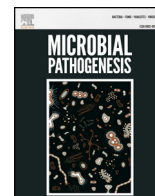




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Phytochemical profiling and antiviral activity of *Ajuga bracteosa*, *Ajuga parviflora*, *Berberis lycium* and *Citrus lemon* against Hepatitis C Virus

Tahir Yousaf^a, Shazia Rafique^b, Fazli Wahid^a, Sidra Rehman^c, Abdul Nazir^a, Javeria Rafique^b, Kashif Aslam^d, Ghulam Shabir^d, Shahid Masood Shah^{a,*}

^a Department of Environmental Sciences, COMSATS Institute of Information Technology, Abbottabad, Pakistan

^b Center for Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan

^c Department of Bio Sciences, COMSATS Institute of Information Technology, Islamabad, Pakistan

^d Institute of Molecular Biology & Biotechnology, Bahauddin Zakariya University, Multan, Pakistan

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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 administered 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

* Corresponding author.

E-mail addresses: tahiryousofzai007@gmail.com (T. Yousaf), shaziarafique@gmail.com (S. Rafique), fazliwahid@ciit.net.pk (F. Wahid), sidra.cemb@gmail.com (S. Rehman), abdulnazeer@ciit.net.pk (A. Nazir), javeria.rafique@gmail.com (J. Rafique), dr.kaslam@gmail.com (K. Aslam), shabirpbg50@gmail.com (G. Shabir), smasood@ciit.net.pk (S.M. Shah).

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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

Table 1
List of selected medicinal plants and herbs.

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

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 rotary 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.

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% CO₂ 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

Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (combination of penicillin & streptomycin), and sodium bicarbonate (0.0037 gm/ml). When reach to 70–80% confluency, subculturing were performed and plates were prepared.

The HepG2 cells were split and plated for antiviral analysis of plant extracts. For this purpose, cells were grown at a density of 3.1×10^5 cells/well in 12-wells culture plates. Briefly, the 70–80% confluent plates media were aspired and washed with 5–8 ml of $1 \times$ Phosphate Buffered Saline (PBS) followed by the addition of trypsin EDTA (Invitrogen life technologies, Carlsbad, USA) to dislodge the cells. Then, 2–3 ml of complete medium (DMEM + 10% FBS + antibiotics) was added to the flask to neutralize the trypsin EDTA. The cells were then shifted with medium to 15 ml falcon tube, centrifuges, counted and seeded at the required density and dislodged the cells into a single cell suspension (10–20 times). Then cells were incubated at 37 °C, 5% CO₂ for 24 h before treatments.

2.5. Toxicity assay

The toxicity assay was performed using trypan blue exclusion method. All plant extracts were subjected to toxicity analysis. For this purpose, HepG2 cells were cultured in 24 well plates at a density of 8×10^4 cells/well. The first 3 wells were taken as control while the other wells were treated with different concentration of plant extracts ranging from 40 µg/ml to 200 µg/ml. After 24 h, media was aspired and wells were washed with $1 \times$ PBS solution and trypsinized with trypsin EDTA to dislodge the cells. After 2 min, complete culture media was added to stop further trypsinization. Cells were suspended into a single cell suspension through generous pipetting. Ratios of 1:1 (10 µl each) of trypan blue and suspended cells were mixed well. After this, 10 µl of the mixture was poured onto hemocytometer and the viable and dead cells were counted on the basis unstained and stained cells, respectively. Initially, percent viability was found out using below formula

$$\text{Percent viability} = 1 - \frac{\text{Number of dead cells}}{\text{Number of total cell}} \times 100$$

In the next step, viability of the control cells was considered as 100% and all other treatments were compared with control.

2.6. Anti HCV analysis of plant extracts

HepG2 cells were seeded in complete media at density of 3×10^5 cells per well in 24 wells plate. The plates were incubated at 37 °C for 24 h. Five wells of the 1st row was seeded with 100 µl of HCV positive serum of known viral load, in which the 5th well was kept as positive control (in which only virus added; no plant extract added) and the 6th was kept as negative control (in which no virus & no plant extract added). Another plate was kept as negative control (no virus, no drug). Each row was treated with separate plant extract, *A. bracteosa* (1st row), *A. parviflora* (2nd row), *B. lycium* (3rd row) and *C. lemon* (4th row) at a concentration of 200 µg/ml except *B. lycium*, which was 20 µg/ml.

After 24 h incubation, total RNA was extracted using MACHEREY-NAGEL nucleic acid extraction kit (MACHEREY-NAGEL GmbH & Co. Germany) according to the manufacturer's instructions. HCV RNA quantifications were determined by Real-time PCR Smart Cycler II system (Cepheid Sunnyvale, USA) using the Sacace HCV quantitative analysis kit (Sacace Biotechnologies Caserta, Italy) according to the manufacturer's instructions.

2.7. Statistical analysis

All experiments were conducted in triplicate to check the reproducibility of the results. All statistical comparisons were made by means of Student's t-test and $p < 0.05$ were considered significant.

Table 3

The phytochemical constituents of the *A. bracteosa*, *A. parviflora*, *B. Lycium* and *C. lemon*.

S. No	Chemical	<i>A. bracteosa</i>	<i>A. parviflora</i>	<i>B. lycium</i>	<i>C. lemon</i>
1	Alkaloids	–	–	+	–
2	Carbohydrates	+	+	+	–
3	Amino Acid	+	+	–	+
4	Flavonoids	+	+	+	+
5	Saponins	–	–	–	+
6	Tannins	–	–	+	–
7	Phenols	+	+	+	+

3. Results

3.1. Phytochemical analysis

Phytochemical analysis of the methanol plant extracts of *A. bracteosa*, *A. parviflora*, *B. lycium* and *C. lemon* revealed the presence of phytoconstituents such as alkaloids, carbohydrates, amino acids, flavonoids, saponins, tannins, and phenols as shown in Table 3. Flavonoids and phenols were detected in all of the above mentioned four tested extracts. Alkaloids and tannins were only present in *B. lycium*, whereas saponins were only detected in *C. lemon*. Carbohydrate and amino acid were detected in all except *C. lemon* and *B. lycium* in which it was not detected, respectively.

3.2. Toxicity assay

Toxic extracts and compounds cannot be used for biological activities. So, it was important to find out the toxic effects of four medicinal plants used in the current study before evaluating for anti-HCV activities. Results have been summarized in Fig. 1. It was observed that *A. Bracteosa*, *A. parviflora*, and *C. lemon* exhibited no significant cytotoxic effects on cell proliferation of liver cells up to concentration of 200 µg/ml. It is worth mentioning that *B. lycium* methanol extract was significantly toxic even at a dose of 40 µg/ml and were extremely toxic at higher doses.

3.3. Anti-HCV activities of the crude methanol extract of selected plants

HCV serum can be used to infect human hepatocyte cultures as reported by Rehman et al. [10]. Antiviral activities of methanol extract of four medicinal plants were tested against HCV and results has been summarized in Fig. 2 for 24 h and Fig. 3 for 48 h treatments. Results

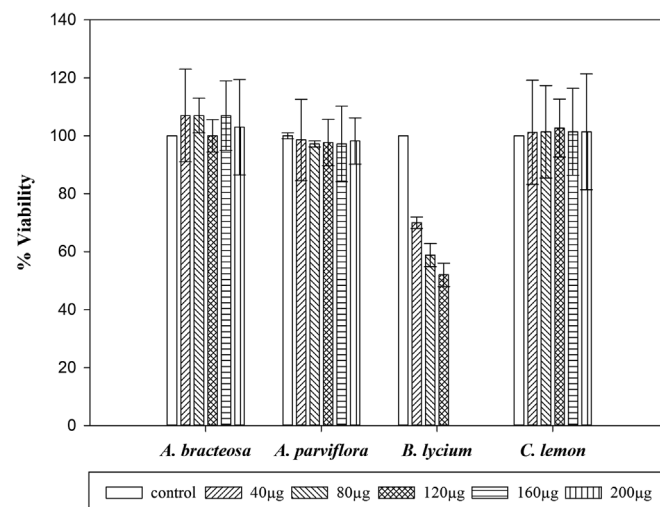


Fig. 1. Cellular toxicity of plant extract via trypan blue exclusion method. *A. Bracteosa*, *A. parviflora*, and *C. lemon* exhibited non-significant cytotoxic effects.

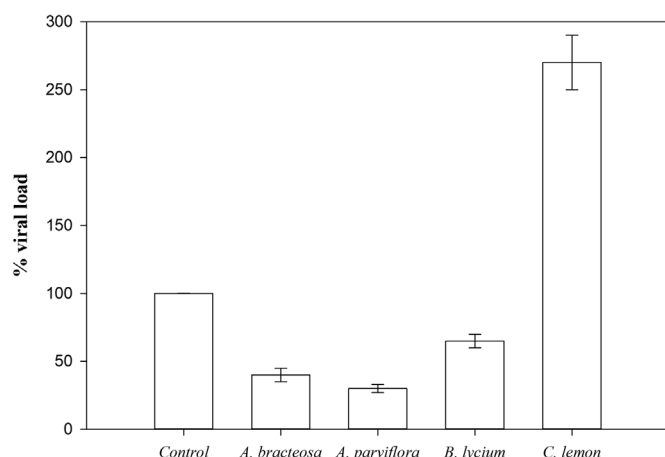


Fig. 2. Anti HCV activity of plant extracts after 24 h treatment. Viral count dramatically decreased in case of *A. parviflora* treated sample followed by *A. bracteosa* and *B. lycium*, while *C. lemon* had shown negative antiviral activity.

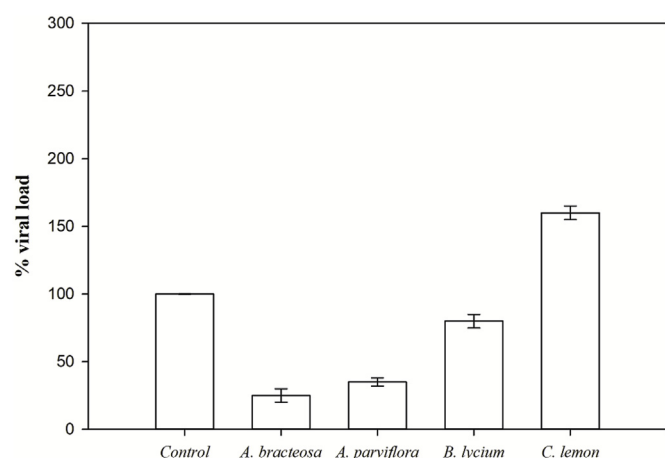


Fig. 3. Anti HCV activity of plant extracts after 48 h treatment. Viral count was decreased up to a very low titer in case of *A. bracteosa* treated sample, followed by *A. parviflora* and *B. lycium*, while *C. lemon* still had negative effects.

showed that after 24 h viral count dramatically decreased in case of *A. parviflora* (70%) treated sample followed by *A. bracteosa* (60%) and *B. lycium* (35%), while *C. lemon* had shown negative antiviral activity and viral count increased by 2.7 times as compared to untreated control. Similarly, Real-time PCR results showed that after 48 h the viral count was decreased up to a very low titer in case of *A. bracteosa* (75%) treated sample, followed by *A. parviflora* (65%) and *B. lycium* (20%), while *C. lemon* still had negative effects having viral count of about 1.6 times as compared to control.

4. Discussion

A large variety of traditional medicinal herbs and plants have been reported to exhibit antiviral activities against different viruses [16,17]. As HCV infect liver, so the idea was to check Pakistani medicinal plants having traditional uses in the treatment of various viral, infectious diseases and liver disorders for the anti-HCV activities. Methanol extract from the leaves of *A. bracteosa*, *A. parviflora*, roots of *B. lycium* and from the pulp of *C. lemon* were checked for their anti-HCV activity. It is always important to know the phytoconstituents of extracts under investigation. Therefore, plants were subjected to phytochemical analysis. The plants under study were also previously analyzed by researchers for phytoconstituents. They had reported that *A. bracteosa* methanol extract is a promising bioactive extract due to presence of polyphenols and phytoecdysteroids. Kayani et al. [18]. The results in

current study agreed as phenols were detected (Table 3). Similarly, previous study reported the presence of aromatic compounds, carbohydrates, glycosides, tannins, alkaloids, polyphenols, quinines and dions, aminophenols, steroids/sterols, flavonoids and terpenoids in *A. Parviflora* methanol extract. In the present study flavonoids, carbohydrates, amino acids and phenols were detected, however alkaloids were not detected (Table 3). The reason for absence of alkaloids could be the difference in season of collection or age of the collected plants in the present and previous study. Likewise, the presence of flavonoids, alkaloids, tannins, carbohydrates was reported in *B. lycium* [19]. All these constituents were also present in the currently investigated extract (Table 3). In a previous study, the phytochemical analysis of citrus juice concentrates revealed the presence of flavonoids and saponins which are detected in the current study [20].

Non-toxic plant extracts can be used for biological activities; therefore, it was important to find out non-toxic doses of all plant extracts. Extracts of *A. bracteosa*, *A. parviflora*, *C. lemon* were found non-toxic at the maximum concentration (200 µg/ml) while the *B. lycium* was observed toxic even at very low dose of 40 µg/ml therefore its concentration was kept at a minimum dose (20 µg/ml).

In past, anti HCV activity has not been reported for *A. bracteosa*, *A. parviflora* extracts but another specie belonging to the same Family *Lamiaceae* has been reported to possess antiviral activity. Ma et al. [21] found that the plant of *A. decumbens* poses potent antiviral activity against respiratory syncytial virus (RSV) with an IC₅₀ value of 131.6 µg/ml. Plant extracts (*A. bracteosa*, *A. parviflora*, *B. lycium*) investigated in current study showed significant activity against HCV at nontoxic concentration, while the *C. lemon* had negative effects in antiviral activity. Antiviral efficacy of tested could be explained based on presence or absence of chemical constituents of plant extracts.

5. Conclusion

From these results, it was concluded that the medicinal plants specially *A. bracteosa* and *A. parviflora* as well as *B. lycium* have a significant ability to combat with the HCV virus and reduce/stop the growth of the virus. These medicinal plants can be used as an alternative treatment option for HCV infection after further pre-clinical and clinical studies. In future, bioassay guided isolation can be performed to isolate bioactive anti-HCV compound(s) from these plants.

Ethics approval and consent to participate

Consent were taken from all participants. This study was approved by Research Ethics Committee, of COMSATS Institute of Information Technology, Abbottabad.

Consent for publication

All author agrees to publish the data.

Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.micpath.2018.03.030>.

List of abbreviations

HCV	Hepatitis C virus
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
EDTA	Ethylene Diamine Triacetic Acid

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