



Vernonia cinerea Extract and Its Silver Nanoparticles in the Making of Indigenous Eyeliner: A Formulation and Evaluation Study

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Abstract: Background, *Vernonia cinerea* Less. (Asteraceae), known as “Poovaamkurunnel” in Malayalam and “Sahadevi” in Sanskrit, is a medicinally significant herb documented in Ayurvedic and other traditional medicine systems for its anti-inflammatory, analgesic, antipyretic, and antioxidant activities, with specific ethnomedicinal application in eye care, including conjunctivitis and as an anti-cataract agent. The present study aimed at using green synthesis for making silver nanoparticles (AgNPs) with the aqueous extract of *Vernonia cinerea* (Vc) to make VcAgNPs (*Vernonia cinerea* extract silver nanoparticles), and to incorporate these into an eyeliner formulation prepared by the indigenous burning method. VcAgNPs were characterized by standard physicochemical techniques. Antimicrobial activity of the extracts and finished products was evaluated against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 6538P. The carbon soot obtained was additionally tested against these organisms and *Candida albicans* ATCC 2091. Microbial safety of the formulations was also assessed. Physical stability studies confirmed that all three eyeliner formulations maintained their properties over six months. The major finding was that although both the Vc and VcAgNPs fresh extracts demonstrated notable antimicrobial activity, the finished eyeliners showed no inhibitory activity against the tested organisms, consistent with the expected degradation of bioactive compounds during the high-temperature burning process. Conclusion, The formulated eyeliners were physically and microbiologically safe over the study period, though they did not retain measurable antimicrobial activity after processing. These preliminary findings suggest the traditional preparation method may compromise the extract’s bioactive properties, and further studies are needed to evaluate the formulation’s broader safety and any potential ophthalmic therapeutic benefit.

Keywords: *Vernonia cinerea* Less; indigenous eyeliner; silver nanoparticles; green synthesis; carbon nanoparticles; cosmetic applications

1. Introduction

Eyeliner is a traditional eye cosmetic preparation made from herbs and minerals, used primarily in Unani, Ayurvedic, and Greco-Arabic medicine for eye care and beautification. It has been documented to be used in ancient Egypt, the Assyrian empire and the Middle East by both men and women [1]. In indigenous cultures, eyeliner is applied to the inner eyelid to maintain eye health [2,3]. Kohl, kajal, and anjanam are all traditional eye cosmetics with ancient origins. However, they differ in composition and application as kohl typically contains antimony sulfide or lead-based compounds, kajal is primarily carbon-based (soot or ghee residue), and anjanam often incorporates Ayurvedic herbal ingredients [4]. Applied on the inner eyelids, anjana maintains eye health and prevents various diseases, and is categorized into *Lekhanjana*, *Ropanjana*, and *Prasadanjana* [5]. Indigenously produced eyeliner also provides a cooling effect [6].

Vernonia cinerea Less. (Asteraceae), is a herb forming part of the Dasapushpam group of plants [7]. The plant grows naturally near roadsides, arid land, and grazing areas, reaching heights of 12–75 cm [8]. Its stems are solid, cylindrical, and hairy, and its dark green leaves are alternate, spiral, or elliptic, with a distinctive odour and bitter taste [9,10]. The flowers are white, pink, or purple, arranged in rounded or flat-topped corymbs [11].



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V. cinerea is traditionally employed across tropical regions, where the whole plant is used in ethnomedicine [12]. In Ayurveda, the whole plant is indicated for the treatment of intermittent fever, filariasis, blisters, boils, and vaginal discharges [8]. Leaf and root decoctions have been used to treat hiccups, fever, kidney problems, and stomach disorders [13]. In traditional Chinese medicine, the plant is employed for fever, sore throat, headache, eczema, mastitis, dysentery, dyspepsia, and infections [14]. The plant has also been reported for potential utility in treating cancer and gastrointestinal disorders [15].

Phytochemical investigations have revealed that *V. cinerea* contains a variety of bioactive compounds contributing to its documented antibacterial, antiviral, antifungal, anti-inflammatory, and antioxidant pharmacological activities [16]. It has also been reported for its anti-cataract activity [17,18]. Traditional kohl demonstrated significantly greater improvement in blepharitis signs and symptoms compared to controls [19].

Silver nanoparticles (AgNPs) synthesized using plant aqueous extracts are favoured due to their easy availability, safe handling, and the presence of multiple active phytochemical compounds that serve as reducing and capping agents [20]. Biosynthesis and characterization of AgNPs using *V. cinerea* aqueous extract has been previously reported, demonstrating antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, and antifungal activity against *Candida albicans* and *Penicillium notatum*. Ramaswamy et al. (2015) previously confirmed antibacterial activity against *S. aureus* and *E. coli* and antifungal activity against *C. albicans* and *Penicillium notatum*, but this and other recent studies have characterized *V. cinerea*-AgNPs only in extract or solution form focussing on their antioxidant potential [20]. AgNPs due to their antibacterial effect, have often been applied in wound disinfection, and attracted attention due to their broad antimicrobial spectrum, and large surface area of action. The optical and electrochemical properties of silver nanoparticles have a bearing on their antioxidant activity [21]. Whether this antioxidant and related antimicrobial activity survives incorporation into a finished, traditionally-processed cosmetic product which has been prepared by the high-temperature indigenous burning method used for kajal and eyeliner has not been investigated. This is a critical gap, since thermal processing is known to degrade many plant-derived bioactive compounds, and the antimicrobial performance of a nanoparticle-containing raw extract cannot be assumed to predict the performance of the finished cosmetic actually applied to the eye.

The present study therefore differs from prior work in that it evaluates *V. cinerea*-derived AgNPs (VcAgNPs) incorporated into a finished eyeliner prepared by the traditional indigenous burning method. It directly compares this formulation against control eyeliners prepared with coconut oil alone and with *V. cinerea* aqueous extract, to isolate the specific contribution of the AgNPs. It evaluates the finished products for antimicrobial activity, microbial safety, and six-month physical stability, addressing whether traditional processing preserves or eliminates the bioactive potential reported for the raw nanoparticle preparation.

2. Materials and Methods

2.1. Collection and Authentication of Plant Material

Fresh whole plants of *V. cinerea* were collected from the precincts of Oushadhi (The Pharmaceutical Corporation (I.M) Kerala Ltd.), Thrissur, Kerala, India. The plant was identified and authenticated by Dr A. K. Pradeep, Curator, CALI Herbarium, University of Calicut. Voucher specimens were deposited in the CALI Herbarium for future reference (Figure 1).



Figure 1. Fresh and herbarium sample of *Vernonia cinerea*.

2.2. Preparation of *V. cinerea* Aqueous Extract

Freshly collected whole plants were washed thoroughly with tap water to remove surface impurities and then rinsed twice with double distilled water. Plant material was cut into small pieces and shade-dried for seven days. The dried material was ground to a coarse powder (as shown in Figure 2) using a laboratory blender and stored in clean, airtight glass bottles at ambient temperature until use [22].

The aqueous extract was prepared by mixing 10 g of coarse powder with 150 mL of distilled water in a 1000 mL glass beaker. The mixture was boiled until 150 mL was reduced to 35 mL of the leaf extract [22], during which the solution colour changed from light yellowish to dark green, indicative of phytochemical solubilization. The extract was cooled to room temperature and centrifuged at 2000 rpm for 15 min at 25 °C. The supernatant was collected, filtered through Whatman No. 1 filter paper, and stored at 4 °C until further use.



Figure 2. Vc coarse powder.

2.3. Green Synthesis of VcAgNPs

AgNPs were prepared by initially mixing 10 mL of *V. cinerea* aqueous extract with 90 mL of 2 mM aqueous silver nitrate prepared from silver nitrate (AgNO_3 , Merck, 99.8%). It was seen to change colour at the end of 24 h signalling formation of silver nanoparticles of the extract. Several incubation periods were assessed: 0, 0.5, 1, 2, 4, 9, and 24 h. In each synthesis trial, 5 mL of *V. cinerea* aqueous extract was added to 2 mM AgNO_3 solution at volumes of 25 mL, 50 mL, and 100 mL, yielding extract-to- AgNO_3 ratios of 1:5, 1:10, and 1:20, respectively as shown in (Table 1).

Table 1. Extract-to- AgNO_3 ratios and incubation times evaluated for the synthesis of VcAgNPs.

<i>V. cinerea</i> Aqueous Extract (mL)	AgNO_3 Solution (mL)	Ratio	Incubation Time (h)
5	25	1:5	0.5, 1, 2, 4, 9, 24
5	50	1:10	0.5, 1, 2, 4, 9, 24
5	100	1:20	0.5, 1, 2, 4, 9, 24

The mixtures were incubated in a water bath at 40 °C for upto 24 h. Formation of VcAgNPs was confirmed by a characteristic colour change from light yellow to dark brown, indicative of surface plasmon resonance. The optimal extract-to- AgNO_3 ratio and incubation time for maximum nanoparticle yield was established through spectroscopic characterization (UV-Vis spectrophotometry).

2.4. Preparation of Eyeliner

Strips of cotton cloth (11 cm × 5 cm) were repeatedly dipped in the *V. cinerea* aqueous extract and shade-dried; this process was repeated for seven days until the cloth attained a green hue. Wicks were fashioned from the treated and untreated strips. A total of 54 cloth wicks were prepared. Cloth wicks of *Vernonia cinerea* extract were 18 in number and cloth wicks of VcAgNPs were also 18 in number. Plain cloth wicks were also 18 in number. The wicks were dipped in Virgin coconut oil and burnt in separate set ups and the carbon soot harvested separately. They were burnt in an earthen container covered with an inverted stainless-steel plate, with

a small amount of water added to the upper surface of the plate to facilitate easy extraction of the carbon nanoparticles collected underneath. Combustion was maintained for three hours, yielding a black soot.

Description of the illustration as shown in Figure 3: To the *V. cinerea* extract solution as well as VcAgNPs solution, clean cotton cloth strips are dipped and shade dried, the process being repeated for seven days. They are made into wicks, dipped in coconut oil and placed in an earthen container with a stainless-steel plate upturned over it. They are burnt to get residue, which is scraped out and mixed with coconut oil to make eyeliner. The residue was carefully scraped and stored in a clean, airtight container. For application as eyeliner, 10 g of the powder was mixed with 2 mL of pure coconut oil. An identical procedure was employed using the VcAgNPs suspension in place of the aqueous extract to prepare a second experimental formulation. A third control formulation was prepared from cotton cloth wicks soaked in coconut oil only.

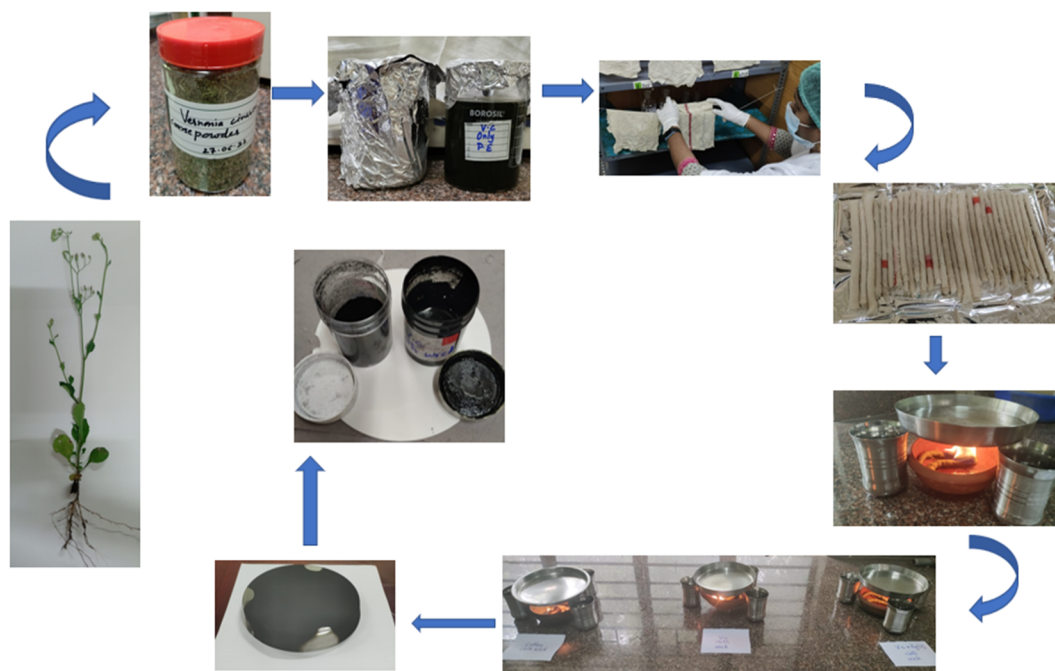


Figure 3. The process of making indigenous eyeliner using wicks dipped in *V. cinerea* extract and its AgNPs.

2.5. Microbial Contamination Assessment

Microbial safety of the finished kajal formulations was assessed using standard plate count and total fungal count methods. Total aerobic microbial count (TAMC) was determined on nutrient agar, total *E. coli* count on MacConkey agar, and total yeast and mould count (TYMC) on Sabouraud Dextrose Agar (SDA). Bacterial plates were incubated at 37 °C for 24–48 h and fungal plates at 25 °C for four days. Colonies were enumerated using a digital colony counter, and results were expressed as colony-forming units per gram (CFU/g).

2.6. Antimicrobial Susceptibility Testing

Antimicrobial activity was assessed using two complementary methods: (i) the agar well diffusion method and (ii) the ditch plate assay. Test organisms included *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538P and *Candida albicans* ATCC 2091 for the well diffusion assay. The cultures were maintained on nutrient agar or Sabouraud dextrose agar slants for bacteria and yeast respectively. 24-h old culture was standardized to a 0.5 McFarland turbidity vial (HiMedia) prior to inoculation. Sterile Mueller-Hinton agar or Sabouraud dextrose agar (for *C. albicans*) plates were inoculated uniformly with the above standardized inoculum using a sterile swab. 6 mm diameter wells were punched into the agar using a sterile cork borer, and 100 µL of the test sample (eyeliner formulation) was introduced into each well. Plates were kept at room temperature for one hour to allow pre-diffusion before incubation at 37 °C for 24–48 h. Zones of inhibition were measured in millimeters after 24–48 h of incubation at 37 °C. In the case of the ditch plate technique used to assess antibacterial activity, a ditch (2–3 mm wide and 7–8 mm length) was cut into an agar plate. Then, 0.05 g powder was mixed with 5 mL of molten agar was mixed well and poured into the ditch to make it plain with the rest of the agar in the plate, and test organisms were streaked perpendicular to the ditch. The plates were incubated at 37 °C for 24 h. Inhibition of bacterial growth towards the ditch is due to antibacterial efficacy. The distance of inhibited growth

from the ditch was recorded as a measure of antibacterial efficacy. All assays were performed in triplicate ($n = 3$) on separate occasions to ensure reproducibility. Results are expressed as mean $\pm$ standard deviation [23].

Sterile coconut oil, a standard antibiotic agent 5 microgram gentamicin for bacteria, and fluconazole for *C. albicans* were used to validate assay sensitivity and allow comparison of the test formulation's antimicrobial efficacy.

2.7. Characterization of VcAgNPs

Synthesized VcAgNPs were characterized by UV-Vis spectroscopy to monitor the surface plasmon resonance (SPR) absorption band indicative of nanoparticle formation. Morphological analysis and elemental composition were determined by Scanning Electron Microscopy with Energy Dispersive X-ray Analysis (SEM-EDAX). Functional group characterization was carried out by Fourier Transform Infrared Spectroscopy (FTIR), enabling identification of plant-derived capping and reducing agents on the nanoparticle surface. The Aeris Research Benchtop X-ray diffractometer (Malvern Panalytical/PANalytical, Almelo, The Netherlands) was used as an experimental method of examining the structure of for structural and phase characterization of nanomaterials.

2.8. Stability Studies

Physical stability of the formulated eyeliner (appearance, texture, colour, consistency, and pH) was evaluated, in accordance with ICH Q1A(R2) guidelines for stability testing of cosmetic formulations. Three independent batches of the eyeliner formulation were prepared and subjected to stability testing to account for batch-to-batch variability. Each parameter was evaluated in triplicate for every batch at each sampling interval, and results are expressed as mean $\pm$ standard deviation (SD).

Samples were packed in their final proposed packaging in sterile Pharmacol containers (HiMedia, Thane, India) was used and stored under room temperature (long-term) storage: $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}/60\% \pm 5\% \text{ RH}$. Samples were withdrawn and evaluated at the following time points: Day 0 (baseline), 1 month, 2 months, 3 months, 6 months and 12 months (long-term stability). At each sampling point, the following were assessed for physical parameters like color, homogeneity, and odor. Microbial load (total viable count) of all time periods was analysed [24].

3. Results

3.1. Green Synthesis and Characterization of VcAgNPs

Nanoparticle formation by green synthesis was characterised using UV-Vis spectroscopy, which is based on the SPR band exhibited at different wavelengths [21]. UV-Vis spectra recorded from the *V. cinerea* aqueous extract with different ratios of AgNO_3 solution at different time intervals of reaction showed a maximum absorption sharp peak at 420 nm at an incubation time of 24 h for the solution with ratios 1:20 (as shown in Figure 4) and 1:10. The highest absorbance peaks in the UV-Vis spectra data indicate the level of formation of AgNPs. However, no distinction peaks were observed for the solutions at a ratio of 1:5.

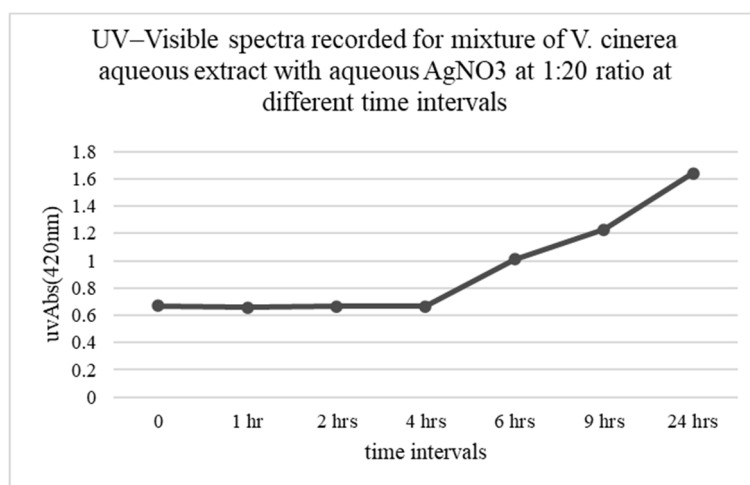


Figure 4. Corresponding graph of the values.

The increase in intensity may be attributed to either a large number of nanoparticles being formed or the generation of silver nanocrystals through the reduction of Ag^+ ions by phytochemicals present in *V. cinerea* whole plant extract. The maximum synthesis of AgNPs was obtained at 1:20 ratio of extract concentration to AgNO_3

solution, pH 6, 2 mM AgNO₃, and an incubation temperature of 40 °C for 24 h (Table 2). The characteristic colour changes of the reaction mixture from light yellow to dark brown confirmed nanoparticle formation via surface plasmon resonance [23].

The traditional eyeliner preparation method employed in this study yielded a fine, black carbonaceous residue with a characteristic texture following the combustion of *V. cinerea* extract-impregnated cotton wicks, VcAgNPs cotton wicks and plain cotton wicks.

Table 2. Time of exposure and absorbance values at 1:20 ratio.

Time of AgNO ₃ Exposure to Form AgNPs	Mean ± SEM
0	0.669 ± 0.000
1 h	0.657 ± 0.053
2 h	0.665 ± 0.025
4 h	0.664 ± 0.031
6 h	1.010 ± 0.277
9 h	1.227 ± 0.351
24 h	1.641 ± 0.132

The residues yielded from the above three types of wicks were almost equal in yield., and upon admixture of 10 g with 2 mL pure coconut oil, produced a smooth, homogeneous preparation with acceptable consistency for ophthalmic application (yields as shown in Table 3). The inclusion of coconut oil (*Cocos nucifera*) as a carrier is consistent with traditional Ayurvedic practice and imparts lubricating, antifungal, and emollient properties to the formulation [24].

Table 3. Carbon soot yield (in gms) from the combustion of three types of wicks kept for burning at equal weights.

	Trial 1 (g)	Trial 2 (g)	Mean (g) ± SEM
Cloth wick only	11.16	11.34	11.25 ± 0.090
Vc + cloth wick	11.86	11.79	11.825 ± 0.035
Vc AgNPs + cloth wick	11.31	11.36	11.335 ± 0.025

We wanted to test whether the combustion of phytochemical-impregnated cloth, which generates the carbon-rich soot, would contain partially pyrolyzed plant metabolites, including carbonized forms of flavonoids and phenolic acids. It was hypothesized that these thermally altered compounds would retain residual pharmacological activities, including antimicrobial properties relevant to ophthalmic safety and efficacy. The VcAgNP plant extract and the carbon soot were further investigated using FTIR, SEM, EDAX and XRD. Two distinct samples were characterised in this study: (1) Plant extract-mediated silver nanoparticles (AgNPs), synthesised using a *V. cinerea*-based aqueous extract (VcAgNP), and (2) Carbon soot (Vc eyeliner), prepared by pyrolysis/combustion of the same plant material. Three measurement sites were collected per sample for EDAX quantification, ensuring statistical reliability. FTIR spectra (SAIF Cochin), SEM micrographs at 10,000× magnification, and EDAX compositional data are discussed below.

The FTIR spectrum of VcAgNPs shows a set of absorption bands spanning 400–4000 cm^{−1}. This shows the complexity of the plant phytochemical with the silver nanoparticles (Figure 5). The broad absorption at 3420 cm^{−1} is characteristic of hydrogen-bonded O–H stretching vibrations, which may be from polyphenols, flavonoids, and carbohydrates that serve as both reducing and capping agents during nanoparticle synthesis [24]. The twin bands at 2920 and 2851 cm^{−1} show the presence of aliphatic –CH₂– chains likely from lipids, waxes, or fatty acids. A notable absorption at 1763 cm^{−1} is attributed to C=O ester stretching characteristic of flavone/flavonoid lactone rings. The band at 1635 cm^{−1} corresponds to C=O stretching (Amide I) and/or N–H bending, indicating possible enzymatic involvement in the bio-reduction mechanism. The 1384 cm^{−1} band is consistent with O–H in-plane bending or nitrate (NO₃[−]) residue from the silver nitrate precursor (AgNO₃). Most critically, the low-wavenumber bands at 418 and 457 cm^{−1} are signatures of the Ag–O bond (metal–oxygen lattice vibrations), directly confirming the formation of metallic/oxide silver nanoparticles. The 824 cm^{−1} band corresponds to out-of-plane aromatic C–H bending, indicating aromatic phytochemicals as the capping agents.

The FTIR spectrum of carbon soot as shown in Figure 6 is simpler than that of the AgNP sample, which is due to the extensive thermal decomposition of plant biomolecules during pyrolysis. The dominant C–H stretching bands at 2925 and 2854 cm^{−1} indicate residual aliphatic hydrocarbon chains retained from the plant precursor, though these are less complex than those in the AgNP sample. The C=O absorption at 1746 cm^{−1} in the soot spectrum arises from oxidised carbonaceous surface groups (ketones, aldehydes, or esters) generated during

incomplete combustion. The bands at 1466 and 1377 cm^{-1} are characteristic of aromatic C=C stretching and C–H deformations in graphitic carbon domains, consistent with the formation of polycyclic aromatic hydrocarbons (PAHs) and carbon structures during pyrolysis. The C–O–C vibrations at 1160 and 1112 cm^{-1} indicate partial surface oxidation of the carbon soot, which is common in plant-derived carbons. The 722 cm^{-1} band corresponds to long-chain C–H vibrations and aromatic C–H out-of-plane bending in the graphitic structure. Critically, the soot spectrum lacks the Ag–O bands at 418–518 cm^{-1} and the broad polyphenol O–H band at $\sim 3400 \text{ cm}^{-1}$, confirming the complete conversion of phytochemical capping agents and the absence of silver in the sample.

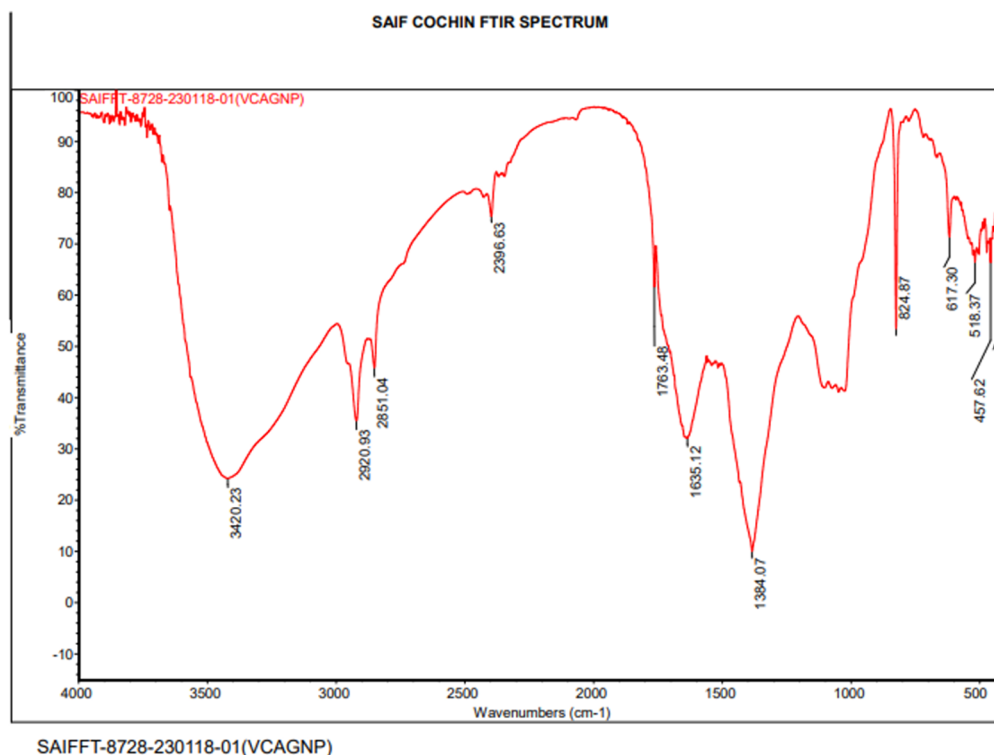


Figure 5. FTIR spectrum of plant extract with AgNPs showed several peaks indicating the complex nature of the biological material.

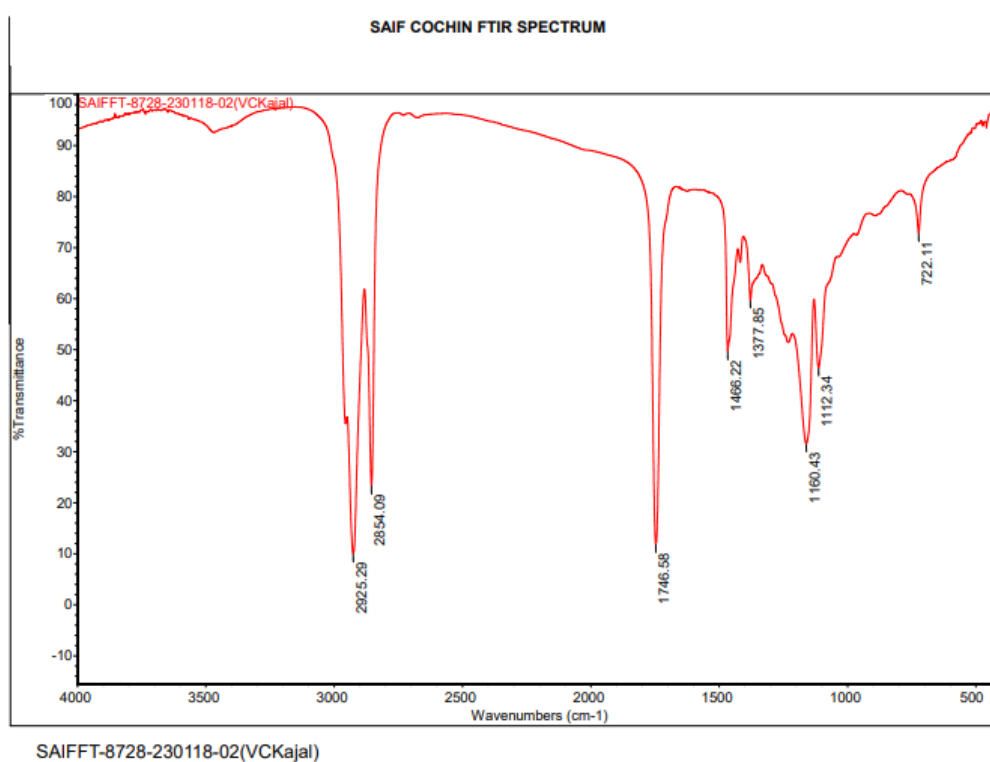


Figure 6. The FTIR spectrum of carbon soot showing simpler arrangement.

SEM-EDAX data of the VcAgNP sample showed a silver content range from 19.7 to 23.9 wt% (Figure 7A) confirming the presence of silver. The high carbon (18–28 wt%) and oxygen (29–34 wt%) contents reflect the organic capping matrix. Potassium (15–21 wt%) and chlorine (3.4–4.0 wt%) are characteristic of plant-derived extracts, originating from intracellular salts and secondary metabolites. Trace amounts of sodium, phosphorus, and sulphur further confirm the plant phytochemical origin of the capping layer.

SEM-EDAX of the soot shows near-pure carbon (99.94–100 wt%) as shown in Figure 7B represents the graphitic core of the soot. Some sites show 87–88 wt% carbon with 7–8 wt% oxygen plus trace silicon, iron, copper, chlorine, potassium, and calcium, which are all characteristic of plant ash contaminants and surface oxidation. There was some trace Ag detected at some soot sites (0.01–0.08 wt%), which can be attributed to a background signal or cross-contamination artefact. It is not indicative of silver nanoparticles. The consistent absence of K, Cl, Na, P, and S in the soot (in contrast to the AgNP sample) confirms complete thermal mineralisation of plant biomolecules during pyrolysis [25].

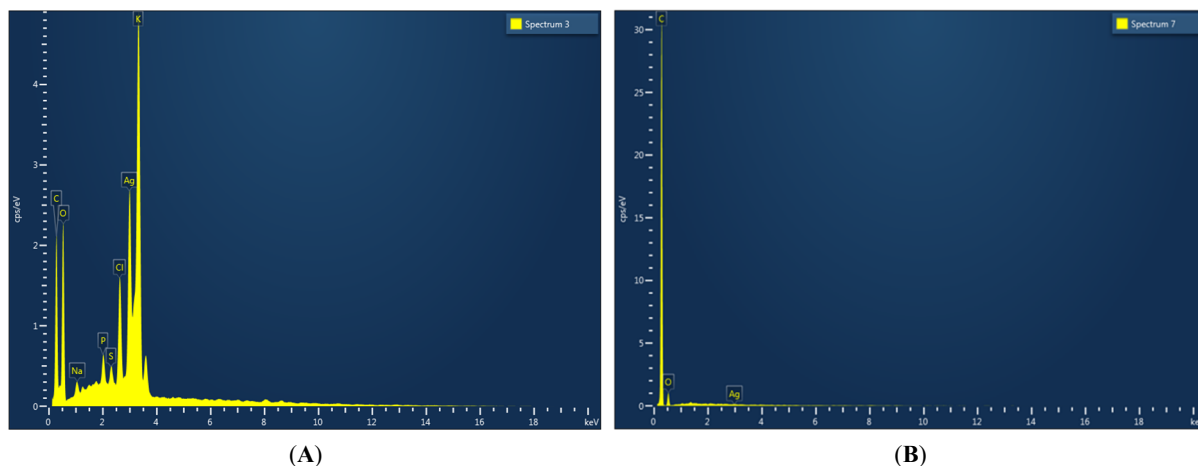


Figure 7. (A) EDAX data of the VcAgNP sample and (B) carbon soot.

The SEM micrograph (20 kV, $\times 10,000$, scale bar = 1 μm) of the VcAgNP extract reveals a bio-agglomerated morphology typical of plant extract-derived silver nanoparticles as shown in Figure 8A. Large, irregular globular masses ($\sim 1\text{--}5\text{ }\mu\text{m}$ across) are visible, representing clusters of the plant matrix in which the nanoparticles are embedded. We can notice bright, discrete crystalline particles of approximately 100–500 nm scattered across the surface. These are consistent with silver nanoparticles characterising their high atomic number, strong backscatter or secondary electron signal. The layered, folded surface texture of the matrix is characteristic of dehydrated phytochemical material, which may consist of polysaccharides or proteins that form the organic capping shell. Some circular pores ($\sim 200\text{--}300\text{ nm}$) are also seen.

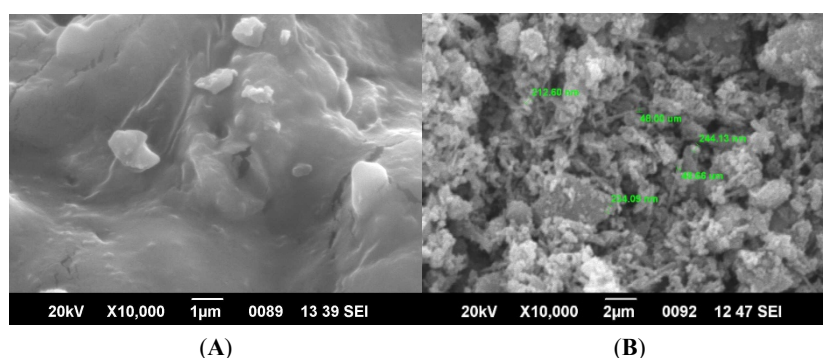


Figure 8. SEM image (A) VcAgNP sample at 10,000 $\times$, (B) soot sample at 10,000 $\times$.

The SEM image (Figure 8B) of carbon soot (20 kV, $\times 10,000$, scale bar = 2 μm , image 0092) presents a completely different microstructure. The material consists of densely packed, irregularly-shaped granular aggregates with a rough texture, which is consistent with soot morphology. The particles display a broad size distribution. The green measurement annotations confirm particle sizes in the range of 212–244 nm for individual granules, with carbon with an average diameter of 48 nm. This morphology reflects the fusion and sintering of

carbon during pyrolysis. Importantly, no bright metallic particles are visible, confirming the absence of silver, consistent with EDAX data.

X-ray diffraction (XRD) analysis is of significance in defining the structural details of the carbon soot and the eyeliner prepared using the carbon soot by mixing with coconut oil. XRD patterns of both S1 (carbon soot) and S2 (carbon particles mixed in coconut oil to form a paste) show no sharp Bragg reflections. The pattern shows only broad humps in the 15–23° 2 θ region, confirming phase-pure, disordered carbon rather than crystalline graphite or detectable impurities. S1 displays two resolved humps at 15.1° and 22.2° ($d = 5.86$ and 4.01 Å), consistent with pre-graphitic short-range order. In S2, this two-hump pattern collapses into a single broader feature at 19.2° ($d = 4.62$ Å), which coincides almost exactly with the β -polymorph short-spacing reflection of crystallized triglycerides (Figure 9A,B).

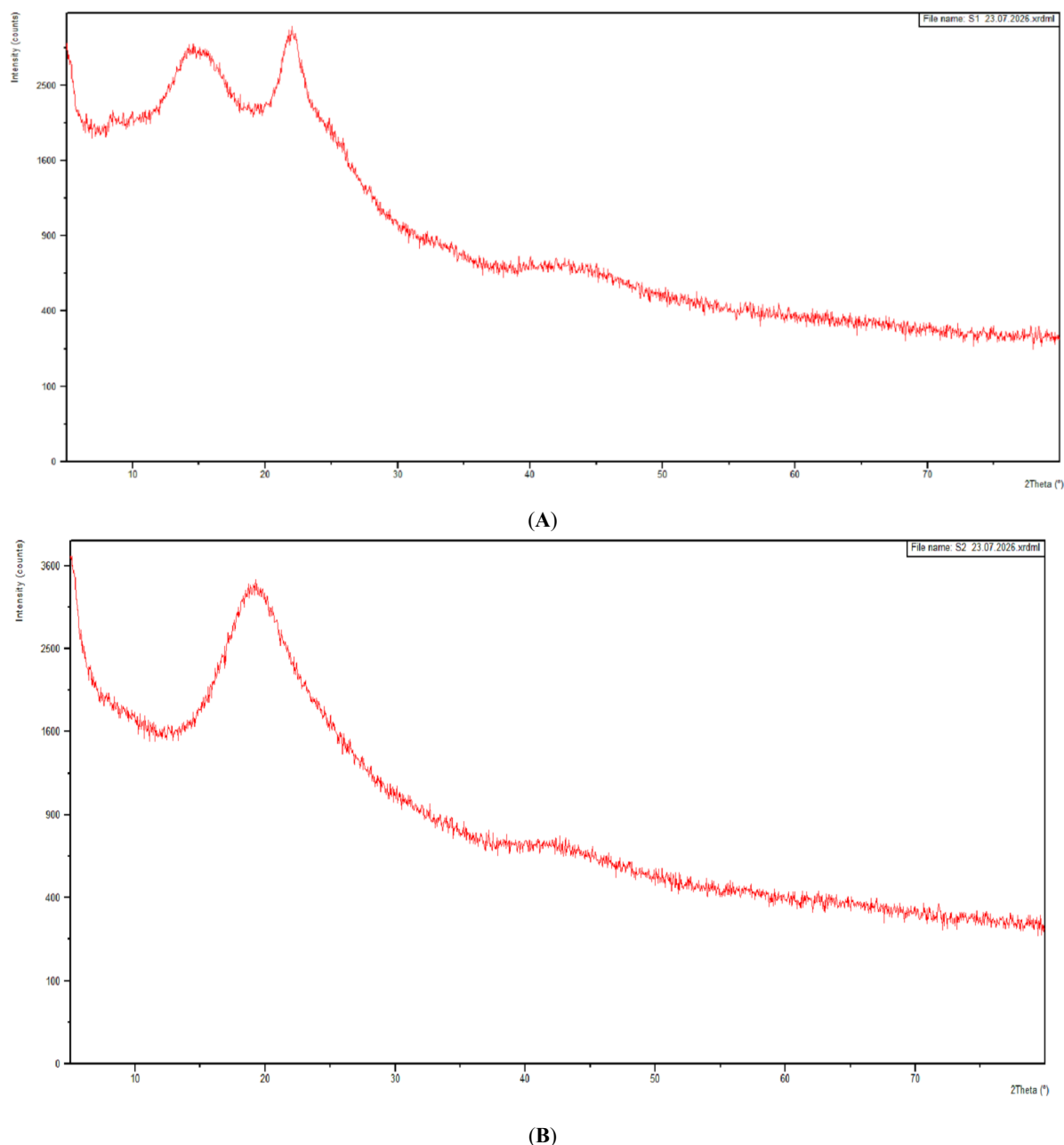


Figure 9. XRD profile of (A) carbon soot and (B) carbon soot + coconut oil.

3.2. Microbiological Quality Evaluation

Microbiological testing of the finished Kajal products was conducted to ensure product safety in compliance with standard pharmaceutical quality benchmarks for cosmetic and semi-ophthalmic preparations. The evaluation encompassed total aerobic microbial count (TAMC), total yeast and mould count (TYMC), and specific enumeration of *E. coli* as a gram-negative indicator organism (Table 4).

Acceptable microbiological limits for cosmetic and medicated Kajal products are set by regulatory standards [9,26] which specify TAMC $\leq 10^3$ cfu/g, TYMC $\leq 10^2$ cfu/g, and absence of *E. coli*. The inclusion of AgNPs in the formulation is expected to contribute to microbial load reduction, given the well-documented broad-spectrum antimicrobial activity of silver nanoparticles against both gram-positive and gram-negative bacteria, as well as fungi [27–29].

All three formulations: cotton cloth wick eyeliner (control), cotton cloth with *V. cinerea* extract, and cotton cloth wicks with VcAgNPs showed colony counts of too few to count (TFTC) at both undiluted and 10^{-1} dilutions for standard plate count and yeast and mould count. The formulations also tested negative for the presence of *E. coli*.

Table 4. Microbial counts of the three eyeliner formulations (done in triplicate). TFTC = Too few to count.

Eyeliner Formulation	TAMC (Undiluted)	TAMC (10^{-1})	TYMC (Undiluted)	TYMC Count (10^{-1})
Cotton cloth wick eyeliner (control)	TFTC	TFTC	TFTC	TFTC
<i>V. cinerea</i> extract eyeliner	TFTC	TFTC	TFTC	TFTC
VcAgNPs eyeliner	TFTC	TFTC	TFTC	TFTC

TAMC: Total Aerobic Microbial Count; TYMC: Total Yeast and Mould Count; TFTC: Too few to count, AgNPs: Silver Nanoparticles.

3.3. Antimicrobial Activity

Well diffusion assay results as shown in Figure 10a,b,d demonstrated that none of the three finished kajal formulations exhibited inhibitory activity against *S. aureus* or *E. coli* [30]. In contrast, coconut oil alone, the VcAgNPs suspension, and 2 mM AgNO_3 solution all showed zones of inhibition. The VcAgNPs suspension exhibited the largest zones of inhibition (21 ± 0.5 mm against *S. aureus*; 20 ± 0.4 mm against *E. coli*), while coconut oil showed modest inhibition against *S. aureus* only (12 ± 0.3 mm). There were no zones of inhibition against *C. albicans*. Ditch plate assay (Figure 10c) confirmed the absence of antimicrobial activity in all finished eyeliner formulations against *E. coli*, *S. aureus*, and *C. albicans* (Table 5) [31].

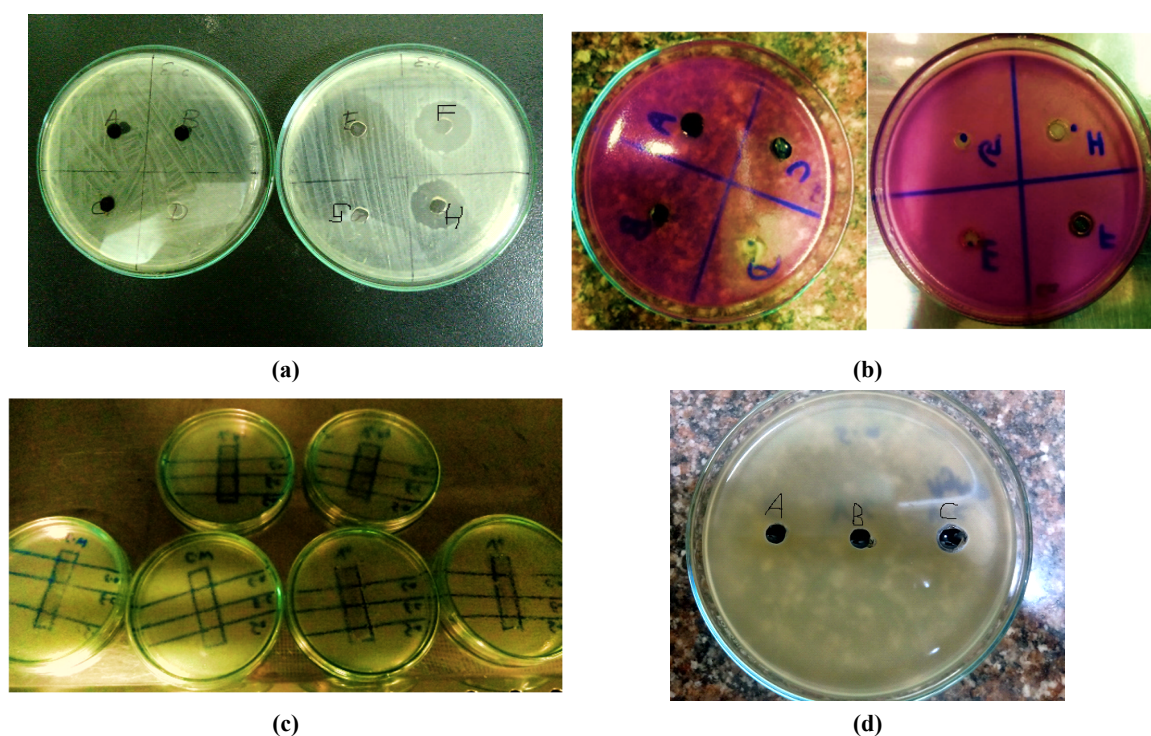


Figure 10. (a) Agar well diffusion studies on *E. coli* with test samples A–H, (b) Agar well diffusion studies on *C. albicans* with test samples A–H, (c) Ditch plate assay of the eyeliner powder material and (d) Agar well diffusion studies on *S. aureus* with test samples A, B and C.

Table 5. Zones of inhibition (mean $\pm$ SD, mm) by agar well diffusion. — = no inhibition observed.

Label	Test Sample	Zone of Inhibition <i>S. aureus</i> (mm)	Zone of Inhibition <i>E. coli</i> (mm)	Zone of Inhibition <i>C. albicans</i> (mm)
A	Cotton cloth wick eyeliner (control)	—	—	—
B	<i>V. cinerea</i> extract eyeliner	—	—	—
C	VcAgNPs eyeliner	—	—	—
D	Coconut oil	12 $\pm$ 0.3	—	—
E	<i>V. cinerea</i> aqueous extract	—	—	—
F	VcAgNPs suspension	21 $\pm$ 0.5	20 $\pm$ 0.4	—
G	Distilled water (negative control)	—	—	—
H	Gentamicin/Fluconazole	18 $\pm$ 0.6	17 $\pm$ 0.5	—

3.4. Stability Studies

Physical stability studies confirmed that all three kajal formulations maintained their appearance in colour, homogeneity and odor over the 12-month observation period under ambient storage conditions, indicating shelf-life stability, obliterating the use of synthetic preservatives. Coconut oil was checked for rancidity [32].

4. Discussion

The present study demonstrates the feasibility of formulating a traditional indigenous eyeliner using *V. cinerea* aqueous extract and its biosynthesised silver nanoparticles (VcAgNPs) by the indigenous burning method. FTIR characterisation, SEM-EDAX data and XRD corroborate the presence of pristine carbon nanoparticles in the carbon soot obtained [28,33]. A central finding of this study is the complete absence of antimicrobial activity in all three finished eyeliner formulations against *S. aureus*, *E. coli*, and *C. albicans* by both agar well diffusion and ditch plate assay [34]. The loss of antimicrobial activity in the finished product is most plausibly attributed to the high-temperature pyrolysis step, during which biologically active AgNPs and phytochemical reducing agents are thermally decomposed [35]. The temperatures generated during combustion (estimated to exceed 400–600 °C in traditional wick-burning systems) are well above the threshold for nanoparticle sintering, organic ligand decomposition, and silver volatilisation, resulting in a carbonaceous residue with no residual silver or bioactive phytochemicals. This highlights that the bioactivity of precursor materials does not necessarily persist through traditional high-temperature processing [36]. Hence there is actually no gain in incorporating *Vernonia cinerea* extract or its AgNP form as wicks which on combustion provide none of the protective effect of raw extract or its silver nanoparticle solution. Instead, future work could look into incorporating a “cold process” (adding AgNPs to the coconut oil after the soot is collected) and look out for its antimicrobial effect.

The microbiological safety profile of all three formulations was good. The inclusion of coconut oil as a carrier may have contributed to product safety as its lauric acid content is known to possess antifungal and antimicrobial properties that may inhibit residual microbial growth during storage [37]. Notably, the satisfactory microbial safety of the plain control (Cotton wick alone) underscores that the burning process itself is sufficient to produce a microbiologically safe product, independent of any plant-based additive.

The shelf-life stability and self-preserving nature of the formulation position it favourably against conventional synthetic eyeliners that rely on preservatives such as benzalkonium chloride (BAK), which have well-documented cytotoxic effects on the ocular surface epithelium. The toxicological safety of *V. cinerea* has been previously established, with no apparent in vivo toxicity reported at tested dosages in both acute and brine shrimp lethality assays [31,38], and therefore it may be added post formulation as against pre formulation. The ethnobotanical profile of *V. cinerea* strongly supports the rationale for its use in an ophthalmic context. Its documented applications in conjunctivitis, filariasis, and inflammation, and the anti-cataract activity of lupeol provide compelling pharmacological grounds for further clinical investigation. Clinical evidence further supports the ophthalmic utility of carbon-based indigenous eyeliners which have reportedly given significantly greater improvement in blepharitis signs and symptoms with traditional kohl compared to controls, indicating that systematic scientific validation of such formulations is warranted.

One approach may be to purify and treat the carbon nanoparticles further and develop them as functionalized carbon dots (FCDs) which are emerging as powerful new antibacterial agents [39]. As these eyeliners are applied to the lash line, the new formulation as carbon dots could actively suppress bacterial infections like blepharitis, conjunctivitis, and lid margin infections. Photoactivated carbon dots have demonstrated strong antibacterial activity against both Gram-positive and Gram-negative bacteria, effectively preventing biofilm formation and disrupting preformed biofilms, while displaying low cytotoxicity toward mammalian cells [39]. These novel approaches need to be investigated.

5. Conclusions

The present study successfully demonstrated the green synthesis and physicochemical characterisation of silver nanoparticles using the aqueous whole-plant extract of *V. cinerea* and their incorporation into an indigenously prepared kajal eyeliner by the traditional Ayurvedic burning method. It established the successful biogenic formation of stable, phytochemical-capped nanoparticles. However, the carbon soot generated by pyrolysis was characterised as a near-pure carbonaceous nanomaterial with disordered graphitic structure, surface oxidation, granular aggregate morphology (212–244 nm), and nanotube-like structures (~48 nm diameter). These findings are structurally distinct from the VcAgNPs and consistent with plant-derived biochar nanoparticles indicating that no trace of silver remains after the combustion thus negating the use of *Vernonia cinerea* incorporation into the wick.

All three eyeliner formulations met regulatory microbiological safety thresholds, and physical stability was maintained. However, for confirming the formulation's suitability as a safe cosmetic product, a HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) assay would be needed for validation. These investigations provide insight into the limitations of traditional high-temperature processing in the preparation of eyeliner.

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