ISSN: 0974-5823 Vol. 7 No. 2 February, 2022

International Journal of Mechanical Engineering

Rose Hibiscus Black Currant Tea Extract Mediated Silver Nanoparticles and its Anti-microbial, Anti-oxidant, Anti-inflammatory and Cytotoxic Effect

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Abstract:

The flowers of Rose, Hibiscus and Black currant plants have bio molecules of medicinal value. In the present study, biosynthesis of silver nanoparticles are carried out. The synthesized nanoparticles are characterized by UV-Vis spectrophotometer, Fourier Transform Infrared Spectroscopy (FT-IR), Energy Dispersive X-ray (EDX) and Scanning Electron Microscopy (SEM). The UV-Vis spectrophotometry revealed a Surface Plasmon Resonance (SPR) at 440 nm which is characteristic for silver nanoparticles. EDX analysis disclosed silver as predominant element (73.2 Wt %) along with other elements. FT-IR spectral analysis showed the functional groups which were responsible for reduction of AgNPs and for capping and stabilization. The antimicrobial assay confirmed good inhibitory activity against *Enterococcus faecalis* and average inhibition was found against *Streptococcus mutans.* Anti-inflammatory activity of the RT-AgNPs revealed 79.1 % of efficacy in 50 µL which was close to the standard 84 %. Anti-oxidant efficiency of the synthesized nanoparticles exhibited 86.9 % in 50 µL which was near value of standard 93.15 % in 50 µL concentration. Brine Shrimp Lethality Assay proved effective cytotoxic activity of RT-AgNPs, mortality rate was only 20 % in 80 µL.

Key Words: Biosynthesis, Silver nanoparticle, Characterization, Antimicrobial, anti-inflammatory, Antioxidant

Introduction

The plant mediated or biogenic synthesis of nanomaterials have gained much appreciation in the field of medicine. The major focus in biosynthesis (different plants and its parts) of nanoparticles by the Nano Biomedicine scientist is because, it is environment-friendly that is, the approach does not use any carcinogenic chemicals, it is cost effective, and often involves a one-step synthesis procedure (Gomathi *et al.*, 2019) and importantly, tests can be performed easily in room temperature and normal atmospheric pressure (Rolim *et al.*, 2019). The biosynthesized nanoparticles have diverse physico-chemical attributions, smaller dimensions and increase their surface area which reflects in its Surface Plasmon Resonance (SPR) (Kandhan *et al.*, 2019). Silver nanoparticles (AgNPs) are extensively used to determine its efficacy against clinical pathogens (Harini *et al.*, 2020) , they are good anti-inflammatory agents that obstruct the secretion of pro-inflammatory cytokines (IL-6, IL-1 beta and TNF-alpha) (Tyavambiza *et al.*, 2021). In the field of oncology silver nanoparticles play a vital role in enhancing the drug efficiency as the drug impregnated AgNPs have better anti-cancer properties (Gomes, Martins and Prior, 2021). The Free radical scavenging activity of the phytochemicals show less bioavailability as they are not effectively absorbed by the intestines, this defect is overcome by the biosynthesized AgNPs which are impregnated with the phytochemicals (Myint *et al.*, 2021). Also, the AgNPs that are protected or covered by organic ligands have gained much more attraction for their biological and technological applications (Bharathi and Bhuvaneshwari, 2019).

Different plant parts such as leaf, stem, root, bark, fruit, seed, flower etc. have been used to synthesize nanoparticles as they are less toxic and surface facets are in the required scale. Few examples of plants that are utilized to synthesize nanoparticles are *Datura stramonium, Galenia African, Capparis spinose, Terminalia mantaly* and *Hypoxis hemerocallidea* (Vanlalveni *et al.*, 2021) (Vasyliev *et al.*, 2020). Flowers are usually the colorful and attractive parts of the plants and also rich in the phytochemicals such as Carotenoids, Flavonoids and Vitamins (Moteriya and Chanda, 2017). Even though all parts of the medicinal plants are beneficial, flowers are comparatively a better choice as they are readily available in every season, and have high pigment concentration of Carotenoids and Flavonoids in the Corolla of the flower, moreover, flowers from the medicinal plants are generally edible and recently considered as "New Vegetable" and accepted as food to enhance the health benefits due to their anti-oxidant activity and have the capacity to counteract certain specific pathologies in Human Being (Benvenuti and Mazzoncini, 2021). Therefore, in the present study, Rose tea extract (Rose Tea) made from the flowers of Rose, Hibiscus and Blackcurrant are used to synthesize AgNPs. The main focus of the study is to experimentally characterize and evaluate the bioactivities (anti-microbial, anti-oxidant, anti-inflammatory and cytotoxic efficacy) of the AgNPs synthesized from Rose tea extract. Rose plant is woody perennial shrub that belongs to family Rosaceae and genus Rosa. Studies have shown a significant anti-obesity, anti-oxidant, anti-bacterial and anti-diabetic activities in Rose flowers of *Rosa kordesii.* The flowers are used for its appreciable quantities of phytochemicals such as terpenoids, phenolics, sugars, fatty acids and their derivatives, aromatics and other polar compounds (Belal *et al.*, 2016) that are involved in bio reduction, capping and stabilization of AgNPs. *Hibiscus rosa sinensis* is

an ornamental and evergreen plant of the family Malvaceae. Young flowers of Hibiscus are used as an aphrodisiac, to treat Liver disorders and to regulate the blood pressure. Hibiscus flowers have abundant Vitamin C in it (Reveendran *et al.*, 2016). *Ribes nigrum* which is commonly called as Black currant belongs to the family Grossulariaceae the pomace extracts predominantly contains phenolic compounds importantly caffeic acid (10.1%), chlorogenic acid (5.4%), protocatechuic acid (15.6%), and minute quantities of flavonoids and aldehydes which mainly used for the reduction, capping and stabilizing the AgNPs (Vasyliev *et al.*, 2020). The synthesized Rose Tea Extract mediated silver nanoparticles (RT-AgNPs) are characterized by various spectrophotometric analysis and microscopic study to determine the size, shape and surface morphology of the R-AgNPs and anti-microbial, free radical scavenging activity, anti-inflammatory and its cytotoxicity efficiency are determined so that it can be used in Nano biomedicine fields.

Materials and Methods

Chemicals

Rose, Hibiscus and Black currant (Rose Tea) powder was collected from Redplum Pvt. Ltd., Haryana, India. The fungi *Candida albicans* and the bacteria *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas* were obtained from microbiology Laboratory Saveetha Dental College, Chennai, India. Silver nitrate,(AgNO3) ascorbic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 3-[4, 5-dimethylthiazole-2-yl]-2, 5- diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and other required Agar media were bought from HI media Laboratories Pvt. Ltd., Mumbai, Maharashtra, India.

Preparation of Rose Tea Extract

Rose tea consists dried flowers of Rose, Hibiscus and Black currant plants. 1 gram of rose tea powder was weighed accurately and taken in a clean glass beaker and 100 mL of distilled water was added to it. The solution was boiled for 10 minutes and allowed to cool. The crude extract was then filtered using sterile Whatman filter paper No 1. The extract was then covered with foil and stored in a refrigerator for further analysis.

Biosynthesis of Silver nanoparticles From Rose Tea Extract

In a clean Erlenmeyer flask, 1 mM (1 milli mole) salt of Silver Nitrate ($AgNO_3$) was taken. To this, 50 mL of distilled water was added and mixed gently so that $AgNO_3$ salt completely dissolved in distilled water. 50 mL of Rose tea extract was measured exactly and mixed in the $AgNO_3$ solution. This solution containing both the tea extract and $AgNO_3$ was kept in a shaker incubator for 72 hours at room temperature until the permanent colour change was obtained which is an indication of bio reduction process of Silver ions (Ag^+) to metallic Silver (Ag^0) which indicates the formation Rose tea extract mediated Silver nanoparticles (RT-AgNPs). The solution is then centrifuged in Lark cooling centrifuge at 8000 rpm for 10 minutes to separate the nanoparticles. After the centrifugation process, supernatant was separated and pellet was collected in a clean tube and capped properly then it was stored in refrigerator for further analysis. The supernatant was poured in a clean glass Petri plate and allowed to dry in a Hot Air Oven at 70^0 C overnight. Once the drying was complete the dried supernatant was also collected in a separate Eppendorf tube and stored in refrigerator for future analytical purpose.

Characterization of RT-AgNPs

The formation of RT-AgNPs was carefully monitored by using a double beam UV-Visible Spectrophotometer (Model – 3375). At constant intervals the absorbance and Peak values were recorded. The functional groups found in the extract which may be responsible for reduction, capping and stabilization of RT-AgNPs was identified and analyzed by FT-IR spectroscopy. The elemental ratio and its composition were evaluated with EDX analyzer, surface morphology and size of the RT-AgNPs were determined by using a Scanning Electron Microscopy.

Result and Discussion

Visual Observation and UV-Vis Spectral Analysis

(A) (B) (C) (D)

Fig 1. Depicts (A) Dried flowers of rose tea (B) Rose tea extract (C) Pale orange colour of rose tea extract added with 1mM of $AgNO_3$ (D) deep brown colour formed after 72 hours of incubation

The primary observation during the formation of RT-AgNPs was the colour change from pale orange to deep brown colour during the 72hours of incubation appeared due to complete reduction of silver ions to silver ($Ag^{+} \rightarrow Ag^{0}$) by the biomolecules of the extract which is shown in Fig 1 (A B C D). UV-VIS readings were recorded to evaluate the rate of bio reduction and formation of RT-AgNPs at constant intervals. The wavelength was set between 250 nm to 650 nm. The free oscillation of the electrons exhibited characteristic Surface Plasmon Resonance (SPR) as band at 440 nm in UV-VIS spectral analysis (Fig 2). SPR band mainly depends on the biomolecules surrounded by the nanoparticles, size and its shape (Varadavenkatesan, Selvaraj and Vinayagam, 2019). A study done in biosynthesis of silver nanoparticles done by (Bindhu *et al.*, 2020) in *Moringa oleifera* flowers revealed SPR band at 429 nm correlates the present study.

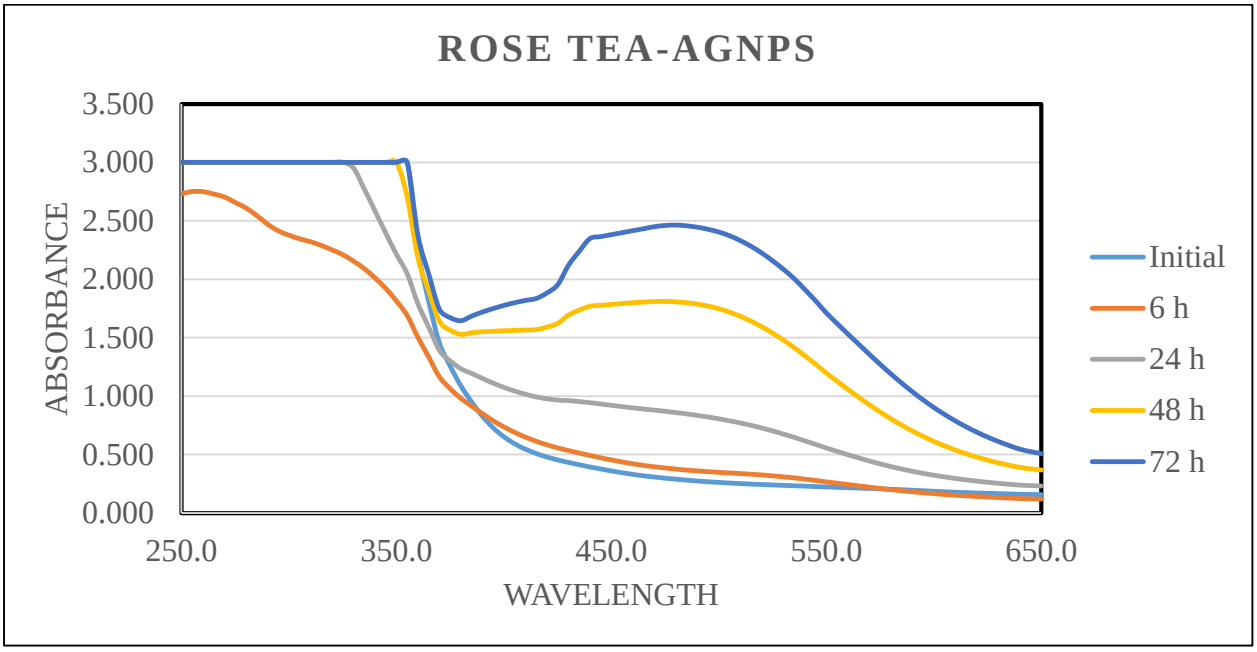


Fig 2. UV-Vis Spectra of RT-AgNPs

Phytochemical Analysis

The phytochemicals present in RT-AgNPs were evaluated by

Alkaline Reagent Test: The presence flavonoids in RT-AgNPs was revealed by Alkaline Reagent. RT-AgNPs were treated with few drops of NaOH solution. Intense yellow colour appeared and turned colourless on further addition of dilute acetic acid confirmed the flavonoids in RT-AgNPs.

Dragondroff's Test: Potassium Bismuth Iodide which is the Dragondroff's reagent was used to analyze the presence of Alkaloids. Appearance of permanent orange red colour confirmed Alkaloids present in the nanoparticles.

Foam Test: Presence of saponins were determined by the formation of foam in RT-AgNPs after the addition of 2 mL of distilled water and shaking the tubes. Foam was formed on the surface of the liquid confirmed the presence of saponins.

Ferric Chloride Test: Polyphenols existence were resolved by adding 3 to 4 drops of Ferric Chloride to RT-AgNPs. A bluish black colour disclosed the presence of polyphenols.

Keller-Killari Test: This test was carried out to confirm the presence of Glycosides. RT-AgNPs were treated with 2 mL of glacial acetic acid having 1 drop of ferric chloride in it and 1 mL of concentrated Sulfuric acid was added. Appearance of brown ring at the top and violet ring in acetic acid layer indicates the presence of Glycosides.

A study conducted in rose (Cendrowski *et al.*, 2017), Hibiscus (Nascimento *et al.*, 2021) and Black currant (Cortez and Gonzalez de Mejia, 2019) also confirmed the existence of flavonoids and other phenolics present in these flowers. These secondary metabolites present in the RT-AgNPs are responsible for the synthesis by reduction, capping and stabilization of RT- AgNPs from the extract.

Table 1: Depicts the phytochemical Analysis of RT-AgNPs

S No	Phytochemical	Present/Absent
1	Flavonoids	Present
2	Alkaloids	Present
3	Saponins	Present
4	Polyphenols	Present
5	Glycosides	Present

FT-IR Analysis for the Identification of Functional Groups in RT-AgNPs

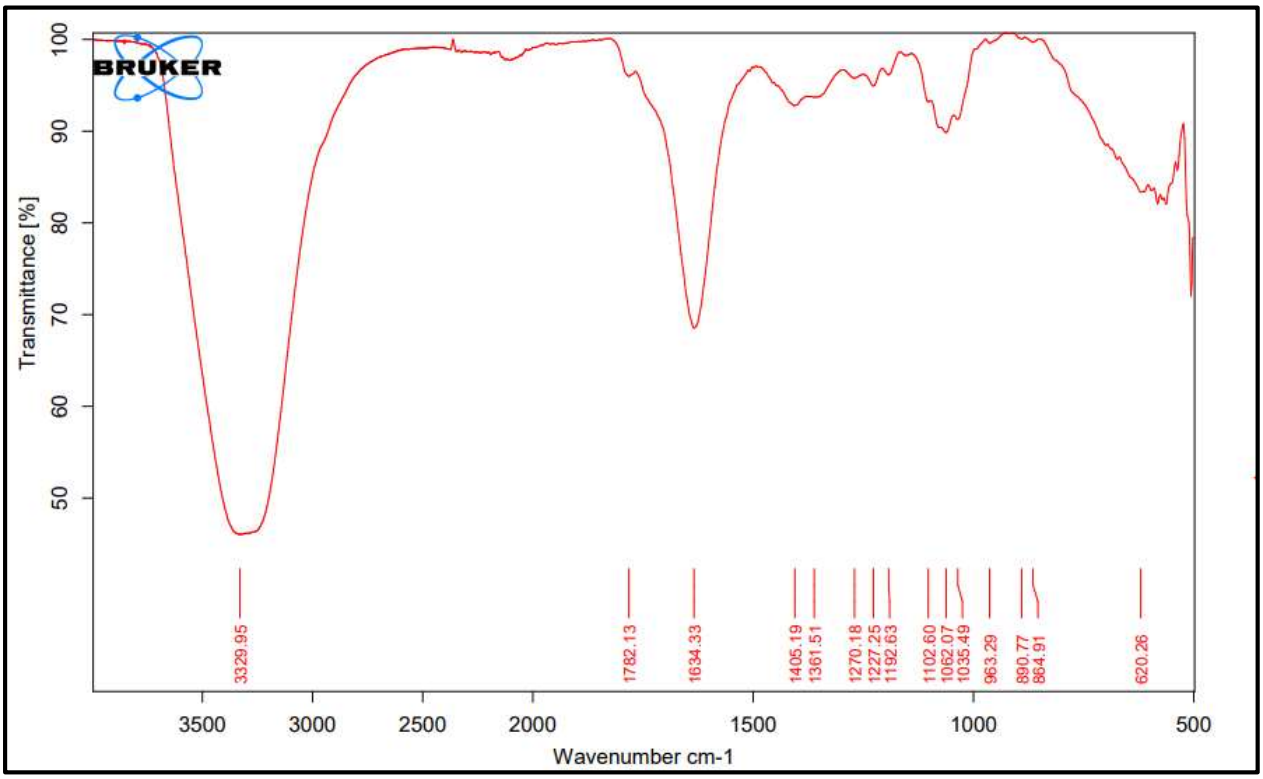
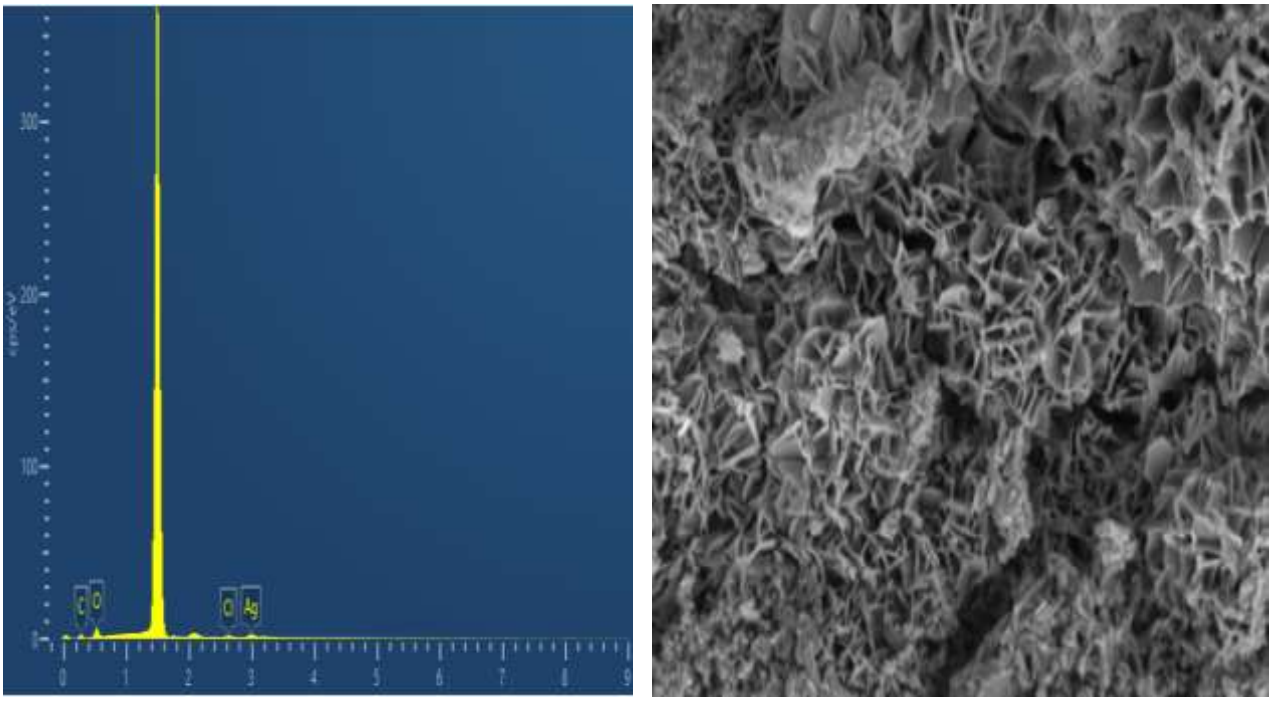


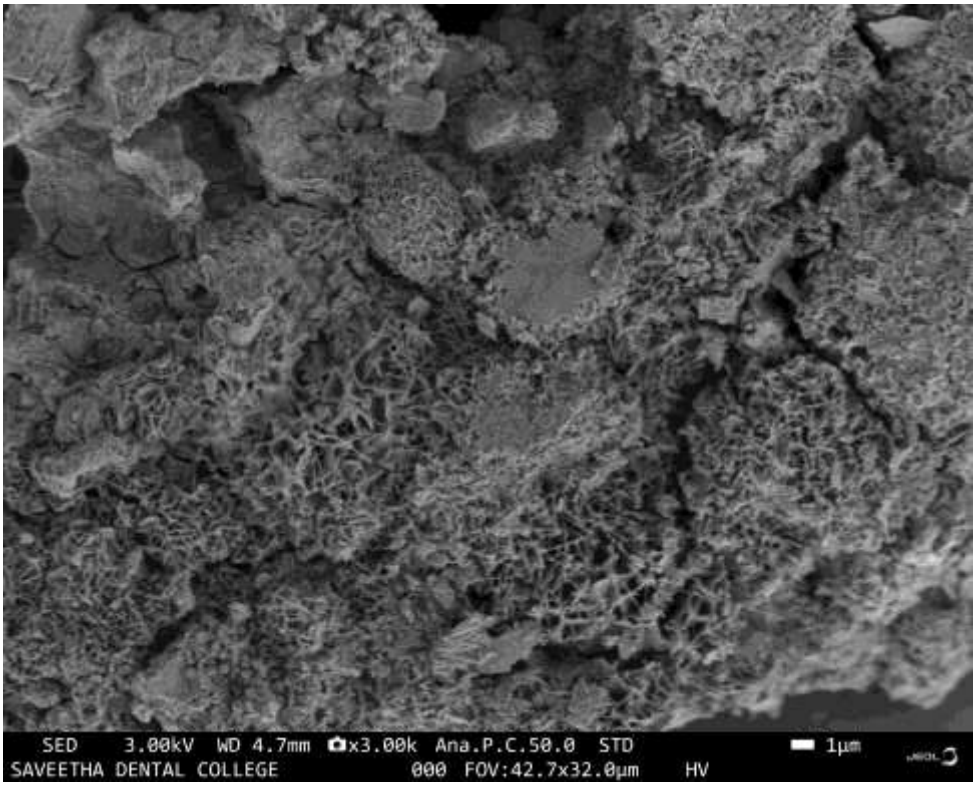
Fig 3. FT-IR spectrum of phyto chemicals found in RT-AgNPs

The FT-IR analysis was performed to identify the functional groups of the biomolecules present in the RT-AgNPs. The absorbance bands were seen in between 500 to 4000 cm^{-1} wave number (Fig 3). Characteristic peaks were seen in 3329.95, 1634.33, 1405.19, 1361.51, 1270.18, 1192.63, 1102.60 and 620.26 cm^{-1}. A strong broad band appeared in 3329.95 cm^{-1} with O-H stretching bond indicates the presence of alcohols (Suriyakala *et al.*, 2022). Medium stretches of C=C bond at 1634.33 cm^{-1} exhibits the presence of conjugated, cyclic alkenes and alkanes in methyl group. The medium band found in 1405.19 cm^{-1} of O-H bond represents the carboxylic acids. The presence of phenols are shown by the medium peak found at 1361.51 cm^{-1} with O-H bending bonds and strong S=O stretches represents the sulfonates and sulfonamides. Aromatic amines and aromatic esters are exhibited by the peaks found at peak in 1270.18 cm^{-1} (Patil *et al.*, 2018). Strong C-O stretching bond appeared at 1192.63 cm^{-1} indicates the tertiary alcohols (Hemlata *et al.*, 2020). Primary and secondary alcohols were seen in 1062.07 and 1102.60 cm^{-1} which were identified as strong C-O stretching bonds. Halo compounds were observed at 620 cm^{-1} as strong C-Br stretching bond. The identified functional groups are responsible for the formation, capping and stabilization of RT-AgNPs (Kuppusamy *et al.*, 2016).

Energy Dispersive X-ray (EDX) and SEM Analysis of RT-AgNPs



(A) **(B)**



(C)

Fig 4. (A) elemental composition of RT-AgNPs in EDX analysis (B) SEM image of RT-AgNPs

The Energy Dispersive X-ray (EDX) analysis was performed to evaluate the weight percentage of the elements present in the emerged RT-AgNPs (Fig 4 A). The analysis manifested Silver as a predominant element along with carbon, oxygen and chlorine which was identified by strong signals exhibited at 3 KeV. The atomic weight percent of silver was (73.2 Wt %), Carbon (2.85 Wt %), Oxygen (19.81 Wt %) and Chlorine (4.14 Wt %). This proves that silver is the predominant metal present along with other naturally occurring elements during the formation of RT-AgNPs from the extract. The results correlated with the previous study made in synthesis of silver nanoparticles using *Cassia angustifolia* flower extract by (Bharathi and Bhuvaneshwari, 2019). The surface morphology and the size of the RT-AgNPs were revealed by the Scanning Electron Microscopy (SEM) technique (Fig 4 B). The morphology was appeared to be spherical with a size of 75 nm in diameter.

Antimicrobial Activity of RT-AgNPs

(A) (B) (C) (D)

Fig 5. Antimicrobial activity of RT-AgNPs – Agar Well Diffusion Assay depicting zone of inhibition (A) *Streptococcus mutans* (B) *Lactobacillus* (C) *Enterococcus faecalis* (D) *Candida albicans*

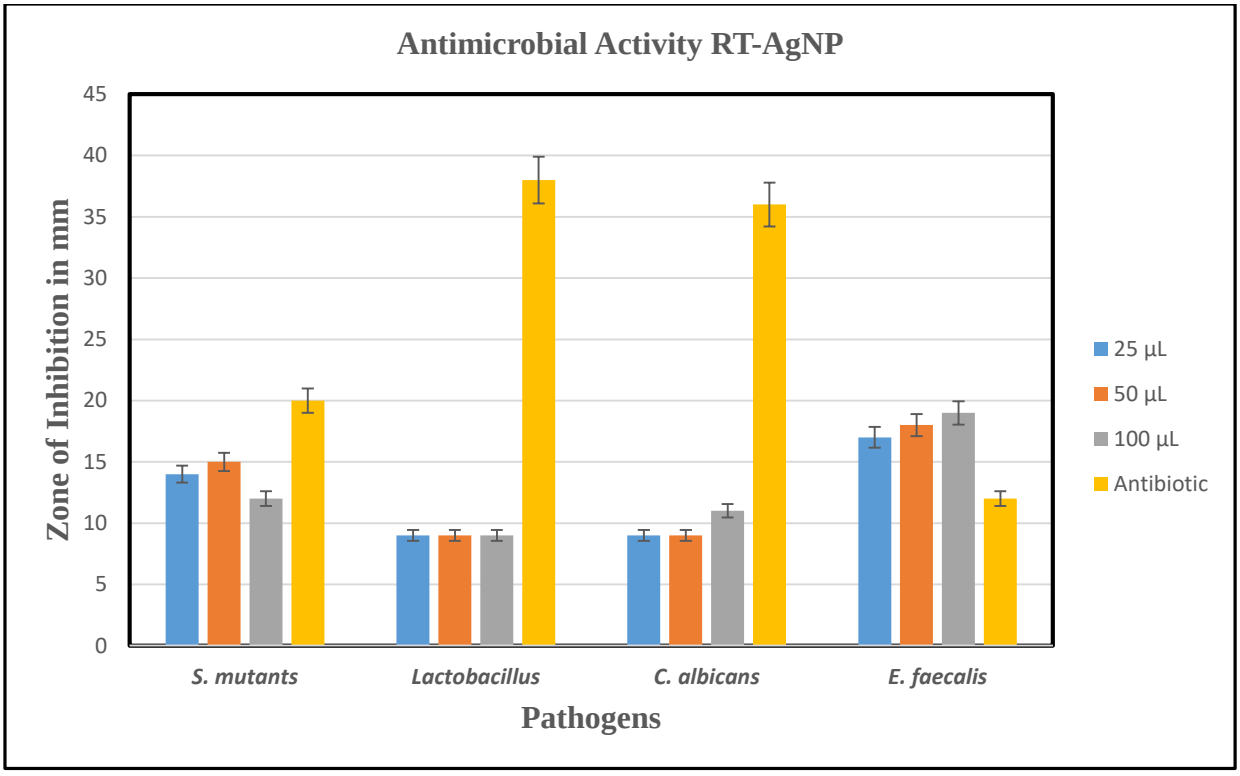


Fig 6. Represents the antimicrobial activity of RT-AgNPs in different concentration against pathogens

Agar Well Diffusion Assay was performed to find the antimicrobial activity of RT-AgNPs. Fig 5 (A B C D) shows the plates that revealed antimicrobial activity against *Streptococcus mutans* (Gram +ve), *Lactobacillus* (Gram –ve), *Candida albicans* and *Enterococcus faecalis* (Gram +ve). The test was performed using sterilized Muller-Hinton agar media poured in sterile petri plates. The media was allowed to solidify. The wells were then made in the media using a sterile polystyrene tip and concentration value was marked as (25µL, 50µL, 100µL and standard). Amoxicillin was used as standard drug for bacteria and Fluconazole for the fungi. The pure broth culture of each microorganism were then swabbed properly in each plate and labelled. In each well the RT-AgNPs and the standard drug were added according to the concentration value mentioned. The plates were then incubated in room temperature for 24 hours. After the incubation period was completed the zones were measured (millimeter) and recorded. Fig 6 shows the RT-AgNPs that revealed a maximum inhibition against *Enterococcus faecalis* when compared to other organisms with a clear zone of 17mm in 25µL, 18mm in 50 µL and 19mm in 100 µL. *Candida albicans* showed no zone in 25 µL and 50 µL and in 100 µL zone was formed and was found to be 11mm in diameter. This differences in the zone formation is due to the differences in the structural composition of cell walls in Gram positive, Gram negative bacteria and in fungi (Gomathi *et al.*, 2019). The antimicrobial potential of RT-AgNPs is because of shape, surface charge, and size and partial size distribution. Smaller nanoparticles with larger surface area exhibits more antimicrobial efficacy as they easily adheres to the cell wall. The possible mechanism of antimicrobial efficacy was due to the cell wall damage, inhibiting enzymatic activity, obstructing DNA replication, production of free radicals that generates Reactive Oxygen Species (ROS) leading to cell death (Paosen *et al.*, 2019). The test confirmed that RT-AgNPs showed good inhibitory effect against *Enterococcus faecalis* and can be used as antibacterial agent after further required studies are made.

Free Radical Scavenging Activity of RT-AgNPs – DPPH Assay

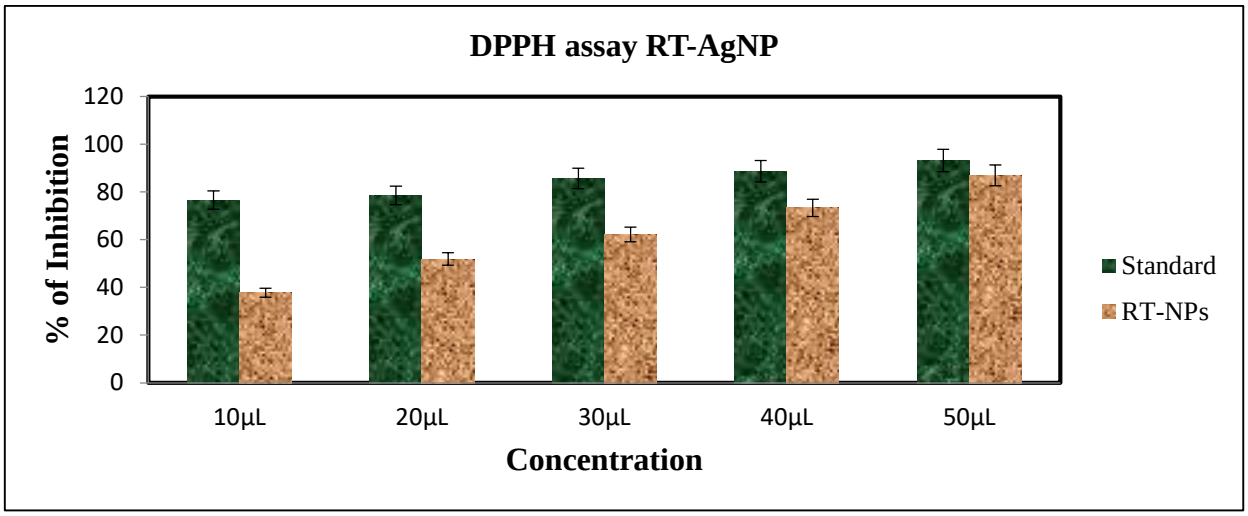


Fig 7. Free Radical Scavenging Activity of RT-AgNPs–DPPH (2, 2-diphenyl-2-picrylhydrazyl) Assay depicting the antioxidant activity in dose dependent manner

DPPH (2,2-diphenyl-2-picrylhydrazyl) Assay was carried out to evaluate the free radical scavenging activity of RT-AgNPs. 1mL of 0.1 mM of DPPH solution and 450 µL of 50 mM of Tris Hcl was added in five tube labelled 10, 20, 30, 40 and 50 µL concentration. To this, RT-AgNPs was added according to the labelled concentration value. Then the tubes were incubated in dark condition for 30 minutes at room temperature. After the incubation period was completed photometric readings were taken at 517 nm using a UV-Vis spectrophotometer. The readings were recorded and percent value of scavenging activity was identified by using the formula,

Inhibition percentage (%) = $\frac{\text{Absorbance of control – Absorbance of test sample}}{\text{Absorbance of control}} \times 100$ ---------- 1.

The scavenging activity of RT-AgNPs increased with increase in concentration. At 10 µL the percent of activity was found to be 37.8 % which was far less than the standard ascorbic acid value 76.56 %. In 20 µL it was 51.9 % and for standard it was 78.52 % in 30 µL the scavenging activity increased further and exhibited 62.2 % but not closer to the standard value 85.63 %. In 40 and 50 µL concentrations the scavenging activity increased firmly to 73.3 and 86.9 % which was closer to the standard 88.68 and 93.15 %. Similar reports were found in a study done by (Singh *et al.*, 2021) in biosynthesized silver nanoparticles from *Carissa carandas* leaf extract. Our study proved that free radical scavenging activity of biosynthesized RT-AgNPs have good antioxidant activity in a dose dependent manner so better results could be obtained in further increase in the concentration of nanoparticles.

Anti-inflammatory Activity of RT-AgNPs Egg White Albumin Denaturation Assay

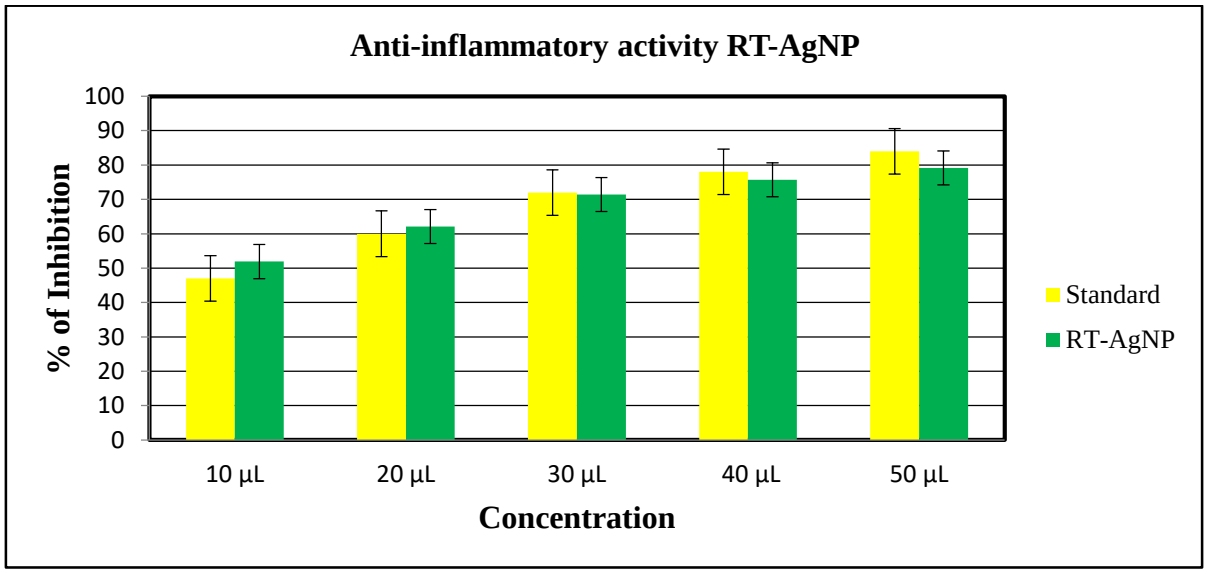


Fig 8 Anti-Inflammatory Activity of RT-AgNPs - Egg White Albumin Denaturation Assay depicts anti-inflammatory activity in a dose dependent manner

Anti-inflammatory activity of RT-AgNPs Egg - White Albumin Denaturation Assay

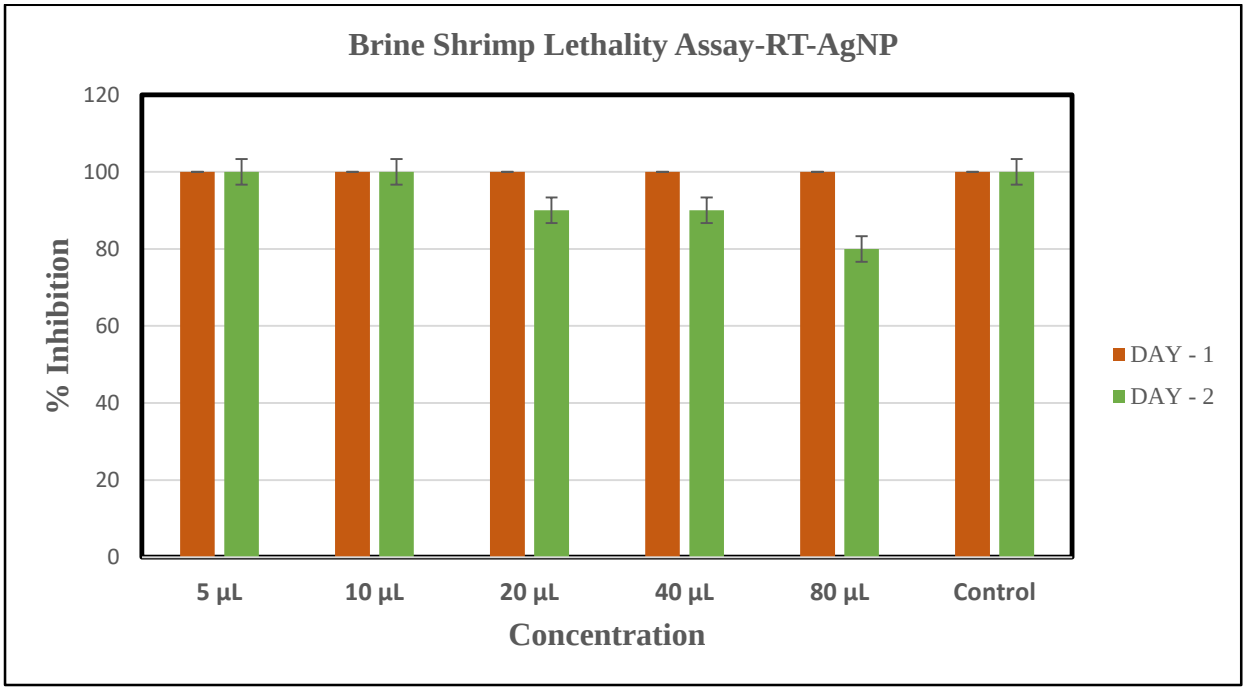
Anti-Inflammatory Activity – Egg White Albumin Denaturation Assay was performed for the estimation of anti-inflammatory efficacy of RT-AgNPs. Five clean test tubes were taken and marked as 10, 20, 30, 40 and 50 µL which represents the concentration of RT-AgNPs. The test was performed by adding 200 µL of egg white albumin in 5 separate test tubes, and 2800 µL of 1X phosphate buffer solution was added in each tube containing egg white. Then RT-AgNPs were added (10, 20, 30, 40 and 50 µL) according to the concentrations value marked on the tubes. The tubes were incubated for 10 minutes. Once the incubation time was finished the tubes were kept in hot water bath and temperature was adjusted to 55^0 C. After that the tubes were allowed to reach the room temperature and photometric OD value was taken using a UV-Vis spectrophotometer at 660 nm wavelength. The results were recorded to estimate the inhibiton activity of RT-AgNPs in percentage by using the formula

Inhibition percentage (%) = Absorbance of control – Absorbance of test sample × 100 ---------- 2.

Absorbance of control

The findings were compared with the standard drug (Diclofenac sodium). The assay confirmed the anti-inflammatory activity of the RT-AgNPs in a dose dependent manner and results were near close to the standard drug value shown in Fig 8. Maximum anti-inflammatory activity of RT-AgNPs was observed in 50 µL concentration which was 79.1 % and 84% for standard drug. A study performed by (NivedaRajeshwaran, Ramamurthy and Rajeshkumar S, 2021) in grape seed oil infused silver nanoparticles to evaluate anti-inflammatory activity also showed a very similar results which was found to be 82 %. The RT-AgNPs proved to be good anti-inflammatory agent biosynthesized in an eco-friendly manner so it would not have any side effects as it is in chemically synthesized drugs. Hence RT-AgNPs could be used as an alternative for chemically produced anti-inflammatory drugs used for inflammatory conditions after analyzing with further required and extended techniques.

Cytotoxic Activity of RT-AgNPs against Brine Shrimp Artemia Nauplii



Brine Shrimp Lethality Assay was performed to estimate the cytotoxic effect of RT-AgNPs in Nauplii of Brine Shrimp Artemia. The test was done by using the 2nd Instar Nauplii in live condition. Salt water was prepared by adding 20 grams of iodine free salt in 200 mL of water and mixed well so that salt get dissolved completely. A clean six well ELISA plate was taken and marked as 5, 10, 20, 40, 80 µL and control. Required volume of salt water was added in all the wells and 10 live Nauplii were added in each well. Then RT-AgNPs was added in each well according to the concentration value marked on the wells (5, 10, 20, 40, 80 µL) respectively and no nanoparticles were added in the control well. The setup was then incubated for 24 hours at room temperature. After the incubation period number of live Nauplii were counted in each well to assess the cytotoxic effect of RT-AgNPs and percentage of Nauplii was calculated using the formula

Inhibition percentage (%) = Number of dead Nauplii / Number of dead nauplii + Number of Live × 100 ----- 3.

At 5 and 10 µL concentration there was no mortality found and in 20 and 40 µL only 10 % mortality found and 90 % of Nauplii were alive. In 80 µL concentration the mortality rate was found to be 20 % and 80 % live Nauplii were found. Control well showed 100 % live Nauplii without any mortality. Similar findings were seen in a study reported by (Soundarajan *et al.,* 2020) in grape seed oil mediated AgNPs which showed 90 % of live Nauplii in second day of incubation at 20 µL the report exhibited more lethality when the concentration was increased to 25 µL but in present study our findings proved to be less lethal to the eukaryotic cells even at higher concentrations (80 µL).

Conclusion

The study concludes that the biosynthesis of silver nanoparticle using Rose, Hibiscus and Black currant tea extract is a simple and environment friendly approach. The phytochemicals found in the extract effectively reduced silver ions to silver and also involved in capping and stabilizing the nanoparticles. The RT-AgNPs revealed good antibacterial activity towards *Enterococcus faecalis* than other organisms which were used in the study. Also, the nanoparticles showed excellent antioxidant, anti-inflammatory activity. The cytotoxic effect was found to be less toxic which proves the biocompatibility against Brine Shrimp Artemia. Thus the RT-AgNPs could be used in nano biomedicine field.

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