In Vivo and In Vitro Evaluation for Antimicrobial and Antioxidant Agents

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Abstract

Recently, increasing public concern about hygiene has been driving many studies to investigate antimicrobial and antiviral agents. However, the use of any antimicrobial agents must be limited due to their possible toxic or harmful effects. In recent years, due to previous antibiotics’ lesser side effects, the use of herbal materials instead of synthetic or chemical drugs is increasing. Herbal materials are found in medicines. Herbs can be used in the form of plant extracts or as their active components. Furthermore, most of the world’s populations used herbal materials due to their strong antimicrobial properties and primary healthcare benefits. For example, herbs are an excellent material to replace nanosilver as an antibiotic and antiviral agent. The use of nano silver involves an ROS-mediated mechanism that might lead to oxidative stress-related cancer, cytotoxicity, and heart diseases. Therefore, existing antibiotic drugs can be replaced with biomaterials such as herbal medicine with high antimicrobial, antiviral, and antioxidant activity. This review paper highlights the antibacterial, antiviral, and radical scavenger (antioxidant) properties of herbal materials. Antimicrobial activity, radical scavenger ability, the potential for antimicrobial, antiviral, and anticancer agents, and efficacy in eliminating bacteria and viruses and scavenging free radicals in herbal materials are discussed in this review. The presented herbal antimicrobial agents in this review include clove, portulaca, tribulus, eryngium, cinnamon, turmeric, ginger, thyme, pennyroyal, mint, fennel, chamomile, burdock, eucalyptus, primrose, lemon balm, mallow, and garlic, which are all summarized.

Introduction

Antioxident :

Today currently lifestyle is faster and busy Antioxidants are substances that may protect your cells against free radicals, which may play a role in heart disease, cancer and other diseases. Free radicals are molecules produced when your body breaks down food or when you're exposed to tobacco smoke or radiation. A diet high in antioxidants may reduce the risk of many diseases (including heart disease and certain cancers). Antioxidants scavenge free radicals from the body cells and prevent or reduce the damage caused by oxidation. The protective effect of antioxidants continues to be studied around the world. Antioxidants can be divided into three groups by their mechanism: (1) primary antioxidants, which function essentially as free radical terminators (scavengers); (2) secondary antioxidants, which are important preventive antioxidants that function by retarding chain initiation; and (3) tertiary antioxidants which are concerned with the repair of damaged biomolecules.

Antimicrobial :

An antimicrobial is an agent that kills microorganisms or stops their growth. Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibiotics are used against bacteria, and antifungals are used against fungiAn antimicrobial agent is defined as a natural or synthetic substance that kills or inhibits the growth of microorganisms such as bacteria, fungi and algae. Antimicrobials (also called biocides and antimicrobial pesticides) are substances that kill or slow the spread of microorganisms such as bacteria, viruses, or fungi, and they are crucial in helping to prevent and stop the spread of harmful microbes. Antibiotic class defines a set of related antibiotics. State-level rates of penicillins, macrolides, cephalosporins, and fluoroquinolones are displayed. Also displayed are all antibiotic classes, which include these four classes plus additional classes not available for release at the state level. Antibiotic class defines a set of related antibiotics. State-level rates of penicillins, macrolides, cephalosporins, and fluoroquinolones are displayed. Also displayed are all antibiotic classes, which include these four classes plus additional classes not available for release at the state level.



Due to increasing concerns about the sustainability of human living, the control of the damaging effects of microorganisms is becoming very important. A wide range of microorganisms exist in a biological balance with the human body and its living environments, but an uncontrolled and rapid growth of microbes can lead to some dangerous problems

In Vitro and In Vivo System

In vitro is Latin for “in glass.” It describes medical procedures, tests, and experiments that researchers perform outside of a living organism. An in vitro study occurs in a controlled environment, such as a test tube or petri dish.In vivo is Latin for “within the living.” It refers to tests, experiments, and procedures that researchers perform in or on a whole living organism, such as a person, laboratory animal, or plant.

**In** vitro

In vitro testing occurs in a laboratory and usually involves studying microorganisms or human or animal cells in culture. This methodology allows scientists to evaluate various biological phenomena in specific cells without the distractions and potential confounding variables present in whole organisms.In vitro testing is a straightforward research methodology. Researchers can perform more detailed analyses and examine biological effects in a larger number of in vitro subjects than they would in animal or human trials.However, although petri dishes and test tubes provide controlled environments for in vitro testing, they cannot replicate the conditions that occur inside a living organism.As a result, it is necessary to interpret in vitro data with caution, as these results do not necessarily predict the reaction of an entire living being.Some examples of in vitro testing include pharmaceutical testing and fertility treatments.

In vivo

Despite positive preclinical results, around [30% of drug candidates](https://www.sciencedirect.com/science/article/abs/pii/S147148921930058X) fail human clinical trials due to causing adverse side effects. An additional 60% do not produce the desired effect.In vivo testing, especially in clinical trials, is a vital aspect of medical research in general. In vivo studies provide valuable information regarding the effects of a particular substance or disease progression in a whole, living organism.The main types of in vivo tests are animal studies and clinical trials.

 Materials and Methods

Chemicals and Reagents

Phenolic compound standards were purchased from Sigma Aldrich (Milan, Italy). All the other chemicals and solvents were purchased from Fluka (Saint-Quentin Fallavier, France) at analytical or HPLC grade and were used as received.

Plant Material and Extract Preparation

*P. lanceolata* was collected in June 2012 in the Royal Park of Capodimonte, Naples, Italy. The voucher specimen (no. 0134) was prepared and identified in our lab and deposited at the Herbarium of the Botanical Garden of the “Federico II” University in Naples, Italy.

Flowers, leaves, and roots of*P*.*lanceolata* were dried separately at 55°C for 48 h in a Carlo ErbaE.28 CL.1 Oven and then ground to a coarse powder. A total of 2 g of powder of each part of the plant was suspended in 20 mL of*Amorosa* water (still mineral water, low in sodium, and ideal for the preparation of baby and toddler food) at 100°C for 3 min. The preparation was infused for 8 min and then was cooled to room temperature, filtered, and evaporated to complete dryness.

To prepare the mouth rinse, 200 g of pulverised material (flowers and leaves) was suspended in 2.0 L of*Amorosa* water following the same procedure previously described. The preparation was filtered, placed in a sterilised 3.0 L glass container, and stored at 4°C before use.

In Vitro Antimicrobial Tests

The bacterial strains used for testing were*L. casei, S. bovis, S. mutans, S. mitis, S. parasanguinis, S. viridans*, and*S. sobrinus*. The strains were from clinical specimens obtained at the Diagnostic Unit of Bacteriology and Micology of the University of Naples “Federico II.” Bacteria were grown on Trypticase Soy Agar with 5% Sheep Blood (TSS; Becton Dickinson, USA) plates at 37°C in 5% CO2 for 48 h.

The minimum inhibitory concentration (MIC) was measured using the standard microdilution method in 96-well polystyrene plates using brain-heart infusion (BHI) medium. The starting inoculum was 5 × 105 CFU mL−1, and the final concentrations of the*P. lanceolata* infusion ranged from 4 to 0.025 mg mL−1. To determine the minimal bactericidal concentration (MBC), 50 *μ*L of bacterial suspension from the wells containing extract concentrations equal to or higher than the MIC were inoculated in 5 mL of sterile BHI medium and incubated for 24 h at 37°C under a 5% CO2 atmosphere.

In Vivo (Clinical) Antimicrobial Tests

The clinical studywas conducted on a sample consisting of forty-four adolescents (24 males and 20 females) enrolled in the Department of Paediatric Dentistry at the University of Naples “Federico II”, Italy, ranging from 12 to 18 years old.

The inclusion criteria were good general health (ASA I: Healthy person; ASA II: Mild systemic disease) and an agreement to comply with study procedures.

Subjects who had used antibiotics or mouth rinses during the 14 days prior to the beginning of the study were excluded.

Only 2 subjects of the 44 were excluded for this reason. However, during the research period, 2 subjects chose to end their participation; thus, 40 subjects completed the entire protocol. Participation in the study was voluntary. All of the parents gave written informed consent after receiving verbal and written explanations of the experimental protocol and study aims. The protocol was approved by the Ethical Committee of the School of Dentistry, University of Naples “Federico II”, Italy.

A controlled random clinical study was conducted. The subjects were divided into 2 groups of 20 subjects, respectively (Group A and Group B). Patients were randomly assigned to test and control groups using blocked randomisation from a computer-generated list.

Salivary counts of*S. mutans* and*L. casei* were estimated using a chair-side test that contained 2 agar surfaces. The blue mitis-salivarius-agar with bacitracin was used to detect the presence of streptococci, whereas the light culture medium, Rogosa agar, was used to evaluate that of lactobacilli.

No special dietary restrictions were imposed on the subjects, and no tooth brushing was allowed for at least 1 h after consuming lunch and dinner. All of the subjects were encouraged to maintain their normal oral hygiene habits. All subjects were given the same brush and tooth-paste for daily oral hygiene.

Saliva was inoculated on a dip slide with selective media for streptococci and lactobacilli. After adding a NaHCO3 tablet to the tube, the dip slides were immediately cultivated at 37°C for 48 h. The tablet releases CO2 on contact with moisture, creating favourable conditions for bacterial growth.

The colonies were identified using a stereomicroscope with ×10 magnification, and the culture density (CFU/mL) was visually compared with the aid of a chart provided by the manufacturer. Bacterial colonies were categorised as low (<105 CFU/mL of saliva) or high (> or = 105 CFU/mL).

Two different mouth rinse formulations were prepared.

(1) Experimental mouth rinse (Group A): an experimental mouth rinse was prepared with an infusion of*P. lanceolata* leaves and flowers, as previously described.

(2) Placebo mouth rinse (Group B): the placebo mouth rinse was prepared with*Amorosa* water, coloured with food dye.

*Experimental Design*. During the experimental period, after collecting the first saliva sample (T0), all of the Group A participants were asked to rinse with 10 mL of the experimental mouth rinse; instead, the participants of Group B were instructed to rinse with 10 mL of a placebo mouth rinse that did not contain phenolic substances, for 60 seconds after performing oral hygiene, 3 times a day (after breakfast, after lunch, and before sleeping) for 7 days.

On the 4th (T1) and 7th (T2) days of treatment, additional salivary samples were collected and immediately incubated to calculate the density (CFU/mL) of*S*.*mutans* and*L*.*casei* for each

*Statistical Analysis*. At the end of the treatments, the data were processed using the Statistical Package for Social Sciences (version 10.0, SPSS Inc., Chicago, Illinois, USA). A regression binary logistic analysis was performed. The statistical significance level was established at

In vivo and In vitro testing of antioxidant

2, 2-Diphenyl -1- Picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The free radical scavenging activity of the samples was determined according to the method of Kumari et al. (2016). A freshly prepared solution of DPPH in methanol (6 × 10-5 M) was used for the UV measurements. The samples of different concentrations (4–64 μg/mL) were added to DPPH solution in 1:1 ratio followed by vortexing. Then, it was allowed to take place in the dark at room temperature. Ascorbic acid and trolox were utilized as a standard. The inhibition percentage of DPPH radical scavenging activity was calculated using the following equation.

Inhibition(%) = [(A0−A)/A0] × 100

Where, A0 is the absorbance of DPPH in the absence of the sample and A is the absorbance of DPPH in the presence of the sample.

The IC50 values (the concentration required to scavenge 50% of the free radical) were estimated from a plot of % inhibition against the concentration of the sample solutions.

Total Reduction Capability

The total reduction capability of samples was determined according to the method of Kumari et al. (2016). 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferric cyanide were added to 1 mL of samples in different concentrations (4–64 μg/mL), followed by gentle mixing. The mixture was incubated at 50°C in a water bath for 20 min. The reaction was stopped by adding 2.5 mL of 10% TCA and the mixture was centrifuged at 4000 rpm for 10 min. From the top layer, 2.5 mL was transferred into the tube containing 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride (FeCl3.6H2O), mixed thoroughly. After 5 min, the absorbance was measured at 700 nm against blank. Trolox and ascorbic acid were taken as a standard.

ABTS+ Radical Cation Decolorization Assay

The antioxidant activities of the extracts were determined by the improved ABTS+ radical cation scavenging ability with the slight modification (Re et al., 1999; Sarma et al., 2016). ABTS+ radical cation was produced by mixing 7 mM 2, 2′- azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2.45 mM potassium persulfate (K2S2O8), incubated at room temperature in the dark. To determine the ABTS radical scavenging activity, 3 mL of ABTS+ solution was mixed thoroughly with 0.2 mL of different concentration (4–64 μg/mL) of extracts. Ascorbic acid and trolox were taken as a standard. The reaction mixture was allowed to stand at room temperature for 6 min.

The percentage inhibition was calculated by the following formula:

%Inhibition = OD of control−OD of sample/OD of control × 100

Hydrogen peroxide (H2O2) Radical Scavenging Activity

Hydrogen peroxide (H2O2) radical scavenging activities of the extracts were determined according to the method of Sarma et al. (2016). Briefly, water extracts of C. asiatica (4–64 μg/mL) were added to 0.6 mL H2O2 (40 mM) with the prepared phosphate buffer (pH 7.4). The reaction mixtures were incubated at room temperature for 10 min. After the incubation, the reaction mixture read at 230 nm against the blank solution with phosphate buffer (pH 7.4). Ascorbic acid and trolox were taken as a standard. The inhibition percentage was calculated based on the formula:

Percentage(%) of inhibition = (A1−A2)/A1 × 100.

Where, A1- absorbance of the H2O2 and A2- absorbance of the reaction mixture with extract.

Nitric Oxide (NO) Radical Scavenging Assay

Nitric oxide (NO) radical scavenging assay was carried out by the method of Kumari et al. (2016). 0.6 mL of 10 mM sodium nitroprusside was mixed with 1 mL of water extract of C. asiatica in different concentration (4–64 μg/mL). The mixture was incubated at 25°C for 150 min, followed by mixing with 1.0 mL of pre-prepared Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dichloride, and 2% phosphoric acid). Ascorbic acid and trolox were taken as a standard. The absorbance was measured at 546 nm. The inhibition was calculated by the following equation:

%inhibition of NO radical = [A0−A1]/A0 × 100.

Where, A0 is the absorbance before the reaction and A1 is the absorbance after the reaction has taken place with Griess reagent.

The decreasing absorbance indicates a high NO scavenging activity.

In vivo Study

Experimental Animals and Ethics Statement

All the experiments were conducted by using laboratory-bred male Wistar albino rats at IASST, Guwahati, Assam, in accordance with the internationally accepted guideline for experimental animals use and the study was approved by the Institute Animal Ethics Committee (IAEC) (1706/GO/c/13/CPCSEA), IASST. All animals were housed under controlled conditions of temperature (24 ± 3°C), relative humidity (60 ± 10%), 12/12 h. light–dark cycle, and water *ad libitum* the animal house of the IASST. Wister rats (male) weighing between 150 and 200 g were utilized for animal experiments.

Acute Toxicity Study

Acute toxicity test was conducted in C3H mice (*n* = 6) following the Organization for Economic Co-operation and Development (OECD) protocol. The animals were kept fasting overnight except water *ad libitum* and administered with a single dose of 5 mg/kg body weight (b.w.) with under observation for a period of 14 days. As per the protocol mandate, (i) if mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose, (ii) If mortality took place for single out of three, the dose was repeated to confirm the toxicity, (iii) if there was no mortality at all, the dose specificity had been raised to the maximum of 2,000 mg/kg b.w.

Induction of Hyperlipidemic, Experimental Design, and Treatment Schedule

For the development of hyperlipidemic conditions, rats weighing between 150 and 200 g were fed with high-cholesterol-diet (HCD), consisting of cholesterol 2%, whole wheat 62.5 g, yellow corn 37.5 g, barley, vitamin B12 one tablet. The cholesterol solution was prepared under the requirement of 25 mg/kg b.w. of rat by dissolving the cholesterol in refined groundnut oil (0.5% w/v) ([Khanna et al., 2002](https://www.frontiersin.org/articles/10.3389/fphar.2016.00400/full#B29)). Each individual animal was given 12 g of diet per day. All groups were subjected to intragastric administration every day till 28 days. After allowing a week for acclimatization, the rats were divided into six groups (six rats per group). Group A: normal diet and water (control). Group B: normal diet + cholesterol (25 mg/kg b. w.)/day. Group C: normal diet + cholesterol (25 mg/kg b.w./day) + fenofibrate (65 mg/kg b.w./day). Group D: normal diet + cholesterol (25 mg/kg b.w./day) + CAE1 (0.25 g/kg b.w./day). Group E: normal diet+ cholesterol (25 mg/kg b.w./day) + CAE2 (0.5 g/kg b.w./day). Group F: normal diet+ cholesterol (25 mg/kg b.w./day) + CAE3 (1 g/ kg b.w./day) up to 28 days.

Collection of Heart, Liver, and Serum

At the end of the experiment, all feed was removed 14 h before anesthesia. After being anesthetized, the heart, liver, kidney from rat were removed and weighed. The livers were collected and stored at -80°C for further analyses. Blood samples were also collected by an intracardiac puncture followed by separation of serum from the blood samples and collection in heparinized tubes. The collected serum samples were mixed gently by inverting 2–3 times and incubation at 4°C for 2–3 h. Plasma was also separated from blood by centrifugation at 2500 rpm for 30 min, which was aliquoted and stored at 4°C until further use ([Feng et al., 2015](https://www.frontiersin.org/articles/10.3389/fphar.2016.00400/full#B13)).

Preparation of Tissue Homogenate

Tissue homogenate (rat brain, liver, heart, and kidney) was prepared in a ratio of 1 g of wet tissue to 10 times (w/v) 0.05 M ice-cold phosphate buffer (pH 7.4) and homogenized by using a Teflon homogenizer. 0.2 mL of homogenate was used for estimation of TBARS. The remaining part of the homogenate was divided into two parts, one part of which was mixed with 10% TCA (1:1), centrifuged at 5000 rpm (4°C, for 10 min) and the supernatant was used for GSH estimation. The other part of the homogenate was centrifuged at 15000 rpm at 4°C for 60 min, the supernatants were used for SOD estimation.

Biochemical Analysis

Blood lipid profile analysis

Total cholesterol, TG, and HDL-C in serum were determined using test kits from Accurex Biomedical Pvt. Limited. Low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) were calculated using Friedwald’s formula ([Friedwald’s et al., 1972](https://www.frontiersin.org/articles/10.3389/fphar.2016.00400/full#B16)).

Evaluation of tissue markers of oxidative stress

Thiobarbituric acid reactive substances were measured as a marker of lipid peroxidation for plasma, heart, and liver tissues by using the procedure described by [Ohkawa et al. (1979)](https://www.frontiersin.org/articles/10.3389/fphar.2016.00400/full#B43), while glutathione (GSH) by [Ellman (1959)](https://www.frontiersin.org/articles/10.3389/fphar.2016.00400/full#B12) and superoxide dismutase (SOD) ([Marklund and Marklund, 1974](https://www.frontiersin.org/articles/10.3389/fphar.2016.00400/full#B39)). Nitrate/nitrites (NO) level was assayed by using the method described by [Green et al. (1982)](https://www.frontiersin.org/articles/10.3389/fphar.2016.00400/full#B19).

Histopathological Evaluation

Rats were fed diets for 4 weeks. Upon termination of the experiment, food was withheld from the rat for 24 h before sacrifice. Rat were then anesthetized, and livers were immediately excised, weighed and stored at 80°C for further use, or fixed in 10% buffered formalin at room temperature for histological analysis. For hepatic histological examination, formalin-fixed liver samples were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E), and subjected to light microscope observation.

Statistical Analysis

The statistical analysis was carried out using the OriginPro 9.0 software packages (OriginLab Corporation, Northampton, MA, USA) and the statistical Pearson’s correlation coefficients by using the OriginPro 6.0 software packages (OriginLab Corporation, Northampton, MA, USA). The results were determined by using one-way ANOVA and *p* < 0.05 were set as significant. IC50 was calculated using GraphPad PRISM, version 6.03 for windows (GraphPad software). All the measurements were performed in triplicate (*n* = 3). Mean values ± SD were calculated.

Results

Identification of Phenolic Compounds

The screening and identification of C. asiatica extract was performed by UHPLC–ESI-MS/MS. The chromatographic runs are illustrated in Figure 1 and Supplementary Figure S1. It reveals the observation of intense peaks at 0–20 min. The peak identification was performed by comparison of the retention time (RT), λ max, and mass spectra of the C. asiatica with the standard compounds and the earlier literature reports. Peaks with RTs (min) of 1.62, 1.72, 1.87, 1.88, 2.24 (peaks 1–5) were identified as the following: gulonic acid (RT 1.62, [M-H]- ion at m/z 195.81, λ max 275 nm), ferulic acid (RT 1.72, [M-H]- ion at m/z 193.81, λ max 275 nm), kaempferol (RT 1.87, [M+H]+ ion at m/z 287.89, λ max 275 nm), chlorogenic acid (RT 1.88, [M-H]- ion at m/z 353.09, λ max 275 nm), and asiatic acid (RT 2.24, [M-H]- ion at m/z 487.34, λ max 275 nm).

Conclusion

In this study, we have evaluated the antioxidant and anti-hyperlipidemic ability of extracts of C. asiatica. Herein, C. asiatica showed promising antioxidant and -antihyperlipidemic activities and also exhibited the highest phytochemical contents. The results of the present study indicate the presence of strong phenolic antioxidants components mainly gulonic acid, ferulic acid, kaempferol, chlorogenic acid, and asiatic acid in C. asiatica extract as evidenced from UHPLC-MS/MS. Additionally, CAE is capable of exhibiting significant anti-hyperlipidemic activities in HCF induced rat by enhancing parameters like antioxidant enzyme, body weight, and decrease serum lipid levels as well as regeneration of hepatic structures. Moreover, the CAE exerted to improve the hyperlipidemia induced hepatic structures by reducing OS, and restoring the antioxidant capacities. Taken together, this study strongly suggests that the CAE treatment might be an efficient way for treatment hyperlipidemia. This study has provided more evidence for the use of C. asiatica as a promising traditional medicine in the therapy of hyperlipidemia. Thus, these plant leaf extracts may be utilized as natural agents in food and pharmaceutical industries. Further studies are needed to isolate and identify the bioactive compounds present in the plant extracts and for the elucidation of their molecular mechanisms.

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