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ANTIBACTERIAL ACTIVITY OF AGRICULTURAL WASTE-BASED ACTIVATED CARBONS AND SILVER-IMPREGNATED ACTIVATED CARBON AGAINST PATHOGENIC *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

The ability of activated carbons from agricultural waste based precursors, *Melaleuca leucadendron* Husk (MLH), *Caesalpinia pulcherrima* husk (CPH), blended activated carbon (BAC-PA) and silver impregnated blended activated carbon (SIBAC-PA) to eliminate and destroy *Staphylococcus aureus* and *Pseudomonas aeruginosa* were tested under Plate Assay and Shake Flask techniques. SIBAC-PA showed the highest antibacterial effect against the two test organisms with inhibition zone diameters of 24 and 27 mm respectively. On using the shake flask technique, it was found that bacterial count started reducing after one hour incubation, while no bacterial growth was detected after 2,3 and 24 hours using the blended activated carbon. Bacterial growth was completely inhibited on using silver impregnated activated carbon at all the tested concentrations (12.5 – 50 mg/ml) after one hour incubation.

KEYWORDS: Activated carbon, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Agricultural waste

INTRODUCTION

Activated carbon, also widely known as activated charcoal or activated coal is a form of carbon which has been processed to make it extremely porous and thus to have a very large surface area available for adsorption and chemical reactions[1]

Materials to be potential precursors of activated carbons should have high carbon content and low inorganic compound levels [2] in order to obtain a better yield during the carbonization processes. This is valid for practically every lignocellulosic waste. A large number of agricultural materials have been used for preparation of activated carbon for example, corncob, corn grain, avocado, mango, orange, and guava seeds[2-4] , lignite[5]and rosewood sawdust[6].

Activated carbon (AC) has been widely used in various industrial areas such as air pollution control, wastewater treatment to remove various pollutants , it is used in filters in gas masks and even in water treatment systems of hospital renal haemodialysis care units[5], [7].

Activated carbon has also been found to have the ability to remove bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* from fresh and potable water systems [7] ,[13].

Activated carbon is one of the most widely used nanoparticles for water purification due to its large surface area and high adsorption capacity [14]. Activated carbon has proven to remove bacteria like Pseudomonas aeruginosa and Escherichia coli from fresh and potable water systems [15, 16]. Despite electrostatic repulsion between negatively charged microorganisms and carbon surfaces, microorganisms attach to activated carbon particles through strong Lifshitz van der Waals forces [17]. Potable water systems are considered low in ionic strength so electrostatic interactions can offer the possibility of enhancing the efficacy of activated carbon to remove microorganisms from water by

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positive charge modification of the carbon surfaces. Once there is a charge reversal, the electrostatic attraction between negatively charged microbial cell surfaces and positively modified carbon particles will be strong [18, 19]. Moreover, modification in the activated carbon particles by coating with a quaternary ammonium compound gives the activated carbon particles bactericidal properties [19] and decreases the possibility of biofilm growth. In addition to the microorganisms charge, the hydrophobicity of the surfaces that come in contact with microbes is important in adhesion [18]

Nanoparticles have attracted great interest in their development as potential antibacterial drugs [20], which has also been reported that many biophysical interactions occur between silver nanoparticles and bacteria including biosorption, nanoparticles decomposition and cellular uptake, with effects bacterial cell membrane damage and toxicity [21,22].

In Europe and in some part of Africa, silver has been used as a water disinfectant and has shown effectiveness against planktonic bacteria [23]. Silver has gained this efficacy through its binding to disulfide or sulfhydryl groups present in the cell wall proteins [24]. Silver has been shown to bind DNA in the nucleus thus causing cell death.

One drawback for the use of activated carbon is the lack of reversibility [25], uncertainty when determining whether capacity has been reached. Silica based nanoparticles used as immobilizers have shown to enhance the non-selective capture of organic contaminants from wastewaters, thus increasing the time of contact between microorganisms and substrate resulting in enhanced biodegradation [26]

The main objective of the present study is to evaluate the antibacterial efficacy of MLH, CPH, BAC-PA and SIBAC-PAC against *Staphylococcus aureus* and *Pseudomonas aeruginosa* strain, where the combination of the activated carbon and silver would take an important antibacterial advantage, due to the strength of these two nanoparticles and these materials used widely for application in water purification.

MATERIALS AND METHODS

Test Organisms

The test organisms used in this study were clinical isolates of bacteria obtained from Department of Microbiology, SHEDA, Abuja. The isolates include:

1. *Staphylococcus aureus*
2. *Pseudomonas aeruginosa*

Maintained on Nutrient Agar slants and stored at 4°C with regular transfers at monthly intervals. For long preservation, the slants were folded with 25% glycerol.

Raw Materials

Activated carbon was washed with deionized water and modified by treatment with phosphoric acid according to [27], Silver Nitrate, $AgNO_3$ (99%, Sigma Aldrich), sodium borohydride ($NaBH_4$, Sigma Aldrich), The M-Endo agar medium and Macconkey agar medium (Sigma Aldrich), deionized water of high purity (Chemistry Department, Ahmadu Bello University, Zaria) was used in all experiments.

Preparation of seed culture

Transfers from single slant cultures (48 hours old) were taken into 50 ml aliquots of the seed medium containing (g/l): beef extract, 1; yeast extract, 2; peptone, 5; sodium chloride, 5 and 1 liter of distilled water. Dispensed in 250 ml Erlenmeyer flasks to initiate growth (OD<1). Standard inoculam of 2% (v/v) were taken from the latter liquid culture after growth for 18 hours at 30°C ± 2 on a reciprocal shaker to start growth in the fermentation flask which is equivalent to 3×10^8 colony forming unit (CFU/ml) according to McFarland scale 0.5.

Preparation of silver nanoparticles

Silver nanoparticles were prepared according to the chemical reduction method adapted by Fang, *et al.,* (2005). 50 ml of $1x10^{-3}$ M silver nitrate was prepared, and then heated till boiling and 5 ml of 1% tri-sodium citrate added drop by drop. The solution was mixed vigorously and heated until the colour changed to pale brown followed by stirring until cooled to room temperature. The aqueous solution was air dried up to 4 days so as to obtain a powdered form of sliver nanoparticles.

Preparation of the adsorbents

The adsorbents were prepared as described by [27]. Husks from CPH and MLH collected after discarding the fruit pulps, were sun dried, crushed and grinded in a ball mill individually. The grinded samples were sieved to obtain the particles of uniform size, 1.0 and 3.0 mm respectively. The ratio of acid to precursor was 2:1 i.e 200 ml of acid w/v for every 100g of sample. The adsorbents prepared were denoted CPH-PA, MLH-PA and BAC-PA throughout the work, where PA is Phosphoric acid.

BAC-PA impregnation with silver nanoparticles

Blended activated carbon (1g) was added to 20 ml of different $AgNO_3$ concentrations (a) 0.1; b) 1 and c) 1.5 mol.L^{-1}) one at a time. After 24 hour of impregnation in the dark, the powder samples were washed with water to remove loosely adsorbed $AgNO_3$, until no $AgNO_3$ was observed in the filtrate. The powder samples collected after decantation was air-dried until the next day. By adding 10 ml of 0.2 mol L^{-1} $NaBH_4$, impregnated $AgNO_3$ was chemically reduced (over 24h) to form Ag particles, then it was washed with water to remove the excess $NaBH_4$ followed by drying [28]. The adsorbent prepared was denoted SIBAC-PA.

Antibacterial test

MLH-PA, CPH-PA, BAC-PAC and SIBAC-PA were tested for their antibacterial effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* under test. If these organisms are killed, as a standard, all other disease-causing organisms are assumed killed.

(a) Sensitivity test of the adsorbents using Agar well Diffusion method

The standardized inocula of the bacterial isolates 0.1 ml were uniformly streaked unto freshly prepared Mueller Hinton Agar (MHA) plates with the aid of a sterile swab stick. Five wells were punched on the agar plates using a sterile cork borer (8 mm) in diameter. About 25 mg of different adsorbents under test were added one at a time in these holes, using one to two drops of sterilized water. They were left at 4°C for 1 hr then incubated at 37 C for 24 hours.

After incubation period, the plates were observed for any evidence of inhibition (zone of inhibition), which will appear as a clear zone that were completely devoid of growth around the wells. The diameter was measured using a transparent ruler calibrated in millimetre.

(b) Plate Assay Method (qualitative test)

Melted M-Endo Agar medium was fortified with 3x108 CFU/ml medium of *Staphylococcus aureus* and *Pseudomonas aeruginosa* equivalent to 0.5 Mcfarland. About 20 ml of the previously prepared seeded agar was then dispensed in petri dishes, solidified by refrigerating for 4 to 6 hours. Seven mm diameter holes were made in the seeded agar using a sterilized cork-borer. 25 mg of different adsorbents under test were added one at a time in these holes, using one to two drops of sterilized water. They were left at 4°C for 1 hr then incubated at 37 C for 24 hours and the antibacterial effect was measured referring to the inhibition zone diameter.

(c) Shake flask test in saline medium (Quantitative test)

For the shake flask test, 50 ml of sterile saline (0.9% NaCl) was inoculated with 1 ml bacterial suspension (3x108 CFU/ml) equivalent to 0.5 Mc Farland. 50 mg of different adsorbents were added to the flasks, one at a time and the contents were stirred on a rotary shaker at ambient temperature. The samples were drawn periodically (0, 1, 3 and 24 hours) from the flask and tested for the number of surviving *Staphylococcus aureus* and *Pseudomonas aeruginosa* by plate count method on M-Endo agar, using standard procedures.

RESULTS AND DISCUSSION

Antibacterial test

(a) Sensitivity test

This is the test for the susceptibility of bacteria to antibiotic but in this case to the adsorbents under study. The result of the sensitivity tests are given in table 3.1 below.

Table 3.1: Sensitivity test of the adsorbents under study

Adsorbents	Mean zones of inhibition *(Staphylococcus aureus)*	Mean zones of inhibition *(Pseudomonas aeruginosa)*
MLH-PA	13	11
CPH-PA	16	14
BAC-PA	18	17
SIBAC-PA	22	24

It can be shown from table 3.1 above that SIBAC-PAC had the highest zone of inhibition followed by BAC-PA, CPH-PA and finally MLH-PA with the least zone of inhibition. This shows that *Staphylococcus aureus* and *Pseudomonas aeruginosa* will be more susceptible to SIBAC-PA and least susceptible to MLH-PA. The sensitivity test has shown that SIBAC-PA will have a high efficacy in the removal of the test organisms under study as a result of the presence of silver nanoparticles in its blend.

(b) Plate Assay Method

In the inhibitory effect of MLH-PA, CPH-PA, BAC-PA and SIBAC-PA, it was revealed that all the tested adsorbents had an antibacterial effect exhibited by the diameters of the inhibition zones.

SIBAC-PA proved to be the most effective antibacterial agent against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with an inhibition zone of 24mm and 27mm respectively followed by BAC-PA (22 mm and 19 mm), CPH-PA (18mm and 15 mm) and finally MLH-PA the least effective (12mm and 14 mm). *Staphylococcus aureus* and *Pseudomonas aeruginosa* adhere only weakly to different adsorbent particles, and the main difference between different types of activated carbons is the number of attractive sites revealed upon traversing of a carbon particle through the outer bacterial surface layer [29], while MLH-PA showed smaller inhibition zone which could be due to the fewer attractive sites revealed in contrast to others which were more efficient in bacterial removal [30]

Table 3.2: Inhibitory effect of the nanoparticles under test against *Staphylococcus aureus* and *Pseudomonas aeruginosa* , using plate assay method.

Adsorbents	*S. aureus*	*P. aeruginosa*
MLH-PA	12	14
CPH-PA	18	15
SIBAC-PA	24	27
BAC-PA	22	19

Several studies have shown that activated carbon has the antibacterial activity against bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* from fresh and potable water systems [31], [32] particles through strong Lifshitz van der Waals forces [33] despite electrostatic repulsion between negatively charged microorganisms and carbon surfaces. The results of the study carried by [34] showed antimicrobial capabilities of activated carbon composite - in which magnetite and silver- were used towards bacteria and on Pseudomonas koreensis and Bacillus mycoides cultures isolated from river water. Potable water systems are considered low in ionic strength so electrostatic interactions can offer the possibility of enhancing the efficacy of activated carbon to remove microorganisms from water by positive charge modification of the carbon surfaces. Once there is a charge reversal, the electrostatic attraction between negatively charged microbial cell surfaces and positively modified carbon particles will be strong [35], [36].

In a trial to test the antibacterial effect of the tested nanoparticles against *Staphylococcus aureus* and *Pseudomonas aeruginosa* under shaken conditions, it was revealed that the number of non-adsorbed viable bacterial cells incubated with different nanoparticles reduced slightly within 1 hour after treatments with activated carbon, and silver impregnated activated carbon at different concentration. However, after shaken for 3 hours, the total viable *Staphylococcus aureus* and *Pseudomonas aeruginosa* count reached to zero with SIBAC-PA but with BAC-PA, MLH-PA and CPH-PA still having some bacterial colonies. Continuously, after contacting for 24 hours, the adsorbents killed all bacterial cells and markedly proved to have bactericidal effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* under test.

The antibacterial effect of SIBAC-PA at different concentrations (20, 40 and 60 mg/ml) against *Staphylococcus aureus* and *Pseudomonas aeruginosa* were tested. Results in table 3.4 below showed no growth of bacteria starting one hour of incubation where the percentage reduction of the total viable count of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was 100 %, while after 3 hours of incubation with activated carbon, 100% reduction in the bacterial count was reported. Therefore, the change after 1 hour of incubation with the adsorbents under test is due to the fact that the loss of cell viability has approached the complete inactivation [37]. It was clear that the amount of silver particles on the activated carbon considered to be the main factor for the complete reduction in the total viable count of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and responsible for the effective antimicrobial activity. The results are in agreement with that obtained by [38], where silver nanoparticles inhibited E. coli and Bacillus sp. growth at low concentrations (0.1 µg/ml), as for: silver targets multiple components in the bacterial cell, and the mechanism behind its antibacterial activity is by weakening DNA replication and inactivating proteins, as a result, resistance to bacteria cannot easily develop [38].

Moreover, other researchers showed that activated carbon-Silver composite have a superior antibacterial activity towards microorganisms due to the synergistic effect of activated carbon and silver nanoparticles [39].

In general, Most of the bacterial reduction and inactivation took place during the first three hours of incubation, and the mortality rate increases continuously with the increase of adsorbent concentration and their antibacterial activities are time and concentration dependent [37]. Thus, Silver nanoparticles showed efficient antibacterial activity against pathogenic *Staphylococcus aureus* and

Pseudomonas aeruginosa that was similar to that found by [40].

The mechanism of inhibitory action caused by silver nanoparticles on *Staphylococcus aureus* and *Pseudomonas aeruginosa* is partially known, where some researchers reported that silver nanoparticles inhibit bacterial growth through binding to the thiol group leading to bacterial inactivation [41]. Moreover, the Gram negative bacteria have a layer of lipopolysaccharides at the exterior that are composed of covalently linked lipids and polysaccharides; they lack strength and rigidity. Negative charges on the lipopolysaccharides are attracted towards the positive charges available on silver nanoparticles [42]. The opposite charges attract each other due to electrostatic forces. So once the nanoparticle comes in contact with the bacterial cell, it either inhibit the cell wall synthesis, damage the cytoplasmic membrane, inhibit nucleic acid and protein synthesis or inhibit specific enzyme systems which result in the complete bacterial inhibition [43].The mechanism by which the nanoparticles are able to penetrate the bacteria is not understood completely, but previous studies suggested that when *Staphylococcus aureus* and *Pseudomonas aeruginosa* are treated with silver, changes took place in their membrane morphology that produced a significant increase in its permeability affecting proper transport through the plasma membrane, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane, resulting in cell death [44]. Moreover, these studies showed that bacterial inhibition caused once silver nanoparticles penetrated inside the bacteria and caused damage by interacting with phosphorus and sulfur containing compounds such as DNA [43].

The antibacterial effect of MLH-PA, CPH-PA, BAC-PA and SIBAC-PA against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was obtained and compared. Plate assays and shake flask methods showed MLH-PA had the lowest antibacterial activities compared to the other prepared nanoparticles.

However, SIBAC-PA at the highest concentration showed the maximum inhibitory effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Therefore, higher concentrations of silver ions cause greater bactericidal effect and antibacterial activities are time and concentration dependant. Most of the bacterial cell number reduction occurred after three hours of incubation and the reduction rate was greatly influenced by the increase of silver ion concentrations and contact time. The present study demonstrates the potential of activated carbon composites for use in water purification and that silver impregnation is very effective in producing potable drinking water. After contact for 24 hours, all nanoparticles killed all bacterial cells, proving their bactericidal effect against pathogenic *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Table 3.3: Total viable count as affected by the exposure time to different activated carbons, using shake flask test

Adsorbents	*S. aureus*		*P. aeruginosa*	
	Contact time (hrs)	CFU/ml x 10^4	Contact time (hrs)	CFU/ml x 10^4
MLH-PA	0	82	0	67
	1	47	1	53
	3	29	3	31
	24	11	24	7
CPH-PA	0	75	0	85
	1	46	1	57
	3	30	3	29
	24	9	24	6
BAC-PA	0	50	0	46
	1	32	1	39
	3	21	3	24
	24	13	24	17

Table 3.4: Total viable count as affected by the exposure time to different concentrations of blended activated carbons, using shake flask test

Adsorbents	*S. aureus*		*P. aeruginosa*	
	Contact time (hrs)	CFU/ml x 10^4	Contact time (hrs)	CFU/ml x 10^4
	0	0	0	0
	1	0	1	0
SIBAC-PA	3	0	3	0
(12.5 mg/ml)	24	0	24	0
	0	0	0	0
	1	0	1	0
SIBAC-PA	3	0	3	0
(25mg/ml)	24	0	24	0
	0	0	0	0
	1	0	1	0
SIBAC-PA	3	0	3	0
(50 mg/ml)	24	0	24	0

It was clear that the amount of silver particles on the blended activated carbon seemed to be the main factor causing the complete reduction in the bacterial count and was responsible for the effective antimicrobial as can be seen in table 3.4 above [45, 46]. Moreover, other researchers showed that activated carbon-Silver composite have a superior antibacterial activity towards *Staphylococcus aureus*, *Pseudomonas aeruginosa* and other tests organisms due to the synergistic effect of activated carbon and silver nanoparticles [39].

CONCLUSIONS

1. Some agricultural wastes can be advantageous when used for the preparation of activated carbons.
2. Activated carbon prepared from MLH, CPH, BAC-PA and SIBAC-PA has physical and chemical properties that support good antibacterial activity.

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