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SYNTHESIS AND OPTIMIZATION OF BIOACTIVE SILVER NANOBIOCONJUGATES FROM PIPER BETLE LEAVES AND THEIR ACTIVE COMPONENT EUGENOL

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ABSTRACT

The leaves of *Piper betle* (Paan), have been shown to have anticancer and antimicrobial properties due to the presence of rich bioactive components, with the most predominant being eugenol. The present study deals with the synthesis of silver nanobioconjugates from the methanolic extract of *Piper betle* leaves and its active compound eugenol. The presence of eugenol in the leaf extract was confirmed by HPLC analysis in the methanolic extract of betel leaves. Since various conditions influence the rapid reduction of Ag^+ to Ag^o, different methods such as microwave heating, water bath heating at 60ºC, sunlight exposure and incubation at 37ºC were applied for the synthesis of nanobioconjugates. The antimicrobial efficiency of the synthesized silver nanobioconjugates from both leaf extract and active compound eugenol were also evaluated on Gram positive (*Staphylococcus aureus*) and Gram negative (*Shigella flexneri*) microorganisms. The results showed that the components in *Piper betle* leaves and active compound eugenol act as good reductants in synthesizing silver nanobioconjugates and these nanobioconjugates exhibit good antimicrobial properties.

KEYWORDS: ***Piper betle*** **leaves, eugenol, silver nanobioconjugates, antimicrobial activity**

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INTRODUCTION

Nanobiotechnology is a multidisciplinary field of research, interspersing technology and biology, with which astonishing advances were made in the field of life sciences such as biomedical application, health care and food industry. The nanoparticles have received special attention due to greater surface area to volume ratio, modified structure with more activity than macromolecules [1]. The nanoparticles synthesis using plant extracts is stated to be the best method, readily scaled up, environment friendly and less expensive when compared with the chemical and physical synthesis [1-2]. Plant-based materials seem to be the best candidates for the rapid synthesis of nanoparticles and they are suitable for large-scale biosynthesis. Plant parts such as leaf, root, latex, seed and stem, have been used for the synthesis of metal nanoparticles of silver, gold, iron oxides and metal alloys [3]. Several products based on silver available ranging as topical ointments and bandages for wound healing, have been proven to be effective in retarding and preventing bacterial infections [4]. 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 [5]. Silver is known for its antimicrobial properties and has been used in various medical fields. Several studies have reported that silver nanoparticles attach to the surface of cell membrane and distressing the cell respiration and permeability process. They also penetrate into the cell easily due their small size and larger surface area, which unique character helps to kill the microorganism [6].The leaves of *Piper betle*, which are deep green heart-shaped commonly referred to as "betel leaves" are traditionally consumed as a mouth freshener in Eastern Asia. They possess strong antimicrobial, antioxidant, anti-inflammatory, anticarcinogenic and antitumor properties due the presence of major phenolic compounds like eugenol [7, 8, 9, 10]. Eugenol is a natural phenolic compound, present in basils, cinnamon and bay leaves [9]. Several studies have reported the pharmacological mode of action of eugenol from medicinal plants such as *Ocimum sanctum*, *Anethum sowa Roxb*, *Pimpinella anisum Linn.*, *Alpinia galanga wild, Salvadora persica Linn.* and *Vetiveria zizanioides* in experimental animal systems [11]. Earlier studies conducted in our laboratory proved *Piper betle* leaf extract to be intrinsically rich in phenols and have antioxidant and hepatoprotective activity [12]. Hence, the present study focused on the synthesis of the silver nanobioconjugates using methanolic extract of betel leaves. The present research was undertaken to explore the influence of *Piper betle* leaves extract on the synthesis of silver nanobioconjugates and its bioactivity against Gram positive and Gram negative bacteria.

MATERIALS AND METHODS

Preparation of betel leaf extract

Fresh leaves of *Piper betle* (Athur variety) (Figure 1) were collected from local market, which was authenticated from TNAU, Coimbatore by Dr. Saraswathi. The leaves were cleaned thoroughly with deinoized water to remove the dust particles. The leaf sample (10g) was cut into small pieces and added to 100 ml of methanol. The mixture was stored at room temperature in the dark for 24 hours with occasional shaking and then filtered through Whatman No. 1 filter paper. The methanolic extract was stored at 4ºC until further experiments were carried out. The presence of eugenol in the *Piper betle* leaves was analyzed using HPLC.

Figure 1
***Piper betle* leaves**

Analysis of betel leaf extract by HPLC

The mobile phase used was water and methanol in the ratio of 40:60 and the flow rate was 1ml/minute at 30ºC. The chromatograms were monitored at a broad spectrum window of 210-600 nm. All the mobile phases and samples were filtered through a 0.45µm nylon membrane filter and the mobile phase was degassed in an ultrasonic bath prior to use. The extracts were dissolved in an appropriate volume of HPLC grade methanol and 20µL of the sample (betel leaves extract or eugenol) 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 VPTM 6.14.

Synthesis and separation of silver nanobioconjugates

The silver nanobioconjugates was prepared by adding 10ml of methanolic extract (betel leaves extract) or 10ml of diluted purified compound (100µg eugenol) to 90ml of 1mM silver nitrate (Sigma) solution. The nanobioconjugates were synthesized under different

conditions and durations. The methods used were microwave heating (10, 20, 30, 40 seconds), heating in water bath at 60ºC (5, 10, 15, 20 minutes), sunlight exposure at 12500-13500 lux (5, 10, 15 and 20 minutes) and at 37ºC to optimize the conditions of maximum yield of silver nanoparticles. After nanobioconjugates obtained were collected by centrifuging at 18,000 rpm for 20 minutes under refrigeration and washed three times with deionized water. The pellet was transferred to a pre-weighed container and dried in a hot air oven at 50ºC. The dried nanoparticle residue was weighed and the difference in weight was taken as the yield.

UV-visible absorption spectroscopy

Nanoparticles posses unique optical properties, which can be evaluated by UV-visible absorption spectroscopy. A volume of 100µl of synthesized silver nanobioconjugates was diluted with 900µl distilled water and subjected to spectral analysis using nanophotometer (Shimadzu-Bio Spec-nano, Japan), in the wavelength range of 220nm - 800nm.

Antimicrobial activity

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 grown in nutrient 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 (Amp) was used as the positive control (50µg) and DMSO as negative control. The standard ampicillin, PAgNPs, plant extract, 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 the Piper betle leaf extract

The HPLC chromatograms of the eugenol standard (Figure 2) and the methanolic extract of *Piper betle* leaves (Figure 2) were recorded and compared with each other. A peak at the retention time of 9.18 minutes in the leaf extract corresponded with the standard peak obtained (at the retention time of 9.2 minutes). This observation proved the presence of eugenol in the methanolic extract of betel leaves. The extract also showed the presence of additional peaks, indicating the presence of other compounds.

Figure 2
HPLC profile of standard eugenol

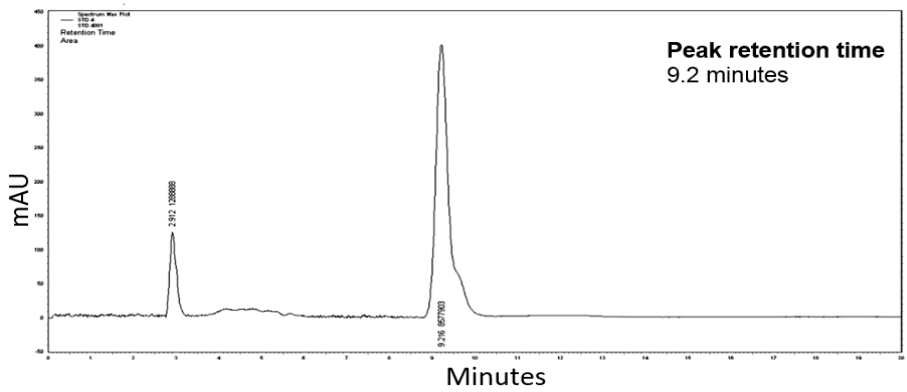
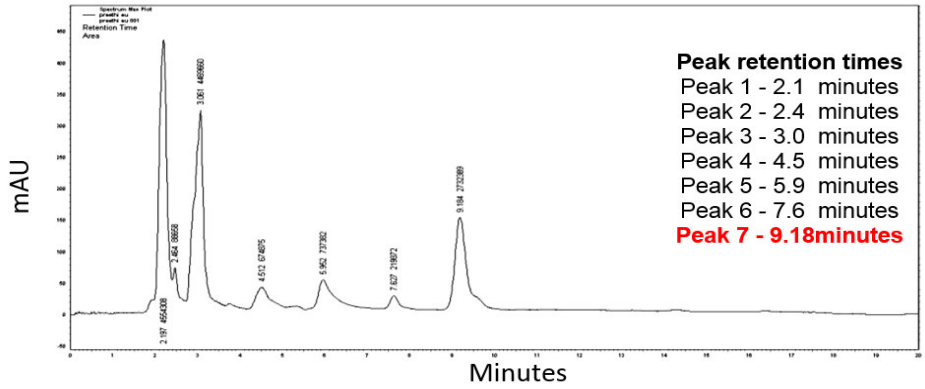


Figure 3
HPLC profile of methanolic extract of *Piper betle* leaves



Thus, having confirmed that the Piper betle leaves contained considerable amounts of the eugenol, the leaves were taken for the study, along with commercially available eugenol for the synthesis of silver nanobioconjugates.

Formation and yield of silver nanobioconjugates

Rapid synthesis of silver nanobioconjugates occurred in all the four methods (microwave, heating, sunlight exposure and incubation at 37ºC) using betel leaf extract. The colour intensity also increased with the exposure and duration of incubation in all the four methods, namely microwave heating (10, 20, 30 and 40 seconds), heating in a water bath at 60ºC (5, 10, 15 and 20 minutes), sunlight exposure (5, 10, 15 and 20 minutes) and 37ºC (Plate 2a-d and Table 1). Among the four different methods, there was a notable colour change, yield and increase in the intensity of colour from 5 to 20 minutes by the sunlight exposure, than the other methods. These observations confirmed that the sunlight exposure for 20 minutes was the best method for the rapid synthesis of silver nanoparticles using *Piper betle* leaf . So the major phenolic compounds of betel leaf, namely eugenol was employed in the study, which also showed the colour changes and confirmed the synthesis of silver nanobioconjugates (Plate 2e).

Plate 2 e
Formation of silver nanobioconjugates from *Piper betle* leaf extract / eugenol

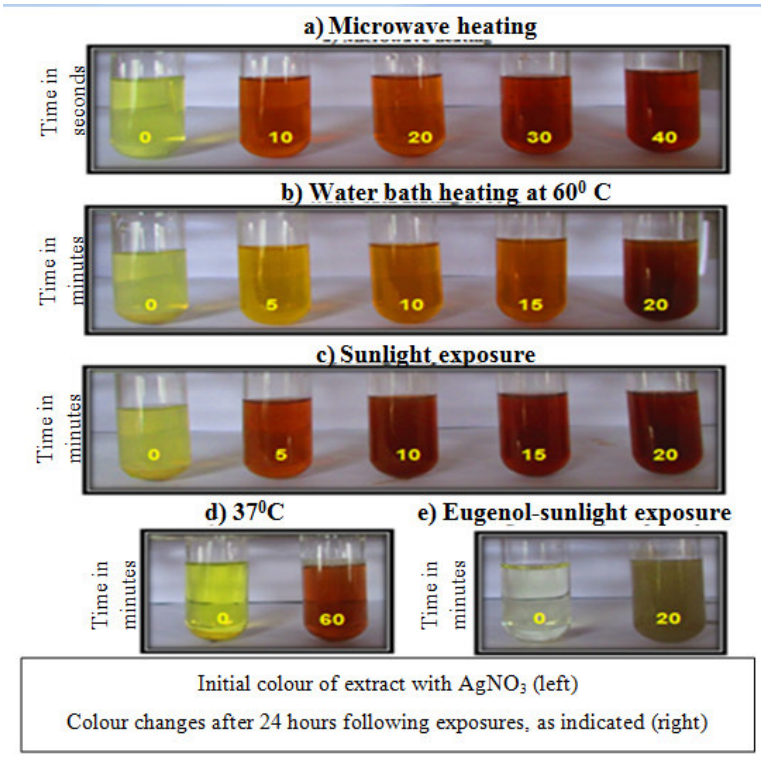


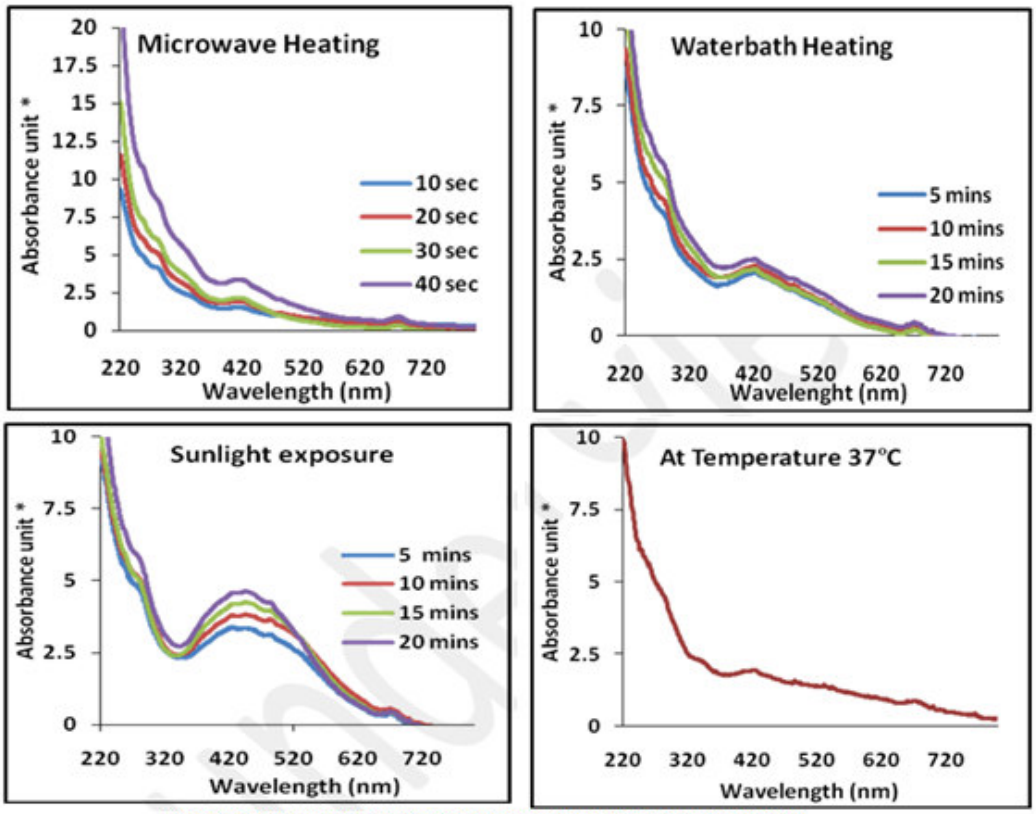
Table 1
Yield of silver nanobioconjugates synthesized from Piper betel leaf extract

S. No.	Method	Duration of Exposure	Yield (mg) from 100ml
1	Microwave heating	10 seconds	15
		20 seconds	23
		30 seconds	32
		40 seconds	35
2	Heating in water bath at 60oC	5 minutes	17
		10 minutes	20
		15 minutes	23
		20 minutes	30
3	Sunlight Exposure	5 minutes	30
		10 minutes	36
		15 minutes	40
		20 minutes	41
4	37oC	1 Hour	25*

Spectral analysis of silver nanobioconjugates

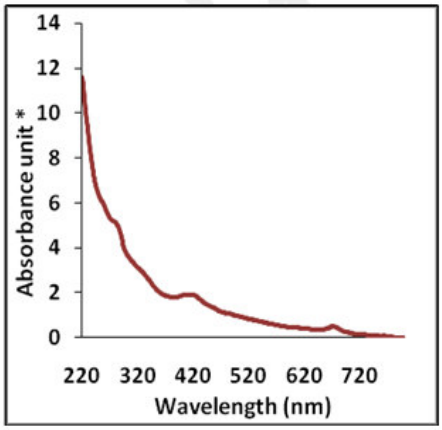
Followed by the colour change and yield, the bioreduction of silver nitrate to silver nanobioconjugates in the presence of plant extract and their component phenolics, by the various methods, was evaluated by UV-visible spectroscopy. All the silver nanobioconjugates synthesized (Figure 3) from betel leaf extract using the four different methods showed distinct peaks at 420nm which are characteristic of AgNPs. The peaks were more pronounced in the sunlight-exposed samples than the other methods, reiterating that this was the best among the methods tested. Therefore, the nanobioconjugates were synthesized from active phenolic compound, eugenol using only sunlight exposure for 20 minutes. The active compounds also showed the characteristic peak at 420nm (Figure 4).

Figure 3
UV-visible spectroscopy study of silver nanobioconjugates



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

Figure 4
Absorption spectrum of silver nanobioconjugates of eugenol

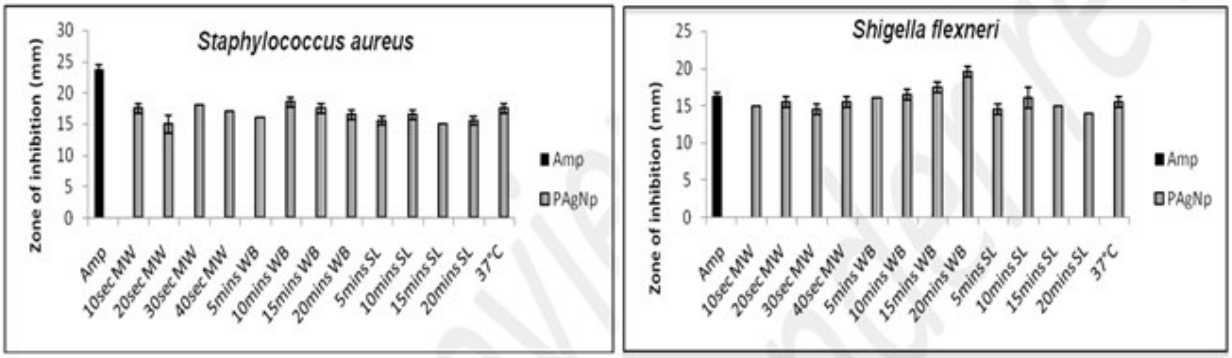


****Absorbance units as recorded in Shimadzu-Bio Spec-nano,Japan***

Antimicrobial activity of silver nanobioconjugates

Having optimized the method of choice for the synthesis of silver nanobioconjugates, before the detailed and systematic characterization of the nanoparticles, it was felt imperative to test if the synthesized nanobioconjugates possessed bioactivity. Additionally, it was also necessary to compare the efficacy of the nanobioconjugate with its unconjugated counterpart, in evoking a biological response. The bioactivity of the synthesized nanobioconjugates was tested as their antibacterial activity. Irrespective of the method of synthesis, all the different nanobioconjugates were tested for their antibacterial activity against a Gram positive (Staphylococcus aureus) and a Gram negative (Shigella flexneri) bacteria, at a dose of 50µg. Appropriate solvent control, positive control (ampicillin) and the raw material (the plant extracts and phenolics, without AgNP synthesis) were included. Based upon the earlier work done in our laboratory with the various concentration of silver nanobiconjugates among that 50µg was optimized with 50% inhibition, which was consider for the study These results proved that the AgNPs synthesized possessed good bioactivity, which was higher than their non-nano counterparts as shown by the distinct zones of inhibition of bacterial growth, both in Gram positive and Gram negative organisms (Figure 5 and Table 2).

Figure 5
Antimicrobial activity of silver nanobioconjugates synthesized from *Piper betle* leaf extract



The values are Mean ± S.D. of triplicates
MW-Microwave, WB-Water bath, SL-Sunlight

Table 2
Antimicrobial activity of silver nanobioconjugates synthesized from eugenol

Groups	Zone of inhibition (mm)	
	Staphylococcus aureus	*Shigella flexneri*
DMSO	0	0
Eugenol	0	1
AgNPs	9	10
Ampicillin	20	15

The values are Mean of triplicates

DISCUSSION

Nanotechnology is emerging as one of the most dynamic disciplines of research, where plants and plant-based products are used extensively to synthesize ecofriendly and activity-enhanced nanoparticles [13]. In the present study, the methanolic extracts of *Piper betle* leaves as well as their major phenolic active principles, eugenol were used to build silver nanobioconjugates using a bottom-up approach. Before this, the presence of the selected phenolics in the chosen plant materials was confirmed by HPLC. The HPLC analysis in the present study showed that the betel leaf extract was rich in eugenol. HPLC fingerprint showed quality consistency of hydroxychavicol and eugenol in *Piper betle* leaves [14]. Banerjee and Shah reported the presence of eugenol in betel leaf using HPLC analysis [15]. The HPLC phenolic compounds profile revealed the presence of eugenol, quercetin, rutin and catechin in the stem of Piper guinneensis [16]. In tune with these studies, in the present study also, the presence of eugenol was evidenced in the Athur variety of *Piper betle* leaves by HPLC. Which was used for the silver nanobioconjugates synthesis. Several researches have been involved in synthesizing of silver nanoparticles using natural sources. The synthesis of silver nanoparticles from leaf extract of *Cassia auriculata* exhibited changes in colour from yellow to brown and the intensity of the brown colour increased in direct proportion to the incubation period, which indicated the reduction of silver nitrate by the extract [17]. The leaf extract of banana, neem and black tulsi showed a colour change from faint light to yellowish-brown to colloidal brown under microwave heating [18]. Our observation in the synthesis of silver nanobioconjugates using betel leaf extract showed a rapid colour change in all the four methods used, in accordance with the above studies, proving the effective synthesis of silver nanobioconjugates. Among the methods, sunlight exposure was found to be the most effective. Following the observation of colour changes and yield, the silver nanobioconjugates were characterized by the UV-visible absorption spectrum, which quantifies the extent of silver nanoparticle formation by the various methods. The nanobioconjugates synthesized from betel leaf extract and eugenol showed the characteristic peak at 420nm. The absorption band is influenced by size, shape, morphology, composition, distribution and stability of the synthesized nanoparticles [19]. Similar characteristic peak at 420nm has been reported using *Artocarpus heterophyllus* Lam seed extract [20], *Plectranthus amboinicus* leaf extract [21], *Haloferax alexandrinus* [22], *Solanum tricobatum*, *Syzygium cumini*, *Centella asiatica* and *Citrus sinensis* plant powder extracts [23]. Our results are in agreement with these reports. Following the successful synthesis of silver nanobioconjugates, their bioactivity was determined as antibacterial effect against the clinical isolates of one Gram positive (*Staphylococcus aureus*) and one Gram negative (*Shigella flexneri*) organisms. The synthesized silver nanobioconjugates from betel leaf and their respective active compounds, eugenol showed potent antimicrobial activity. Silver nanoparticles have been shown to exhibit powerful antimicrobial properties [24]. Many reports have investigated the antimicrobial activity of silver nanobioconjugates. The AgNPs obtained from *Abutilon indicum* leaf extract showed highly potent antibacterial activity towards Gram positive (*Staphyloccocus aureus* and *Bacillus subtilis*) and Gram negative (*Salmonella typhi* and *Escherichia coli*) microorganisms [25]. Similarly, in another study on the *in vitro* antibacterial screening of AgNPs synthesized from leaf extract of *Ocimum sanctum*, higher inhibitory action was observed on *E. coli* and *S. aureus,* followed by *B. subtilis* and *P. aeruginosa* [26]. The silver nanoparticles synthesis from *Argyeria nervosa* extract showed activity against

Staphyloccocus aureus and *Bacillus subtilis* [27]. AgNPs have been shown to bond with the DNA of microorganisms, destroy the replication process and bind with the cell wall, which paralyse the cell permeability, ending in cell death [28]. These reports lend credibility to our results, which strengthened that the synthesised silver nanobioconjugates along with phenolic compound eugenol from betel leaf extract was more efficient in comparison with the plant extract alone against the microorganisms. It is possible that the nanoparticles cross the membrane easily and deliver the phytochemicals that cause antibacterial effects.

CONCLUSION

In this study, *Piper betle* leaf extract and eugenol were conjugated to silver ions to generate bioconjugates in the nanoscale. Among the different methods employed for the synthesis (microwave heating, at 60ºC in water bath, exposure to bright sunlight and incubation at 37ºC), exposure to sunlight resulted in the maximum yield of the nanobioconjugates of all the materials. The optimal duration of sunlight exposure was for 20 minutes. The AgNPs synthesized exhibited good bioactivity against both the organisms tested. The findings of the study, thus, validate and strengthen the method of synthesis of silver nanobioconjugates from *Piper betle* leaves and its major polyphenol (eugenol) using a rapid, inexpensive and eco-friendly method.

CONFLICT OF INTEREST

Conflict of interest declared none.

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