Periodico di Mineralogia (2013), 82, 1, 89-100 DOI: 10.2451/2013PM0005

PERIODICO di MINERALOGIA
established in 1930

An International Journal of
MINERALOGY, CRYSTALLOGRAPHY, GEOCHEMISTRY, ORE DEPOSITS, PETROLOGY, VOLCANOLOGY
and applied topics on *Environment*, *Archaeometry* and *Cultural Heritage*

Characterization of some tunisian clays to be used as antidiarrhoeic agents

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Abstract

Clays are used as non-specific anti-diarrhoeic materials in several commercial pharmaceutics. Their employment is based on the high sorptive capacities of special clay minerals, in particular smectites and fibrous clays. This use greatly increases the value of these materials. This study was made in order to determine the possible use of some Tunisian clays for the treatment of diarrhea. With this aim, the mineralogical and chemical compositions of the studied samples were determined and subsequently, cation exchange capacities and specific surfaces areas were measured. Finally, the specific adsorption of two micro-organism responsible of this disorder (*Echerichia coli* and *Staphylococcus aureus*) was studied and compared to the adsorption of the dye (methylene blue) mainly used to quantify the ability of a pharmaceutical-grade clay as anti-diarrheic material. According to the mineralogical characterization, the studied samples contain variable amounts of phyllosilicates, with minor quantities of quartz and calcite. In particular, four samples (AYD1, AYD2, HMD1 and BRD1) are mainly made up of smectite (> 66%), whereas the rest (OMV1, SDA1, CAA1 and CBL9) are illitic and kaolinitic clays. Chemical analysis revealed relatively high contents of Fe_2O_3. Smectite and illite-kaolinite rich samples showed, as expected, higher sorptive capacities of the studied micro-organism, with values reaching 95% of *Staphylococcus aureus* and 65% of *Escherichia coli* retained after 90 minutes of interaction. These results are in agreement with those obtained with mehtylene blue, showing promising abilities of most of the studied Tunisian clays to be used in the treatment of these gastric disorders.

Key words: Tunisian clays; adsorption; bacteria; anti-diarrhoeic.

Introduction

Diarrhoeal disease is either an acute or chronic pathological state characterized by an increase in the fluidity of the fasces and the frequency of their evacuation. Its causes are food poisoning, defective intestinal absorption, allergic states and mainly caused by bacterial infections (Carretero, 2002). The most important way of controlling the diseases caused by bacteria is antibiotic treatment. However, abuse or overuse of antibiotics causes various side effects and also results in the emergence and increase of bacteria resistant to antibiotics (Kunin, 1993). It is necessary to develop new types of antibacterial agents that can replace antibiotics as feed additives (Cai-Hong Hu and Mei-Sheng Xia, 2006).

Clays has been shown in studies in vitro to protect the intestinal mucosal barrier and to adsorb toxins, bacteria, and virus (Carretero et al., 2006). Clays are used as non-specific anti-diarrheic materials in several commercial pharmaceutics. Their employments are based principally on their high specific area and their absorption capacity, chemical inertness and low or not toxicity for the patient. This power is already known for a long time, since medicine of the antique put it to profit in preparations of adsorbents for the intestinal treatments. This power of adsorption is most interesting in therapeutic.

One aim of this study is to describe and evaluate some Tunisian clays for possible uses as antidiarrhoeic agents. Another aim is the application of the usual techniques for the description and characterization of some Tunisian clays (CEC, specific surface, XRD, geochemistry, grain size distribution), to improve and to complete the requirements in the principal Pharmacopoeias (European Pharmacopoeia, 2005; US Pharmacopoeia, 2004a).

Materials and methods

The clayey materials investigated in this work come from different deposits located in different region of Tunisia (Figure 1). The smectitic clays were collected in Jebels Aïdoudi (AYD1, AYD2) and Berda (BRD1), belonging to the Aleg formation (Burrolet, 1956) raging in age from Coniacian to low Campanien, and in Jebel Hamadi (HMD1) belonging to the middle member of Abiod formation ranging in age from Campanien to Maastrichtian (Fakhfakh et al., 2007).

The illite-kaolinite clays are sampled in Wealdien facies of El Hamma region (OMV1) ranging in age from Hautervian to Barremien, sample SDA1 (M'Rabet, 1981) collected from Bouhedma formation in Jebel Sidi Aïch, CAA1 sample was collected in Sejnanae region from numedien Oligocene in age., and CBL9 sample come from Sidi Berka formation (Pliocene in age) of Menzel Temim region (Figure 1).

All samples were dried at 60 °C, sieved to 63 μm

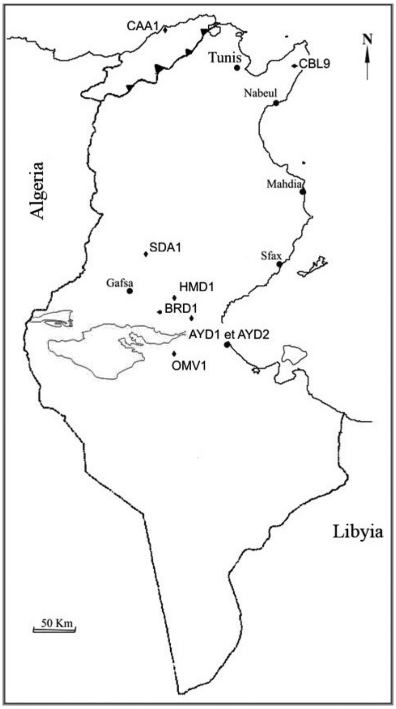

Figure 1. Localization of studied clay sites: CAA1, CBL9, SDA1, BRD1, HMD1, AYD1, AYD2 and OMV1.

and kept in a dry controlled environment for at least 48 h before testing.

Mineralogy and geochemistry

Mineralogical analyses of bulk samples were carried out by X-ray diffraction (XRD), using Philips X′ Pert equipment and Cu Kα radiation. Oriented aggregates were treated with ethylene glycol and heated to 500 °C for 2 h.

The quantitative mineralogical analysis was carried out using information from chemical analyses and from XRD, according to the mineralogical peculiarities of each sample. The values obtained by the classic method (area measurement of peaks and reflective power, e.g. Schultz, 1964; Biscaye, 1965; Barahona, 1974) were corrected according to the chemical composition of the rock, following Torres-Ruiz et al. (1994).

Chemical analyses were done by ICP-ES after $LiBO_2$ fusion. The loss-on-ignition was evaluated as percentage weight difference in one sample heated at 100 °C and 1000 °C. Trace elements were measured in ICP-MS Perkin Elmer® SCIEXElan-5000 equipment, with Rh as internal standard (5% accuracy for 5 ppm).

Morphology, particle size and pore distribution

A Zeiss® DSM 950 scanning electron microscope (SEM) was used to study the morphology of the clay particles. The pore access size and surface area were then determined by mercury injection porosimetry (MIP) using a Micromeritics Autopore III model 9410 porosimeter with 414 MPa maximum pressure, capable of measuring pores between 0.003 and 360 μm in diameter. Grain size distribution was analysed using a Micrometrics Sedigraph 5100, which measures particles up to 150 μm in diameter dispersed in a liquid.

Microbiological analysis

Culture studies designed for microbiological examination involved transfer of colony forming units (CFU) to a nutrient medium. In all cases, clay suspensions were made up with a known amount of clay and the aggregates broken up with a Waring blender using a sodium metaphosphate ($Na_6O_{18}P_6$) as dispersing agent (Viseras et al., 2006). Serial dilutions were then made and replicate 1 ml portions were transferred to each culture medium for incubation. The presence of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella* were also determined by adjustments to media and conditions (selective media and enrichment techniques). When possible, single plate colonies were counted. The number of single colonies was equated to the number of single CFU in the suspension. If plate count was not appropriate, dilution counts were made and the highest dilution that could still provide growth was equated with the more probable number (MPN) of CFU. Three replicates were made of each inoculation for three appropriate serial dilutions.

Pharmacopoeial tests

- pH measurement: according to pharmacopoeia specifications for clay (European Pharmacopoeia, 2005), the pH values of clay water suspensions (2 g/50 ml) were measured after 2 min continuous stirring.
- Sediment volumes (European Pharmacopoeia, 2005) or gel formation (US Pharmacopoeia, 2004a); supernatant volume of suspensions were prepared with 6g of each clay in 200 ml of water using a high-speed mixer at 10,000 rpm and measured after 24 h repose.
- Swelling power: apparent sediment volumes in suspensions of 2g clay in 100 ml were quantified after 2 h.
- Loss in desiccation: in an empties crucible puts 1g of clay, dried at 105 °C for 24 h to measure the difference of weight of a crucible before and after the desiccation of one gram of clay.

Cation-exchange capacity (CEC) and individual *exchangeable cations determination*

Prior to conducting CEC studies, samples were washed thoroughly with deionised water to remove extraneous cations. Then, clay powders (1 g) were dispersed in 25 ml 1M aqueous solution of tetramethylammonium bromide to displace the constituent cations. The dispersions were shaken overnight at 50 rpm in a water bath at 25.0 ±1.0 °C and then filtered. The cations in solution were determined by atomic absorption (Na^+, Ca^{2+}, Mg^{2+}) or atomic emission (K^+) spectroscopy (Perkin Elmer spectrophotometer 5100 mod.) and the CEC was calculated as the sum of exchangeable cations, expressed in meq/100 g dry clay.

Methylene blue adsorption

The official method for the evaluation of the non-specific anti-diarrhoeic capacity of an adsorbent comprises a measure of its capability for the adsorption of methylene blue (MBA test, United States Pharmacopoeial Convention, 2005). The major limitations of this test are associated with the chemical particularities of the dye. Methylene blue is an organic base combined with an acid, the dimensions being 130-135 X 20-25 Å. methylene blue is a basic blue dyestuff. The formula is $C_{16}H_{18}N_3SCl$, with a corresponding molecular weight of 319.87 g/mol, and the structure is reported in Figure 2.

Methylene blue in aqueous solution is a cationic dye, $C_{16}H_{18}N_3S^+$, which absorbs to negatively charged clay surfaces (Hang and Brindley, 1970). At low concentrations, the molecules in solution are unassociated but when they reach concentrations over 5-10 mol/l, associations of two or more molecules result. The Methylene blue

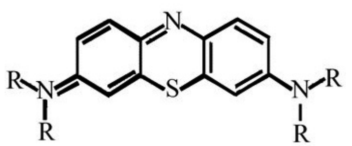

Figure 2. Scheme showing the structural formulas of methylene blue (R = CH3).

employed as adsorbat in this study, is a Biotechnica product with 95% of purity.

A 25 ml of clay/water dispersion (10% w/v) were agitated for 20 min at 400 rpm, with 80 ml of Methylene blue/water solution (0.1% w/v). Exact concentrations of dye aqueous solutions were determined by means of an UV-visible spectrometer operating at the wavelength 640 nm (Cerezo et al., 2001) .

Adsorption bacteria tests

To demonstrate in vitro adsorption capacity of suspension clay against bacteria *Escherichia coli* and *Staphylococcus aureus*, 150 ml of Tunisian clay's preparation were placed in a 250 ml beaker which contained a plastic coated magnetic agitator. 1 ml of a 24 hour broth culture of *Escherichia coli* or *Staphylococcus aureus* was added to the clay preparation and the beaker placed on a magnetic agitator. After five minutes of stirring, 1 ml of clay suspension was removed for culturing. This was repeated at 30 minute intervals for 90 minutes. This experiment was repeated five times to show that the phenomenon was genuine and reproducible.

Results and discussions

Mineralogical composition of the studied samples is indicated in Table 1. The clay sample have an important rate of phyllosilicate (> 71%). According to the mineralogical results two groups of clays were distinguished, the first one (AYD1, AYD2, HMD1 and BRD1) is mainly made up of smectite (> 66%) whereas the rest (OMV1, SDA1, CAA1 and CBL9) are illite-kaolinite rich clays (illite between 13 and 73%, kaolinite between 9 and 58%).

Carbonates exist in almost all samples (13% calcite and 3% dolomite in HMD1 or 8% calcite and 2% dolomite in BRD1) although normally their values are low. Quartz is also present, although their total contents do not exceed 8%, with the exception of the illite-kaolinite rich clay samples (28% and 11% quartz respectively for CAA1 and SDA1).

Chemical analyses of all samples are reported in Table 2 (major elements) and Figure 3 (trace elements). The major elements of the samples revealed higher contents of CaO in smectite rich clay, obviously related to the abundance of carbonate minerals present in the samples.

Iron oxides are abundant in almost al the samples, except for CAA1 (2.27 wt%) and SDA1 (3.83 wt%). High content of K2O was observed for the samples OMV1, SDA1 and CBL9 attribute to the abundance in illite.

Regarding the trace elements, attention should be drawn to the amount of Pb and As, the pharmaceutical limits for clay being respectively 25 ppm and 3 ppm for internal use and 50 ppm and 5 ppm for external use. Al clays clearly fulfil the established limit for external and internal use. The exception is observed for CAA1 this clay is outside the limits for internal use (Figure 3).

Microbiology

Microbiological studies reveal that all samples are free from pathogenic bacteria, the total amount falling within that permitted by regulations (Table 3). USPC (2005) and EPC (2005) respectively establish total aerobic acceptance limits of 5,000 UFC/g and 1,000 UFC/g, respectively for materials to be used in the preparation of non-sterile pharmaceutical products (US Pharmacopoeia, 2005g). Sample SDA1 has values over these limits and should therefore be sterilized before use. None of the samples were

Table 1. Mineralogical composition of the studied samples.

	Phyl	Ill	Ka	Sm	Qz	Cal	Dol	Gy	Fd
AYD1	89	3	9	77	5	5	0	0	1
AYD2	95	0	1	94	4	0	0	0	1
BRD1	82	8	8	66	5	8	2	0	3
HMD1	81	1	2	78	3	13	3	0	0
CAA1	71	13	58	0	28	1	0	0	0
CBL9	85	49	20	16	9	3	0	0	3
OMV1	92	83	9	0	8	0	0	0	0
SDA1	83	73	10	0	11	5	0	0	1

(Phyl = phyllosilicates, Sm = smectite, Ill = illite, Ka = kaolinite, Qz = quartz, Fd = feldspars, Cal = calcite, Dol = dolomite, Gy gypsum)

Table 2. Chemical compositions of the studied clays in oxides wt%.

Ech inf 63 µ	SiO_2	Al_2O_3	Fe_2O_{3T}	CaO	MgO	Na_2O	K_2O	TiO_2	P_2O_5	MnO	LOI
AYD1	49.02	20.61	7.41	3.97	2.29	1.8	1.75	1.02	0.34	0.04	11.27
AYD2	53.93	21.73	8.27	0.64	2.38	1.64	1.58	1.06	0.36	0.03	7.98
BRD1	49.37	20.51	8.18	6.37	2.82	0.71	1.8	1.00	0.43	0.02	8.34
HMD1	44.62	13.68	5.42	12.98	3.17	1.05	1.04	0.64	0.6	0.03	16.16
CAA1	67.02	19.35	2.27	0.07	0.42	0.22	1.6	1.17	0.13	< 2 ppm	6.6
CBL9	52.6	18.95	9.13	3.22	1.57	0.61	2.52	0.97	0.31	0.11	9.2
OMV1	53.3	21.66	7.56	0.2	2.3	0.67	5.87	0.93	0.09	0.03	6.6
SDA1	64.43	17.27	3.83	0.08	1.46	1.35	4.99	0.93	0.12	0.01	5.0

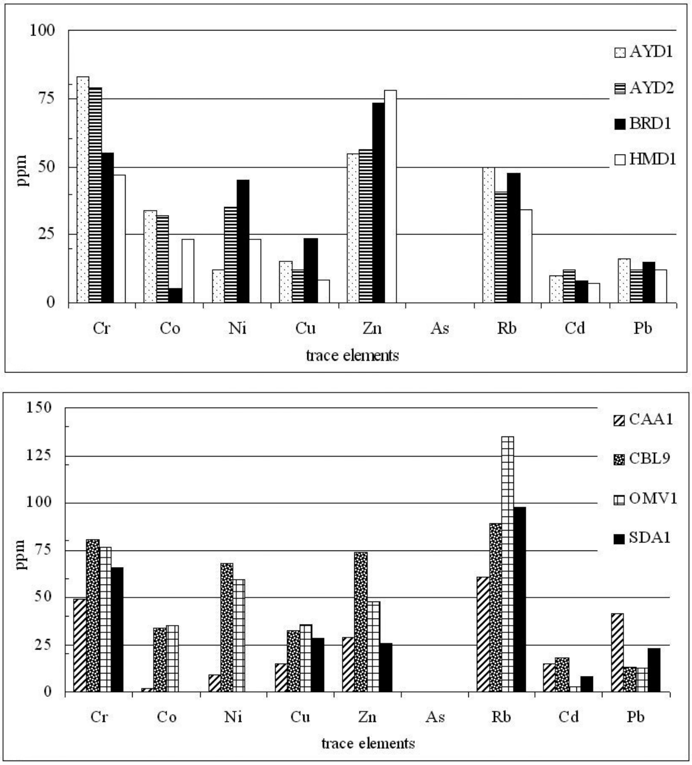

Figure 3. Average trace elements content of the studied samples.

Table 3. Results of microbial analyses (No. UFC/g).

	AYD1	AYD2	HMD1	BRD1	CAA1	CBL9	OMV1	SDA1
Total aerobic	55	55	653	60	746	332	170	1,420
Yeasts and Mildews	0	100	0	550	200	0	0	90
E. coli	0	0	0	0	0	0	0	0
S. aureus	0	0	0	0	0	0	0	0
P. Aeruginosa	0	0	0	0	0	0	0	0
Salmonella	0	0	0	0	0	0	0	0

contaminated by *E. coli*, *P. aeruginosa* or *S. aureus*.

Grain Size distribution

The size distribution determined in the grain fraction less than 63 µm of the studied clays shows that only CBL9, SDA1 and CAA1 have almost less than 47% of particles under 1 µm (Figure 4). The highest frequencies of such particles (> 1 µm) were found in samples BRD1, AYD2, AYD1, HMD1 and OMV1. It must be notified that all samples have more than 70% of

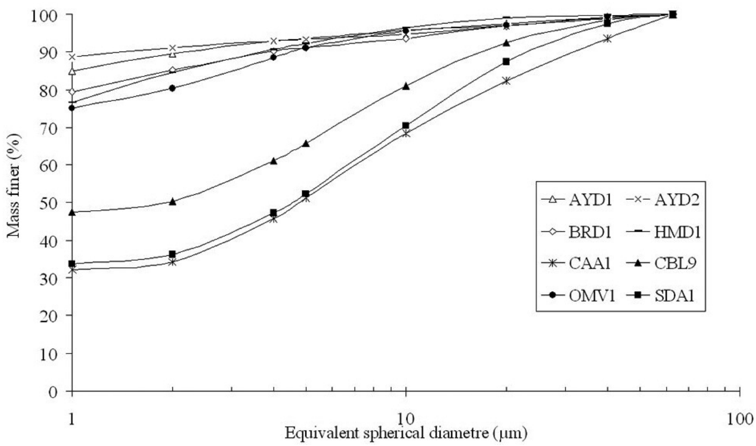

Figure 4. Grain size distribution curves of the studied clays.

particles whose size is lower to 10 μm.

Porosity

Porosity of the samples varies from 51.77% in sample CAA1 to 60.47% in sample CBL9 (Table 4). Porosimetric distribution is characterized by the presence of one main type of pores with r < 2 μm and 5 < r < 12 μm (Figure 5). Nevertheless, some studied clay (BRD1, HMD1, CBL9) show another peak with 18 < r < 38 μm. It is difficult to classify these pores, as the size limits chosen by different authors are arbitrary, depending on the instrumental technique used. Pore size ranges for macro porosity (pore size > 50 nm), meso porosity (2 nm < pore size < 50 nm) and microporosity (pore size < 2 nm) are defined accordingly to the international of pure and applied chemistry IUPAC Gold Book classification McNaught and Wilkinson (1997) and Rouquerol et al. (1999), the pores of the clay studied here should be considered macro pores.

Table 4. Porosity parameters of the studied samples.

	Cumulative intrusion volumes (mL/g)	Specific surface area by weight (m^2/g)	Apparent Porosity (%)
AYD1	0.5983	41.18	60.34
AYD2	0.4666	31.9	55.66
BRD1	0.5356	34.83	54.14
HMD1	0.5276	40.48	55.82
CAA1	0.4946	27.47	51.77
CBL9	0.6085	27.65	60.47
OMV1	0.6130	11.54	52.45
SDA1	0.5604	40.66	58.13

Pharmacopoeial tests

The pH values of the aqueous dispersion meet the pharmacopoeial requirement, with values between 8.3 and 9.5; exception is observed for the CAA1 and SDA1 (6.2 and 6.4). The supernatant volume prepared for sediment volume measurement reveals only a few differences between samples. The value obtained for swelling power of the samples fell within the expected values for smectite (> 22 ml) and for illite kaolinte (< 4 ml) (Table 5).

CEC and Exchangeable Cation

The results (Table 6) show that cation exchange capacities of illite-kaolinite clays are lower (8.81-25.08 meq/100 g) than those of smectite clays reaching 55.81 meq/100 g.

The values of cation exchange capacities make the Tunisian clay sampled suitable for an

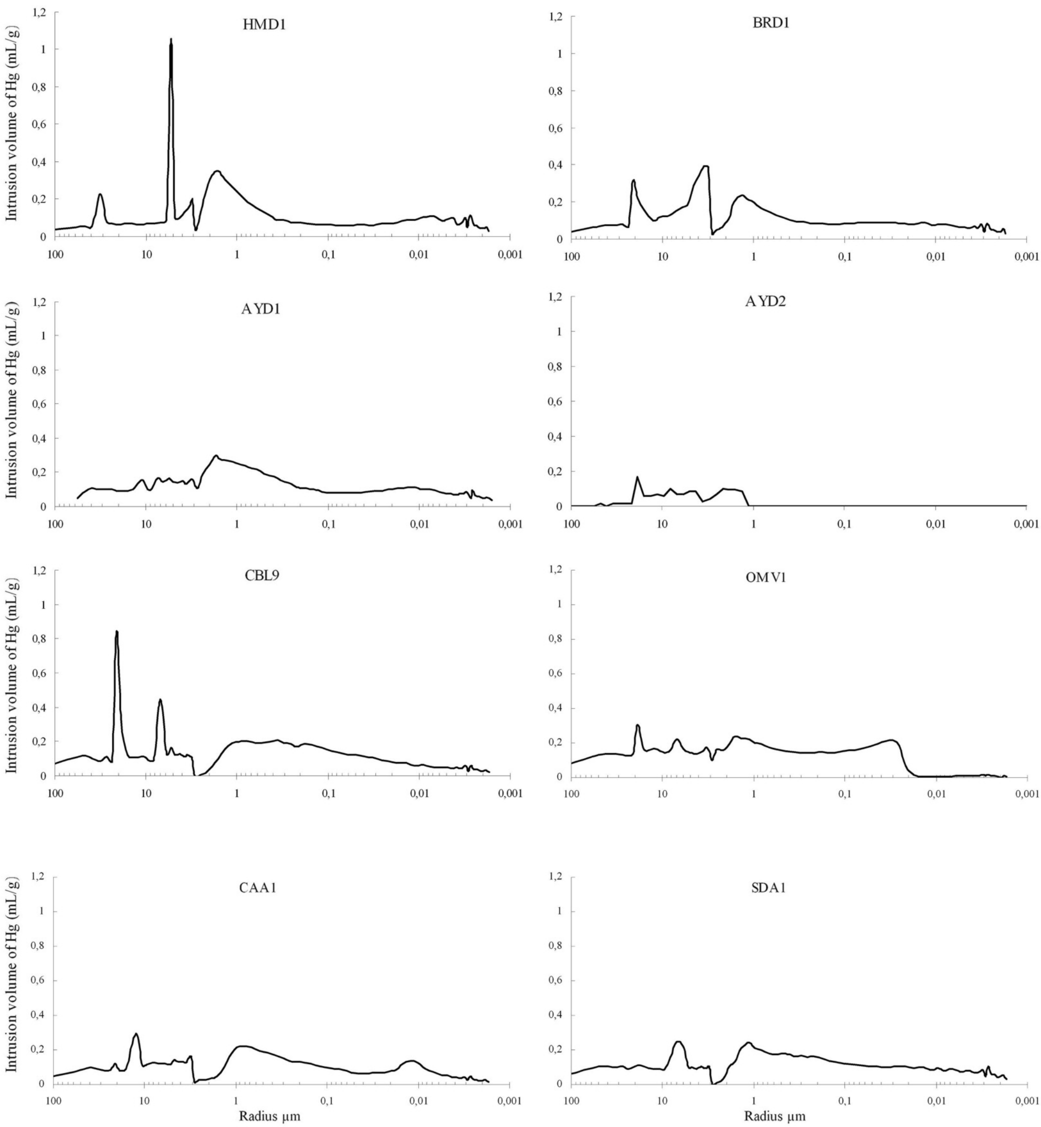

Figure 5. Porosimetric profiles of the studied clays.

utilization as active principles (antidiarrhoeics, gastric bandaging, adsorbents of toxins, bacteria and viruses) and pharmaceutical excipients (agent of suspension, stabilizer and binding) (Carretero M., 2002).

In this case, the main exchangeable cation for CBL9 and HMD1 is Ca^{2+}, allowing these samples to be classified as calcium clays. For the other clays, the main exchangeable cations are: for BRD1 Na^{+} and Mg^{2+}, for AYD2 Na^{+} and Ca^{2+} for AYD1 is Na^{+} (Table 6).

Table 5. Properties of the water suspensions.

	pH values	Supernatant volume	Swelling power	loss in desiccation
AYD1	9.5	0.5	24	4.75
AYD2	8.3	0	37	9.16
BRD1	9.4	2	20	5.17
HMD1	8.8	80	22	6.52
CAA1	6.2	0	2	1.51
CBL9	8.9	0.5	8.5	4.86
OMV1	8.5	0.5	4	2.48
SDA1	6.4	0.5	2	3.74

Bacteria adsorption

The adsorption on clays sampled occurs in different way according to the nature of bacteria and clays. In fact, adsorption kinetics of *Staphylococcus aureus* (Gram+ organism) (Figure 6) showed that the maximum of adsorption of this bacteria occur after 90 minutes of interaction. For BRD1 clays this maximum is reached sooner (60 minutes of interaction). With *Escherichia coli* (Gram- organism) (Figure 7), it was also shown that the maximum of adsorption is reached after 90 minutes of interaction with the studied clays,

Table 6. Exchangeable cation (meq/100 g) of the studied samples.

	Ca^{2+} (meq/100 g)	Mg^{2+} (meq/100 g)	K^+ (meq/100 g)	Na^+ (meq/100 g)	CEC (meq/100 g)
AYD1	20.64	3.28	4.00	27.84	55.77
AYD2	25.6	3.92	2.73	23.57	55.81
BRD1	10.11	18.74	2.57	19.63	51.04
HMD1	29.45	6.375	6.91	17.73	60.46
CAA1	1.21	3.95	0.44	3.21	8.81
CBL9	14.05	5.83	1.08	4.11	25.08
OMV1	4.36	7.33	1.62	9.48	22.79
SDA1	3.82	5.24	1.12	4.13	14.33

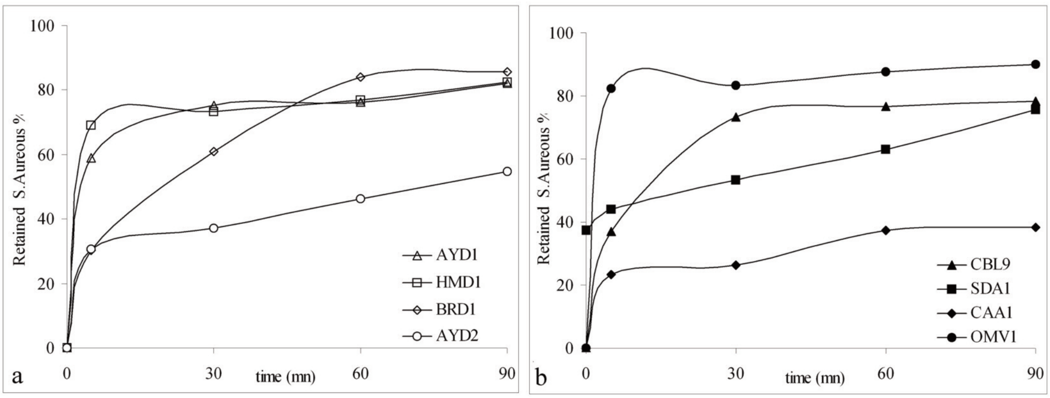

Figure 6. Percentage of *Staphylococcus aureus* adsorbed by smectite rich clays (a) and illite-kaolinite rich clays (b).

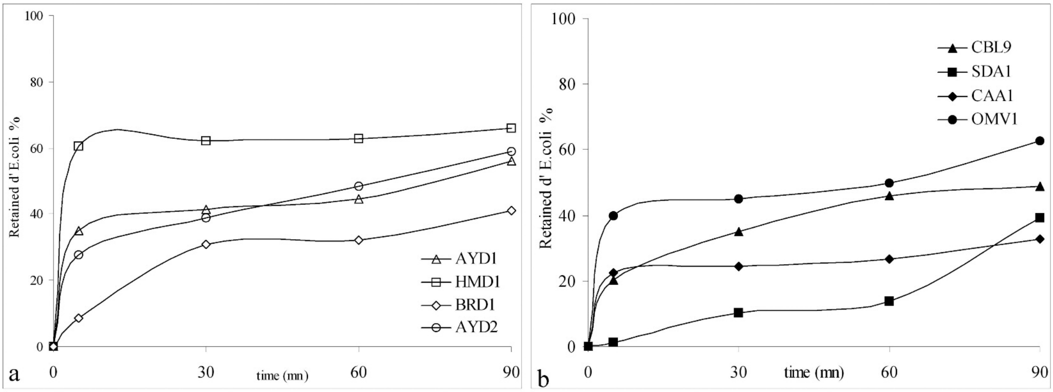

Figure 7. Percentage of *Escherichia coli* adsorbed by smectite rich clays (a) and illite-kaolinite rich clays (b).

except HMD1, for which this maximum (about 60%) is reached after immediately.

These results indicated that by the employed in vitro method, it has been possible to demonstrate that the smectite rich clays suspension can remove significant numbers of certain gram positive bacteria (*S. aureus*) and gram negative bacteria (*E. coli*). Whereas, the illite-kaolinite rich clays are able to adsorb, with the same efficiency, both bacteria Gram+ and Gram-.

Two possible mechanisms for the observed reductions in bacterial counts are proposed. One model involves the adsorption of the bacteria from solution and their immobilization on the surface of the clay rendering them unable to replicate (Herrera, 2000). The second model, inactivation of bacteria by clays may involve killing the bacteria.

Few studies focus on the adsorption of bacteria on clay minerals which are the most active inorganic colloidal components in soils, and the interaction mechanisms between them are still not known (Stotzky, 1989; Jiang et al., 2007). The main reasons for the lack of data on bacteria adsorption on clay minerals were the difficulty in the separation of bacteria in suspension from that adsorbed by clay minerals since the sizes of bacteria and clay minerals are in the same order (Chenu and Stotzky, 2002). Affinity of bacteria for the clays may involve both, electrostatic attraction and hydrophobic interaction. Under physiologic conditions, bacterial cell walls are negatively charged due to functional groups such as carboxylates present in lipoproteins at the surface. At physiological pH, clays exhibit a net negative charge (Stotzky, 1989). Under these conditions, bacteria would not normally be significantly attracted to these clays.

The difference observed in the percentage of adsorption of the two bacteria *Staphylococcus aureus* and *Escherichia coli* can be owed to the structural difference of their envelopes (Hassen et al., 2003). In fact, cell membrane of most of the Gram+ bacteria is thick covered by a porous peptidoglycane layer. Whereas cell membranes of Gram- bacteria are thin and surrounded by two membranes. The outer membrane functions as an efficient barrier permeable because it contains lipopolysaccharides (LPS) and porins.

Methylene blue adsorption

Research on methylene blue adsorption with different pretreatments leads to sample classification according to ability to adsorb the dye and, indirectly, ability to retain micro-organisms.

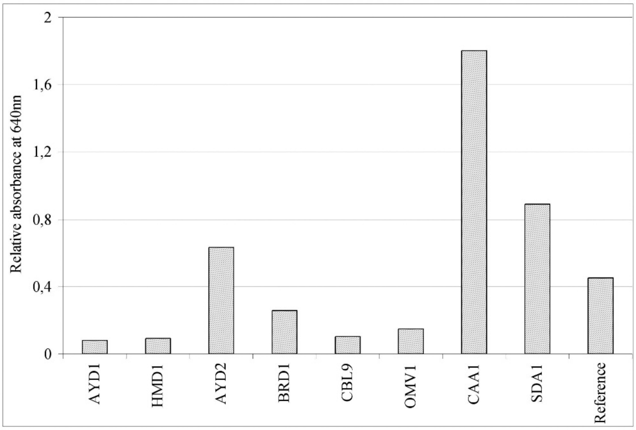

Figure 8. Methylene blue adsorption of the studied clays.

The results shown in Figure 8 correspond to the official test under pharmacopoeial regulation. Figure 8 presents the values of Methylene blue adsorption capacity expressed as absorbance of the tested solution vs. absorbance of the 0.15 µg/ml solution. Absorbance is inversely related to MBA values. As observed, the capacity was higher for the majority of samples except for AYD2, CAA1 and SDA1.

Conclusions

The suitability of the Tunisian clays sampled for pharmaceutical applications is established. The studied samples are divided in two groups. The first is mainly made up of smectite (AYD1, AYD2, HMD1 and BRD1) and the second (OMV1, SDA1, CAA1 and CBL9) contain illite and kaolinite as essential clay minerals.

The chemical composition, and particularly the trace element content, fulfil the pharmacopoeial requirement regarding Pb and As as toxic elements. The values obtained for sediment volume and the swelling power of the samples fell, according to the European Pharmacopoeia, within the expected values for smectite and kaolinite clays, exception for SDA1 and BRD1. The values obtained for sediment volume and the swelling power of the samples fell, according to the European Pharmacopoeia, within the expected values for smectite and kaolinite clays. The values of cationic exchange capacity and specific surface area give the studied smectitic and illitic kaolinitic clays efficient for utilization as pharmaceutical excipient (agent of abeyance, stabilizer and binding) and as active principles (gastric bandaging, adsorbants of toxins, bacteria and viruses). In fact, the studied clays adsorbe efficiently bacteria *Staphylococcus aureus* 77 *Escherichia coli*. This adsorption is selective in the case of smectitic clays, when illitic kaolinitic clays, they are effective in the adsorption of the two types of bacteria.

Acknowledgements

This paper benefited by a review of anonymous referees and of a careful handling of the editor.

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Submitted, June 2012 - Accepted, February 2013