IN VITRO HEMOLYTIC AND ANTIOXIDANT ACTIVITY OF COMMERCIALLY AVAILABLE JOSHANDA SAMPLES

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Abstract

Joshanda is a polyherbal formulation of Unani origin widely used in Pakistan to treat the common cold, bronchitis and inflammation as a substitute for expensive pharmaceutical drugs. Due to its common use, it is important to evaluate the efficacy and toxicity of the active ingredients of joshanda. Hemolytic activity is an important indicator of the relationship between joshanda constituents and biological molecules at the cellular level. Thus, the present study was designed to check the hemolytic and antioxidant activity of different joshanda samples commercially available in Pakistan. All joshanda samples showed significant antioxidant potential in DPPH radical scavenging assay with activity ranging between 92.87 to 81.67% at the composition commonly used. Hemolytic assay showed lysis of human red blood cells ranged between 30.46 to 38.32% at normally consumed concentrations. Phytochemical analysis of the samples indicated the presence of flavonoids, saponins, alkaloids, phenolics, glycosides, terpenoids, and sterols, while quinones were absent in all the samples. The study concluded that the polyherbal formulation is an important source of antioxidants that are responsible for its pharmacological activities. But at the time, hemolytic activities observed demands detailed cytotoxicity testing of these joshanda samples.

Keywords: antioxidant activity, DPPH, hemolysis, joshanda, red blood cells.

Introduction

Medicinal plants are used to discover and develop novel drugs due to the presence of active principles responsible for their medicinal value. These active principles are used in different forms like infusions, syrups, decoctions, creams and infused oils (Sofowora, 1993). There are many different systems to prepare drugs and one of them is the Unani system of the utilization of medicinal plants in drug preparation. Unani system of medicine based on plants and herbs has been used for centuries to treat various diseases, particularly in developing countries. Due to their high cost and side effects, modern allopathic medicines are not preferred by the majority of the population in these countries. Herbal medicines, due to their cheap rates, easy availability and lesser side effects, are widely accepted in these countries (Chan, 1993; Said, 1996; Soomro *et al*., 2011). In herbal medicines, different plant parts or whole plants are used to make medicines, but sometimes different types of plants and herbs are collectively used as a medicine that has a combined effect on disease. These herbal medicines are known as polyherbal medicine (Parasuraman *et al.,* 2014).

Joshanda is also a polyherbal formulation of Unani origin (Greco-Arab). The word joshanda means "prepared by boiling". It is made up of many herbal plants each of which has its own medicinal values. This herbal tea is commonly used to treat inflammation of the mucous membranes of nasal and air passages (Azmi *et al*., 2010), such as conditions of cold, fever, flu and catarrh. Joshanda is widely used in Pakistan for the treatment of bronchitis, common cold, flu and inflammation of the respiratory tract. Joshanda is also reported for antioxidant (Soomro *et al*., 2011), anti-inflammatory (Khan *et al*., 2012), anti-bacterial (Azmi *et al*., 2010), anti-pyretic, anti-leshmatic, antimicrobial, phytotoxic, and cytotoxic activities (Abdullah *et al*., 2014). The medicinal plants mostly used for the preparation of this polyherbal formulation are mentioned in **table 1** (Vohora, 1986).

Table 1: Medicinal plants used for joshanda preparation (Vohora, 1986)

Plant name	Common name	Family	Plant part used
Althea officinalis	Khatami	Malvaceae	Seeds
Cordia latifolia	Sapistan	Boraginaceae	Dried fruit
Glycyrrhiza glabra	Mulethi	Fabaceae	Dried roots
Malva rotundifolia	Khubbazi	Malvaceae	Seeds
Onosma bracteatum	Gaozaban	Boraginaceae	Leaves
Viola odorata	Banafsha	Violaceae	Herb
Zizyphus jujuba	Unaab	Rhamnaceae	Dried fruit

Since most plants have medicinal properties, it is important to evaluate their efficacy and toxicity risks as the toxicity of biologically active molecules is a critical factor in drug design. Hemolytic activity is an important factor to consider in this regard since it gives the basic information on the relationship between phytochemicals and living organisms at the cellular level (Kalita *et al*., 2011). Keeping in view the wide use of joshanda all over the world, the present study was designed to check the hemolytic and antioxidant activity of joshanda. Different samples of commercially used joshanda were selected for the study and were tested for their phytochemical composition and pharmacological activities. Furthermore, literature survey revealed that this is the first report of hemolytic activity of different joshanda samples available in the market according to the best of our knowledge.

Materials and Methods

Collection of joshanda samples

The five different samples of joshanda were purchased from the local market. Three samples were in the raw form and named A, B and C, while two others were in the form of processed powder for instant use named D and E.

Extraction

The samples were prepared according to the given instructions. Samples A, B and C were prepared by adding 5 g of each sample to

150 mL of boiled water in a flask to form a herbal tea-like solution. While samples D and E were prepared by boiling 35 g of each sample in 300 mL of distilled water till the water was reduced to half of the original volume to get the joshanda extract. These joshanda extracts (dilution 1) were used for further analysis and were stored at 4°C. Six different concentrations of each sample were prepared by double dilution method using sample 1 and seven dilutions of each joshanda sample were prepared for analysis (dilutions 1, 2, 3, 4, 5, 6 and 7).

Phytochemical analysis

Each joshanda sample was tested for the presence of selected phytochemicals considered to be important contributors in biological activities of medicinal plants using standard methods as outlined by Harborne (1978).

Alkaloids

a) Dragendorff's test: Alkaloids were detected by adding 4-6 drops of Dragendorff reagent in 1 mL of joshanda sample. Formation of orange-yellow precipitates confirmed alkaloids in solution.

b) Tannic acid test: Each joshanda sample (1 mL) was mixed with solution of tannic acid (1 mL). Buff-colored precipitates were indicative of alkaloids in solution.

Glycosides

a) One mL of each joshanda sample was added to 10% NaOH solution. Yellow coloration indicated glycosides in the sample.

c) One mL of respective joshanda sample was added in 1 mL of KOH solution (10%). The formation of brick red precipitates confirmed glycosides.

Flavonoids

a) Alkaline Reagent Test: Each joshanda sample was treated with NaOH solution (10%). The solution turned dark yellow in color that disappeared after addition of dil. acid indicating flavonoids in the solution.

b) 3 mL of each joshanda sample and 4 mL of 1% KOH solution were mixed in a test tube. The dark yellow color of the solution indicated flavonoids.

c) Added a few drops of aluminum chloride to 1 mL of each sample. Flavonoids were confirmed by yellow coloration of mixture.

d) Added 2-3 drop of conc. HCl to 1 mL of the individual joshanda samples and mixed. HCl produced red color in the solution indicating the presence of flavonoids.

Saponins

Froth Test: Added 2 mL of water to 2 mL of sample and shaken vigorously for 15 min. The formation of a foam layer of 1 cm confirmed saponins.

Phenols

a) Ferric Chloride Test: Mixed 1 mL of 5% $FeCl_3$ solution with 2 mL of sample. A deep blue color confirmed the presence of phenols.

b) Nitric acid test: Each joshanda sample was mixed with dilute HNO_3. A reddish to yellow coloration indicated phenols.

Tannins

a) Precipitate test: 2 mL of each sample was boiled with 1 mL of HCl (1% aqueous).

The formation of red precipitates confirmed tannins.

b) Ferric chloride test: Added 3 drops of 5% $FeCl_3$ to 1 mL of the test sample. A blue or greenish-black coloration which changed to olive green after addition of $FeCl_3$ indicated tannins.

c) Potassium dichromate test: Mixed 5 mL of each test sample with 2.5 mL of 10% potassium dichromate solution. The formation of yellow precipitates indicated tannins in solution.

Steroids

Added 5 drops of conc. H_2SO_4 to 1 mL of the test sample, blue-green color confirmed the presence of steroids.

Terpenoids

a) Added 5 drops of conc. H_2SO_4 to 1 mL of each sample solution. A blue-green coloration indicated the presence of terpenoids.

b) Mixed 5 mL of joshanda sample, 3 mL of conc. H_2SO_4 and 2 mL of chloroform. Formation of a reddish-brown interface confirmed terpenoids.

Sterols

Mixed 5 mL of sample and 2 mL of chloroform followed by addition of 3 mL of conc. H_2SO_4 carefully along the side of test tube to form a layer. A reddish-brown color at the fused surface indicated terpenoids.

Quinones

1 mL of conc. HCl and 1 mL of sample solution were mixed together. The appearance of yellow precipitates confirmed the presence of quinones.

Estimation of phenolic content (TPC)

TPC of all the joshanda samples were determined by the spectrophotometric method (Cliffe *et al*., 1994). About 20 µL of the test sample, 158 µL of distilled water and 100 µL of Folin-Ciocalteu's reagent were mixed and shaken well. The mixture was kept at room temperature for 5 min followed by the addition of 300 µL of 25% Na_2CO_3. The mixture was incubated for 30 min at 40°C temperature and absorbance was determined at 750 nm. Phenolic content was estimated by the calibration curve prepared by using gallic acid as a standard. The total phenols were expressed as mg gallic acid equivalent (GAE)/g dry extract (dE).

Estimation of total flavonoid content (TFC)

For the determination of total flavonoid content in the joshanda samples under consideration, the aluminum chloride colorimetric method was used. For analysis, 250 µL of each joshanda sample, 400 µL of distilled water and 90 µL of $NaNO_2$ (5%) solution were mixed together and left for 5 min at room temperature. Finally, 600 µL of 1M NaOH and 180 µL of 10% $AlCl_3$ were added to the above mixture and the volume was raised to 3 mL with distilled water. The absorbance of this reaction mixture was recorded at 510 nm (Dewanto *et al*., 2002). For estimation of TFC, the calibration curve of quercetin was used and the results were expressed as mg quercetin equivalent (QE)/ g dE.

Estimation of tannin content

Tannin content was estimated by following the protocol of Ram and Mehrotra

(1993). About 0.1 mL joshanda sample, 0.5 mL Folin-Denis reagent, 1 mL 35% Na_2CO_3 and 7.5 mL water were mixed and allowed to stand for 30 min. Absorbance of this mixture was read at 725 nm using UV-Visible spectrophotometer. Tannin content was calculated using a tannic acid calibration curve and was expressed as mg Tannic acid equivalent (TAE)/g dE.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant activity of each joshanda sample was determined using DPPH radical scavenging assay (Hatano *et al.*, 1998). To perform the assay, 5 mL of 0.05 mM DPPH was mixed with 50 µL of different dilutions (1, 2, 3, 4, 5, 6, 7) of each joshanda sample and incubated for 30 min at room temperature. The absorbance of each reaction mixture was recorded at 517 nm using methanol as a blank. The % inhibition of DPPH radical (I %) was calculated using the following formula:

DPPH scavenging activity (%) =

$$\frac{[Abs_{control} - Abs_{extract}]}{(Abs_{cotrol})} \times 100$$

Hemolytic activity by HRBC method

In vitro hemolytic activity was carried out by following the method of Yang *et al*. (2005).

Preparation of cell suspension

Fresh human blood (10 mL) from a healthy individual was collected in heparinized tubes and was stored at 4°C. Prior to use blood sample was centrifuged for 15 min at 3000 rpm. The supernatant containing plasma and leucocytes was carefully removed without disturbing the red blood cells (RBCs). RBCs were washed 3-4 times with fresh isosaline (0.85% NaCl w/v, pH 7.2) to remove the plasma. Finally, 10% v/v suspension of RBCs was prepared in isosaline.

Preparation of joshanda samples

For joshanda samples, A, B and C, 1 g of each sample was dissolved in 30.667 mL of Phosphate buffer saline (pH 7.4) individually. Whereas, for samples E and D, 1 g of each sample was dissolved in 8.57 mL of saline buffer. All the samples were further diluted in the same saline buffer.

Hemolytic activity assay

To carry out the hemolysis activity assay of RBC suspension was mixed with different dilutions of each joshanda sample (0.5 mL) and incubated for 30 min in an incubator at 37°C. The reaction mixture was centrifuged for 10 min at 1500 rpm. The content of free hemoglobin released during hemolysis was quntified by measuring the absorbance at 540 nm. Distilled water and phosphate buffer saline were used as positive and negative control giving maximal and minimal hemolysis respectively. The degree of hemolysis was calculated using the following formula:

$$\text{Hemolysis (\%)}: \frac{At-An}{Ac-An}\ x\ 100$$

Where

A_t represents absorbance of joshanda sample.

A_n represents absorbance of the negative control
A_c represents absorbance of the positive control

Results and discussion

Polyherbal formulations, also called herb-herb combination, are used worldwide due to their medicinal and therapeutic values. In case of individual plants the active phytochemicals are not adequate enough to attain the required therapeutic effects. In polyherbal formulations multiple herbs combined in a specific ratio produce enhanced therapeutic effect with decreased toxicity that might be due to synergism. Polyherbal formulations have expressed high effectiveness in several diseases even at high doses (Kerole *et al.,* 2019). Joshanda, the most common polyherbal formulation used in Asian countries was under taken for the present study to check the antioxidant and hemolytic effects of different joshanda samples commonly used in Pakistan. The samples were also subjected to phytochemical estimations.

Qualitative analysis of phytochemicals

Phytochemical screening was done with all these five commercially available joshanda samples. Samples A, B, C, D and E contained flavonoids, phenolics, saponins, tannins, glycosides, alkaloids, sterols, quinones, terpenoids and steroids. The results are summarized in **table 2**.

Table 2 Qualitative phytochemical analysis of joshanda samples

Phytochemicals	Sample A	Sample B	Sample C	Sample D	Sample E
Flavonoids	+	+	+	+	+
Saponins	+	+	+	+	+
Alkaloids	+	+	+	+	+
Phenolics	+	+	+	+	+
Glycosides	+	+	+	+	+
Terpenoids	+	+	+	+	+
Quinones	-	-	-	-	-
Sterols	+	+	+	+	+

+ = presence; - = absence

Total phenolic, flavonoid and tannin contents

The results of total phenolic, flavonoid and tannin contents are summarized in **figure 1**. High phenolic content was observed in samples A, B and C while in samples D and E the contents were significantly low. Phenolics are secondary metabolites and play important role in growth, pigmentation and reproduction. They are reported for antitumor, anti-carcinogenic, anti-allergic and anti-inflammatory activity. Antioxidant and vasodilatory activities are reported to be strongly related with high phenolic content (Burns *et al*., 2000; Kalaivani and Mathew, 2009). Thus, high phenolic content in samples A, B and C indicates positive health effects of these samples being a source of phenolic compounds widely known for their health benefits.

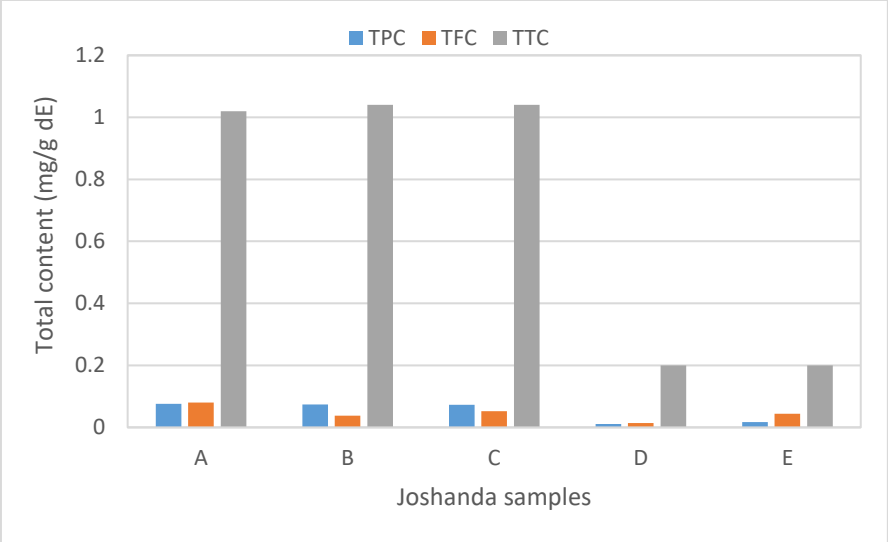

Figure 1 Total phenolic, flavonoid and tannin contents in different joshanda samples

A difference in flavonoid contents of all the joshanda samples was observed. The highest flavonoid content was determined in sample A followed by samples C and B while the flavonoid content in samples D and E was significantly lower. Flavonoids are secondary metabolites in plants with reported antioxidant, antimicrobial, anticancer, anti-inflammatory and anti-allergic activities (Janicijevic *et al.*, 2007).

Determination of total tannin content also showed the same pattern as observed for phenolics and flavonoids. Tannin content was higher in samples A, B and C and significantly low in samples D and E. In general, all the joshanda samples had high tannin contents than phenolic and flavonoids.

DPPH radical scavenging activity assay

Significant antioxidant activity of all the joshanda samples was observed at all the tested dilutions. At composition commonly used for treatment highest activity was observed for sample A (92.87% inhibition) and the least was for sample B (81.67% inhibition) (**Figure 2**). Even at the lowest dilution used the DPPH scavenging potential ranged from 56.78% for sample E to 78.48% for sample C. The antioxidant activity was dose-dependent as

it showed a decrease in activity with a decrease in concentration. Free radicals are produced in living cells during normal metabolic reactions, but if accumulated in the body due to overproduction, these can lead to mortal diseases including hepatic disorders, arthritis and cancer (Sarmah and Baishya, 2014). Phenolics, flavonoids, carotenoids, and tannins present in medicinal plants act as natural antioxidants and have an important role in suppressing the damaging effects of these radicals (Mondal *et al.*, 2005). Synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene are frequently used during oxidative stress but are reported to have carcinogenic liver destructive effects (Politeo *et al.*, 2007). Therefore, plant-based natural antioxidants with lesser side effects are preferred over synthetic antioxidants (Muhammad *et al.*, 2012).

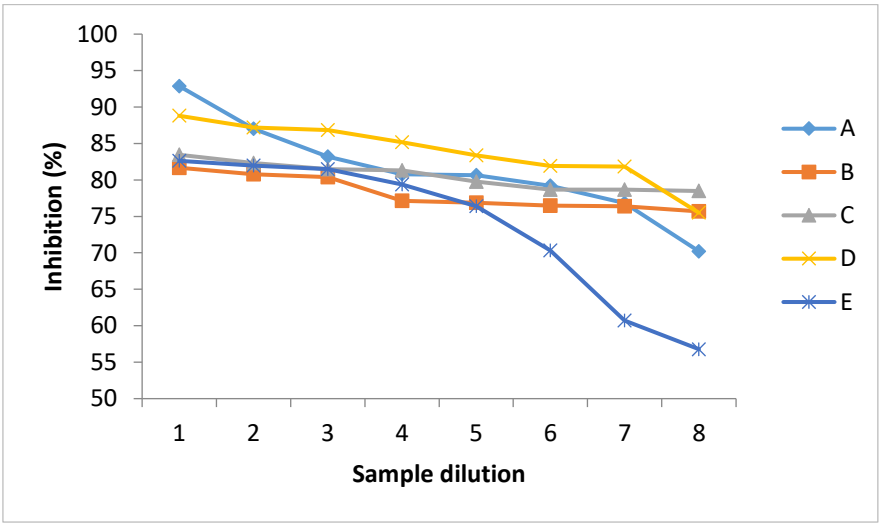

Figure 2 DPPH radical scavenging activity of different joshanda samples

Joshanda, as a natural polyherbal decoction, can be an excellent source of treating these free radicals with a wide range of antioxidants derived from different medicinal plants that might react better in a synergistic manner targeting different radicals at a time (Kerole *et al.*, 2019). All the joshanda samples produced excellent results in DPPH scavenging assay retaining the potential even after dilution that shows the antioxidant power of the constituents contributed by different plants used in the formulation.

Hemolytic activity assay

Hemolysis is the degradation of red blood cells or erythrocytes. There are various nonspecific mechanisms that can cause hemolysis. Hemolytic activity is caused due to the formation of pores in cell membranes which can change the membrane permeability or it can be due to change in the activities of calcium-magnesium and sodium-potassium ATPase (Sovadinova, 2011). Plant phenols are also reported to cause oxidation of hemoglobin which can cause hemolysis by changing hemoglobin to methemoglobin (Bukowska and Kowalska, 2004). It is important to measure hemolytic activity because it indicates the

presence of cytotoxicity (Greco *et al.*, 2020). The *in vitro* hemolysis test is also used to determine the toxicity of different plants (Gandhi and Cherian, 2000). In drug formulations, determination of hemolytic potential is an important parameter to check whether a bioactive drug is safe to be used in medical applications or not (Kalaivani *et al*., 2010). In the hemolysis assay, all the samples showed lysis of human RBCs ranging from 30.46 to 38.32% at concentrations normally used. The extent of hemolysis was related to the concentration so that with a decrease in concentration the rate of hemolysis decreased (**Figure 3**). However, in diluted form, all the samples (B to E) were non-toxic with hemolytic activity as low as 12.58 to 18.2% while for sample A the extent of hemolysis at the lowest concentration was 34.94%. At the same dilution, the antioxidant activity of these samples ranged from 76.38 to 83.39% indicating that these samples can be safely consumed in diluted form while still retaining their antioxidant potential.

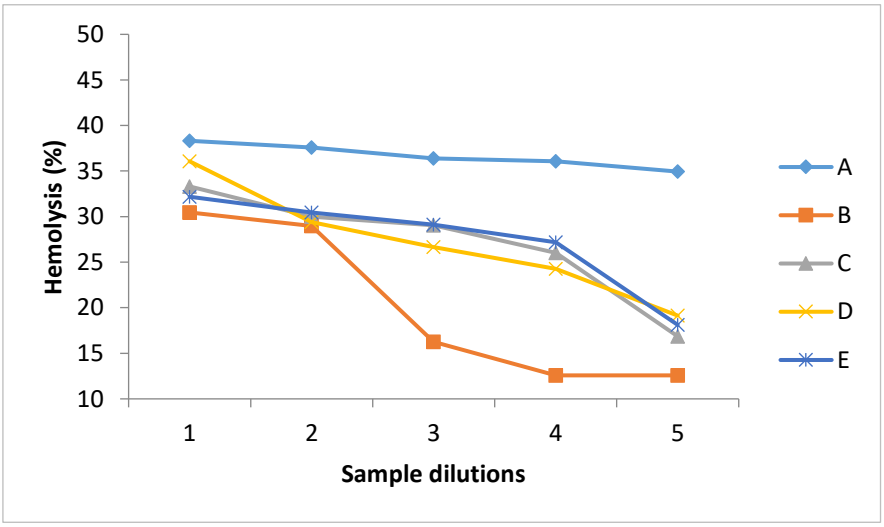

Figure 3 Hemolytic activity of joshanda samples at different dilutions

Conclusion

All the joshanda samples showed a strong antioxidant activity which may be due to the presence of biologically active compounds including phenols, flavonoids, tannins *etc*. All the samples in the diluted form are safe to be used with very low hemolytic activity and retaining high antioxidant activity. Further analysis of these samples using other *in vitro* and *in vivo* methods is suggested.

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