positive control doxorubicin.

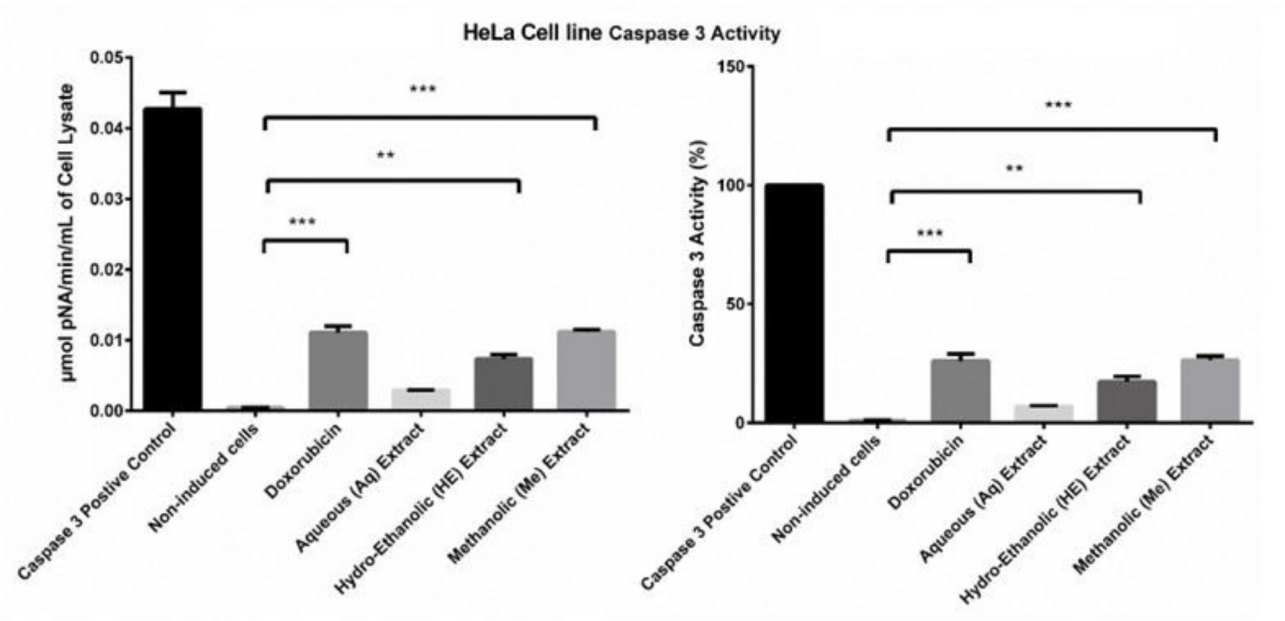


Figure 3. Caspase-3 activity of PUF extracts and doxorubicin in HeLa cell lines.
**=p<0.01vs. non-induced cells; ***=p<0.001vs. non-induced cells (One-way ANOVA followed by post-hoc test).

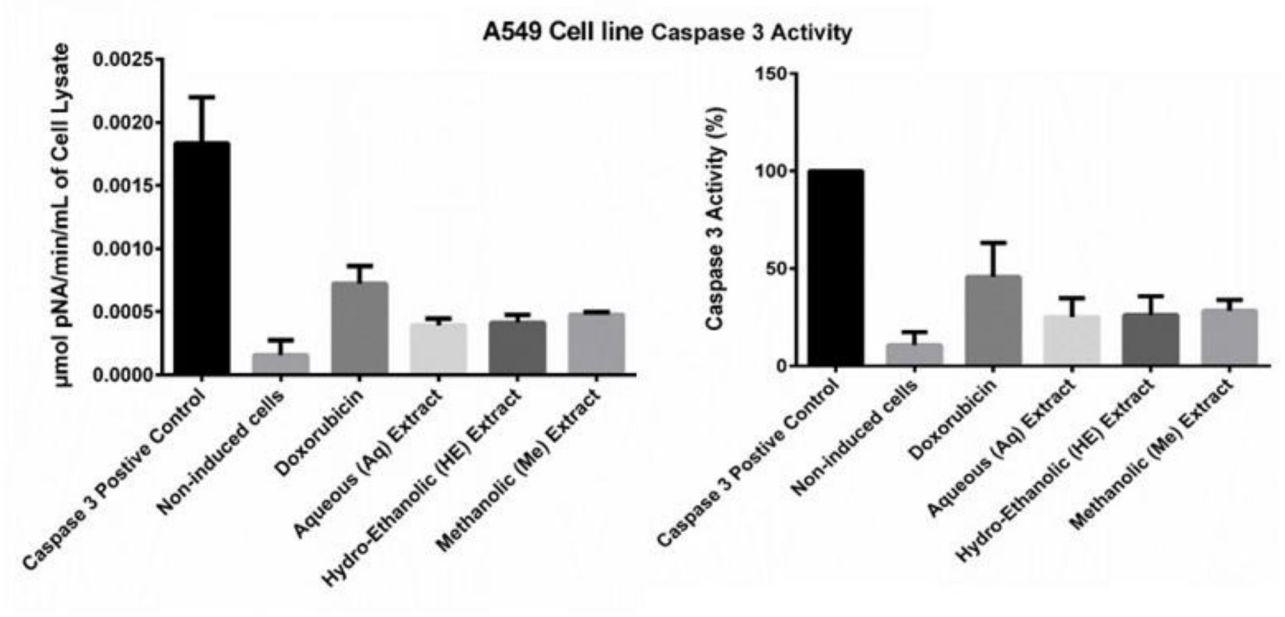


Figure 4. Caspase-3 activity of PUF extracts and doxorubicin in A-549 cell lines; no statistically significant difference was observed in any drug treatment group compared to non-induced cells (One-way ANOVA followed by post-hoc test).

In A-549 cells, although there was an increase in caspase-3 activity in doxorubicin as well as PUF extracts treated cells compared to non-induced control cells, but no statistically significant difference was found in any treatment group in comparison with non-induced control cells (Figure 4). In both the HeLa and A-549, the effect of the caspase-3 positive control was considerably higher than in any treatment group.

4. Discussion

There is a resurgence of interest in drug discovery from traditional sources, particularly from medicinal plants, across the globe. These bioactive phytoconstituents compounds are supposed to work through diverse yet coordinated mechanisms, including free radical scavenging, quenching of ROS, inhibition of oxidative enzymes and related mechanisms and reduces the risk of pathological consequences[23]. Cancer is a deadly disease that is rapidly growing and one of the new global health issues since there are insufficient effective early detection methods and suitable treatments[24]. The available chemotherapeutics strategy for treating cancer is costly, has side effects, and may also interfere with genes ability to operate normally. Therefore, a method of therapy that is both safe and effective is highly required to prevent the spread and growth of cancer. An economic, efficient, and safer way to stop the spread of malignant cells is offered by several traditional systems including *Ayurveda, Unani* medicine, and other traditional systems. It is already established that bioactive constituents derived from natural sources have remarkable pharmacological activities and arrest the growth of wide varieties of cancer[25,26]. Scientific validation of folkloric claim is need of hour for widespread and safe use of such promising traditional medicine. In view of this, we evaluated the *in vitro* anticancer potential of a traditional *Unani* formulation (PUF), mentioned in the classical *Unani* literature. Further, anti-oxidant activity of the PUF and its ingredients was also assessed as potential health benefit in pathological condition.

The qualitative analysis of PUF confirmed the presence of alkaloids, total phenols, flavonoids, glycosides in aqueous, hydro-ethanolic and methanolic extract. TPC content of the Aq extract of PUF and its constituent is higher than that of the HE and Me extracts. Higher content of TPC in aqueous compared to methanolic extract is justified owing to hydrophilic nature of phenolic compounds and is in agreement with earlier report with other plants[27]. Aq extract of PUF or any of its constituent typically has less TFC than HE or Me extracts. This finding is in agreement that the majority of flavonoids are lipophilic in nature[28]. Methanolic extract may provide better effect as antioxidants on the body since it improves the extraction of lipophilic antioxidant molecules as compared to water[27].

The results showed that PUF had significant quantity of total phenolic contents as well as flavonoid contents which are known for scavenging free radicals. Among six individual ingredients of PUF analysed, *T. chebula* has highest TPC and TFC, whereas *A. albus* has the lowest content. The TPC, TFC of PUF and its ingredients specially *T. chebula, L. stoechas* positively correlates with observed antioxidant activity. It is recognized that phenolic and flavonoid contents have antioxidant action, which counteracts free radical damage[29]. The possibility exists for natural substances like flavonoids, which function as antioxidants but also inhibit cancer in other ways, to be more important in therapy[30]. One of the most significant flavonoid anticancer mechanisms is thought to be their immunomodulatory action, which includes (a) upregulating the activating receptors on Natural Killer (NK) cells to increase their activity and cytotoxicity toward tumour cells, (b) lowering regulatory T cell activity against tumour cells and enhancing the cytotoxicity of cytotoxic T lymphocytes[31,32].

The DPPH, ABTS, and FRAP assays supported the antioxidant scavenging activity of PUF as well as its ingredients. The investigated traditional *Unani* formulation PUF have demonstrated a remarkable antioxidant potential which may be accountable at least in part, for the reported anti-cancer activity of PUF. The antioxidant properties of PUF might inhibit cancer progression, while its cytotoxic potential could target cancer cells, promoting apoptosis and cell death[33]. Distinguished *Unani* physicians documented a degenerative process termed *Iḥtirāq,* (unwanted combustion) signifying heightened metabolism or 'combustion,' acknowledged as a primary pathology contributing significantly to chronic diseases such as cancer. Oxidative stress can be viewed as a molecular manifestation of *Iḥtirāq*[34].

The investigation on the anti-proliferative effect of PUF extracts, resulted IC_{50} values ranging from 28.50±1.53 µg/mL to 207.86±1.24 µg/mL in HeLa, A-549, MCF-7, B16F10 cancer cell. The treatment with methanolic extract of PUF significantly repressed the growth of B16F10 cells at the lowest concentration (*i.e.,* 28.50±1.53 µg/mL) which was almost equivalent to positive control doxorubicin (IC_{50} value, 24.62±0.19

µg/mL). The MTT data revealed that the methanolic extract of PUF possesses greater potential to produce cytotoxicity on HeLa, MCF-7, A-549, and B16F10 cells than hydro-ethanolic and aqueous extract. A preliminary study conducted on the same formulation reported that the IC_{50} in the range of 1000 µg/mL in MCF-7 Mannan *et al*., which are quite high compared to our observed values of IC_{50} in MCF-7 cells (136.63±3.58, 129.10±1.43 and 91.08±8.81 µg/mL for Aq, HE, and Me extract, respectively)[35]. The difference may be primarily due to 48 h incubation period in the present study compared to 24 h incubation period reported by Mannan *et al.*[35]

Different extracts and cell lines have varying degrees of cytotoxicity. As per the guiding document by American National Cancer Institute (NCI) USA, mentions that botanicals or crude extracts are deemed cytotoxic if they achieve an IC_{50} of 20 or 10 µM after 48 to 72 hours, respectively. With an IC_{50} upper limit of 30 µg/mL, the NCI considers a crude extract as a potential candidate for further purification[30,36]. The PUF methanolic extract IC_{50} comes within this threshold of NCI in B16F10 cells. However, some authors like Ayoub *et al*., and Canga *et al*., proposed higher concentrations and a herbal extract could be cytotoxic at concentrations as high as 100 µg/mL[37,38]. In the present study, IC_{50} values of PUF methanolic extract are 103.54±5.20, 91.08±8.81, and 28.50±1.53 µg/mL, in A-549, MCF-7 and B16F10 cells, respectively. PUF hydro-ethanolic extract IC_{50} are 129.10±1.43 and 127.55±6.11 µg/mL in MCF-7 and B16F10 cells. Thus, both methanolic and hydro-ethanolic extracts of PUF may be viewed as containing secondary metabolites with potential anti-cancer properties, warranting further research for the development of targeted cancer therapies.

Aq, HE and Me extracts of individual ingredients of PUF were also individually tested in MCF-7 cells for cytotoxicity. IC_{50} value of methanolic extract *A. albus* was highest among all extracts, which suggested its lowest cytotoxicity potency in MCF-7 cells. Whereas, IC_{50} value of methanolic extract of C. reflexa was lowest (34.02±7.92 µg/mL), among all extracts, which suggested its highest cytotoxicity potential in MCF-7 cells. Moreover, in MCF-7 cells, the IC_{50} value of methanolic extract of C. reflexa (IC_{50} value, 34.02±7.92 µg/mL) was found almost equal to doxorubicin (IC_{50} value 30.61±0.55 µg/mL) positive control. As with compound PUF, methanolic extract of all individual ingredients was found to be most potent, followed by hydroethanolic and aqueous extracts with the exception of *A. albus* where this pattern was reversed. Almost all the ingredients of PUF or their phytoconstituents were reported as cytotoxic in cancer cells or in animal tumour model. The IC_{50} values of aqueous extract of *T. chebula* was reported to be 108.5µg/mL in MCF-7 cells[39]. This is in agreement with our observed IC_{50} value *i.e.,* 133.91±5.10 µg/mL. A recent investigation into ethanol and ethyl acetate extracts revealed a notable cytotoxic impact on MCF-7 cells, which varied with both dosage and duration[40].

Ali *et al*., reported that methanolic and aqueous extract of stems *C. reflexa* indicated significant cytotoxicity potential against human lung cancer H-1299 cells (IC_{50} values 93.50 and 902 µg/mL, respectively) and MCF-7 (IC_{50} values 104.99 and 99.99 µg/mL, respectively)[41]. A small difference in IC_{50} values of *C. reflexa* observed in our study in MCF-7 cells might be on account of differences in extraction methodology as well as the incubation period (24 h *vs.* 48 h) used for MTT assay. The aqueous extract of *C. reflexa* was found to trigger apoptosis in Hep3B cells by enhancing p53 and BAX (pro-apoptotic), while reducing Bcl-2 and survivin (anti-apoptotic factors)[42]. Cytotoxic activity of ethanolic extract of *P. vulgare* on A375 cells was evaluated by Tabeshpour *et al* and it has been reported that the IC_{50} was 123 µg/mL and there was a significant increase in apoptosis in melanoma cells at this concentration[43].

Moreover, Rajkumar *et al*., reported the cytotoxicity potential of the *P. kurroa* in MDA-MB-435S, PC-3, and Hep3B cells with IC_{50} values for aqueous (100.81 ± 8.3, 63.93 ± 3.2, 122.70 ± 7.8) and methanolic extracts (19.00 ± 5.2, 72.13 ± 2.8, 32.61 ± 10.7), respectively[44]. Aqueous dichloromethane (1:1) and n-hexane extract of *P. kurroa* were reported to be cytotoxic against MCF-7, SKBR3, MDA-MB-231, and SKOV3). *In vitro* and *in vivo* studies conducted by Lou *et al*., on picroside II revealed that it significantly reduced the migration and invasion of MDA-MB-231 cells[45]. Aboalhaija *et al*., reported that out of aqueous, methanol, ethanol, ethyl acetate, and chloroform extracts of *L. stoechas*, the most potent cytotoxicity was shown by ethanolic extract with IC_{50} values of 104.1 µg/mL on MCF-7 and

214.5 µg/mL on MDA-MB-231 cells, respectively[46]. The researchers also noted that the ethanol extract led to a 43.29% reduction in tumour size among the treated group, contrasting with a 58.9% increase in tumour growth observed in the control group mice[46]. This reported IC_{50} value of ethanolic extract of 104.1 µg/mL is in consonant with present observed IC_{50} values of 100.12±10.33 and 94.57±1.09 µg/mL in aqueous and hydroethanolic extracts, respectively in MCF-7 cell lines. HepG2 and BJ cell lines showed more than 90% cytotoxicity at 5 mg/mL of herbal extract. *L. stoechas* spp. *Luisieri* showed the strongest anti-proliferative effect against HepG2 cells[47]. Initial research on *A. albus* indicated cytotoxic effects of both methanolic and aqueous extracts at a concentration of 1,000 µg/mL against the MCF-7 cells. *A. bisporus* extract administration was shown by Latif *et al.*, to reduce tumour biomarkers, enhance antioxidants, and reduce lipid peroxidation in SD rats treated with DMBA-induced breast cancer[49]. Bioactive principles of *A. blazei* interact with immune mechanisms in cancer therapy and antiangiogenic effect is one its possible antimetastatic mechanism[50].

The Caspase-3 enzyme belongs to a group of endoproteases that regulate signalling networks involved in inflammation and apoptosis. There is growing evidence that caspase-3 also regulates the growth and maintenance of homeostasis in normal and cancerous cells[51]. In the current investigation, caspase-3 activity was performed to confirm the apoptosis. Aqueous, hydroethanolic, and methanolic extract of PUF and doxorubicin showed about 6.79%, 17.32%, 26.33%and 26.12% of caspase-3 activity in HeLa cell lines compared to caspase-3 positive control while non-induced cells showed about 0.99% activity. In A-549 cells, there was 25.17%, 26.20%, 28.24%and 45.64% of caspase-3 activity compared to caspase-3 positive control while non-induced cells showed 10.62% activity. Increased caspase-3 activity following treatment with PUF and doxorubicin indicated promotion of caspase-3 mediated apoptosis in both cell lines while the effect was more pronounced in HeLa cells.

These *Unani* herbal remedies could potentially enhance the anticancer activity and mitigate the adverse effects of traditional medications. Some of the natural substances directly inhibit cancer cells, causing them to die, return to normalcy (a process known as differentiation), or just cease growing. Other natural substances will stop the spread of cancer by altering the local environment in a way that makes it difficult for it to bleed, invade, or metastasize[30]. Therefore, combination of natural substances, like compound *Unani* formulation tested here, appeared to be a better therapeutic strategy against stubborn conditions like cancer.

5. Conclusion

The findings of present investigations showed that PUF has significant antiproliferative activity against MCF-7, HeLa, A-549, and B16F10 cell lines. Data of the present study validate the traditional indication of this *Unani* formulation in cases of *Saraṭān*. Oxidative stress can be viewed as a molecular manifestation of the *Unani* concept of *Iḥtirāq* which may be one of the causative factors of cancer. The current investigation found that PUF and its ingredients are rich in antioxidant polyphenols and flavonoids, which are at least in part, responsible for observed anti-cancer effect of PUF in the tested cell lines. The findings presented here are encouraging signs for the possible use of this formulation in chemopreventive and therapeutic contexts. Identifying bioactive phytochemicals responsible for the observed effects and investigating their precise mechanisms of action at cellular and molecular levels using *in vivo* experimental models would be a significant area for future research.

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7. Author Contribution

Conceptualization: GMH, MU; Resources: SSR, EV, TA, MU, DKD, NA and YIM; Methodology, Analysis and Visualization: SSR, EV, DKD, GMH, TA; Writing: DKD and GMH; Review and Editing: All; Supervision: GMH, MU, YIM, and DKD; Project administration: GMH, YIM and NA. All authors have read and agreed to the published version of the manuscript.

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