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BIOSYNTHESIS AND BIOACTIVITY OF SILVER NANOBIOCONJUGATES FROM GRAPE (*VITIS VINIFERA*) SEEDS AND ITS ACTIVE COMPONENT RESVERATROL

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ABSTRACT: The seeds of *Vitis vinifera* have been shown to have antioxidant, anticancer and antimicrobial properties due to the presence of rich bioactive phenolic components like resveratrol. The research deals with the synthesis of silver nanobioconjugates from the methanolic extract of grape seeds and the pure compound resveratrol. The presence of resveratrol was confirmed by HPLC analysis in methanolic extract of various parts of grape fruits such as skin, pulp and seeds of two varieties (pink and black). As higher amount of resveratrol was present in the seed extract of pink grapes, it was selected for the silver nanobioconjugate synthesis. Various conditions were applied to influence the rapid reduction of Ag^+ to Ag^o, such as microwave heating, water bath heating, sunlight exposure and incubation at 37°C for nanobioconjugate synthesis. The reduction of silver ions was confirmed by color changes, adsorption spectroscopy (280-800nm) and percentage yield. Among the four different methods, sunlight exposure proved to be rapid in the synthesis and the yield of nanobioconjugates was also high. Therefore, this method was chosen for the synthesis of silver nanobioconjugates using resveratrol. The bioactivity of the synthesized silver nanoconjugates were also evaluated through antimicrobial activity. The results showed that the resveratrol in grape seeds extract can act as good reductant and stabilizer for the silver nanobioconjugate synthesis, which also exhibits potent bioactivity.

INTRODUCTION: Synthesis of NPs involve two approaches, namely top-down and bottom-up. In the top-down approach, the nanoparticles are synthesized from a suitable bulk material, and size reduction is achieved by physical and chemical methods. In case of the bottom-up approach, the nanoparticles are built from the smaller particles by chemical and biological methods. Both are traditional methods to synthesize the nanoscale materials [1, 2].

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The green production of nanoparticles using naturally occurring agents such as plant extract, microorganisms and biodegradable sources like reductant and capping agents, have made a remarkable attraction toward nanotechnology. These methods have led to the fabrication of a number of inorganic nanoparticles, mainly the metal nanoparticles. Plant-based materials seem to be the best candidates for the rapid synthesis of nanoparticles and they are suitable for large-scale biosynthesis [3].

Various researchers have studied the exploitation of numerous plant and plant-based materials for the biosynthesis of nanoparticles, which is considered as a green technology that does not involve any harmful chemicals [4]. The nature of the plant extract, its concentration, the concentration of the metal salt, pH, temperature and contact time have been recognized as the necessary factors involved in the rapid production of nanoparticles, their yield and other characteristics [5].

Over the past two decades, nanomedicine has been a vehicle for the development of pharmaceutical formulations. Nanomedicine focuses on the application of nanotechnology in medicine for diagnosis, prevention, detection and treatment of various diseases. There are diverse types of nanoparticles that have been synthesized for biomedical field, comprising of dendrimers, solid lipid nanoparticles, liposomes, peptide nanoparticles, poylmersomes, nanospheres and inorganic nanoparticles such as carbon nanotubes, gold, silver, zinc oxide and magnetic nanoparticles [6]. Among these, the inorganic nanoparticles have become very desirable as drug delivery systems because of their ideal characteristics [7].

Since the nineteenth century, silver-based compounds have been used in many antimicrobial applications. Among the various nanoparticles, nanosilver is one of the nanomaterials with the highest degree of commercialization and has been used for more than 150 years. Due to their size and specific surface area, the uses of silver nanoparticles are innumerable in various fields such as textiles, food packaging, waste water treatment and medical devices [8]. AgNPs synthesized from plants have proven that silver capped with the functional groups present in the compounds in plant extract, which act as antioxidant agents, enhance the biological activity like antimicrobial effect. The AgNPs synthesized from *Mimusops elengi*, *Pongamia pinnata*, *Artemisia nilagirica* and *Morinda citrifolia* showed higher inhibitory activity against multi-drug resistant microorganisms [9].

Grapes are one of the world's most important fruit crops. It is consumed raw and used for preparing wine, jam, juice and jelly. Moreover, grapes and grape-based products have been found to have rich antioxidant properties, which was suggested for beneficial effects against a variety of diseases. The major active compound in grapes has been identified to be resveratrol[10]. The present study focussed on the green synthesis of silver nanoparticles from anticancer phenolic-rich fruit source, namely *Vitis vinifera* and its major component resveratrol. As the initial phase of the study, the presence of the phenolics in the fruits was confirmed by HPLC analysis. Following the confirmation, different methods were tried for the synthesis of silver nanoparticles to find the optimal method.

MATERIALS AND METHODS:

Preparation of grape extract:

Grapes are available in two major varieties in the local market, namely, the pink and black varieties. Fresh pink (Plate 1) and black (Plate 2) grapes were purchased from the local market of Coimbatore. The grapes were washed and air dried by spreading on tissue paper to remove the water droplets. The whole fruit, as well as individual parts of the fruits, were used for the extraction in order to determine where the maximum yield of phenolic could be obtained. The skin, pulp and seeds were dissected and collected separately from both the varieties. The individual parts or the whole fruit (25g each) of both the varieties were individually macerated with 50ml of methanol. The extraction mixtures were kept in an ultrasonic bath for 30 minutes, and centrifuged at 500 g for 20 minutes. The extracts were stored at 4ºC for further experiments. The presence of resveratrol in the various component parts and the whole grape extract was analyzed by HPLC.

PLATE 1: *VITIS VINIFERA* FRUITS (PINK VARIETY)

PLATE 2: *VITIS VINIFERA* FRUITS (BLACK VARIETY)

Analysis of grape extracts by HPLC:
All the mobile phases and sample were filtered through a 0.45µm nylon membrane filter and the mobile phase was degassed in an ultrasonic bath prior to use. The extracts / compounds were dissolved in an appropriate volume of HPLC grade methanol and 20µL of the sample was injected into the reverse phase C18 column of the HPLC (Shimadzu, 100A, 250mm × 4.60mm particle size). Detection was carried out using a photodiode array detector (Prominence SPD-M20A).

Chromatographic data were obtained and processed with the software of LC-WorkStation VP^{TM} 6.14. The mobile phase used was water and methanol in the ratio of 15:85 and the flow rate was 0.8ml/minute at 40°C. The chromatograms were monitored at a broad spectrum window of 210-600 nm. The peaks were identified by comparing their retention time and UV-Visible absorption with the standard compound resveratrol.

Synthesis and separation of silver nanobioconjugates:
The silver nanobioconjugates were prepared by adding 10ml of methanolic extract (grape seed extract) or 10ml of diluted purified compound (containing 100µg resveratrol) to 90ml of 1mM silver nitrate (Sigma) solution. The nanobioconjugates were synthesized in different conditions and durations, namely microwave heating (10, 20, 30 and 40seconds), heating in water bath at 60°C (5, 10, 15 and 20minutes), sunlight exposure (5, 10, 15 and 20minutes) and at 37°C. Then the reaction mixture was kept under dark for 24 hours. The nanobioconjugates were separated by centrifugation at 3500g for 20minutes and washed thrice with deionized distilled water. The purified nanobioconjugates were allowed to dry, weighed and stored for further analysis.

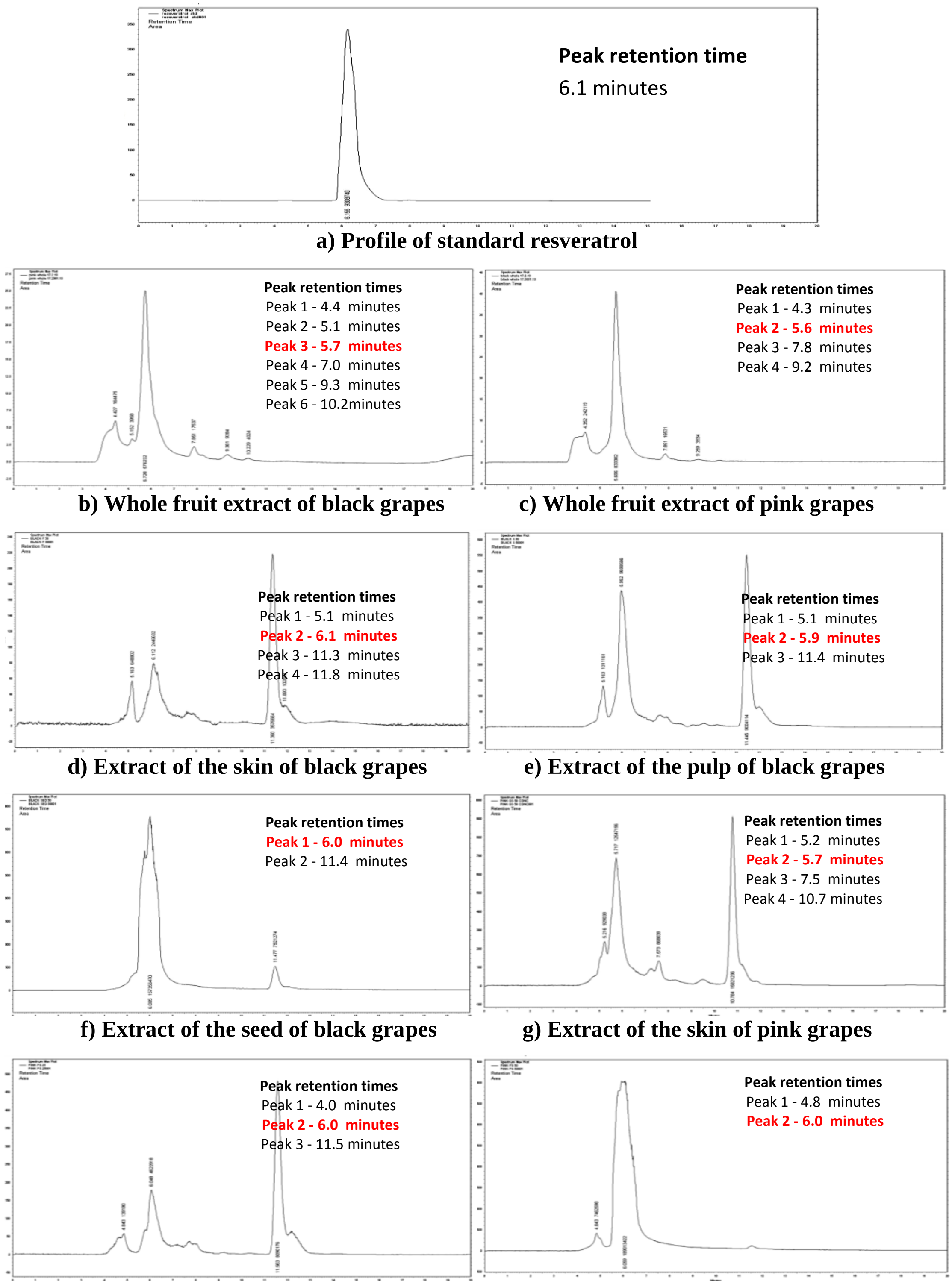
Characterization of silver nanobioconjugates:
The rapid bioreduction of silver nitrate to silver nanobioconjugates was preliminarily observed by recording the color changes and yield. Then to determine the properties of nanobioconjugates, the nanobioconjugates were scanned using an ultraviolet-visible nanophotometer (Shimadzu BioSpec-nano, Japan) in a wavelength range from 220 nm to 800 nm.

Bioactivity of silver nanobioconjugates:
The antimicrobial activity of synthesized silver nanobioconjugates were assessed by well diffusion method. Both the Gram positive and Gram negative microorganisms were used namely, *Staphylococcus aureus* and *Shigella flexneri* respectively. The bacterial cultures were maintained on nutrient broth. The nutrient agar was prepared and poured into sterile petri plates. When the medium solidified, bacterial suspensions were uniformly spread over the surface of nutrient agar plates. Sterile gel puncture was used to make wells in the agar. All the synthesized silver nanobioconjugates and plant extract were individually dissolved in DMSO (dimethyl sulfoxide) at the concentration of 50µg/10µl. The standard ampicilllin was used as the positive control (50µg) and DMSO as negative control. The standard ampicillin, AgNPs, seed extract / resveratrol, DMSO were added to the punctured wells and incubated at 37°C for 24 hours. After incubation, the zones of inhibition were measured in mm.

RESULTS:

HPLC analysis of *Vitis vinifera* fruit extracts:
The HPLC profile of the standard resveratrol (**Fig. 1.a**) was compared with those obtained with the methanolic extracts of the whole fruit of the black (**Fig. 1.b**) and pink (**Fig. 1.c**) varieties of *Vitis vinifera.* Standard resveratrol showed a single peak at the retention time of 6.1 minutes. The fruit extracts showed a single major peak at 5.7 (black variety) and 5.6 (pink variety) minutes respectively, which spilled over the retention time of 6.1 minutes. Additional minor peaks were present in the extract, indicating the presence of other compounds. The reason for the slight shift in the peak is not known and may be attributed to the presence of very closely related compounds.

Following this, in order to identify the specific part of the fruit that is rich in resveratrol, the fruits were dissected into their component parts (skin, pulp and seeds) and extracted into methanol and subjected to HPLC. The skin, pulp and seeds of the black grape variety showed multiple small and big peaks. The peaks that corresponded to resveratrol appeared at 6.1 minutes in the skin (**Fig. 1.d**), 5.9 minutes in the pulp (**Fig.1.e**) and 6.0 minutes in the seeds (**Fig. 1.f**). The peak corresponding to resveratrol was the only major peak in the seeds, while other peaks were observed in the skin and the pulp. A similar trend was observed in the pink grape variety also. The peaks that corresponded to resveratrol appeared at 5.7 minutes in the skin (**Fig. 1.g**), and 6.0 minutes in the pulp (**Fig. 1.h**) and the seeds (**Fig. 1.i**). Among the two varieties, this peak area was slightly higher in the pink variety than the black variety. Additionally, the number of seeds per fruit was also higher in the pink variety than the black variety. Therefore, the seeds of the pink fruits of *Vitis vinifera* were taken for the preparation of the silver nanobioconjugates.



a) Profile of standard resveratrol

b) Whole fruit extract of black grapes **c) Whole fruit extract of pink grapes**

d) Extract of the skin of black grapes **e) Extract of the pulp of black grapes**

f) Extract of the seed of black grapes **g) Extract of the skin of pink grapes**

h) Extract of the seed of black grapes **i) Extract of the skin of pink grapes**

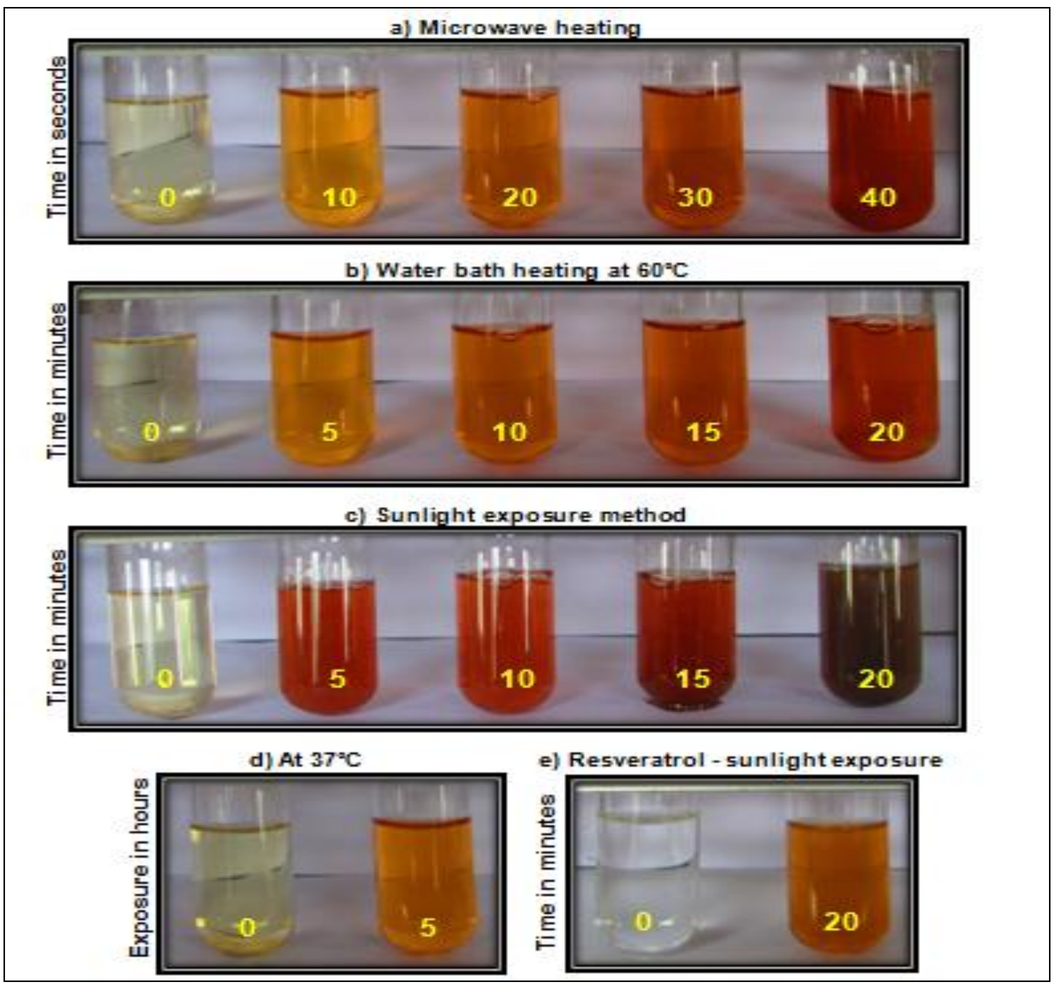
FIG. 1: HPLC PROFILE

Formation and yield of silver nanobioconjugates:

The biosynthesis of silver nanobioconjugates of *Vitis vinifera* seed extract was confirmed by the colour change from yellow to intense brown, which was recorded by visual observation. Rapid synthesis and colour intensity increased with the exposure and duration of incubation in all the four methods (microwave heating, heating in a water bath at 60°C, sunlight exposure and incubation at 37°C) (**Plate 3a-d**). Followed by the color changes, the yield of the nanobioconjugates found to be increased with the duration of exposure in all the methods. However, there was a marked increase in the nanoparticle synthesis pattern of grape seed extract (**Table 1**) under sunlight exposure, which showed the maximum yield at 20 minutes. Hence, the respective major phenolic compound of grape seed extract, namely resveratrol was employed in the synthesis of silver nanobioconjugates under sunlight exposure for 20 minutes. Which also showed the colour changes and confirmed the synthesis of silver nanobioconjugates.

TABLE 1: YIELD OF SILVER NANOBIOCONJUGATES SYNTHESIZED FROM *VITIS VINIFERA* SEED EXTRACT

S. No.	Method	Duration of Exposure	Yield (mg) from 100ml
1	Microwave heating	10 seconds	10
		20 seconds	15
		30 seconds	26
		40 seconds	32
2	Heating in water bath at 60°C	5 minutes	25
		10 minutes	28
		15 minutes	31
		20 minutes	40
3	Sunlight Exposure	5 minutes	32
		10 minutes	39
		15 minutes	45
		20 minutes	48
4	37°C	5 Hour	15



Initial colour of extract with $AgNO_3$ (left)
Colour changes after 24 hours following exposure, as indicated (right)

PLATE 3: FORMATION OF SILVER NANOBIOCONJUGATES FROM *VITIS VINIFERA* SEED EXTRACT / RESVERATROL

Spectral analysis of silver nanobioconjugates: The reduction of silver nitrate in the presence of grape seed extract and its component, resveratrol under various conditions applied (microwave heating, heating in a water bath at 60°C, sunlight exposure and incubation at 37°C) was evaluated by UV-visible spectroscopy. This technique is very important for the analysis of metal nanoparticles and provides information about the optical properties of the synthesized nanosize silver particles [11, 12]. All the silver nanobioconjugates synthesized from grape seed extract and its component resveratrol showed distinct peak at 420nm, indicated the characteristic of AgNPs (**Fig. 2**, **3**). Among the various methods, the peaks were more pronounced in the sunlight-exposure, reiterating that this was the best among the methods analyzed.

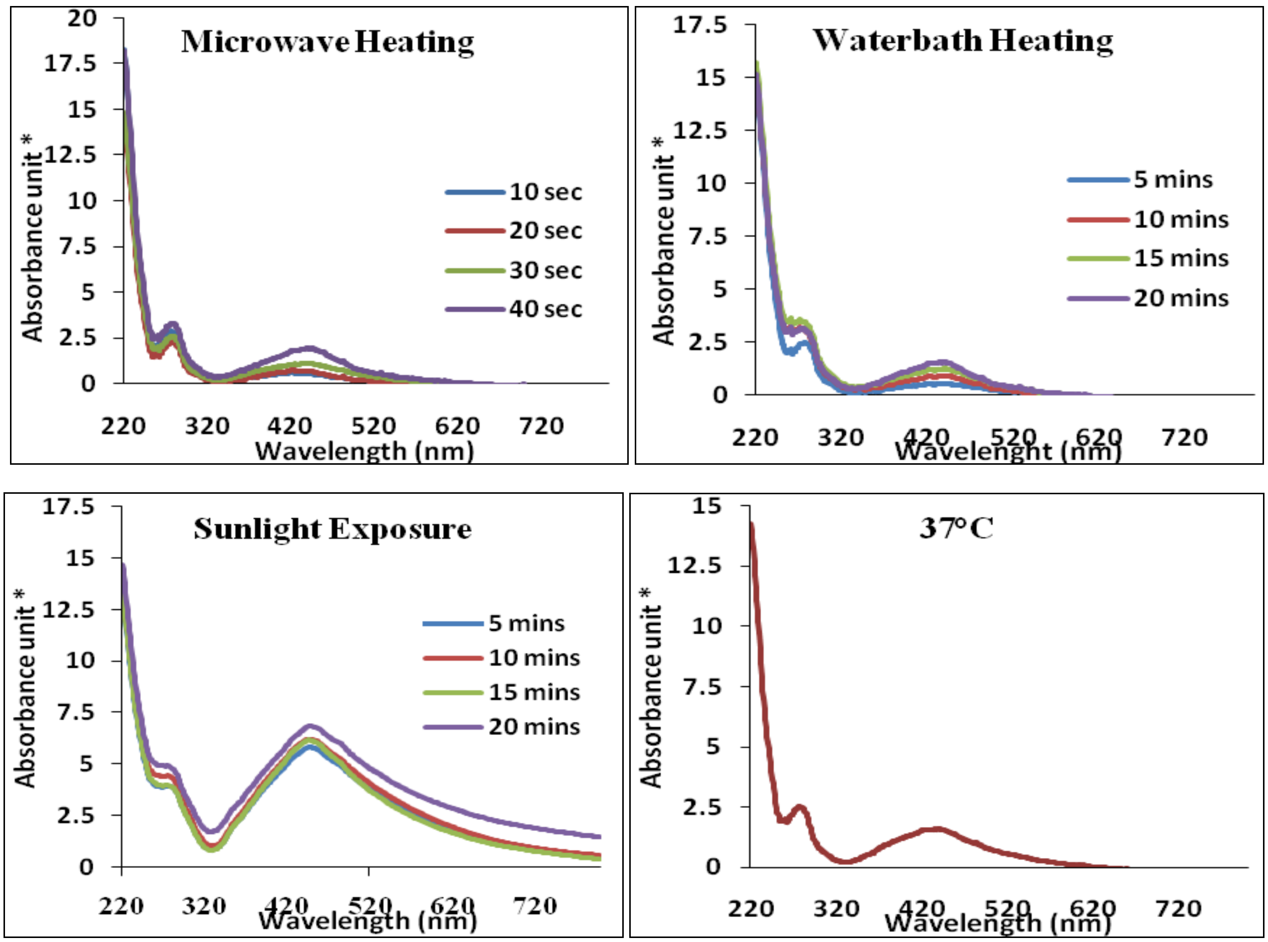
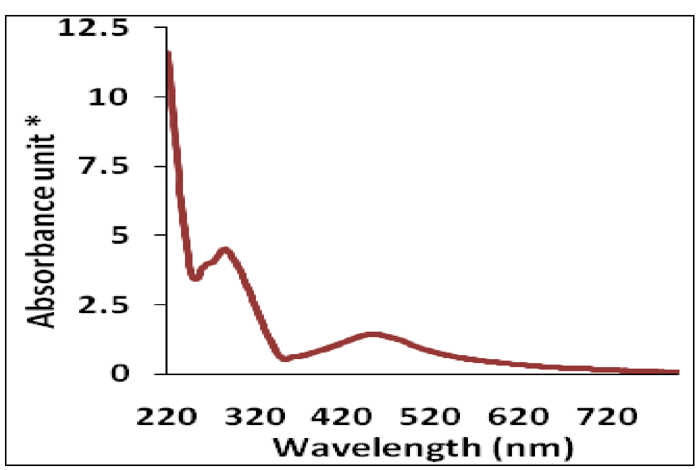


FIG. 2: ABSORPTION SPECTRUM OF SILVER NANOBIOCONJUGATES OF *VITIS VINIFERA* SEED EXTRACT

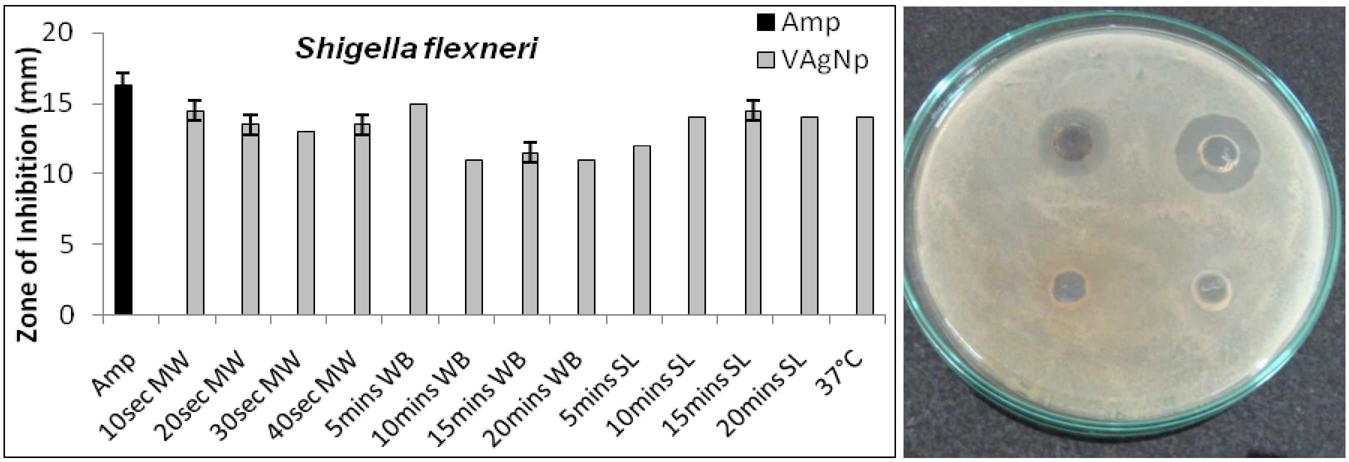


* Absorbance units as recorded in Shimadzu-Bio Spec-nano, Japan

FIG. 3: ABSORPTION SPECTRUM OF SILVER NANOBIOCONJUGATES OF RESVERATROL

Antimicrobial activity of silver nano bioconjugates:

The method of choice for the synthesis of silver nanobioconjugates have optimized, so the bioactivity was tested for the synthesised nanobioconjugates against Gram positive and Gram negative microorganisms. The efficacy of silver nanobioconjugates was compared with it unconjugated counterpart. Appropriate solvent control (DMSO), ampicillin (positive control), unconjugated seed extract/compound and nanobioconjugates synthesized using four different methods were tested at a dose of 50µg. The results clearly showed that the unconjugated seed extract/compound did not show any inhibition, where as the nanobioconjugates synthesized were effective antibacterials against both Gram positive and Gram negative organisms (**Fig. 4** and **Table 2**).



The values are Mean ± S.D. of triplicates

MW - Microwave, WB - Water bath, SL - Sunlight, VAgNP - Silver Nanobioconjugates from grape seed, Amp - Ampicillin, VE - Seed Extract, DMSO - Dimethyl sulfoxide

FIG.4: ANTIMICROBIAL ACTIVITY OF SILVER NANOBIOCONJUGATES SYNTHESIZED FROM *VITIS VINIFERA* SEED EXTRACT

TABLE 2: ANTIMICROBIAL ACTIVITY OF SILVER NANOBIOCONJUGATES

Groups	Zone of inhibition (mm)	
	Staphylococcus aureus	***Shigella flexneri***
DMSO	0	0
Resveratrol	1	1
AgNPs	10	11
Ampicillin	21	15

DISCUSSION: Our country is a chief and rich source of medicinal plants and plant products. The practices of herb-based traditional medicine is wide spread and the plants have been extensively utilized in Ayurveda. Recent development in nanomedicine gained attention towards the use of plants and fruits to synthesize nanoproducts, due to their versatile acceptability in various fields [13]. Among the various metal nanoparticles, silver and gold nanoparticles, provide a great platform in the biomedical filed as biosensing, biomolecular recognition and targeted drug delivery system [14]. In this study, the methanolic extracts of Vitis vinifera seeds, as well as their major phenolic active principles resveratrol were used to build silver nanobioconjugates. The presence of resveratrol in the grape fruits was confirmed by HPLC before the synthesis of silver nanobioconjugates. All the parts of the grapes, of both pink and black varieties, namely skin, pulp and seeds, contained considerable amounts of resveratrol. Among the three parts, the seeds of the pink variety showed the highest amount of resveratrol, and was, hence, used in this study.

Resveratrol is a naturally occurring polyphenol detected in more than seventy plant species, including *Vitaceae* spp, legumes, peanuts and Sorghum bicolor [15]. The presence of resveratrol was confirmed by HPLC in the whole fruit [16], skin[17], pulp and seed [18]. Similarly, using HPLC analysis, various phenolic compounds such as gallic acid, procyanidin, quercetin, resveratrol and viniferin were reported from the fractions of grapes stem [19] and anthocyanidins, flavan-3-ols, phenolic acids and resveratrol in red wines prepared from various grape varieties [20]. Our study also showed considerable quantities of resveratrol in the parts of pink and black grapes, with the seeds of the pink variety showing the highest peak in HPLC. Following the confirmation of active compound, resveratrol in grape seeds, they were used for the silver nanobioconjugate synthesis.

Among the four different methods (microwave heating, water bath heating at 60°C, sunlight exposure and incubation at 37°C) sunlight exposure showed the most marked effect, with rapid colour change and high yield. Several researches have been involved in synthesizing of silver nanoparticles using natural sources. Numerous reports are available on the synthesis of AgNPs using fruit extract as environment friendly substitutes to chemical methods. The instant green synthesis of silver nanoparticles using *Terminalia chebula* fruit extract [21], *Dillenia indica* fruit extract [22], *Solanum lycopersicums* [23] and *Emblica officinalis* [24] were confirmed by the formation of brown colour.

A study conducted using grape fruit extract also showed an immediate change in colour from pale pink to grayish brown, as observed in our study[25]. Our observation in the synthesis of silver nanobioconjugates using grape seed extracts showed a rapid colour change in all the four different conditions applied, among the methods, sunlight exposure was found to be the most effective.

The nanoparticles synthesized using light, when compared to chemical synthesis, gives more benefit of uniform distribution of the reducing agent in the whole mixture [26]. A similar research work conducted in our laboratory using methanolic extract of betel leaf extract to synthesis gold nanoparticles under sunlight exposure was one of the best methods to synthesize nanoparticles [27], which corroborated with the results we obtained. Thus, our results showed that exposure to sunlight generates rapid and high yielding nanobioconjugates than the other methods, which is an inexpensive, potential and best process for the synthesis of silver nanobioconjugates. Following the observation of colour changes and yield, the silver nanobioconjugates were analyzed by the UV-visible absorption spectrum, which confirm the formation of silver nanoparticles. The characteristic peak of grape seed extract mediated synthesis, found at 440 nm, has also been reported by many researchers working with AgNPs. This absorption band was observed for AgNPs synthesised with *Mukia maderaspatana*, *Kedrostis foetidissima* and *Cayratia pedata* [28], *Euphorbia helioscopia* Linn [29], seed extract of *Calendula officinalis* [30], seed extract of *Vigna radiate* [31] and *Elettaria cardamomom* seed extract [32].

Our results are in agreement with these reports. Based on the rapid colour change, yield and the high absorbance characteristic peak, the maximum extent of silver nanobioconjugates was observed to occur under sunlight exposure for 20 minutes. Hence, bioactivity was determined as antibacterial effect against the clinical isolates of one Gram positive (*Staphylococcus aureus*) and one Gram negative (*Shigella flexneri*) organisms for the synthesis of silver nanobioconjugates.

Among the VARIOUS noble metals, silver has been the metal of choice in the field of biological systems and medicine. AgNPs are the most commonly used nanomaterial in consumer products, representing about a quarter of all marketed nanomaterials. Silver nanoparticles have been shown to exhibit powerful antimicrobial properties [33]. Many reports have investigated the antimicrobial activity of silver nanobioconjugates.

The silver nanoparticles synthesized from the aqueous leaf extract of *Ocimum sanctum Linn* also showed prominent activity on Gram negative human pathogenic *Salmonella typhimurium*, *Escherichia coli* and *Pseudomonas aeruginosa* [34]. The antibacterial activity of the silver nanoparticles synthesized from rhizome extract of *Alpinia galangaby* by microwave irradiation was investigated against the Gram positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* and against the Gram negative bacteria *Vibrio cholerae* and *Salmonella paratyphi*, which showed higher antibacterial activity in both Gram positive and Gram negative organisms [35].

Silver nanoparticles have been shown to be able to penetrate into the bacterial cell membrane, interact with sulfur-containing and phosphorus-containing compounds, such as DNA, and cause damages inside it. It has been reported that DNA loses its replication ability and cellular proteins become inactivated after interaction with silver nanoparticles, which lead to the cell death [36]. Thus, our results showed that all synthesized AgNPs from betel leaf extract and its component, eugenol posses antimicrobial activity.

CONCLUSION: In this study, the method of synthesis of silver nanobioconjugates was optimized using the methanolic extract of *Vitis vinifera* seeds, as well as their active phenolic component, eugenol. Thus, validate the method of sunlight exposure is a rapid, inexpensive and ecofiendly. The synthesized bioconjugates were also bioactive, as reflected by their antibacterial effect. Additionally, it was observed that the nanobioconjugates of extract and compound were more effective than their non-conjugated counterparts, indicating potential biomedical applications.

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