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RESEARCH ARTICLE

Isolation, Characterization and Quantitation of Photoactive phases of Titanium (IV) oxide in skin-lightening products

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ABSTRACT:
Titanium (IV) oxide (TiO_2) is used as a physical blocker of ultraviolet (UV) radiation in many skin-care products. Absorption of TiO_2 through the skin is likely to interact with viable tissues because UV radiation absorption generates toxic reactive oxygen species such as hydroxyl radicals. Studies on the acute toxicity of TiO_2 nanoparticles in mammals indicate that intra-tracheal instillation, intraperitoneal injection or oral instillation of TiO_2 particles to the animals evoke an inflammatory response as well as certain histopathological changes. Ultrafine particles of the anatase form of titanium (IV) oxide, which are smaller than 0.1 microns, are pathogenic. In this work eight skin-lighteners containing TiO_2 from South African market were studied. The TiO_2 was extracted by a fusion technique and quantified by inductively coupled plasma-optical emission spectrometry (ICP-OES). Sequential solvent extraction was employed to isolate TiO_2 particles for characterization employing high-resolution transmission electron microscopy (HR-TEM) and powder X-ray diffraction (PXRD). All samples considered in this study meet agreeable TiO_2 % (m/m) levels as specified by all health regulatory bodies. Both forms of TiO_2: anatase and rutile, were found to be present. Most samples contained nano-TiO_2 in the particle size range of 16.23 nm to 51.47 nm that could lead to detrimental effects. The fact that the anatase form, known for its photocatalytic activity, was present, is a cause for concern.

KEYWORDS: Quantitation, Anatase, Rutile, Nano-TiO_2

INTRODUCTION:
Physical blockers like titanium (IV) oxide (TiO_2) present in most skincare products have been shown to photo induce degradation of organic sunscreens, enzymes, and DNA[1,2]. Studies on the acute toxicity of TiO_2 nanoparticles in mammals indicate intra-tracheal instillation, intraperitoneal injection or oral instillation of TiO_2 particles to animals evoke inflammatory response and histopathological changes[3]. In cultured macrophages, TiO_2 nanoparticles change the integrity of cell membrane and phagocytic activity[4].

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The reduction in cell viability, morphological alterations, compromises antioxidant system, intracellular ROS production, and significant DNA damage in cells exposed to TiO_2 nanoparticles signifying the potential of nanoparticles to induce cytotoxicity and genotoxicity in cultured human amnion epithelial (WISH) cells[5].

TiO_2 absorbs about 70 % of incident UV, and in viable aqueous environments this may lead to generation of hydroxyl radicals. These free radicals may initiate oxidative reactions presenting possible undesirable mutagenic effects.[6] demonstrated that, if the sunscreen agent TiO_2 illuminated with appropriate UV light it interacts with DNA or RNA and is, can cause serious damage. A recent study showed that even some modified TiO_2 particles specifically developed and marketed for sun-care, skin-care, and colour cosmetic formulations,

still retain photocatalytic activity[7]. Mild cytotoxic response of TiO_2 nanoparticles has been reported and linked to induction of DNA damage.[8] observed significant induction in micronucleus formation, reduction in glutathione, concomitant increase in lipid hydroperoxide and reactive oxygen species (ROS) generation demonstrating mild cytotoxic potential. Though induced ROS and oxidative stress may lead to oxidative DNA damage, micronucleus formation may form the basic mechanism of TiO_2 nanoparticle genotoxicity[8].

Oxidative and nitrative stress causes nitration of the protein tyrosine, a post-translational modification linked to the onset or progression of diseases, such as cardiovascular diseases neurodegenerative diseases, and inflammation. The presence of tyrosine nitration in diseased conditions is an indication of the generation of peroxynitrite ($ONOO^-$) *in vivo* produced from the very fast reaction of nitric oxide (NO) and superoxide ($O_2^{\bullet-}$) radical.[9] recently demonstrated the physiological potential of nano-TiO_2 to photocatalyse protein nitration in mouse skin homogenate. Tyrosine nitration is reported in several cutaneous pathological effects: contact hypersensitivity, systemic sclerosis, cutaneous inflammation, and thermal injury[9]. The anatase form of TiO_2 can greatly increase the formation of free radicals when exposed to sunlight and water in sunscreens. Studies indicate nano-anatase TiO_2 (1-100 nm) is highly photo-reactive, and thus hazardous. Nano-anatase TiO_2 in sunscreens was shown to react with sunlight and break down coatings on steel roofs at a rate 100-fold more than normal sunlight[10]. With the same effectiveness, nano-anatase is likely to attack viable tissues if it comes in contact.

The nano-particulate range of TiO_2 in cosmetics possess a danger to infants and children with thinner, developing skin and people having broken skin. Nanoparticulate TiO_2 is widely used in sunscreen products to boost the SPF. Another area of application is in skin-lightening preparations. Skin-lighteners are designed to reduce the formation of melanin in the skin and thus the skin is left without adequate protection from the deleterious effects of UV radiation. Consequently, these products contain TiO_2 to afford broad-spectrum protection. From the foregoing it is apparent that the amount, particle size, and form of TiO_2 in a formulation needs to be controlled; European cosmetic, toiletry and perfumery association (COLIPA) set the maximum allowable concentration of TiO_2 in sunscreens as 25% (m/m)[11]. However, most health regulatory bodies worldwide to date do not specify particle size limits. In the present work isolates, quantitate, and characterize the phases of titanium (IV) oxide present in skin-lightening products in the South African market.

MATERIALS AND METHODS:

Reagents:

Titanium (IV) oxide (TiO_2) (99.8% – Analytical Reagent Grade) from Riedel-de Haën A.G., Seelze-Hannover, was used for the preparation of standard solutions. Analytical grade sulphuric acid (H_2SO_4) (98.0%) was supplied by Associated Chemical Enterprises (Pty) Ltd, Johannesburg, South Africa, and BDH Chemicals Ltd, Poole, England. Potassium hydrogen sulphate ($KHSO_4$) was supplied by BDH Chemicals Ltd, Poole, England. A total of eight skin-lightening products containing TiO_2 were purchased from retail outlets in Durban, South Africa.

Quantitation of TiO_2:

Preparation of standard solutions*:*

A mass of 0.05g of TiO_2 (> 99%) was weighed and dissolved in 100mL of hot concentrated H_2SO_4 (> 98%), with constant stirring for 12 h to make a standard stock solution of 300mg mL^{-1} of Ti^{4+}. The stock solution was used to make working standards in the range 2 mg dm^{-3}–10mg dm^{-3}. All Standards were purchased from Capital Labs, South Africa).

Preparation of samples:

Different masses of the skin-lightening samples in the range of 0.4 - 0.6g were weighed into a fused silica crucible and placed into an electrical furnace (Natalab supplies, South Africa) with the temperature set at 600 ºC for three hours to give carbon-free ash. The ash was allowed to cool in a desiccator for 10 min, and then about 0.5g of $KHSO_4$ was added to it. The crucible containing the ash residue and $KHSO_4$ was heated over a Bunsen burner for 15 min to fuse the mixture. The molten product was then dissolved in hot, concentrated H_2SO_4 and the solution transferred to a beaker. This solution was strongly heated to ensure complete solubilization of the TiO_2. The sample solutions were then diluted with deionised water to 100mL. A ten-fold dilution was done for samples that did not fall within the range of the calibration standards. All samples were analysed in triplicate.

Inductively coupled plasma-optical emission spectroscopy analysis:

Analysis of TiO_2 in sunscreens was as prescribed by[12]. An inductively coupled plasma optical emission spectrometer Perkin Elmer (Optima 5300 DV) fitted with an auto-sampler was used for the quantitation of TiO_2 and the data was processed by Perkin Elmer Win Lab 32 software. The instrument was programmed to sample each standard and sample five times in radial view mode. Other operating conditions were: argon gas flow rate of 1.5 L min^{-1}, auxiliary and nebulizer gas flows at 0.2 L min^{-1} and 0.8 L min^{-1} respectively. The pump flow rate was set at 1.5mL min^{-1} with the plasma

radiofrequency working at 1300W. The data were acquired at a wavelength of 337.279nm.

Method validation:
The method validation was done by spiking a TiO_2-free sample with about 10mg of TiO_2. A mass of ~ 0.150g of the spiked sample was accurately weighed into a fused silica crucible. The crucible was then put in an electrical furnace at 600°C for three hours after which it was placed in a desiccator for 10 min to cool. To the cooled carbon-free ash residue a mass of 0.50g of $KHSO_4$ was added and fused over a Bunsen burner for 15 min. The molten product was dissolved in hot, concentrated H_2SO_4 and made up to 100mL with deionized water it was then subjected to a ten-fold dilution. The diluted sample was subjected to ICP-OES analysis. The standards were analyzed in between sample runs to check on instrument signal response and precision. An intra- and inter-day analysis were performed based on the precision of the standards analysis within the day of analysis and between days of analysis.

Data analysis:
The calibration data was analyzed with Microsoft Excel© 2007 tool pack. The limit of detection (LOD) and limit of quantitation (LOQ) was calculated from the results of the linear calibration curve of the standards. The results were expressed as mean ± SD.

Characterization of TiO_2:
Extraction of TiO_2:
Samples containing TiO_2 were washed with solvents of varying polarity indices to isolate crystalline particles. A mass of ~ 0.2g of the sample was weighed into a beaker and washed firstly in 200mL dimethylformamide with ultrasonication for 2 h. The solvent with the dissolved organics was filtered through Whatman 1 filter paper and the remaining solid residue was then re-washed with fresh solvent in the order: methanol, acetone, and chloroform. The order varied depending on the sample matrix. Each wash was similarly filtered until crystalline TiO_2 could be observed. The isolated crystals were then dried in an electric oven at 100°C for one hour.

Characterization by PXRD:
PXRD analyses were done by using a Bruker D8 Advance diffractometer equipped with an Anton Paar XRK 900 reaction chamber, a TCU 750 temperature control unit, with CuK_α radiation at 40 mA; 40 kV and 1.5405 Å. The diffractograms were collected over a 2θ of 10.000° - 89.893° range at a goniometric velocity of 0.034° min^{-1} at 25°C. The spectral data was accumulated and processed by using Diffrac^{plus} basic XRD Wizard2.8 software. The diffraction peaks of crystalline phases were compared with standard anatase and rutile reported in the JCPDS database. The particle size of TiO_2 extracted by the sequential solvent system was estimated from the width, of diffraction peaks, calculated by using Scherrer's equation:

$$\tau = \frac{K\lambda}{\beta cos\theta}$$

where K is Scherrer's constant (0.89): shape factor, λ is the X-ray wavelength used (1.5405 Å), β is the width at half maximum intensity (FWHM) in radians of the diffraction peak measured at 2θ, θ is the Bragg angle, and τ is mean size of the crystalline particles.

Characterisation by high resolution transmission electron microscopy:
Samples for high resolution transmission electron microscopy (HR-TEM) observation were prepared by dispersing the extracted TiO_2 powders in an absolute ethanol solution under ultrasonic irradiation. The dispersed TiO_2 was then deposited on carbon-copper grids. The crystallite sizes and shapes were observed by HR-TEM on a JEOL JEM-2100 microscope at 200 kV. The structure resolution of the microscope was set at 0.2 nm.

RESULTS AND DISCUSSION:

The TiO_2 content of the eight skin-lightening products investigated in this work was determined by ICP-OES. The analytical method had a linear working from 0.48 to 2.5 mmol dm^{-3} as observed from the calibration curve of the standards. The correlation coefficient of the calibration curve (determined in triplicate) was 0.999.

Table 1 shows the amounts, particle size, and phases of the TiO_2 analyzed in this work. The LOD was calculated by using equation 1:

$$LOD = 3S_{y/x}/b \quad (1)$$

where $S_{y/x}$ is the standard error of the slope and b is the slope of the calibration curve [13]. The limit of detection was 0.06518 mg dm^{-3}. The LOQ from this data was calculated using equation 2:

$$LOQ = 3.3\,LOD. \quad (2)$$

Table 1: Average percentage concentration, particle size and phase of TiO_2 in the skin-lightening samples.

Sample	*TiO_2 % (m/m)	Particle size/nm	Phase of TiO_2
B	6.90 ± 0.01	16.23 ± 0.31	Rutile/anatase
E	7.47 ± 1.24	26.39 ± 1.79	Rutile
G	5.65 ± 0.01	45.03 ± 1.27	Rutile
L	3.04 ± 0.01	22.86 ± 4.14	Rutile
C	2.83 ± 0.01	44.42 ± 2.00	Anatase
I	3.35 ± 0.00	58.70 ± 0.38	Anatase
J	2.86 ± 0.01	42.59 ± 5.35	Anatase
K	3.73 ± 0.01	51.67 ± 6.56	Anatase

* Each value is an average of three replicates (mean ± SD).

The limit of detection at this wavelength was 0.2151mg dm^{-3}. The recovery test using spiked samples gave a mean recovery of 98.8% and the signal stability was determined by the intra- and inter-day analysis. The intra-day analysis using an authentic standard gave an RSD% of 0.10% and an inter-day value of 0.10% thereby indicating very high precision.

The percentage composition of titanium (IV) oxide in these samples was in the range of 2.83% to 7.47% (Table 1). These were all well below the COLIPA allowable 25% (m/m) maximum concentration of titanium (IV) oxide in a cosmetic formulation[11]. Most of the samples contained approximately 3% (m/m) TiO_2, which when compared with the maximum allowed limit is low.

The PXRD characterization of the samples gave signals at 2θ values: 25.22, 37.73, 38. 45, 47.82 and 54.95° characteristic of anatase, at 27.33 37.73, 41.10, 54.10 and 68.69° characteristic of the rutile phase of TiO_2 (see Figure 1).

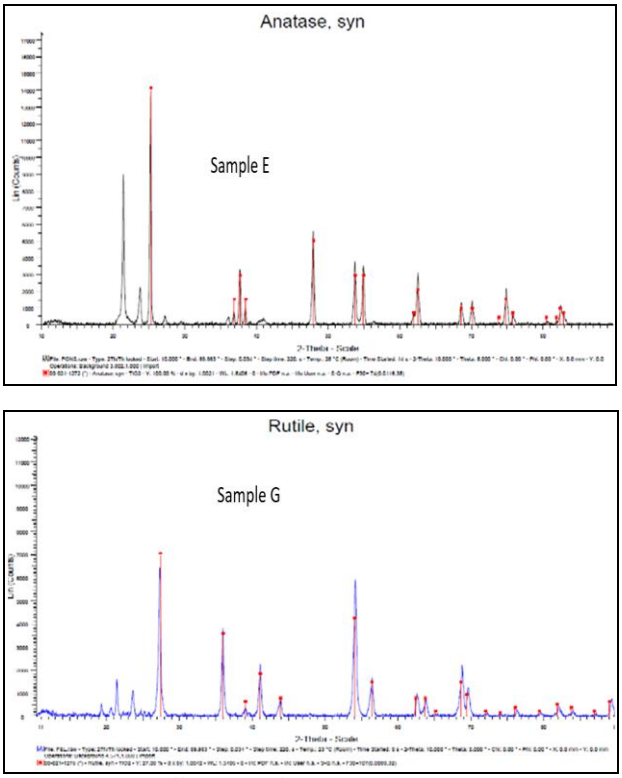

Figure 1: X-ray diffactogram for sample E (anatase) and sample G (rutile) superimposed on library difractograms of anatase and rutile.

The crystallite size estimation was based on the Scherrer equation. The Scherrer formula can provide a good estimate of the particle size but a variety of factors can contribute to the width of a diffraction peak. Besides crystallite size, the most important of these are usually inhomogeneous strain and instrumental effects. If all of these other contributions to the peak width were zero, then the peak width would be determined solely by the crystallite size and the Scherrer formula would apply. If the other contributions to the width are non-zero, then the crystallite size can be larger than that predicted by the Scherrer formula, with the peak broadening coming from the other factors. The eight samples gave crystallite sizes in the range of 16.23nm to 58.38 nm (see Table 1). These all fall within the nano-dimension.

Analysis of the high-resolution transmission electron microscopy images also revealed grain sizes in the nano range (see Figures 2 and 3).

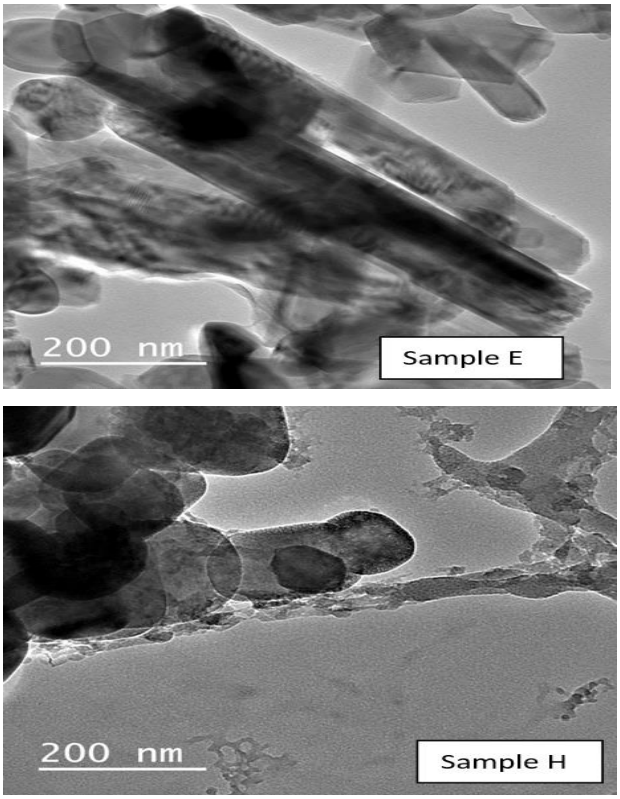

Figure 2: HRTEM images of samples E and showing well-defined crystalline TiO2.

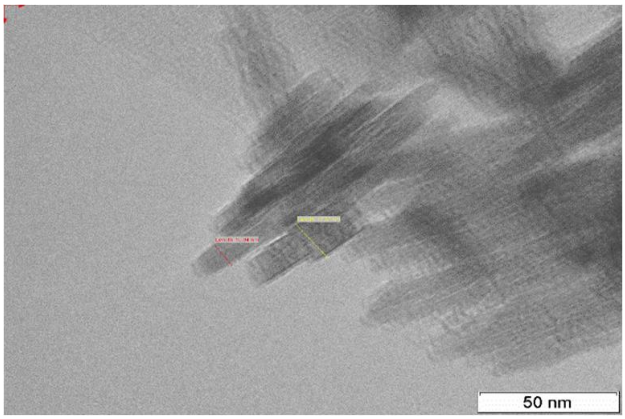

Figure 3: Particle size measurement for sample A observed using high-resolution transmission electron microscopy

This range is useful for the attenuation of UV radiation. Attenuation is the combined effect of absorbing and scattering of the incident light. Because nano-TiO_2 absorbs more UV light than it scatters compared with pigmentary grade TiO_2, it is preferred in most sunscreen preparations. Also, in this size range, it does not produce a whitening effect on the skin and thus it is more aesthetically appealing. The two methods of characterization thus proved helped useful in crystallite size approximation. However, the measurement from the HR-TEM depends on the particle dispersion and it is not apparent on the form of TiO_2 being measured. Whereas with the PXRD both particle size and form of TiO_2 could be obtained by library match. In this work, three samples displayed pure anatase signals indicating that the samples contained majorly anatase and four displayed rutile signals. One sample, however, showed mixed signals of anatase and rutile thereby showing a mixture of the two in the samples (Table 1).

DISCUSSION:

Samples investigated in this work contained TiO_2 acting as a sunscreen. The high refractive index of TiO_2 makes it useful for this purpose. The refractive indices of the rutile and anatase forms of TiO_2 are 2.75 and 2.54 respectively[14]. In this study, the crystallite size range was from 16.23 – 58.70nm (Table 1) as determined by PXRD. The particles of TiO_2 in the size range < 100nm are referred to as nano-TiO_2 and those > 100nm are known as pigmentary-TiO_2. The nano-TiO_2 have shown to be excellent UV filters or blockers because they have strong UV light absorbing capabilities and resistance to discolouration under UV irradiation. This advantage enhances its stability and ability to protect the skin from UV light[15]. Hence, nano-TiO_2 particles are frequently used in cosmetics because they scatter visible light less than pigmentary-TiO_2 while still providing UV protection. The majority of sunscreens intended for infants or people with delicate skin use are often based on TiO_2 and/or ZnO, because these physical UV filters are believed to cause less skin irritation than other UV absorbing agents.

However, nano-TiO_2 is naturally photocatalytic because when they absorb UV light electrons are excited and promoted for anatase to the conduction band across the 3.2 eV bandgap. This excitation is induced by UV light of wavelengths below 385 nm. The excited electrons promoted from the valence band (v_b) to the conduction band (c_b) generate Ts1wo mobile charged species; negatively charged single electrons (e^-) and positively charged spaces called holes (h^+) (chemical equation 1). The electron and hole pair (e^-/h^+) (equation 2) may recombine or migrate rapidly to the particle surface. At the surface, the electrons and holes may participate in chemical reactions with adsorbed chemical species. Two possible reactions may take place at the surface. The e^- may react with dissolved oxygen gas (O_2) and h^+ with hydroxyl (OH^-) ions or water (H_2O), to form superoxide (O_2^-) or hydroxyl ($^{\cdot}OH$) radicals:

$$TiO_2 + h\nu \rightarrow TiO_2\ (e^-/h^+) \quad (1)$$

$$TiO_2\ (e^-/h^+) \rightarrow e^-\ (c_b) + h^+\ (v_b) \quad (2)$$

$$e^-\ (c_b) + O_2 \rightarrow O_2^{\cdot -} \quad (3)$$

$$h^+\ (v_b) + OH^- \rightarrow {}^{\cdot}OH \quad (4)$$

$$h^+\ (v_b) + H_2O \rightarrow {}^{\cdot}OH + H^+ \quad (5)$$

The O_2^- and, in particular, the $^{\cdot}OH$ radicals formed are the active agents for the degradation of organic compounds:

$$^{\cdot}OH \text{ or } O_2^{\cdot -} + \text{substrate} \rightarrow \text{photo-oxidized products}[16]. \quad (6)$$

Also, the excited electrons may return to their ground state, emitting energy, or escape from the particle (chemical equation 2). Escaped electrons may initiate oxidative reactions in nearby molecules, generating free radicals (chemical equation 3 - 5). Free radicals may cause further damage to skin cells or interact with other sunscreen components producing chemical species with undesirable effects (equation 6). The fear is that this could lead to cancer in the skin.

The probability of photo-electron promotion and generation of e^-/h^+ pairs is the phase of the nano-TiO_2 crystal. In this work, both phases of TiO_2, namely, anatase and rutile were identified in the skin-lightening products. It is known that rutile is more photostable than anatase. This arises from the size dependence on the orbital character of the conduction band of anatase TiO_2 nanocrystals. It is known that the appearance and predominance of unoccupied states derived from the hybridization of the antibonding Ti 4s and O 2p band are observed when the nanoparticle size approaches the exciton radius (ca. 1nm). Such extended hybridization of O 2p with Ti 4s compared to narrow directional 3d in rutile demonstrates a confinement effect in anatase TiO_2 nanocrystals, a factor in electron excitation upon UV irradiation. The presence of s-hybridized band gap states controls the interfacial electron transfers and reduces the back reaction[14]. This may create an avalanche of escaped electrons that may attack viable skin cells via the generation of free radicals as illustrated above. On this account, rutile should be the preferred phase of TiO_2 for use in cosmetic preparations. In this study samples C, D E and H showed characteristic peaks of the anatase in the XRD diffractograms (see Figure 1) an evidence that

anatase is still used in some skin-lightening preparations. The forms of TiO_2 present in these samples were not indicated on the packet labels. This is a major concern because anatase TiO_2 is a very active photocatalyst and should not be used in cosmetic preparations. Secondly, as shown by[7] even surface-modified TiO_2 still retains photocatalytic activity.

The question of the percutaneous penetration of TiO_2 has drawn a lot of attention, especially after topical application. Table 2 shows the relationship between particle size and possible viable tissue penetration by the nano-range particles. In the worst-case scenario the particle range of TiO_2 found in this work (16 – 59nm, Table 1) are likely to enter viable tissue should they be in contact with any of these body tissues.

Table 2: Particle size and entry into the human body

Nanoparticle Size/nm	Entry Point
70	Alveolar surface of lung
50	Cells
30	Central nervous system
20	No data yet

(http://www.organicmakeup.ca/titaniumdioxide.asp (accessed on 14/10/2020)

Animal studies indicate that subjects who routinely apply sunscreens with micronized TiO_2 show that the skin can absorb microfine particles[9,17]. The samples investigated in this work all have TiO_2 in the nano-range (< 100 nm) (Table 1). The penetration of nano-TiO_2 into the cells may lead to photocatalysis within the cell, causing DNA damage after exposure to sunlight.

[18]and[19] have shown that a Ti^{+4} solution stimulates neutrophils and increases the quantity of released O_2^- anions. The authors showed that the cytotoxic effect of Ti particles is size-dependent and that they must be smaller than that of cells. Animal model studies have shown the ingested titanium accumulates in the liver DNA leading to histopathological changes and hepatocyte apoptosis[5,20].

However, some studies show that there is no deeper penetration of topically applied TiO_2 into viable skin tissue[21]. The same study indicated that there is possible penetration of TiO_2 into the open skin parts around the follicles. This is a pointer that compromised skin surface may be susceptible to TiO_2 penetration. The effects of viable tissue incorporated TiO_2 include induction of ROS reactions that can lead to DNA mutations and cell death[22]. There are reports that TiO_2 particles isolated from commercial sunscreen products induced DNA strand breaks and other lesions in DNA plasmids and human cells[20]. It can therefore be inferred that the presence of TiO_2 in sunscreen formulations can initiate or lead to photo-oxidative damage of the skin. Though, other investigations have shown that coarse or fine particles of TiO_2 to be safe and effective at deflecting and absorbing UV light, protecting the skin[23,24]. But consumers should avoid using products with nano-pigments, either in sunscreens or colour cosmetics if they have any wounds or broken skin. Such preparations should be used with caution on the children where the skin is thinner and more permeable.

Most of the investigated products in this work contained a combination of TiO_2 with organic UV-filters tert-butylmethoxy dibeznoylmethane, 2-ethylhexy-*p*-methoxy cinnamate, and benzophenone-3. There is the possibility that TiO_2 may photocatalyse the photodegradation of these UV filters. Several reports indicate loss of photo-absorption efficacy of these UV filters in the presence of TiO_2[25]. The photoproducts resulting from the TiO_2 photocatalyzed reactions of the organic UV filters lead to a loss of photoprotection and a potential risk to the skin. Besides, the toxicities of the resulting photoproducts are not known.

To inhibit the effects of TiO_2 on the organic macromolecules and other substrates the surface of the TiO_2 may require deactivation. The surface deactivation of nano-TiO_2 like the one found in this work may afford thin-film uniform surface coating on the particles. However, such surface modifications have been found ineffective in photo-oxidative reactions[7]. The structural modification of the TiO_2 crystalline lattice by the introduction of impurities has been shown to reduce the photo-activity of TiO_2. The choice of the transition metal (dopant) determines the photo-response of the doped TiO_2. Recently, it was demonstrated that manganese-doped TiO_2 had enhanced UVA absorption, less degradation of other organic constituents of the formulation and a reduction in a free radical generation [26]. However, there is no guarantee that surface coating or doping completely deactivates TiO_2.

CONCLUSIONS:

This study aimed to isolate, characterize and quantitate the amount of TiO_2 present in the eight skin-lightening preparations. The percentage composition of TiO_2 in these skin-lightening agents was found to be in the range of 2.83 % to 12.47 % (m/m). Both anatase and rutile forms of TiO_2 were found present in the nano range (16.23 nm to 51.67 nm). Since anatase TiO_2 is a potent photocatalyst it should not be used in such topical skin preparations. This is more so since it has been shown that surface modification does not eliminate this photocatalytic activity.

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