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**Original Article**

# In-Vitro Anticancer and Free Radical Scavenging Potential of Compound Formulation Used in Unani System of Medicine

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**ABSTRACT**

Cancer is one of the leading cause of mortality in India as well as worldwide. The management of cancer by conventional therapy has shown life threatening adverse effects. The researchers are now exploring the natural way of treatment. Unani system of medicine have rich literature for cancer and many compound formulations have been described in this system. Unani system of medicine is based on holistic approach and treat human being as a unit with natural herbs, mineral and animal origin drugs.

An important compound Unani formulation (CUF) from the literature has been chosen to explore the Unani claim of its anticancer activity. The phytochemical constituents were assessed using standard phytochemical screening method. Antioxidant property of this formulation was assessed by DPPH assay. The DPPH free radical scavenging assay was carried out by colorimetric method and ascorbic acid was taken as a positive control.

Three different extracts of CUF on different concentrations were used to screening on human breast cancer (BCC) MCF-7 cell line. For the estimation of in-vitro cytotoxic potency of the investigated extracts was assessed on MTT assay by using trypan blue method and paclitaxel was used as the standard. Hydro-ethanolic (HE) extract showed highest free radical scavenging activity among all extracts. DPPH Assay showed substantial antioxidant activity of these extracts in hydro-ethanol extract at 1µg concentration of CUF. The CUF showed antioxidant and anticancer activity. The claim made by Unani physician has been proved.

**Keywords** Anti-cancer Activity, Antioxidant, Breast cancer cell line, MCF-7, *Saratan,* DPPH Assay, MTT Assay

## 1. INTRODUCTION

Cancer is a multigenic and multicellular disease that can arise from all types of cells and organs with a multi-factorial etiology (Baskar *et al.*, 2012). It has capacity to overrun other tissues (metastasis) through direct cell migration or through the blood and lymph systems. Cancer is a leading cause of death worldwide (Davis CP., 2019; Anonymous, 2019; Ahuja *et al.*, 2013; Vorobiof DA., 2007). Approximately 70% of total death occurs due to cancer and more in low and middle income countries (WHO, 2018). An estimated 9.6 million deaths occur in 2018 due to cancer as per WHO data. The second most common cancer is breast cancer having around 2.09 million cases (Urruticoechea *et al.*, 2010). Several clinical trials have been found in Unani medicine in developing medications for chronic systemic diseases (Sunyana Jain *et al.*, 2009).

In the classical Unani literature *Saraṭān* word is used for cancer which is an Arabic word meaning crab and literally used from ancient times (131-200 A.D) (Alam MA *et al.*, 2017). Humoral theory which is described by Hippocrates, comprises of four humours (*Khilṭ*/body fluids) viz. blood, phlegm, yellow bile and black bile. If anyone or more than one is imbalance in these body fluids results disease condition (Samarqandi AN, 2007; Sudhakar, 2009; Quraeishi AH, 2002). Unani scholars described *Saraṭān* as "*Waram al-Sulb Sawdāwī*" a type of *Sawdāwī* (black bile) swelling. The excessive production and accumulation of *Sawdā* (black bile) in a particular organ site is the main reason for development of *Saraṭān* (Samarqandi AN, 2007; Sudhakar, 2009; Quraeishi AH, 2002; Majusi, 2010; Tabri M, 1997; Samarqandi AN, 2009; Ibne-al-Qif-al-Maseehi, 2000). Spread of *Saraṭān* is very fast due to involvement of vessels (Okunlola A *et al.*, 2007). The description of *Saraṭān* (carcinoma) was also available in many books written by *Rāzī, Ibn Sīnā, Al-Qamarī, Majūsī* and *Jālīnūs* (Al- Qamri, 2008; Diamandopoulos. GT, 1996; Weiss. L, 2000; Bernard *et al.*, 2003; Ibne Rushd, 1987).

In Unani system various drugs have been described for the

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treatment of cancer such as Aftimoon (*Cuscuta reflexa* Roxb.), Elva (*Aloe vera* L.), Ghariqoon (*Agaricus albus* L.), Haldi (*Curcuma longa* L.), Asgandh (*Withania somnifera* L.), Zift (*Pinus gerardiana* Wall.), Halela (*Terminalia chebula* Ritz.), Balela (*Terminalia belerica* Roxb.), Bisfayej (*Polypodium vulgare* L.), Ustukhudoos (*Lavandula stoechas* L.), Tahlab/Spirulina (*Arthrospigra maxima*), Kutki (*Picrorhiza kurroa* Royle ex Benth.) and Hulba (*Trigonella foenum-graceum* L.) (Majoosi, 2010; Razi, 2002; Rampuri, 2011; Nadkarni, M. 2010; Kirtikar & Basu, 1999a; Kirtikar & Basu, 1999b; Kumar A, 2014). These single drugs have been evaluated for anticancer activity and found to be very effective. These drugs are also used for various other diseases from long time and still being used.

There are a wide range of studies on individual Unani drugs for their efficacy and phytochemical activities for their use in alternative medicine (Ignacimuthu *et al.*, 2006) however; no attention has been paid to elucidate the efficacy of compound formulations in Unani medicine which needs to be scientifically validated. Novel approaches to cancer therapy should include discovery of such new therapeutics, with natural products which are the best reservoirs of medicinal values with minimal side-effects.

In modern medicine, the treatment of cancer includes chemotherapy and radiotherapy which have many side effects on the patient's body and also generate a lot of free radicals. Quick production of free radicals can act as a precursor for oxidative damage to bio-molecules and may cause diseases such as cancer, cardiovascular diseases, neurodegenerative diseases and premature ageing (Young *et al.*, 2001). Antioxidants are believed to have an important role in maintenance of human health by rendering protection against the free radicals.

## 2. MATERIALS AND METHODS

The present study was conducted at National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad. The plant materials used in the study were identified and authenticated by the Botanist of NRIUMSD, Hyderabad and voucher specimen no. Halela Siyah (SMPU/CRI-Hyd 13568), Aftimoon (SMPU/CRI-Hyd 13569), Bisfayej (SMPU/CRI-Hyd 13570), Ustukhudoos (SMPU/CRI-Hyd 13571), Kutki (SMPU/CRI-Hyd 13572), Ghariqoon (SMPU/CRI-Hyd 13573) and Namak Siyah (Black salt) were deposited in the survey of medicinal plant herbarium.

### 2.1 Preparation of compound Unani formulation and its extracts

The CUF prepared according to the composition of the formulation given by, '*Ali Ibn 'Abbās Majūsī*, in the *Kāmil al-Ṣanā'a* (Majoosi, 2010; Mannan *et al.*, 2020). The formulation consists of seven ingredients shown in table 1. Each ingredient was powdered separately and mixed together to obtain the formulation which was used to prepared extracts in different solvents. Aqueous (Aq), Hydro-Ethanolic (HE: 50:50), and Methanolic (Me) extracts were obtained by mixing 10 gm of powdered form in 100 ml with respective solvent system for overnight stirring at 50 rpm in rotatory Shaker Incubator at 37 °C. Then the mixture was filtered with Whatman filter paper No.1 and the filtrate was subjected to rotatory vacuum evaporator. The resultant dried forms of extracts were stored in desiccators until used for assays.

**Table 1.** Composition of Compound Unani formulation (CUF).

| S.No. | Plant species | Common name | Part of used |
|---|---|---|---|
| 1 | *Terminalia chebula* Ritz. | Halela | Fruit |
| 2 | *Cuscuta reflexa* Roxb. | Aftimoon | Whole plant |
| 3 | *Polypodium vulgare* L. | Bisfayej | Root |
| 4 | *Lavandula stoechas* L. | Ustukhudoos | Whole plant |
| 5 | *Picrorhiza kurroa* Royle ex Benth. | Kutki | Rhizome |
| 6 | *Agaricus albus* L. | Ghariqoon | Fungal species |
| 7 | Black salt | Namak Siyah | Crystals |

### 2.2 Qualitative Phytochemical Analysis

The extract of compound Unani formulation used in this study were analysed for the presence of various phytochemicals such as alkaloids, terpenoids, tannins and saponins etc by appropriate methods (Kaileh *et al.*, 2007).

**2.2.1 Test for Alkaloids**: 0.5 g of the extract was added to 5 ml of 1% aqueous HC1 on a steam bath. This was filtered and 1 ml of the filtrate treated with a few drops of Dragendorff's reagent (Potassium bismuth iodide solution) (Hamuel J, 2012). The orange brown precipitate indicates the presence of alkaloids (Vaghasiya *et al.*, 2011; Aynehchi *et al.*, 1982; Kokate *et al.*, 2017).

**2.2.2 Test for Tannins**: 0.1% $FeCl_3$ was added to the extract and presence of brownish green or blue-black colouration specifies the existence of tannins (Adeyemi *et al.*, 2014).

**2.2.3 Test for Saponins**: The extract was mixed with 1ml of distilled water in a test tube and it was shaken vigorously for 15 min. The formation of stable foam of 1cm layer was an indication for the presence of saponins (Vaghasiya *et al.*, 2011).

**2.2.4 Test for Terpenoids**: The extract was dissolved in 2 ml of chloroform and evaporated to dryness. To this 3 ml of concentrated $H_2SO_4$ was added and heated for about 2 minutes. A greyish colour indicated the presence of terpenoids (Farooq U *et al.*, 2014).

**2.3 Antioxidant assay:** The DPPH (2, 2-dipheny-1-picryl-hydrazyl-hydrate) free radical scavenging assay was carried out for the evaluation of the anti-oxidant potential. This method was assessed according to the method already reported with slight modification (Katiyan, S.S *et al.*, 2016). Briefly, an aliquot of 80 µL of 0.1mM DPPH in methanol were mixed with 20 µL of test extracts, with different concentrations in a micro test plate. The reaction mixture was kept in dark for 20 minutes and the absorbance was determined after 30 min at 517 nm with a TECAN multimode reader using ascorbic acid as a positive control.

**2.4 Cytotoxicity Assay on Human Cancer Cell Line:** For the estimation of *in-vitro* cytotoxic potency of the investigated extracts an established microtiter plate assay was used on human cancer cell line MCF-7. The MCF-7 cell line were grown in Dulbecco's Modified Eagle's Medium (DMEM) with FBS 10%

and mixed antibiotics. The culture was sustained at 37 ºC in CO2 incubator (Kaileh *et al.*, 2007). This calorimetric assay is based on the conversion of the yellow tetrazolium bromide MTT (3-(4, 5-dimethylthiazoly-2)-2,5-diphenyltetrazolium bromide), to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells.

Cells were seeded in 96 well plated. At the density of 10,000 cells/well in 100 µL medium, allowed to grow overnight with various concentration of the indicated extracts. After 24 hrs incubation 20 µl of MTT reagent has been added to cells and incubated for an additional 4 hrs followed by 80 µL of DMSO solution was added to the well to solubilize MTT crystals. The plates were incubated for overnight at 37 °C. The readings were taken at 517 nm using a TECAN multimode micro-plate reader. The potency of cell growth inhibition for each extract has been expressed as an IC50 value which defines as the concentration that will cause a 50% loss of cell growth.

**2.5 Statistical analysis**: The results represented were calculated and data was expressed as mean ± standard deviation (SD). Significance was assumed at a probability value of less than or equal to 0.05. Graph Pad Prism 5.0 software and Microsoft Excel 2010 were used for the statistical and graphical evaluations.

## 3. RESULTS

**3.1 Yield of extracts:** 10 g of CUF yielded 18.33% of crude aqueous extract, 30.81% of crude Hydro-ethanol extract and 31.28% of crude Methanol extract.

**3.2 Qualitative phytochemical analysis:** It was observed that almost all extracts of CUF shows good content of alkaloids, tannins, terpenoids and saponins. The results show the presence of therapeutically active compounds in the plant used (Table 2).

**Table 2**. Phytochemicals in the different extracts of CUF.

| Type of Extract | Alkaloids | Terpenoids | Tannins | Saponins |
|---|---|---|---|---|
| Aqueous Extract | + | + | + | + |
| Hydro-ethanolic Extract | + | + | + | + |
| Methanol Extract | + | + | + | + |

**3.3 Antioxidant potential:**
The ability of different extracts of CUF to scavenge DPPH free radicals at different concentrations (0.1 µg, 1 µg, 10 µg, 100 µg, 1000 µg) were measured for the calculation of IC50 values using GraphPad Prism 5.0 software and compared with ascorbic acid and showed antioxidant potential in all extracts. The lower IC50 value suggests stronger scavenging activity (Table 3). The hydro-ethanolic (HE) extract of CUF showed the best antioxidant potential among the three extracts (Fig. 1)

**Table 3.** Antioxidant activity different extracts of CUF using DPPH assay

| Concentrations (µg) | Aqueous. Ext. of CUF | Hydro ethanol Ext. of CUF | Methanol Ext. of CUF | Ascorbic acid |
|---|---|---|---|---|
| 0.1 | 10.77±0.56 | 2.73±0.28 | 3.41±0.42 | 31.35±0.45 |
| 1 | 15.54±0.54 | 5.27±0.27 | 16.2±1.29 | 35.74±0.90 |
| 10 | 23.57±0.36 | 22.42±1.00 | 22.46±0.46 | 61±0.47 |
| 100 | 47.57±0.65 | 41.48±1.54 | 38±00.86 | 79.88±0.81 |
| 1000 | 57.77±0.69 | 58.03±1.99 | 53.34±2.67 | 120±0.47 |

Values represent Mean ± SD; all extracts were done in triplicates.

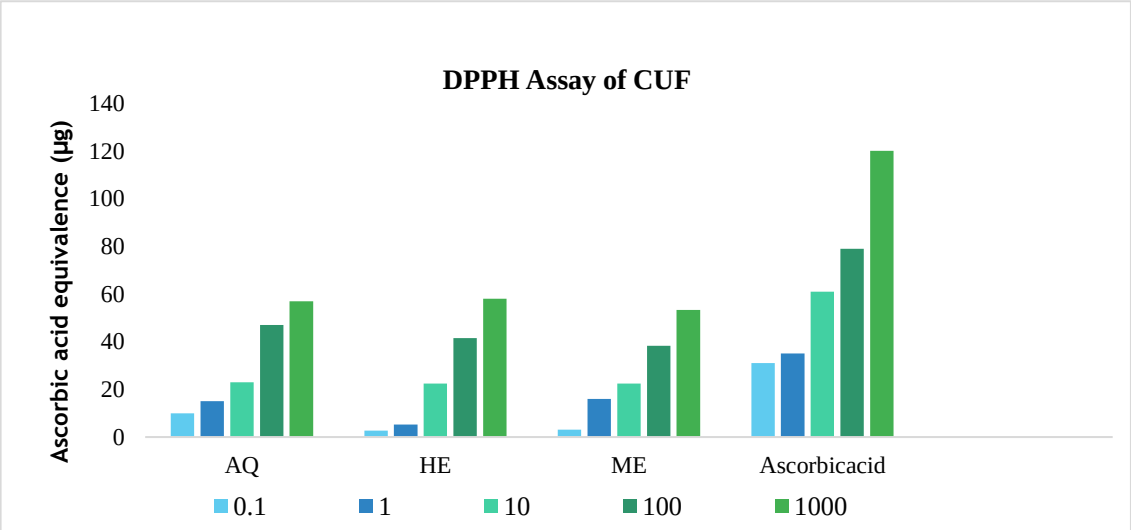


**Fig 1.** DPPH scavenging activities of aqueous (AQ), hydro-ethanol (HE) and methanol (Me) extracts of CUF with AAE (ascorbic acid equivalence) in µg. Data expressed as mean ± SD of n = 3 samples ($P < 0.05$).

**3.4 Cytotoxicity:** The cytotoxic activity of the CUF was evaluated on Breast cancer (MCF-7) cell line using the MTT assay. Cell viability decreased with increased concentration of extracts compared with paclitaxel as the control cytotoxic drug. In the present study, low cytotoxic effect of three different extracts of CUF was found against MCF-7 cell line in comparison to paclitaxel. The IC50 values for all the extracts were higher nearly 1000 µg ml-1 when compared to paclitaxel with IC50 value of 35.01 µg/ml. Among all the three extracts methanol extract showed higher anti-cancerous activity followed by hydro-ethanol and aqueous extracts (Fig. 2).

The aqueous, hydro-ethanol and methanol extracts of Compound Unani formulation (CUF) demonstrated remarkable anticancer activity against MCF-7 cell line. The cytotoxic activity was measured at different concentration from 0.1 to 1000 µg ml-1 in logarithmic scale and compared with paclitaxel the control cytotoxic drug. All three extracts have exhibited almost 50 % of cell death, at 0.1 and 1 ug/ml concentration with respect to control. At the concentration of 10 ug/ml aqueous extract (Aq) showed 38%, Methanolic extract (Me) and hydro-ethanol extract (HE) showed 22% of cell death. At the concentration of 100 ug/ml Aq ext. showed 44%, HE ext. showed 35% and Me. ext. showed 33% of cell death. At the concentration of 1000 ug/ml, Aq ext. showed 51%, HE ext. showed 53% and Me ext. showed 57% cell death. Decreased in cell growth were observed after 48 hrs of incubation, respectively as compared to the untreated control cells. The result showed cytotoxic effect of this formulation against MCF-7 cell line in comparison to paclitaxel. Different extracts showed different cytotoxic effects at different concentration.

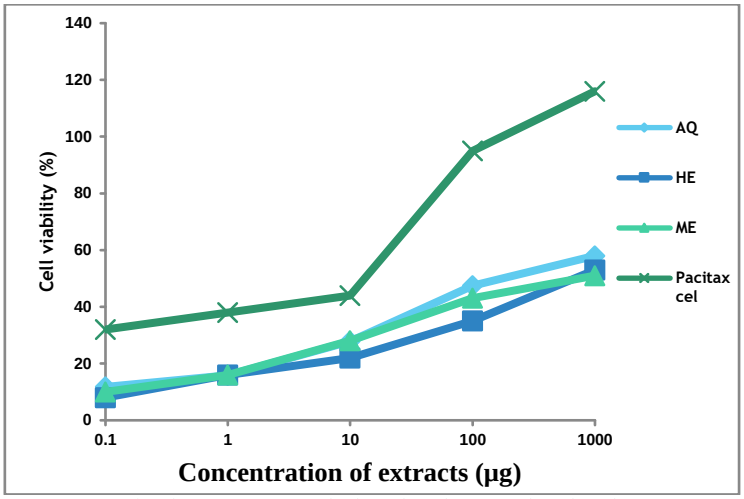


**Fig. 2.** The % inhibition by different concentration for aqueous (AQ), hydro-ethanol (HE) and methanol extracts (ME) compared with the paclitaxel.

## 4. DISCUSSION

The present study was planned to evaluate anticancer and antioxidant activities of compound Unani formulation (CUF) to validate the claim for its anticancer activity as mentioned in Unani literature. The phytochemical screening of different extract was also done to assess the pharmacological active compounds in this formulation. Although this CUF has been mentioned for treatment of cancer and was used Unani physician for long time but no scientific data is available for its validation. An attempt has been made to validate the Unani claim by exploring its anticancer activities by modern methods. It is a preliminary attempt to explore the future research on this formulation for clinical use of it.

Many single drugs have been investigated for the same purpose such as *Picrorrhiza kurroa* (*Kutki*) which has shown anti-carcinogenic and anti-tumor activity (Joy, KL *et al.*, 2000; Jeena *et al.*, 1999). It also decrease levels of lipid peroxidases and hydroperoxidases, free radical producing agents, and help facilitate the recovery of a powerful antioxidant in the liver needed to prevent oxidative damage (Chakraborti *et al.*, 1974). The Ethanol and chloroform extract of *Cuscuta reflexa* have shown significant antitumor activity and increased the life span of tumor bearing mice (Udavant *et al.*, 2012; Chatterjee*et al.*, 2011). The aqueous extract of *Cuscuta reflexa* has shown anti-HIV activity and methanol extract shows anti-bacterial and free radical scavenging activity (Mahmood *et al.*, 1997). The antioxidant activity of water and ethanol extracts of lavender (*Lavandula stoechas* L.) was studied (Gulcin, 2004). An essential oil from its leaf was found to be active against cancer cell line COL-2 (9.8 µg/mL) and weakly active against LNCaP (17.6 µg/mL), while the chloroform extract of the same plant was found to be highly active against cancer cell line P-388 (1.4 µg/mL) (Gören *et al.*, 2002). Phytochemical analysis of the plant extracts revealed the presence of pharmacological active constituents such as alkaloids, saponins, terpenoids, and tannins. Alkaloids have been associated with medicinal uses for centuries and may be responsible for their cytotoxicity (Nobori T *et al.*, 1994; Yahaya U *et al.*, 2019; Sofowora, 1993).

Antioxidants protect the body and maintain health from constant and unavoidable risk of reactive oxygen species (Fridovich I, 1998). Accordingly, in the present study all extracts showed a concentration dependent scavenging activity in DPPH assay. The better scavenging activity was shown by all extracts in 1µg concentration. The antioxidant and cytotoxic activities of these extracts may be considered as a source of anticancer drug. It is assumed that antioxidant activity may play a role in prevention of cancer progression; while the cytotoxic potential, on the other hand, might be used against cancer cells, thereby directing them towards apoptosis and cell death (Rajkumar V *et al.*, 2011). Thus, the consumption of natural antioxidant from food supplement and traditional medicines including the Unani system of medicine will help in combating various diseases with fewer side effects or without any side effect.

## 5. CONCLUSIONS

The result of this study revealed the presence of therapeutically active compounds such as alkaloid, tannin, saponins etc. The Compound Unani formulation (CUF) has shown antioxidant and cytotoxic activity on MCF-7 cell line as compared with the standard paclitaxel. The aqueous extracts of CUF have shown best activity and it supports the use of natural drugs in its pure form by Unani Physicians. It is a preliminary study which may guide the future research of this formulation in experimental animals and clinical trial at last. The characterization of active compound responsible for its action can be done to prove its pharmacokinetics and pharmacodynamic. As per Unani concept natural drugs are similar to human body so they are generally not harmful to the body and easily digested and metabolised by the body without producing harmful secondary metabolites. This is the strength of Unani system of medicine and it should be promoted.

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## CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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