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Original Article

In vitro Biological Activity of (Anti-inflammatory, Anti-oxidant) Pomegranate Peel Extract Mediated Titanium Dioxide

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Pomegranate peel is considered to be a reservoir of biologically active compounds, the presence of which provides anti-inflammatory and antioxidant properties to the peel extract. Titanium is a wonder metal with broad range of application in all aspects of life. Titanium dioxide is the most common compound of titanium. The nano particles of titanium dioxide have low toxicity, which forms the basis for its broad range of applications including cancer therapy, targeted drug delivery, photodynamic therapy, antibacterial effect, role in bone formation. In the present study green synthesis of pomegranate peel extract mediated titanium dioxide ($PPETiO_2$) was carried out and it's anti-inflammatory, anti-oxidant properties were evaluated. The aim of the present study was to evaluate the biological activity (anti-inflammatory, antioxidant) of green synthesized pomegranate peel extract mediated titanium dioxide ($PPETiO_2$). The main objective of this research was to prepare green synthesized pomegranate peel extract mediated titanium dioxide and to analyse the biological activity namely anti-oxidant activity, and anti-inflammatory activity. The anti-inflammatory activity was evaluated using the bovine serum albumin denaturation assay (BSA), the egg albumin denaturation assay (EA) and was compared with diclofenac as standard. Antioxidant activity was measured using the 2,2 Diphenyl-1-Picryl hydraxylhydrate (DPPH) assay, the H_2O_2 assay, the ferric reducing antioxidant power (FRAP) assay. Comparison made with ascorbic acid as standard. The green synthesized pomegranate peel extract mediated titanium dioxide showed antioxidant and anti-inflammatory activity. It was directly proportional to the concentration of the extract. Protein denaturation causes inflammation. The green synthesized PPETiO2 composite inhibited the protein denaturation and it was concentration dependent. The study concluded that the green synthesized PPETiO2 has Anti-inflammatory and Anti-oxidant activity and it is concentration dependent.

Introduction

Dental implants made from titanium and its alloys are commonly used in dentistry due to their ability to naturally form a thin and strong oxide layer on their surface, which promotes osseo-integration - the process by which the implant fuses with the surrounding bone tissue [1,2]. The osteoconductive property of TiO_2 has been evaluated it was found that the pure or pigmented forms of titanium dioxide are bio compatible and possesses excellent osteoconductive properties and can be successfully used to augment bony defects[3]. Adithya et al. synthesized titanium dioxide nanoparticles using Mucuna pruriens seed extract which showed anti-inflammatory and anti-oxidant properties[4]. Rajesh and coworkers reported that the TiO_2 nanoparticles synthesized by Paniculata showed the increased concentration of the anti-oxidant activity [5]. Researches have shown that surface nanotopography has a positive effect on bone cell behaviour in laboratory settings [6]. Nanostructured materials have the ability to increase surface area, and modify the surface topography to resemble natural bone. This can improve the interaction between bone cells and implants. Studies have also shown that the use of

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titaniumdioxide (TiO_2) nanotubes can enhance osteoblast adhesion by up to 400%, and leads to higher levels of alkaline phosphate and mineralization compared to non-modified TiO_2 [7].

The ability of crystalline TiO_2 to promote biological activity is related to the presence of surface hydroxyl groups and induced negative charges. These factors attract calcium and phosphorus ions from body fluids to the implant surface. Research findings suggest that the transformation of the titanium dioxide from anatase to rutile results in a significant decrease in the rate of dissolution of metallic ion in simulated body fluid [8].

The incorporation of TiO nanoparticles (TiONPs) into calcium phosphate cement has been shown to enhance the mechanical strength of bone defect repairs. The nanometric thickness of TiO_2 nanotubes (TiO_2NTs) results in an increased surface area and porous structure, which promotes cell adhesion and improves the bone regeneration capacity [9]. The surface topography of dental implants has a significant impact on osseointegration. Research has shown that dental implants coated with TiO_2 nanotubes (TiO_2NTs) via anodic oxidation and loaded with BMP2 (bone morphogenetic protein 2) exhibited a high level of osseointegration [10]. Jin and his coworkers discovered and reported that, when TiO_2NTs with a diameter of less than 100 nm were used, osteoblast adhesion was improved. Furthermore, it increased the activity of the alkaline phosphatase enzyme, demonstrating the ability of TiO_2NT coated orthopaedic implants to interact with bone tissue and produce new bone [11].

The distinctive biochemical composition of pomegranate includes more than 124 phytochemicals, which play a crucial role in its diverse range of health benefits such as anti-oxidants, anti-inflammatory, and antimutagenic properties [12]. Anthocyanins a flavanoid found in peel and juice of pomegranate are responsible for the anti-oxidant, anti-inflammatory, and antiproliferative properties of pomegranate. Furthermore, punicalagin, an ellagitannin found in pomegranate, also plays a significant role in providing the above mentioned health benefits [13]. Consuming pomegranate juice can increase the level of anti-oxidants up to threefold and hinder the production of harmful substances such as DNA oxidation products, reactive nitrogen species, and lipid peroxidation. Moreover, it can also help eliminate reactive oxygen species through its scavenging properties [14-16]. Extracts obtained from pomegranate are powerful vehicles of anti-oxidants and anti-inflammatory compounds, which can effectively aid in the restoration of bone health [17].

Consumption of pomegranate peel derived dietary extract has the potential to enhance both bone mass and the microarchitecture of bone tissue, particularly during the onset of osteoporosis [18]. The extract derived from pomegranate fruit has been found to be effective against osteoarthritis by enhancing cartilage stiffness and physical fitness, it also reduces the activity of cartilage catabolizing enzymes and reinforce the body's anti-oxidative defence system [19]. Research has shown that the inclusion of pomegranate peel extract (PPE) in the diet can improve bone mineralization by elevating the activity of bone maker proteins, like alkaline phosphatase (ALP), as well as osteogenic transcription factors [20]. Researchers have also pointed out that by discovering the specific constituents present in pomegranate extracts, it may be possible to explore novel approaches for developing effective preventive therapies for arthritis. The anti-oxidant and anti-inflammatory properties found in pomegranate fruit compounds could potentially be harnessed as functional food to prevent bone loss and inflammatory arthritis. There is ample evidence in the literature that highlights the ostoconductive properties of TiO_2. The osteogenic potential of different pomegranate extracts due to the presence of abundance of biologically active compounds has also been reported. These compounds are abundantly found in the pomegranate peel.The identification and evaluation of compounds that possess anti-oxidant and anti-inflammatory properties can offer novel therapeutic prospects in the realm of bone regeneration. Therefore it becomes imperative to explore their potential therapeutic applications, especially in the context of bone regeneration. In the present study, green synthesized pomegranate peel extract mediated titanium dioxide ($PPETiO_2$) was prepared and its biologic properties namely anti-oxidant and anti-inflammatory were evaluated. The objectives were to prepare the PPE, green synthesis of pomegranate peel extract mediated titanium dioxide ($PPETiO_2$) and to evaluate the anti-oxidant and anti-inflammatory properties.

Materials and Methods

Preparations of pomegranate peel extract

The process involved cleaning freshly purchased Ganesh variety pomegranate fruits by washing them with water and a diluted 'Koparo Clean' vegetable and fruit wash solution. After drying with a cotton cloth, the pericarp was separated and air-dried. The dried peel was then ground into a coarse powder using a 'SS 304 multi milli machine'. Then, 2g of the powder was mixed with 100mL of distilled water using a magnetic stirrer, and the mixture was heated at 60 to 80 degrees Celsius for 15 to 20 minutes using a heating mantle. After heating, the mixture was filtered through Whatmann No. 1 filter paper and the filtered extract was further compressed to a volume of 5mL.

Green synthesis of PPE mediated TiO_2 nanoparticles

50 ml of PPE was used, to that 0.35gm of titanium oxide mixed in 50ml distilled water was added, it was then kept on a magnetic stirrer at 600RPM for 48 hours, after 48 hours it was centrifuged at 8000 RPM for 10minutes, the green synthesized PPE mediated TiO_2 nanoparticles pellet was collected and the supernatant was discarded.

Anti-inflammatory activity

Bovine serum albumin denaturation assay (BSA)

To determine the anti-inflammatory activity of green synthesized $PPETiO_2$, the albumin denaturation assay was carried out with some modifications based on the convention proposed by Muzushima and Kabayashi. Bovine serum albumin (1% aqueous solution) was mixed with different volumes (10µL, 20µL, 30µL, 40µL, 50µL) of green synthesized $PPETiO_2$ and the pH was adjusted to 6.3 using a small amount of 1N hydrochloric acid. After incubation at room temperature for 20 minutes, the samples were heated at 55°C for 30 minutes in a water bath, then cooled and the absorbance was measured at 660 nm using a spectrophotometer. Diclofenac Sodium was used as the standard, while Dimethyle sulfoxide (DMSO) was used as a control. Percentage of protein denaturation was determined utilizing following equation,

$$\% \text{ inhibition} = (Abs._{control} - Abs._{sample})/Abs._{control} \times 100$$

Egg albumin denaturation assay

To prepare the test solution, 2.8ml of freshly manufactured phosphate buffered saline at pH 6.3 was mixed with 0.2ml of hen's egg albumin extract to create a total volume of 5ml. The green synthesized $PPETiO_2$ was then prepared at different

concentrations (10µL, 20µL, 30µL, 40µL, 50µL). The mixture samples were heated in a water bath at 37°C for 15 minutes, and then allowed to cool to room temperature. The absorbance was measured at 660 nm. Diclofenac sodium was used as the positive control.

Anti-oxidant activity

The anti-oxidant capacity of green synthesized PPETiO_2 was assessed using three different methods, including two based on free radical scavenging and one based on measuring iron-reducing capacity.

22 Diphenyl 1 picryle hydraaxile hydrate assay (DPPH)

The first method used a commercially available free radical, DPPH, which is soluble in methanol, and the decrease in absorbance at 515 nm was used to measure the antioxidant activity. For DPPH assay the green synthesized PPETiO_2 was prepared at various concentrations (10µL, 20µL, 30µL, 40µL, 50µL) and mixed with 1ml of 0.1mM DPPH in methanol and 450µl of 50mM Tris HCl buffer at pH 7.4. The mixture was then incubated for 30 minutes, and the reduction in the amount of DPPH free radicals was measured based on the absorbance at 517nm. BHT was used as the control. The percentage of inhibition was calculated using the following equation:

$$\% \text{ inhibition} = (Abs._{control} - Abs._{test\ sample})/Abs._{control} \times 100$$

Vitamin E was used as a positive control in both methods.

Hydroxyl radical scavenging assay (H_2O_2)

The experiment followed the Halliwell method with slight modifications, using freshly prepared solutions. The reaction mixture of 1.0mL contained 100µL of 28mM 2-deoxy-2-ribose (dissolved in phosphate buffer 7.4), 500µL of green synthesized PPETiO_2 at various concentrations (10µL, 20µL, 30µL, 40µL, 50µL), 200µL of 200µM $FeCl_3$ and 1.04mM EDTA (1:1 v/v), 100µL of H_2O_2 (1.0mM), and 100µL of ascorbic acid (1.0mM). The reaction mixture was incubated at 37°C for 1 hour, and the extent of deoxyribose degradation was measured by the TBA reaction. The absorbance was measured at around 532nm against the blank solution, and Vitamin E was used as a positive control.

Ferric reducing antioxidant power assay (FRAP)

Reagents used for FRAP assay are a) Acetate buffer 300 mM, pH 3.6: 3.1g sodium acetate trihydrate was taken and 16 ml of glacial acetic acid was added to it and the volume was made up to 1 L with distilled water. b) TPTZ (2, 4, 6-tripyridyl-s- triazine): (M.W. 312.34), 10 mM in 40 mM HCl (M.W. 36.46). c) FeCl3. 6 H2O: (M.W. 270.30), 20 mM. The working FRAP reagent was prepared by mixing a, b and c in the ratio of 10:1:1 just before the testing. Standard was ferrous sulphate heptahydrate (FeSO4. 7 H2O): 0.1 - 1.5 mM in methanol. All the regents were procured from Merck (Germany) company. 3.6 mL of FRAP solution was combined with 0.4 mL of distilled water and incubated at 37°C for 5 minutes. This solution was then mixed with different concentrations of green synthesized PPETiO_2 (10µL, 20µL, 30µL, 40µL, and 50µL) and incubated for 10 minutes at 37°C. The absorbance of the reaction mixture was measured at 593 nm. A calibration curve was created using five concentrations of $FeSO_4$,$7H_2O$ (0.1, 0.4, 0.8, 1, 1.12, and 1.5 mM), and the absorbance values were calculated for the sample solutions in the same way.

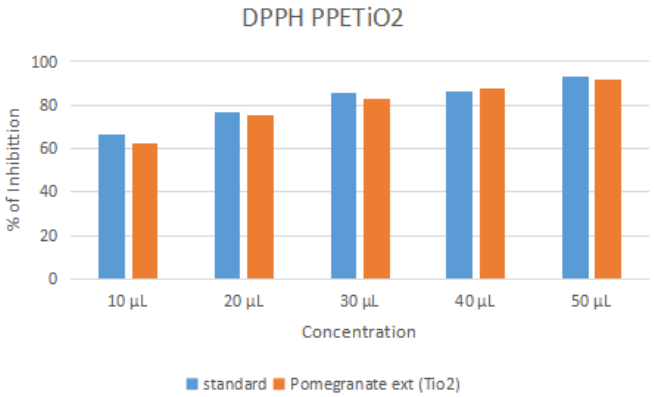

Figure 1: Antioxidant activity DPPH - PPE TiO_2

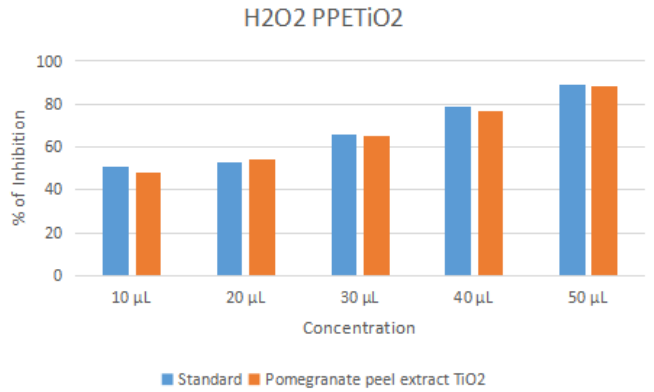

Figure 2: Antioxidant activity H_2O_2 - PPE TiO_2

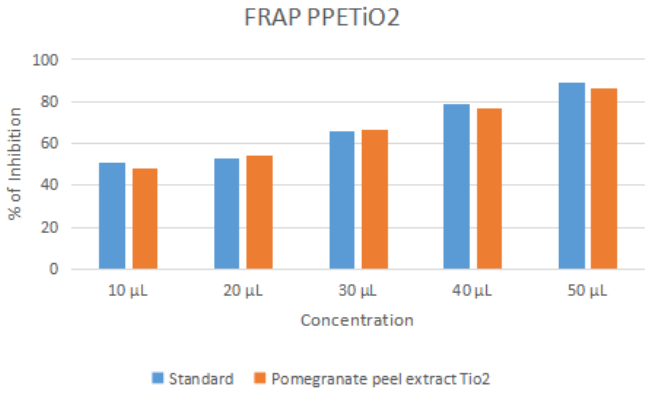

Figure 3: Antioxidant activity FRAP - PPE TiO_2

Results

In the present study, the percentage of inhibition for anti-oxidant activity for five concentrations of green synthesized PPETiO_2 using DPPH (517nm) was as follows: 10µL (62.34%), 20µL (75.26%), 30µL (82.84%), 40µL (87.42%) and 50µL (92.13%). For standard it

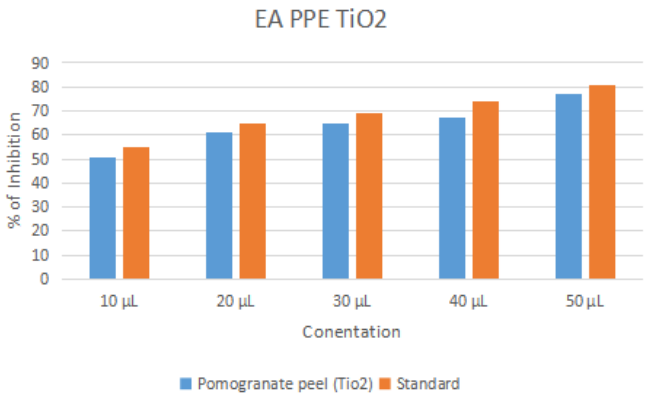

Figure 4: Anti-inflammatory activity EA - PPE TiO_2

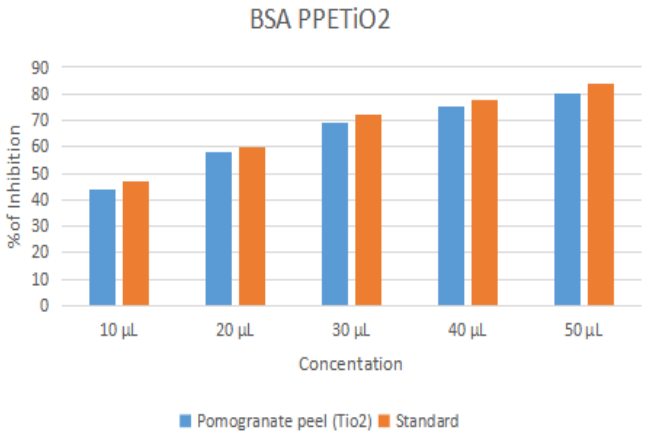

Figure 5: Anti-inflammatory activity BSA - PPE TiO_2

was 10µL (66.25%), 20µL (76.52%), 30µL (85.63%), 40µL (88.68%) and 50µL (93.15%) (figure1).

For H_2O_2 assay the percentage of inhibition was as follows for green synthesized PPETiO_2 10µL (48.32%), 20µL (54.21%), 30µL (65.37%), 40µL (76.55%) and 50µL (88.39%). For standard it was 10µL (51.1%), 20µL (52.9%), 30µL (66.1%), 40µL (78.8%) and 50µL (88.9%) (figure2).

In FRAP the percentage of inhibition of green synthesized PPETiO_2 at various concentrations were10µL (48.32%), 20µL (54.21%), 30µL (66.37%), 40µL (76.55%) and 50µL (86.39%) for standard drug it was 10µL (51.1%), 20µL (56.9%), 30µL (66.1%), 40µL (78.8%) and 50µL (88.9%) (figure 3).

In the present study, anti-inflammatory activity was measured by measuring the percentage of inhibition of egg albumin denaturation (EA). Percentage of inhibition by green synthesized PPETiO_2 of five concentrations is as follows: 10µL (51%), 20µL (61%), 30µL (65%), 40µL (67%) and 50µL (77%). For standard it was 10µL (55%), 20µL (65%), 30µL (69%), 40µL (74%) and 50µL (81%) (figure 4).

For the bovine serum albumin denaturation assay (BSA) percentage of inhibition by green synthesized PPE TiO_2 10µL (44%), 20µL (58%), 30µL (69%), 40µL (75%) and 50µL (80%) for the standard drug it was 10µL (47%), 20µL (60%), 30µL (72%), 40µL (78%) and 50µL (84%) (figure5).

Discussion

When titanium is used in living organisms, the oxide layer that forms on its surface plays an essential role in promoting its biocompatibility. The oxide layer prevents the metal from reacting with surrounding tissues and cells, which reduces the risk of inflammatory reactions and toxicity [21,22]. Additionally, the unique properties oxide layer make it useful in various medical applications, such as implants, drug delivery systems, and tissue engineering scaffolds [23]. The combination of titanium and its unique properties oxide layer makes it a promising material for biomedical applications and has led to significant advances in the field of biomedicine [24,25].

Aditya et al. utilized Mucuna pruriens seed extract to synthesize TiO_2 nanoparticles. The resulting nanoparticles exhibited notable anti-inflammatory and anti-oxidant activities, which were more potent and less toxic compared to other tested substances [4]. Rajesh et al. have reported that TiO_2 nanoparticles synthesized using A.paniculata have antioxidant activity presently nanoparticles are widely used for anti-oxidant activity to improve their biomedical applications. According to the study conducted by Sharma and colleagues, TiO_2 mediated with grape seed exhibited powerful and effective anti-oxidant properties, indicating its potential utilization in various medical applications [26]. Yixing Ren and co-authors observed that TiO_2 nanoparticles exhibited cytotoxic effects on cells; however, they also found that these nanoparticles facilitated the mineralization and maturation of osteoblasts [27]. Samyuktha et al. reported that titanium dioxide nanoparticles (TiO_2-NPs) have several advantages, including their eco-friendliness, ease of synthesis, and affordability. Additionally, TiO^2-NPs have been found to possess various beneficial properties such as antibacterial, antifungal, antiviral, and antioxidant activities, which further increase their potential applications [28].

Researches have also pointed out that titanium dioxide nanoparticles, specifically titania nanotubes (NTs), have the ability to remain stable and maintain their structure when used as a surface coating on implants. In addition to their stability, these nanoparticles can act as a drug delivery system to regulate drug release in implants [29]. The porous nature of TiO_2 NTs can also promote bone regeneration and repair, making them beneficial for enhancing implant functionality [30-34]. Pomegranate is known to contain a wide range of biologically active compounds, which contribute to its anti-inflammatory and anti-oxidant properties. In fact, the peel of the pomegranate is considered to be the richest source of these biologically active compounds compared to any other part of the fruit [35,36]. Several studies have proved that pomegranate peel extract exhibits notable antioxidant properties, suggesting its potential use as a natural antioxidant source in combating pathologies or diseases induced by autoxidation. This highlights its promising applications in the health food, nutraceutical, and pharmaceutical industries for a range of purposes [37].

In the present study, pomegranate peel extract of Ganesh variety was used for the green synthesis of PPETiO_2. The antioxidant activity was measured using 22Diphenyl 1 picryle hydraaxile hydrate assay (DPPH), H_2O_2 assay and ferric reducing antioxidant power assay (FRAP)the standard used was asorbic acid, all the three assays

showed that the antioxidant property of the green synthesized PPE TiO_2 was similar to the standard used for each concentration of the extracts tested. It was also found that the antioxidant activity increased as the concentration was increased from 10µL to 50µL this shows that the antioxidant properties increased with increase in concentration ie the antioxidant activity is dose dependent and the aqueous peel extract is able to provide the same antioxidant similar to the standard.

The findings of this study are consistent with those of other studies conducted by Aditya et al., Rajesh et al., Sharma et al., Samyuktha et al., and Yixing Ren et al., which also evaluated the biological properties of TiO_2 nanoparticles and found their potential for use in various medical applications. It can also be concluded that the addition of TiO_2 did not diminish the anti-oxidative property of PPE, as the values obtained were similar to the standard used.

Anti-inflammatory

The pomegranate plant is known for having high concentrations of polyphenols in its peel, juice, flowers, and seeds. These polyphenols are converted by intestinal bacteria into urolithins, which have been shown to possess anti-inflammatory properties [38]. Research conducted by Pohl et al. demonstrated that pomegranate's biologically active components, present in the fruit's peel, juice, and extracts, can affect the expression of a protein that signals inflammation in cancer cells [39]. These findings suggest that pomegranate may have potential anti-inflammatory and anticancer properties. Rasheed et al reported the use of polyphenol-rich pomegranate fruit extract or compounds derived from it for the treatment of inflammatory diseases by suppressing basophils and mast cells activation. Basophils and mast cells are immune cells that play a key role in initiating and perpetuating inflammatory responses. By suppressing their activation, pomegranate fruit extract or its compounds may help to reduce inflammation in the body. This suggests that pomegranate may have potential as a natural remedy for inflammatory diseases [39].

The present study used egg albumin denaturation assay and bovine serum albumin denaturation assay were used to measure the anti-inflammatory property of green synthesized PPETiO_2. Denaturation of proteins can lead to inflammation, so inhibiting this denaturation can have anti-inflammatory effects. The evaluation of green synthesized PPETiO_2 on its anti-inflammatory activity showed that the extract was able to inhibit protein denaturation in both assays. The percentage of inhibition was similar to the control, which was diclofenac sodium, and the anti-inflammatory activity increased as the concentration of green synthesized PPETiO_2 increased. These findings suggest that the anti-inflammatory activity of green synthesized PPETiO_2 is concentration dependent. Moreover, the results obtained for green synthesized PPETiO_2 were comparable to the commercial drugs used, indicating that green synthesized PPETiO_2 possesses anti-inflammatory properties similar to diclofenac sodium, a commonly used anti-inflammatory drug. The result of the present study revealed that green synthesized PPETiO_2 have anti-oxidant and anti-inflammatory properties similar to that of control used and it increased as the concentration of PPE increased ie it is concentration dependent. It is also seen that addition of TiO_2 did not affect anti-oxidant and anti-inflammatory properties as the results obtained was similar to that of control used.

Conclusion

Pomegranate's unique biochemical profile, which includes over 124 bioactive components, supports its antioxidant and anti-inflammatory properties. The study found that the antioxidant activity of green synthesized PPETiO_2 was comparable to the control used and was directly related to the concentration. Additionally, the ability of green synthesized PPETiO_2 to suppress protein denaturation demonstrated its anti-inflammatory potential, which was also concentration dependent. These findings suggest that green synthesized PPETiO_2 possesses both anti-oxidant and anti-inflammatory characteristics that vary with concentration. However, further testing is necessary before pomegranate and its bioactive compounds can be used alone or in combination with other compounds as a therapeutic agent for treating degenerative disorders such as osteoporosis, rheumatoid arthritis, and its potential utilization in bone regeneration and various medical applications.

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