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PRECLINICAL EVALUATION OF CROTON OIL FORMULATIONS FOR ITS HAIR GROWTH POTENTIAL

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Keywords:

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ABSTRACT: Traditionally, Croton tiglium seed oil is reported to be very effective for hair loss. Thus, the aim is to study the effectiveness of *Croton tiglium* with different oils in the treatment of alopecia. The study will help develop the pharmacological profile of herbal formulation, which is being reported scientifically for the first time. The herbal formulations (formulation1, formulation2, formulation3) were formulated by mixing croton oil with olive oil and pure coconut oil. For the primary irritation test, 1 ml quantity of formulations was applied over the respective test sites of one side of the spine. The test sites were observed for erythema and edema for 48 hours after application. A physical parameter study was done for hair length, density, and quantitative histomorphometric studies. Biochemical estimation was done using different biochemical parameters; this oil was applied on shaved skin area of rats for 21 days once a day and hair length, serum total protein, and total testosterone were measured. From observation and results, it can be concluded that all the 3 formulations have potential hair growth activity, however; formulation 3 was observed to be more potent than other formulations in promoting hair growth. Since, all the formulations in the present investigation show good hair growth promotion activity. Hence, the study will act as the source of referential data that will be useful for performing human trails on these formulations' hair growth promotion activity.

INTRODUCTION:

Alopecia: Alopecia is the medical term for hair loss or baldness. It is a health condition in which hair is lost from some or all body areas, usually from the scalp.

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Occurrence: A systemic review of the epidemiology of *Alopecia areata* indicated worldwide lifetime incidents around 2% [1].

Some smaller studies indicate a slight female-to-male gender bias, which may be due to higher female concern regarding hair loss and subsequent treatment. Male patients are more likely to be diagnosed in childhood, while females are more likely in adolescence [2]. A study has shown that 58 % of the male population suffers from male androgenic alopecia at age 30-50 years [3]. *Alopecia areata* in North Indians showed a preponderance in

men (M:F = 2:1), and most persons with disease (88%) were below 40 years of age. Onset in childhood was more frequent in girls or women, but the incidence of severe alopecia was higher in boys or men with onset at an earlier age [4].

Types of Alopecia: Alopecia is classified into several categories based on hair loss pattern and causes. The two major forms i.e., Androgenic *alopecia* and *Alopecia areata* are of main concern.

Drugs used for Alopecia:

Allopathic: Minoxidil, Anthralin, Corticosteroids, Cyclosporine, Methotrexate, Finasteride [5, 6] are available for the treatment of alopecia (both Androgenic and areata), but not single and multiple drug therapy is giving satisfactory and permanent results to the alopecia patients, besides the number of side effects are associated with the use of these synthetic compounds, including erythema, scaling, pruritis, dermatitis, itching, etc. and are also not cost-effective and thus there occurs the need to move toward the herbal therapy as it has less side effect and economically affordable. Nutritional support, DHT blockers and 5-α- reductase blockers are the proposed mechanism of action for these herbal remedies [7].

Herbal: Turmeric, Fenugreek, Ginger, Nagarmotha Holy Basil, Bhringra [8] *Croton tiglium* (jamalgota) generally used for its purgative action has also been found to show potential effects in the treatment of alopecia [9, 10]. The herbal medicine includes herbal materials, herbal preparation and finished product containing active ingredients, parts of the plant or other plant materials. Traditional medicine is "the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness" [12]. Large sections of the population in developing countries rely on traditional practitioners and herbal medicines for their primary care. In Africa, up to 90% and in India, 70% of the population depend on traditional medicine to help meet their healthcare needs. In China, traditional medicine accounts for around 40% of all health care delivered, and more than 90% of general hospitals in China have units for traditional medicine [11]. WHO has defined the herbal system of medicine as "the sum total of all the knowledge and practices, whether applicable or not, used in the diagnosis, prevention, and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation whether verbally or in writing" [11].

Many plants are described for their hair growth promotional activity in Ayurvedic preparations. Traditional healers from Maharashtra have also reported *Croton tiglium* to be very effective for hair loss when used with different oils. Thus the motive is to study the effectiveness of *Croton tiglium* with different oils in treating alopecia. The study will help develop the physicochemical, phytochemical and pharmacological profile of herbal formulation, which is being reported scientifically for the first time.

MATERIAL AND METHODS:

Plant Material and Extraction: The raw material *i.e.*, croton seeds, were procured from the market and authenticated (Herbarium sheet no. 1055) and identified by Dr. Dongarwar from the Department of Botany, R.T.M. Nagpur University, Nagpur, India.

Chemicals and Reagents: Minoxidil [Mintop, 2% lotion] (Dr. Reddy's Lab, Hyderabad) and all other diagnostic kits and solvents used for experimental works were of AR grade.

Animals: Swiss Albino Mice of either sex weighing 20-30 g were obtained from the Animal House (Reg. No 92/1999/CPCSEA Dated - 28/04/1999) (Reg. No IAEC/UDPS/2018/44.), Department of Pharmaceutical Sciences, RTMNU, Nagpur and were kept under standard lab condition. The animals were allowed to acclimatize to the environment for 7 days before the commencement of experiments. The Central Animal Ethical Committee of RTMNU Nagpur University approved the experimental protocol. The grouping of animals was based upon individual studies and the preparations were given in the form of oil and was applied topically.

Anti-microbial Study [13]: For evaluation of antimicrobial activity, 2 different microbial cultures i.e., *Staphylococcus aureus* (clinical isolate) and *Candida albicans* (clinical isolate) were procured

from Rajiv Gandhi Center of Biotechnology, RTMNU, Nagpur.

An autoclave prepared and sterilized the nutrient agar medium and Sabouraud dextrose agar medium. In an aseptic room, they were poured into sterile petri plate to a uniform depth of 4mm and were allowed to solidify at room temperature. After solidification, the test organism was inoculated with the help of a sterilized inoculating loop of bacterial and fungal culture or suspension, respectively. This provides a uniform surface for the growth of bacterium/fungi and is used for antibacterial and antifungal sensitivity studies. After inoculation, the hole was created using a sterilized cork borer. Then the 50µl of each sample was immersed in the solidified agar so that the zone of inhibition was not overlapping. Plates were kept at room temperature for half an hour for the diffusion of the sample into agar media. The organism-inoculated petri dishes were incubated for 24 hours (bacterial culture at 37ºC and fungal culture at 28ºC). After the incubation period was over, the zone of inhibition was measured. Gresofulvin was used as a standard for the study.

Preparation of Oil Formulations:

Formulation 1: The seed of *Croton tiglium* was accurately weighed, and the oil was extracted through the Soxhlet apparatus using petroleum ether as solvent. The oil extracted was solely in the formulation and was stored in an amber-colored glass bottle for further study.

Formulation 2: Accurately weighed quantity of the croton oil (oil was extracted through Soxhlet apparatus using petroleum ether as solvent) was taken and was mixed with an accurately weighed quantity of pure olive oil procured from the market by cold mixing using a magnetic stirrer and the preparation was stored in an amber colored glass bottle for further study

Formulation 3: Accurately weighed quantity of the croton oil (oil was extracted through Soxhlet apparatus using petroleum ether as solvent) was taken and was mixed with accurately weighed quantity of pure coconut oil procured from the market, by cold mixing using magnetic stirrer and the preparation was stored in amber colored glass bottle for further study.

Proposed Invasive Techniques [14]:

Induction of Alopecia (Testosterone Induced): Alopecia was induced in mice using testosterone. 2 ml of Testosterone solution (1% w/w) prepared as a suspension in aqueous carboxyl cellulose solution, was injected subcutaneously into all the animals for 21 days. At day 17, a patch of hair from the dorsal area was removed using Veet Hair Removal Cream. The animals were divided into 6 groups, each containing 6 mice, in which one served as a normal group, the second served as control group, the third served as standard group, and the other three served as the treatment group.

Treatment Protocol: After the induction of alopecia for 21 days, the treatment started by applying 0.5 ml of the formulations to the respective groups. Minoxidil (2%) was selected as the standard drug and was also applied topically at a dose of 0.5ml. The treatment continued for 21 days. The hair regrowth at 7, 11, 15, 18 and 21 days after the beginning of topical application was calculated using the following hair growth score: score 0: no hair growth observed; score 1: less than 20% growth observed; score 2: 20% to less than 40% growth observed; score 3: 40% to less than 60% growth observed; score 4: 60% to less than 80% growth observed; score 5: 80% to 100% growth observed.

Primary Skin Irritation Test: Three healthy Mice, weighed 25-30gm were selected for the study. Each mice was caged individually food and water given during the test period 24 hours prior to the test. The hair from the back of each mice of 1 cm^2 was shaved on the side of the spine to expose sufficiently large test areas; 1 ml quantity of formulations was applied over the respective test sites of one side of the spine. The test sites were observed for erythema and edema 48 hours after application.

Physical Parameter Study:

Hair Length Determination: Hairs were plucked randomly from the shaved area of all mice 7, 14, and 21 days after beginning the treatment.

The length of 10 hairs was measured and the average length was determined. The results are expressed as the mean length ± S.D. of 10 hairs.

Hair Density: A hole of 1 cm 2 was made on the cardboard. The card board set on the desired depilated area (where hair fall patched observed) on the back of the mice after 25 days of depilation. The hair was trimmed of desired depilated area and the hair was cut with the seizure. The hair was count manually.

Quantitative Histomorphometrical Studies [15]: One rat from each group was forfeited after 21 days of treatment.) Skin biopsies were obtained from the shaved portion and preserved in 10% formalin. Sections of tissues were implanted in paraffin wax and sectioned into a thickness of 10µm. The sectioned tissues (T.S. and L.S.) were stained with hematoxylin and eosin, and the follicular hair phases were examined under a trinocular microscope (Leica Microscope, Model DM, Germany). Individual hair follicles were confined to specific hair cycle stages (telogen or anagen I–VI), based on the classification of Chase.

Biochemical Estimation: All the biochemical parameters, Estimation of protein and DNA content, Estimation of Catalase, and Estimation of lipid peroxidation were studied on the 15th operative day, the tissues from each were removed and each tissue was divided into three parts for the following study:

Procedures of Biochemical Estimation:

Preparation of Homogenate for Estimation of Protein and DNA Content: For protein estimation, tissue was first homogenized in 5% trichloro acetic acid and then centrifuged. The pellets were then washed with 10% trichloro acetic acid and resuspended in 5% trichloro acetic acid. It was then kept for 15 min in a water bath maintained at 90°C. The contents were centrifuged and the precipitated proteins were suspended in 0.1 M Tris–HCl, pH 7.4.and the protein content was estimated.

Estimation of Protein Content [16]:

Principle: Itis based on the principle that copper forms a complex with protein (tyrosine major and tryptophan to minor extent) and causes reduction of Folin Ciocalteu reagent (phosphotungstic and phosphomolybdic acid) forming blue color having absorptivity maximum at 600nm.

Reagents: Alkaline copper reagent, Copper sulfate: 20mg/L, Sodium potassium tartrate: 20mg/L, Sodium carbonate: 20g/L, Sodium hydroxide: 40g/L, Folin Ciocalteu reagent (FCR): 2N.

Procedure: To 0.5ml of above homogenate, 6.0ml of alkaline copper reagent was added. This was then mixed properly and allowed to stand for 10 minutes. To this, 0.5 ml of Folin Ciocalteu reagent was added with proper mixing and allowed to stand for 30 minutes. Finally, absorbance was taken at 600nm. A standard curve was prepared by using standard bovine serum albumin in the concentration range of 10, 20, 40, 80 and 160 microgram/ml.

Estimation of DNA Content [17]:

Principle: It is based on the principle that diphenylamine forms a complex with deoxyribose DNA moiety having absorptivity maximum of 600nm.

Reagents: Perchloric acid: 0.5M, Burton Reagent (Diphenylamine: 15g, sulfuric acid: 0.25M, Acetaldehyde: 0.05ml/L)

Procedure: To the tissue sample, enough perchloric acid was added to cover the sample. It was then heated in a water bath for about 90 minutes at 70-80ºC. This was then centrifuged at 300 rpm to remove the cellular debris. To 1 ml of the above supernatant, 2 ml of Burton reagent was added, mixed properly and allowed to stand for 18 hours at 30ºC. Finally, the absorbance was taken at 600nm. The standard curve was prepared following the same procedure by using a standard DNA sample in the concentration range of 40, 80, 120, 160 and 200 microgram/ml.

Estimation of Catalase [18]:

Principle: It is based on the principle that the hydrogen peroxide in the phosphate buffer (pH 7.0) reacts with the diluted sample causing the release of the catalase enzyme, which gets absorbed at 240 nm.

Reagents: Phosphate buffer, Hydrogen peroxide: 6%

Procedure: To 1 ml of supernatant, 2.25 ml of phosphate buffer was added, followed by 0.65ml of H_2O_2. The absorbance was taken at 240 nm and compared with 6% H_2O_2. First reading was not constant.

Estimation of Lipid Peroxidation [18]:

Principle: It is based on the principle where MDA (Malonyl dialdehyde) forms a complex with TBA in presence of sodium dodecyl sulphate (SDS) and n-butanol-pyridine mixture which gets absorbed at 532nm.

Reagents: Sodium dodecyl sulphate (SDS)**,** 20 % acetic acid solution (pH 3.5), Thiobarbituric acid**,** N-butanol and pyridine (15:1, v/v).

Procedure: To 0.2 mL of tissue homogenate, 0.1 mL of 8.1 % SDS, 0.75 mL of 20 % acetic acid solution (pH 3.5) and 0.75 mL of 0.8 % aqueous solution of TBA was added in stoppered tubes. The mixture was made up to 2 mL with distilled water, and then heated in an oil bath at 95 °C for 60 min.

After cooling with tap water, 0.5 mL of distilled water and 2.5 mL of mixture of n-butanol and Pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 3000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured against blank containing 0.2 mL of distilled water in place of sample.

Statistical Analysis: The experimental results are expressed as mean ± SEM, with six animals in each group, followed by a two-way analysis of variance (ANOVA). Newman-Keuls test for multiple comparisons was applied to determine the statistical significance between different groups. GraphPad Prism, version 5 software, was used for all statistical analyses. P values <0.05 were considered to be significant.

RESULTS:

Antimicrobial Evaluation: Synergistic effects were observed with Formulation 1, formulation 2 and formulation 3 and Griseofulvin against *Candida albicans* and *Staphylococcus aureus* for antimicrobial studies formulation 1 found 1.9mm and 3 mm zone of inhibition against *Candida albicans* and *staphylococcus aureus.* For Formulation 2 zone of inhibition for *Candida albicans* and *staphylococcus aureus* were 1.2 mm and 1.5mm whereas for formulation 3; 0.9mm and 1mm and for Grisofulvin zone of inhibition found to be 11mm and 10mm thus formulation 1 showed maximum antibacterial activity compared to formulation 2 and formulation 3.

Pharmacological Studies: For pharmacological studies grouping of animals done like Normal, Control, Standard (Minoxidil 2%) and for formulation 1, 2 and 3. Six numbers of animals were used for each group. All three oil formulations of formulation 1, formulation 2 & formulation 3 showed Hair growth activity. Qualitative observation for hair growth and hair length is given in **Tables 2** and **3**. Formulation 3 depicted the most potent hair growth promotion property from all the tested formulations, which was quite comparable with Standard Minoxidil **Fig. 1.**

TABLE 1: FORMULATIONS 1, 2 AND 3

Sr. no.	Ingredient	Weight (ml)
1	Croton oil	50
2	Croton oil+ Olive oil	19.54+30.46
3	Croton oil+ Coconut oil	19.54+30.46

TABLE 2: QUALITATIVE OBSERVATION OF HAIR GROWTH

Sr. no.	Groups	Time taken to initiate the growth (In Days)	Time taken to complete the growth (In Days)
01	Normal	5	12
02	Control	13	24
03	Standard (2% Minoxidil)	08	18
04	Formulation 1	09	21
05	Formulation 2	08	21
06	Formulation 3	08	19

TABLE 3: MEAN HAIR LENGTH IN MM

Sr. no.	Groups	Mean hair length in mm
01	Normal	11±0.78
02	Control	05±1.47
03	Standard (2% Minoxidil)	10±0.89*
04	Formulation 1	08±1.16*
05	Formulation 2	08±1.16*
06	Formulation 3	10±0.88*

Values are mean ±S.E.M. (n = 6). Where a: P < 0.05 vs. Normal control.

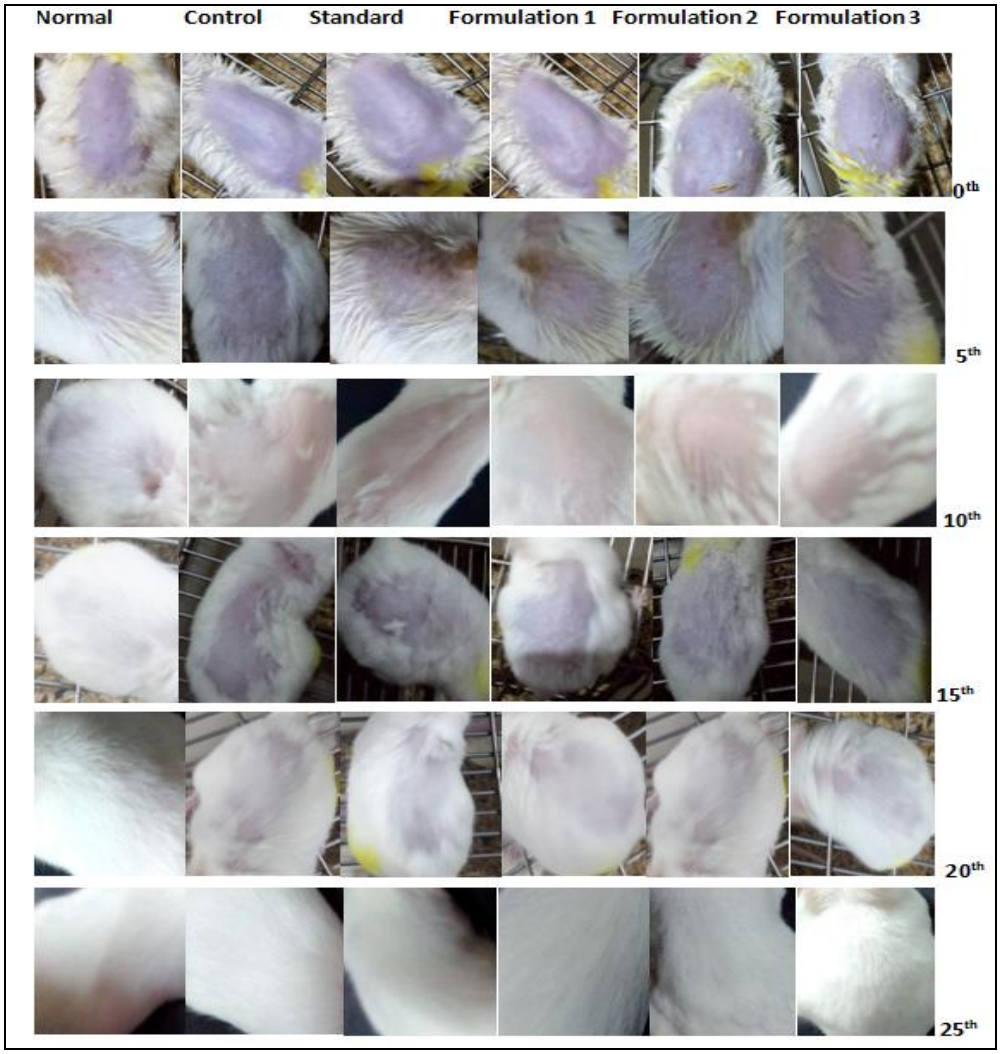

FIG. 1: DAY WISE COMPARISON OF CONTROL, STANDARD, FORMULATION 1, FORMULATION 2, FORMULATION 3 OIL FORMULATION AT DIFFERENT INTERVAL

Biochemical Analysis: From Biochemical analysis estimation it is observed that Protein content (mg/100mg), Catalase content (mcg/g per mg of protein) and DNA content (mg/100g) in formulation 3 is highest as compared to formulation 1 and 2.

Lipid peroxidation (LPO) (MDA mol/unit mg of protein) shows highest quantity in formulation 1 is highest as compared to formulations 1 and 3 (MDA nmol/unit mg of protein) **Table 4.**

Hormonal Analysis: In the testosterone induce Alopecia experimental model, it was noted that testosterone caused a disorder in the telogen stage during hair growth cycles in dermal papilla cells.

Testosterone administration caused remarkable suppression of hair regrowth in these mice; however, on treatment with all the 3 formulations; there was a significant increase in the hair growth promotional activity **Table 5, Fig. 2, Fig. 3, Fig. 4.**

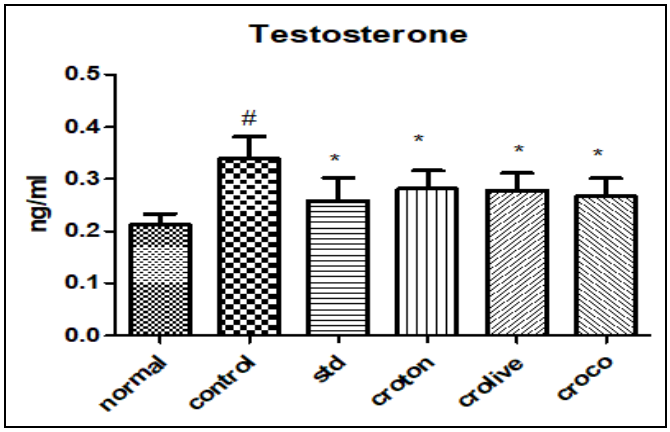

FIG. 2: TESTOSTERONE LEVEL OF THE TREATMENT GROUP IN COMPARISON TO THE NORMAL AND CONTROL. Values are expressed as mean ±S.E.M. (n = 6), followed by One Way ANOVA analysis by Newman- Keuls test Where# represent <0.05 vs Normal and * represent <0.05vs control.

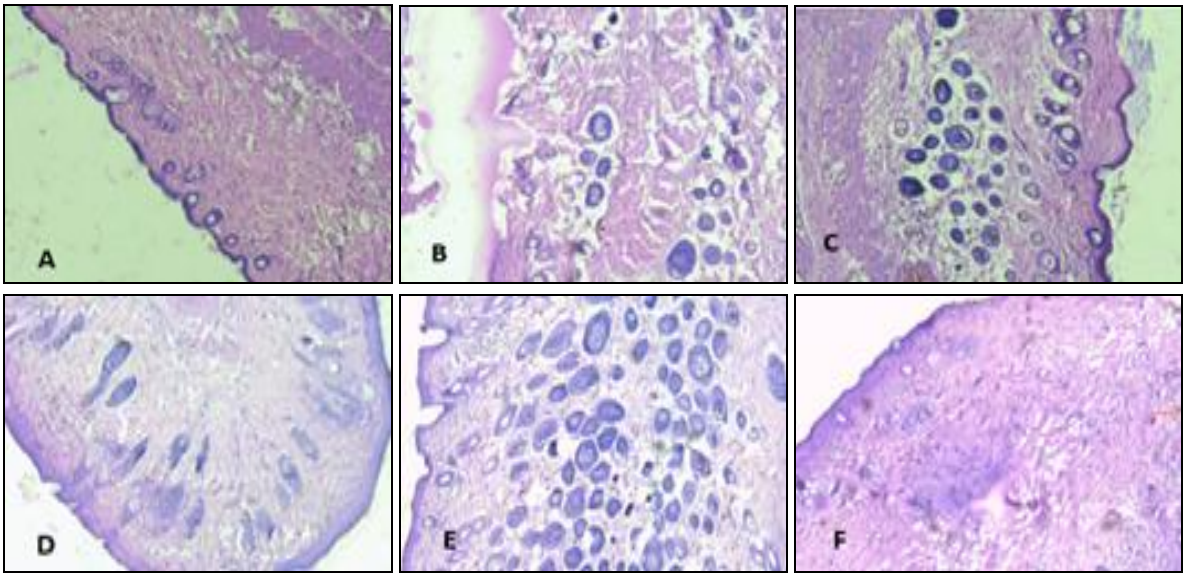

FIG. 3: L.S. SECTION OF THE SKIN TISSUE AFTER-TREATMENT WHERE A. NORMAL, B. CONTROL, C. STANDARD, D. CROTON, E. CROTON + OLIVE, AND F. CROTON + COCO. INDIVIDUAL HAIR FOLLICLES WERE CONFINED TO SPECIFIC HAIR CYCLE STAGES (TELOGEN OR ANAGEN I–VI). THE PRESENCE OF HAIR FOLLICLES TOWARDS THE EDGE OF THE SKIN OF THE CONTROL GROUP IS LESS AS COMPARED TO THE NORMAL AND THE TREATED GROUP SHOWS THE PRESENCE OF MATURE HAIR FOLLICLE

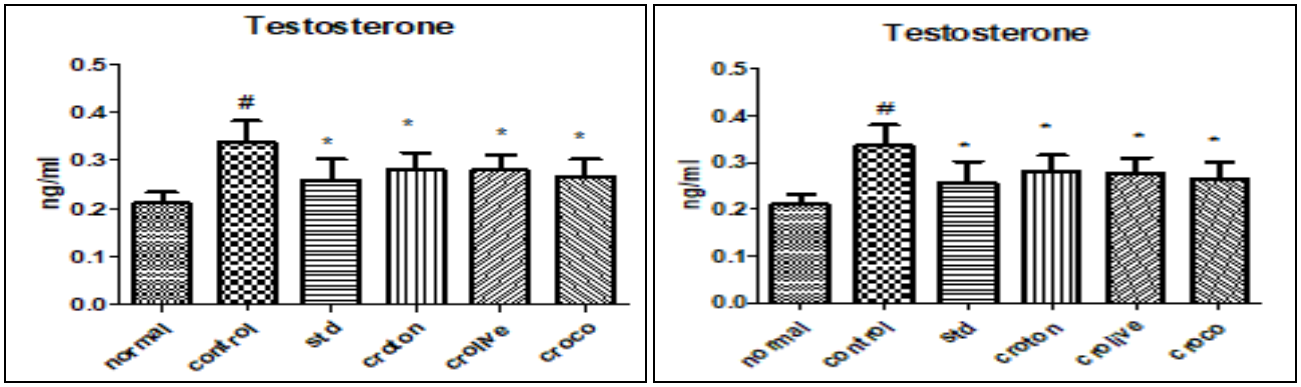

FIG. 4:

TABLE 4: BIOCHEMICAL ESTIMATIONS

GROUPS	Normal	Control	Standard	Formulation 1	Formulation 2	Formulation 3
Protein content(mg/100mg)	7.727± 0.380	6.447*± 0.339	8.071*± 0.31	8.828*± 0.403	8.311*± 0.333	9.645*± 0.333
DNA content(mg/100g)	0.170± 0.020	0.083± 0.005	0.164± 0.004	0.154± 0.004	0.134± 0.013	0.163± 0.005
Catalase content(mcg/g per mg of protein)	3.888± 0.332	2.754± 0.291	3.734± 0.254	4.740± 0.372	4.079± 0.258	5.531± 0.284
Lipid peroxidation (LPO) (MDA nmol/unit mg of protein)	34.928± 3.41	46.291*± 4.43	30.448*± 2.53	34.874*± 3.44	34.32*± 1.998	28.986*± 1.572

Values are expressed as mean ±S.E.M. (n = 6), followed by One Way ANOVA analysis by Newman- Keuls test where a: P < 0.05 vs. Normal control.

TABLE 5: DATA OF HORMONAL ANALYSIS

Groups	Normal	Control	Standard	Croton	Croton + Olive	Croton + Coco
Testosterone	0.211±0.021	0.338±0.043	0.258±0.044	0.281±0.034	0.278±0.032	0.266±0.034

Values are expressed as mean ±S.E.M. (n = 6), followed by One Way ANOVA analysis by Newman- Keuls test where a: P < 0.05 vs. Normal control

Histopathological Analysis: The histopathological data shows the most follicles in the tissue treated with formulation 3 compared to the other two formulations.

DISCUSSION: The hair growth promotion study on Mice revealed that the time taken for complete hair growth was 19 days in Formulation 3, 21 days in Formulation 1 and in Formulation 2. In the comparative antimicrobial study, formulation 3 and Minoxidil showed better hair growth potential as compared to other formulations. In the testosterone induce Alopecia experimental model, it was noted that testosterone caused a disorder in the telogen stage during hair growth cycles in dermal papilla cells. Testosterone administration caused remarkable suppression of hair regrowth in these mice; however, on treatment with all the 3 formulations, there was a significant increase in the hair growth promotional activity (Matsuda *et al.*, 2001).

From the biochemical parameters like protein, DNA, catalase and lipid peroxidation, it can be concluded that all the formulations have significant activity, out of which formulation 3 has the most potent activity comparable to the standard Minoxidil. Also, the histopathological data shows the most follicles in the tissue treated with formulation 3 compared to the other two formulations.

After a keen study of all the parameters and pharmacological evaluation, it can be concluded that all 3 formulations have potential hair growth activity. However, formulation 3 was observed to be more potent than other formulations in promoting hair growth.

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