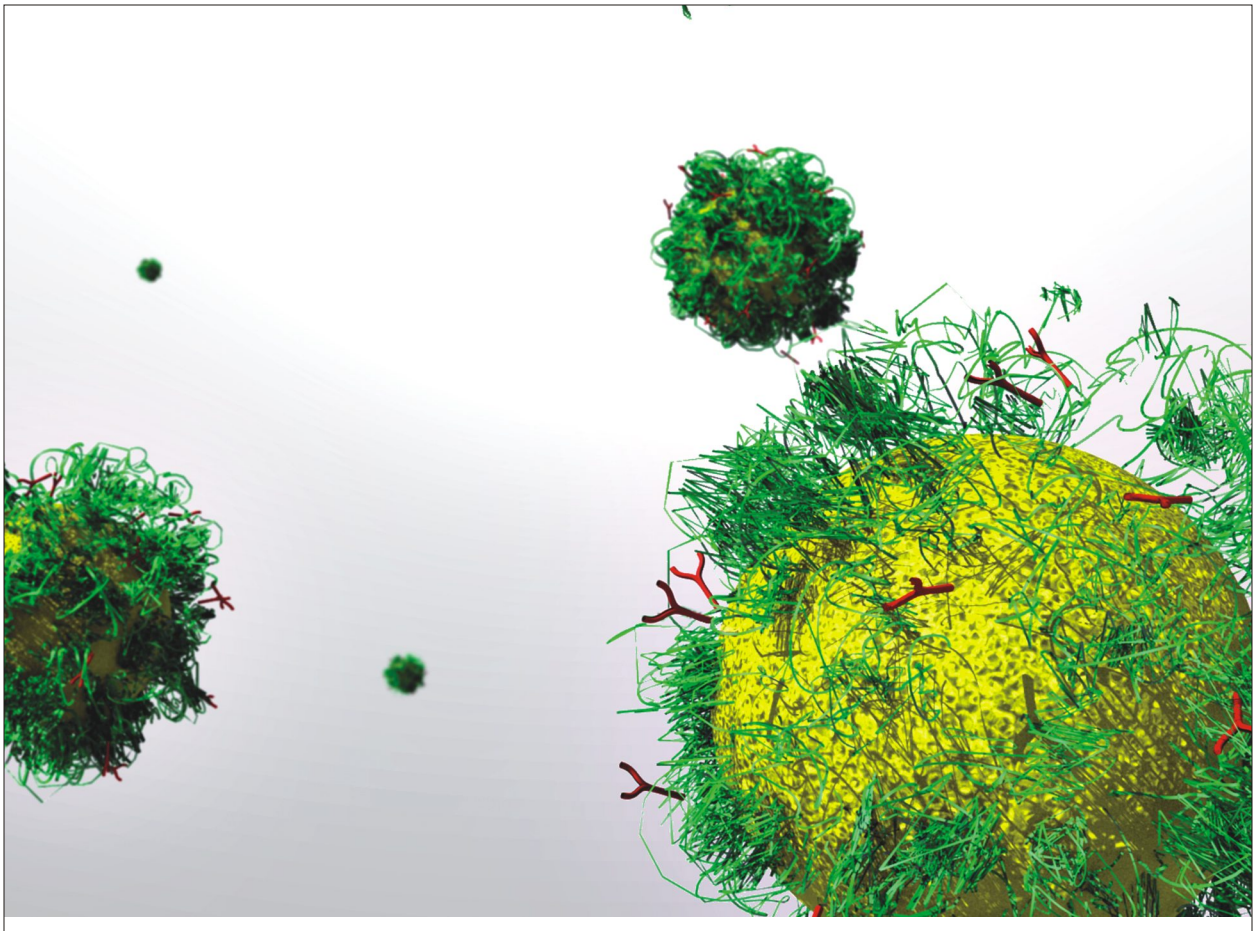

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In vitro Antioxidant Activity of Itrifal Kishneezi: A Unani Formulation

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ABSTRACT

Unani system of medicine (Unanipathy) originated in Greece, enriched by Persians and Arabs and now became an integral part of Alternative medicinal systems of India. Itrifal Kishneezi is a Unani medicine prescribed for gastric problems, head ache and used as a stimulant. The present study was taken up as there is no published scientific data available. The drug was tested for antioxidant activity, as there is growing evidence of role of free radicals in disease progression in number of diseases and benefits of concomitant antioxidant administration. Itrifal Kishneezi was tested for DPPH$^{\bullet}$ free radical scavenging and Fe^{2+} metal ion chelating activity using UV-Vis spectrophotometer. It showed considerable *in vitro* antioxidant activity in a dose dependent manner.

Key words: Unani, Itrifal Kishneezi, DPPH$^{\bullet}$, ferrozine, free radicals, antioxidant

INTRODUCTION

Unani system of medicine (Unanipathy) originated in Greece based on the principles propounded by Galen, a Greek Practitioner and was called Galenic. After him many Arab and Persian scholars enriched the system and became Unani (Ahmad and Akhtar, 2009). Now it has become a part of Indian traditional system of medicine (Anupama *et al*., 2009).

Itrifal Kishneezi (IK) is used in unani for chronic catarrh, gastric problems-flatulence, indigestion, hyperacidity; head ache, eye pain and as a stimulant (Gupta, 2003; Kabeeruddin, 1989; Rehman, 1985). IK contains Terminalia chebula (Myrobalan)-black Myrobalan (unripe fruit-0.435 g), yellow myrobalan (fresh ripe fruit-0.435 g) and brown myrobalan (dried ripe fruits-0.435 g); *Terminalia belerica* (0.435 g); *Coriandrum sativum* (0.435 g); clarified butter (ghee-0.866 g) and honey (6.953 g).

Terminalia chebula is shown to have antibacterial, antifungal (Inamdar *et al*., 1959), purgative (Miglani *et al*., 1971), hypolipidemic (Khanna *et al*., 1993), anthelmintic (Dwivedi *et al*., 2008) and anticancer activities (Kaur *et al*., 1998; Saleem *et al*., 2002). It is used for flatulence, constipation, diarrhoea, dysentery, cyst, digestive disorders, vomiting, enlarged liver and spleen, cough and bronchial asthma, metabolic harmony; as diuretic and antioxidant (Kokate *et al*., 2002; Evans, 2006; Khare, 2007; Chattopadhyay and Bhattacharyya, 2007). *Terminalia belerica* has

purgative, astringent, antipyretic, hypotensive actions and is also used in diarrhoea, dyspepsia, biliousness, cough, bronchitis, upper respiratory tract infections, tropical pulmonary eosinophilia, helminthiasis and allergic eruptions (Kokate *et al*., 2002; Evans, 2006; Khare, 2007). It is shown to have hepatoprotective (Anand *et al*., 1997; Jadon *et al*., 2007), antidiabetic, antioxidant (Sabu and Kuttan, 2009) and antisalmonella (Madani and Jain, 2008) activities. *Coriandrum sativum* is used as stimulant, stomachic, carminative, anti-spasmodic, diuretic, hypoglycemic, anti-inflammatory, bactericidal, larvicidal and in measles, aerophagy, gastroenteritis, bleeding piles, neuralgia, cephalalgia, spermatorrhoea and viral infections (Kokate *et al*., 2002; Evans, 2006; Khare, 2007). It is shown to have antidiabetic (Swanston-Flatt *et al*., 1990; Gray and Flatt, 1999), antifertility (Al-Said *et al*., 1987), hypolipidemic (Chithra and Leelamma, 1997), antioxidant (Chithra and Leelamma, 1999) and hypotensive (Medhin *et al*., 1986) activities. Clarified butter increases intelligence, enhances memory, enhances bioavailability and is an appetizer. It is an antioxidant and is also useful in psychological disorders and epilepsy (Shastri, 1998). Honey is useful in cough, sore throat, acidity, diabetic ulcers, colitis, inflammation (Bilsel *et al*., 2002; Al-Waili, 2004), wound healing (Molan, 2006) and as a sweetener (Shastri, 1998).

Studies showed the significance of oxidative stress, mitochondrial dysfunction and free radicals in aging and in the pathogenesis of many diseases viz., autoimmune disorders, cancer, radiation injury, parkinsonism, alzheimer's disease, multiple sclerosis, myocardial infarction, atherosclerosis, diabetes, peptic ulcer, epilepsy, depression, nephrotoxicity, smoking induced respiratory disorders, etc. (Christensen and Somers, 1996; Halliwell and Gutteridge, 1999; Sharma and Sharma, 2008; Flerov and Shalyapina, 2009). Oxidative stress is caused by - Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). ROS are either free radicals (superoxide anion radical $^1O_2^{\bullet}$, $^{\bullet}OH$) or non radicals that are oxidizing agents and/or easily convert into radicals (HOCl, HOBr, O_3, ONOO-, H_2O_2). Similarly RNS are either free radicals ($NO^{\bullet}$, $NO_2^{\bullet}$) or non radicals (HNO_2, N_2O_4). These are capable of damaging nucleic acids, lipids, proteins and carbohydrates and also can cause DNA damage, cellular damage and neuronal death (Halliwell and Gutteridge, 1999; Gulcin *et al*., 2003). Cells contain two types of natural defense systems-enzymes to detoxify (viz., superoxide dismutase, catalase and peroxidase) and antioxidants (vitamins C and E, glutathione, ferritin and uric acid). Saturation of these defense systems causes oxidative stress (Arzimanoglou *et al*., 2002). The defense mechanisms act by removing oxygen or decreasing local oxygen concentration, removing catalytic metal ions, ROS and RNS, quenching or scavenging initiating free radicals, breaking the chain of initiated sequence, enhancing endogenous antioxidant defenses by up-regulating expression of genes encoding the antioxidant enzymes, repairing oxidative damage caused by radicals, increasing elimination of damaged molecules and not repairing excessively damaged molecules in order to minimize introduction of mutation (Wood *et al*., 2006). Thus therapies aimed at reducing oxidative stress may ameliorate tissue damage and favorably alter the clinical course (Costello and Delanty, 2004; Anupama *et al*., 2010).

In the present study we have taken up two *in vitro* antioxidant models-free radical scavenging activity and metal chelating activity, to test the antioxidant potential of IK.

MATERIALS AND METHODS

The present study was carried out during spring/summer, 2010 at Sultan-ul-Uloom College of Pharmacy, Hyderabad, India.

Drug: IK was obtained from M/s Hamdard (Wakf) Laboratories, Ghaziabad, Uttar Pradesh, India and was dissolved in methanol and filtered.

Chemicals: DPPH$^{\bullet}$ (1,1-Diphenyl-2-picrylhydrazyl radical) was obtained from Sigma, New Delhi, India. L-Ascorbic Acid (AA), Ferrous Chloride ($FeCl_2$), ferrozine, disodium Ethylene Diamine Tetra Acetate (EDTA) and solvents were obtained from sd-fine Chemicals, Mumbai, India. All the chemicals were of analytical grade.

Free radical scavenging activity: The free radical scavenging activity of IK was measured employing the method of Blois (1958). To 1 mL of different concentrations (1, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg mL^{-1}) of IK, 1 mL of 0.1 mM solution of DPPH$^{\bullet}$ in methanol was added and stirred vigorously on vortex mixer. The reaction mixture was kept in dark for 30 min and the absorbance was measured at 517 nm using UV-Spectrophotometer (Shimadzu, Japan). A control containing 1 mL water and standard containing different concentrations of AA (1-100 μg mL^{-1}) were prepared to which DPPH$^{\bullet}$ was added as above. All measurements were made in triplicate and their means were taken. Percentage inhibition was calculated using the formula:

$$\text{Inhibition } (\%) = \frac{(A_o - A_s)}{A_o} \times 100$$

where, A_o and A_s are absorbencies of control and sample/standard respectively. IC_{50} values introduced by Brand-Williams *et al.* (1995) were calculated using online software of TMDU Chemical Biology Database.

Metal chelating activity: The chelation of ferrous ions was estimated by method of Dinis *et al.* (1994). To the tubes containing 1.7 mL of deionized water, 50 μL of 0.2 mM $FeCl_2.4H_2O$ and 50 μL of different concentrations of IK (20, 40, 60, 80 and 100 μg mL^{-1}) were added mixed and kept aside for 1 min. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine, mixed on a vortex mixer and after 10 min the absorbance of the solutions were measured at 562 nm in a UV-Vis Spectrophotometer (Shimadzu, Japan). All tests and analyses were made in triplicates. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:

$$\text{Inhibition } (\%) = \frac{(A_o - A_s)}{A_o} \times 100$$

where, A_o and A_s are absorbencies of control and sample/standard respectively. IC_{50} values introduced by Brand-Williams *et al.* (1995) were calculated using online software of TMDU Chemical Biology Database.

Statistical analysis: Results were presented as Mean±SD. Statistical analysis was performed using Graphpad instat software. The values $p<0.05$ were considered significant after performing Duncan's multiple range test.

RESULTS AND DISCUSSION

Free radical scavenging activity: The free radical scavenging activity of IK was evaluated through its ability to quench the DPPH$^{\bullet}$ using ascorbic acid as reference. The results are shown in Table 1. IK showed free radical scavenging activity and the IC_{50} values of IK and AA were found to be 25.14 and 21.23 μg mL^{-1}, respectively (Fig. 1).

Metal chelating activity: IK interfered with chelation of Fe^{2+} ions in a dose dependant manner reducing the intensity of the color of Fe^{2+}-ferrozine complex. Results are shown in Table 2.

Table 1: DPPH$^{\bullet}$ scavenging activity of Ascorbic Acid (AA) and Itrifal Kishneezi (IK)

	Inhibition of DPPH$^{\bullet}$ radical (%)			
	AA		IK	
Concentration (μg mL^{-1})	Mean	±SD	Mean	±SD
0	0.0	0.0	0.0	0.0
1	8.8	9.2	4.8	3.2
10	24.1	4.7	15.6	5.3
20	49.1	5.6	34.3	5.9
30	64.9	1.7	61.1	4.8
40	86.1	0.5	84.4	4.7
50	95.6	0.2	93.3	2.2
60	95.7	0.1	95.2	2.1
70	96.9	0.2	96.2	2.2
80	97.2	0.2	95.9	1.1
90	97.2	0.1	96.9	0.9
100	97.3	0.2	98.8	0.9

$p<0.05$ Duncan's multiple range test

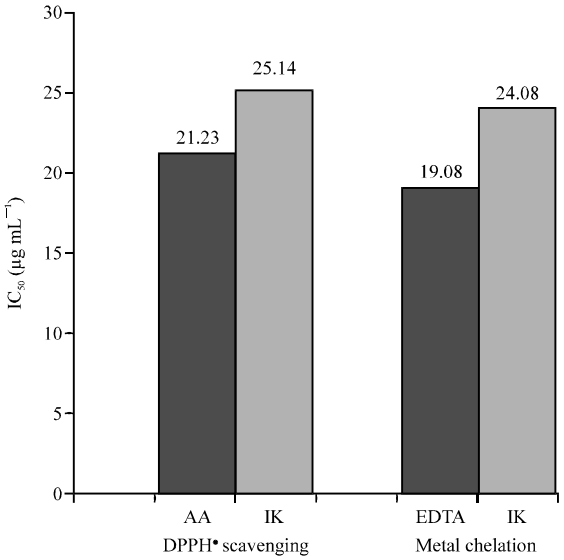


Fig. 1: IC_{50} values of Itrifal Kishneezi in DPPH$^{\bullet}$ scavenging and metal chelating activities

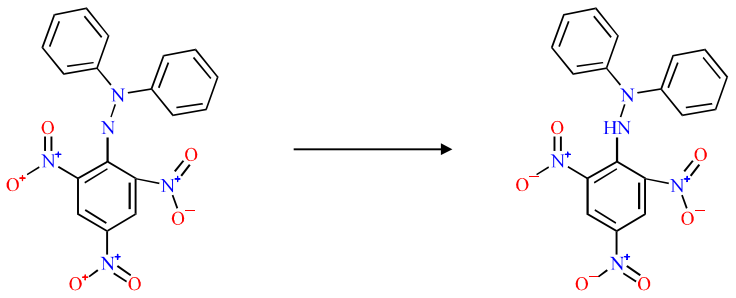
Table 2: Metal chelating activity of EDTA and Itrifal Kishneezi (IK)

Concentration ($\mu g\ mL^{-1}$)	Inhibition of metal chelation (%)			
	EDTA		IK	
	Mean	SD	Mean	SD
0	0.0	0.0	0.0	0.0
20	58.5	0.4	38.7	3.1
40	98.6	0.3	77.1	6.6
60	98.7	0.2	92.9	4.1
80	98.9	0.1	95.4	7.2
100	99.0	0.1	96.4	8.6

$p<0.05$ Duncan's multiple range test

Concentration for 50% inhibition of metal chelation (IC_{50}) by IK was found to be 24.08 $\mu g\ mL^{-1}$ and that of disodium EDTA was 19.08 $\mu g\ mL^{-1}$ (Fig. 1).

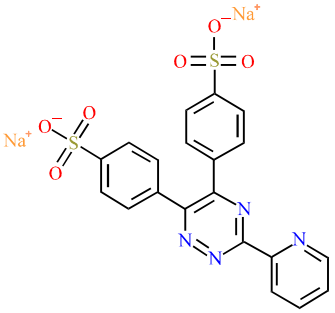
Studies indicate that the Reactive Oxygen Species (ROS) can induce epithelial dysfunction and macrophage activation resulting in the release of cytokines and growth factors that stimulate matrix remodeling and proliferation of smooth muscles. Oxidation processes are also involved in the cross linking of collagen fibers resulting in long term vascular constriction (Huq, 2007). DPPH$^{\bullet}$ is considered to be a model of lipophilic radicals which initiate lipid auto oxidation (Dinis *et al*., 1994). DPPH$^{\bullet}$ is characterized as a stable free radical by virtue of delocalization of the spare electron over the molecule as a whole so that the molecules do not dimerize, as would be case with other free radicals. The delocalization also gives rise to deep violet color, characterized by an absorption band at 517 nm. When a solution of DPPH$^{\bullet}$ is mixed with a substance that can donate hydrogen atom, it reduces to DPPHH (1,1-Diphenyl-2-picrylhydrazine, pale yellow color from the picryl group still present) (Molyneux, 2004).



DPPH$^{\bullet}$ (517 nm)
(1,1-diphenyl-2-picrylhydrazyl radical)

DPPHH
1,1-diphenyl-2-picrylhydrazine)

Metal chelating activity (Brand-Williams *et al*., 1995) is claimed as one of the antioxidant mechanisms, since it reduces the concentration of bivalent transition metal ions which act as catalysts in lipid peroxidation leading to formation of hydroxyl radicals and hydrogen peroxide decomposition reactions via fenton reactions (Halliwell, 1997). Ferrozine quantitatively forms complexes with Fe^{2+}, however in presence of chelating agents, the complex formation is disrupted and the dark red color (562 nm) of the complex decreases. The color reduction allows the estimation of chelating activity of co-existing chelator. IK quenched the DPPH$^{\bullet}$ and interfered with chelation of Fe^{2+} ions in a dose dependant manner.



Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine)

There is no published research evidence available on the activity of Itrifal Kishneezi, though most of its ingredients are widely studied for antioxidant activity (Chattopadhyay and Bhattacharyya, 2007; Chithra and Leelamma, 1999; Sabu and Kuttan, 2009). Some of the ingredients of the present formulation are rich sources of tannins and flavonoids which are phenolic compounds and may be responsible for its antioxidant activity (Uddin *et al*., 2008). The medicinal properties of plants have been centre of attraction for researchers in recent scientific developments throughout the world, due to their potent antioxidant properties, no side effects and economic viability (Gupta and Sharma, 2010). Unani medicines are formulated generally from natural resources and are well tolerated and this system of medicine needs to be deeply explored for the benefit of mankind.

CONCLUSIONS

Unani medicines are used extensively, but as they lack modern scientific evidence, they are not accepted by conventional medicine practitioners. Itrifal Kishneezi showed antioxidant activity by its free radical scavenging and metal chelating activities and also might reduce the free radical generation, quench the radicals already formed and inhibit neuronal and other free radical mediated damages. Further studies are required to determine its mechanism of action and *in vivo* studies.

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