Response Surface Methodology Optimized Ultrasonic Polyphenolic Fractions of Selected *Buxus* Species as Green Reductants for Synthesis of Antibacterial and Antiradical Silver Nanoparticles

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Summary: The current study reports synthesis, characterization, and bioactivities (antibacterial and antioxidant) of silver nanoparticles (AgNPs) using *Buxus wallichiana* and *Buxus sempervirens* polyphenolic fractions (PPF). A response surface methodology (RSM) optimized ultrasound assisted extraction technique was used for extraction. Binary solvent systems [solvent system-I (70% ethanol and 30% water), solvent system-II (50% ethanol and 50% water), and solvent system-III (30% ethanol and 70% water)] at time (60, 90 min) and temperature (30 °C, 45 °C, 60 °C) were utilized for obtaining PPF. In AgNPs synthesis, reacting 1:1 to 1:15 proportions of the PPF and the silver salt solution, a proportion of 1:5 (PPF from SS-I), produced a higher yield of AgNPs as indicated by the increased absorbance intensity at the characteristic surface plasmon resonance peak (λmax 421 nm) in UV–Vis spectroscopic analysis compared to other tested ratios. Further, the synthesized AgNPs possessed an average size of 7.64 nm to 8.04 nm. Furthermore, the study reports that *B. wallichiana* and *B. sempervirens* contained high total phenolics and total flavonoids content (TPC and TFC). The PPF of *B. wallichiana* and *B. sempervirens* extracted with solvent system-I (SS-I) at 45 °C for 90 min, contained the highest TPC of 244.5±4.70 mg gallic acid equivalent/g (mg GAE/g) and 199.8±3.99 mg GAE/g, respectively. In addition, the TFC were found to be 182.7±5.11 mg quercetin equivalent/g (mg QE/g) and 203.7±4.92 mg QE/g, respectively. Assessing the antibacterial activity, the AgNPs and the PPF exhibited significant bacterial inhibition against *Staphylococcus aureus* and *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli* with inhibition zone ranging from 8.00 mm to 20.0 mm. The antioxidant activity was found high ranging with IC_{50} values of 0.02 to 0.09 mg/mL. This is the first report describing green synthesis of biologically active AgNPs via *B. wallichiana* and *B. sempervirens* RSM optimized PPF.

Keywords: Response surface methodology; Ultrasound assisted extraction; *B. wallichiana*; Saponins; Silver nanoparticles.

Introduction

Metals/metal oxides nanoparticles (NPs) possess a wide range of application in medicines, sensing, electronics, water purification, environmental remediation, drug delivery, cosmetics, pharmaceutics, food, energy, automobiles, beverages, textiles, and agriculture [1–3]. Plasmonic silver NPs (AgNPs) with distinctive optical properties play a significant role as potent antimicrobial agents in medical fields, industries including pharmaceuticals, cosmetics, food, textile, and environmental remediation [4, 5]. These have also been reported to display significant anti-inflammatory [6], anti-diabetic [7], antioxidant, and anticancer [8, 9] activities. Therefore, the researchers are focusing to develop biologically active NPs.

NPs can be synthesized using chemical, physical, and biological/green methods. Chemical synthesis requires the use of synthetic reducing/stabilizing agents whereas physical methods apply high-energy input, external temperature and pressure for NPs synthesis, which produces harmful byproducts and are nontoxic to environment. Perhaps, these methods have limitations due to environmental concerns and energy consumption [10]. On the other hand, green synthesis using plants, microbes, and algae, has reduced toxicity, safer, cost effective (requires less energy), reduced global warning, sensible use of natural resources/agricultural waste, and ecofriendly [11]. Because of the high demands for eco-friendly methods, now-a-days researchers are focusing on NPs green synthesis.

Plants containing bioactive phytochemicals as reducing agents have been widely used to synthesize various NPs under different response factors (solvent, temperature, time, and pH). According to previous literature, plant extracts due to stability, efficiency in neutralizing metals, and simplicity in scaling up, have been proved to be superior biological media [12–14]. Phytoconstituents (polyphenols, flavonoids, saponins, terpenoids, and alkaloids) present in the plant extracts act as reducing/stabilizing/capping agents in the production

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of NPs where metal ions are reduced to neutral metallic state [15]. Polyphenols due to hydroxyl (–OH) groups, play important role as potent reducing, antioxidative, anticancer, COX inhibitory/anti-inflammatory, and antimicrobial agents [16]. Plants as rich source of safer and green reducing agents (phytochemicals), the current study was aimed to synthesize AgNPs of various optimized PPF of *B. wallichiana* and *B. sempervirens.*

B. wallichiana (Buxaceae family), a rich source of saponins, alkaloids, carbohydrates, polyphenols, flavonoids, terpenoids, steroids, and glycosides, has been used as diuretic, diaphoretic, purgative, sedative, hair growth, urinary infections, amoebic dysentery, chronic skin issues, and chronic constipation [17–19]. *B. sempervirens* containing steroidal alkaloids, tannins, and saponins, flavones, wax, resin, lignin, and minerals (potassium, magnesium, phosphorus, iron, and silicon), is used as to cure rheumatism, epilepsy, malaria, intestinal problems, toothache, hemorrhoids, urinary tract infections, bronchitis, and several skin complications [20]. It has been traditionally used in lowering fever and hair growth [21, 22]. Phytoconstituents isolated from the subject *Buxus* species have been reported to exhibit significant antioxidant, antibacterial and antiprotozoal activities [23, 24].

Plant polyphenols extracted via several extraction techniques have been played a key role in AgNPs synthesis [16, 25–30]. Among the extraction techniques, ultrasound assisted extraction yields efficient bioactive polyphenolics by shortening extraction time at lower temperature, and mechanical and thermal effects [31–33]. For example, the PPF of *Punica granatum* leaves produced AgNPs of 24.0–55.8 nm [16]. Similarly, spherical AgNPs of the sizes 12.0–38.0 nm of significant antioxidative and antibacterial potential using tea polyphenols were synthesized [30]. Several other recent studies such as Anwar et al. (2019) [26], Mariychuk et al. (2020) [28], Jamila et al. (2021) [25], Saad et al. (2021) [29], and Malik et al. (2022) [27] reported significantly bioactive AgNPs from polyphenolic compounds. Hence, from the results, the role of polyphenols in AgNPs formation is deduced.

Taking the plants as rich source of bioactive polyphenols, ultrasound assisted extraction as preferred technique for extraction of polyphenols, role of polyphenols in AgNPs synthesis, and lack of nanomaterial study on the subject *Buxus* species, into account, the current study was designed to synthesize AgNPs from RSM optimized PPF of *B. wallichiana* and *B. sempervirens*. The PPF were extracted with solvent system-I, SS-I (70% ethanol and 30% water), solvent system-II, SS-II (50% ethanol and 50% water), and solvent system-III, SS-III (30% ethanol and 70% water) at different time (60, 90 min) and temperature (30 °C, 45 °C, 60 °C). The study further analyzed total phenolic content (TPC) and total flavonoid content (TFC) in various fractions. Furthermore, based on the traditional uses in skin diseases, the antibacterial and antioxidant activities of the PPF and the subject synthesized AgNPs were assessed.

Experimental

Samples collection

B. wallichiana was collected from Tirah, Khyber Pakhtunkhwa (Pakistan) whereas *B. sempervirens* was obtained from Peshawar (Khyber Pakhtunkhwa, Pakistan) (Fig 1). After collection, the samples (leaves) were cleaned from any solid undesirable material, dried in shade at room temperature (25–27°C), and powdered using grinder. The powdered samples were stored in dry zipper plastic bags until analysis.

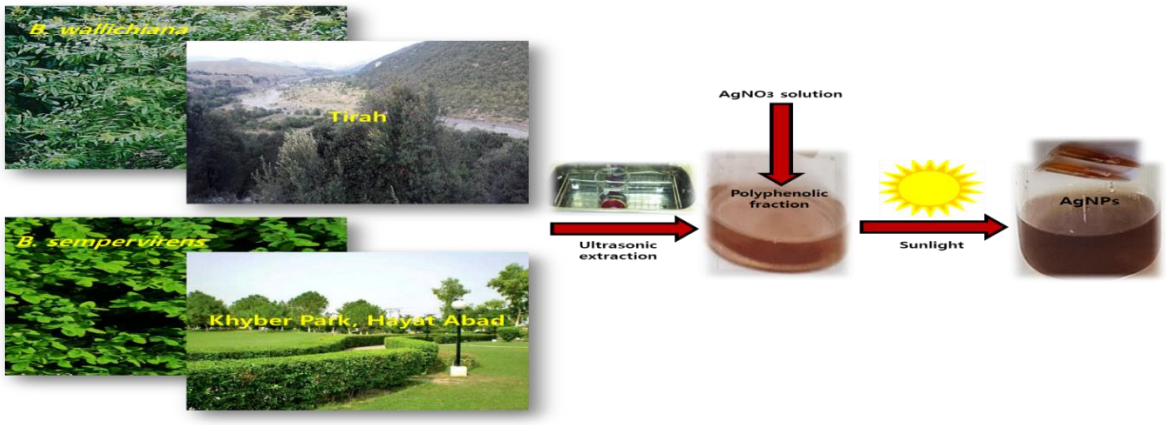


Fig **1:** *B. wallichiana* and *B. sempervirens* (collected from Tirah, Khyber Agency and Hayatabad, Peshawar) of) mediated silver nanoparticles synthesis (under sunlight) using response surface methodology (RSM) optimized ultrasound assisted PPF.

Reagents, chemicals, and instrumentation

Chemicals including ethanol, deionized water, Folin-Ciocalteu's reagent, aluminium chloride (99.9% pure), sodium nitrite (99.9% pure), gallic acid (99% pure), quercetin (99.9% pure), $AgNO_3$ (99.9% pure), DPPH (99.9% pure), ABTS (99% pure), Mueller-Hinton agar and broth (MHA and MHB), bacterial Gram-positive and Gram-negative strains, streptomycin (99.9% pure), and vancomycin (99.9% pure), were purchased from Merck (Germany) and Sigma-Aldrich (USA). The purities of the chemicals/reagents were selected as per their suitability to ensure reproducibility, minimize potential interference from impurities, and align with standard practices for nanoparticle synthesis and bioactivity assays. PPF from *B. wallichiana* and *B. sempervirens* were extracted using three different solvent systems (deionized water and ethanol in different volumes) by an ultrasonic bath (China). For quantification of TPC and TFC, confirmation of AgNPs synthesis, and antioxidant activity evaluation, a UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan) was used. For the identification of functional entities of the constituents present in the extracts and that involved in AgNPs synthesis, an FT-IR spectrometer was used. Sizes and morphology of the synthesized AgNPs were determined by FE-SEM (S-4800, Hitachi, Japan) and TEM (Phillips CM12, Netherlands). For SEM analysis, the operating conditions were set as accelerating voltage (15 kV), magnification (x2.0k), working distance (7.7 mm), and high lens mode. Also, For TEM analysis, the samples were stained using uranyl acetate stained samples placed on copper grid were analyzed by TEM at 120 kV.

Optimization of extraction conditions by RSM (Experimental design)

The current study proposes extraction of TPC and TFC of *B. wallichiana* and *B. sempervirens* optimized by RSM. RSM is an advanced statistical design of experiments optimizing the response by exploring the relationship of several independent variables to the response variables, and reduce the cost and time for the proposed research work. To optimize extraction, four independent and three dependent variables were considered. The independent variables were time (60, 90 min), temperature (30 °C, 45 °C, 60 °C), ethanol (70%, 50%, 30%), and water. The response variables included TPC (mg standard equivalent/g sample), TFC (mg standard equivalent/g sample), and antioxidant activity (mg/mL). From this, three different solvent systems (ethanol:water) in ratios [70:30 (solvent system-I, SS-I), 50:50 (solvent system-II, SS-II), and 30:70 (solvent system-III, SS-III)] at time 60 and 90 min and temperature (30 °C, 45 °C, and 60 °C) were used to extract PPF of *Buxus* species.

Ultrasound assisted extraction

Considering the optimized conditions from RSM, approximately 5.0 g of sample per 50 mL of the solvent systems (I to III) at time 60 and 90 min and temperature 30 °C, 45 °C, and 60 °C were extracted via ultrasound assisted procedure in a temperature controlled ultrasonic cleaner/bath. From this ultrasound assisted RSM optimized extraction, 18 different polyphenolic rich fractions were obtained. The fractions were filtered, and stored for further analysis of TPC and TFC, AgNPs synthesis, and evaluation of antioxidant and antibacterial activities.

Synthesis of AgNPs

Green synthesis of AgNPs was performed using RSM optimized hydroalcoholic polyphenol fractions of *B. wallichiana* and *B. sempervirens*. Hydroalcoholic extracts (18 fractions) were mixed with $AgNO_3$ salt solution in 1:5 ratio, and reacted under sunlight at different intervals (0 min to 180 min). Synthesis of AgNPs was represented by the visual change of appearance of dark brown colour, which indicated the reduction of Ag^+ ions to Ag^0 metal, and hence, AgNPs synthesis. Further, the AgNPs were confirmed by appearance of SPR band in the region of 400-440 nm. The AgNPs solutions were centrifuged, vacuum dried, and stored for further analysis and characterization.

Quantification of TPC and TFC

A slightly modified Folin-Ciocalteu's assays of Jamila et al. (2013) [34] has been adopted to quantify TPC. Briefly, mixtures of 1.0 mL of PPF (1.0 mg/mL), 5.0 mL Folin-Ciocalteu's reagent (10%), and 4.0 mL of Na_2CO_3 (7.5%) were incubated at room temperature (25 to 28 °C) for 2 h in dark. After incubation, absorbances of the mixture samples were measured at 765 nm. A calibration curve was established with different concentrations (0.01 to 0.5 mg/mL) of gallic acid (GA) as a standard. The TPC was reported as mg gallic acid equivalent per gram (mg GAE/g) extract.

For TFC quantification, a slightly modified aluminium chloride colorimetric assay (Jamila et al. 2013) [34] was adopted. Briefly, 0.5 mL samples (PPF), 6.1 mL distilled water, 0.3 mL of 5% $NaNO_2$, 0.6 mL of 10% $AlCl_3$, and 2.0 mL of 1.0 M NaOH

solutions were reacted for 6 min and then measured the reaction mixtures at 725 nm. A calibration curve was established for standard (quercetin) by plotting absorbance versus concentration ranges (0.01 mg to 0.5 mg/mL). The results of TFC were expressed as mg quercetin equivalent per gram (mg QE/g) of extract.

Antibacterial activity

Antibacterial activity of the optimized PPF and AgNPs was evaluated using established protocol of Muller-Hinton agar/broth well as well as disc diffusion (DD) methods [35]. The tested bacterial strains included *S. aureus*, *B. subtilis*, *P. aeruginosa*, and *E. coli*. Briefly, in DD assay, MHA while spreading on the media surface, was inoculated with uniform bacterial suspension. The dried filter discs (6.0 mm) impregnated with 0.04 mL solution of extracts, PPF, and AgNPs (2.0 mg/mL), and standards (streptomycin and vancomycin) were incubated overnight (24 h) by placing on the growth agar surface at 37 °C. The diameter of the inhibition /clear zone around filter discs was measured in mm.

Antiradical/antioxidant activity

Antiradical potential of the PPF and AgNPs were evaluated using DPPH and ABTS assays by a modified procedure of Jamila et al. (2021) [25]. In brief, 1.0 mL of samples in triplicate, and 2.0 mL of DPPH solution (1.0 mM) were mixed. The reactions mixtures were incubated overnight (dark) at 25 to 28 °C. Then, the mixture samples' absorbance(s) were recorded at 517 nm. In ABTS radical scavenging activity, 1.0 mL ABTS (2.0 mM) and 1.0 mL potassium persulfate (2.0 mM) solutions were mixed, diluted with deionized water (to 1.1±0.02 absorbance at 734 nm), and incubated in dark for 16 h. A 0.5 mL of reaction samples and 2.5 mL of diluted ABTS solution were mixed, and the absorbance(s) (734 nm) of the mixture samples were recorded after 6 min of reaction time. Standard solutions of gallic acid and quercetin were used for comparison. The results were expressed as half-maximal inhibitory concentration (IC_{50}) values in mg/mL, which were calculated by plotting %inhibition against concentration range in GraphPad Prism software.

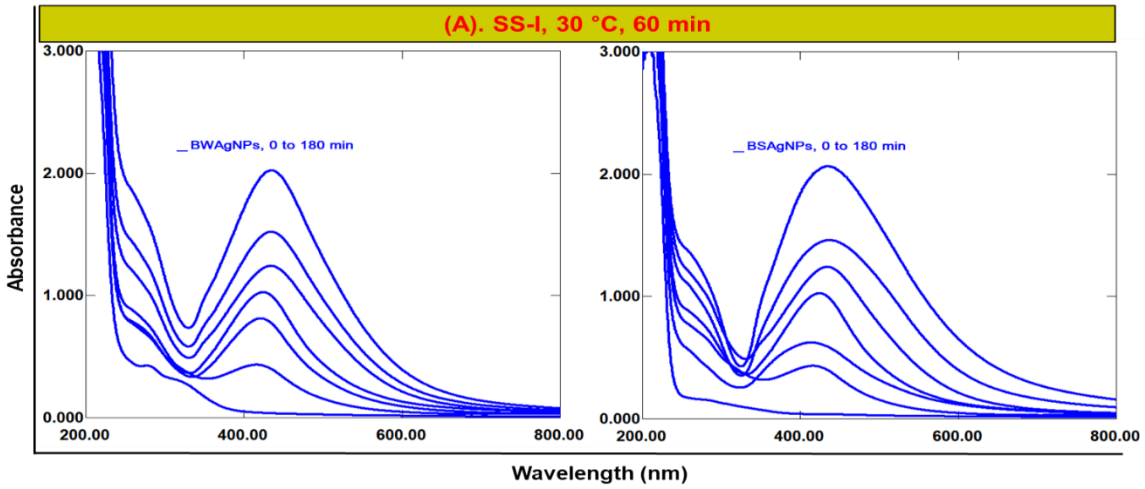
Statistical analysis

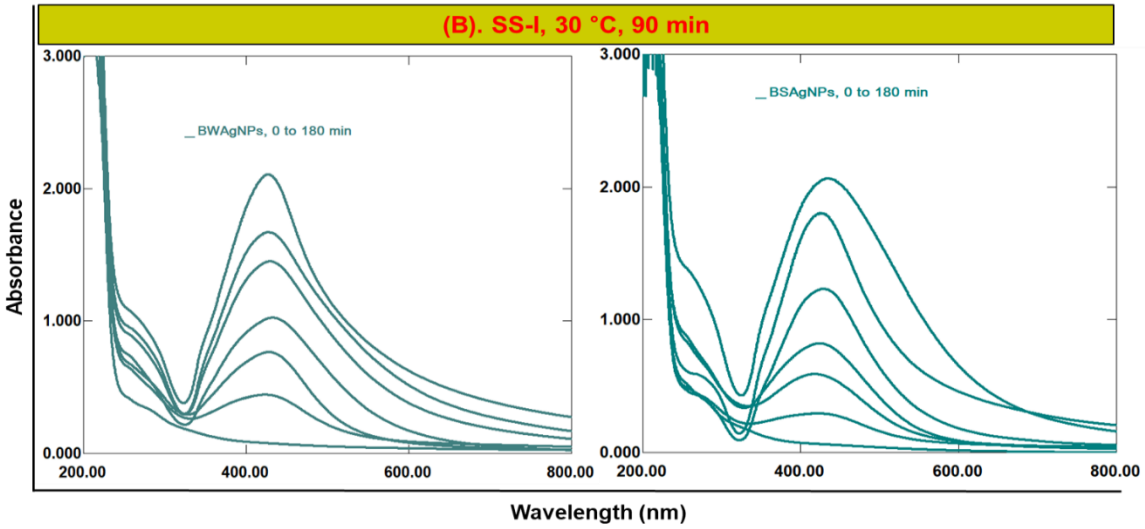
The statistical tests (triplicate) were expressed as mean ± standard deviation. The TPC, TFC, TSC, antibacterial and antioxidant activities of the PPF and AgNPs were quantified and where $P < 0.05$ were considered as significant. All the statistical analysis was performed by an SPSS software, version 20 (IBM, New York, USA). To optimize the method and to select the best parameter for yielding high TPC from *Buxus* species, the statistics of RSM was performed using Design Expert 12 (Stat Ease Inc., Minneapolis, USA).

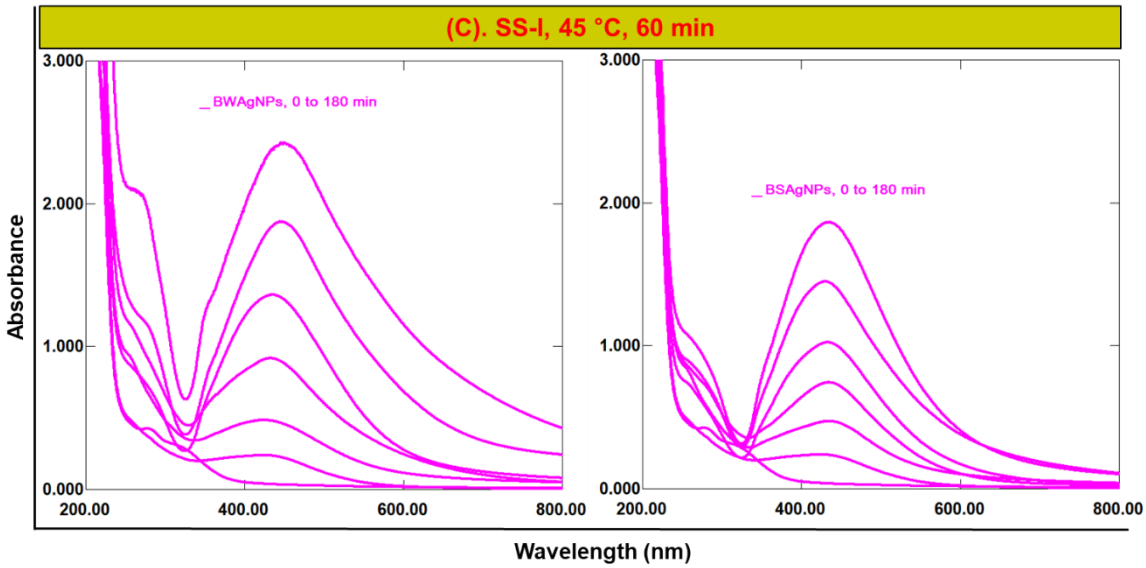
Results and Discussion

Synthesis and characterization of BWAgNPs and BSAgNPs

AgNPs of the *B. wallichiana and B. sempervirens* RSM optimized PPF were synthesized by reacting different proportions (1:1 to 1:15) of the fractions and freshly prepared $AgNO_3$ salt solution. Experimental trials indicated that a proportion of 1:5 reacted under sunlight, yielded a high content of AgNPs as indicated by a sharp intense surface plasmon resonance (SPR) peak at λmax 421 nm (Fig 2). Hence, the subject proportion (1:5) and a parameter of sunlight was selected to synthesize BWAgNPs and BSAgNPs of all the PPF extracted with different solvent systems. The results depicted that the AgNPs synthesized using fractions of SS-I were high in yield (Fig 2) followed by that of SS-II (Fig 3). However, AgNPs biosynthesized via SS-III fractions were insignificant (Fig 4). The confirmation of AgNPs synthesis was monitored by visual colour changes as well as appearance of SPR bands at time intervals of 0 to 180 min (Fig 1). In UV-Vis measurements, AgNPs exhibited strong SPR band in a range of λmax 420-435 nm. Similarly, proceeding to other fractions for AgNPs synthesis, the intensity of broadening of SPR band was observed as the TPC decreased. The comparison of AgNPs production was based on λmax and intensity of SPR band where the best and stable AgNPs exhibit low λmax in 420-435 nm range with sharp/high intensity SPR. Plant polyphenols extracted via several extraction techniques have been played a key role in AgNPs synthesis [16, 25-30]. Among the extraction techniques, ultrasound assisted extraction yields efficient bioactive polyphenolics by shortening extraction time at lower temperature, and mechanical and thermal effects [31-33]. For example, the polyphenolic fractions of *Punica granatum* leaves produced AgNPs of 24.0–55.8 nm [16]. Similarly, spherical AgNPs of the sizes 12.0–38.0 nm of significant antioxidative and antibacterial potential using tea polyphenols were synthesized [30]. Several other recent studies such as Anwar et al. (2019) [26], Mariychuk et al. (2020) [28], Jamila et al. (2021) [25], Saad et al. (2021) [29], and Malik et al. (2022) [27] reported significantly bioactive AgNPs from polyphenolic compounds. Hence, from the results, the role of polyphenols in AgNPs formation is deduced. As shown by various literature studies [11, 25-30], the AgNPs synthesized via RSM optimized PPF were stable, well dispersed, and smaller in size.


(A). SS-I, 30 °C, 60 min
_ BWAgNPs, 0 to 180 min
_ BSAgNPs, 0 to 180 min
Absorbance
Wavelength (nm)


(B). SS-I, 30 °C, 90 min
_ BWAgNPs, 0 to 180 min
_ BSAgNPs, 0 to 180 min
Absorbance
Wavelength (nm)


(C). SS-I, 45 °C, 60 min
_ BWAgNPs, 0 to 180 min
_ BSAgNPs, 0 to 180 min
Absorbance
Wavelength (nm)

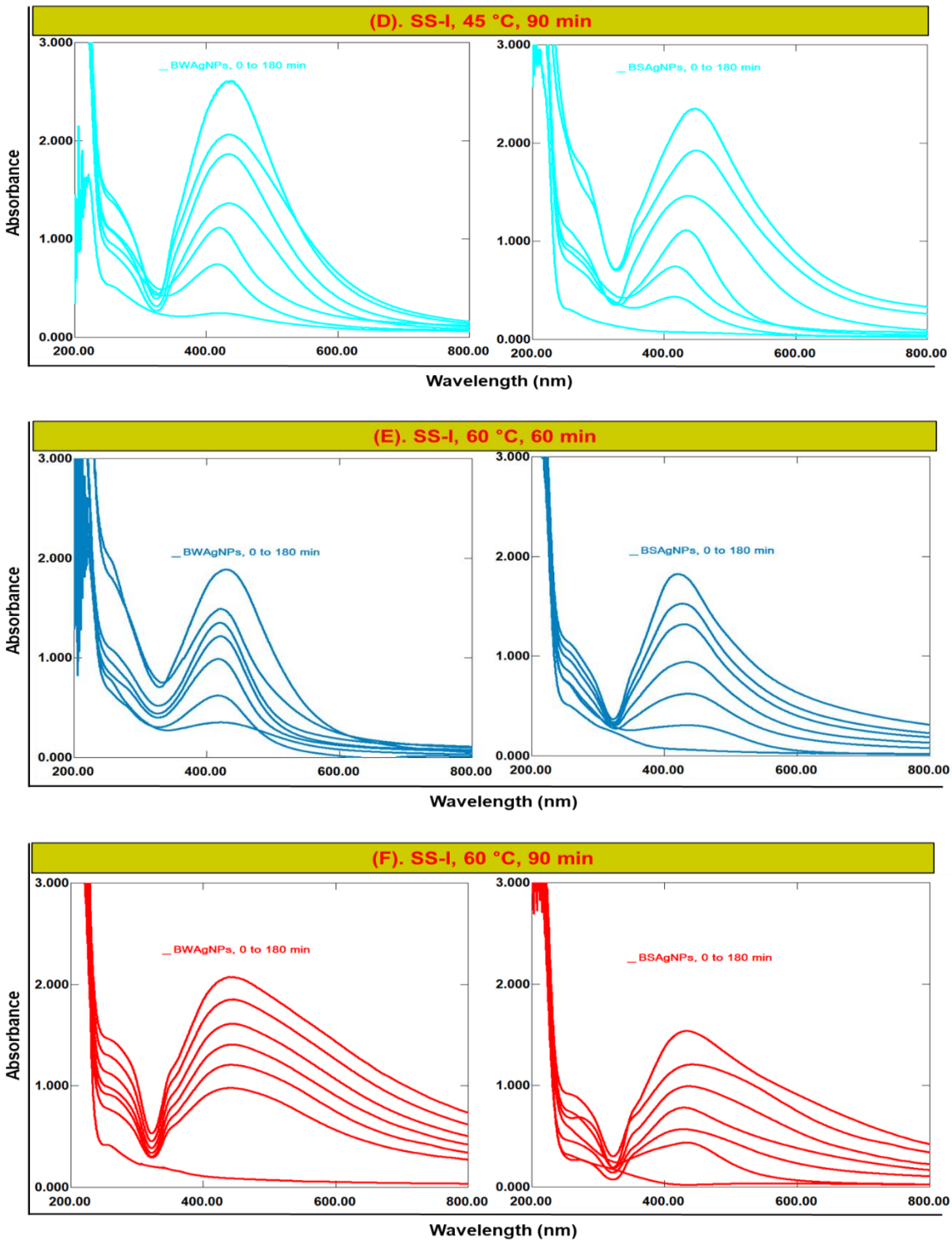
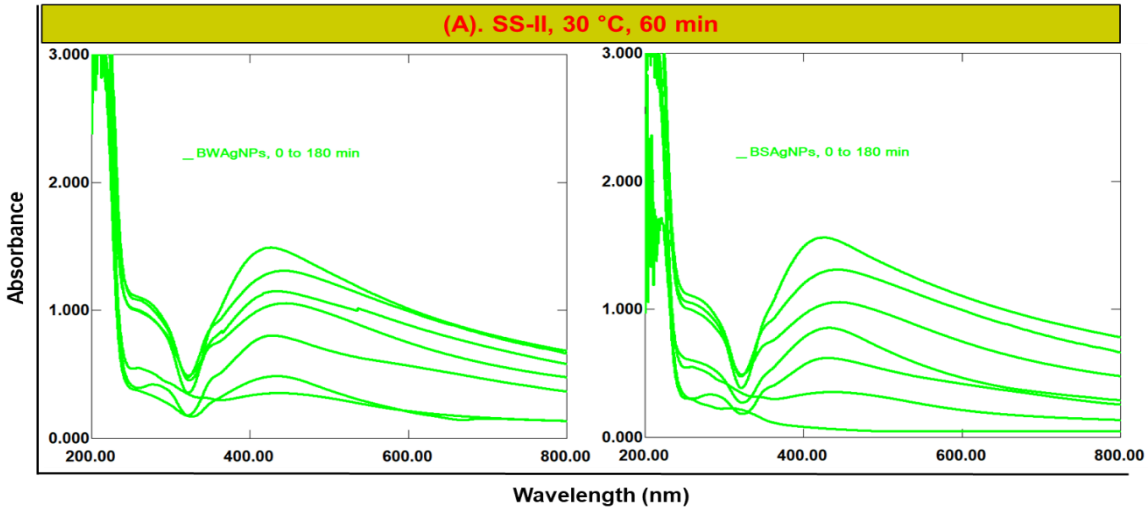
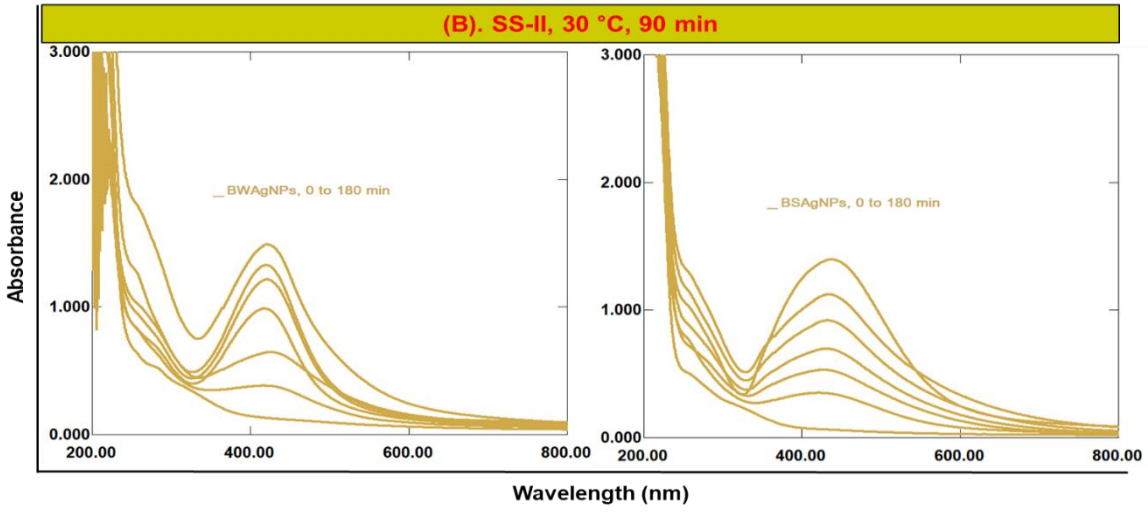
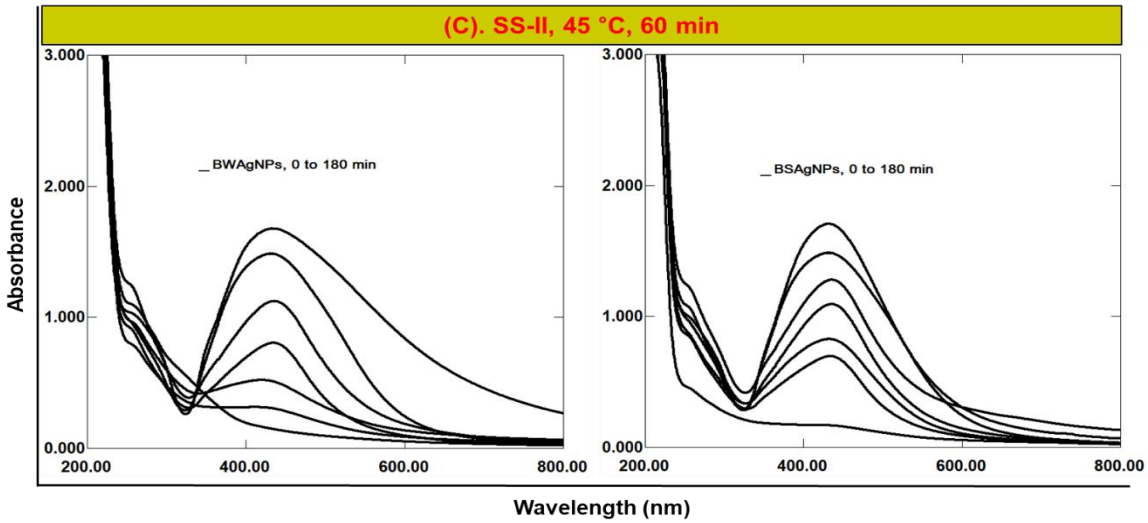


Fig **2:** Successive UV-Visible spectral graphs of *B. wallichiana* silver nanoparticles (BWAgNPs) and *B. sempervirens* silver nanoparticles (BSAgNPs) synthesized in a proportion of 1:5 of PPF extracted with solvent system-I, SS-I (70%ethanol:30%water) at 30 °C, 45°C, 60 °C for 60 and 90 min, and silver nitrate salt solution under sunlight.


(A). SS-II, 30 °C, 60 min
_BWAgNPs, 0 to 180 min
_BSAgNPs, 0 to 180 min
Absorbance
Wavelength (nm)


(B). SS-II, 30 °C, 90 min
_BWAgNPs, 0 to 180 min
_BSAgNPs, 0 to 180 min
Absorbance
Wavelength (nm)


(C). SS-II, 45 °C, 60 min
_BWAgNPs, 0 to 180 min
_BSAgNPs, 0 to 180 min
Absorbance
Wavelength (nm)

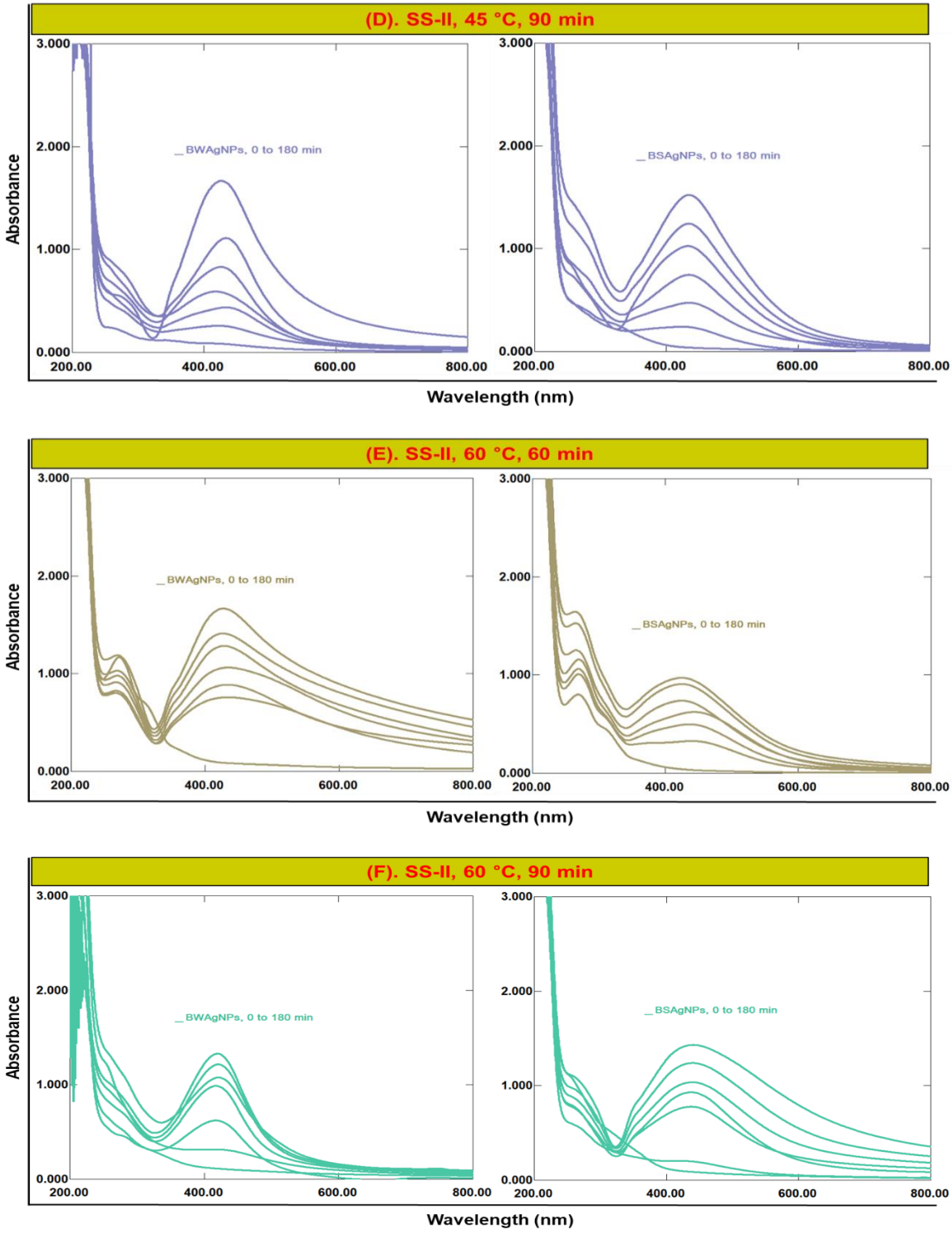
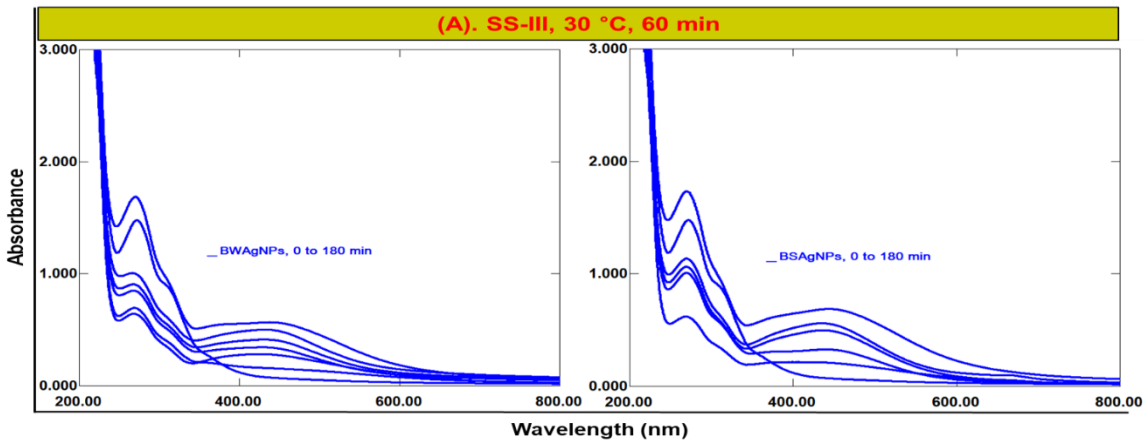
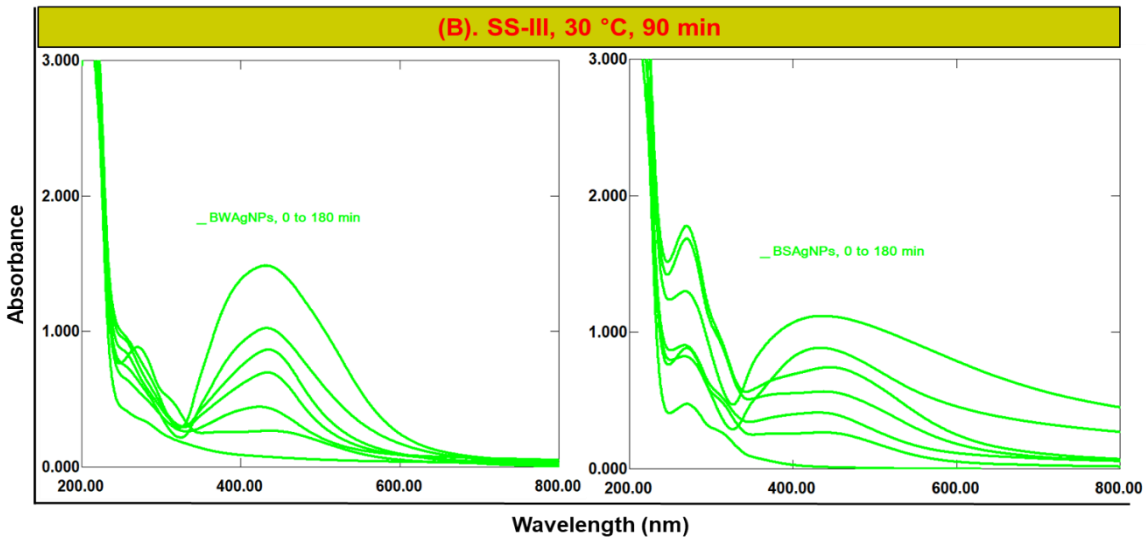
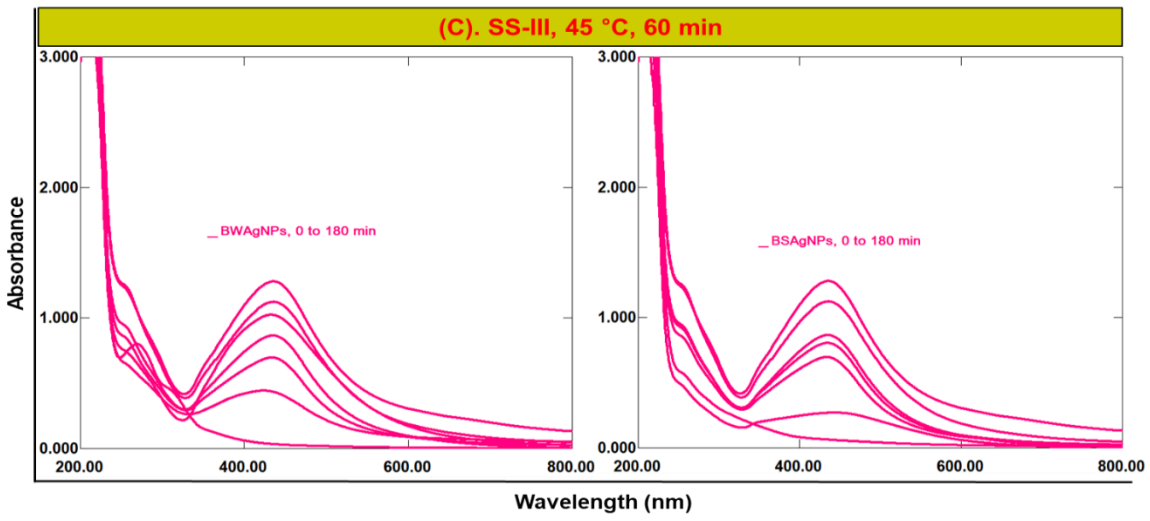


Fig 3. Successive UV-Visible spectral graphs of *B. wallichiana* silver nanoparticles (BWAgNPs) and *B. sempervirens* silver nanoparticles (BSAgNPs) synthesized in a proportion of 1:5 of PPF extracted with solvent system-II, SS-II (50%ethanol:50%water) at 30 °C, 45°C, 60 °C for 60 and 90 min, and silver nitrate salt solution under sunlight.


(A). SS-III, 30 °C, 60 min
Absorbance
3.000
2.000
1.000
0.000
_ BWAgNPs, 0 to 180 min
_ BSAgNPs, 0 to 180 min
200.00
400.00
600.00
800.00
Wavelength (nm)


(B). SS-III, 30 °C, 90 min
Absorbance
3.000
2.000
1.000
0.000
_ BWAgNPs, 0 to 180 min
_ BSAgNPs, 0 to 180 min
200.00
400.00
600.00
800.00
Wavelength (nm)


(C). SS-III, 45 °C, 60 min
Absorbance
3.000
2.000
1.000
0.000
_ BWAgNPs, 0 to 180 min
_ BSAgNPs, 0 to 180 min
200.00
400.00
600.00
800.00
Wavelength (nm)

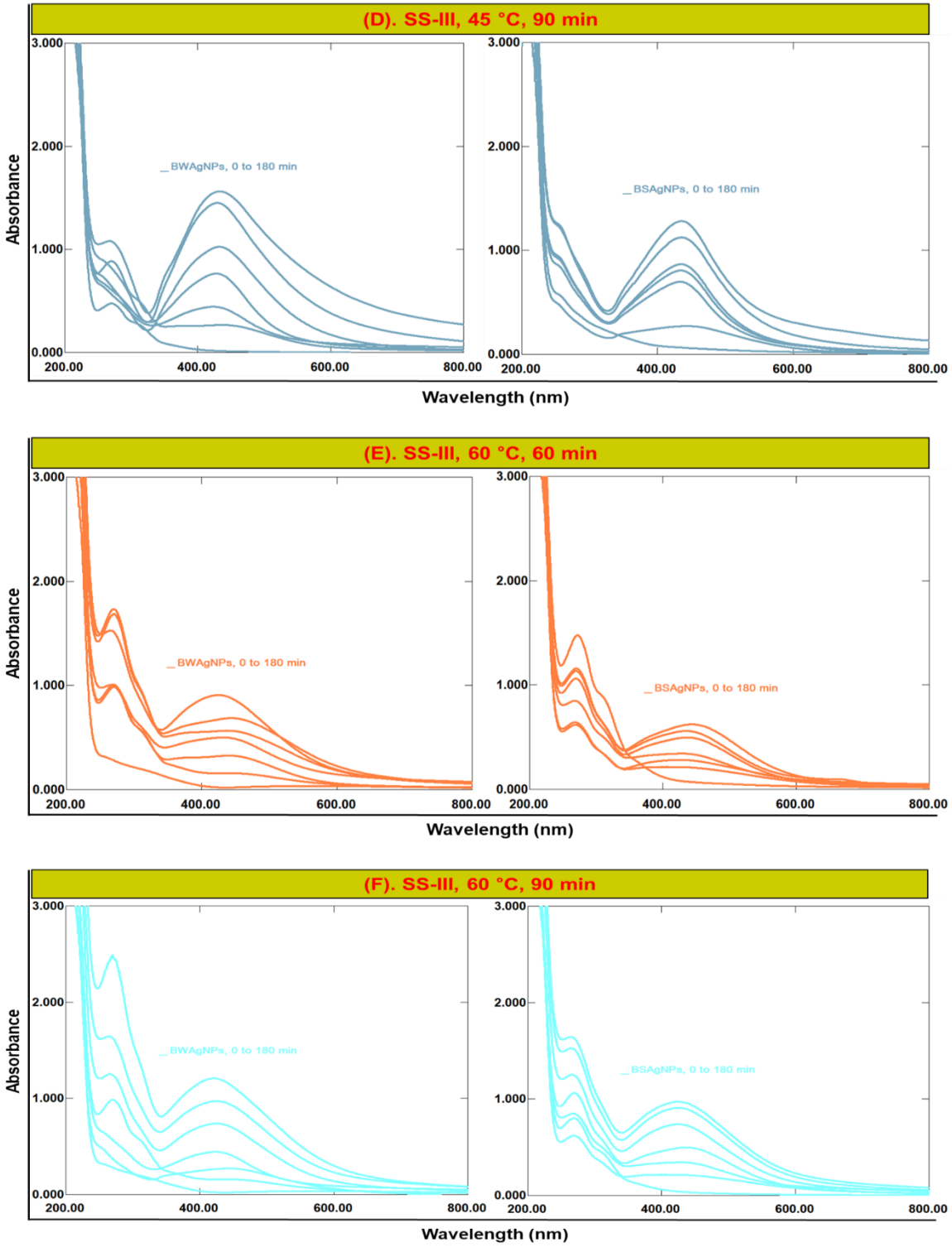
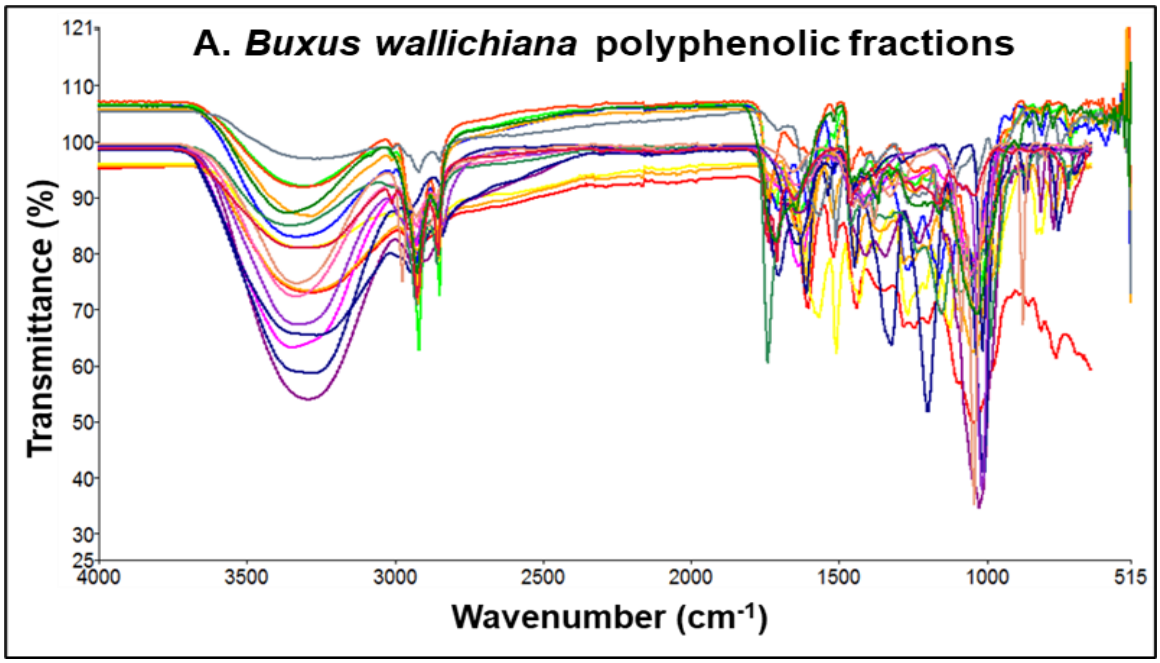
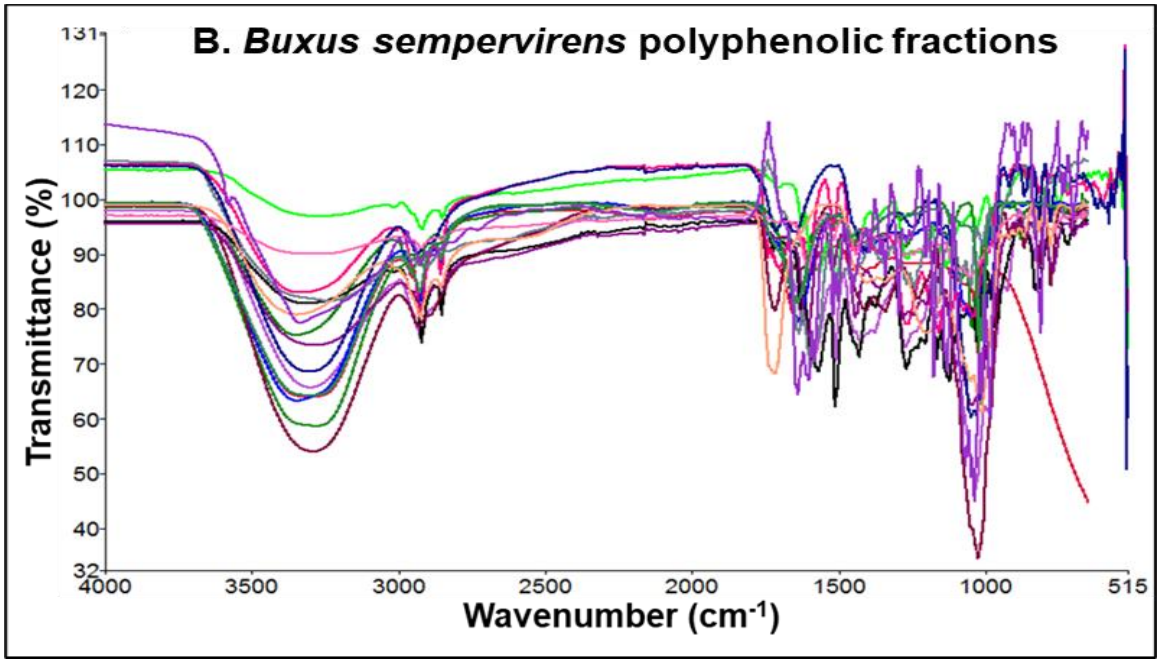


Fig 4: Successive UV-Visible spectral graphs of *B. wallichiana* silver nanoparticles (BWAgNPs) and *B. sempervirens* silver nanoparticles (BSAgNPs) synthesized in a proportion of 1:5 of PPF extracted with solvent system-III, SS-III (30%ethanol:70% water) at 30 °C, 45°C, 60 °C for 60 and 90 min, and silver nitrate salt solution under sunlight

FT-IR analysis provides information on the functional groups of the phytochemicals present in the plant extracts/fractions, which are involved in AgNPs synthesis to reduce or cap the metal ions ($M^{+/++}$) to neutral metal (M^0) [36]. From the FT-IR spectral analysis of the *Buxus* PPF (Fig. 5), it is revealed that the characteristic absorption frequencies exhibit at 3400-3300 cm^{-1}, 2900-2800 cm^{-1}, 1700-1550 cm^{-1}, and 1100-1000 cm^{-1} assigning to hydroxyl (–OH), amine (–NH), (–CH), carbonyl (–CO), and carboxyl (–COOH) groups, which support the presence of phenolic compounds in the subject fractions [37].





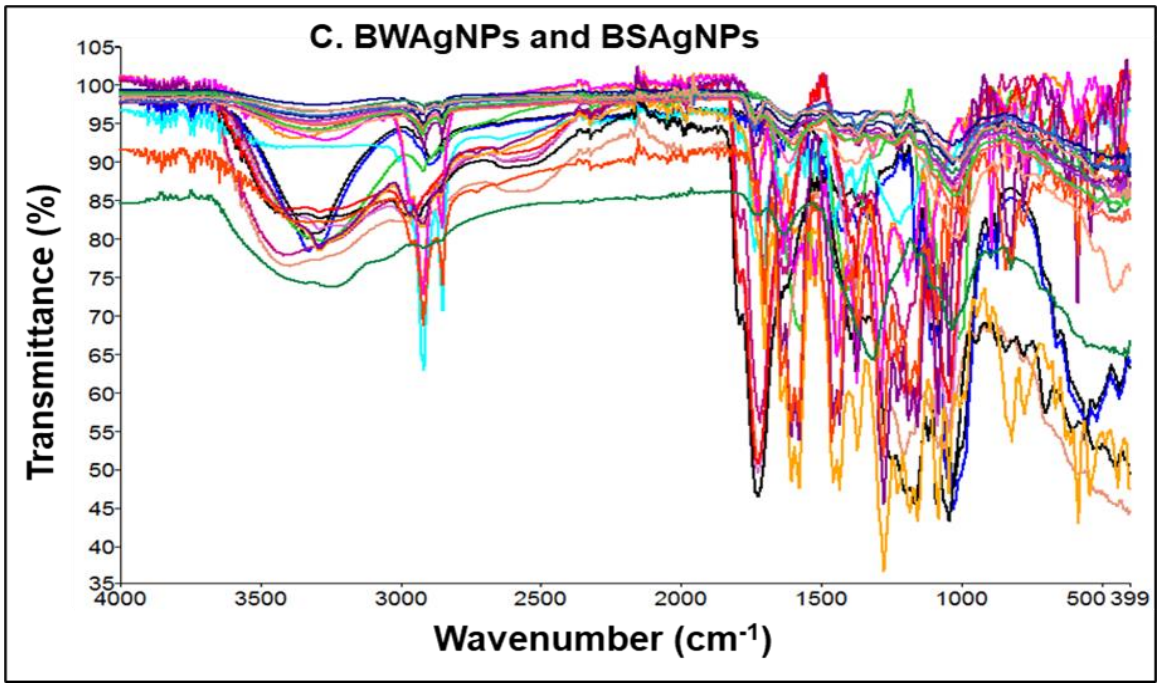


Fig 5. FT-IR spectra of (A) *B. wallichiana* PPF (B) *B. sempervirens* PPF, and (C) BWAgNPs and BSAgNPs.

Moving on to the FT-IR spectral analysis of BWAgNPs and BSAgNPs (Fig 5), it is important to note that there are some variations such as diminished intensities or disappearance in the characteristic FT-IR absorption frequencies of the spectra. This supports the involvement and binding of the characteristic groups (–OH and –COOH) and aromatic structures in AgNPs synthesis [38].

The morphology (size and shape) of the produced AgNPs by SEM and TEM analyses (Fig 6) depicts that the AgNPs mediated with the PPF (SS-I, 45 °C, 90 min) of *B. wallichiana* and *B. sempervirens* exhibit an average size of 7.64 nm (BWAgNPs) and 8.04 nm (BSAgNPs). As from the polyphenolic content analysis, the highest yield of TPC and TFC were obtained with SS-I. This indicates that the AgNPs obtained with the fractions which are rich in TPC and TFS play an important role in the production of small sized AgNPs. Furthermore, TEM images (Fig 6) indicated well-dispersed and spherical AgNPs formation.

Quantification of TPC and TFC

As per the literature, polyphenolics present in *Buxus* species have been reported to be extracted with a limited number of methods. Hence, in this study, *B. wallichiana* and *B. sempervirens* PPF were extracted with 18 ultrasound assisted extraction procedures optimized by RSM. The PPF were evaluated for TPC, TFC, and TSC. According to the results (Table 1), TPC were high in the fractions obtained with SS-I (70% ethanol, 30% water). *B. wallichiana* have shown the highest TPC (244.5±4.70 mg GAE/g) extracted with SS-I at 45 °C and 90 min time. From the results, it can be seen that TPC decreases with the increase in temperature and % of water. Regarding *B. sempervirens* with SS-I, for TPC (199.8±3.99 mg GAE/g), a same order as that of *B. wallichiana* was observed. Time is an important parameter for determining the extraction yield of the TPC. To increase the yield of polyphenolics, an extraction time play significant role in ultrasound assisted extraction. However, too long extraction period decomposes the target phenolic compounds. According to Table 1, the extraction yield of the TPC of *B. wallichiana* and *B. sempervirens* increased with time and reached the highest value (459.5±8.70 mg GAE/g and 401.8±7.99 mg GAE/g, respectively) at 90 min. After this time, the extraction yield went to a downward trend. Therefore, for the subject analysis, the extraction time was set at 90 min. Regarding temperature, the higher temperature leads to higher extraction yield, however, it may also negatively affect the extraction because with increasing the temperature, the heat-sensitive compounds such as polyphenols decompose. Therefore, in the current study, three extraction temperatures; 30 °C, 45 °C, and 60 °C were selected where a high yield of TPC was obtained at 45 °C.

Table-1: Total phenolic content (TPC) and flavonoids content (TFC) in RSM optimized ultrasound assisted PPF of *B. wallichiana* and *B. sempervirens*

Solvent system	Temperature (°C)	Time (min)	TPC (mg GAE/g extract)		TFC (mg QE/g extract)	
			B. wallichiana	*B. sempervirens*	*B. wallichiana*	*B. sempervirens*
I	30	60	173.7±2.06^{l}	171.2±2.74^{l}	91.2±3.70^{g}	98.6±3.04^{g}
		90	215.1±2.13^{m}	209.9±3.07^{m}	107.8±2.93^{j}	120.8±4.81^{i}
	45	60	297.2±2.59^{n}	268.7±2.04^{n}	159.6±4.99^{k}	163.8±3.59^{j}
		90	459.5±8.70^{o}	401.8±7.99^{o}	182.7±5.11^{l}	203.7±4.92^{k}
	60	60	155.8±3.89^{j}	139.6±4.01^{h}	81.7±2.18^{e}	100.3±3.36^{h}
		90	167.3±4.02^{k}	151.5±3.19^{j}	101.6±3.69^{i}	119.5±2.06^{i}
II	30	60	130.1±3.03^{g}	127.7±2.01^{f}	82.3±2.11^{e}	89.0±2.72^{e}
		90	139.0±2.11^{h}	140.3±3.14^{h}	91.8±2.94^{g}	97.6±4.72^{g}
	45	60	144.8±3.73^{i}	145.5±2.50^{i}	100.6±3.01^{i}	101.3±4.17^{h}
		90	157.3±3.60^{j}	159.3±2.86^{k}	106.7±3.71^{j}	105.8±3.10^{i}
	60	60	121.8±3.59^{f}	110.2±2.18^{e}	91.8±2.83^{g}	92.3±3.17^{f}
		90	132.7±3.77^{g}	133.0±3.93^{g}	97.8±3.60^{h}	99.5±2.78^{g}
III	30	60	105.2±2.01^{b}	89.8±3.01^{a}	73.9±2.15^{c}	76.0±1.73^{c}
		90	118.3±3.40^{e}	99.4±2.10^{c}	78.5±3.11^{d}	82.7±3.03^{d}
	45	60	111.4±2.99^{c}	101.7±2.00^{c}	88.3±2.84^{f}	89.0±2.04^{e}
		90	121.8±3.81^{f}	105.5±2.94^{d}	92.2±3.01	96.8±4.05g
	60	60	100.8±3.09^{a}	87.1±2.49^{a}	59.8±4.92^{a}	65.9±4.03^{a}
		90	114.2±3.91^{d}	96.9±3.18^{b}	67.1±4.85^{b}	73.8±4.00^{b}

Values are mean ± standard deviations of three measurements

The superscript letters in a column represent significantly different values ($p < 0.05$) by Tukey's and Duncan's multiple range tests

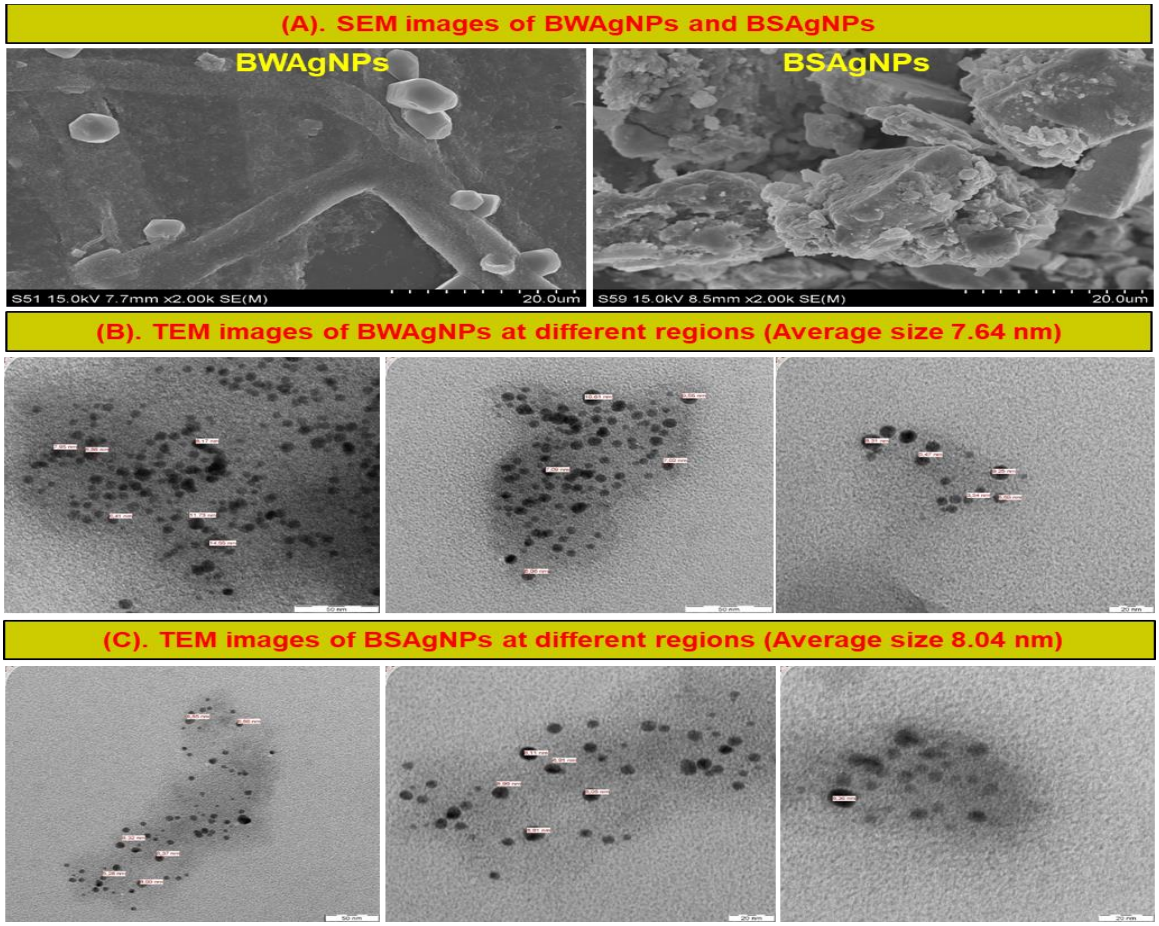


Fig **6:** (A) Scanning electron microscopy, SEM, and (B & C) transmission electron microscopy, TEM images of *B. wallichiana* and *B. sempervirens* silver nanoparticles (BWAgNPs and BSAgNPs) synthesized in a 1:5 proportion of polyphenolic fraction extracted with solvent system-I, SS-I (70%ethanol:30%water) at 45 °C for 90 min, and silver nitrate salt solution under sunlight.

In present study, similar to the profile of TPC, the high content of TFC [182.7±5.11 mg QE/g (*B. wallichiana*) and 203.7±4.92 mg QE/g (*B. sempervirens*)] were shown by the fractions extracted at 45 °C with SS-I for 90 min. The overall TFC (Table 1) for the subject species obtained were ranged as 59.8±4.92 mg QE/g to 182.7±5.11 mg QE/g for *B. wallichiana*, and 65.9±4.03 mg QE/g to 203.7±4.92 mg QE/g for *B. sempervirens.*

For both *B. wallichiana* and *B. sempervirens*, the highest TPC and TFC values were obtained using solvent system I at 45 °C for 90 min, whereas lower values were generally observed with solvent system III across all tested conditions. Overall, moderate extraction temperature (45 °C) combined with longer extraction time favored higher polyphenol and flavonoid yields compared to lower or higher temperatures. ANOVA results confirmed the model's significance, with low *p* values, while the regression coefficient (R^2) indicated strong agreement between the predicted and experimental values obtained during model development.

Antibacterial activity

Antibacterial activity (Fig 7) against selected bacterial (Gram-positive and Gram-negative) strains were investigated for polyphenols rich fractions and the synthesized AgNPs. The detailed inhibition zone values for the ultrasound assisted fractions are listed in Table S1 (supplementary material). Among the polyphenol fractions (Fig 7), the fractions extracted with SS-I at 45 °C for 60 and 90 min had the highest inhibition zones ranging from 10.0 mm to 16.0 mm against the subject bacterial strains. In addition, the AgNPs have more significant antibacterial activity ranging from 9.00 mm to 20.0 mm against *S. aureus*, 9.00 mm to 19.0 mm against *B. subtilis*, 9.00 mm to 14.0 mm for *P. aeruginosa*, and 9.00 mm to 13.0 mm for *E. coli* (Fig 7, Table S2). The antibacterial activity of the synthesized AgNPs may be attributed to multiple interrelated mechanisms, for example, particle size or surface area-to-volume ratio. Smaller AgNPs possess a higher surface area-to-volume ratio, which enhances their contact with bacterial cell membranes disrupting membrane integrity, increase permeability, and cause leakage of intracellular components. Additionally, AgNPs can penetrate bacterial cells, where they may bind to proteins and nucleic acids, interfering with vital cellular processes. The generation of reactive oxygen species (ROS) further contributes to oxidative stress, damaging cellular structures and ultimately leading to cell death. These mechanisms collectively explain the potent antibacterial effects observed in the present study, particularly for AgNPs of smaller size.

Antioxidant activity

An antioxidant activity (DPPH and ABTS) of *Buxus* PPF were analyzed using DPPH and ABTS assay. For the antioxidant activity of ultrasound assisted PPF (Fig 8A & B), the DPPH and ABTS %inhibition was found to be dose-dependent. The IC_{50} values of DPPH and ABTS assays ranged from 0.103±0.01 mg/mL to 0.195±0.12 mg/mL for *B. wallichiana*, and 0.108±0.05 mg/mL to 00.196±0.09 mg/mL for *B. sempervirens*. Comparing with that of the standard gallic acid and quercetin, the IC_{50} values of the standards were 0.019±0.005 mg/mL and 0.010±0.003 mg/mL for DPPH radical, respectively (Table S3, supplementary material). In addition, in ABTS assay, the antioxidant capacity was found of the same trend as seen in DPPH assay. However, compared to DPPH, ABTS was highly reduced by the subject samples and exhibited significantly lower IC_{50} values against ABTS radical. The IC_{50} values of gallic acid and quercetin (standards) in ABTS assay were 0.0087±0.0004 mg/mL and 0.0072±0.0005 mg/mL, respectively.

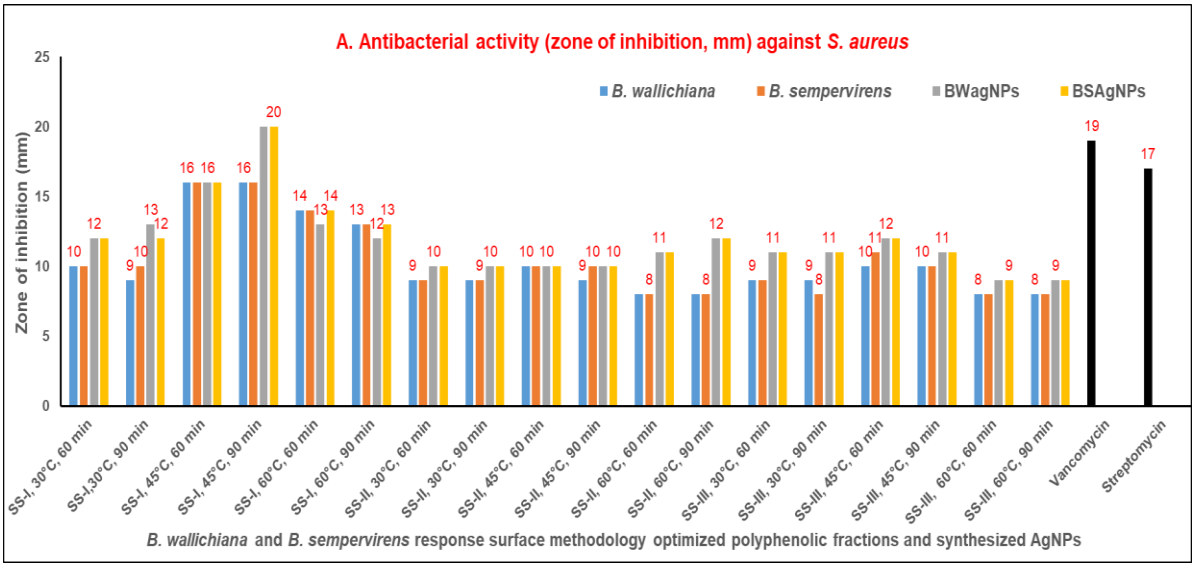
Proceeding the assays with the synthesized AgNPs, as compared to the extracts, the NPs displayed potential antiradical (DPPH and ABTS) activities (Fig 8C & D, Table S4, supplementary material). BWAgNPs and BSAgNPs inhibited DPPH with IC_{50} values of 0.056±0.004 to 0.084±0.006 mg/mL, and 0.064±0.005 mg/mL to 0.083±0.006 mg/mL, respectively. Furthermore, in ABTS assay, the subject AgNPs potently inhibited the radical with IC_{50} values of 0.018±0.002 mg/mL to 0.071±0.002 mg/mL by BWAgNPs, and 0.027±0.002 mg/mL to 0.079±0.003 mg/mL by BSAgNPs.

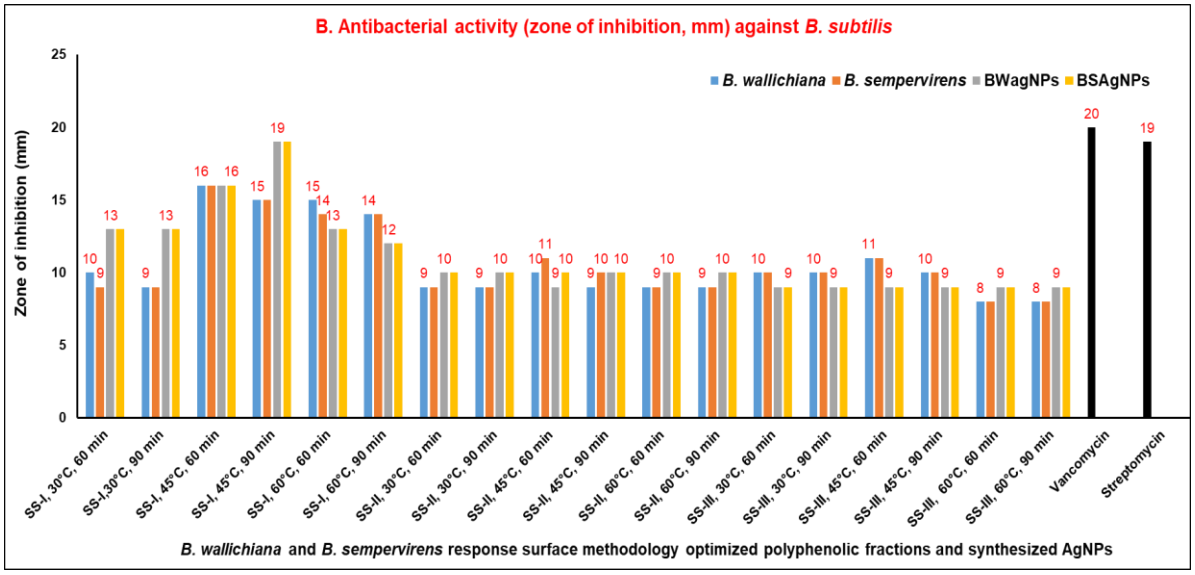
In the study, a positive Pearson correlation coefficient (*r*) of the TPC with DPPH and ABTS radical assays was found having values of 0.80 and 0.82, respectively. Furthermore, the "*r*" values of TFC with antioxidant activities were 0.78 (DPPH) and 0.79 (ABTS). This indicates the strong correlation of TPC and TFC with the DPPH and ABTS radical assays of the antioxidant activities.

To extract polyphenols from plants, several extraction procedures including Soxhlet, reflux, microwave, supercritical fluid, and ultrasound assisted extraction have been used. Among them, compared to the conventional extraction techniques, ultrasonic assisted extraction leads to efficient polyphenolics extraction from plant materials and food products by shortening extraction times at lower temperatures, strong cavitation, and mechanical and thermal effects. In addition, this extraction procedure does not only contribute to increase polyphenols yield, but also leads to potent and significant

biological activities of the extracted polyphenols [31-33]. Flavonoids, a class of phenolics, have received a great attention from scientists and researchers as potent antioxidants, anti-inflammatory and anticancer agents [32, 37, 39, 40]. Investigation of the phenolic and flavonoids content of the two *Buxus* species represented their high content.

Regarding the involvement of polyphenols in AgNPs synthesis, previous investigation reported the best AgNPs synthesis via polyphenols rich fractions [16, 30, 41]. Dark brown color was observed for the mixtures containing fractions of high TPC (obtained with SS-I), which could be associated with the high AgNPs content/synthesis. In our study, a high content AgNPs obtained with the rise in TPC of the reaction mixtures was consistent in UV–Vis spectroscopic analysis. Besides the concentration/volume of reducing agent, time also affected the AgNPs content in which an increase in the peak intensity/absorbance was observed with increasing time intervals from 30 to 180 min. The AgNPs obtained with SS-I fractions (45 °C and 90 min) were the highest in content as represented by the sharp intense SPR band as compared to that of all other fractions. Several previous studies highlighted the role of polyphenols in AgNPs synthesis [11, 25-30].





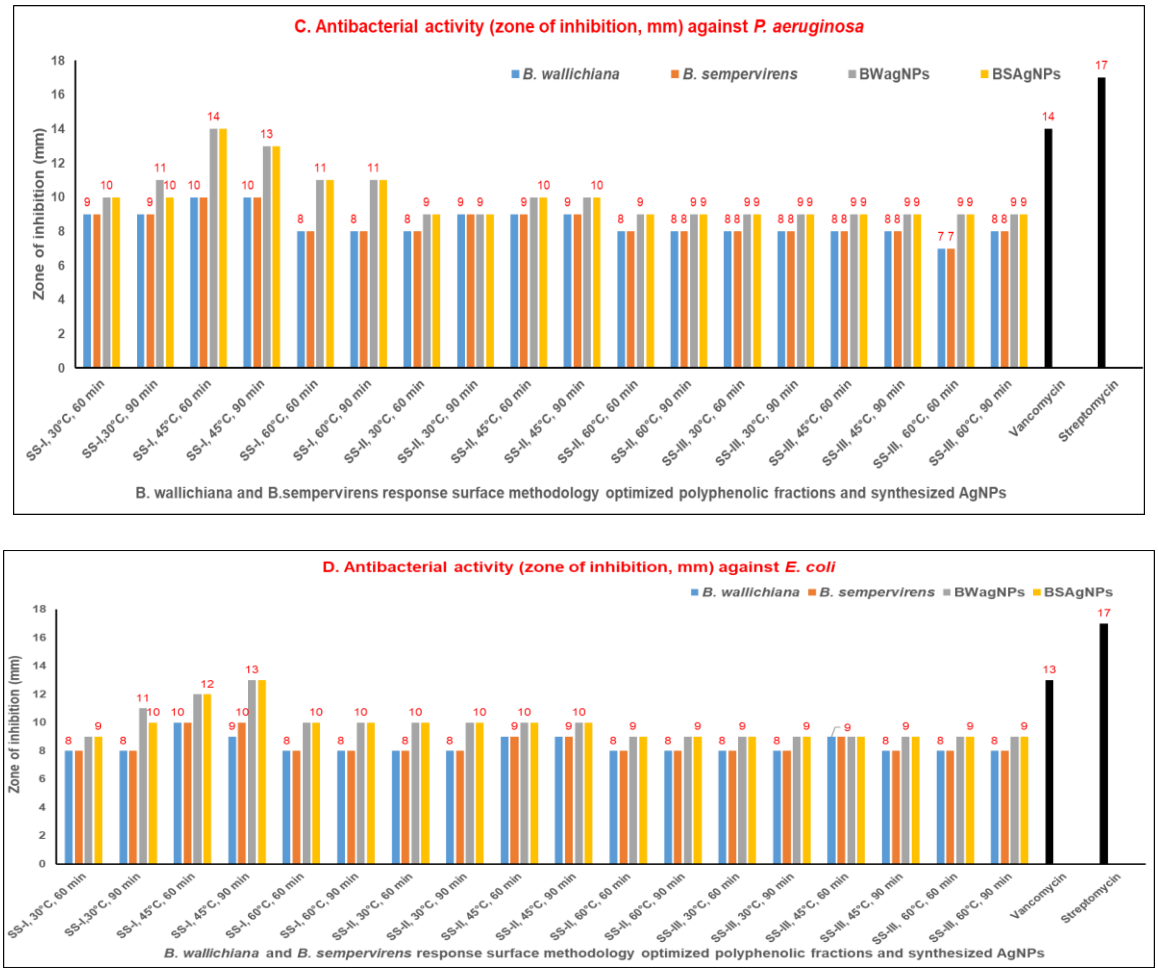
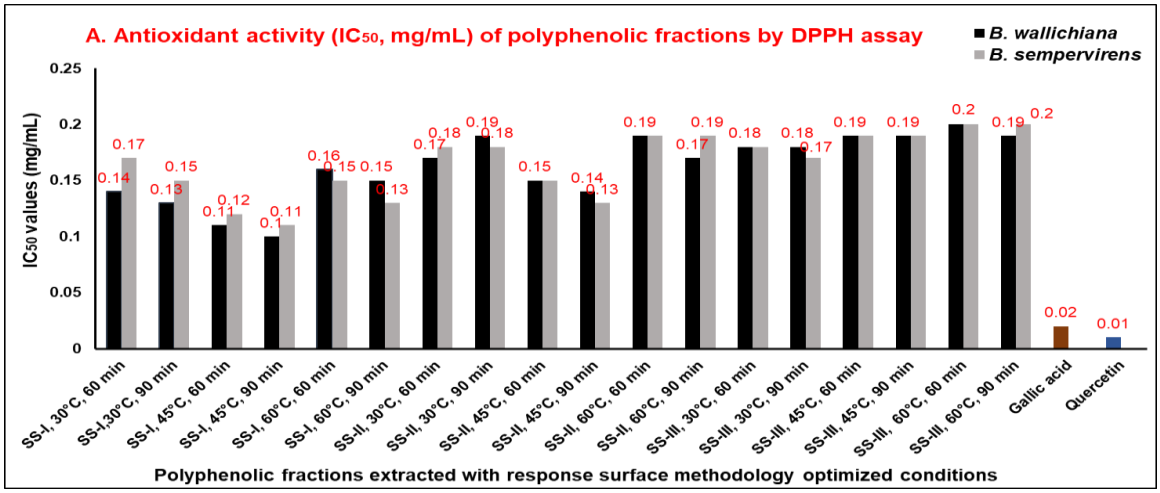
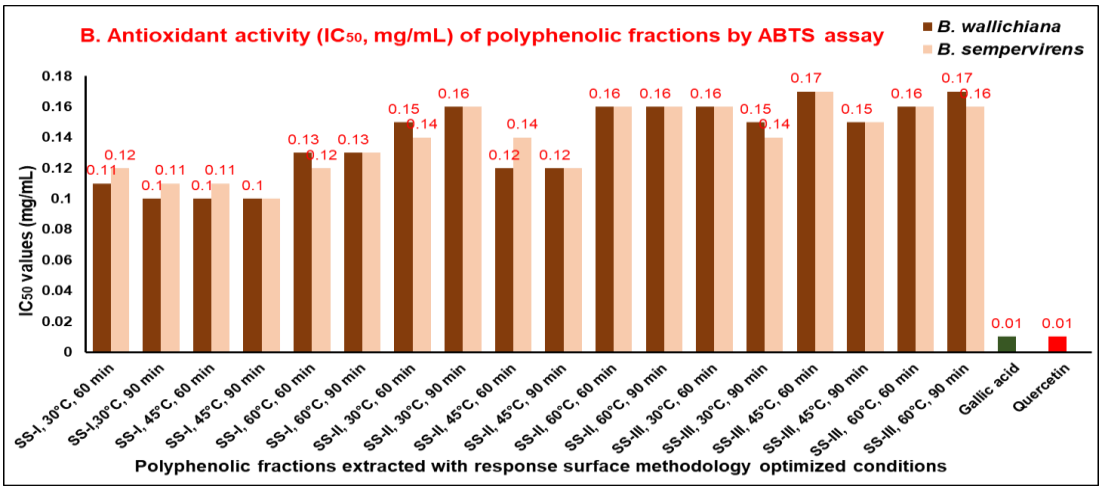
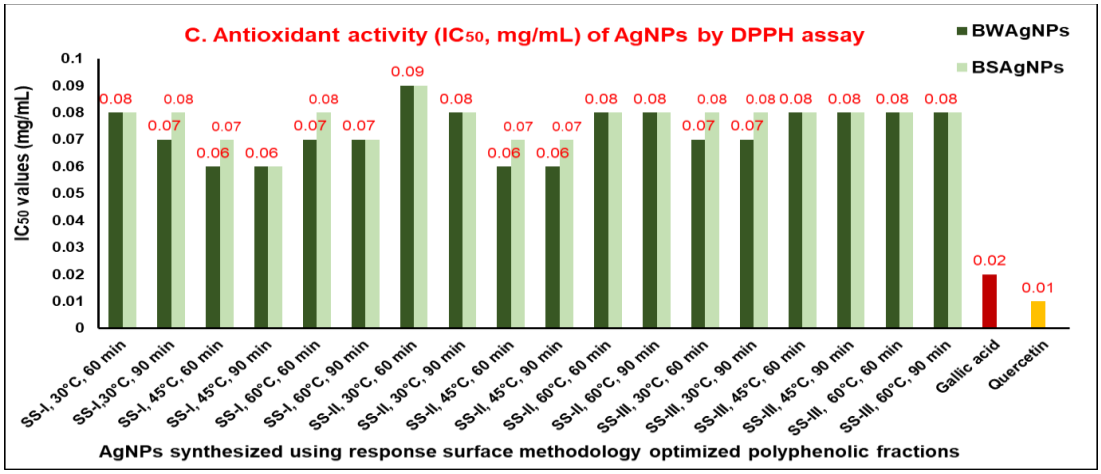


Fig **7:** Antibacterial activity (zone of inhibition, mm) of PPF and the synthesized AgNPs against (A) *S. aureus*, (B) *B. subtilis*, (C) *P. aeruginosa*, and (D) *E. coli.*







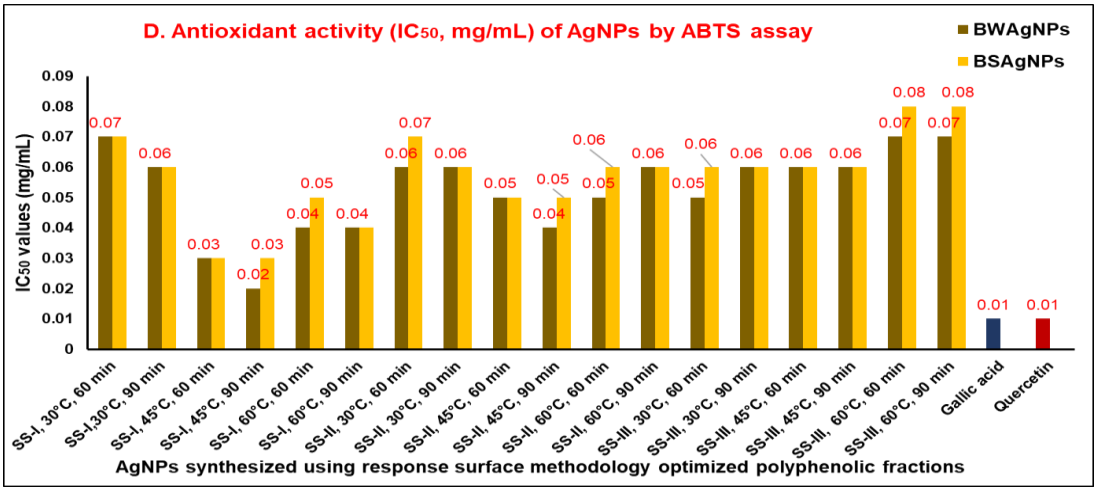


Fig 8: Antioxidant activity (IC_{50} values, mg/mL) of (A) PPF by DPPH assay, (B) PPF by ABTS assay, (C) AgNPs by DPPH assay, and (D) ABTS assay.

Parameters such as temperature, time and solvent systems greatly affected the TPC and TFC and the stability of AgNPs. For example, the SS-I, which contained ethanol and water in 70:30 at 45 °C and 90 min, resulted in the high yield of TPC, TFC, and ultimately AgNPs exhibiting the lowest λmax and the highest intensity. The AgNPs produced under the subject conditions were stable up to 20 days. Applying temperature higher than 45 °C caused a bathochromic shift and broadening of SPR band, which indicates the agglomeration and destabilization of AgNPs at high temperature [16, 28, 29].

FTIR spectroscopy is a reliable and sensitive method for the detection of composition of phytochemicals/class of natural products. Therefore, in this study, the possible functional groups in different extracts and PPF were determined using peak values of IR radiation. This FT-IR spectroscopic analysis confirmed the absorption frequencies for O–H/N–H, C–H, C–O, and C=C stretching and bending vibrations of phenolic compounds [36, 37].

Regarding the antibacterial activity of PPF, to the best of authors' knowledge, there is no antibacterial study on an optimized ultrasonic PPF of the subject *Buxus* species and the corresponding AgNPs. From the results, it was found that most of the PPF were either resistant or had low bacterial inhibitory potential against all the tested microorganisms. The study shows that the optimized fractions and the mediated AgNPs exhibited appreciable antibacterial properties that highly inhibit the growth of Gram-positive as well as Gram-negative bacterial strains and could serve as a valuable source of potential antibacterial agents.

Plant phenolics play a key role as antioxidant, anticancer, and antimicrobial agents. Antioxidant activity is attributed to the presence of –OH groups, which donate hydrogen, and thus, reduces the free radicals, which ultimately prevents the radical chain reactions. Several spectrophotometric antioxidant assays have been utilized in the analysis of antioxidant potential of foods, plants, and herbs. The antioxidant study conducted on the PPF of *Buxus* species revealed significant activity, which was found strongly correlated to TPC and TFC of the species. Several previous studies determined the close correlation of antioxidant activities with TPC and TFC [40, 42, 43]. The study further indicated that total phenolics are major contributors for antioxidant activity. These results suggested that the strong antioxidant effects observed in these extracts and PPF could be due to the high yield of TPC and TFC.

Conclusions

The RSM optimized ultrasound assisted extraction was applied to obtain PPF from *B. wallichiana and B. sempervirens*. The optimal conditions on which higher yield of PPF obtained were solvent system-I (SS-I) consisting of 70% ethanol and 30% water, 45 °C, and 90 min. The TPC and TFC were high in ultrasound assisted PPF obtained with different solvent systems in order of SS-I > SS-II > SS-III. Solvent system-I produced prominent AgNPs, which exhibited significant antibacterial and antioxidant activities. The zones of bacterial strains inhibition against *Staphylococcus aureus* and *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli* were significant with inhibition zone ranging from 8.00 mm to 20.0 mm. Moreover, PPF and synthesized AgNPs significantly inhibited DPPH and ABTS free radicals (IC_{50} values of 0.02 to 0.09 mg/mL). Hence, the study concludes that PPF of *B. wallichiana and B. sempervirens* and their corresponding AgNPs can potentially be used as a source of natural bacterial inhibitory and antiradical agents.

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