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IN-VITRO ANALYSIS OF *CINNAMOMUM VERUM* FOR FORMULATION OF BIO-ACTIVE COSMETIC GEL

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Keywords:

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ABSTRACT: Plants have always been traditionally used to address various disorders. Due to their rich phytochemical profile, they have been employed in a gamut of skin care and concomitant treatment of disorders. In this Study, we screened the aqueous (CAE), hydroalcoholic (CHE) and ethanolic (CEE) extract of leaves of *Cinnamomum verum* for its activities The antioxidant potential of the extracts were determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Potential) assays. The extracts showed good activity with increasing concentration. Further, extracts were screened for anti-Inflammatory property by HRBC membrane stabilization assay and Inhibition of Protein Denaturation. The results of anti-inflammatory activity were compared with standard control values. CHE was found to exhibit better activities as compared to the other two extracts and was subsequently screened for Tyrosinase inhibition, Elastase inhibition and Hyaluronidase inhibition activity. CHE showed moderate anti-inflammatory and Hyaluronidase-inhibition activity. CHE can therefore be used as a potential bioactive component in skin care products.

INTRODUCTION: India is the largest producer of medicinal herbs. [1] Rigveda, one of the oldest ayurvedic literatures written around 2000 B.C mentions the use of Cinnamon (*Cinnamomum verum*), Ginger (*Zingiber officinale)*, Sandalwood (*Santalum album*) and others in medicinal preparations [2]. Cinnamon has been used for many purposes According to World Health Organization (WHO), more than 80% of the world's population relies on traditional medicines for their primary health care requirements [3].

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The term cinnamon commonly refers to the dried bark of *C. zeylanicum* and *C. aromaticum* used for the preparation of different types of chocolate, beverages, spicy candies and liquors. Moreover, cinnamon is used in various savory dishes, pickles, soups, and Persian sweets. Cinnamon bark, leaves, flowers and fruits are used to prepare essential oils, which are destined for use in cosmetics or food products. Moreover, according to traditional Chinese medicine (dating roughly 4000 years), cinnamon has been used as a neuroprotective agent and for the treatment of diabetes. [4] Free oxygen and reactive oxygen species (ROS) are constantly formed in the human body. Free-radical mechanism is implicated in pathology of certain human diseases including cancer, malaria, atherosclerosis rheumatoid arthritis, neurodegenerative diseases and in inflammation [5].

Studies also revealed that the phenolic compounds present in the spices and herbs play a vital role in their antimicrobial activities. Growing interest with this in herbal medicines, many phytochemical bioactive compounds from different medicinal plants have shown many pharmacological activities including anti-inflammatory activity and anti-oxidant activity [6].

Several studies have demonstrated that both skin-aging and anti-wrinkle effects are significantly correlated with decreased elastase activity. Damage to the skin is believed to lead to reduced skin elasticity and the linearity of dermal elastic fibers, inducing wrinkling and sagging. Elastase is the enzyme that is capable of breaking down elastin, an insoluble elastic fibrous protein that, together with collagen, determines the mechanical properties of connective tissue [7].

There is an imperative need for the development of novel, potent, safe and cheap cosmetics from herbal products as anti-ageing agents [8].

Although Hyaluronidase is enzyme that destroys the natural hyaluronic acid created in the body, therefore disabling it from binding collagen and elastin molecules. This prevents the formation of new collagen and elastin and causes premature wrinkling and sagging. Levels of Hyaluronidase are increased by free radicals [7]. It has been desired to develop a material capable of inhibiting hyaluronidase without causing any toxicity to human, notably in the absence of irritation or any other adverse effects on the skin [8].

Plant extracts that have a good inhibitory effect on melanin formation may be a good choice for the cosmetic purposes of whitening facial skin and protection against skin darkening. In addition, they have relatively fewer side effects [10]. The main action behind the pigment-reducing effect of plant extracted flavonoids may be the ROS-scavenging properties and the ability to chelate metals at the active site of metalloenzymes [11].

MATERIALS AND METHODS:

Materials: All the chemicals used were of analytical grade, Spectrophotometer (Cary 50, Varian), SCDA and SDA (HiMedia), Bovine Serum Albumin (Sigma Aldrich)

Methods:

1. Collection of Plant Material: The plant *Cinnamomum verum* was collected from a botanical Garden in Kopri, Thane District, Maharashtra in the month of January 2016. The leaves were taken and they were shade dried at room temperature. Later the plant material was powdered separately. The powder was then subjected to extraction of phytochemicals and these extracts were used throughout the investigations [11].

2. Extraction using different solvents: Extraction was carried out using Soxhlet apparatus. The dried powdered leaves were weighed. 15 grams were used for each of the extraction using solvents distilled water, ethanol: water (70:30) and ethanol. All the three extracts were kept at room temperature throughout the investigations.

3. Phytochemical Screening: All the extracts were used directly (as such) for each of the following tests.

Test for flavonoids: To 2 mg of the extracts few drops of NaOH was added to give intense yellow colour, which further decolorizes on addition of few drops of concentrated HCl, confirms the presence of flavonoids.[12]

Test for saponins (Foam Test): To 2mg of the extracts, 5mL of distilled water was added and shaken for the formation of froth which confirms the presence of saponins. [14]

Test for cardiac glycosides: 2mg of extracts was treated with 2 mL of glacial acetic acid containing a drop of $FeCl_3$ solution. This was under layered with 1 mL of concentrated H_2SO_4. A brown ring obtained at the interface indicates the presence of de-oxy sugar characteristics of cardenolide.[13]

Test for terpenoids: 2 mg of extracts was treated with 2 mL of chloroform and concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colour formation at the interface confirms the presence of terpenoids. [12]

Test for tannins: 2mg of extracts was boiled in 2mL of water for 5-10 minutes and filtered. Ferric chloride (0.1%) was added to this and a brownish green or blue black colouration formed confirms the presence of tannins.[14]

Test for phenols (Ferric Chloride Test): To 10mg of the extracts add 3-4 drops of $FeCl_3$ was added and was checked for the appearance of bluish black precipitate. [14]

4. Anti-oxidant activity:

a. DPPH Assay: Radical scavenging activity of plant extracts against stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined by the slightly modified method of Brand-Williams *et al.*, 1995. The experiment was carried out in triplicates. DPPH reacts with an antioxidant compound, which can donate hydrogen, and reduce DPPH. The change in colour (from deep violet to light yellow) was measured at 517 nm on a UV visible light spectrophotometer [15].

The solution of DPPH in methanol 6×10^{-5} M was prepared fresh addition. 0.5 mL of this solution was mixed with 0.5 mL of different extracts of plants prepared in methanol with concentration ranging from 0.1mg/mL to 1mg/mL for CAE, 0.1mg/mL to 0.5mg/mL for CHE and 0.1mg/mL to 0.5mg/mL of CEE. The samples were kept under dark conditions for 30 minutes at room temperature and the decrease in absorbance was measured at 517nm. The negative control consisted of 0.5mL DPPH in 0.5mL of methanol. Percent radical scavenging activity was calculated by the following formula.

$$\text{Percent Radical Scavenging Activity} = \frac{\text{ABSORBANCE OF NEGATIVE CONTROL} - \text{ABSORBANCE OF SAMPLE}}{\text{ABSORBANCE OF NEGATIVE CONTROL}} \times 100$$

b. FRAP Assay: The reducing power of the individual plant extracts as well as their mixture was determined according to the method of Oyaizu in 1986. The experiment was carried out in triplicates. 0.5 mL of different extracts of plants prepared in methanol with concentrations ranging from 0.1mg/mL to 1mg/mL for CAE, 0.1mg/ mL to 0.5mg/ mL for CHE and 0.1mg/mL to 0.5mg/ mL for CEE was mixed with 0.5 mL of 0.2 M sodium phosphate buffer (pH 7.4). The mixture was then incubated at 50 ^{o}C for 20 minutes. After incubation, 0.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 minutes. 0.5 mL of supernatant was mixed with distilled water (0.5 mL) and ferric chloride (0.1 mL, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Colour blank were prepared in a similar way but without the addition of Ferric Chloride .[16]

5. Total Viable Count (TVC): The TVC was estimated by the pour plate method. Stock solutions of 1mg/mL of all the extracts were prepared in 0.9% Saline. From this stock working solutions of 0.1mg/mL and 0.01mg/mL were prepared. These two dilutions were further used to check bioburden by total viable count. Both the dilutions of all the extracts were poured in sterile Petri dishes aseptically. 20mL media of SCDA (Soybean Casein Digest Agar) and SDA (Sabouraud Dextrose Agar) were added to the plates over the plant extracts-saline solutions. Plates were rotated to ensure complete mixing of the agar and sample. Agar was solidified at room temperature. Plates were incubated for 72 hours at 37 °C for SCDA and room temperature for SDA plates.[17]

Agar and Diluent Sterility Control: Each batch of agar or diluent used for plating was evaluated to determine that the media or diluent is sterile. Two plates were prepared by pouring 20mL of molten, tempered agar SCDA and SDA into sterile Petri dishes. Agar was allowed to solidify. Plates were incubated at respective temperatures. After the 72 hour incubation period, plates were checked for microbial growth.[17]

6. Anti-Inflammatory activity:

a. *In vitro* Human Red Blood Cells (HRBC) membrane stabilization assay: The anti-inflammatory activity of various extracts of leaves of *Cinnamomum verum* was assessed by *in vitro* HRBC membrane stabilization method. Blood was collected from Pathology lab.

Preparation of HRBC suspension: The collected blood was mixed with 1M PBS (pH 7.40) (1:4 *i.e.* Blood: PBS) and was centrifuged at 4 °C at 2700rpm for 2 minutes. The concentration of HRBC suspension required for assay is $1x10^8$cells/mL.

Different concentration ranging from 0.1mg/mL to 1mg/mL for CAE, 0.1mg/mL to 0.5mg/mL for CHE and 0.1mg/mL to 0.5mg/mL of CEE were prepared in hyposaline and 0.5mL of each of these were added to a mixture containing 0.025mL of

HRBC suspension, 0.475mL of hyposaline (0.25%). All the assay mixtures were incubated at 37°C for 30 minutes and centrifuged at 1700rpm for 10mins. The negative control consisted of 0.025mL of HRBC suspension and 0.975mL of hyposaline. The negative control was subjected to same experimental conditions as the sample. The released hemoglobin content was measured spectrophotometrically at 560nm.[19]

The percentage of protection was calculated then by the formula as given below:

$$\text{Total Protection} = \frac{\text{ABSORBANCE OF NEGATIVE CONTROL} - \text{ABSORBANCE OF SAMPLE}}{\text{ABSORBANCE OF NEGATIVE CONTROL}} \times 100$$

b. Protection against Protein Denaturation Assay*:* The experiment was described by the method described by Banerjee *et al.*, 2011 with slight modifications. Different concentration of the extract ranging from 0.1mg/mL to 1 mg/mL for CAE, 0.1mg/mL to 0.5mg/mL for CHE and 0.1mg/mL to 0.5mg/mL of CEE were prepared in phosphate buffer saline (pH 6.3). The reaction mixtures (0.5mL) consisted of 0.4mL bovine serum albumin (1% aqueous solution) and 0.1mL of plant extracts (different concentrations). pH was adjusted at 6.3 using a small amount of IN HCl. The samples were incubated at 70 °C for 10 min. After cooling the samples 0.5 mL phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 280nm. For negative control 0.1 mL distilled water was used instead of plant extracts.[19]

The percentage Protection against Protein Denaturation Assay was calculated as:

$$\text{Total Protection} = \frac{\text{ABSORBANCE OF NEGATIVE CONTROL} - \text{ABSORBANCE OF TEST}}{\text{ABSORBANCE OF NEGATIVE CONTROL}} \times 100$$

7. Statistical Analysis: For each result mean and standard deviation was calculated.

RESULTS AND DISCUSSIONS:

1. Nature and Colour of Extracts of *Cinnamomum verum*:

TABLE 1: NATURE AND COLOUR OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

Extract	Texture	Colour	Percent Yield
Cae	Sticky	Brown	13.33%
Che	Sticky	Dark Yellow	15.08%
Cee	Sticky	Greenish Brown	08.49%

2. Phytochemical Profile:

TABLE 2: PHYTOCHEMICAL PROFILE OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

Test No	Tests	CAE	CHE	CEE
1	Flavonoids	+	++	++
2	Saponins	++	+	-
3	Tannins	+	++	++
4	Terpenoids	+	++	-
5	Phenols	+	++	++
6	Cardiac glycosides	++	+	+

Key: (+ = Less), (++ = Abundant), (- = Absent)

The phytochemical screening showed different components in different quantities in the three extracts of *Cinnamomum verum.* The CEE contained more phenolic compounds than leaf aqueous extract and leaf hydroalcoholic extracts. The CEE has a similar trend to the leaf ethanolic profile found in the literature [20]. The presence of these phytochemicals viz. Flavonoids, terpenoids, tannins, cardiac glycosides is associated with medicinal values such as antioxidant, anti-inflammatory, anti-diabetic ability of plant extracts.

3. Anti-Oxidant Assay:

a. DPPH Assay: The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical scavenging activity. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, it then leads to a loss of this violet colour. Free radical scavenging activities of the various extracts are presented in **Table 3**.

TABLE 3: DPPH ASSAY SHOWING MEAN ANTIOXIDANT ACTIVITY OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

Concentration mg/mL	CAE	CHE	CEE
0.05	17.00 ± 0.021	42.37 ± 2.821	68.45 ± 1.484
0.1	33.46 ± 1.173	73.19 ± 0.466	82.81 ± 0.021
0.2	59.93 ± 0.707	93.55 ± 0.586	94.74 ± 0.162
0.5	90.88 ± 2.644	98.03 ± 2.715	94.62 ± 1.965

All data are shown as the means ± SD for triplicate determination in same extracts

A lower IC_{50} value indicates higher antioxidant activity. [21] The CEE exhibited remarkable antioxidant activities. The IC_{50} of CEE, CHE and CAE were 0.0421mg/mL, 0.0597mg/mL and 0.1831mg/mL, respectively. The IC_{50} value of the

standard Ascorbic acid was 0.0457mg/mL. In this study, DPPH radical scavenging activity of test samples increased with increasing its concentration**.** So the DPPH Radical Scavenging Ability of different leaf extracts and Standard Ascorbic acid was in the order,

CEE> Std ascorbic acid>CHE >CAE

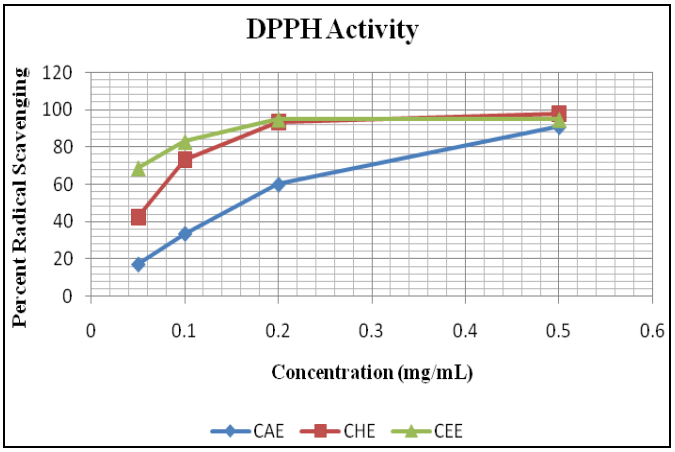

FIG. 1: GRAPH SHOWING PERCENT SCAVENGING ACTIVITY OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

b. FRAP assay: A simple, automated test measuring the ferric reducing ability of the essential oils, the FRAP assay, is presented as an accurate method for assessing "antioxidant power." Ferric to ferrous ion reduction at low pH causes a coloured ferric-ferrous complex to form. FRAP values are obtained by comparing the absorbance change at 700nm in test reaction mixtures with those containing ferrous ions in known concentration. The FRAP value of the *CAE, CHE and CEE* increased with increasing concentration. FRAP assay is widely used in the evaluation of the antioxidant component in dietary polyphenols. [21]

TABLE 4: FRAP ASSAY SHOWING ANTIOXIDANT CAPACITY OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

Concentration mg/mL	CAE	CHE	CEE
0.1	0.23 ± 0.024	0.23 ± 0.006	0.34 ± 0.020
0.2	0.36 ± 0.022	0.35 ± 0.005	0.53 ± 0.002
0.3	0.42 ± 0.032	0.45 ± 0.041	0.57 ± 0.028
0.4	0.50 ± 0.013	0.54 ± 0.008	0.95 ± 0.034

All data are shown as the means ± SD for triplicate determination in same extracts

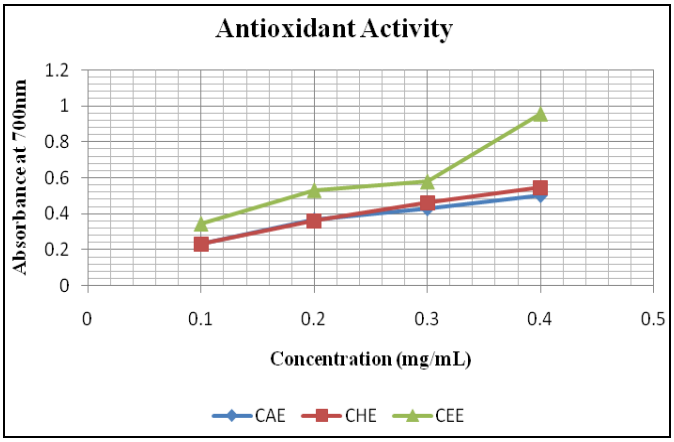

FIG 2: GRAPH SHOWING FRAP RESULTS OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

In a paper presented by Mazimba *et al.*, 2015, the DPPH scavenging ability and reducing power (FRAP) depended on the phytochemical profile and was found that, the stem bark methanolic extract (IC_{50}=0.765mg/mL) had better DPPH scavenging ability than leaves methanolic extract (IC_{50}= 0.100mg/mL). [22]

3. Total viable count: Bioburden level indicates the quality of the plant extracts. The plates were incubated for a total period of 48 hours. Absence of bacterial and fungal growth indicated the absence of microbial contamination in the investigational samples.

TABLE 5: TOTAL VIABLE COUNT FOR *CINNAMOMUM VERUM* LEAF EXTRACT FOR CHECKING THE PRESENCE OF BACTERIAL/ FUNGAL CONTAMINATION

Extract used for test	Test for bacteria		Test for fungi-moulds	
	0.1mg/mL	0.01mg/mL	0.1mg/mL	0.01mg/ml
CAE	No Growth	No Growth	No Growth	No Growth
CHE	No Growth	No Growth	No Growth	No Growth
CEE	No Growth	No Growth	No Growth	No Growth

4. Anti Inflammatory Assay:

a. *In vitro* HRBC membrane stabilization assay: From the present study, the leaf extract of *C. verum* was subjected to *in vitro HRBC membrane stabilization assay* for various concentrations. The percentage stabilization of different extracts is shown in the table below. [23] The extract demonstrated a significant anti-inflammatory activity at all the concentrations tested as compared to control (Ibuprofen). The percentage membrane stabilization increased with the increase in concentration of the extract.

It was found that CHE (IC_{50}=0.164mg/mL) had better protection ability as compared to the CEE (IC_{50}=0.401mg/mL) and CAE (IC_{50}=1.45mg/mL). So, the percent protection ability of the three plant extracts and standard ibuprofen (IC_{50}=0.340 mg/mL) was in the order,

CHE > Ibuprofen> CEE >CAE

TABLE 6: HRBC MEMBRANE STABILIZATION ASSAY SHOWING ANTI-INFLAMMATORY ACTIVITY OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

Concentration mg/mL	CAE	CHE	CEE
0.2	16.51 ± 0.678	57.28 ± 0.254	37.35 ± 0.629
0.4	17.87 ± 0.202	57.84 ± 0.282	51.85 ± 0.084
0.6	21.33 ± 0.471	60.03 ± 0.028	58.69 ± 0.841
0.8	26.42 ± 0.466	62.69 ± 1.972	72.56 ± 0.636
1.0	40.02 ± 2.446	66.96 ± 0.452	77.50 ± 0.424

All data are shown as the means ± SD for triplicate determination in same extracts

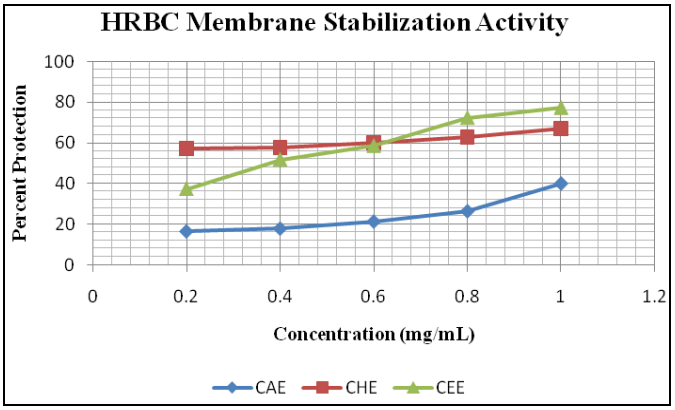

FIG. 3: GRAPH SHOWING HRBC MEMBRANE STABILIZATION CAPACITY OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

In a paper presented by Jain *et al.*, 2012, the in-vitro anti-inflammatory activity in various concentrations of bark extract of *Cinnamomum zeylanicum* was checked and the IC_{50} value was found between the concentrations 0.4 -0.6 mg/mL. [23]

4b. Inhibition of Protein Denaturation Assay: The percentage inhibition of various concentrations of the three extracts of *Cinnamomum verum* is given in the table below (**Table 6**). [23] These values were compared with standard Ibuprofen. The percent inhibition increased with increased concentration. The CHE exhibited an IC_{50} value of 0.87mg/mL followed by CEE and CAE with IC_{50} values of 0.929 mg/mL and 2.7 mg/mL respectively. The activity of all these extracts were comparable with that of standard ibuprofen, which had an IC_{50} value of 1.98mg/mL

CHE > CEE > Std ibuprofen > CAE

TABLE 7: INHIBITION OF PROTEIN DENATURATION ASSAY SHOWING ANTI-INFLAMMATORY ACTIVITY OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

Concentration mg/mL	CAE	CHE	CEE
0.2	09.22 ± 0.098	20.99 ± 0.784	14.92 ± 0.304
0.4	28.74 ± 0.120	25.45 ± 0.141	16.01 ± 0.434
0.6	36.16 ± 0.721	38.05 ± 0.671	17.92 ± 1.244
0.8	43.13 ± 0.615	40.41 ± 2.276	20.44 ± 0.084
1.0	50.07 ± 0.410	61.76 ± 0.579	26.50 ± 0.721

All data are shown as the means ± SD for triplicate determination in same extracts.

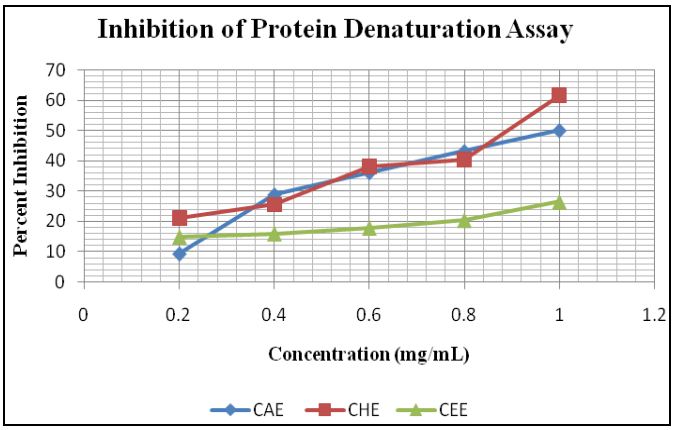

FIG. 4: GRAPH SHOWING INHIBITION OF PROTEIN DENATURATION OF ALL THREE EXTRACTS OF *CINNAMOMUM VERUM*

In a paper presented by Jain *et al.*, 2012, *in-vitro* anti-inflammatory activity by Inhibition of Protein Denaturation Method in various concentrations of bark extract of *Cinnamomum zeylanicum* was checked and the IC_{50} value was found between the concentrations 0.2mg/mL -0.4 mg/mL.[23]

CONCLUSION: In the present study, *Cinnamomum verum* was investigated for its anti oxidant, anti inflammatory and inhibition of protein denaturation potential. Further, its cosmetic

potential was evaluated by means of inhibition studies against Tyrosinase, Elastase and Hyaluronidase The phytochemical profile showed different components in different samples extracted in different solvents (Water, Ethanol, 70% Ethanol). The highest anti-oxidant activity and ferric reducing potential was found in leaf ethanol extract followed by leaf hydroalcoholic and leaf aqueous extracts. Whereas highest anti-inflammatory activity was reported in leaf hydroalcoholic extract. The extracts showed absence of bacterial and fungal contamination.. The extracts showed low anti microbial activity against the tested pathogens. From the results it was found that CHE had good anti-inflammatory activity and also good anti-oxidant activity.

So, the CHE was screened for anti-aging, ski whitening and moisturizing capacity. The extract therefore shows potential to be used in formulations for skin care, namely skin lightening, anti ageing and increasing the moisturization. The hydroalcoholic extract was further used to prepare a water-based gel which was tested for pH, consistency in colour and appearance. The results obtained revealed the possibility of using the test plant as natural sources of antioxidants, anti-inflammatory agent, skin whitening agent, anti-ageing agent and moisturizing agent which may be beneficial for human skin care and health care. These activities of *Cinnamomum verum* also indicate that routine flavoring of food with spices could help the body to relieve oxidative stress as well.

Further studies need to be performed to understand the extract mechanisms such as wound healing, cytotoxicity, among others. Further scope is to screen the gel for clinical trials.

CONFLICT OF INTEREST: The manuscript is an original article which has not been submitted for publication elsewhere. All the authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects.

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