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## Agile, simplified sonication mediated one- pot aqueous extraction and antibacterial assessment of predominant Korean mushrooms

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### Abstract

Solvent based extractions have always held the upper hand when it comes to mushrooms. Assimilating the fact that mushrooms are a part of culinary components cooked in water and not in solvents; solvent based extraction becomes a priority. Effective water based extraction stretches 24h, leaving space for prospective improvising through analytical interference. We have demonstrated the effective downsizing of the extraction time from 24h to 2min via sonication based extraction strategies. Water bath based method could achieved effective extraction at 30 min, while further enhancement was seen through the use of probe sonication approach tot 2 min. The extraction efficiency was tested based on the antibacterial activity of mushroom extracts against two pathogens, *Streptococcus mutans* and *Pseudomonas a eruginosa*. The systematic optimization of the sonication approaches and the comparison of their effectiveness versus the conventional approaches are demonstrated. The bioactive components in the extracts obtained via the different extractions have been characterized using biochemical characterizations as well as GC-MS analysis. The enhanced extraction and potent role of butanoic acid, hexadecanoic acid, octadecanoic acid and 1,2-benzenedicarboxylic acid are confirmed to be behind the success behind the sonication mediated extraction.

**Key words:** extraction; mushrooms; sonication; water extraction; antibacterial

### Introduction

For time immemorial, mushrooms have been extensively known for their nutritive and medicinal roles. Mushrooms form an intergral part of the Asian cuisine, dominating countries such as China, Japan and Korea. With increased knowledge on the positive aspect of mushrooms, more recently there has opened a huge market for mushrooms. In terms of not only nutrition but also their specific aroma or texture, mushrooms

have risen from a delicacy to a routine everyday regular food. The demands are growing on basis of the fact that mushroom encompass a huge diversity of biomolecules established for not only nutritional but exceptional medicinal properties too. Previous studies have reported that mushrooms are rich in proteins, polysaccharides, fibers, and minerals, yet low on lipid and caloric contents [1-3]. Mushrooms are also a good source of natural antibiotics, through the low molecular weight (such as terpenes, steroids, sesquiterpenes ) and high molecular weight compounds[4]. These bioactive compounds have been proved in the past for their anti-inflammatory, antiviral, antidiabetic, immunomodulatory antioxidant, antitumor and antimicrobial properties [4-8].

Researchers have reported that mushrooms extracts from either of the fruiting body or mycelium show a wide range of antimicrobial and antioxidant activities [9-11]. Turkoglu *et al.* 2007 [12], have reported the ethanolic extract of *Laetiporus sulphureus* (Bull.) Murrill showed antioxidant activity which positive correlates with their phenolic and flavonoid contents. Their corresponding antimicrobial activity, whereby they strongly inhibit the growth of Gram positive bacteria but decreased inhibition of Gram negative bacteria is also reported. Numerous organic solvent based mushroom extracts have been trialed and reported for their antimicrobial activity. These include: ethyl acetate and hexane extracts of *Agaricus bisporus* and reports on their antimicrobial activity against *Micrococcus luteus*, *Bacillus subtilis* and *Bacillus cereus* [13]. Further, an aqueous extract of *Cordyceps sinensis* has been demonstrated to inhibit the growth of *Bacillus subtilis* and *Streptococcus epidermidis* [10]. Based

on current status of the literature survey on this research area, it appears that the Shiitake mushroom (*Lentinus edodes*) emerges as the most studied mushroom species, Aida *et al.* 2009 [14], reported a correlation that the shiitake extracts exhibit higher antibacterial activity than the broad spectrum antibiotic, ciprofloxacin. Hirasawa *et al.* 1999 [15], has further demonstrated that the Shiitake extracts have pronounced inhibitory action on varied groups of bacteria including; *Streptococcus* spp., *Actinomyces* spp., *Lactobacillus* spp. and *Pseudomonas aeruginosa*.

According to Kitzberger *et al.* 2007 [9], extraction techniques play a vital role in the preselection of substances or components of interest. Extraction methods thus far employed sequential extraction[13, 15] followed by parallel analysis; some approaches made use of single solvent based extraction, while the extraction time was observed to be inconsistent ranging from 5 to 72 hours [16-18,9,11]. Some approaches have used special equipments to assist extraction: bath sonication [19] and supercritical fluid extraction which is for solute extraction [9]. The advantages of these techniques are that they are more effective and use lesser solvent. It is recognized that high intensity ultrasound can promote mass transfer of different products and processes [20]. Despite the high temperature induction which can lead to formation of free radicals on the cell membranes, ultrasound sonication probes are proposed for plant extraction processes due to its high efficiency, reproducibility and lower solvent consumption [20-22].

Prevalently, classical organic solvents such as ethanol, methanol, chloroform, ethyl

acetate and dichloromethane had been used for extraction of active compounds from mushrooms. Organic solvents are reported to be better solvents when it comes to extraction and several researchers have proved that ethanol was the best extractive solvent that shows maximum antimicrobial activity [18, 23]. However, Bala *et al.* 2011 [19], have reported that ethanolic and water extracts of two Nigerian edible mushrooms revealed similar antimicrobial activity. In addition, the drawback of organic solvents is its toxicity towards bacterial cells, which will affect the observations on the antimicrobial activity. Also, looking at the culinary point of view, people don't cook mushrooms in organic solvents, but water, thus a water based extraction is inevitable for practical purposes. Compared to organic solvents, the universal solvent water is safer and easier to implement. But, a literature sweep reveals that water based extraction of mushrooms for testing their antibacterial property is not much reported nor published.

The present study aims at looking into the antimicrobial activity of five predominantly used Korean mushrooms, which are consumed by a majority of the population as part of their daily diet. These mushrooms are usually consumed as soups or steamed in water mediums, therefore, the extraction process for the extraction of the antimicrobial properties if any, based on water extraction has been worked out in detail. A comparison of the water based extraction against the conventional ethanolic extraction has also been made. The use of an accelerated sonication based extraction methodology based on water bath type sonication and probe type ultrasonication has been established. The antioxidant properties, bioactives like phenols, polysaccharides,

flavonoids, and proteins have been estimated and correlated to the success of the extraction process. The antibacterial properties of these extractions have been tested against *Streptococcus mutans* and *Pseudomonas aeruginosa*. For the first time, we optimize a sonication based procedure for aqueous extraction of antimicrobial compounds from mushrooms in less than 5 min compared to the conventional process which requires more than 24h.

**Materials and Methods**  
**Samples** Fresh fruiting bodies of 5 predominant commercial mushrooms species used in Korean cuisine namely: *Pleurotus ostreatus*, *Agricus bisporus*, *Lentinula edodes*, *Pleurotus eryngii*, *Flammulina velutipes*, were purchased from the local supermarket in Seoul, Korea. These will be referred to by codes M1, M2, M3, M4 and M5, in the order represented above, in the following sections. After collection, each mushroom was freeze-dried and then ground into powder. The samples were stored in sealed plastic bags at room temperature for further use.

#### **Reagents and apparatus**

Folin-Ciocalteu reagent, gallic acid, rutin were obtained from Sigma-Aldrich. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Wako. Acridine orange was purchased from Alfa Aesar. All the other solvents and reagents were of analytical grade purity. Water was collected from a Mili-Q water purification system. Water bath sonication and probe ultrasonication were performed using a Bath Sonicator (JAC-2010, 300W/60 Hz) and Probe Sonicator (Bandelin GM 2200, 200W/20 kHz), individually. The absorbance value was measured using a Shimadzu UV-1700

spectrophotometer. The fluorescence imaging were conducted using a Olympus FluoView™ FV1000 confocal laser scanning microscope (CLSM), OLYMPUS AMERICA INC. Corporate Center Drive, Melville, NY, USA. The bacterial cells were observed using a field emission scanning electron microscopy (JSM-5410LV). For GC analysis, a Shimadzu GCMS-QP2010 SE with Rts-5MS column (Restek, 30 m×0.25 mm, id×0.25 µm film) was used.

## **Extraction Procedures**

### **Ethanol and Water Extraction**

Ethanol is reported to be a promising solvent for conventional extraction, for extraction of total phenolic compounds from mushrooms [24]. Water extraction too is known to contain high amounts of polysaccharides and soluble protein compounds [25]. In order to compare the difference between organic solvent extraction and water extraction, in present study, both extractions were attempted. samples preparation for water and ethanol extraction followed Barros *et al.*, 2006 [16] with brief modifications. Two grams of lyophilized mushroom powder were immersed in 100 ml distilled water (for water extraction) or 70% ethanol (for ethanol extraction) for 24 hours at room temperature with magnetic stirring. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C. The residue was then re-extracted. The supernatants were combined and evaporated on a rotary evaporator at 40°C to approximately 20 ml and made up to 25 ml. The extracts were stored at -20°C until analysis.

### **Sonication based water extraction**

2g of sample in 100 ml of water was sonicated for different sonication time (30 min, 60 min, and 180 min). The resulting suspension was centrifuged and evaporated as described above.

2g of the respective mushroom powder was suspended in 100 ml of water and sonicated for 1 min, 2 min and 5 min respectively with varying sonication frequencies of 10%, 20%, 50% and 100%. The resulting suspension was evaporated following the same protocol mentioned above.

## **Biochemical Characterization**

### **Determination of total phenolic compounds**

Phenolic compounds in the mushroom water extract were analyzed based on the spectrophotometric method described by Loots *et al.*, 2007 [26]. Briefly, 50  $\mu$ L of mushroom extract was mixed with 1150  $\mu$ L of distilled water and then 200  $\mu$ L of dilute Folin-Ciocalteu reagent was added. The mixture was mixed thoroughly and allowed to stand for 7 min at room temperature. Then, 600  $\mu$ L of 20% sodium carbonate aqueous solution was added to this mixture. The reaction was incubated for 60 more minutes and the absorbance was measured at 765 nm against the blank using a spectrophotometer. Different concentrations of gallic acid (0.0325-0.5 mg/ml) were used to construct the calibration curve. The results were expressed as mg of gallic acid equivalents per gram dry weight (mg GAE/ g DW).

### **Determination of total flavonoid compounds**



The total flavonoid content in the different mushroom extractions were estimated by a colorimetric assay according to the method of Chang *et al.*, 2002 [27] with slight modifications. Briefly, 0.5 mL of mushroom extract was mixed with 0.1 mL of 10% aluminum chloride and 4.3 mL of distilled water. The mixture was incubated for 30 min at room temperature. Absorbance was then measured at 415 nm. Rutin was used to obtain the standard curve (15.15-500  $\mu\text{g/mL}$ ). And the final results were expressed as  $\mu\text{g}$  rutin equivalent per gram dry weight ( $\mu\text{g RE/g DW}$ ).

#### **Determination of total polysaccharides**

Total polysaccharides were also measured by a colorimetric assay based on Masuko *et al.*, 2005 [28]. The stock mushroom extract was diluted 100 times using distilled water. 0.5mL of the diluted sample was mixed with 1.5 mL of concentrated sulfuric acid and the mixture was shaken for 30 min at room temperature. After that, 0.3 mL of 5% phenol solution was added and the mixture was heated for 5 min at 90 $^{\circ}\text{C}$  in a water bath, followed by reading the absorption value at 490 nm. The calibration curve was construed using standard D-glucose (7.8125-250  $\mu\text{g/mL}$ ) solution.

#### **Determination of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity**

The free radical scavenging activity of the mushroom extracts was determined following the protocol reported by Chung *et al.*, 2000 [29]. 0.25 mL of each mushroom extract was mixed with 2.5 ml of 0.1mM DPPH and made up to 3 mL with distilled water. The mixture was vortexed and incubate for 80 min at room

temperature in a dark place. The absorbance was read at a wavelength of 517 nm. The mushroom extract was replaced by distilled water served as control. The free radical scavenging activity on DPPH was calculated by the following equation:

$$\text{Free radical scavenging effect\%} = (1 - \text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

### **Evaluation of antibacterial activity**

The antibacterial activity of the mushroom extracts were determined via turbidometric assay using the spectrophotometer and also total viable count method using plate count technique. Two bacteria strains *Streptococcus mutans* 11823 (ATCC 25175) purchased from Korean culture center of microorganisms, Seoul, South Korea and *Pseudomonas aeruginosa* (KACC 11085) were used as test organisms. Before, testing the antibacterial activity, all the mushroom extracts were filtered using 0.20  $\mu\text{m}$  sterilized bacterial filter (Minisart). *S. mutans* was cultured on Bacto™ Brain Heart Infusion broth and BBL™ Brain Heart Infusion Agar and *P. aeruginosa* was cultured on Difco™ Nutrient Broth and Difco™ Nutrient Agar.

5 mL quantities of BHI broth/Nutrient broth were inoculated with *S. mutans* or *P. aeruginosa* respectively and mushroom extracts (concentrations varying from 500, 1000, 2000  $\mu\text{L}$ ) added and incubated in a shaker cum incubator at 35°C overnight. Absorbance was measured at 600 nm using a spectrophotometer. The total viable count (TVC), indicating the number of bacteria that survived after the mushroom extract interaction, was enumerated by plate count method. The TVC was represented as cfu/mL (colony forming unit /mL) [30]. Figure 1 gives the schematic work flow of

this study.

### **Post exposure analysis of bacteria using Confocal laser scanning microscopy (CLSM)**

To 500  $\mu\text{L}$  of the interacted sample, 100 $\mu\text{L}$  of acridine orange (0.1% solution in distilled water) was added and incubated in dark for 10 min. After 10 min, the unbound stain was removed by centrifugation at 5000 rpm for 10 min. This washing was repeated thrice and the acridine orange stained cells were finally suspended in 500 $\mu\text{L}$  sterile distilled water. Then 10 $\mu\text{L}$  of the respective cell suspensions were laid on glass slides and covered with a cover slip and viewed using a fluorescence microscope. Acridine orange (Alfa Aesar, CAS:10127-02-3), a fluorescent dye, differentially stains single stranded RNA and double stranded DNA, fluorescing orange when intercalated with the former and green while complexing with the latter. Thus, the number of orange fluorescing cells depicts the actively metabolizing cells and the green fluorescing cells the dead cells [31].

### **Field emission scanning electron microscopy (FE-SEM)**

FESEM (JEOL, JSM-5410LV) at an accelerating voltage of 2 kV was used to image the damage incurred on the bacterial cells, following interaction with M3 mushroom extract. 500  $\mu\text{L}$  *S. mutans* and *P. aeruginosa* cells were pelleted via low speed centrifugation and the supernatant discarded and the cell pellet washed with sterile water thrice and resuspended in 500  $\mu\text{L}$  of sterile water. 10  $\mu\text{L}$  of each cell pellet

suspension was laid on aluminium tapes (dried overnight at 50°C) and mounted on the stubs using carbon tape. Samples were sputter coated with platinum and viewed using FESEM.

### **Gas Chromatography -Mass Spectrometry analysis (GC-MS) analysis**

The chemical constituents in the water extract (WE), bath sonication extract (WEBS), probe ultrasonication extract (WEPUS) of M3 (which showed the highest antimicrobial activity) was identified by GC-MS technique [32, 33]. Our samples were analyzed after TMS derivatization. 1 mL of water extract samples were mixed with chloroform (1/10, v/v). The organic layer was collected and evaporated to dryness for GC-MS analysis. Another set of samples were prepared in ethanol, followed which it was evaporated to dryness. Then both the two residues were incubated with 100 µL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 900 µL of pyridine at 70 °C for 1h and analyzed using GC-MS.. Injector and detector was set at 275°C and 300 °C, respectively. Helium was used as the carrier. Column temperature was held at 70 °C for 10 min, then increased to 300°C at the rate of 5°C/min and maintained for 20 min. Mass spectra were obtained via electron impact mode. The identification of compounds was done based on comparison of their fragmentation pattern and retention time consulting the NIST library.

## **Results and discussion**

### **Antimicrobial activity of conventional extractions**

Conventional extraction procedures involving ethanol based extraction and water extraction were used for extracting the bioactive components in the five different

mushrooms. Following the 24h extraction procedure, the mushroom extracts were tested for their antibacterial activity against the oral pathogen, *S. mutans*. Four different concentrations, 30 $\mu$ L/mL, 150 $\mu$ L/mL, 300 $\mu$ L/mL and 500 $\mu$ L/mL were tested. Figure 2 gives the results of this study, as observed from the results obtained from the spectrophotometric method, compared to the control, 300  $\mu$ L/mL concentrations of M1, M2, M3, M4 and M5 extracts showed significant antimicrobial activity. It can be observed that in some cases the bacterial counts are even more than the control. This is because the extracts show no inhibitory properties, rather they serve as an additional nutritional source for the bacteria and hence they grow better in here than in the control. With respect to EE, M1, M3 and M5 showed significantly higher antibacterial activity. In case of WE, M2, M3 and M5 yielded significant results. However, compared to EE, WE showed less antibacterial activity. This has also been reported earlier in literature. Jonathan and Fasidi, 2003 [18] have reported the effective extraction of antimicrobial compounds using organic solvents, indicating ethanol to be the most effective amidst others tested. However, the organic solvents by themselves are toxic to bacteria and are known to disrupt bacterial cell membranes [34], therefore solvent based extractions for antibacterial studies would have their own limitations. Also, with respect to the practical application, human consumption of mushrooms does not involve the use of solvents, but water. Hence, in order to increase the practical implication of the study, it is necessary to look for water based extraction methods or increase the efficacy of the water based extractions. This would facilitate the harnessing of the full potential of the antimicrobial bioactive compounds

contained in these edible mushrooms. Hirasawa *et al.*, 1999 [15] have shown that the aqueous extract from *L.edodes* possesses antibacterial activity against *S.mutans*. Our results also indicate that WE of M3 (which is *L. edodes*) showed the highest total phenolics and DPPH scavenging activity and correspondingly the highest antibacterial activity compared to the rest.

The antibacterial activity of the WE's of the five mushroom samples were further investigated in more detail against another gram negative pathogen, *P. aeruginosa*. As observed from Fig. 3A (a & b), M1, M2, M3, M4 and M5 showed significant inhibitory activity on *P. aeruginosa* compared to *S. mutans* (Fig. 3B (a & b)). Also, it was interesting to note that the variation in the trend from the spectrophotometric method to the TVC method was distinctly high. The possible colored mushroom extracts and also the likelihood that the mushroom extracts did not inhibit growth but rather led to cell death via cell interaction based mechanisms, are probable explanations. Also, since the spectrophotometric method is a turbidity based method, it is not a standalone technique for evaluating the live/dead condition of cells (since the dead cells will also add to the turbidity of the assayed suspension). That is the reason why the TVC method was also attempted to validate the results. Via the TVC method, we get more accurate information of the number of living cells following any antibacterial treatment.

Distinct difference in the antibacterial activity of the mushroom extracts on *P. aeruginosa* and *S. mutans* was observed. *P. aeruginosa* as observed from Fig. 3A (b) was susceptible to all five mushroom extracts, while *S. mutans* (Fig.3B (b)) appeared

to be less susceptible to all extracts, showing marginal inhibition even at high concentrations such as 500 $\mu$ L/mL in case of M1, M3 and M5. *P. aeruginosa* is a Gram-negative common clinical pathogen, while *S. mutans* is a Gram-positive pathogen responsible for dental caries. Figure S1 shows total inhibition of *P.aeruginosa* by M1, M3 and M5 extracts at 300  $\mu$ L/mL concentrations (Fig S1A (a)), while *S. mutans* (Fig. S1B (b)) showed only just then one order difference even at 500 $\mu$ L/mL. Thus, all these results indicate that there is well defined specificity in the bactericidal activity exhibited by the mushroom extracts and that most of the mushroom extracts studied did not display broad spectrum antibacterial properties. Of all the extracts, M3 apparently showed trends close to broad spectrum antibacterial activity. Previous studies have demonstrated that aqueous extracts of *L. edodes* (M3 in our study) showed broad spectrum antimicrobial activities [35]. Of the five mushroom studied, M3 is the most reputed and most characterized mushroom with respect to its antibacterial properties. Water extracts of M3 are well reported for their antibacterial activity. Water soluble polysaccharides and lenthionine, a cyclic organosulfur compound are believed to be the compounds driving the antibacterial activity of M3 [15]. Few other researchers have emphasized specifically that bacteria related to oral infectious disease (e.g. *S.mutans*, *Prevotella intermedia*) were more sensitive to shiitake extract [15, 36]. The differences could be ascribed to the differences in the cell wall components of the gram positive and gram negative bacteria and also their relative susceptibility to the bioactive compounds of the various extracts [37-44], which will be detailed later.

### **Water extraction via bath sonication (WEBS)**

Other extraction techniques were sought, to enhance the extraction efficiency of the WE method, since the conventional WE approach took more than 24h and hence was highly time consuming. Two sonication based techniques were employed for furtherance of the WE technique in terms of time as well as efficiency. Earlier studies have demonstrated that the extraction efficiency varied significantly depending on the sonication time and ultrasound amplitude [45]. Therefore, in the current study, WEBS was carried out at different time intervals of 30min, 1h and 3h. The results of the WEBS sonication time optimization are shown in Fig. 4. As can be observed in case of M3 extracts against *P. aeruginosa*, significant extraction of antimicrobial bioactive compounds was achieved even as less as 30 min via WEBS approach. The extraction efficiency is confirmed by the bacterial inhibition results obtained. Thus, WEBS sonication could bring about a 23.5h reduction in the extraction time compared to WE method.

### **Water extraction via probe ultrasonication (WEPUS)**

Probe based sonication was also attempted for reducing the extraction time and its efficiency further. The sonication time and the frequency were varied from 1min, 2min and 5 min with 10%, 20%, 50% and 100% frequency respectively. Fig. 5 gives the results of these optimization studies for the WEPUS method using *P. aeruginosa*. As observed from Fig. 5A the results showed that the extraction efficiency did not



increase proportionally with increasing sonication frequency or time. This is shown by the decreasing antibacterial activity with increasing time and sonication frequency. The plate count method (Fig.5B) also confirms this trend. With respect to *S. mutans* too, a similar trend was confirmed (Fig, S2), where not much improvement in the antimicrobial property was observed with increasing neither frequency nor time. Hence the optimum conditions for ideal extraction were optimized at 20%-2min. The rest of the experiments reported use this optimum WEPUS condition.

Figure 6 represents the demonstration of the optimized WEBS and WEPUS techniques against the conventional WE method using M3 extract. In case of *P. aeruginosa*, both WEBS and WEPUS techniques showed results equivalent to that obtained from the 24h WE method. As observed from Fig. 6A, highly significant antibacterial activity was observed compared to the control ( $10^{10}$  cfu/mL) to 0 cfu/mL in the test samples (absolute inhibition). With respect to time what activity was observed after a 24h extraction process in the WE method and after 30 min in the WEBS method was achieved at 2min by the WEPUS method. Fig. 6B gives the results obtained against *S. mutans*. Although absolute inhibition was not observed in case of *S. mutans*, the WEBS and WEPUS techniques apparently showed an order of magnitude enhanced antibacterial activity compared to the WE method. Thus, the use of sonication based extraction strategies has unequivocally resulted in reducing the 24h process to 30 min and 2 min. The most effective extraction method could be laid in the order of WEPUS> WEBS > WE.

### **Antibacterial examination via imaging techniques**

The live dead condition of the bacterial cells following treatment with the mushroom extracts by WE, WEBS and WEPUS methods was studied using CLSM imaging technique using acridine orange. Acridine orange is a fluorescent dye which is generally known for its nucleic-acid selective fluorescence characteristics. The fluorophore intercalates into doubled-strand DNA to produce green fluorescence. Simultaneously, it also could stack on the phosphate radical of single-strand DNA or RNA to produce orange to red fluorescence due to the electrostatic attraction. The normal cell nucleus will exhibit red color in cytoplasm and green color in DNA, while during the process of cell damage leading to cell death, green color fluorescence will be evident [46]. Control and WE, WEBS and WEPUS treated *P. aeruginosa* are shown in Fig 7A. Compared to the untreated cells (Fig.7A(a)), the WE (Fig.7A(b)), WEBS (Fig.7A(c)) and WEPUS (Fig.7A(d)) showed decreased cell numbers and also increasing green fluorescence indicating the increase in dead bacterial cell number with treatment. Similar trends, however with lesser magnitudes were observed in *S. mutans* too (Fig.7B (a-d)). A more advanced imaging method namely Field Emission Scanning Electron Microscope (FE-SEM), was used to assess the damage at a higher resolution. FE-SEM is known to be an efficient tool providing stereoscopic images which help us to unravel surface features of bacteria and cell damages following treatments more intricately. Previous researchers have illustrated that the mode of action is through: I) interference with the synthesis of the cell wall or proteins, even chromosome replication or through II) modifying the permeability of plasmatic

membranes [46, 47, 48]. In the present study, FE-SEM was used to explore the damage incurred by the mushroom extract on the bacteria cells. It can be observed for Fig. 8A(a) that compared to the *P. aeruginosa* control, evident disruption of cells and cell morphology is evident following WE interaction (Fig 8A(b)). FESEM, further throws more evidence to the enhanced antibacterial property of WEBS and WEPUS approaches, as shown in Fig 8A(b &c), where the total cell shape and integrity have been totally compromised and only cell debris could be evidenced. In case of *S. mutans* the FESEM results clearly show that the cell damage is comparably lesser than that imaged on *P. aeruginosa*. This clearly connects the results obtained earlier (indicating the resistance of *S. mutans* to the mushroom extracts), with those obtained by this imaging method too. Fig 8B(a-d) shows that compared to the control and WE, the WEBS and WEPUS approaches showed significant cell damage, as evidenced by the broken and damaged cells and ruptured cells. Especially with WEPUS interaction (Fig. 8B(d)), extensive damage is evident.

The CLSM and FESEM imaging studies confirm that the cells have been damaged due to the mushroom extracts. The results show that the M3 extracts significant antibacterial property and inhibitory property. The results confirm that WEBS and WEPUS approaches indeed were not only rapid innovations but also exhibited enhanced antimicrobial activity compared to the 24h conventional WE technique.

### **Characterization of bioactive compounds**

In order to understand the reason for the enhanced antibacterial activity of the extracts, it is necessary that we determine the effective extraction of the bioactive compounds. The total phenols, flavonoids, DPPH and polysaccharides which are usually behind antibacterial properties, were assayed. Table 1 clearly reveals that there was a marked increase in the total phenols, flavonoids and DPPH levels in the order WE < WEBS < WEPUS in case of M1, M2, M3, M4 and M5. This is the same order of inhibition observed for the bactericidal activity too. In *L. edodes* (M3) polysaccharides, proteins, terpenoids, phenolics, were reported to have an effect on treating different infections as well as on the inhibition of various bacteria. Table 2 gives the consolidated list of the bioactive compounds determined from WE, WEBS and WEPUS M3 extracts. As observed from the table compared to WE, clearly WEBS and WEPUS extracts showed enhanced extraction efficiency (2-3 fold increase compared to WE) of total phenolics, flavonoids and DPPH and polysaccharides. This is actually the reason for the enhanced bioactivity observed in the WEBS and WEPUS methods. Actually in present analysis, the total phenolics in the aqueous extract were even higher than in ethanol extract for M3. Similar results were reported by another group [49]. Barros *et al*, 2007 [50] reported that the antioxidant and antimicrobial activities of *Laetiporus sulphureus* could be strongly correlated to phenol and flavonoid contents. Other studies indicate that the phenolic constituents of medicinal plants play an important role in determining the antimicrobial characteristics, because they can lead to cell membrane lysis and inhibit protein synthesis as well as interact with proteolytic enzymes [Cowan, 1999 [51]. It appears that the total phenolic

composition determines the antibacterial activity. Other researchers have reported such a positive correlation between total phenolics and antioxidant activity and antimicrobial properties too [52, 53]. Polysaccharides are also said to play a vital role in the antimicrobial properties of mushroom extracts. It is reported that the antimicrobial potential of *Lentinula edode* was due to the presence of some functional compounds such as eritadenine and lentinan, a water soluble polysaccharide. According to Hirasawa *et al.*, 1999 [15], the main component in organic solvent extract from shiitake is lenthionine, a cyclic organosulfur compound, which were identified to inhibit the growth of bacteria [27, 54]. It is thus confirmed that WEPUS approach could lead to effective extraction not only in terms of reduced extraction time, but also in the effective extraction of the bioactive components which accelerate the antimicrobial potential of these mushroom extracts.

#### **GC-MS analysis of bioactive compounds from extracts**

In order to identify the potential antibacterial compounds in M3 (which was found to be the most promising of the five mushroom extracts tested), its extracts were volatilized in two different solvents, namely chloroform and ethanol. The chemical composition obtained from subjecting the ethanol based extracts to GC-MS are shown in Fig. 9 for WE (a-1), WEBS (b-1) and WEPUS(c-1). As clearly evident from the GC-MS spectra, significant increase in peak number and intensity was observed in case of WEBS and WEPUS compared to WE. Table S1 details the comprehensive identification of each of the numbered peaks for WE approach shown in Fig. 9(a-1), Table S2, for WEBS (Fig.9(b-1)) and Table S3 for WEPUS(Fig.9(c-1)). Figure

9(a,b,c-2) gives the GC-MS spectra obtained from chloroform for WE, WEBS and WEPUS respectively and their corresponding peak ids are given in Tables S4, S5 and S6 respectively.

We selectively emphasize on the predominant peaks obtained which have been reported earlier to have relevance to antimicrobial activity and their effective extraction. Previous researchers have showed that xylitol has beneficial properties including antibacterial property with significant inhibitory activity against oral pathogens [55, 56]. This is the reason why nowadays, it had been used in various medicines and for dental products and gums and mints. In Fig. 9(a-1), WE extracts peak 13 and peak 14 were identified to belong to Xylitol, WEBS Fig. 9(b-1), and WEPUS Fig. 9(c-1), extracts show similar Xylitol peaks at peak positions 18 (WEBS) and 11, 15 (WEPUS) respectively. It is interesting to observe that inspite of the 2min extraction time, the same peak intensity of Xylitol is present in WEPUS as in the 24h WE approach. The other peaks pertain to amino acids, nucleotides and organic acids (malic acid) which give the umami flavor. Sugars like mannose, glucose, arabinose, fructose were also present, according to [57], these free sugars contribute to a special flavor of shiitake. Propanonic acid is reported to have more effective antimicrobial activity than lactic acid, because of its acidic property which can cause intracellular acidification and protein denaturation [58]. Propionic acid was identified at peak positions of 2 in WE (Fig.9 (a-1)) and 1 in WEBS (Fig. 9 (b-1)).

In the chloroform samples (Fig. 9(a-2, b-2, c-2) from WE, WEBS and WEPUS, the major compounds expected are fatty acids, including unsaturated fatty acid. Previous

studies have indicated that butanoic acid, 1, 2-benzenedicarboxylic acid, hexadecanoic acid and octadecanoic acids are responsible for potential antimicrobial activity in mushroom extracts [59]. Alves, et al., 2013 [60] have identified 2,4-Dihydroxybenzoic acid and protocatechuic acid as phenolic compounds that show relatively higher antibacterial activity against a vast majority of gram positive and gram negative bacteria. The role of fatty acids in antimicrobial activity and unsaturated fatty acids showing more inhibition against gram positive bacteria have been reported. These researcher explain that the capacity of antibacterial activity depends on the carbon chain length and the concentration [58].

As observed in Fig. 9(a-2) showing the spectra obtained from WE extracts, show low intensity peaks of its non-polar components. Table S4 gives the peak identifications of the WE extracts in chloroform. Peak 1 in Fig. 9(a-2), peak 2 in Fig. 9(b-2) and peak 1 in Fig. 9(c-2) correspond to butanoic acid. Peak 3 & 6 (WE) and peak 6 & 10 (WEBS) and 2, 7 (WEPUS) in Fig 9(a, b, c -2) are hexadecanoic acid. While Peak 4 & 7 (WE) and peak 7 & 8 (WEBS) and peaks 3,4,5& 8 (WEPUS) in Fig 9(a, b, c -2) belong to octadecanoic acid Peak 5 in Fig. 9(a-2), peak 9 in Fig. 9(b-2) and peak 6 in Fig. 9(c-2) correspond to 1,2-benzenedicarboxylic acid. All these non-polar moieties are hold high reputation as antimicrobial agents. In WEPUS, method highly effective extraction of major non-polar groups is confirmed. Thus, from our investigations we could say that the success of the WEPUS method was due to the effective extraction of these bioactive non-polar groups. Also, it appears that these non-polar moieties play a distinct role in the antimicrobial activity of mushroom extracts, evidenced by

their domination in the GC-MS analysis.

### Conclusion

WEPUS method has been demonstrated to lead highly efficient extraction of antibacterial bioactive compounds from aqueous mushroom extracts. The extraction time has been slashed down to 2min from the conventional 24 h extraction. Significant enrichment of the bioactive compounds in the extract has been demonstrated to be the reason for the enhanced bioactivity via the WEPUS technique. The bioactivity of M3 extracts is ascertained to the non-polar components of the extract based on GC-MS analysis.

### Acknowledgement

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**Table 1. Characterization of bioactive compounds from the mushroom extracts**

Mushroom extracts		Total phenolics (mg GAE/ g DW)	Flavonoids (mg RE/ g DW)	DPPH ( $\mu$ mol TE/ g DW)
<b>WE</b>	M1	2.49 $\pm$ 0.310	0.18 $\pm$ 0.006	3.48 $\pm$ 1.047
	M2	2.55 $\pm$ 0.030	0.79 $\pm$ 0.060	7.33 $\pm$ 0.230
	M3	3.94 $\pm$ 0.040	0.18 $\pm$ 0.009	9.31 $\pm$ 0.038
	M4	1.75 $\pm$ 0.070	0.13 $\pm$ 0.009	4.18 $\pm$ 0.307
	M5	3.08 $\pm$ 0.290	0.34 $\pm$ 0.009	16.20 $\pm$ 0.269
<b>WEBS</b>	M1	3.05 $\pm$ 0.600	0.60 $\pm$ 0.145	8.22 $\pm$ 0.115
	M2	5.04 $\pm$ 0.210	1.36 $\pm$ 0.047	14.30 $\pm$ 0.268
	M3	7.49 $\pm$ 0.088	1.46 $\pm$ 0.032	8.07 $\pm$ 0.663
	M4	1.96 $\pm$ 0.100	0.26 $\pm$ 0.025	5.08 $\pm$ 0.038
	M5	2.38 $\pm$ 0.230	0.57 $\pm$ 0.063	4.73 $\pm$ 0.230
<b>WEPUS</b>	M1	6.91 $\pm$ 0.221	1.06 $\pm$ 0.101	4.33 $\pm$ 2.750
	M2	6.69 $\pm$ 0.972	3.57 $\pm$ 0.133	3.96 $\pm$ 0.988
	M3	7.36 $\pm$ 0.114	1.39 $\pm$ 0.046	12.17 $\pm$ 0.589
	M4	4.94 $\pm$ 0.854	0.28 $\pm$ 0.026	12.50 $\pm$ 4.628
	M5	6.78 $\pm$ 0.088	1.048 $\pm$ 0.144	1.905 $\pm$ 0.468

**Table 2. Consolidated list of bioactive compounds found in M3 as a function of the different extraction techniques**

Mushroom extracts	Total phenolics (mg GAE/g DW)	Flavonoids (mg RE/g DW)	DPPH ( $\mu$ mol TE/g DW)	Polysaccharides (mg GE/g DW)
<b>WE</b>	3.94 $\pm$ 0.040	0.18 $\pm$ 0.009	9.31 $\pm$ 0.038	0.36 $\pm$ 0.005
<b>WEBS</b>	7.49 $\pm$ 0.088	1.46 $\pm$ 0.032	8.07 $\pm$ 0.663	0.61 $\pm$ 0.011
<b>WEPUS</b>	7.36 $\pm$ 0.114	1.39 $\pm$ 0.046	12.17 $\pm$ 0.589	0.76 $\pm$ 0.026

**Figure Captions**

Fig1. Schematic work flow followed in the study

Fig 2 Graph showing comparison of antibacterial activity on *S. mutans* by conventional ethanol extraction (EE) and water extraction (WE) from M1 (*Pleurotus ostreatus*); M2: *Agricus bisporus*; M3: *Lentinula edoeds*; M4: *Pleurotus eryngii*; M5: *Flammulina velutipes*.

Fig 3 Antibactericidal activity of the extracts against A: *Pseudomonas aeruginosa* B: *Streptococcus mutans* evaluated by (a) Spectrophotometric methos (b) plate count method.

Fig. 4 Optimization of WEBS using M3 extracts against *P. aeruginosa*

Fig. 5 Optimization of WEPUS approach using M3 against *P. aeruginosa* evaluated by (a) Spectrophotometric methos (b) plate count method.

Fig 6. Graph showing the comparative antimicrobial activity exhibited by WE, WEBS and WEPUS techniques against A: *Pseudomonas aeruginosa*; B: *Streptococcus mutans*.

Fig 7. Post exposure imaging of A:*Pseudomonas aeruginosa* and B: *Streptococcus mutans* cells using Confocal laser scanning microscopy following incubation with M3 extracts (a) control; (b) WE; (c) WEBS (d) WEPUS. Cells stained with acridine orange. Live bacteria are red; dead bacteria are green.

Fig 8. FE-SEM images of A:*Pseudomonas aeruginosa* B: *Streptococcus mutans* treated with M3 extracts (a) control; (b) WE; (c) WEBS (d) WEPUS .

Fig 9. GC-MS of M3 extracted via (a) WE; (b) WEBS (c) WEPUS. (a,b,c-1). Samples diluted in ethanol; (a,b,c-2). Samples diluted in chloroform.

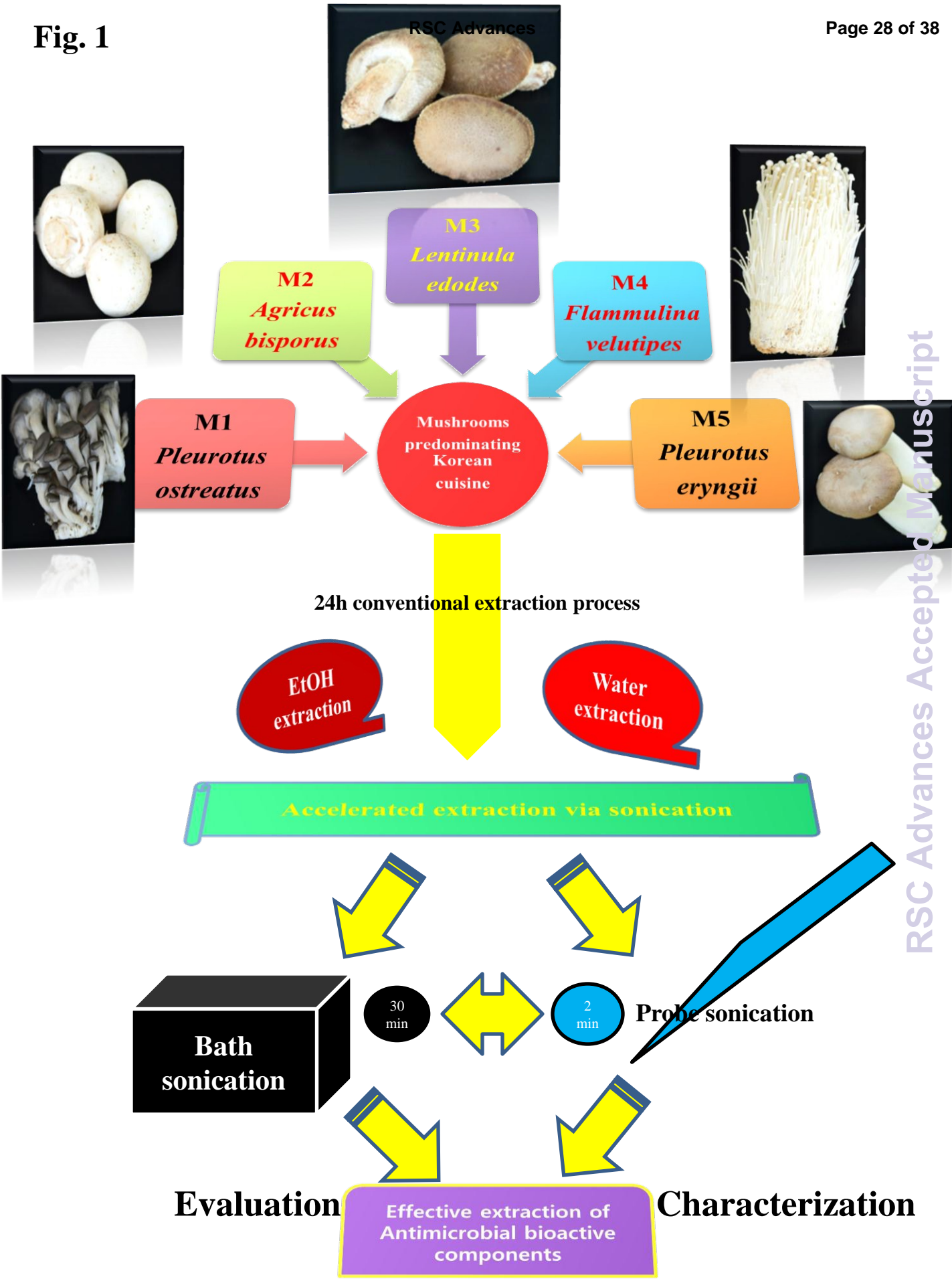


Fig. 2

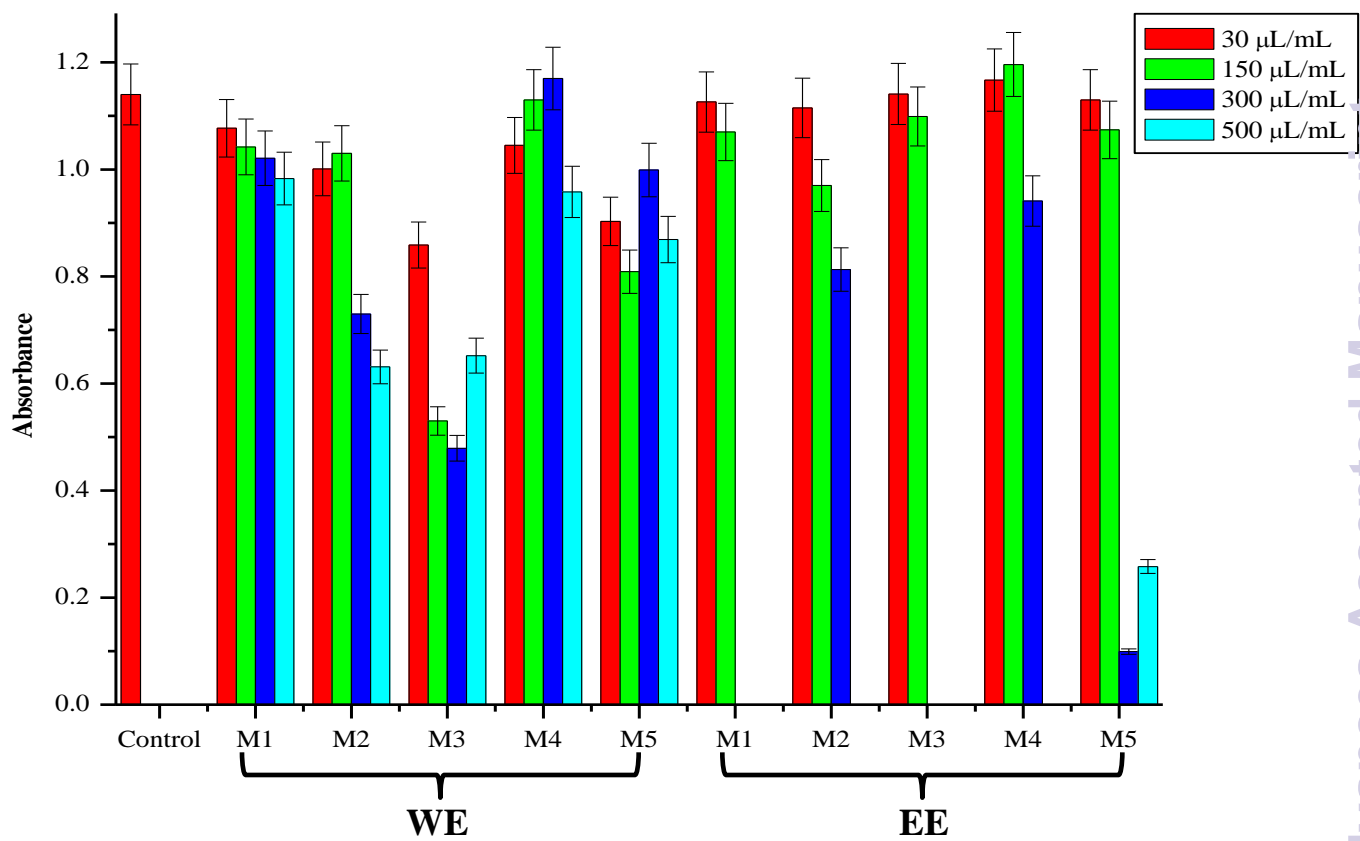


Fig. 3A

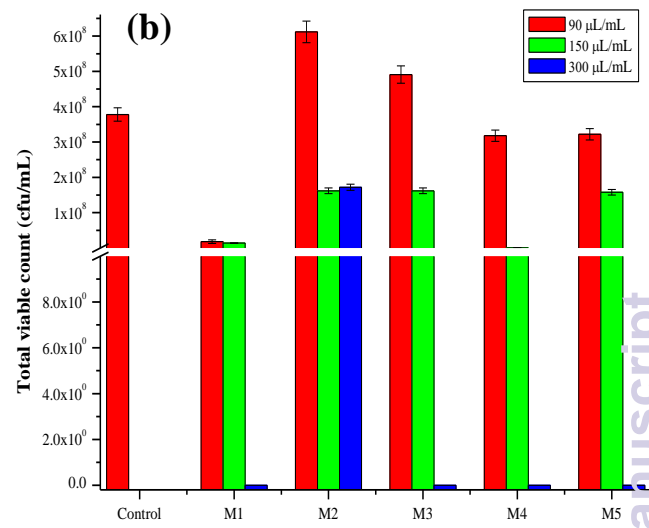
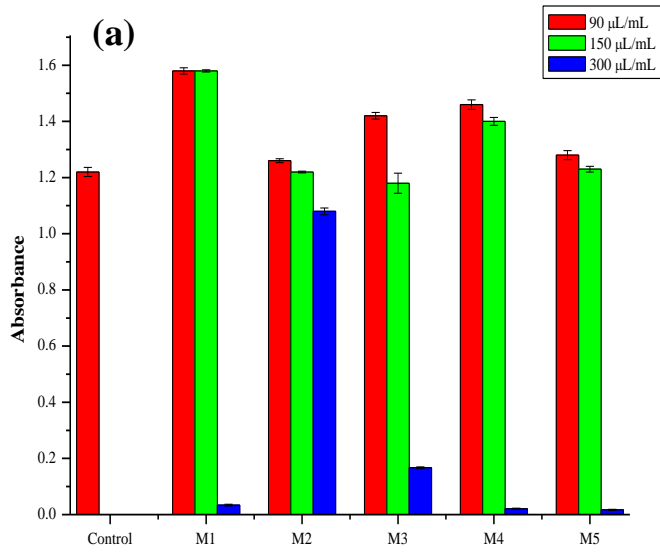
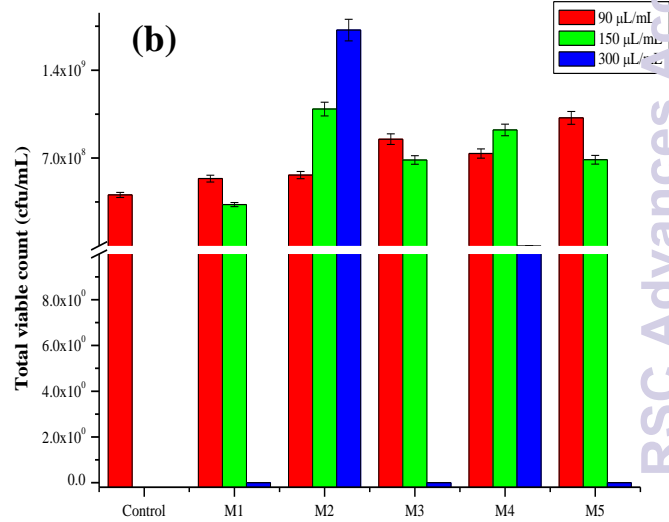
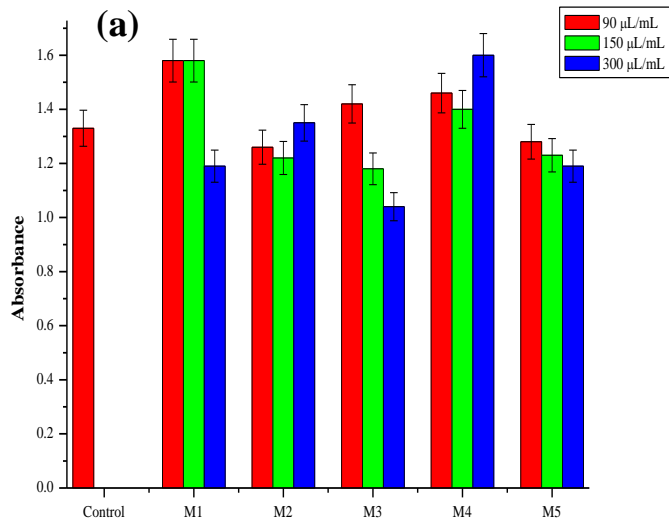


Fig. 3B



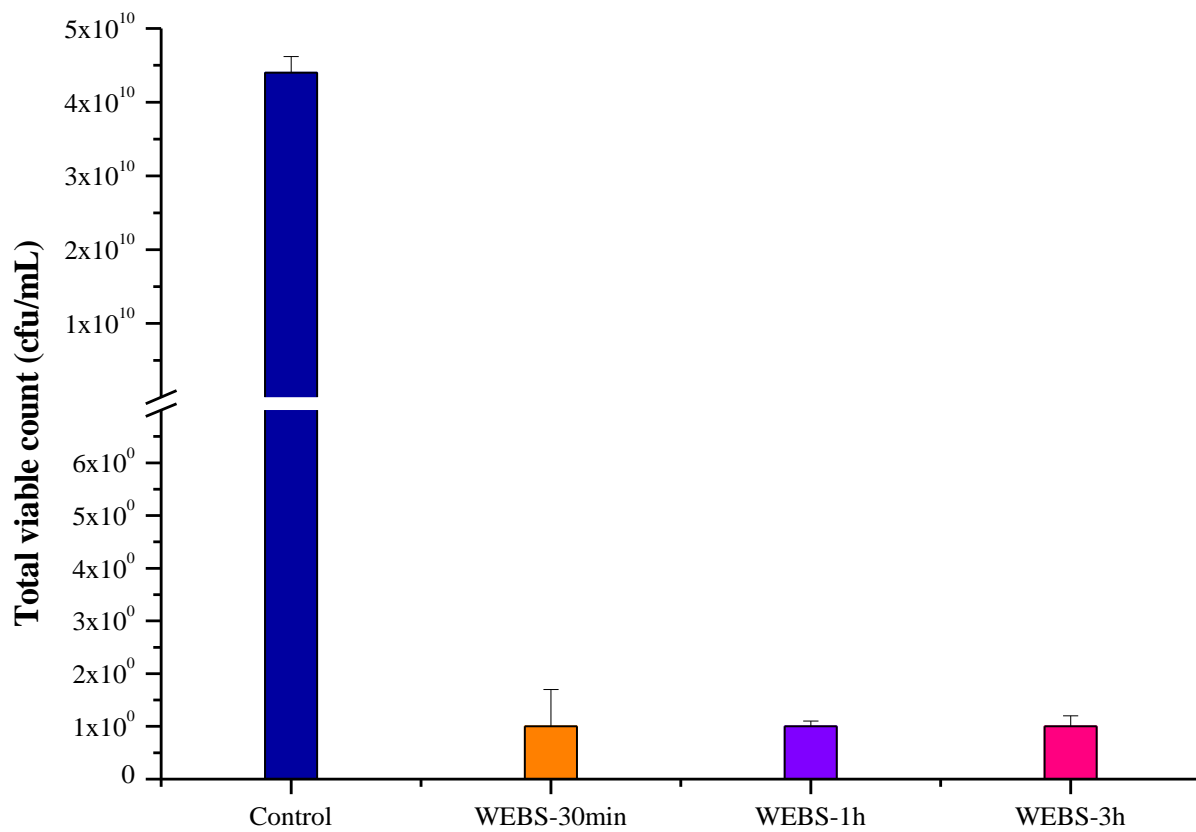
**Fig. 4**



Fig. 5A

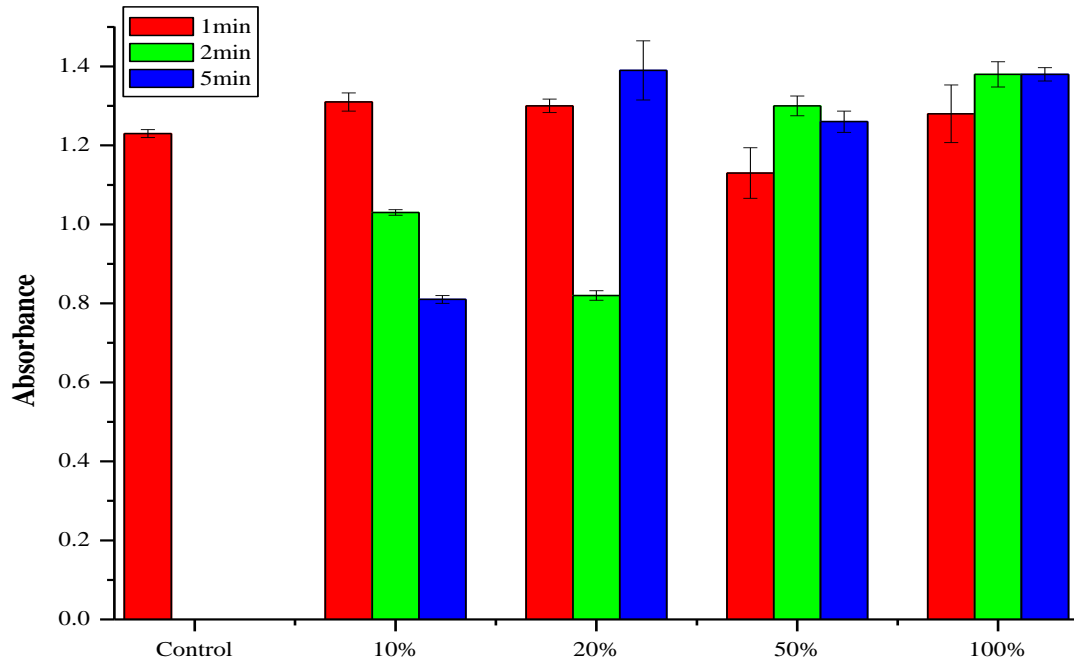
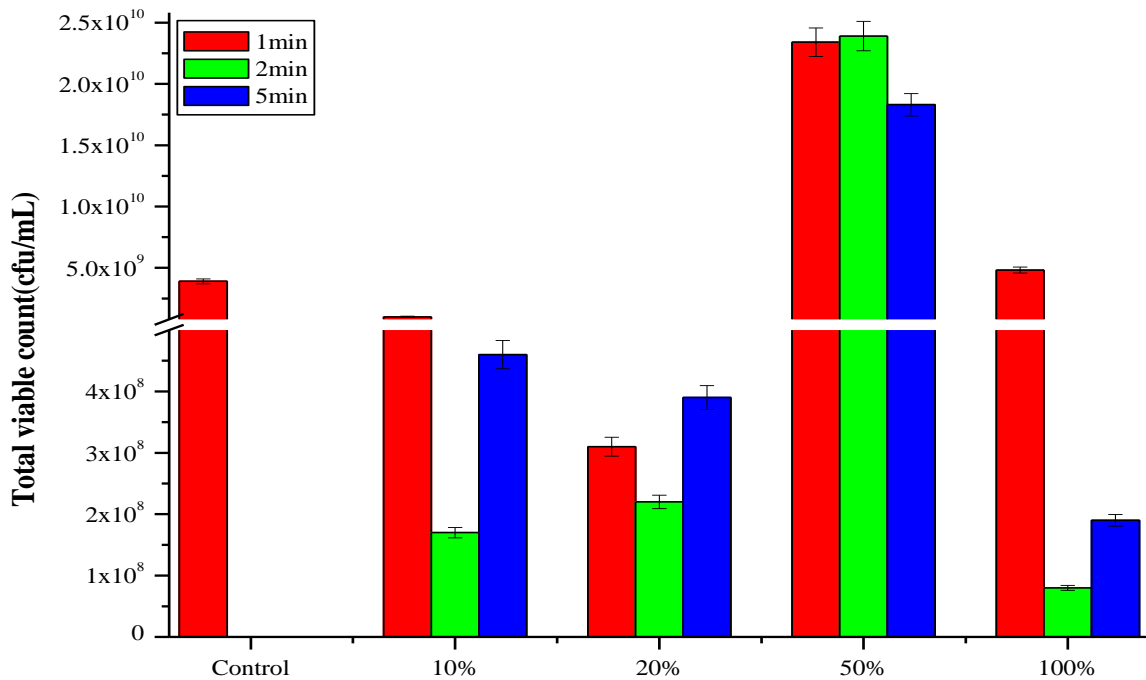
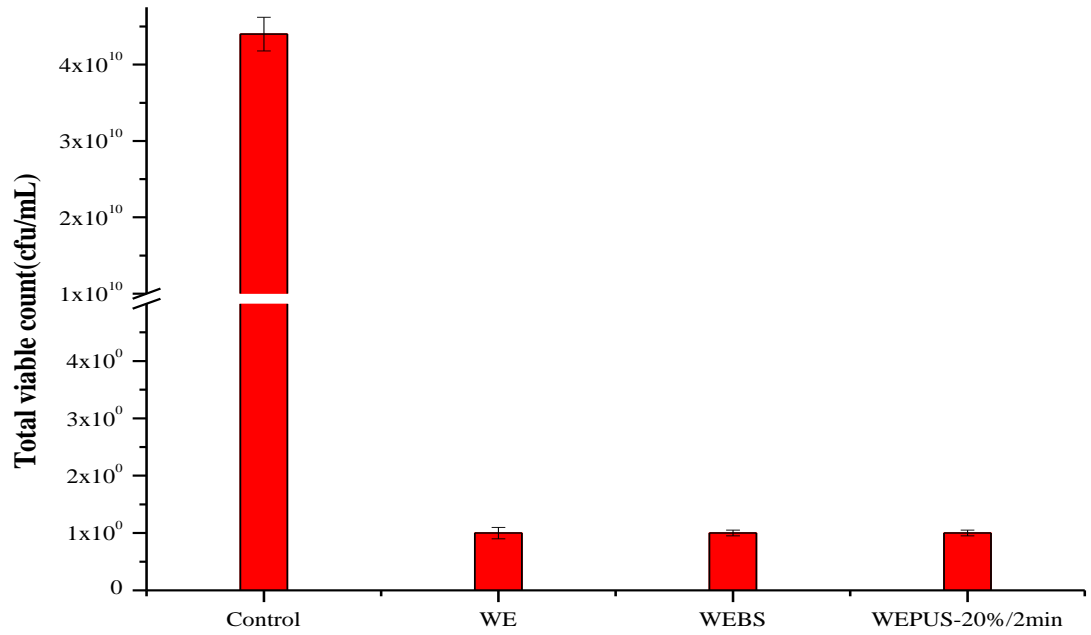
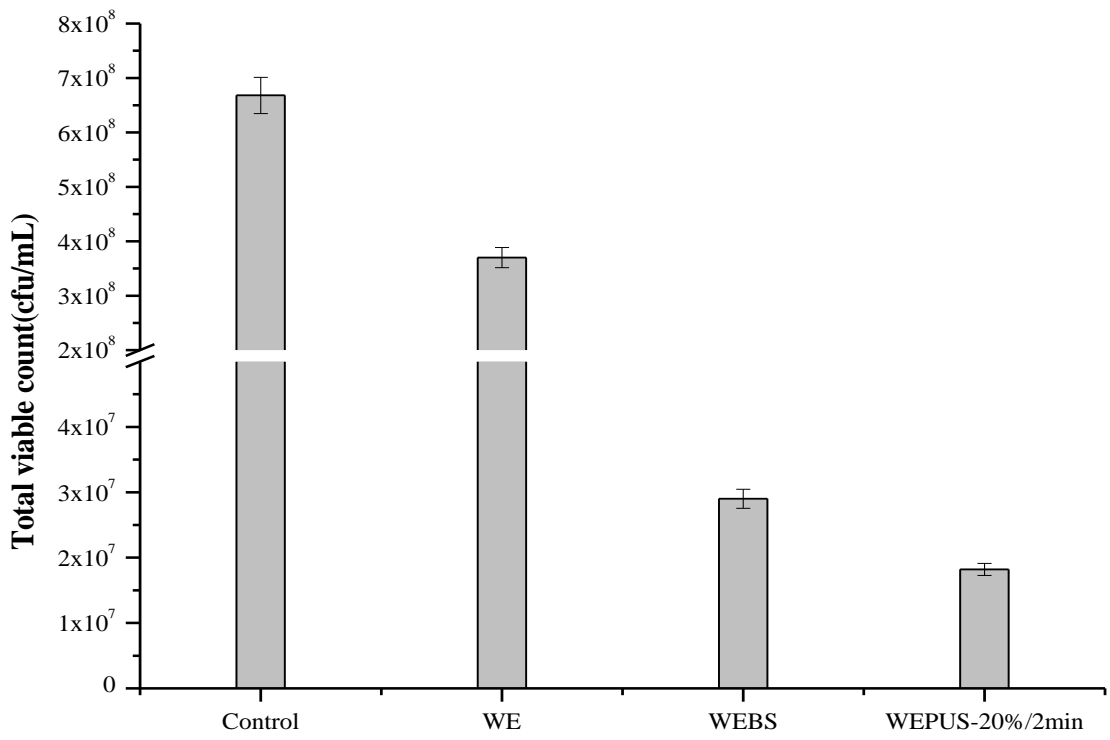


Fig. 5B

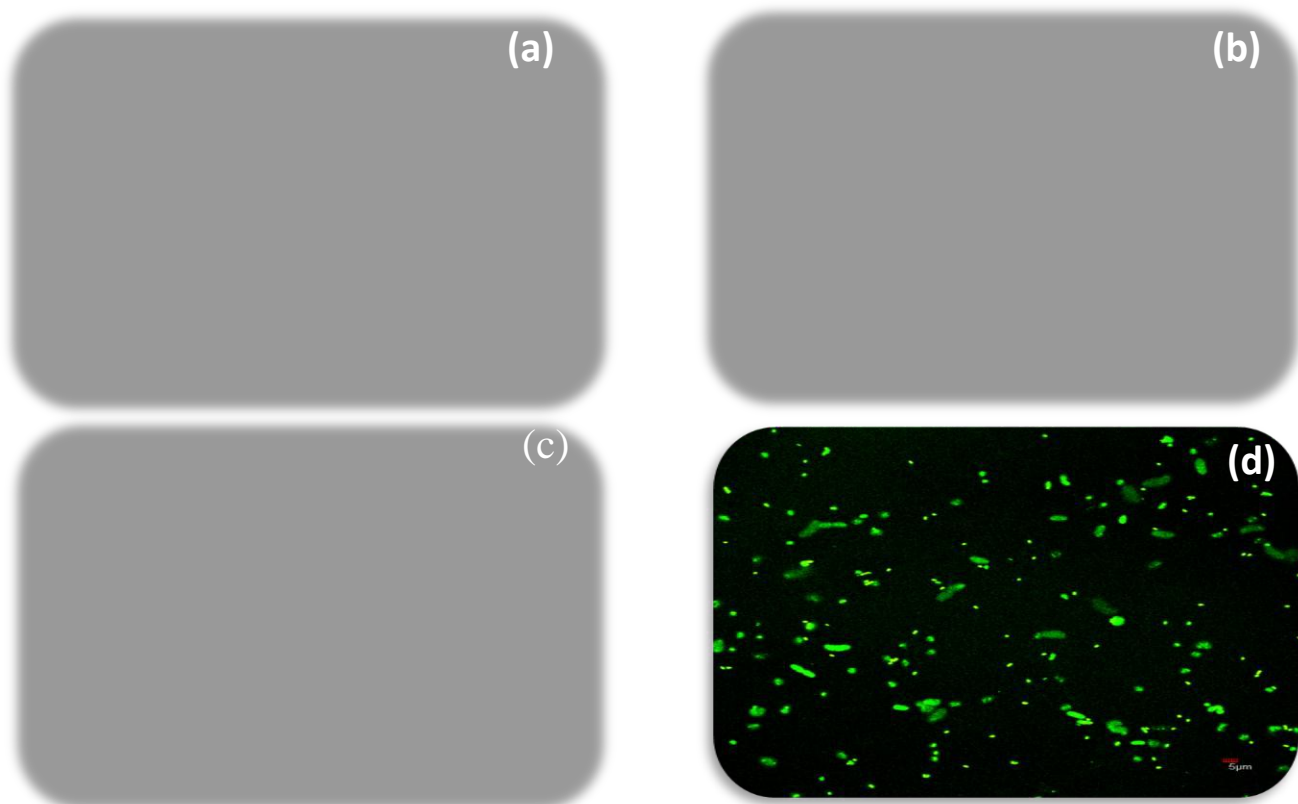
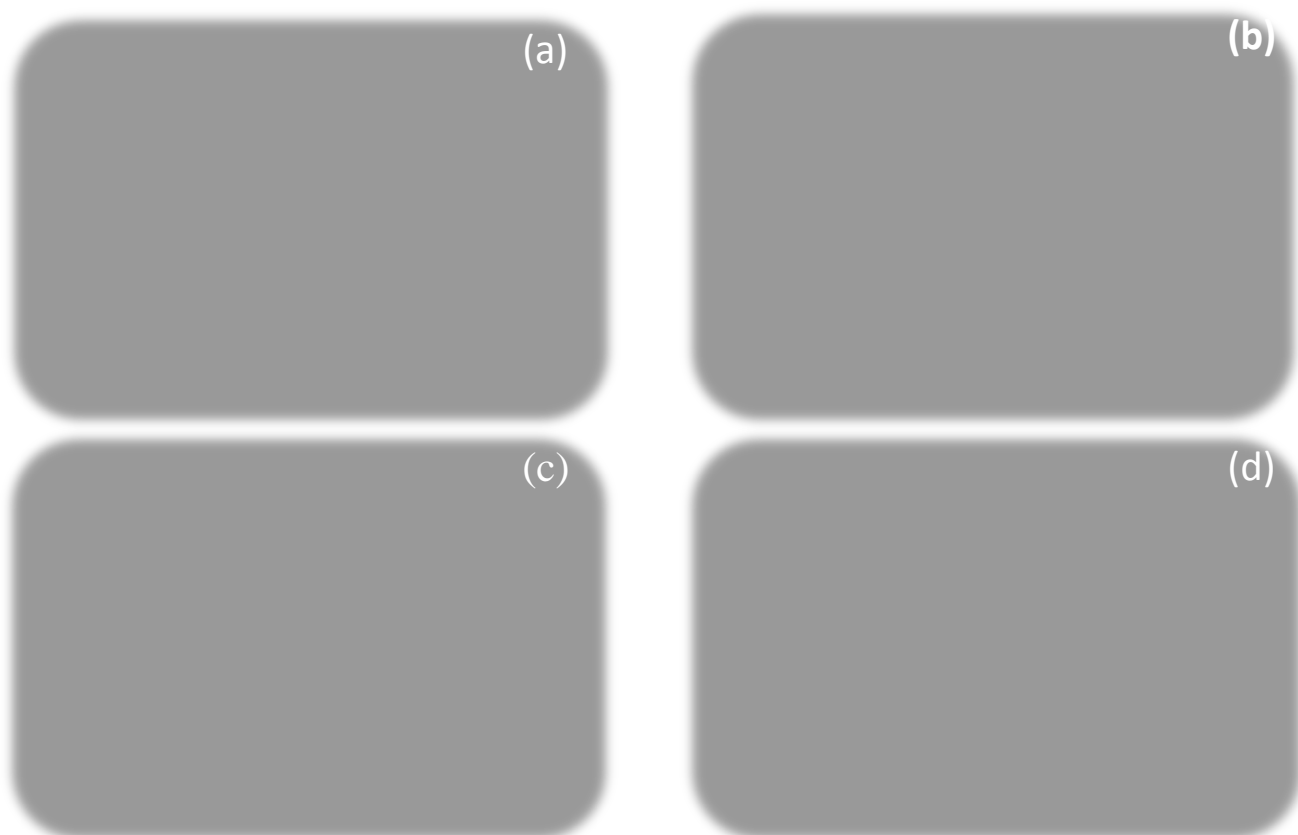




**Fig. 6B**



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**Fig. 7A****Fig. 7B**

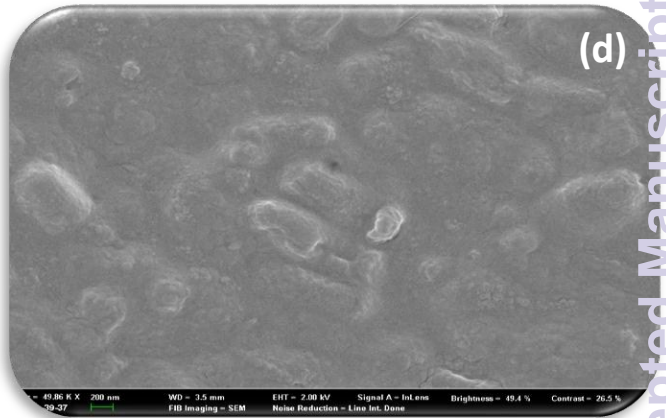
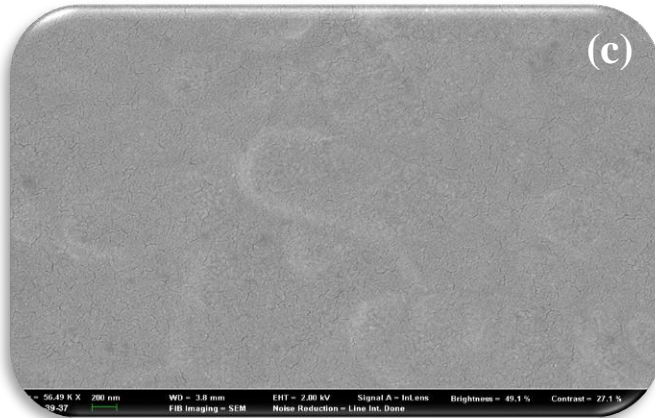
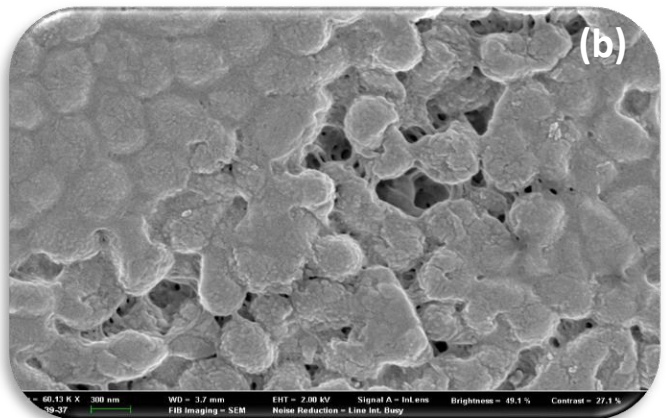
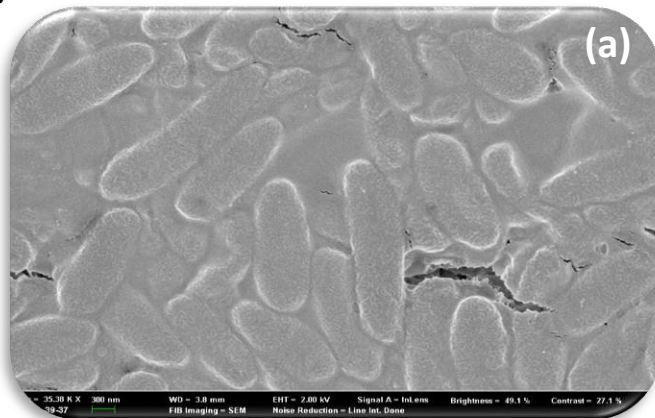
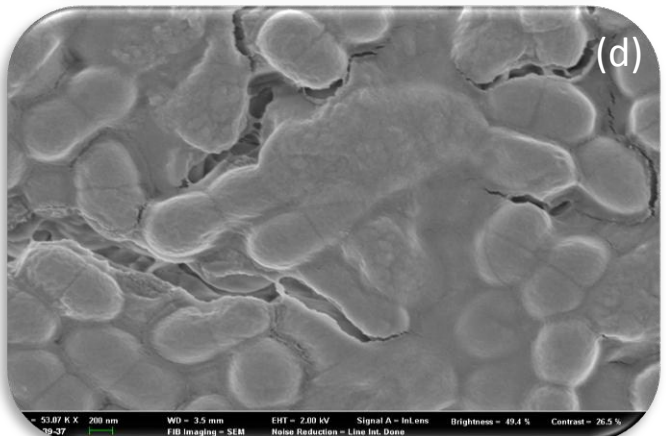
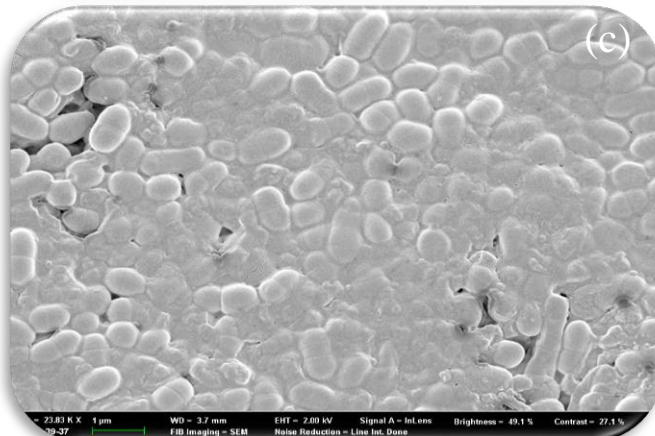
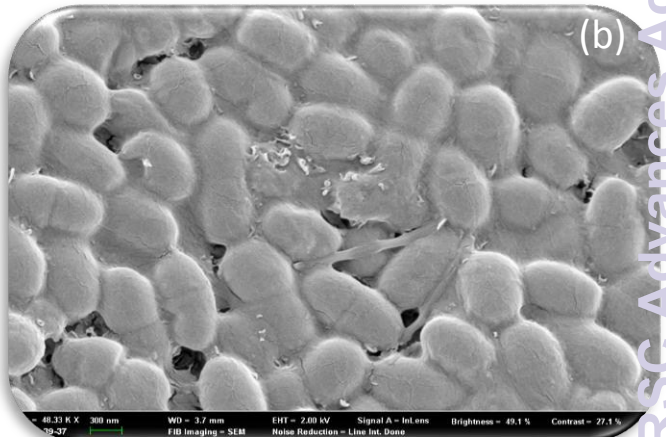
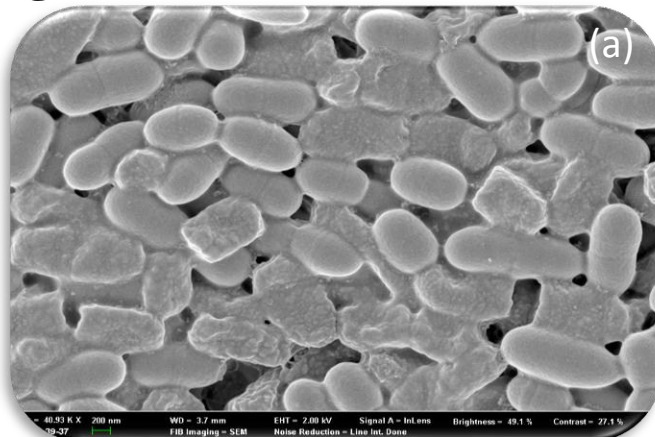
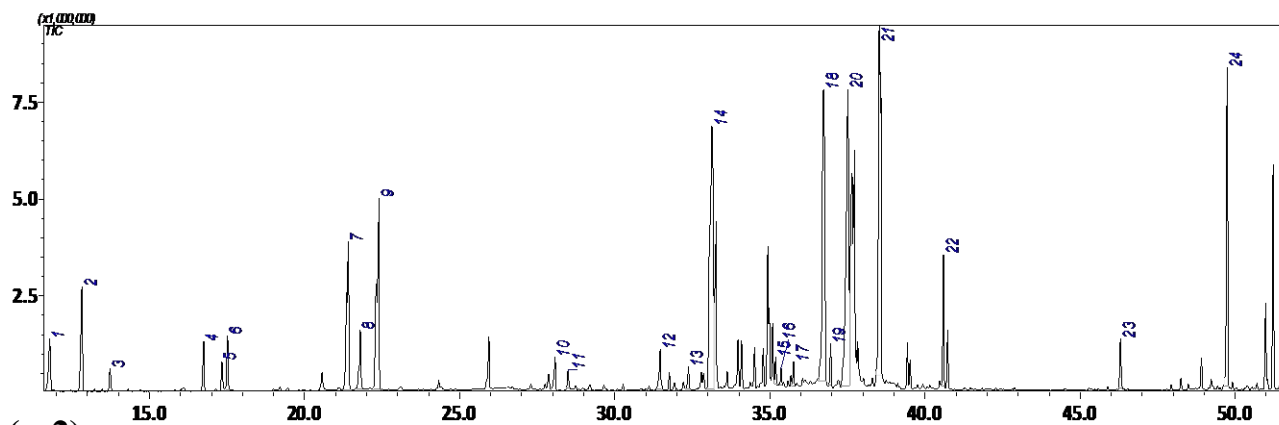


Fig. 8B

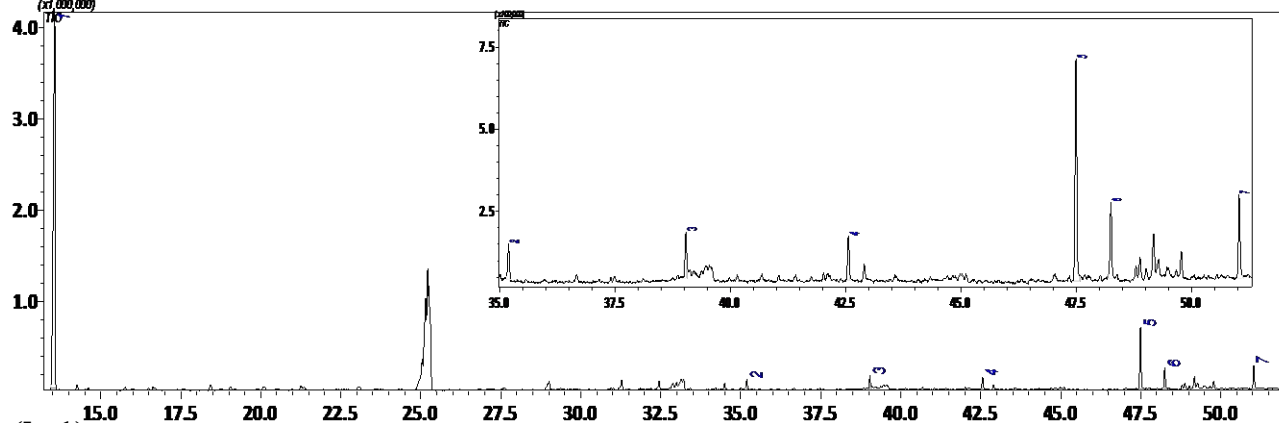


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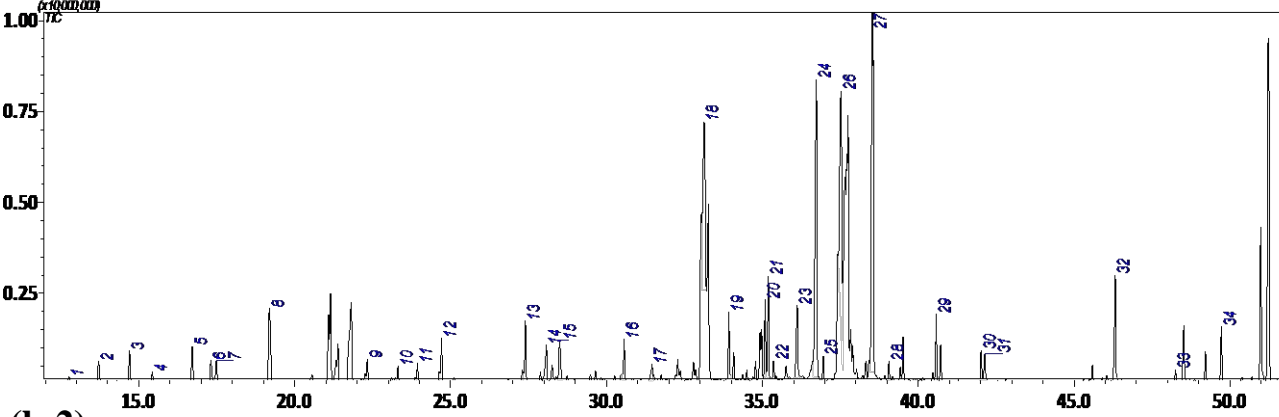
(a-1)



(a-2)



(b-1)



(b-2)

