



FORMULATION AND EVALUATION OF ANTIOXIDANT CREAM BASED ON *MARKHAMIA TOMENTOSA* ETHANOLIC EXTRACT AND *CITRUS SINENSIS* OIL

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ABSTRACT

Premature aging is a common problem that occurs globally due to continuous exposure to the sun and agents of oxidative stress like free radicals which are ubiquitous. This study aims to formulate an antioxidant cream based on *Markhamia tomentosa* ethanolic extract and *Citrus sinensis* oil. Seven cream formulations A1-A7 were prepared. Fusion technique was utilized in the formulation of the creams which were all water-in-oil emulsions. Formulations A1-A7 were characterized for pH, viscosity, organoleptic tests, spreadability, skin irritancy, antioxidant tests and centrifuge test. The pH of the formulations A1-A7 was found to be in the range of 6.3 to 7.9. It was found that the creams were smooth, homogenous and formed an easily spreadable non-greasy film on the skin surface with no irritation on dermal application. Formulation A7 had the highest spreadability $116.7 \pm 0.93 \text{ mm}^2\text{g}^{-1}$ while formulation A2 had the lowest spreadability $92.9 \pm 0.10 \text{ mm}^2\text{g}^{-1}$. At a concentration of $800 \mu\text{g/mL}$ the antioxidant activity of *Markhamia tomentosa* extract (75%) was close to that of Ascorbic acid (95%) which was the standard. Formulation A1 had the highest antioxidant activity while formulation A7 had the lowest due to the absence of *M. tomentosa* extract in its composition. All formulations passed the centrifuge test. A combination of the *Markhamia tomentosa* leaf extract and *Citrus sinensis* oil conferred a synergistic activity which led to potent antioxidant activity which is necessary for preventing oxidative stress and dermal photoaging. This herbal antioxidant formulation serves as a prototype that can be developed and translated to a marketable formulation for pharmaceutical application.

KEYWORDS: Aging; Oxidative; Stress; Formulation; *Markhamia Tormentosa*; *Citrus senensis*.

INTRODUCTION

Aging is a natural yet complex physiological process which involves changes in aesthetic, structural and functional properties of the skin. Aging can be accelerated by intrinsic or external factors [1]. Intrinsic factors that promote aging include genetic programming and excessive activity of fibroblast cells. Intrinsic factors are normally inherited and not much can be done to control their direct effect on aging. However, the effects of extrinsic-

environmental factors such as ultra-violet rays from the sun, pollution, chronic stress and poor diet can be halted, as these factors ultimately lead to the production of free radicals which are also known as reactive oxidative species. Excessive production of these reactive oxidative species in comparison to the body's natural antioxidant defense system can lead to oxidative stress which leads to aging [2]. Simply put oxidative stress is the imbalance between oxidants and antioxidants in the skin [3].

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Antioxidants are molecules that assist in the retardation and inhibition of oxidation, stopping free radicals from cellular damage in the skin thereby slowing down the process of cutaneous or dermal aging [4]. The use of naturally sourced plant extracts as antioxidant for the prevention of premature cutaneous aging has been of great research interest. This is mainly because naturally sourced plant extracts can deactivate free radicals leading to restoration of the skins reductive oxidative specie balance preventing inflammation and skin aging [5]. Medicinally active plants also give a great source of lead bioactive molecules with minimal side effects in comparison to synthetic drug compounds. Literature has shown that ethanolic extracts of plants can be more potent than the plant parts themselves. This is because the process of preparation of ethanolic extracts allows the concentration of the phyto-constituents responsible for the plants bioactivity [6].

Markhamia tomentosa is a flowering plant from the family Bignoniaceae. *Markhamia tomentosa* is a tree up to 15m high, it is mostly found in forests and is indigenous to tropical areas such as Nigeria, and other parts of Africa or Asia. Traditional practitioners utilize the root, barks and leaves for treatment of several ailments such as diarrhoea, backache, gout, scrotal elephantiasis, rheumatoid arthritis and skin diseases. The use of *Markhamia tomentosa* as an antioxidant is a novel concept. Preliminary studies carried out on the leaves of the plant showed the presence bioactive compounds such as phenols, terpenes, steroids, saponins and flavonoids. Pentacyclic triterpenoids including pomolic acid, oleanolic acid, tormentic acid and β -sitosterol have been isolated from the stem bark of *Markhamia tomentosa*. Phenols have been reported to possess potent antioxidant activities [7].

Citrus senensis is a fruit that belongs to the family Rutaceae and is commonly called sweet orange. It is a plant hybrid of pomelo and mandarin orange. The *citrus sinensis* tree is 10m in height and is grown almost anywhere in the world. The fruit can act as a laxative, anthelmintic, and is useful for stress relief. It also possesses anti-inflammatory, antibacterial and antioxidant properties. The major antioxidant property of the fruit is attributed to the presence of ascorbic acid and flavonoids. *Citrus sinensis* oil can be extracted from the peel of the fruit, the oil can be used in flavouring, as fragrance and for aromatherapy [8]. *Citrus sinensis* oil has a high concentration of ascorbic acid - a water-soluble free radical scavenger important for the producing collagen, which is the substance responsible maintaining the skins integrity. *Citrus sinensis* oil also works as a permeation enhancer by ensuring enhanced delivery of bioactive substances

through the percutaneous surface promoting penetration and flux [9].

The deployment of two herbal antioxidants is a viable approach to stop symptoms due to oxidative stress induced aging of the skin and may also offer a synergistic activity. This work was carried out to ascertain the abilities of a plant sourced bioactive substances to suppress oxidative stress by elevating the presence of antioxidants in the skin [10]. Not enough literature and scientific investigations on the use of herbal based formulations as antioxidant exist, neither is there any existing scientific work on *Markhamia tomentosa* and *Citrus sinensis* based herbal cream as an antioxidant formulation. This study therefore fills a knowledge gap in the field of dermatological aging. The purpose of this study is to develop antioxidant cream containing *Markhamia tomentosa* ethanolic extract and *Citrus sinensis* oil as base. This work is important because premature aging is a common problem that occurs globally due to continuous exposure to agents of oxidative stress like free radicals which are ubiquitous [11]. This poly-herbal formulation will serve as an antioxidant that will not only protect the skin from the effects of reactive oxygen species but enhance an overall healthy skin condition irrespective of skin type, level of exposure, or age [12].

MATERIALS AND METHODS

Materials

The following materials were used in this study. Amaranth dye (Sigma Aldrich, St. Louis USA), stearic acid (Surfachem, UK), cetostearyl alcohol (Pure Nature, NZ), soft paraffin (Unicorn petroleum, India), liquid paraffin (DBS Chemicals, India), hard paraffin (Okchem, China), triethanolamine (Merck, Germany), carbopol (Okchem, China), methyl parabene and propyl paraben, (Sigma Aldrich, St. Louis USA), 1,1-diphenyl-2-picrylhydrazyl (Okchem, China), ethanol (All school Labs, Nigeria).

Extraction

Fresh leaves of *Markhamia tomentosa* sourced from Ikire, Osun State in Nigeria were identified by Mr Nodza at the Herbarium of the Department of Botany and Microbiology, University of Lagos. A voucher specimen (8056) was prepared and deposited for the plant. *M. tomentosa* leaves were then air dried for three days after which the leaves were oven dried at 40°C and triturated to powder. The powdered plant material (150 g) was macerated with absolute ethanol for 72h at room temperature. The extract was filtered and concentrated using an oven at 40°C. It was then stored in an amber bottle at 8°C. Fresh peels of *Citrus*

sinensis were cleaned and preheated in distilled water at 80°C for 2h. The orange peels (200g) were then transferred into a steam distillation flask containing 400mL of water. The distillate was collected in a separating funnel and the dense oil layer was collected and stored in an air tight amber coloured bottle at 8°C [13].

Formulation development

Stearic acid which acts as the emulsifier and other oil soluble components (cetostearyl alcohol, soft paraffin, liquid paraffin and hard paraffin) were dissolved as the oil phase and heated to 80 °C. The preservatives (methyl paraben and propyl paraben) and other aqueous components (triethanolamine, water and ethanol extract of *M. tomentosa* (EEMT)) were also heated to 80 °C. The aqueous phase was added in portions to the oil phase with continuous stirring. Carbopol Ultrez was dissolved in water and utilized as a thickener, it was added to the cream base. The *Citrus Sinensis* oil extract (CSOE) was added after the temperature dropped to (45 ± 0.5) °C (Table 1) [14].

Determination of the emulsion type

To determine the emulsion type, drops of Amaranth dye was added to the cream (1g) and was examined for phase type using microscopy (Eclipse E100 Nikon TX, USA). A drop of the cream was placed on as microscopic slide and observed, the globules appeared red due to staining by the amaranth dye and the continuous phase remained colourless [15].

Microscopic examination

A micrograph for each formulation (A1-A7) was obtained by preparing a smear of each formulation on separate slides and covering them over with cover slips. The slides are viewed on the microscope [15].

pH of the formulations

The pH of the formulations A1-A7 was measured using a pH meter. The electrode was in contact with the formulation for 45 sec to permit equilibration. Measurements were performed in triplicate [16].

Rheological evaluation

The viscosity of the formulations (A1-A7) was determined at 25 °C at 10–50 rpm using a Spindle 4.0, cone and plate viscometer (DV-E Digital viscometer, Brookfield Engineering Laboratories, Middleboro, USA). Measurements were performed in triplicate [12].

Organoleptic test

Formulations (A1-A7) were evaluated for homogeneity by assessing their visual appearance and texture. The appearance of the cream was judged and graded by its

colour, pearl essence and smoothness. Emolliency and slipperiness were also evaluated [15].

Spreadability

The spreadability was expressed as time (seconds) taken for two slides to slip off from the cream, placed in between them, under a load weight (70g). The time in which the upper glass slide moves over the lower slide to cover a distance of 10cm is noted. Spread ability can be calculated using the formula [11].

$$S = M \frac{L}{T} \dots\dots\dots (1)$$

M = Weight tied to the upper slide, L = Length of glass slide, T = Time taken to separate the slides.

Skin irritancy test (Draize Test)

The study was approved by the College of Medicine, University of Lagos Health Research Ethics Committee (CMUL HREC number: CMUL/HREC/07/22/1066). An area of up to 1 cm² of human skin was marked and a fixed amount of formulation (A1-A7) was applied to the specified area and the time lapse noted. It was checked for signs of irritancy such as rash, redness or swelling at regular intervals for 24h and reported [15].

Antioxidant activity of the *M. tomentosa* compared with the standard (Ascorbic Acid) using DPPH assay

The free radical scavenging activity of *M. tomentosa* leaf extract and Ascorbic acid were evaluated and analyzed spectro-photometrically. The radical scavenging assay of the samples against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical via UV absorbance at 517nm was carried out [14]. The absorbance was recorded after 30 min. The percentage antioxidant activity was calculated using Formula (2).

$$\% \text{ Antioxidant Activity} = \frac{\text{Absorbance}_{517 \text{ control}} - \text{Absorbance}_{517 \text{ sample}}}{\text{Absorbance}_{517 \text{ control}}} \times 100 \dots\dots\dots (2)$$

In-vitro antioxidant activity of the formulation A1-A7 using DPPH assay

The radical scavenging activity of the formulations (A1-A7) against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical through UV absorbance at 517 nm, using ascorbic acid as standard and ethanol as control was carried out. The formulations were assayed spectrophotometrically. Exactly 100mg of each formulation and ascorbic acid was extracted utilizing absolute ethanol via a separating funnel. About 2mL of the test sample was dissolved in

ethanol and introduced at different concentrations (5-25mg/mL) to the ethanolic solution of DPPH (100mmol/L, 2mL. Absorbance was noted at 517nm at 30 min [14]. The percentage antioxidant activity was calculated using Formulation (2).

Centrifugation test

The stability of the freshly prepared formulations A1-A7 were determined with centrifugation test at 3,500 rpm for 10minutes. The appearance and phase separation were assessed by macroscopic observation [16].

Statistical analysis

To determine the level of significance ($p < 0.05$), statistical analyses were performed using a one-way ANOVA test followed by Bonferroni's multiple comparisons test (if applicable) performed on GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA) [17].

RESULTS

Organoleptic test

Formulations A1 and A2 were found to be pale yellow, while formulations A3 and A4 were off white in colour. Formulations A5 - A7 were white in colour. All the formulations had pleasant citrus characteristic smell. The pH of the formulation was found in range of 6.3 to 7.9. It was found that the cream was homogenous, smooth, non-greasy film on the skin surface and was easily spreadable, moisturizing the human skin.

Skin irritancy

Draize test showed that there were no alterations in the physiological condition of the skin, neither was swelling observed on the human skin after 24h. No rash or allergic reaction was observed as well indicating that formulations A1-A7 were safe for dermal application.

Spreadability

Formulations A1-A7 were easily spread on dermal application, as no resistance to flow was observed. Formulation A7 had the highest spreadability while A2 was the lowest.

Microscopic examination and determination of emulsion type

The micrographs of each formulation reflected their granular features (Figure 3). The granular structure

and intermolecular arrangement is shown in Figure 3. Formulations A4 and A7 were evenly distributed. Amaranth dye staining indicated that all the formulations were water-in-oil emulsions. There was absence of coalescence in the micrographs studied. The globule size range of each formulation is shown in Figure 4.

Rheological evaluation

Formulation A7 had the highest viscosity at the shear rate of 50 rotations per minute, while formulation A2 had the lowest viscosity. Formulations A1-A7 exhibited non-Newtonian, pseudoplastic flow under shear stress at 10-50 rotations per minute. It can be seen in Figure 4 that as shear rate increased the viscosity of the formulations decreased. In this case, the rheological behaviour is typical of two phase systems such as emulsions.

Antioxidant activity of the *M. tomentosa* compared with the standard (Ascorbic Acid) using DPPH assay

DPPH radical interacts with ideal reducing agents which lose colour stoichiometrically after which the number of electrons consumed are measured at 517 nm. The percentage DPPH radical scavenging activity of the standard, Ascorbic acid was 95% at 800 µg/mL as seen in Figure 5. The percentage DPPH radical scavenging activity of the *M. tomentosa* leaf extract was 75% at a concentration of 800µg/mL, showing that *M. tomentosa* is capable of DPPH radical scavenging activity.

In-vitro antioxidant activity of the cream using DPPH assay

The DPPH antioxidant activity of ascorbic acid at 100 µg/ml was 82.73% which was the highest. All formulations possessed a level of radical scavenging activity. The formulation with the highest in-vitro antioxidant activity at all concentrations is formulation A1 and the lowest was A7.

Centrifugal test

All formulation (A1-A7) did not show any phase separation after 3500 rpm centrifugation for 10 minutes. Therefore, all formulations were considered homogenous under experimental conditions. The instability of the formulations is known to affect the solubility and therapeutic activity of the bioactive ingredients. All formulations were stable throughout this investigation.

Table 1: Formula for preparing antioxidant cream formulations (A1-A7), utilizing ethanolic extract of *Markhamia tomentosa* and *Citrus sinensis* oil.

Ingredients	A1	A2	A3	A4	A5	A6	A7
EEMT(g)	0.008	0.008	0.008	0.008	0.008	0.008	-
CSOE (mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Stearic acid (g)	2.5	5.0	2.5	5.0	2.5	5.0	2.5
Cetostearyl alcohol (mL)	3.75	5.0	3.75	5.0	5.0	3.75	5.0
Liquid Paraffin (mL)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Hard Paraffin (g)	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Soft Paraffin (g)	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Methyl Paraben(mL)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Propyl Paraben(mL)	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Triethanolamine (mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Carbopol Ultrez (g)	1.0	1.0	0.5	0.5	1.0	1.0	1.0
Purified water (mL) to	100	100	100	100	100	100	100

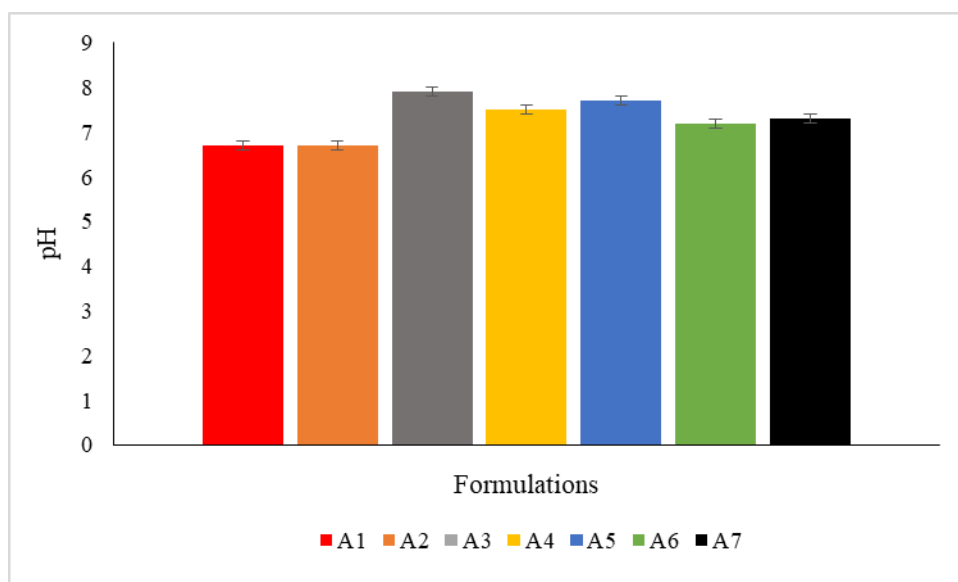


Figure 1: pH reading for antioxidant formulations A1-A7. Values are expressed as mean \pm SD (n=3).

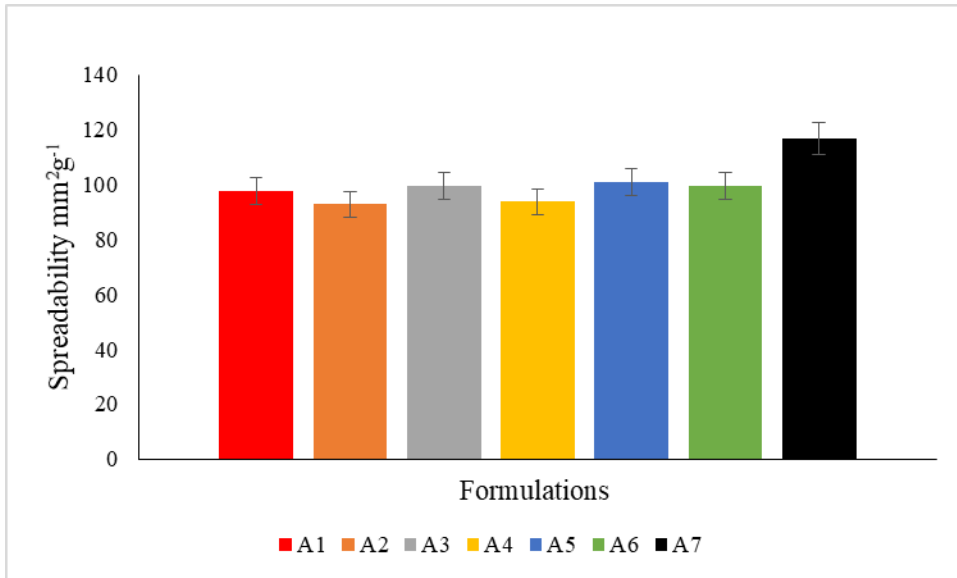


Figure 2: The values for the spreadability of formulations A1-A7, values are expressed as mean \pm SD (n=3).

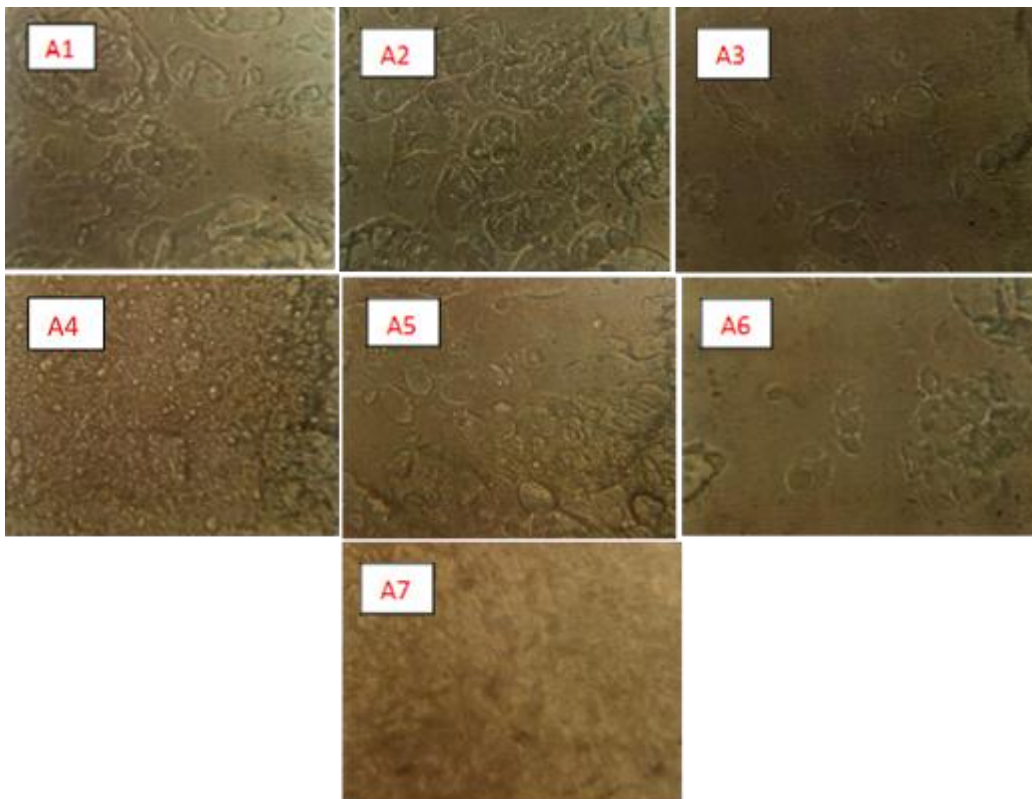


Figure 3: Photomicrographs of the varying formulations (A1-A7).

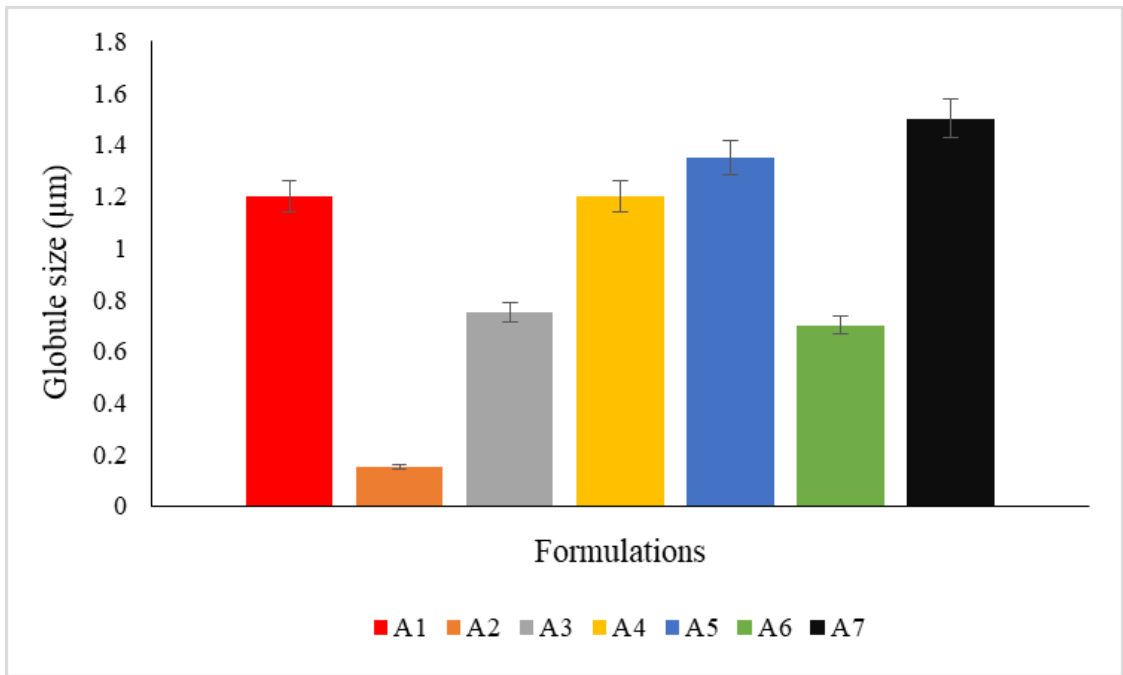


Figure 4: Average globule sizes of the varying formulations (A1-A7). Values are expressed as mean \pm SD (n=3).

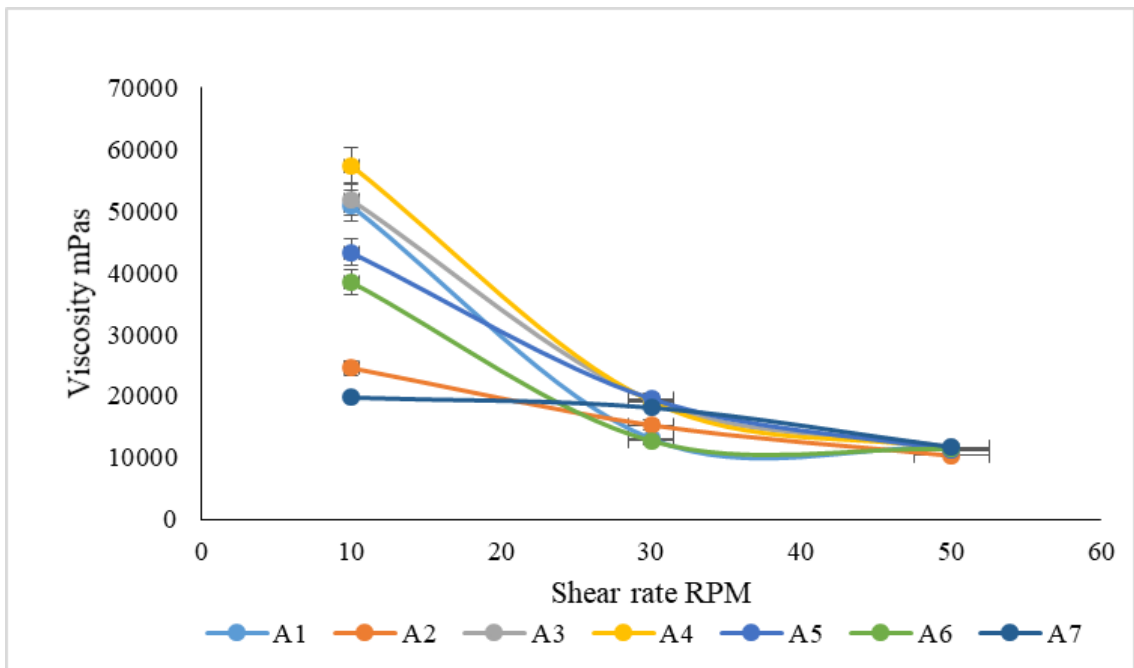


Figure 5: Viscosity plot showing the effect of shear rate on the viscosity of formulation A1-A7. Values are expressed as mean \pm SD (n=3).

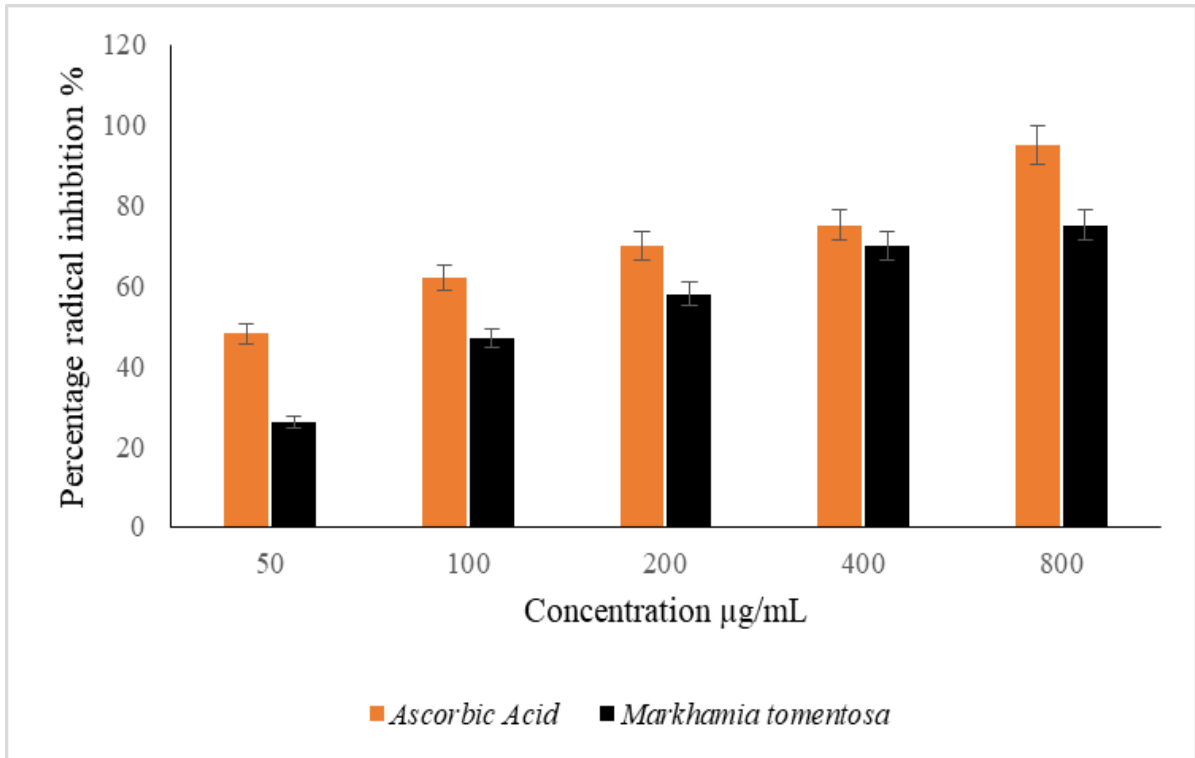


Figure 6: Bar Chart of percentage radical inhibition against concentration (µg/ml) of *Markhamia* leaf extract and (standard) ascorbic acid. Values are expressed as mean ± SD (n=3).

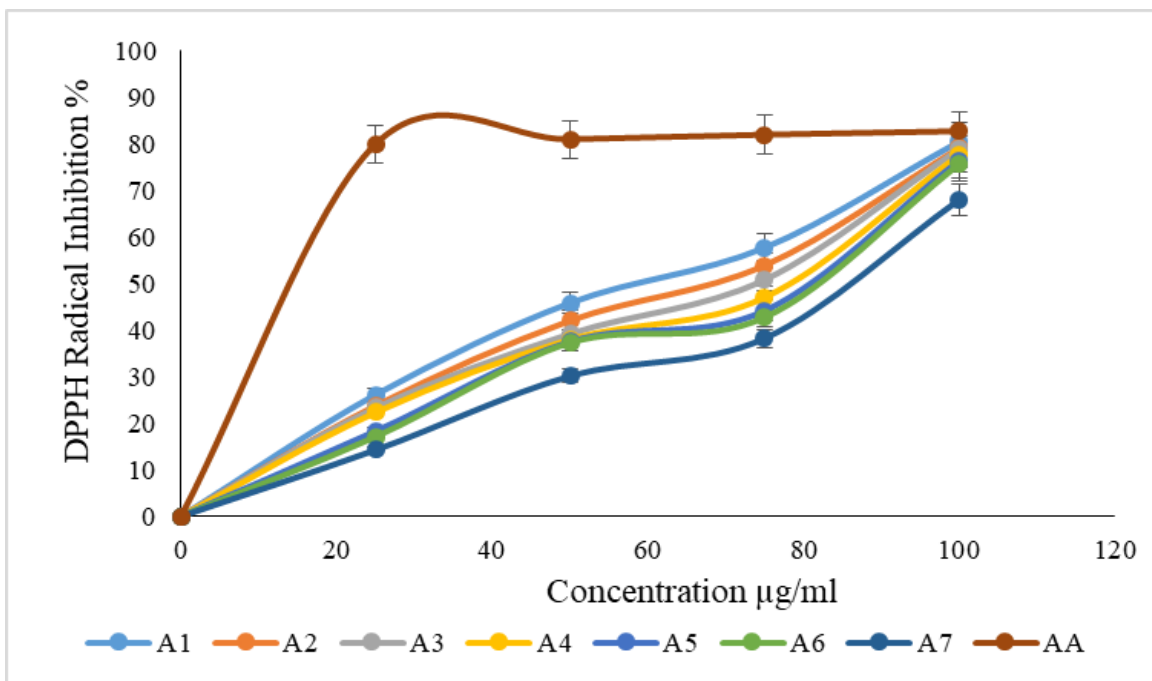


Figure 7: Percentage DPPH radical inhibition against concentration (µg/ml) for formulations A1-A7. Values are expressed as mean ± SD (n=3).

DISCUSSION

Interests in herbal medicines and its use has been on the increase over the years due to its efficacy, acceptability and the fact that it has lesser side effects in comparison to synthetic agents. *Markhamia tomentosa* leaf extract has been known to contain flavonoids and tannins which are phenolic compounds responsible for its antioxidant activity [18]. *Citrus sinensis* possess ascorbic acid which has significant antioxidant activity, when extracted as oil may also serve as a permeation enhancer. It also synergistically potentiates the antioxidant effect of *Markhamia tomentosa* [19]. The role of antioxidants in protection from free radical damage cannot be over emphasized hence its use in preventing aging process. This topical antioxidant formulation not only mops up free radicals but ultimately provides protection from photo-aging and prevents skin cancer [20].

Formulations were all well prepared without any observation of cracking or phase separation. The citrus fragrance observed in all formulations may be due to the presence of *Citrus sinensis* oil. *Citrus sinensis* oil contains 92.5% limonene which is responsible for its fragrance. The emulsion type was detected as a water-in-oil emulsion, this emulsion type is desirable for creams as it helps to seal in moisture and enhance absorption of medicaments topically [21]. In Figure 2, A7 had the highest spreadability. Micrographs depicting the microstructures of the creams showed that there was no coalescence indicating stability of the emulsion system. The pH of the skin is about 6.0, the pH of the respective formulations ranged from 6.3-7.9, its closeness to the skin buttresses the fact that the cream is safe for dermal use [22].

Centrifugation is when a system, in this case a biphasic system such as an emulsion is subjected to the centripetal force impacted by a centrifuge recreating *in-situ* stress condition. A centrifuge model can replicate stress conditions by increasing the gravitational acceleration within its field [23]. All formulations were found to be stable after the centrifuge test with no coalescence or phase separation observed. The average globule size gives an indication of the level stability of the respective formulations. Larger globule sizes mean higher chances of coalescence and eventually phase separation. In Figure 4, the low average globule size in formulation A2 was due to the high concentration of emulsifier (stearic acid) and presence of the gelling/ thickening agent carbopol. Formulation A7 had the largest globule size due to the low concentration of stearic acid and comparatively higher

concentration of the softening agent, cetostearyl alcohol [24].

The viscosity of the formulations varied depending on their compositions. The high concentration of stearic acid in formulation A2 contributed to its low viscosity as stearic acid acts as a surfactant and softening agent in creams. A7 had the highest viscosity and this is due to the absence of the *Markhamia tomentosa* leaf extract indicating that the *M. tomentosa* ethanolic leaf extract influenced the texture and viscosity of the formulations [25]. Viscosity of the creams was reduced as shear rate increased confirming the spreadability of the formulations for dermal application. Viscosity gauges the resistant to flow occurring by the inner friction due to relative movement by layers of fluid within a bi-phasic system [26]. In this case, the formulations are water-in-oil emulsions and are therefore non-Newtonian fluids hence viscosity is dependent on shear stress due to shear thinning. Shear thinning occurs when the thickness of the formulations reduces as the shear stress increases [27].

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a dark purple crystalline compound made up mainly of stable free radical molecules. DPPH free radical technique is an oxidant scavenging assay based on electron-transfer [28]. DPPH gives a dark violet solution when dissolved in ethanol. The free radicals are stable at room temperature and can be reduced in the presence of an antioxidant molecules, to give a translucent ethanol solution [29]. The percentage antioxidant activity of *M. tomentosa* leaf extract was close to that of the standard Ascorbic acid at all concentrations (50-800 µg/mL) in Figure 6. In Figure 7 the formulation with the highest in-vitro antioxidant activity at all concentrations is formulation A1. The lowest was A7 due to the absence of *M. tomentosa*. The little antioxidant activity observed in A7 may be due to the presence of *Citrus sinensis* oil. DPPH radical inhibition was higher in all formulations that contained *M. tomentosa* and *Citrus sinensis* oil as it gave a synergistic effect to the activity of *M. tomentosa*, which has confirmed DPPH radical scavenging activity. Hence as the *M. tomentosa* leaf extract performs its antioxidant function, the *Citrus sinensis* oil synergizes its antioxidant effect and acts as an ideal protectant and repair agent for the skin [30].

CONCLUSION

The herbal antioxidant creams A1-A7 were successfully formulated. The antioxidant formulations varied in colour from pale yellow to clear white and the pH of the formulations was within

the safe limits for application to the *stratum corneum*. Formulations were easily spreadable. Rheological behaviour of all formulations was non-Newtonian (shear thinning). All formulations were stable throughout the centrifuge test. Formulation A1 had the highest antioxidant activity. A combination of the *Markhamia tomentosa* leaf extract and *Citrus sinensis* extract oil conferred a synergistic activity that provided the potent antioxidant activity necessary for preventing oxidative stress, dermal photo aging and skin cancer. This herbal antioxidant formulation serves as a prototype that can be developed and translated to a marketable formulation for therapeutic application.

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