Exploring its immunogenicity and antioxidant potential of protein from *Mangifera indica* and *Syzygium cumini*

Gurpreet Singh, Shubham Tyagi, Aashrika Gupta, Abhishekh Tripathi, Karishma Ghosh, Amit Gupta*

Department of Biotechnology, Graphic Era (Deemed to be) University, Dehradun, India

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**ABSTRACT**

India is considered to be one of the rich repositories of medicinal and aromatic plants, which are mainly used as starting raw materials for drug manufacturing and perfumery products. More than thirty percent of various medicinal plant species are routinely used for various medicinal purposes. In this study, our major objective is to evaluate its immunogenicity and antioxidant potential of protein extracted from leaves of these two medicinal plants, i.e. *Mangifera indica* and *Syzygium cumini*. In order to achieve this objective, immunopharmacological studies were conducted and extracted protein from leaves (using Tris HCl and ice cold acetone) and confirming its protein content through the Lowry test. In addition, immunogenicity (using the standard vaccine, i.e. Typhoid vaccine) and antioxidant (using DPPH [1,1-diphenyl-2-picrylhydrazyl] assay) based studies were evaluated using a variable concentration of protein. The results indicate that proteins showed immunogenic effect against typhoid vaccine and also showed antioxidant effect as compared to control. In short, proteins serve as a natural antioxidant and also showed immunogenicity, which may be useful in preventing free radical-induced diseases and also claimed its immunoboosting properties as well. Overall, these studies reveal that protein possesses potential benefits in terms of immunogenicity (against specific protein antigen) and antioxidant effect.

*Corresponding Author*

Name: Amit Gupta
Phone: 
Email: dr.amitgupta.bt@geu.ac.in

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**INTRODUCTION**

Medicinal plant products are routinely applied in the traditional type of medicinal practices since prehistoric times. These medicinal plants are able to synthesise various compounds in the form of primary and secondary metabolites for performing various functions e.g. defence against various groups of insects along with microbial agents that are responsible for causing diseases (Gupta et al., 2014; Negi et al., 2003; Gupta and Shah, 2016). According to the literature, various medicinal plant based products showing less adverse effects while some of them are too much, especially for both humans and animals. Now a day, these medicinal plants considered them as future of medicine but some of them becoming endangered species (Balaraju et al., 2009; Bapat et al., 2008). In literature, various principles (hepatoprotective, cardioprotective, etc.) have been reported in the form of compounds extracted from various medicinal plant products. Still, these are unable to enter into the clinical trials (Guidelines for Clinical Trials on Pharmaceutical Products in India, 2001; Good Clinical Practices Guidelines for clinical trials in Ayurveda, 2013).

India is considered to be one of the rich repositories of medicinal and aromatic plants. The major contribution of these medicinal plant products in
developed countries (e.g. USA, around 25%) but in developing countries (e.g. India), the contribution level is more than 80%. In other words, medicinal plant products showed more importance, especially is seen in India in terms of economy level as compared to the rest of the world. So, these medicinal plant products are applied in modern system of medicine, including the health care system, which is mainly depending on indigenous systems of medicine (Shelton et al., 2016; Claeson et al., 2000).

One of the medicinal plants, i.e. Mangifera (Mangoes; family Anacardiaceae) consists of 30 species and showed various medicinal properties. In literature, mangiferin (polyphenolic antioxidant; glucosyl xanthone) considered as a strong antioxidant along with immunomodulatory, anti-inflammatory and anti-diabetic activities. In addition, leaves and flower contained essential oil and its major components (i.e. humulene, elemene, ocimene, linalool, nerol etc.) as already mentioned in the literature whereas fruit pulp contains vitamins A and C, β-carotene and xanthophylls (Gupta and Chaphalkar, 2016b,c, 2015). In contrast, Syzygium cumini (Myrtaceae) possess medicinal properties (anti-hyperglycemic, hypolipidemiant, anti-inflammatory, cardio-protective, and antioxidant) and this activity is mainly due to the presence of bioactive compounds that are reported in different parts of the plant (Gupta and Chaphalkar, 2014; Bansode et al., 2016). The major objective of this study related to medicinal plant products should be drug development and provides a beneficial effect against various human pathogens or disorders. In this regard, we focused on various medicinal plant products, especially Mangifera indica and Syzygium cumini and exploring its primary metabolites, especially protein and determined its immunogenicity (using standard vaccine) and antioxidant potential.

MATERIALS AND METHODS

Collection of plant material

Fresh leaves of Mangifera indica and Syzygium cumini were collected in January 2020 from Graphic Era Deemed to be University, Dehradun. The identification of plant species was provided from Forest Research institute, India. The fresh leaves were dried in a shady area and then powdered and stored at 4°C until extraction and analysis.

Phytochemical analysis

Fresh leaves extracts were taken and macerated in PBS using mortar and pestle. The suspensions were shaken for 20 min at 4°C. After filtration, aqueous extract was collected and confirmed the presence of secondary metabolites (i.e. flavonoids, terpenoids, alkaloids and saponins) using various qualitative tests.

Extraction and determination of protein through HPLC

In this study, leaves powder (2 g) of Mangifera indica and Syzygium cumini were taken in two different flask and then add extraction buffer (i.e. 20 mM Tris HCl, pH 7.2) dissolved in PBS. Incubate leaves powder along with extraction buffer for 5 minutes at room temperature and then centrifuged (6000 rpm; 10 minutes at 4°C). After centrifuging, collect the supernatant and then add an equal or similar volume of ice cold acetone. Incubate the solution for 10-15 minutes at room temperature and then centrifuged. Collect the pellet and washed with ice cold acetone to remove the pigments, including lipids as well (Gupta and Chaphalkar, 2016a). Finally, the protein concentration of leaves powder was determined by using the Lowry method using BSA as standard. In contrast, proteins from these medicinal plants were also determined through HPLC (Agilent Technologies) with a photodiode array detector. The influence of wavelength (266 nm), mobile phase (acetone and methanol), column C18, flow rate (0.6 ml/min), run time (30 min), temperature (25 °C), sample load (20 μl) and solvent system used (acetonitrile and water in the ratio of 1:9) affecting the separation of proteins. Elution with acetone and methanol in a C18 (25 cm × 4.6 mm i.d.) column allowed us to separate proteins from medicinal plants with high resolution and short analysis time (Laemmli, 1970; Hames and Rickwood, 1990).

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To study the SDS-PAGE protein profile, unidimensional SDS-PAGE, (10% separating gel and 5% stacking gel) was carried out using a mini-vertical system. For this Mangifera indica (10 μl) and Syzygium cumini (10 μl), the protein was loaded in each sample well along with 10 μl sample buffer containing bromphenol blue as tracking dye. In this study, the marker was also incorporated into the gel to determine the molecular weight of the bands. The gels were run at a constant voltage of 100 V for 3 hrs followed by staining in coomassie brilliant blue overnight. Relative mobility (Rm) of the protein bands was determined, and Zymograms were constructed. The gel was photographed and stored in 3% acetic acid (Laemmli, 1970; Hames and Rickwood, 1990).

Immunogenicity assay

Informed consent letter was obtained from human healthy volunteers in order to assess its immuno-
Figure 1: HPLC analysis. Extraction of protein from leaves of medicinal plant products (Mangifera indica and Syzygium cumini) using Tris HCl and ice cold acetone

Figure 2: SDS PAGE. Lane A (Ladder; 10 \( \mu l \)), Lane B (Mangifera indica; 10 \( \mu l \)) and Lane C (Syzygium cumini; 10 \( \mu l \))

Antigenicity activity in human whole blood samples (10^6 cells/ml, 100 \( \mu l \)) using the variable concentration of protein from these medicinal plants. Lyses human whole blood samples were cultured for 24 h in carbon dioxide incubator with variable concentration of protein containing an optimized concentration of typhoid vaccine (25 \( \mu g/ml \); 10 \( \mu l \)) in phosphate buffered saline (PBS) containing heat-inactivated Fetal calf serum (FCS, 10 %) along with penicillin and streptomycin (100 \( \mu g/ml \)) in tissue culture 96 well plate. After incubation, MTT dye was added and further incubates for 2 h. centrifuging the plate, supernatant was discarded and observed formazan crystals settled at the bottom. Dissolve these formazon crystals using dimethyl sulphoxide (DMSO) solution and then analyzed its optical density (OD) at 570 nm (Gupta and Chaphalkar, 2015, 2014).

Antioxidant activity
This activity was assessed through DPPH free radical assay using protein from medicinal plants. In this assay, proteins from leaves were reacted with the stable DPPH radical in an ethanol solution.
Figure 3: Immunogenicity assay. Lyses human whole blood were cultured with variable concentration of protein from Mangifera indica and Syzygium cumini along with standardized concentration of typhoid Vaccine. Values were expressed as Mean ± S.E. The difference between control and treatment group is determined through one way ANOVA test (*P<0.05; **P<0.01 and ***P<0.001).

Figure 4: An antioxidant assay using DPPH. This assay was conducted to check the free radical scavenging ability of the extracts against DPPH free radical using fixed/optimized concentration of protein (i.e. 630 μg/ml and 580 μg/ml respectively) and its values are expressed as Mean ± S.E.

The reaction mixture consisted of protein (500 μl), absolute ethanol (3000μl) and DPPH radical solution (300 μl dissolved in 0.5 mM ethanol). For these studies, DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep violet to light yellow) were read [Absorbance (Abs)] at 517 nm after 100min of reaction using a UV-VIS spectrophotometer. For these studies, ethanol (3.3 ml) and sample (0.5 ml) selected as blank whereas control solution contained ethanol (3.5 ml) and DPPH radical (0.3 ml) solution. The scavenging activity percentage (AA%) was determined by using this equation i.e. Antioxidant activity = 100 - [(Sample absorbance - Blank absorbance) x100/absorbance control] (Kamboj et al., 2019).

**Statistical analysis**

The difference between control and treatment group of protein from these two medicinal plants is determined through one way ANOVA test (Bonferroni multiple comparison test).

**RESULTS AND DISCUSSION**

**Protein content and HPLC**
These studies showed the presence of protein content extracted from the leaves of *Mangifera indica* (580 μg/ml) and *Syzygium cumini* (630 μg/ml) which is confirmed through the Lowry test. Further confirmation of protein content on the basis of retention time through HPLC, as shown in Figure 1.

**SDS Page**

These studies revealed the presence of thick bands in case of *Mangifera indica* (45-55 kDa, 85-95 kDa and 130-145 kDa) and thin bands in *Syzygium cumini* (45-50 kDa, 90-95kDa, 130 kDa and 150 kDa) (Figure 2).

**Immunogenicity assay**

The effect of protein extracted from *Mangifera indica* and *Syzygium cumini* and determining its immunogenicity assay in whole human blood using typhoid vaccine as shown in Figure 3. The results revealed that protein at lower concentration showed immunogenic effect as compared to standard and control. Typhoid vaccine used as the standard for these studies and showed stimulation as compared to control.

**Antioxidant activity**

The protein was also evaluated for determining its antioxidant study. The antioxidant activity of the protein was investigated by DPPH assay and showed significant enhancement in protein content as compared to control (Figure 4).

The utility of various medicinal plant products which played an important role in Ayurveda, Unani and modern system of medicine. In general, more than 80% of the world population utilizes drugs, derived from medicinal plants for their health security. In Africa, India and other countries have rich floristic yielding herbal drugs. When we look into the world market where medicinal plant products are used in the form of drugs, pharmaceutical products, fragrances including flavours, dyes and other necessary ingredients (*Gupta et al.*, 2014; *Negi et al.*, 2003; *Gupta and Shah*, 2016; *Balaraju et al.*, 2009), these products are commercialized and its market value exceeds several billion dollars per year. In other words, these medicinal plants are beneficial for human health care and reported them as rich resources of ingredients in the form of primary and secondary metabolites which can be used in drug development (*Gupta et al.*, 2014).

A part from that, these medicinal plant products play a critical role in the development of human cultures around the whole world. Moreover, medicinal plant products are also considered them as a better source of nutrition and recommended for its therapeutic values. In view of these studies, we worked on crude extract of *Mangifera indica* and *Syzygium cumini* especially leaves for determining its primary metabolites especially protein and evaluating its molecular weight through SDS-PAGE and also observing its immunogenicity and antioxidant activity.

Phytochemicals are reported in medicinal plant products and these are identified as more effective analogues or agents against various infectious diseases as mentioned in the literature. In this regard, we determined the primary (proteins) and quantified its protein through the Lowry test and determined its molecular weight through SDS-PAGE. In contrast, HPLC is considered as one of the most valuable quality assessment tools for the assessment and evaluation of materials. In both cases, a protein extracted from crude leaves using SDS-PAGE is most economical and extensively used biochemical technique for the analysis of molecular weight of the proteins. From these studies, we found that *Mangifera indica* and *Syzygium cumini* plant leaves showed similar pattern of protein bands in the SDS-PAGE electrophoresis. In addition, greater attention has been given to immunogenicity (using typhoid vaccine) and antioxidant potential of *Mangifera indica* and *Syzygium cumini* using variable concentration of protein. In immunogenicity based studies, human whole blood exposed with variable concentration of protein along with standardized concentration of typhoid vaccine. These studies revealed the presence of protein at a very low concentration showed immunogenic effect against typhoid vaccine. In other words, these findings indicated that protein from these medicinal plants was immunogenic and capable to induce antigen specific immune response. These studies claimed as an alternative plant-based protein as adjuvant candidate from these two medicinal plants could be developed against typhoid vaccine.

In continuation of these studies, our group also determining its antioxidant potential of protein from medicinal plants through DPPH assay. This assay is mainly considered as the most valuable type of technique in order to assess its antioxidant profile through spectrophotometry method (*Kamboji et al.*, 2019). In other words, the DPPH assay (free radical method) is based on electron-transfer, which ultimately produces a violet coloured solution in ethanol. So, free radical is readily stable at room temperature, which is reduced in the presence of an antioxidant molecule, giving rise to colourless ethanol solution. Overall, these studies showed that protein from these medicinal plants exhibited antioxidant activity. In short, protein from leaves of *Mangifera indica* and *Syzygium cumini* showed
immunogenicity against typhoid vaccine and also claimed its antioxidant potential.

**CONCLUSIONS**

These studies suggested the presence of protein from these medicinal plants showed enhancement in antigen specific immune response against the typhoid vaccine and also claimed its antioxidant activity as well. Further immunological investigations of protein should be done through *in vivo* assessment in mice models for determining its cytotoxicity and also act as a way towards vaccine adjuvant development against infectious diseases.

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**Conflict of Interest**

No conflict of interest exists.

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