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Bioactivities of Cholistani harmal (*Peganum harmala*) with exploration of bioactive phytochemicals

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Abstract

Most plant secondary metabolites serving as bioactive compounds, help to promote human health. The current project was designed to investigate medicinal attributes of *P. harmala* collected from Cholistan desert. Qualitative analysis exhibited high concentrations of alkaloids and flavonoids. Leaves were extracted with different solvents like water, ethanol, acetone, chloroform and *n*-hexane; all these extracts were subjected to analysis of bioactivities; and their synergistic effect was also investigated. Among various extracts, *n*-hexane extract and synergistic mixture exhibited quite high values of phenolic contents and other bioactivities. Finally, the bioactive compounds were characterized by RP-HPLC analysis. Synergistic mixture of these extracts exhibited highest phenolic contents (311.56±3.01 mg GAE/g), followed by *n*-hexane extract (368.45±3.12 mg GAE/g). *n*-Hexane extract and synergistic mixture exhibited highest DPPH free radical scavenging (89.25±0.88% for *n*-hexane and 90.25±0.92% for synergistic mixture), inhibition of linoleic acid peroxidation (72.23±0.45% for *n*-hexane and 80.12±0.68% for synergistic mixture) and β-carotene bleaching potential (78.35±0.65% for *n*-hexane and 82.42±0.48 % for synergistic mixture). Moreover, antibacterial activity was evaluated by disc diffusion and cytotoxicity by haemolytic assay. Synergistic mixture (27.2±0.2*mm* against *B. fastidiosus*; 22.1±0.1*mm* against *E. coli*) and *n*-hexane extract showed highest antibacterial (25.1±0.3*mm* against *B. fastidiosus*; 20.6±0.2*mm* against *E. coli*). Lowest cytotoxicity was obtained for *n*-hexane extract (3.25±0.02%) and synergistic mixture (2.65±0.02%) which recommended their usage in medicinal formulations. HPLC analysis of synergistic mixture of all *P. harmala* extracts exhibited the presence of seventeen bioactive compounds. Thus, *P. harmala* leaves can be very effectively used for treatment of numerous diseases.

Keywords: Cholistan, *P. harmala*, Antioxidant, Antibacterial, Cytotoxicity, HPLC.

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1. Introduction

ROS such as hydroxyl radical (HO•), superoxide anion radicals (•O_2^-), peroxyl (R•O_2), hydroperoxyl (H•O_2) and singlet oxygen ($^1O_2$) are commonly produced during respiratory metabolism. These reactive species exhibit damaging effect in living organisms; may initiate degenerative chain reactions leading to damage of different biochemicals such as lipids, proteins and DNA. Such oxidative dissociation of important biomolecules may lead to many chronic dysfunctions such as variations in signal transduction, gene expressions and mutagen [1-3].

Cancer is one of the major degenerative diseases which is responsible for high mortality rates in poor countries. It has been found by modern researchers that one of the major causes of cancer and other degenerative diseases includes reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive chemicals are produced in living organisms as by-products of oxidative metabolism. These unwanted side products are mainly produced during respiratory metabolic functions, also by exogenous environmental factors such as exposure to ionizing radiations, heat, smoke, heavy metals, and pathogens [4].

It has been found that many medicinal phytochemicals are synthesized in plant tissues as secondary metabolites and are the main factors which are responsible for medicinal values of such plants. More than 300 medicinal phytochemicals have been characterized and

reported by modern researchers. These include phenolic acids, flavonoids, tannins, carotenoids, terpenoids, saponins and sterols. These natural pharmaceutical compounds are of different chemical structures and their concentration is different in different species. Moreover, biosynthetic pathways for these natural products are also different from each other [5-6].

Antioxidants, not only, protect the living organisms against many fatal diseases but also increase the shelf life of food products by preventing rancidity, adulteration and discoloration executed by auto-oxidation. Auto-oxidation is a spontaneous process carried out in food items in aerobic atmosphere under the influence of heat and light. Thus, antioxidant compounds are very crucial to increase the stability of foods thereby prolonging the expiry periods for these preserved food items [7-9]. Aromatic plants which produce large amounts phytochemicals, are the basis of modern food and pharmaceutical industries. Allopathic medicines based on synthetic drugs were being used for the treatment of many chronic diseases since early ages, but many of these medicines have been proved to be carcinogenic. That is why regional medicinal plants have attracted the attention of modern researchers. Modern pharmacists are keen to manufacture natural herbal based medicines in order to avoid the chances of cancer and other chronic diseases by synthetic medicines [10-12].

Peganum harmala, also known as harmal or esfand, is a flowering plant located in Asia, Middle East and North Africa. Harmal flowers are white in color. Harmal can be very effectively applied as brain tonic and for curing insecticidal and viral diseases. Fire fumes of this plant can be used as mosquito repellant. Asthma and many respiratory diseases can be effectively treated by a mixture of grind powder of harmal seeds and honey. Joint pains can be very effectively relieved by applying its leaf decoction [13-14].

The current study was aimed to explore antioxidant, antibacterial and cytotoxic attributes of Cholistani *P. harmala* extracts. The compounds responsible for these bioactivities were chemically characterized by HPLC analysis.

2. Materials and methods

2.1. Collection and extraction of plant

Fresh sample of *Peganum harmala* (family: *Zygophyllaceae*) was collected from Cholistan desert, Bahawalpur, Pakistan. Leaves were separated manually, washed, shade dried and ground to fine powder which was extracted with deionized water, ethanol, acetone, chloroform and *n*-hexane. All extracts were concentrated under reduced pressure and stored at 4ºC until further analysis [15-16].

2.2. Qualitative analysis

2.2.1. Flavonoids

Small portion of harmal leaf powder was heated with ethyl acetate over a steam bath, filter and filtrate was shaken with dilute ammonia solution. Yellow coloration indicated the presence of flavonoids [17-18].

2.2.2. Alkaloids

A little fraction of leaf powder was defatted and extracted with dil. HCl on a boiling water bath, centrifuged for 10 min at 3000 rpm, filter and filtrate was treated with few drops of Mayer's reagent. White to yellowish precipitate represented alkaloids [18-19].

2.2.3. Saponins

Harmal leaf powder was shaken with water in a test tube, then warmed in a water bath. Persistent of froth indicated the presence of saponins [17-18].

2.2.4. Tannins

Harmal leaf powder was stirred with 10mL of distilled water, then filtered and ferric chloride reagent was added to the filtrate, a blue-black precipitate was an indication of tannins [19].

2.2.5. Terpenoids

Leaf powder (0.5g) was extracted with 5mL methanol. 2mL of the extract was treated with 1 mL of 2,4-dinitrophenyl hydrazine in the presence of 2M HCl. A yellow-orange coloration indicated the terpenoids [17-19].

2.2.6. Steroids

Leaf powder (0.5g) was extracted with methanol which was treated with 0.5mL of acetic acid anhydride in ice bath, then mixed with chloroform and then concentrated sulphuric acid. Appearance of reddish-brown ring indicated the presence of steroids [18-19].

2.2.7. Glycosides

Leaf powder (0.5g) was extracted with methanol which was treated with dil. HCl; and heated in a boiling water bath for 30 min. 5mL of Fehling's solution was added and the mixture was boiled. Brick red precipitate represented glycosides [17-19].

2.2.8. Anthraquinones

Leaf powder (0.5g) was extracted with benzene, which was treated with 10 % ammonia solution. Appearance of violet coloration indicated anthraquinones [17-18].

2.3. Total Phenolic Contents

Total phenolic contents of all extracts were measured using Folin–Ciocalteu reagent according to a reported method of Kalpoutzakis et al. [20] with minor changes. Each plant extract was mixed with sodium carbonate (2mL, 7.5%) and Folin–Ciocalteu reagent (2.5mL, 10%). The mixture was incubated for 30min at room temperature, then absorbance was recorded at 765nm using an UV-VIS spectrophotometer (IRMECO, Geesthacht/Germany, Model 5000). Total phenolic contents were quantified based on standard curve for gallic acid (2-200 ppm) and expressed as mg gallic acid equivalents (GAE)/g of dry extract [21].

2.4. Antioxidant Activities

2.4.1. Free radical scavenging activity

The antiradical activity of each extract was evaluated following a spectrophotometric method of Wanjala et al. [22] Each extract (3mL) at varying concentrations (1-5000µg/mL) were added to methanolic solution of DPPH (1mL, 0.1mM) and allowed to stand in the dark for 30min at room temperature. Then the absorbance of solution was measured at 517nm using UV- Visible

Spectrophotometer (IRMECO 5000) and antioxidant activity was calculated as:

$$\% \text{ Reduction(DPPH)} = \left[1 - \frac{\text{absorbance of the sample}}{\text{absorbance of control}}\right] \times 100$$

The concentration of extract required to neutralize 50% radicals, expressed as IC_{50} (µg/mL), was also calculated [23].

2.4.2. β-Carotene assay

Antioxidant potential was also examined by β-carotene bleaching assay following Liu et al. [24] method. About 1mL of β-carotene solution (0.2 mg/mL chloroform) was treated with 0.02mL of linoleic acid and 0.2 mL of 100% Tween20. The mixture was evaporated at 40°C for 10 minutes using a rotary evaporator to remove chloroform, then diluted with 100mL distilled H_2O followed by vigorous agitation to form an emulsion. Aliquots of this emulsion were transferred into different test tubes containing 0.2mL of different samples of extracts having varying concentrations (1µg/mL-2.5mg/mL). The tubes were shaken and incubated at 50°C in a water bath, then absorbance was measured at 470 nm.

Antioxidant activity was calculated using the following equation:

$$\text{Antioxidant activity} = 1 - \left(\frac{A_o - A_t}{A_0^o - A_t^o}\right) \times 100$$

where A_0 and A_0^o were the absorbance values measured at the initial time of incubation for the samples and controls while A_t and A_t^o were the absorbance values for samples and controls, respectively at 120 minutes. The EC_{50} values were also calculated. Gallic acid and BHT were used as positive standards [25].

2.4.3. Lipid peroxidation inhibition

The antioxidant activity of *P. harmala* leaves extracts were also evaluated in terms of inhibition to lipid peroxidation following an assay reported by Bektašević et al. [26] Ethanolic solution of each extract was mixed with linoleic acid (0.5mL, 2.51%) solution and 1 mL of sodium phosphate buffer (50mM, pH 7); then incubated for autoxidation at 40°C. The degree of oxidation was evaluated by taking out aliquots every 12 h and adding ammonium thiocyanate solution, $FeCl_2$ solution (0.1 mL, 20 mM in 3.5% HCl) and 9.7 mL of 75% ethanol. The mixture was allowed to stand for 3 min and peroxide value was assessed by taking absorbance at 500nm using UV- Visible Spectrophotometer (IRMECO 5000). The antioxidant activity was measured as:

$$\% \text{ Inhibition} = \left[1 - \frac{\text{As at 120 h} - \text{As at 0 h}}{\text{Ac at 120 h} - \text{Ac at 0 h}}\right] \times 100$$

Where A_s and A_c is absorbance of sample and negative control, respectively. Percentage inhibition and IC_{50} values of all extracts were compared with BHT and gallic acid used as positive standards [27].

2.5. Antibacterial activity

Antibacterial activity of all solvent extracts of *P. harmala* leaves was evaluated by disc diffusion assay using three bacterial strains, kindly provided by Department of Microbiology, University of Agriculture, Faisalabad. The media was poured into petri dishes under aseptic conditions and spread with sterilized glass rod. All three bacterial strains were cultured in Muller Hinton broth overnight and diluted to get 10^8 CFU/mL of each bacterial suspension. 0.2mL of broth culture of the bacteria was aseptically introduced and evenly spread using bent sterile glass rod on the surface of gelled sterile Muller-Hinton agar plates. Paper discs impregnated with all extracts, Tetracycline and Gentamicin (as positive controls) and DMSO (as negative control) were placed on seeded agar plates. The plates were incubated at 37°C for 24 hours. After 24 hours antibacterial activities were measured as diameter of the zones of inhibition and compared with those of positive and negative controls [28-29].

2.5.1. Minimum inhibitory concentration (MIC)

The minimal inhibitory concentrations (MIC) of different *P. harmala* extracts were evaluated using broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). The tests were performed in 96 well-plates by two-fold serial dilution method to achieve a decreasing concentration range of 400µg/mL – 3.125µg/mL. 50 µL each extract in DMSO was added into the wells of Mueller Hinton agar plates that were already seeded with standardized inocula (10^8 CFU/mL) of the bacterial suspensions. The plates were incubated at 37°C for 24 hours. The least concentration of the samples with no visible growth was taken as the MIC [30-31].

2.6. Cytotoxicity

Cytotoxicity was evaluated by haemolytic potential applying the well documented Yasir et al. [32] method. Fresh heparinised human blood (3 mL) was softly mixed and dispensed into a sterile falcon tube (15 mL) and centrifuged at 850 *g*. The sticky pellet was separated from the supernatant and washed with phosphate buffer saline (PBS 2% *v*/*v*) solution three times, to maintain the pH to ~7.4. All types of extracts, at varying concentrations (0.5, 5.0 and 50 mg/mL), were mixed separately with diluted blood cell suspension (180 µL) in separate microfuge tubes, each tube was incubated at 37 °C for 35 min with continuous agitation (180 rpm). After incubation, supernatant (100 µL) from each tube was mixed with 900 µL cooled, sterile PBS in separate microfuge tubes and retained on ice (5 min). Absorbance was then measured at 576 nm, using Triton-X 100 (0.1%) as the positive control; then haemolytic activity was quantified using the formula:

$$\% \text{ Haemolysis} = (Hb_{abs} / Hb_{abs\ 100\%}) \times 100$$

where Hb_{abs} is the absorbance of sample and $Hb_{abs100\%}$ is the absorbance of positive control [33-34].

2.7. HPLC analysis

The phenolic compounds present in different *P. harmala* extracts were chemically characterized by RP-HPLC according to a modified method of Khalid et al. [35]. Extract samples were filtered through a 0.45 µm PTFE syringe tip filter using a 20µL sample loop. A RP-HPLC

system equipped with a Shimadzu PDA detector, reverse phase (RP C-18) column (Interstsil ODS-5 µm x 4.6 mm x 250 mm), a quaternary pump, and an online vacuum degasser, was used for the separation, identification and quantification of phenolic compounds in harmal leaf extracts. The flow rate was set at 0.8 mL/min at 37°C. To perform this study a gradient of two mobile phases was used. Solvent A was prepared by water:acetonitrile:trifluoroacetic acid at 90:10:0.1% (v/v) and solvent B at 10:90:0.06% (v/v). Elution was performed using following gradient: 0-10 min: 10-35% B, 10-20 min: 35-42 % B and 20-30min: 42-100% B. After each run, the system was reconditioned for 10 min before analysis of the next sample.

The detection was carried out at 280 nm for hydroxybenzoic acids and flavanols, at 320 nm for hydroxycinnamic acids and 360 nm for flavonols [35-36].

2.8. Statistical analysis

Experimental results obtained in this study were presented as mean ± S.D. of three parallel determinations. These means were analyzed by two-way analysis of variance (ANOVA) using Minitab 2000 Version 13.2 statistical software (Minitab Inc. Pennysalvania, USA). Differences among values for different types of extracts were considered statistically significant at the 5% confidence level [37]. IC_{50} values for DPPH free radical scavenging, β-carotene assay and inhibition of lipid peroxidation were calculated using linear regression analysis [38].

3. Results and discussion

Qualitative analysis is very handful tool to detect the presence of different phytochemicals in plant extracts. In this project, qualitative analysis was carried out on fresh *P. harmala* leaf powder. Results in Table 1 demonstrated that flavonoids and alkaloids were present in very high concentration as reported by Soleimani et al. [39], tannins and terpenoids were found in medium concentration, steroids and saponins were detected in low amount while glycosides and anthraquinones were not found in *P. harmala* leaf.

3.1. Extract yield

Extraction is one of the most important phenomena for liberating various bioactive phytochemicals from plant tissues. These phytochemicals may act as antioxidant, anti-cancerous and antibacterial agents [40-41]. The comparison of extract yield for different solvents has been presented in figure 1. Results demonstrated that aqueous extracts depicted lowest extract yield (56.24±0.84%) while *n*-hexane presented highest (82.4±1.34%) extract yield. The variation in extract yield was due to variation in polarities of extracting solvents. It was found that as the polarity of solvent decreases, extraction capability increases [42].

3.2. Total phenolic contents

Phenolic compounds constitute one of the largest classes of natural antioxidants. Chemically, they range from a simple phenol to a large size complex polymer molecule. They are found in all parts of vegetative plants including flowers, fruits, vegetables, seeds, cereals and grains. These bioactive compounds are actually plant secondary metabolites synthesized in plants from pentose phosphate and phenyl propanoid shikimate pathways. The qualitative and quantitative variations among different plants are result of different factors like soil composition, maturity stage, growing conditions and post-harvest conditions [43-44].

Figure 1 summarizes the comparison of phenolic contents of different types of extracts of *P. harmala* leaves. It was found that *n*-hexane extract exhibited highest phenolic contents (368.45±3.12 mg GAE/g dry extract) quite comparable to aqueous extracts of *P. harmala* seeds (348.82±2.79 mg GAE/g dry weight) as found by Abolhasani et al. [45]; while aqueous extract of *P. harmala* leaf showed the presence of minimum concentration of phenolic compounds (190.22±0.84 mg GAE/g dry extract), however, still greater than methanolic extract (61.55±0.84 mg GAE/g dry extract) as reported by Mazandarani et al. [46] and hydro alcoholic extract (61.5±0.8 mg GAE/g dry extract) of *P. harmala* seeds as shown by Davoodi et al. [47]. This might be due the fact that many of *P. harmala* phenolic compounds are hydrophobic in nature and hence mainly extracted by *n*-hexane.

3.3. Free radical scavenging activity

Oxidation mostly leads to production of free radicals which does rest of the damage in living organisms. In this assay, the ability of plant extracts to capture free radicals was evaluated. 1,1-Diphenyl picryl hydrazyl is violet colored free radical. In this process, this free radical is neutralized by an antioxidant (plant extract) to a yellow colored non-radical molecule i.e. diphenyl picryl hydrazine. Such inhibition of free radical is achieved either by accepting an electron from free radical or donating a proton to radical by antioxidant species present in plant extract [48-49]. Table 2 demonstrated the results for antioxidant activities of different *P. harmala* leaf extracts. It was found that *n*-hexane extracts exhibited highest free radical scavenging (89.25±0.88%) amongst all solvents used which was quite similar to aqueous extracts of *P. harmala* seeds (90%) as determined by Abolhasani et al. [46], thus showing that *n*-hexane has very suitable polarity to extract maximum number of bioactive compounds from *P. harmala* leaf. Aqueous extract depicted least DPPH scavenging activity (67.25±0.72%), which is comparable to methanolic extract of *A. arvensis* (68.3%) as depicted by Barros et al. [50]; showing that only few bioactive compounds of *P. harmala* are hydrophilic. Moreover, synergistic mixture of all extracts exhibited highest (90.25±0.92%) free radical scavenging potential which was very much comparable to standard antioxidants such as ascorbic acid (88.36±0.75%) and BHT (90.52±0.91%). The concentration of plant extracts required to scavenge 50% of free radicals i.e. IC_{50} values were also evaluated. *n*-Hexane extract (11.25±0.36 µg/mL) and synergistic mixture (9.36±0.24 µg/mL) showed lowest IC_{50} values quite similar to methanol extract of *P. harmala* aerial parts (IC_{50}=11.98µg/mL) as evaluated by Souri et al. [51]; exhibiting their highest free radical scavenging activity which was also comparable to reference standards i.e. ascorbic acid (10.22±0.18 µg/mL) and BHT (9.28±0.18 µg/mL).

3.4. Lipid peroxidation inhibition

There is enhanced production of reactive oxygen species (ROS) under the influence of light and other

oxidative environmental factors. ROS including hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^{1}O_2$), super oxide anion ($\dot{O}_2$) and hydrogen peroxide (H_2O_2) trigger chain reaction leading to many fatal diseases in living organisms. These ROS also cause spoilage of food by carrying out lipid peroxidation. In order to reduce the spoilage of food stuffs, the food products must be fortified with antioxidants obtained from natural resources [52-53]. The results of this essay have also been presented in table 2 which depicts the %age inhibition and IC_{50} (concentration of plant extracts required to inhibit 50% of linoleic acid peroxidation) of various extracts of *P. harmala* leaf. Among different types of extracts, again *n*-hexane exhibited highest percentage inhibition (72.23±0.45%) to linoleic acid peroxidation which was very close to that of methanol extract of *P. harmala* leaf (75.9 ± 0.3 %) as examined by Hayet et al. [54]. Synergistic mixture of all extracts depicted maximum percentage of linoleic acid peroxidation inhibition (80.12±0.68%) which was quite comparable to standard antioxidants i.e. gallic acid (83.22±0.69%) and BHT (84.52±0.78%). Moreover IC_{50} values were found in line with % inhibition values: *n*-Hexane (502.20±4.58) and synergistic mixture (423.26±4.98) showed quite closer values to gallic acid (402.32±5.22) and BHT (392.58±4.22).

3.5 β-Carotene bleaching assay

The environmental factors such as light, ionizing radiations, smoke, heat and pollution lead to oxidative stress in living organisms. Under such extensive oxidative circumstances, the electron transport chain of aerobic organisms become overload and result in prediction of large number of free radicals and ROS. These highly reactive molecules initiate chain reactions which ultimately lead to a number of degenerative diseases like aging, cancer, cardiovascular and neurodegenerative diseases [55]. This assay evaluates the antioxidant activity of plants extract by measuring its ability to inhibit bleaching of β-carotene by linoleate free radical. In this assay, environmental oxidative factors generate a free radical from linoleic acid by abstracting one of proton from diallylic methylene group. The highly unsaturated molecule of β-carotene is then attacked by linoleic acid free radical and other free radicals; thereby orange color of β-carotene begins to fade. However in the presence of a plant extract having strong antioxidants, the orange color of β-carotene is retained for larger period. Thus, in this assay, the antioxidant potential of a plant extract is evaluated in terms of its ability to hinder the bleaching of β-carotene orange color by linoleate and other free radicals [25-56]. Results demonstrated (Table 2) that *n*-hexane extract exhibited highest (78.35±.65%) β-carotene bleaching activity quite comparable to hydro alcoholic extract (78 %) of *P. harmala* seeds as reported by Abolhasani et al. [45]. Synergistic mixture also showed quite high (82.42±0.48%) antioxidant potential of β-carotene bleaching assay which was quite comparable to those of gallic acid (82.22±0.66) and BHT (81.98±052%). IC_{50} values of *n*-hexane extract (218.45±3.02 µg/mL) and synergistic mixture (196.32±2.45 µg/mL) were also in line with those of gallic acid (195.42±2.88 µg/mL) and BHT (202.33±1.98 µg/mL) but lesser than dichloromethane extract (313.18±6.25 µg/mL) of *A. biennis* [57].

3.6. Antibacterial activity

Plants are enriched by various phytochemicals that exhibit potent pharmacological properties. These plants secondary metabolites show antibacterial potential through different mechanisms. Some of them bind through hydrogen bonding or nonspecific interactions with crucial enzymes of metabolic process. While some may trap important metabolites of living processes of microbes thus paralyzing their lives [58-59]. Results of antibacterial activity have been tabulated in Table 3. Antibacterial activity of different extracts against three bacterial strains i.e. *B. fastidiosus*, *E.coli* and *S. typhi* was evaluated in terms of diameters of inhibition zones in disc diffusion assay. It was found that *n*-hexane exhibited highest antibacterial potential against all tested strains (25.1±0.3*mm* against *B. fastidiosus*; 20.6±0.2*mm* against *E. coli*). Synergistic mixture of all extracts showed even higher antibacterial activity (27.2±0.2*mm* against *B. fastidiosus*; 22.1±0.1*mm* against *E.coli*). Antibacterial potential of *n*-hexane extract and synergistic mixture of all extracts were quite comparable to standard antibiotics i.e. tetracycline (27.5±0.2*mm* against *B. fastidiosus*; 22.3±0.2*mm* against *E.coli*) and gentamicine (26.5±0.1*mm* against *B. fastidiosus*; 22.1±0.3*mm* against *E.coli*). These results were also very comparable to antibacterial inhibition zones of *P. harmala* extracts (18.7±3.5*mm* against *M. tuberculosis*) at 200 mg/mL[47], *Mentha piperita* (18*mm* against *S. aureus*)[60-61] and cold water extract (17.25±0.14*mm* against *B. subtilis*) of *Moringa oleifera* leaves [60].

3.6.1. Minimal inhibitory concentration (MIC)

The minimum concentration of plant extracts required to inhibit bacterial growth is an important factor to evaluate effectiveness of a plant extract. Lesser values of MIC demonstrate greater effectiveness of plant extract and vice versa. Results for antibacterial MIC have also been presented in Table 3. Again *n*-hexane and synergistic mixture presented lowest values of MIC, depicting their highest effectiveness against all tested bacterial strains. Their MIC values are quite comparable to standard antibiotics i.e. tetracycline and gentamicine. Such results of minimal inhibitory concentration are closer to previous reports of Rahman et al. [62] for MIC of ethanol extract of *Moringa oleifera* leaves against *Shigella shinga* (916 µg/mL) and *Pseudomonas aeruginosa* (458 µg/mL); and methanolic extract of *Mentha piperita* against *Klebsiella pneumoniae* (256 µg/mL) and *Serratia odorifera* (512 µg/mL) [61].

3.7. Cytotoxicity

Heamolytic assay was applied to evaluate cytotoxicity of different *P. harmala* leaf extracts. Sometimes, in addition to beneficial medicinal compounds, plant extracts also possess toxic metabolites and harmful compounds such as saponins. Such plants extracts, despite high concentration of therapeutic components, cannot be recommended for oral applications. The toxic compounds in these extracts prove to be harmful for human blood cells, as saponins interact to destroy various lipid molecules present in cell membrane, thereby rupturing the cell membrane, hence destroying erythrocytes. Smaller percentage heamolytic activity means lesser the quantity of toxic chemicals is present in plant extracts.

Table 1: Phytochemical constituents of *P. harmala* leaf.

Sr. No.	Bioactive Compound	*P. harmala* leaves
1	Flavonoids	+++
2	Alkaloids	+++
3	Saponins	+
4	Tannins	++
5	Terpenoids	++
6	Steroids	+
7	Glycosides	-
8	Anthraquinones	-

+++ Present (high concentration); ++ Present (mediun concentration); + Present (low concentration); - Absent

Table 2: Comparison of different antioxidant activities (%age and IC_{50}) of various *P. harmala* leaf extracts with standard antioxidants.

Extracting Solvent	Free Radical Scavenging		β-Carotene Assay		Linoleic acid Inhibition	
	%age scavenging	IC_{50} (µg/mL)	%age activity	IC_{50} (µg/mL)	%age inhibition	IC_{50} (µg/mL)
Deionized water	67.25 ± 0.72^{d}	99.51 ± 0.96^{de}	58.22 ± 0.66^{d}	523.50 ± 4.98^{e}	55.58 ± 0.52^{de}	832.24 ± 4.52^{e}
Ethanol	68.69 ± 0.68^{d}	89.50 ± 0.88^{d}	58.45 ± 0.64^{d}	517.22 ± 5.23^{e}	59.25 ± 0.48^{d}	798.56 ± 5.22^{d}
Acetone	$75.\ 25\pm0.78^{c}$	49.52 ± 0.66^{c}	70.24 ± 0.71^{bc}	322.54 ± 4.22^{cd}	65.22 ± 0.62^{c}	694.75 ± 6.10^{c}
Chloroform	81.51 ± 0.94^{b}	28.45 ± 0.42^{b}	72.45 ± 0.75^{b}	298.85 ± 3.22^{c}	66.23 ± 0.66^{c}	682.21 ± 5.23^{c}
n-Hexane	89.25 ± 0.88^{a}	11.25 ± 0.36^{a}	78.35 ± 0.65^{ab}	218.45 ± 3.02^{b}	72.23 ± 0.45^{b}	502.20 ± 4.58^{b}
Synergistic Mixture	90.25 ± 0.92^{a}	9.36 ± 0.24^{a}	82.42 ± 0.48^{a}	196.32 ± 2.45^{a}	80.12 ± 0.68^{a}	423.26 ± 4.98^{a}
Gallic Acid	---	---	82.22±0.66	195.42±2.88	83.22±0.69	402.32±5.22
Ascorbic Acid	88.36±0.75	10.22±0.18	---	---	---	---
]BHT	90.52±0.91	9.28±0.18	81.98±0.52	202.33±1.98	84.52±0.78	392.58±4.22

Values are mean ± SD of three replications.

IC_{50} values were calculated by linear regression analysis.

Different letters in superscript represent significant differences ($p < 0.05$) among different extraction solvents.

Table 3: Antibacterial activity (inhibition zone) and MIC of different *P. harmala* leaf extracts against selected pathogens.

Extract Type	Bacterial Pathogens					
	B. fastidiosus		***E. coli***		***S. typhi***	
	Inhibition Zone (*mm*)	**MIC (µg/mL)**	**Inhibition Zone (*mm*)**	**MIC (µg/mL)**	**Inhibition Zone (*mm*)**	**MIC (µg/mL)**
Deionized water	18.4±0.2^{e}	512±0.00^{e}	19.2±0.2^{c}	512±0.00^{e}	10.5±0.2^{bc}	1024±0.00^{e}
Ethanol	19.0±0.1^{e}	256±0.00^{d}	20.1±0.3^{b}	256±0.00^{c}	10.2±0.2^{c}	1024±0.00^{e}
Acetone	20.3±0.2^{d}	256±0.00^{d}	19.3±0.1^{c}	128±0.00^{b}	10.4±0.1^{bc}	512±0.00^{c}
Chloroform	22.4±0.2^{c}	256±0.00^{d}	19.8±0.2^{bc}	128±0.00^{b}	10.8±0.3^{b}	512±0.00^{c}
n-Hexane	25.1±0.3^{b}	128±0.00^{c}	20.6±0.2^{b}	64±0.00^{a}	11.8±0.1^{a}	256±0.00^{a}
Synergistic Mixture	27.2±0.2^{a}	32±0.00^{a}	22.1±0.1^{a}	64±0.00^{a}	12.1±0.2^{a}	256±0.00^{a}
Tetracycline	27.5±0.2	32±0.00	22.3±0.2	128±0.00	12.0±0.1	1024±0.00
Gentamicin	26.5±0.1	32±0.00	22.1±0.3	64±0.00	11.6±0.2	512±0.00

Values are mean ± SD of three replications.
Different letters in superscript represent significant differences ($p < 0.05$) among different extraction solvents.

Table 4: HPLC profile of bioactive compounds of synergistic mixture of different extracts of *P. harmala* leaf.

Peak No.	RT(min)	Proposed Compounds	Molecular Formula	Concentration (µg/mL)
1	2.97	Ascorbic acid	$C_6H_8O_6$	261.97±2.8
2	3.37	Caftaric acid	$C_{13}H_{12}O_9$	258.47±2.7
3	4.39	Rosmeric acid	$C_{18}H_{16}O_8$	270.12±2.3
4	5.47	Kaempferol	$C_{15}H_{10}O_6$	255.33±2.8
5	22.16	Gallocatechin	$C_{15}H_{14}O_7$	270.03±1.9
6	22.91	Caffeic acid	$C_9H_8O_4$	263.30±2.6
7	23.24	3,4-O-Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	264.59±2.1
8	23.56	Unknown	_____	263.89±2.2
9	23.92	Chrysin	$C_{15}H_{10}O_4$	268.14±2.6
10	24.40	Catechin	$C_{15}H_{14}O_6$	270.17±2.8
11	25.02	Chlorogenic acid	$C_{16}H_{18}O_9$	276.46±2.9
12	25.62	Sinapic acid hexoside	$C_{17}H_{22}O_{10}$	327.41±3.1
13	26.19	Unknown	_____	356.52±3.1
14	26.45	Kaempferol-O-dirhamnoside	$C_{27}H_{30}O_{14}$	494.80±3.4
15	26.95	Chicoric acid	$C_{22}H_{18}O_{12}$	448.52±3.7
16	27.61	Orientin	$C_{21}H_{20}O_{11}$	803.10±4.6
17	28.24	Chebulic acid	$C_{14}H_{12}O_{11}$	503.83±4.1
18	29.22	Malvidin-3-O-glucoside	$C_{23}H_{26}O_{12}$	538.18±3.8
19	29.73	Di-O-methyl ellagic acid	$C_{16}H_{10}O_8$	515.47±3.9

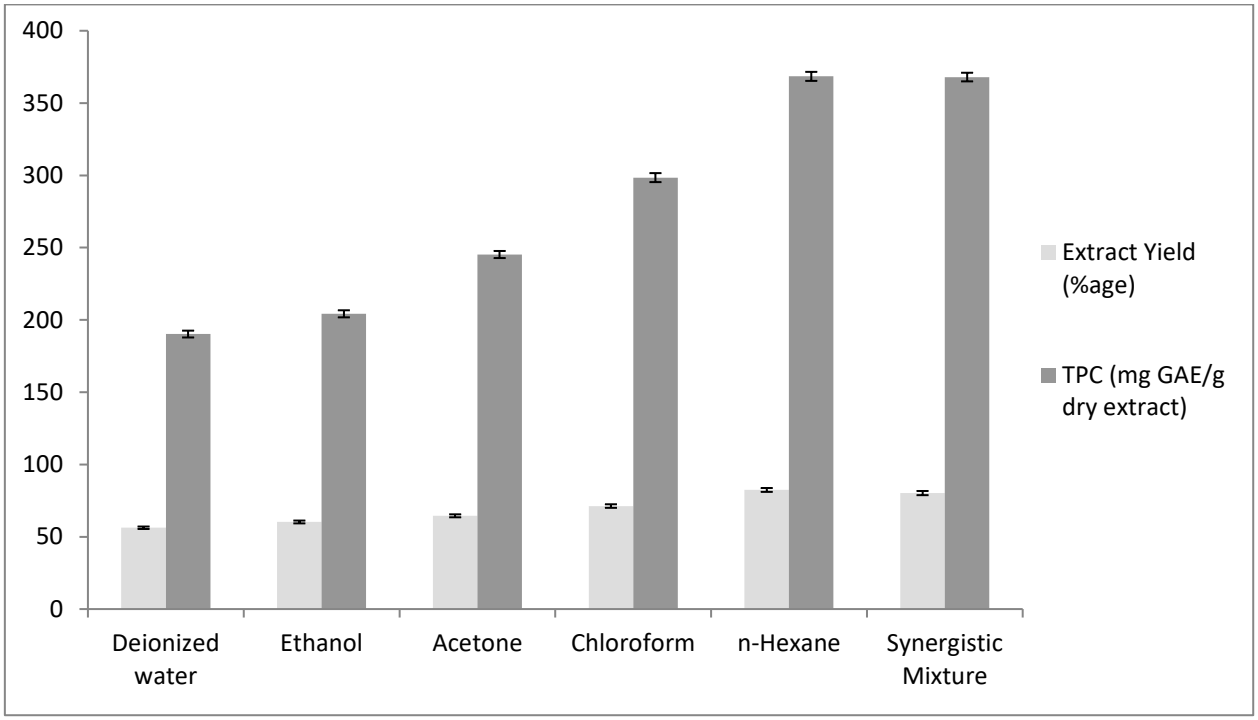
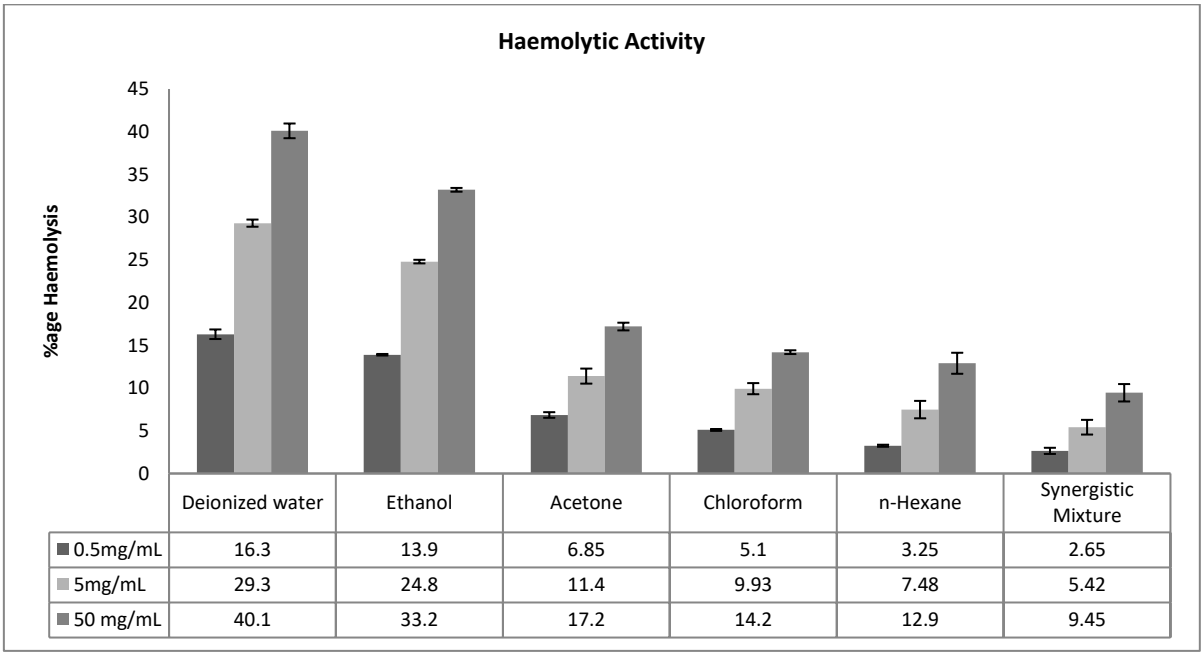


Figure 1: Comparison of extract yield and phenolic contents of different extracts of *P. harmala* leaf.



	Deionized water	Ethanol	Acetone	Chloroform	n-Hexane	Synergistic Mixture
0.5mg/mL	16.3	13.9	6.85	5.1	3.25	2.65
5mg/mL	29.3	24.8	11.4	9.93	7.48	5.42
50 mg/mL	40.1	33.2	17.2	14.2	12.9	9.45

Figure 2: Haemolytic activity of different extracts of *P. harmala* leaf at varying concentrations.

Thus, plant extracts having high %age haemolysis cannot be used in medicinal formulations [62-63]. Figure 2 shows comparison of percentage haemolytic activity of various extracts of *P. harmala* leaf at varying concentrations (0.5 mg/mL; 5 mg/mL and 50 mg/mL). *n*-Hexane extract and synergistic mixture presented quite low values of percentage haemolytic potential (3.25±0.12% at 0.5mg/mL and 2.65±0.36% at 0.5mg/mL, respectively), depicting their high effectiveness in medicinal formulations. These hemolytic potential were quite comparable to those of *H. pinifolia* (2.07±0.63% at 1.0 mg/mL) [62] and soyasaponins (2.78±1.15% at 150 µg/mL) [64]. This can be explained by the fact that these extracts might have highest concentrations of bioactive compounds and least amounts of toxic metabolites. The bioactive compounds present in these extracts protect human erythrocytes against damaging effects of toxic metabolites and saponins which might be present in plant extracts. All extracts of *P. harmala* leaf exhibited dose dependent haemolytic activity; as dose of extract increases, haemolytic potential also increases which might be due to presence of high concentration of toxic compounds.

3.8. Analysis of phenolics by HPLC

Synergistic mixture of all extracts of *P. harmala* leaf showed nineteen peaks. Seventeen of them were identified using standard bioactive compounds (Table 4), and was found belonging to phenolic acids, flavonoids, their glycosidic conjugates and other bioactive compounds.

Peak 1 (RT 2.97min) represents ascorbic acid, a strong antioxidant.

3.8.1. Phenolic acids and their conjugates

Peaks 2 (RT 3.37min), 3 (RT 4.39min), 6 (RT 22.91min), 11 (RT 25.02min), 15 (RT 26.95min) and peak 17 (RT 28.24min) represented different phenolic acids such as caftaric acid, rosemeric acid, caffeic acid, chlorogenic acid, chicoric acid and chebulic acid while some peaks corresponded to conjugates of phenolic acids. Peak 5 (RT 22.16min) represented gallocatechin, a conjugate of gallic acid and catechin; peak 7 showed 3,4-O-dicaffeoylquinic acid (a combination of two caffeic acid and one quinic acid residues); peak 12 (RT 25.62min) indicated sinapic acid hexoside while peak 19 (RT 29.73min) represented di-o-methyl ellagic acid, a conjaute of an ellagic acid residue with two methyl groups.

3.8.2. Flavonoids and their conjugates

Peaks 4 (RT 5.47min), 9 (RT 23.92min), 10 (RT 24.40min) and peak 16 (RT 27.61min) indicated the presence of different flavonoids like kaempferol, chrysin, catechin and orientin, respectively. Peaks 14 (RT 26.45min) and 18 (RT 29.22min) showed the existence of two glycosidic conjugates of flavonoids such as kaempferol-O-dirhamnoside and malvidin-3-O-glucoside, respectively.

4. Conclusions

The current project was designed with the aim to explore various medicinal attributes of *P. harmala* belonging to desert i.e. Cholistan. *P. harmala* leaves exhibited high concentration of flavonoids and alkaloids, intermediate concentrations of tannins and terpenoids, low concentrations of saponins and steroids while glycones and anthraquinones were found to be absent. Results demonstrated that among different *P. harmala* leaf extracts, *n*-hexane exhibited highest phenolic contents, antioxidant and antibacterial activities. Moreover, such extracts exhibited lowest cytotoxicity which favors its oral usage. Synergistic mixture of various *P. harmala* extracts also depicted most potent antioxidant and antibacterial activities; and lowest cytotoxicity to human erythrocytes. Results of chemical characterization by HPLC represented that various medicinal and antibacterial potential of *P. harmala* were due to 17 different bioactive compounds belonging to phenolic acids, flavonoids and their conjugates with carbohydrates which are components of plant cells. It was concluded from current study that *P. harmala* leaves can be effectively used for designing functional foods and in medicinal formulations against various degenerative diseases.

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