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Phytochemical profiling and antiviral activity of *Ajuga bracteosa, Ajuga parviflora, Berberis lycium* and *Citrus lemon* against Hepatitis C Virus

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ABSTRACT

Hepatitis C is a serious health issue and cause liver disorders in millions of people. Available therapeutic agents require long term administration with numerous side effects. Therefore, there is a dire need to find alternative treatment options for this disease. Since ancient times, medicinal plants are widely used to cure various diseases with no or less harmful effects. Therefore, this study was designed to find out phytochemicals and investigate antiviral activity of methanol extract of *Ajuga bracteosa, Ajuga parviflora, Berberis lycium* and *Citrus lemon* against Hepatitis C Virus (HCV infection). Phytochemical analysis of the plant extract was performed using various chemical tests. Toxicity of the plant extract was determined against using trypan blue exclusion method. Antiviral activity of the selected plant extract was find out against HCV infected HepG2 cells. For this purpose, HepG2 cells were seeded with HCV positive and negative serum and nontoxic doses of plant extract for 24 and 48 h. After this RNA was extracted and viral load was determined using Real-time PCR. Phytochemical analysis showed the presence of flavonoids and phenols in all plant extracts while amino acids, alkaloids and tannins were present in *B. lycium* and saponins were detected in *C. lemon*. Toxicity assay showed that all plant extracts were nontoxic at maximum concentration of 200 μg/ml except *B. lycium,* which showed mild toxicity at 40 μg/ml and were extremely toxic at 60 μg/ml and above doses. Real-time PCR quantitation result revealed that after 24 h treatments *A. parviflora* showed highest antiviral activity, followed by *A. bracteosa*, while *B. lycium* extract had low (35%) and *C. lemon* has no antiviral effects. The 48 h treatments showed an increase antiviral activity by *A. bracteosa* followed by *A. parviflora* and *B. lycium* while *C. lemon* showed negative effect. Our results depicted that mentioned plants might be used as an alternative therapeutic regime or in combination with existing treatments against HCV.

1. Introduction

Plants the primitive source of food are utilized for centuries to cure diseases by the human being in different forms. Medicinal plants are valuable source of compounds that are inclusive part of drug administrated presently. Natural product plays vital role in the cure of bacterial and tumour diseases. But their role in case of viral disease is still limited with a few examples such as in coronavirus, coxsackievirus, dengue virus, enterovirus, herpes simplex, measles virus respiratory syncytial virus, influenza, human immunodeficiency, hepatitis B and C [1]. The main advantage of using natural molecules from plant extracts is their low cost, with no need of chemical synthesis. This mode of production might lead to less expensive treatments, available for populations of low-income countries.

Ajuga bracteosa (*A. bracteosa*) and *Ajuga parviflora* (*A. parviflora*) belongs to Ajuga genus and Lamiaceae family. *A. bracteosa* is native to the hilly areas of Pakistan, China, India and Malaysia. In Pakistan, it is found in the northern hilly areas locally known as trakha boti (bitter herb). It has many ethnopharmacological uses as its leaves been used to make herbal medicine to cure diabetes, malaria and digestion related problems [2]. Moreover, leaves extract has been traditionally used to remove toxicity from the blood. The root extract has also been used for the curing of digestion related issues [3]. *A. parviflora* is found in Pakistan, Kashmir, and Afghanistan within the low temperature and hilly

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areas [4]. It was reported that this plant has been traditionally used to cure fever, asthma, HCV, jaundice [5], arthritis, cancer and wounds [6]. This plant also has been used in the treatment of eye irritation, poisoning from insect attack, stomach pain and liver tissue damage [7]. *Berberis lycium* (*B. lycium*) naturally grow in all regions of Himalayas and also found in temperate and semi temperate regions of Pakistan, Nepal, Bangladesh, Afghanistan and India. In Pakistan, they were found in the area which lies between 900 and 2900 m like Azad Kashmir, Khyber Pakhtunkhwa, Punjab and Baluchistan. It is used to cure disease like liver disorders, abdominal pain, skin diseases, cough and ophthalmic. *Citrus lemon* (*C. lemon*) comes under the family rutaceae and grown all over the world. It has been used against cancer, to reduce the cytotoxicity of low density lipoprotein and to prevent edema of the legs.

Hepatitis C is a liver inflammatory disorder caused by Hepatitis C Virus (HCV). HCV is a tiny (55-65 nm in size) virus having an envelope, a positive ssRNA genome and belongs to the *Flaviviridae* family. HCV is present in chronic form in around 130-150 million peoples around the world and are responsible of 33% death alone as a member of hepatitis group [8]. In Pakistan, HCV sero-prevalence among the general adult population is 6.8%, while active HCV infection was found in approximately 6% of the population [9]. Currently, the most common treatment for HCV is the combination of ribavirin and pegylated interferon-α. This treatment is given for 24-48 weeks depending on viral genotype and races [10]. The long term uses of this treatment produces severe side effects including transient virologic response, hemolytic anemia and teratogenic effects [11,12]. Hence, it is needed to find new and alternative ways of treatment for HCV with lower side effects and higher efficacy.

The purpose of the study was to find out more effective alternative therapeutic option with reduced or no side effects. An antiviral regimen which could be less expensive and easily available to all, especially to the people who cannot access the current HCV treatment aim of our basic study. The results demonstrated that 3 out of 4 selected plants had a notable anti-HCV activity. These plants can be subjected to further bioassay guided isolation of anti-HCV compounds which may ultimately leads to an alternative HCV treatment.

2. Materials and methods

2.1. Plants collection, identification and extraction

All plants were selected based on their medicinal uses by the local community for the treatment of viral and other infectious diseases. Three medicinal plants, *A. bracteosa*, *A. parviflora* and *B. lycium* were collected from Dir Lower Malakand division, Khyber Pakhtunkhwa, Pakistan, while *C. lemon* was collected from Ajman, United Arab Emirates (Table 1). Plants were identified by Dr. Abdul Nazir, plant taxonomist at Department of Environmental Sciences, COMSATS Institute of information technology Abbottabad, Pakistan and the voucher specimen was placed in Islamabad herbarium at Quaid-i-Azam University, Islamabad. The plants were washed with tap water and dried at room temperature in the shade. At 8-12% moisture level, the plant materials were grounded and weighted. Then, extraction was performed using previously reported method with some modifications [13]. Briefly, 100 gm of each plant material was soaked in 750 ml of 70% methanol for 10 days. The mixture was filtered using a mousseline cloth in order to remove large waste particles of the plant, followed by filtration with Whatman Filter Paper 42. The extract was concentrated using a rotatory evaporator at 50 °C. The dried samples were weighted and stored. Stock were prepared by dissolving 20 mg of extract in 1 ml of Dimethyl sulfoxide and kept at -20 °C for further use.

Table 1
List of selected medicinal plants and herbs.

S. No	Botanical names	Common names	Plants parts used	Acronyms used
1	*Ajuga bracteosa*	Naeel kanti/Booti	Leaves	*A. bracteosa*
2	*Ajuga parviflora*	Ratti Buti	Leaves	*A. parviflora*
3	*Berberis lycium*	Sumbalu	Roots	*B. lycium*
4	*Citrus lemon*	Nimbu	Pulp	*C. lemon*

2.2. Phytochemical analysis of plant extracts

Phytochemical analysis of the plant extract was done as per standard protocols [14,15]. In short, Mayer's test was performed for the detection of alkaloids. In which the 3 ml of extracts were separately dissolved in 70% HCl and filtered. Then, Mayer's reagent was added to the filtrates and appearance of yellow color precipitate indicated the presence of alkaloids. Fehling's test was used for the detection of carbohydrates. In this, 0.5 g extracts were dissolved in 5 ml of distilled water and filtered. Then, the filtrates were hydrolyzed with 70% HCl, neutralized with alkali and at last heated with Fehling's A and B solutions. The presence of reducing sugars was indicated by the formation of red precipitate. For the detection of saponins, Foam test was applied in which 0.5 gm of extract was dissolved in 2 ml of distilled water and well shaken. The production of foam, and its persistence up to 10 min indicated the presence of saponins. Phenols were detected with Ferric Chloride test in which 2 ml of extracts were treated with 2–3 drops of 10% ferric chloride solution. Formation of bluish black color indicated the presence of phenols. Tannins were detected with Gelatin test. It was performed by adding 1% gelatin solution containing sodium chloride to the extract. The presence of tannins was indicated by the formation of white precipitate. For the detection of flavonoids (Lead Acetate test), 2 ml of extracts were treated with 2–3 drops of lead acetate solution. Formation of yellow color precipitate indicated the presence of flavonoids. Ninhydrin test was used for the detection of amino acids. Ninhydrin solution was added to the extract and boiled for a few min. The formation of blue color indicated the presence of amino acids.

2.3. Serum collection

Fourteen chronically infected patients (diagnosed based on serum HCV RNA by PCR) with HCV 3a were selected based on consent without any age and gender discrimination (Table 2). Sera were taken from patients at the Genome Center of Center for Applied Molecular Biology, Lahore, Pakistan and stored at -80 °C. Only those patients were selected which were negative for Hepatitis B Surface Antigen.

2.4. Cell culture, splitting and plating

HepG2 cells were purchased from American Type Culture Collection (USA). These cells were maintained at 37 °C in a 95% air and 5% CO_2 humidified atmosphere. Cells were cultured in complete Dulbecco's

Table 2
Demographic and virological characteristics of patients selected for the study.

S. No.	Lab I. D	Age	Gender	Viral Count
1	My5-83	70	M	1816479
2	My5-291	65	M	1179648
3	My5-315	45	F	2235839
4	My5-393	45	F	1284927
5	My5-534	60	F	1284927
6	My5-543	27	M	1118764
7	My5-544	43	M	2086270
8	My5-545	46	F	4788642
9	My5-547	33	M	1043923
10	My5-557	55	F	1362111
11	My5-1549	72	M	20353001
12	My5-1551	52	M	172523
13	My5-1552	45	M	243892
14	My5-1553	32	M	227577