IN VITRO ANTI-SKIN AGEING BENEFITS OF CLERODENDRUM PANICULATUM LEAF EXTRACTS.

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ABSTRACT

Clerodendrum paniculatum (Family: Lamiaceae) or Hanuman kireetam is a well-known ayurvedic plant but less explored in India. The objective of the present study was to check the anti-skin ageing benefits of methanol and water extracts of C. paniculatum leaves In vitro. The Collagen-I, Elastin and Hyaluronidase assays was performed on Human dermal fibroblast cells (HDF). The antioxidant potential of C. paniculatum leaf water (CPW) and methanol extracts (CPM) was checked by reducing power assay. The CPM and CPW extract was non-toxic to the cells with CTC₅₀ above 200µg/mL. CPM and CPW showed good anti-oxidant activity comparable to Ascorbic acid at 200µg/mL. The Collagen and Elastin content was increased in the extract treated cells as compared to control. The Hyaluronidase inhibition confirmed increased hydration in cells for CPM and CPW respectively. HPLC chromatogram profile of CPM and CPW was developed using Acetonitrile: water as mobile phase in the ratio of 70:30. This study confirmed the presence of flavonoids, Quercetin and Rutin, as biomarkers in CPM extracts and CPW. Both water and methanol extract showed good skin anti-ageing activity in HDF. The study overall substantiates our objective of anti-skin ageing activity of CPW and CPM leaf extracts, and can be explored as potential ingredient in skin care formulations.

KEYWORDS: Clerodendrum paniculatum, Quercetin, Rutin, Anti-ageing.
INTRODUCTION

*C. paniculatum* (Pagoda flowers) (family- *Lamiaceae*) commonly known as *Hanuman kireetam* is a plant, widely distributed in the tropical and warm temperate regions of the world. It was reported as important source of diet in North eastern part of India. *Clerodendrum spp.* was reported for antimicrobial, antiinflammatory, antimalarial, antidiabetic, anticancer and analgesic activities. [1] Different chemical compounds have been reported in different species of *Clerodendrum* which includes Benzoic acid, Cabsurin, and Quercetin. [2] Different solvent extracts of leaves of *C. paniculatum* showed potent anti-microbial activity against selected Gram-negative bacteria. [3] The methanolic and chloroform extracts of the leaves of *C. paniculatum* has been reported for antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. [4]

In humans, the skin is the major visible organ and is affected due to ageing process. There are two different types of ageing, chronological ageing and photo ageing. [5] Collagen, Elastin and Hyaluronic acid levels decrease during ageing process, leading to a loss of elasticity and hydration in the skin which results in visible wrinkles. [6] Higher levels of reactive oxygen species triggers activation of Collagenase, Elastase and Hyaluronidase, which can further contribute to skin ageing. [7] The free radical scavenging activity and enzyme inhibitory activity of the *Clerodendrum* plant were reported for restoration of skin elasticity and slowing down the wrinkling process. [8] Polyphenolic antioxidants, mainly flavonoids are known to disrupt age-associated deterioration in physiological function and prevent many age-related diseases. [9] Flavonoids mainly Quercetin and Rutin are reported for their antioxidant activity and are modulator of cellular signaling processes to exert cell protective properties. They reduce the accumulation of the ageing marker, lipofuscin. [10]

The present study was aimed to evaluate the *C. paniculatum* leaf extract for restoration of skin Collagen, Elastin and Hyaluronic acid. In order to substantiate the efficacy of this extract, it was investigated by *in vitro* methods of reducing power assay, Collagen, Elastin ELISA and Hyaluronidase Inhibition assays. The extracts were standardized using HPLC for presence of flavonoids.
MATERIALS AND METHODS

Materials

Plant Material
Fresh leaves of *C. paniculatum* (Batch number. NPD/137/2014) were collected from Mangalore, Karnataka and identified as well as authenticated by Dr. Kannan, botanist, Department of Pharmacognosy, The Himalaya Drug Company, (Bangalore, India). The leaves of *C. paniculatum* were coarse powdered after complete shade drying.

Invitro Cell Culture Kit and Chemicals
The cell culture experiments were performed using Human dermal fibroblast (HDF) and Mouse embryo fibroblast (NIH 3T3), which were obtained from National Centre for Cell Sciences (NCCS, Pune, India). DMEM—high glucose (Sigma, Bangalore, India), fetal bovine serum (Sigma, Bangalore, India), Penicillin (100 U/mL) (Sigma, Bangalore, India) and Streptomycin (Hi-Media, USA), Sodium bicarbonate (Sigma, Bangalore, India), Phosphate buffer (pH 6.6) (Sigma, Bangalore, India), Trichloroacetic acid (Sigma, Bangalore, India), Ascorbic acid (Sigma, Bangalore, India), Collagen content kit (Blue Gene Biotech, China), Human Elastin kit (My Biosource, USA), Hyaluronidase kit (Krishgen Biosystems, USA), Acetic acid (Sigma, Bangalore, India), Rutin (Sigma, Bangalore, India), Quercetin (Sigma, Bangalore, India), Methanol (Merck, USA) and Acetonitrile (Merck, USA).

Extraction of water and methanol extract
Successive Methanol and water extracts of *C. paniculatum* were prepared by extraction of 500 g of air dried coarse leaf material. The herb solvent ratio was maintained at 1:6. The Methanol (CPM) and Water (CPW) extracts of the *C. paniculatum* was used for further study. The yield for CPM and CPW was found to be 20% w/w and 25% w/w respectively.

Cell viability in HDF and NIH 3T3 cells by MTT assay
The cytotoxic effect of CPW and CPM extracts was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide MTT assay in HDF and NIH 3T3 cells. Both the cells (1X10^4 cells/mL) were grown in 96 well plates in respective media treated with different concentration of CPW and CPM extracts of (200-1.56 µg/mL for 24 hours. The cells were added with 10 µL of MTT (5 mg/mL) to each well, followed by incubation for 4 hours at 37°C. The supernatant was removed and 100 µL of dimethyl sulfoxide (Merck) was added to each well to dissolve the Formazan product. Absorbance was determined at 540 nm spectrophotometrically using ELISA reader (Synergy HT multi detection micro plate reader).
The CTC$_{50}$ value of extracts cytotoxicity was calculated and non-toxic concentration were taken for further experiment.

**ANTIOXIDANT ASSAY**

**Reducing power assay**

The antioxidant potential was checked using reducing power assay on CPM and CPW extracts. 500 $\mu$L of Ascorbic acid (positive control) was made into concentration of 0-1000 $\mu$g/mL from 10mg stock solution by subsequent dilution method. The samples CPM and CPW were also made into similar concentration simultaneously. 500$\mu$L of 0.2 M phosphate buffer (pH6.6) was added to all 2mL tubes. 500$\mu$L of 1% Potassium ferricyanide solution was added to all the tubes. It was incubated at 50°C for 20 minutes. It was incubated and allowed to cool for 5 minutes at room temperature. 400$\mu$l of 10% Trichloroacetic acid was added to all the tubes. It was centrifuged at 10000 rpm for 20 minutes. 500$\mu$L of supernatant was transferred to fresh 2mL tubes. 500$\mu$L of distilled water was added to it. 300$\mu$L of these samples were transferred into 96 well plates. The optical density was noted at 700nm in ELISA reader.

**Collagen ELISA**

Collagen-I ELISA Kit was used for the quantitative measurements of collagen-I (Blue Gene Biotech, China) in cell supernatants of human dermal fibroblast (HDF). After treatment with the test samples, CPM and CPW, the HDF cells were incubated for 96 h at 37°C. The cells were harvested and the cell supernatant was used for ELISA. ELISA was carried out as per the instructions provided in the kit. Color developed in proportion to the amount of collagen, was measured at 450 nm. Ascorbic acid was used as standard at 100µg/mL concentration.

**Elastin ELISA**

Human Elastin (ELN) ELISA Kit was used for the quantitative measurements of Human Elastin (My Biosource, USA) in cell supernatants of human dermal fibroblast (HDF). After treatment with the test samples, HDF cells were incubated for 96 h at 37°C. The cells were harvested and the cell supernatant was used for ELISA. ELISA was carried out as per the instructions provided in the kit. Color developed in proportion to the amount of collagen, was measured at 450nm. The samples CPM and CPW were tested at 100µg/mL concentration.
Hyaluronidase assay

Hyaluronidase ELISA Kit was used for the quantitative measurements of Hyaluronidase (Krishgen Biosystems, USA) in cell supernatants human dermal fibroblast (HDF). After treatment with the test samples, HDF cells were incubated for 96 h at 37°C. The cells were harvested and the cell supernatant was used for ELISA. ELISA was carried out as per the instructions provided in the kit. Color developed in proportion to the amount of Hyaluronic acid, was measured at 540 nm. The samples were tested at 100 µg/mL concentration.

HPLC

HPLC was carried out to standardize the CPM and CPW extracts. 10 mg of standard Rutin and Quercetin was weighed in a 10 ml volumetric flask separately. 7 ml of methanol was added and sonicated to dissolve. The volume was made up to the mark with methanol. 0.2 ml of the stock solution was pipetted out into another 10 ml volumetric flask and the volume was made up with 50% methanol. The solution was filtered through 0.45µ syringe filter. The first 2mL of the filtrate was discarded and subsequent filtrate was used for the analysis. 500mg of the test substance (CPM and CPW) was weighed in a 250ml of round bottomed flask, 30ml of 50% methanol was added and refluxed at 80°C ± 2°C for 30 minutes. It was cooled to the room temperature and the extract was transferred to a 50ml volumetric flask. The volume was made up to the mark with 50 % methanol and the solution was filtered through 0.45µ syringe filter. The first 2mL of the filtrate was discarded and subsequent filtrate was used for the analysis.

The Shimadzu HPLC instrument with Column Ascentis Express C18 (100 X 4.6 mm), Particle size: 2.7µ was selected for the experiment. Mobile phase was developed using Acetonitrile: water in ratio of (70:30) and 0.2% Acetic acid in HPLC grade water. The flow rate was maintained at 1 mL per minute and the detection wavelength was identified at 354 nm. Column temperature was maintained at 40°C and runtime was established as 32 minutes. The HPLC instrument was stabilized with the mobile phase till the baseline was satisfactory. The working standard solution was injected and the chromatogram was recorded. The samples of CPM and CPW were injected separately and chromatogram was recorded against the individual working standards.

RESULTS AND DISCUSSION

*C. paniculatum* leaf methanol (CPM) and water extracts (CPW) was prepared using methanol and demineralised water. The cytotoxicity and antioxidant activity was performed to check
the preclinical safety and radical scavenging activity due to free radicals formed in cells. The Collagen content, Elastin content and Hyaluronidase activity was performed to substantiate the claim of human skin anti-ageing activity. The activities have been rationalized with HPLC analysis of the CPW and CPM with standard reference compound of Quercetin and Rutin. The HPLC profile confirmed the presence of Rutin and Quercetin in CPM and CPW. From ANOVA all the in vitro based activities were found to be significant (p<0.005) and least significant difference was calculated at 5% level of significance.

**Cytotoxicity assay of CPW and CPM extracts**

This assay was performed to assess the cytotoxicity of water and methanol extract of *C.paniculatum*. The cytotoxic activity of CPM and CPW was analyzed by MTT assay. The results showed that increase in toxicity concentration up to 200µg/mL did not reduce the cell viabilities in all tested concentration in NIH 3T3 cells and HDF cells. The toxicity at highest concentration tested (200µg/mL) for CPM and CPW was found to be 44.09 ± 0.107 % and 8.76 ± 0.141 in NIH 3T3 cells as shown in Fig. 1. The toxicity at highest concentration tested (200 µg/mL) in HDF cell lines as shown in Fig. 2 for CPM and CPW was found to be 40.65 ± 0.300 µg/mL and 34.77 ±0.320 µg/mL. The p value for CPM and CPW found to be p<0.0004 and 0.0001 respectively. The result was found to be statistically significant at P<0.05.

![Graph showing cytotoxicity assay of CPW and CPM extracts](image)

**Fig. 1**: Assessment of Cytotoxic activity of test sample on NIH 3T3 cell lines Data are represented as mean ± SEM (p≤0.05)
Reducing power assay

It was reported that antioxidants can reduce Fe$^{3+}$ to Fe$^{2+}$ by donating electrons. Amount of Fe$^{2+}$ can be assessed by measuring OD at 700 nm. The reducing capacity of a compound acts as a major indicator of antioxidant potential. The antioxidants have been also reported to have various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging. [11] This study confirms the correlation between antioxidant and anti-aging activity. The water and methanol extracts of *C. paniculatum* were checked for antioxidant activity using Ferric Reducing Antioxidant potential method. The figure explains the significant antioxidant activity. Reducing power is associated with antioxidant activity and serves as a significant reflection of the antioxidant activity. In the present study, CPM and CPW were evaluated for its reducing power activity. The results as shown in Fig. 3 confirmed that CPW and CPM had good anti-oxidant activity with absorbance of 2.247 ± 0.102 nm and 2.213 ± 0.101 nm at 250 µg/mL respectively. These samples showed the dose dependent increase in the reducing power activity at all concentration. Ascorbic acid was used as the standard in the present study, which showed the absorbance of 0.990 ± 0.102 nm at 250 µg/mL and showed the dose dependent increase in the reducing power activities at all the test concentrations.
Collagen ELISA for extracts
Collagen is reported to be the major component of the skin dermis, which gets degraded by the enzyme collagenase. Inhibition of collagenase enzyme delays the process of formation of pre-collagen fibers and leads to wrinkling. This study was performed to check whether there is increase in collagen content after treatment with CPM and CPW extracts. The individual methanol and water extracts of *C. paniculatum* (CPM and CPW) was used for collagen-I content determination. The cells treated with standard, Ascorbic acid (100µg/mL) showed 10.86 ± 0.141 ng/mL. *C. paniculatum* methanol extract(CPM) and *C. paniculatum* water extract (CPW) in Fig. 4 showed maximum collagen content of 11.97 ± 0.224 ng/mL and 11.78 ± 0.217 ng/mL of Collagen -1 respectively which is marginally higher compared to standard. The result was found to be statistically significant P≤0.05.

Fig. 3: Reducing power assay for methanol and water extracts of *C. paniculatum* compared to standard .Data are represented as mean ± SEM (p≤0.05) vs. control

Fig. 4: Collagen-I content determination for water and methanol extracts of *C. paniculatum* as compared to standard data are represented as mean ±SEM (P≤0.05)
Elastin ELISA for extracts
Elastic fibers of the dermis are reportedly composed of an elastin protein. Elastic fibers maintain their elastic function for a lifetime; various enzymes, mainly matrix metalloproteinases (MMPs), are able to cleave to elastic fiber molecules which lead to breakdown of elastin protein and leads to reduced elasticity of skin.\textsuperscript{[14]} The study was performed to check the elastin content in extracts. The individual water and methanol extracts of \textit{C. paniculatum} (CPW and CPM) was used for Human Elastin content determination. in Fig. 5 whereas CPW and CPM showed elastin content of 42.75 ± 0.14 and 46.95 ± 0.240 pg/mL so this extract can be used for further in vitro skin anti-ageing based studies. The result was found to be statistically significant P≤0.05.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{elastin_content.png}
\caption{Elastin content assay for Methanol and water extracts of \textit{C. paniculatum}}
\end{figure}

Hyaluronidase assay for extracts
Hyaluronic acid (HA) degradation in extra cellular matrix, leads to decrease in skin hydration with an eventual increased tissue permeability that is attributed for the spreading property. Hyaluronidase inhibitors are thus potent regulators that maintain HA homeostasis and are reported for anti-ageing and other age related disorders.\textsuperscript{[13]} The study was performed to check for Hyaluronidase inhibitory property of CPW and CPM extracts. The cells treated (100µg/ml) in Fig. 6 showed percentage inhibition of 37.4 ± 0.14 % and 34 ± 0.21 % for Hyaluronidase enzyme. The result was found to be statistically significant P≤0.05.
Fig. 6: Hyaluronidase inhibition assay for water and methanol extracts of *C. paniculatum*

**HPLC Chromatogram for CPM and CPW**

Flavonoids mainly Quercetin and Rutin are reported for their antioxidant activity and are modulator of cellular signaling processes to exert cellular protective properties.[10] In this study we have checked for presence of these flavonoids in CPM and CPW extracts which are reported for age defying benefits. The HPLC chromatogram of standards Quercetin and Rutin(Fig. 7 and Fig. 8) was compared with CPM and CPW as shown in Fig. 9 and Fig. 10 showed specific peak at retention time of 19.04 and 11.33 respectively. The content of Quercetin and Rutin in CPM was 0.06% w/w and 0.05% w/w whereas in CPM it was found to be 0.01% w/w and 0.02% w/w respectively.
CONCLUSIONS

This is the first study to investigate the in vitro anti-ageing activity of C.paniculatum leaf water and methanol extracts. The free radical scavenging activity by reducing power assay and ELISA based assays of Collagen-I, Elastin and Hyaluronidase inhibition suggest that they can help restore skin elasticity, hydration and thereby prevent the wrinkling process. The plant extracts was found to be non-toxic at tested concentration and CTC$_{50}$ value of CPM and CPW extracts was found to be above 200μg/mL. The plant extracts promote synthesis of Collagen-I and Elastin content in HDF cells. They also inhibited Hyaluronidase which thereby increases skin hydration in HDF cells. The results of HPLC confirmed presence of flavonoids which also plays major role in antioxidant activity which has direct correlation to anti ageing activity. The in vitro results suggest that CPM and CPW extracts are potent anti-ageing herbal ingredient for building skin structural proteins targeting mainly Collagen, Elastin and Hyaluronic acid. The extract as such or in combination with other polyherbal
blend in topical formulation can be an effective formula to prevent damage of the skin for different clinical conditions mainly for skin vitality and rejuvenation.

DECLARATION OF INTEREST
The authors report no declarations of interest.

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