Chapter-4

In-vitro assessment of antioxidant potential of Unani formulations
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Treatment of cyclophosphamide results in the production of reactive oxygen species, which causes peroxidative damage to urinary bladder and other vital organs (Patel, 1987; Sulkowska et al., 1998). Role of tissue antioxidants becomes important in the prevention of such damage. A number of natural products and synthetic compounds have been shown to reduce CP toxicity mainly due to their antioxidant action (Haque et al., 2003; Abd-Allah et al., 2005; Topal et al., 2005; Tripathi and Jena, 2009; Cui et al., 2010). GSH and other antioxidants are depleted in the patient treated with CP which further depletes when an infection is ensued due to immunosuppressive effects of CP (Angulo et al., 2002, Tsai-Turton et al., 2007). Mutagenicity, clastogenicity, cytotoxicity and carcinogenicity are inhibited by antioxidant compounds formed in abundance in plants (Hochstein and Atallah, 1988; Tripathi and Jena, 2009; Aqil et al., 2011).

Most chemopreventive compounds and their analogs and derivatives are initially of plant origin and inhibit spontaneous and chemical mutagenesis in a variety of in vitro and in vivo test systems (Sengupta et al., 2004; Akinboro et al., 2011). Herbal extracts and their purified compounds with antioxidant
potential have shown protective effects on CP-induced toxicity (Davis and Kuttan, 2000; Haque et al., 2001; Bin Hafeez et al., 2001; Ramadan et al., 2011). Bhatia et al. (2006b) have reported in vitro antioxidant activity of Juglans regia extract and also demonstrated its protective effect against urinary bladder toxicity caused by CP treatment. Oxidative stress has been linked to cancer, aging, ischemic injury, inflammation and neurodegenerative diseases (Parkinson's and Alzheimer's). ROS such as superoxide radical $\left(O_2^\cdot\right)$, hydroxyl radical $\left(OH^\cdot\right)$, peroxyl radical $\left(ROO^\cdot\right)$ and nitric oxide radical $\left(NO^\cdot\right)$, attack biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury associated with aging, atherosclerosis and carcinogenesis (Halliwell and Gutteridge, 1986; Kuang et al., 2011; Yang et al., 2012).

The original 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay was based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce the radical cation, in the presence or absence of antioxidants. This has been criticized on the basis that the faster reacting antioxidants might also contribute to the reduction of the ferryl myoglobin radical (Plotnikov et al., 2009). A more appropriate format for the assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants. Addition of antioxidants to the pre-formed radical cation reduces ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus, the extent of decolorization as percentage
inhibition of the ABTS$^*$ radical cation is determined as a function of concentration and time.

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^*$) is a free radical, stable at room temperature, which produces a purple colour solution in methanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured methanol solutions. Plant extracts or any natural compound with higher content of flavonoids and phenolic acid exhibited the stronger DPPH radical scavenging activity (Yuan et al., 2009). Kalim et al. (2010) has studied in vitro antioxidant capacity of 10 plants which are generally used in Unani system of medicine. These plants showed significant potential for preventing oxidative DNA damage and radical scavenging activity. In vitro studies on plant and its active constituents provide good insight into the in vitro antioxidative property of gingerol an active constituent of ginger (Dugasani et al., 2010). These herbal drugs play an important role in prevention of toxicity of drugs.

In Unani medicine, single drugs or their combinations in raw form are preferred over compound formulations. The naturally occurring drugs used in this system are symbolic of life and generally free from side effects. Such drugs, which are toxic in crude form, are processed and purified in many ways before use (Mukherjee and Wahile, 2006; Ansari et al., 2010; Rasheed and Gupta, 2010). Evaluation of new uses of Unani drugs other than the known traditional uses has been going on and physicochemical and phytochemical evaluation of old drugs being done to know their usage in other disease (Ajazuddin and
Najmi et al. (2005) has evaluated the in vitro antioxidant capacity of Jigrine, a Unani formulation, by DPPH radical scavenging capacity and found it to have good antioxidant capacity and the in vitro results have been supported by showing hepatoprotective activity in vivo.

In this chapter data of study on the in vitro antioxidant activity of Unani drugs as assessed by DPPH and ABTS free radical generation assays are reported. The two Unani herbal preparations are i) Jawarish amla sada and ii) Habbe khabsul hadid which contains a significant percentage of Emblica officinalis and Trigonella foenum-graceum, respectively.

Material and Methods

Unani drugs were dissolved in normal saline.

Measurement of in vitro antioxidant activity of Unani drugs

In vitro DPPH assay

DPPH* assay was performed using the procedure of Hamauzu et al. (2005). A solution of 0.1 mM DPPH in methanol was prepared, and 4 ml of this solution was added to 0.2 ml aliquote of the drug at different concentrations. The decrease in absorbance at 517 nm was measured at 60 min. A control was added with 0.2 ml of distilled water instead of the extract. DPPH radical scavenging capacity of the extract was calculated as IC$_{50}$ graphical method.
In vitro ABTS assay

The ABTS decolourisation assay was performed using the procedure of Re et al. (1999). The ABTS$^*$ radicals were generated by the oxidation of ABTS (7 mM) in deionized water with potassium persulfate (2.45 mM) mixed in ratio of 2:1 and kept in the dark at room temperature for 16 h. The ABTS$^*$+ solution was diluted with ethanol to obtain absorbance of $0.700 \pm 0.020$ at 734 nm. ABTS$^*$+ (3 ml) solution was mixed with the Unani drugs (0.1–1 mg/ml) in a 1-cm path-length cuvette and the decrease of absorption was measured at each 30-s interval for 6 min at 734 nm. Radical scavenging activity was calculated as 50% inhibition (IC$_{50}$) derived from the curve of absorbance versus concentration of plant extract.

Results

Table 4.1 shows the IC$_{50}$ values of two drugs in DPPH and ABTS assays. The data show that both the Unani drugs are effective in scavenging the ABTS radical more than DPPH. However, assay between drugs JAS showed more antioxidant activity than HKH in ABTS assay.
Table 4.1: DPPH and ABTS assay - IC50 values

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<tr>
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<th>Jawarish amla sada</th>
<th>Habbe khabul hadid</th>
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<tr>
<td>DPPH IC50 (mg/ml)*</td>
<td>0.728 ± 0.027</td>
<td>0.813 ± 0.039</td>
</tr>
<tr>
<td>ABTS IC50 (mg/ml)*</td>
<td>0.648 ± 0.052</td>
<td>0.720 ± 0.062</td>
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*Results are average of triplicate data with Mean ± S.D.

Discussion

Many investigators have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases like aging process etc. (Stajner et al., 1998; Sanchez-Moreno et al., 1999; Malencic et al. 2000; Mukherjee, 2002). Numerous plant constituents have proven to possess free radical scavenging or antioxidants activity (Aruoma and Cuppett, 1997; Steinrut et al., 2011). In this study data of the DPPH and ABTS*+ assay show promising antioxidant scavenging capacity of Unani drugs, JAS and HKH. JAS showed more scavenging activity for ABTS*+ radical. Re et al. (1999) has reported the IC50 of vitamin C (ascorbic acid) as 0.99 ± 0.04 at 6 minutes. This gives an idea about the JAS capacity to scavenge ABTS*+ radical is calculated to be 0.648 which is lower than the ascorbic acid. It may be because of different constituents in this herbal
compound formulation which contains around 7% *Emblica officinalis* (Amla), a rich source of ascorbic acid.

With the above result, it can be concluded that the Unani herbal preparations which contain a mixture of medicinal herbs are good free radical scavengers. Therefore, they are good candidates for reducing ROS-related damage in tissues. Najmi et al. (2005) have reported that Jigrine, Unani traditional medicine protects rats against hepatopathy caused by drugs and it showed improvement in biochemical parameters like AST, ALT, and urea in serum, TBARS, and glutathione in liver and whole blood glutathione. Betel nut (*Areca catechu*) is an herbal product which is used traditionally against inflammatory disorders in the Unani system of medicine. Khan et al. (2011) have evaluated the anti-inflammatory and analgesic activities of the crude extract of betel nut and its respective fractions and found to be effective against paw edema, formalin-induced nociception and acetic acid-induced writhing assays in rats. Betel nut also showed free radical scavenging activity in DPPH assay. A compound preparation STW5 was used for management of Irritable Bowel Syndrome (Rahimi and Abdollahi, 2012). It was found that compound preparations were more efficacious than single ones. The efficacy of compound herbal preparations may be because of different mechanisms of action such as anti-inflammatory, prosecretory activity, and affecting gastrointestinal motility (Rahimi and Abdollahi, 2012). JAS and HKH are also compound drugs. Therefore, these drugs showed good antioxidant activity in in-vitro assays.
Thus, providing beneficial data for their further in vivo evaluation in animal studies.

In the present study, two Unani drugs Jawarish amla sada and Habbe khab sul hadid were investigated. These drugs have shown a moderate free radical scavenging capacity and found to be a little more effective in scavenging the ABTS radical. JAS has shown a lower IC50 which indicates its possible efficacy as an antioxidant in vivo also.