Antibacterial activity of materials synthesized from clay minerals

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The antibacterial activity of silver and cationic surfactant modified smectites from North Patagonian, Argentina, were tested in growth inhibition of *Escherichia coli* bacteria by the test of susceptibility on solid medium. The exchange reactions were carried out using Ag^+ and hexadecyltrimethylammonium (HDTMA) cations in larger amounts than those conventionally required to form monolayer on the montmorillonite surface. The antimicrobial activity by the tetracyclines continued after the adsorption on the montmorillonite surface. In contrast, the untreated montmorillonite did not show any antibacterial activity against *E. coli*. The excellent activity shown by the Ag^+, HDTMA, tetracycline (TC) and minocycline (MC) adsorption complexes provides a promising field of clay mineral application in pharmaceutical applications.

Keywords: montmorillonite; adsorption complexes; antibacterial agents

1. Introduction

The development of materials with the ability to inhibit bacterial growth is a subject of major interest due to their potential use in several products as paints, kitchenware utensils and hospital instruments [1]. In this regard, the intercalation of organic substances with antibacterial activity into layered inorganic solids provides a useful and convenient method to prepare organic–inorganic hybrids that contain properties of both the inorganic host and organic guest in a single material [2].

The use of antibiotics in preventive medicine and treatment of several infections turned these compounds into some of the most widely used in the human and animal health care. Tetracycline (TC) and minocycline (MC) are compounds with common chemical structure and pharmaceutical actions and they are very frequently used against gram-positive and gram-negative microorganisms. Other antibacterial agents are metal ions such as Ag^+, Cu^{2+} and Ni^{2+} and quaternary ammonium compounds, used as skin antiseptics and disinfectants. The research on the antimicrobial properties of montmorillonites modified with heavy metals has been focus of interest because of the increasing bacterial resistance that the antibiotic overuse has caused [3].

Clays are natural materials used in human and veterinary health formulations like excipients or active substances. The interaction between the drug and its excipients may delay the drug release and therefore, its absorption, by lowering the drug levels in the blood. This feature can be favorable when the slow, controlled desorption of the drug has a positive effect upon its therapeutic action [4]. Among these materials, montmorillonites have been the most extensively used minerals in this field of application. Montmorillonites exhibit high cation exchange capacity, large specific surface and colloid properties that give rise to optimum adsorbents of organic and inorganic substances.

Metallic ion-exchanged montmorillonite dispersed in water has shown to attract bacteria by electrostatic forces [5]. Furthermore, montmorillonites and fluoromicas intercalated with organic compounds with antibacterial effects such as cetylpyridinium, cetyltrimethylammonium, norfloxacin and tetracyclines, have been also studied. The organo-montmorillonites and fluoromicas showed excellent antibacterial activities [6, 7].

This study focuses on microbiological activities of several complexes formed among tetracyclines, quaternary ammonium compounds and silver with montmorillonites, in its natural and modified form. Natural clays were collected from large deposits located in the Northern Patagonia, Argentina and they showed no antibacterial activity.

2. Clay Minerals. Properties and Applications

2.1 Structural properties and adsorption capacity

Clay minerals are natural material with particle size < 2 μm. Smectites, classified as 2:1 phyllosilicate clays, have a crystal lattice unit formed by one alumina octahedral sheet sandwiched between two silica tetrahedral sheets (Fig. 1).

The ion substitution or the site vacancies at the tetrahedral and/or octahedral sheets gives rise to a negatively charged surface. The exchangeable cations between the layers compensate the negative charge and may be easily exchanged by other metal cations, explaining the high ion exchange capacities of these minerals (70–120 meq/100 g). Due to this crystalline arrangement, smectites are able to expand and contract the interlayer while maintaining the two dimensional crystallographic integrity. The interlayer between units contains positive cations and water molecules. Montmorillonite is some of the members of the smectite family [8].

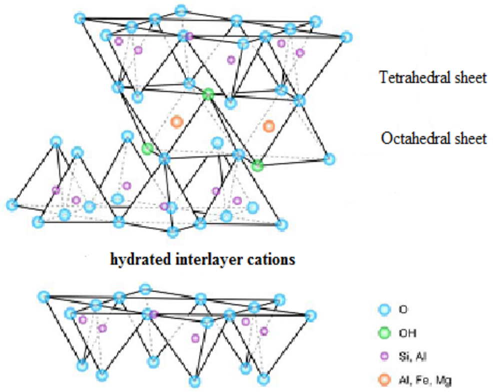

Fig 1 Layer structure with hydrated interlayer cations

Properties such as colloidal particle size, crystalline structure, high specific surface area, charge and swelling capacity confer smectites optimum rheological behavior and excellent adsorption capacities of inorganic and organic substances. In particular, the electrically charged surface of clay controls the interaction with other environmental ions, molecules, polymers, microorganisms and particles. These processes have various technological applications.

2.2 General Applications

Clay minerals are used in a wide variety of industrial applications [9, 10] due to two important and contrasting properties:

- inertia and stability
- reactivity and catalytic activity.

The widespread use of clay minerals responds to their extensive distribution in the environment, the relatively simple extraction and economic feasibility they present. Large volumes of these materials are used in filtration processes of, bleaching and clarification of various systems for animal feed pellets, in pesticides formulation and in water purification. They are also used as fats and oils adsorbents, and as catalyst or support catalysts in several organic syntheses [11, 12].

The modification of the surface properties is of importance to extend the clay applications and has therefore received great interest [13]. The hydrophobic modification of the montmorillonite can enhance the thermal, rheological, and mechanical properties of the resultant materials.

Although the modification of clay minerals can be performed by several methods, the ion exchange of the inorganic cations with organic cations, usually with quaternary ammonium compounds, allows changing the surface properties from hydrophilic to hydrophobic. Hence, these obtained organoclays thus obtained have been extensively investigated for hydrophobic contaminants immobilization.

The grafting clays with silanes have attracted great attention and were demonstrated to be a successful technique to improve the performance of clay-based nanocomposites.

2.3 Clays and Pharmaceuticals

A large number of minerals are used as active ingredients and excipients in pharmaceutical preparations as well as in cosmetic products, including the phyllosilicate (e.g. smectite, palygorskite, sepiolite, kaolinite, talc, mica).
The therapeutic activity of these minerals is controlled by their physical and physico-chemical properties as well as their chemical composition, chemical inertness and low or null toxicity. Minerals may be administered orally (e.g. antacids, gastrointestinal protectors, antidiarrhoeaics, osmotic oral laxatives, homeostatics, direct emetics, antianemics, etc) and topically (e.g. antiseptics, disinfectants, dermatological protectors, anti-inflammatories, local anesthetics, decongestive eye drops, etc) [4].

The use of clay minerals and non-silicate minerals as excipients in pharmaceutical formulations has been described by many authors [14, 15].

The drug–excipients interaction may affect the drug stability and release. The main concept in modified delivery technology is that any pharmaceutical dosage system should be designed to provide therapeutic levels of drug to the action site and keep them constant throughout the treatment [16].

The mineral–drug interaction can be used to control the release of active ingredients. Here the minerals first serve as a carrier, and then as releasers of the active ingredient [17].

3. Antibacterial agents

Antibiotics are natural compounds synthesized by living organisms to inhibit the growth of other organisms. Due to their therapeutic and prophylactic qualities to humans and animals, antibiotics have received widespread use all around the world [18].

Tetracyclines (TCs) comprise a group of natural and semi- synthetic products that inhibit the synthesis of bacterial proteins. They show an important activity towards a variety of microorganisms and consequently, they are used as broad-spectrum antibiotics in human, animals and in some areas of agriculture [19].

Among metallic elements, heavy metals such as silver, zinc, copper, mercury, tin, lead, bismuth, cadmium, chromium, and thallium have exhibited antibacterial properties. In particular, the active form of Ag^+ precipitates proteins, inhibits a variety of enzymes, and has a negative effect on transport systems in membranes [20]. Silver has been incorporated onto glass surfaces, zeolites, and into catheters where it shows very promising antibacterial activity.

Alkylammonium cations, especially those of quaternary amines, also exhibit antibacterial activity used as both skin antiseptics and disinfectants [21, 22]. They generally contain a positively charged hydrophilic ammonium group and a long hydrophobic alkyl chain. These compounds seem to have antibacterial action by disrupting their cell walls and/or inner bilipid membranes, which causes the cytoplasmic membrane contents of the cell to leak out. The most active alkylammonium cations contain alkyl chains between 8 and 18 carbons in length. For instance, compounds with 16 carbon alkyl chains are effective against both Gram-positive and Gram-negative bacteria [23].

4. Activity of antibacterial substances adsorbed on montmorillonite

4.1 Experimental assays

4.1.1 Starting materials for sample preparation

A representative sample of natural smectite (Es) was collected from North Patagonian, Argentina. The chemical analysis and physicochemical properties of the smectite were previously reported and the content of montmorillonite (98%) and quartz (2%), were obtained by X-ray diffraction (XRD). The sample had a cation exchange capacity (CEC) of 1.00 meq/g determined by the ammonium acetate method, a specific surface area of 607 m^2/g measured by adsorption of ethylene glycol monoethyl ether and the exchangeable cations were mainly Na^+ [24]. The chemical and microbiological compositions correspond to materials that are suitable for inclusion in pharmaceuticals formulations [25].

Chemical modification of the smectite was a functionalization of natural clay mineral with silanizing agent 3-aminopropyltrimethoxysilane (APS) covalently bound. The reaction time was 6 h at 80 ºC, using an ethanol/water solution as solvent. Subsequently, the silanized clays (Es-APS) were washed three times with fresh solvent solution. Throughout the whole process, the solid was stirred for 5 h, allowing sedimentation.

The synthesis of surfactant-modified clay minerals with hexadecyltrimethylammonium (HDTMA) was carried out by treating clays with a solution of the cationic surfactant (CH_3-$(CH_2)_{15}(CH_3)_3N^+Br^-$) in warm deionized water containing an amount of organic cation equivalent to 0.20 and 1.50 times the clay mineral. The suspensions were shaken for 3 h at 80ºC, centrifuged and washed with deionized water until they were free of bromide ions as indicated by $AgNO_3$. The solid material was dried at 60 °C and ground with an agate mortar. Smectite modified with 0.20 and 1.50 CEC with HDTMA were denoted as Es-0.2HDTMA and Es-1.5HDTMA, respectively.

4.1.2 Sample preparation

Es-APS-Ag^+ and Es-Ag^+ adsorption complexes were prepared by putting the clay (1.70 g/L) into contact with an $AgNO_3$ solution (1060 mg/L) for eight days. The quantity of adsorbed Ag^+ after the initial contact with the mineral was 114.70 mg/g for Es-APS-Ag^+ and 63.00 mg/g for Es-Ag^+. The system was centrifuged at 4000 rpm and the recovered complexes were washed three times using distilled sterile water and applying agitation until the dispersion was homogenized. After each washing, centrifugation was applied (15 min) and the adsorption complexes were resuspended to an adequate volume of distilled sterile water to obtain the corresponding dispersions. Systems were prepared by duplicate and pH was not monitored.

Adsorption complexes of tetracycline (TC) and minocycline (MC) were prepared from a montmorillonite dispersion (0.90 g/L) and different volumes of stock antibiotic solutions (0.76 mmol/L). After shaking for 24 h, the tubes were centrifuged at 8000 rpm for 30 min and the supernatant was removed to determine the antibiotic concentration in equilibrium. The TC-montmorillonite complexes (0.46 mmol/g and 0.76 mmol/g) and MC-montmorillonite complex (0.50 mmol/g and 0.70 mmol/g) were washed three times with 40 mL of aqueous 0.01 M NaCl solution using high magnetic stirring for 30 min. The dispersions were centrifuged at 9000 rpm for 15 min and the washed adsorption complexes were redispersed in 0.01 M NaCl to obtain an antibiotic concentration of 1.33 mg/mL.

The amount of TC adsorbed was calculated by UV–vis spectroscopy by establishing the difference between the initial and the final concentrations in the supernatant. To determine the Ag^+ ions concentration in a solution, the Volhard Method was used. This method uses a titration with potassium thiocyanate and Fe^{3+} as indicator in acidic medium. Once all the silver ions have reacted, the slightest excess of thiocyanate reacts with Fe^{3+} to form a dark red complex.

4.1.3 Antibacterial test

Antibacterial activity was evaluated by susceptibility testing on solid medium using *Eschericchia coli* ATCC 25922. Petri plates containing 20 mL of the solidified Muller Hinton were inoculated with 0.1 mL of the suspension of 10^6 bacteria/mL of *E. coli* ATCC 25922 and spread with a sterile Digralsky spatula. Once the inoculum was dry, 3 µL of tetracycline solution (1.33 mg/mL) and 5 µL of washed antibacterial agent-montmorillonite dispersions were added. The plates were then incubated at 37 °C for 24 h and then the inhibition zone diameter was measured. The determinations were made for each antibacterial agent and its corresponding complexes by duplicate. A montmorillonite dispersion (900 mg/L) without antibiotics was analyzed in the agar plate to evaluate possible antibacterial activity.

4.2 Adsorption studies on smectite

The amount of adsorbed Ag^+ from aqueous solutions on natural (Es) and modified smectite (Es-APS) is summarized in Fig. 2.

The Ag^+ adsorption is 2.5 times greater for Es-APS than for Es within the analyzed concentration range. This can be explained by the presence of propylamine groups on the Es-APS surface that represents sites for the Ag^+ cations to interact through the formation of complexes with the metal.

TC adsorption on natural smectite under pH 5 is plotted in Fig. 3. TC adsorption reaches values of 230 mg/g corresponding to 0.48 CEC of the mineral.

Assays conducted under diverse pH conditions showed that the TC capture on the smectite surface depends on the pH conditions. Low pH values in the aqueous media favored the TC adsorption due to the prevalence of the cationic species [26].

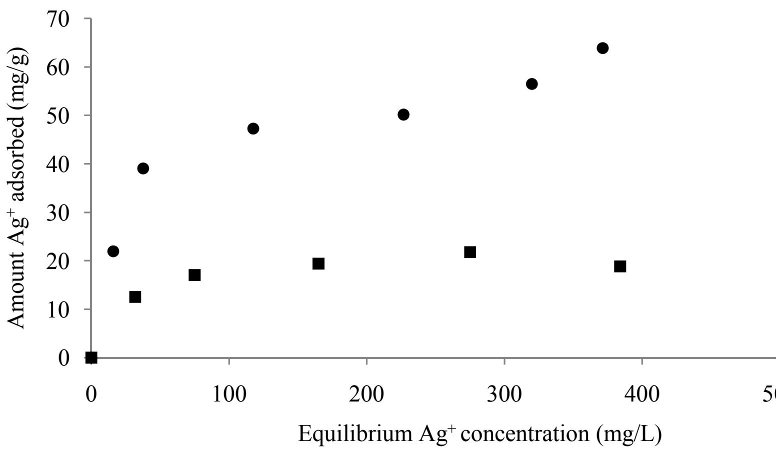

Fig 2 Adsorption of Ag^+ on natural smectite (■) and modified smectite (●).

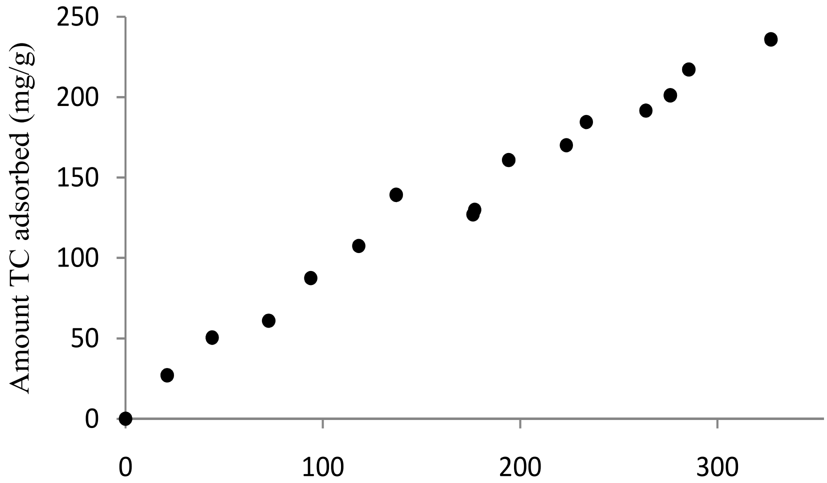

Fig 3 TC adsorption on natural montmorillonite.

From the obtained adsorption results, it can be seen that the natural montmorillonite exhibits a greater affinity towards the organic (TC) in contrast to the inorganic (Ag^+) substance. The highest values of adsorption were 0.50 and 0.16 CEC and mineral, respectively.

4.3 Antimicrobial effect of Ag^+ adsorbed to the clay mineral

Figure 4 illustrates, as an example, the microbial susceptibility assays for the TC solution (1.3 g/L) and the dispersions of the Es-APS-Ag^+ complex in water (25 g/L and 50g/L). Obtained results are summarized in Table 1.

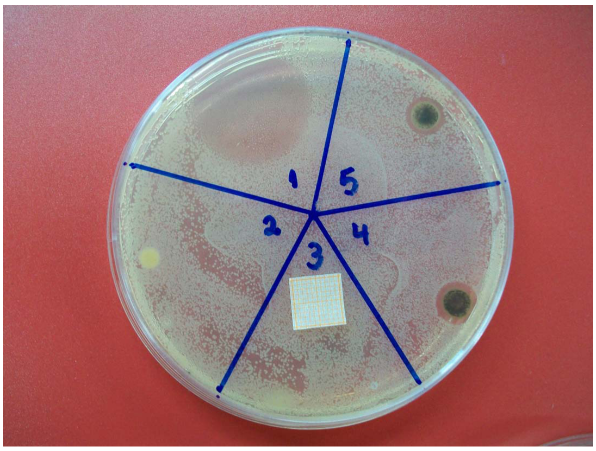

Fig. 4 Comparison of the inhibition zone test: TC solution (1), natural smectite (2), Es-APS-Ag complex dispersion (25 g/L) (4) and Es-APS-Ag complex dispersion (50 g/L) (5).

No antibacterial activity was detected for the natural smectite. The inhibition zone studies indicate that both silver–smectite samples showed suitable antibacterial activity against *E. coli*, with a mean diameter that increased along with the Ag^+ content of the system. Since the modified smectite adsorbed a greater quantity of Ag^+ than the natural smectite, the Es-APS-Ag^+ complex showed to be a stronger antibacterial agent than Es-Ag^+. This suggests that diffusion of this agent took place from the treated clays into the agar.

The inhibition zone for all treated clays was consistently smaller than those for $AgNO_3$. This could be because the Ag^+ diffuses less efficiently from the mineral surface or is tightly bound to the clay surface.

The antibacterial properties of silver-exchanged montmorillonites have been attributed to the attraction, by electrostatic forces, of the negatively charged membrane of the bacteria to the clay surface, where the positive charged silver ions kills the bacteria or inhibits their replication [5, 6].

Table 1 Zone of inhibition test results; all reported data correspond to the mean value

System	Quantity of Antibacterial Agent (mg)	Inhibition zone diameter (mm)
TC	$6.5\ 10^{-3}$	24.0
$AgNO_3$	$1.48\ 10^{-3}$	10.0
$Es\text{-}APS\text{-}Ag^{+}$	$1.03\ 10^{-3}$	7.0
	$2.57\ 10^{-3}$	8.3
	$1.29\ 10^{-2}$	10.0
	$2.57\ 10^{-2}$	10.0
$Es\text{-}Ag^{+}$	$5.93\ 10^{-4}$	6.0
Es	--	0

4.4 Antimicrobial effect of smectite exchanged with quaternary amonium salts

The results of the zone of inhibition tests of exchanged smectite with different cation exchange percentages of HDTMA are given in Table 2.

Table 2 Zone of inhibition test results, all reported data correspond to the mean value

Sample	Inhibition zone diameter (mm)
TC solution (1.33 mg/mL)	24.0
Es	0
Es-0.2HDTMA	0
Es-1.5HDTMA	6.0

The smectites that were exchanged with a quantity of surfactant superior to that of the mineral CEC exhibited suitable antibacterial activity against *E. coli*.

The results are consistent with previous reports in which the antibacterial activity of adsorbed surfactants was found to depend on the amount of surfactant on the surface, and these immobilized surfactants are generally less effective than those studied in aqueous systems [27].

The intercalation of cationic surfactants not only changes the surface properties from hydrophilic to hydrophobic but significantly increases the anion adsorption capacity especially when surfactant loading exceeds the clay CEC. The resulting adsorption of surfactant molecules via hydrophobic bonding [28] and the positive charge of ammonium will attract anions, altering the permeability of the cellular membranes and allowing intercellular ions and low molar mass metabolites to diffuse out.

4.5 Antimicrobial properties of tetracycline and minocycline-montmorillonites

Figure 5 illustrates the antimicrobial effect of the adsorption complexes of TC-natural montmorillonite for two quantities of adsorbed antibiotic: 0.46 mmol/g (0.44 CEC) and 0.76 mmol/g (0.73 CEC). TC solutions and montmorillonite complexes had the same antibiotic concentrations (1.3 g/L).

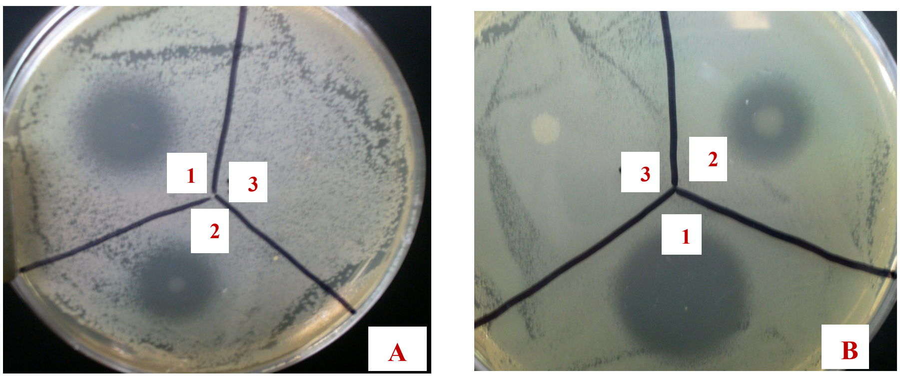

Fig. 5 Inhibition zone test. **A:** 1: TC solution (1.3 mg/mL), 2: TC-montmorillonite (0.46 mmol/g) y 3: montmorillonite dispersion. **B:** 1: TC solution (1.3 mg/mL); 2: TC-montmorillonite (0.76 mmol/g), 3: montmorillonite dispersion.

The inhibition diameters of the dispersions of the adsorption complexes were smaller than those obtained for the TC solution having the same antibiotic concentration. Moreover, along with the increase of the quantity of TC adsorbed to the mineral, there was a considerable increase of the inhibition zone diameter (Table 3).

In the inhibition zone corresponding to the TC-montmorillonite adsorption complexes, a mineral deposit was found in the halo center. These observations would indicate that the TC desorbs from the mineral surface, distributes within the culture media and then, exhibits its antibacterial activity. The quantity of antibiotic that remains adsorbed on the mineral explains the smaller inhibition halos shown by the adsorption complexes in comparison to those from the antibiotic solutions.

Table 3 Zone of inhibition test results; all reported data correspond to the mean value

Sample	Inhibition zone diameter (mm)
TC solution (1.3 g/L)	21.0
TC- montmorillonite (0.46 mmol/g)	14.0
TC- montmorillonite (0.76 mmol/g)	19.0
montmorillonite dispersion	0
MC solution (0.65 g/L)	20.0
MC–montmorillonite (0.50 mmol/g)	15.0
MC–montmorillonite (0.70 mmol/g)	17.0

Table 3 summarizes the antimicrobial effect of the MC-montmorillonita adsorption complexes for two quantities of adsorbed antibiotic: 0.50 mmol/g and 0.70 mmol/g. MC solutions and montmorillonite complexes had the same antibiotic concentrations (0.65 g/L).

TC and MC-montmorillonites exhibited a good antibacterial activity. Results indicate that TC and MC could desorbs from the clay mineral and diffuse into the agar plate.

5. Concluding remarks

The silver and quaternary ammonium surfactant-modified clays showed suitable inhibition properties over *E. coli* growth by the test of susceptibility on solid medium, whereas the samples of natural clay mineral showed no antibacterial activity.

Complexes formed between natural clays and tetracyclines exhibited optimum antibacterial activity against *E.coli*. The inhibition diameters of these complexes were slightly smaller than those for TC and MC solutions. These substances would desorbs from the mineral to display their antibacterial activity, while the amount of antibiotic explains the smaller inhibition halos that the adsorption complexes showed in comparison with those from the antibiotic solutions.

The optimum bacterial inhibition shown by the synthesized materials can be considered to be an interesting material for scientific research of antibacterial composites and provides a promising field of application in several areas related to human and animal health.

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