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Evaluation of anti-elastase and antioxidant activity in antiaging formulations containing terminalia extracts

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ABSTRACT
Most *in vitro* efficacy studies of creams are based on estimation of active constituents as solubility is the limiting factor. Our aim was to establish a method for efficacy testing of both anti-elastase and antioxidant activity in final formulations and to compare the same with the activity of the active ingredients to ascertain whether the native activity remains unaffected, enhanced or is compromised in any way. The actives evaluated in the antiaging formulations included the crude extracts of *Terminalia arjuna* and *Terminalia chebula*, either alone or in combination on the activity of porcine pancreatic elastase and to reduce 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). The results showed that the *T. arjuna* formulation had higher inhibitory activity of elastase (IC_{50} 6.6 mg/ml) while *T. chebula* formulation depicted better antioxidant activity (IC_{50} 0.032 mg/ml). The combination cream had excellent anti-elastase (IC_{50} 2.8 mg/ml) and antioxidant properties (IC_{50} 0.14 mg/ml). Further the cytotoxicity of the two extracts was tested on 3T3 cell line and both extracts were found to have negligible inhibition up to 80 µg/ml.

Keywords: anti-elastase, antioxidant, DPPH, antiaging, *Terminalia arjuna*, *Terminalia chebula*, cytotoxicity

1. Introduction
The two distinct types of aging that exist may be due to genetic inheritance implicated in intrinsic (internal) involving cellular senescence and extrinsic (external) aging, such as photoaging [1, 2]. A consequence of both however is the production of free radicals at whose door the blame is laid for the loss of skin elasticity; one of the classical aging characteristics. Skin firmness and elasticity is mainly contributed to by elastin [3], a dermal protein which is a constituent of the connective tissue (CT). Over time, the metabolism of the CT proteins slows down accompanied by an increase in enzymatic activity, particularly elastase, which breaks down elastin [4]. One way to prevent such a loss of elasticity is to use active ingredients that are able to inhibit these enzymes [5] and we concentrated on evaluation of the anti-elastase activity.
Another molecule of interest is the antioxidant which functions by contributing an additional oxygen molecule to free radicals. In the absence of antioxidants, the free radicals will acquire an oxygen molecule from another biological molecule and this leads to tissue damage and eventually to skin aging. Kim and colleagues [6], have described the various reactive oxygen species (ROS) which cause skin damage. For the most part, free radicals are chemically unstable and are a normal by-product of metabolic processes, but have a negative effect on the body as they are detrimental to cells. It is thus logical to incorporate compounds with free radical scavenging activity in an antiaging formulation [7]. We estimated antioxidant activity by the DPPH assay. DPPH is a stable free radical in methanol and is purple-coloured. The odd electron in DPPH becomes paired with hydrogen from a free radical scavenging antioxidant to form reduced DPPH-H. The resultant decolorization from purple to yellow can be detected spectro-photometrically.
Various studies have already reported multi faceted biological activities of the *Terminalia* species, the anti-oxidant and anti-diabetic potential among the most commonly reported [8, 9, 10]. Others have rationalized that a formulation containing *Terminalia* extracts would be highly beneficial in an anti-aging and skin protective context [11, 12]. The majority of *in vitro* studies on anti-elastase and antioxidant activities are based on the evaluation of the actives. We incorporated the ethanolic extracts of *T. arjuna* and *T. chebula* in anti-aging formulations and

comparatively evaluated the *in vitro* anti-oxidant activity and anti-elastase activities of the same against the actives. This would bring further insight for efficacy and pre-clinical studies before clinical trials are undertaken.

2. Materials and methods

i. Preparation of extracts

The dried bark peels of *T. arjuna* and dried fruits of *T. chebula* were powdered, weighed and extracted with 50% ethanol at 50 °C. The hydrolysates were filtered through Whatman's filter paper (number 41), evaporated to dryness, powdered and stored under dry conditions. *Areca catechu* was powdered and subjected to cold extraction in 70% ethanol. The filtrate was filtered, vacuum dried and stored under dry conditions.

ii. Chemicals

Porcine pancreatic elastase (PPE), N-succinyl-(Ala) 3-p-nitroanilide, DPPH and α-tocopherol were purchased from Sigma. Culture media DMEM, trypan blue and Sulforhodamine B (SRB) were procured from Sigma St. Louis, MO, USA. Fetal bovine serum (FBS), Biowest, [Cat. No. S1810], penicillin-streptomycin was from Cell clone [Cat No. CC4007]. All other reagents were from Qualigens (Analytical grade) or Merck.

iii. Formulation

Table 1: The cream was formulated as per Table 1.

Ingredients	100 gm
Stearic acid	7
Cetyl alcohol	0.8
Stearyl alcohol	0.8
Mineral oil	3
Silicone oil	2
Glyceryl monostearate	2
Beeswax	1
Propyl paraben	25
Methyl paraben	0.25
Triethanolamine	1
Glycerine	4
EDTA	0.1
Water	q.s.

Different ways of incorporation of extracts were tested. It was found that incorporating the extracts at 45 °C after completion of the emulsification process was suitable and this was adapted for all formulations.
The *T. arjuna* and *T. chebula* extracts were incorporated at 20% in the formulation while the combination cream had *T. arjuna* and *T. chebula* in a proportion of 1:2 and integrated at 20%.

iv. Anti-elastase activity

Antielastase activity was evaluated spectrophotometrically as per Lee *et al*., [13] with minor modifications. In brief, the kinetics of the enzymatic conversion of N-succ (Ala) 3-Nitroanilide to p-nitroaniline by PPE was measured spectrophotometrically at 405 nm (Cary UV 50).
For the creams, different solubilisation procedures using Methanol, Ethanol, Iso-propyl alcohol, Chloroform, Cremaphore and Acetone were used either neat or in various proportions with the Tris-HCl buffer. The one that worked best without loss of enzymatic activity was 70% Acetone. The final reaction mixture contained 700 µl of 100 mM Tris-HCl buffer (pH 8.0), 100 µl test sample dissolved in 70% acetone, 50 µl of 0.1 mg/ml PPE and 40-50 µl 0.2 mM N-succ (Ala) 3- Nitroanilide as substrate. Pre-incubation was for 20 minutes. The test reaction contained different concentrations of the samples instead of the buffer and was assayed by starting the reaction with the addition of substrate and incubating for 30 minutes at 25 ºC. Appropriate blanks containing the test reaction without the enzyme were concurrently run. The control reaction was the uninhibited enzymatic reaction where the test sample was replaced by 100 µl of 70% acetone. The amount of enzyme left uninhibited was detected by reacting it with the substrate to give the final product. Higher the amount of product formed, lower was the inhibitory effect of the sample.

A. catechu dissolved in 70% acetone was used as the positive control [14].

The calculation was as follows:

% anti-elastase activity =

$$\frac{[\text{OD of Control} - (\text{OD of Sample} - \text{OD of blank})]}{\text{OD of Control}} \times 100$$

The results were expressed as mean ±SD of three independent experiments in triplicates.
The concentrations of the samples versus % anti-elastase activity were plotted for the IC_{50} values.

v. Antioxidant activity

The free radical scavenging activity was assayed spectrophotometrically as per the method described by Blois [15] and modified as per Nia *et al*., [16]. Briefly, 9 X 10^{-5} M solution of DPPH in methanol was added 1:1 to the test samples. The stock concentration of 10mg/ml was diluted to different testing concentrations for each sample. The reaction was allowed to proceed at room temperature under dark conditions and the OD was read at 519nm after 30 minutes in the UV/Vis spectrophotometer (Cary UV 50). α-Tocopherol was used as the positive control. Appropriate blanks of the test samples were used. The control reaction was carried out with the solvent replacing the test sample.
The calculation was as follows:

% DPPH radical scavenging activity =

$$\frac{[\text{OD of Control} - (\text{OD of Sample} - \text{OD of blank})]}{\text{OD of Control}} \times 100$$

The results were expressed as mean ±SD of three independent experiments in triplicates. The concentrations of the samples versus % DPPH radical scavenging activity were plotted for the IC_{50} values.

vi. Cytotoxicity on 3T3 cell line

The cell line used in this work was NIH 3T3 procured from the National Centre for Cell Science (NCCS), Pune, India;

and propagated in DMEM supplemented with 1% penicillin-streptomycin and 10% heat-inactivated FBS at 37 ºC in a humidified incubator at 5% CO_2. Cell viability was ascertained by the trypan blue dye exclusion method [17] followed by the cytotoxicity experiments by the Sulforhodamine B (SRB) assay [18]. The SRB assay was based on total well protein content at 24 hours. Cells were seeded at a density of 1×10^5 cells/ml in 96-well flat-bottomed tissue culture plates (Nunc) and pre-incubated for 24 hrs.

After treatment with a range of concentrations of both the extracts and using Sodium Dodecyl Sulphate (SDS) as the positive control, cells were fixed with 30% TCA, processed for SRB assay and read at 540 nm (Thermo Labsystems; Ascent Software). The experiments were set up in triplicates and repeated at least five times.

The calculation of Percent Cell Viability was as follows:

$$\text{Percent Cell Viability} = \frac{\text{O.D. of test}}{\text{O.D. of control}} \times 100$$

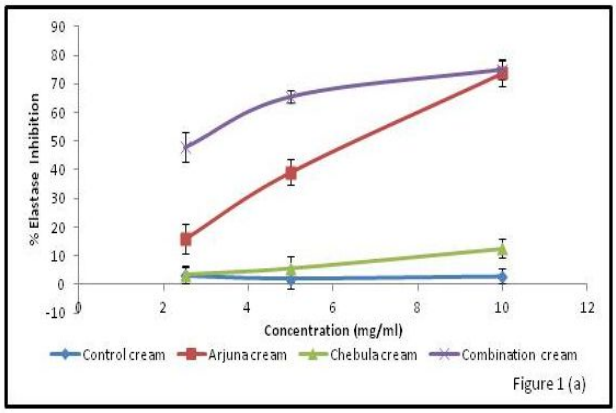
The results are expressed as mean ± standard deviation (S.D.) and the IC_{50} (inhibitory concentration) values were calculated from the dose response curves.

vii. Statistical Analysis

The data was subjected to the two-way analysis of variance (ANOVA) across groups and dose variants where $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Anti-elastase activity



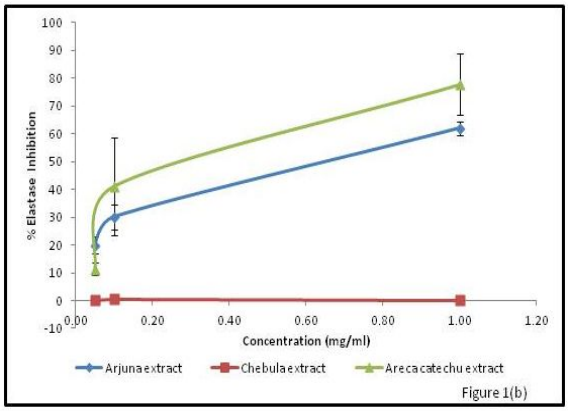


Fig 1: represents the anti-elastase activities of the various extracts and formulations using the control cream without any actives as the experimental placebo and Areca catechu extract as the positive control. While the control and T. chebula creams did not depict any anti-elastase activity, the combination cream had the best activity in terms of enzyme inhibition. The difference due to samples (*T. arjuna*) is significant $p<0.05$ but not concentration dependent.

The anti-elastase and antioxidant activities of the Terminalia species are already well documented. Our aim was to incorporate these extracts into formulations followed by *in vitro* evaluation of anti-elastase, antioxidant and cell inhibition activities. In both assays, the control cream did not exhibit either enzyme inhibition or DPPH reducing properties. In the anti-elastase experiments, the *T. chebula* extract as well as formulation did not show any activity. Thus there was no interference due to our method of evaluation of the cream. Consequently the enzyme inhibition was entirely contributed to by the *T. arjuna* extract.

Table 2: Represents the values at which 50% of enzyme inhibition was achieved

Samples	IC_{50} (mg/ml)
Control cream	Nil
Arjuna cream	6.60
Chebula cream	Nil
Combination cream	2.80
Arjuna extract	0.64
Chebula extract	Nil
Areca catechu extract (Positive Control)	0.28

3.2 Antioxidant activity

Free radicals can impact the skin in three main ways. They can alter the lipids in cellular membranes, which in turn affect the cell structure and control the passage of nutrients and other molecules; they can genetically modify the cells, which maybe disease causing or can make skin liable to premature aging. The protein expression of healthy functional collagen and elastin fibers may thus be altered.

Free radicals also lead to the development of cross-linkage between collagen fibers in the dermal component of the skin. This results in the formation of wrinkles and sagging accompanied by loss of skin tone. The enzymes that produce collagen are attacked by free radicals, thus molecules which bestow a protective function are exploited for anti-aging properties. In a related context, free radicals play a complex role in the inflammatory cascade, [19] thus antioxidants would logically be implicated in an anti-inflammatory function [20].

In the DPPH experiments, both the extracts seemingly played a synergistic role with varying antioxidant profiles as the combination cream had a low IC_{50} value although the *T. chebula* extract probably played a more dominant role.

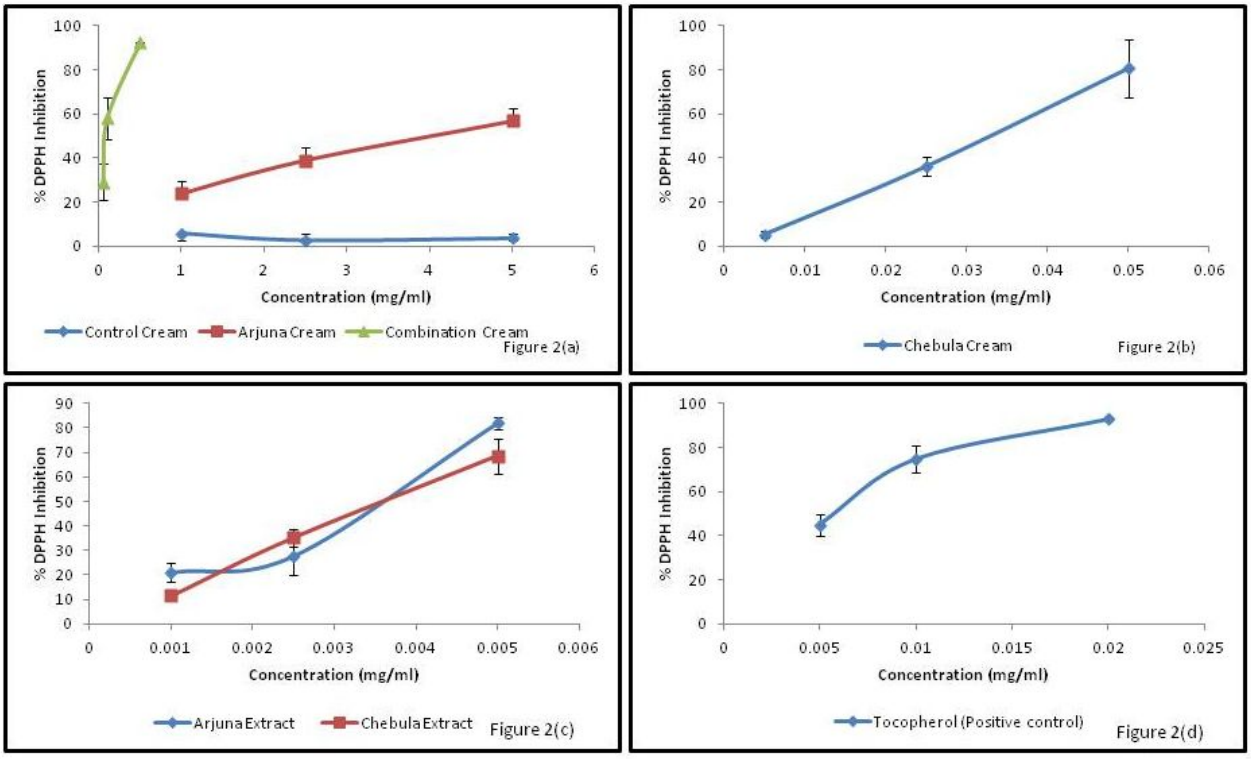


Fig 2: reveals that the T. chebula cream had the best DPPH reduction activity, while both the extracts had IC_{50} values quite close to the positive control. The difference due to samples was significant $p< 0.05$ for the control cream, *T. arjuna* cream and combination cream (Fig 2a); difference due to samples as well as concentrations was significant $p<0.05$ for T. chebula cream (Fig 2b).

Table 3: Represents the IC_{50} values of DPPH radical scavenging activity.

Samples	IC_{50} (mg/ml)
Control cream	Nil
Arjuna cream	4
Chebula cream	0.032
Combination cream	0.14
Arjuna extract	0.0036
Chebula extract	0.0036
Tocopherol (Positive Control)	0.006

Rane. and Mengi [21] studied the effect of ethanolic extracts and tannins isolated from *T. arjuna* for wound healing activity in incision and excision models *in vivo* , after oral and topical application in the form of a hydrogel. Results suggested a significant increase in the rate of healing and the tensile strength of the wounds after oral administration as well as topical treatment in the treated animals compared to the control group. Others have reported on the favorable response of Terminalia extracts on fibroblasts and accelerated wound healing [22, 23, 24].

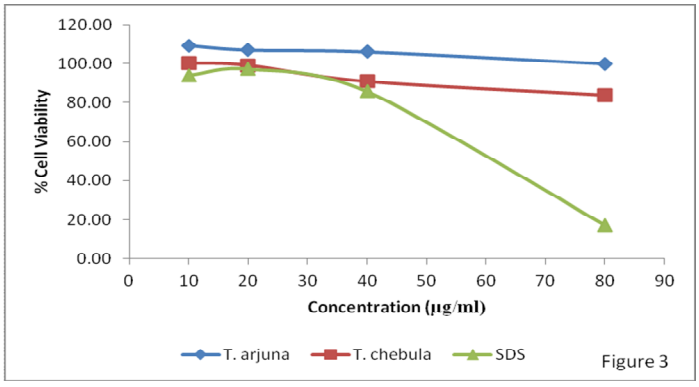


Fig 3: depicts the negligible inhibition of proliferation of 3T3 cell line by the two extracts, while the positive control SDS had an IC_{50} value of 61 µg/ml. The difference between the two extracts on cell proliferation was not significant ($p>0.05$).

4. Conclusion

Overall, the combination cream had both desirable anti-elastase and antioxidant properties, while the cytotoxicity experiments did not reveal any major inhibition or proliferation effects and seems to be at an optimal level. Thus the combination cream could probably be a good lead candidate for an anti-aging formulation. We have harnessed the cosmetic application of the biological activity of these medicinal plants. The methods described to evaluate the *in vitro* activities of the final formulations can be further exploited for efficacy testing and cosmetic evaluation before pre-clinical studies.

5. Conflict of Interest Statement

The authors declare no conflict of interest.

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