

Bio-chemical analysis of *Datura stramonium* extract

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SUMMARY. *Datura stramonium* is an annual plant which belongs to the *Solanaceae* family. *Datura stramonium* is also a toxic plant, with toxicity given by the presence of alkaloids hyoscyamine, atropine and scopolamine. This study performs phytochemical and antibacterial analysis of ethanolic extracts from *Datura stramonium*. Fresh plants have been used in order to obtain *Datura stramonium* tincture. The quality index was 1:5, the concentration of ethanol being 90%. Based on this tincture a qualitative and quantitative phytochemical analysis was performed through thin layer chromatography and high performance liquid chromatography. By thin-layer chromatography the qualitative alkaloids, such as atropine and scopolamine from *Datura stramonium* extract, have been identified. By high performance liquid chromatography with reversed phase the alkaloids quantity from *Datura stramonium* herbs was assessed and that is 1.7 mg/mL. The plant extracts were tested on Gram negative bacteria *Escherichia coli* and on Gram positive bacteria *Staphylococcus aureus*. Both tested strains showed resistance but for *E. coli* a higher inhibition was observed at all samples containing *Datura* extract.

Key words: atropine, bacterial susceptibility, *Datura stramonium*, High Performance Liquid Chromatography with reversed phase, scopolamine, thin-layer chromatography.

Introduction

Datura stramonium is an annual plant which belongs to *Solanaceae* family and emits an unpleasant odor because of the presence of tropane alkaloids (Kumar,

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2009). *Datura stramonium* is a toxic plant, also the toxicity is given by the presence of alkaloids: hyoscyamine, atropine and scopolamine. Iranbakhsh *et al.*, (2006) determined the quantities of scopolamine and atropine in different stages of growth. The largest amount of scopolamine is found in leaves in the vegetative period, and the smallest amount is found in the root in the vegetative stage. In addition the greatest quantity of atropine is found in the petiole in the vegetative period, and the smallest quantity is found in the seed (Sreenivasa *et al.*, 2012). The total concentration of alkaloids in *Datura stramonium* leaves is 0.2 – 0.5%. More than 70 alkaloids were identified in different parts of the plant, but the main alkaloids are: hyoscyamine, atropine, scopolamine (Fig. 1) (Schmelzer and Gurib – Fakin, 2008; Mann, 2008). Hyoscyamine is a major component when the plant is highly developed. Besides this, the plant also contains scopolamine, atropine, tropine, belladonnine, leucine, glutamic acid, enzymes, citric acid, malic acid, etheric oil, mineral salts, etc.

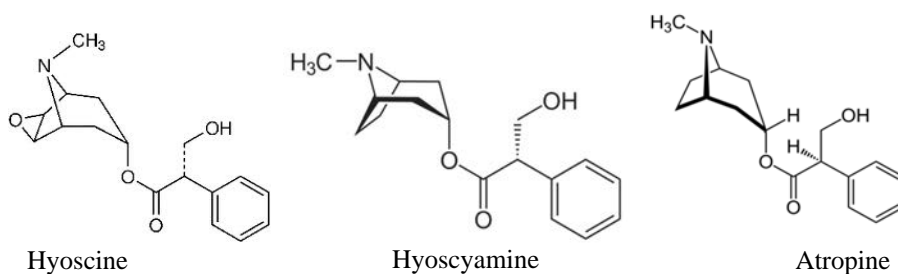


Figure 1. Chemical structure of tropane alkaloids.

Atropine is a tropane alkaloid, isomer of hyoscyamine. It is found in different concentrations in *Hyoscyamus niger*, *Datura stramonium*, *Brugmansia suaveolens*, *Atropa bella-donna*, *Duboisia myoporoides*. The molecular formula is $C_{17}H_{23}NO$. Atropine has two major actions. One of the actions affects the central nervous system (CNS) and provokes an airway stimulation. On the other hand this alkaloid depresses the smooth muscle and the secretory glands which are innervated by the parasympathetic nerves. The central action of atropine is sedative (in paralysis, agitation, Parkinson disease). Peripheral action of atropine refers especially to the secretion of the respiratory glands, bronchial muscles, heart, gastro-intestinal tract, urinary tract (Kasture and Wandodkar, 2008). When it is administrated orally the atropine is absorbed well in the organism and it is eliminated rapidly, the excretion is complete in approximately 24 hours. The normal dose should be 0.4 mg/day by the oral tract or parenteral. If the dose is more than 3 mg it can provoke mental and behavioral changes.

Atropine biosynthesis begins with the amino acid L-phenylalanine. Through transamination the -NH_2 group is transferred and it forms pyruvic acid who in turn is reduced to phenyl lactic acid. Through a series of reaction tropine and litorin is formed which undergoes a rearrangement initiated by the enzyme *p450* forming hyoscyamine aldehyde. Through a dehydrogenation reaction hyoscyamine is formed and through racemization passes into atropine (Behçet, 2014). Atropine is cleaved by the atropinesterase enzyme to the tropic acid in the roots of the plant, *Datura stramonium*.

Hyoscyamine (daturine) is an ester of tropic acid with atropine, being an amino alcohol. It is a secondary metabolite also found in the leaves of *Datura stramonium*. The molecular formula is $\text{C}_{17}\text{H}_{23}\text{NO}_3$. After the plant is dried, the structural composition is modified and hyoscyamine goes through the levogirous isomer called atropine. Hyoscyamine is an antagonist of muscarinic acetylcholine receptors having 98% of the anticholinergic power of atropine.

The place and the hyoscyamine biosynthesis in the plant is similar to atropine (Roberts and Wink, 2013). Hyoscyamine is used in gastrointestinal disorders, spasms, ulcers, pancreatitis, cholics and cystitis. Also it is used in heart problems as well as controlling Parkinson's disease symptoms (Schmelzer and Gurib – Fakin, 2008).

Scopolamine (hyoscine) is a toxic alkaloid met at species of the *Solanaceae* family respectively in *Hyoscyamus niger*, *Brugmansia suaveolens*, *Datura stramonium*, *Duboisia myoporoides*, *Atropa bella-donna*. Scopolamine takes part of the tropane alkaloids class, alkaloids which contain nitrogen in heterocycle and are synthesized from amino. The molecular formula is $\text{C}_{17}\text{H}_{21}\text{NO}_4$. Scopolamine is a hallucinogenic substance if is administrated in high doses, which can even lead to coma or body death (Sweta and Lakshimi, 2015). It has medical properties, frequent are used for motion sickness, it is administrated after surgery because it prevents nausea and vomiting, also it is used as a sedative. Recently it has been shown to be useful as a detoxifier because it has significantly reduced the desire for the use of heroin and its effects (Liu *et al.*, 2013). It is used in intestinal cramps (Alvarez and Marconi, 2011), it has pharmacological properties on the CNS, on the gastrointestinal system, in shortness of breath, and it is also administered to people suffering from Parkinson's disease (Yang *et al.*, 2014). Scopolamine takes part in the class of anesthetic drugs, adjuvants, antiemetics, gastrointestinal and anti-vertigo. The pathway of penetration into the body is transdermal.

The scopolamine biosynthesis can be obtained in 3 steps: a) Beginning from the ornithine amino acid in the presence of the enzyme ornithine decarboxylase is obtained putrescine which in the presence of the enzyme putrescine N-methyl transferase result the N – methyl putrescine. Through a spontaneous reaction tropinone is formed, which in the presence of the tropinone reductase 1 enzyme leads to the obtaining of tropine. b) Starting from shikimic acid, phenylalanine, phenyl pyruvic acid and tropic

acid are obtained. c) The products from step a and b (tropine and tropic acid) leads to the obtaining of hyoscyamine which is transformed in scopolamine by hyoscyamine 6 β hydroxylase enzyme (Facchini, 2001).

The place where biosynthesis begins can be determined through locating alkaloid enzyme of synthesis (Indra, 2012). Thus, the *h6h* enzyme which converts hyoscyamine into scopolamine and *pmt* enzyme which initiates the transformation of putrescine into N-methyl putrescine are found in the pericycle of the root. The pericycle is a thin layer which is found in between the endoderm and the phloem (Dubrovsky and Rost, 2012) and scopolamine is synthesized in the pericycle being translocated through the xylem to aerial parts where it accumulates in the vacuoles (Chandra, 2012; Roberts, 2007; Hashimoto *et al.*, 1991). The mechanism of alkaloids translocation from the root to the aerial parts is controvertible (Kanegae *et al.*, 1994). Scopolamine translocation towards aerial parts of plants is realized with the help of the xylem. More precisely from pericycle the scopolamine passes into xylem. Scopolamine will be translocated with sap xylem to aerial parts of plant (Kandoth *et al.*, 2010). An important factor on which scopolamine depends it is light. Studies made on root crops of *Scopolia carniolica* indicated the fact that the roots kept in darkness strongly stimulates the synthesis of scopolamine. In this article it was realized qualitative and quantitative chemical analysis of the ethanolic extract of *Datura stramonium* by two methods: Thin layer chromatography (CSS) and High Performance Liquid Chromatography with reversed phase (HPLC). Than was determined antimicrobial activity of the *Datura* extract both on *Gram positive* and *Gram negative* bacteria.

Materials and Methods

1. Extraction protocol from *Datura stramonium*. For *Datura stramonium* fresh plants and blossoming herb were used. The extract passes through a series of stages, beginning with the grinding of the used part, up to obtaining the final extract. If all parameters correspond then the entire quantity of the grinded plants is weighed and then the pharmacopoeia quality index is established. The quality index was 1:5, the concentration of ethanol being 90% because it is used the fresh herb. The herb of *Datura stramonium* along with the alcohol mixture were stirred, cleaned and left to macerate for five days in the concentration of ethanol established. During this time the density was determined. It was then filtered and after the analysis it was sealed. *Datura stramonium* extract was obtained from PlantExtract in Rădaia, Cluj County and for compounds analyze from extract of *Datura stramonium* the German Pharmacopoeia was used (HAB, 2011).

2. Identification of the compounds in the extract of *Datura stramonium* by thin-layer chromatography (TLC). Thin layer chromatography is a qualitative method which consists in dividing into three equal areas on the chromatographic plate

and resemble of the compounds in the mixture with specific standards or by identifying compounds based on the values of retention factor. Thin layer chromatography can also be a quantitative method by correlating the concentration with the peak area. In order to analyze the compounds of the *Datura stramonium* extract by TLC a silica gel plate was used with a fluorescent indicator of 254 nm, with a thickness of 0.25 mm and 7x13 cm dimensions. The migration distance is 100 mm, and the eluent is a mixture of ammonia, purified water and acetone (3:7:90, v/v). From the extract, 20 µl were applied.

As standards were used scopolamine hydrobromide (5 mg/mL), atropine sulfate (15 mg/mL) dissolved in 10 mL of methanol and then applied in volumes of 10 µl. The plate was dried at 100-105 °C and was sprayed with diluted Dragendorff reagent and finally with sulfuric acid 0.05 mol/l until the red and red-orange bands appear from yellow to brown. The chromatogram is visualized in visible light.

3. Alkaloid quantification, namely the atropine and scopolamine content in the *Datura stramonium* ethanolic extract using High Performance Liquid Chromatography with reversed phase (HPLC). High performance liquid chromatography is an analytical method whereby the substances in a mixture are identified, separated and dosed. HPLC is an instrument comprising the solvent (constitutes the mobile phase, is used to transport the substance to be analyzed), the pump (continuously pumps the solvent), injector (introduces the sample into the system), chromatographic column (contains the stationary phase), detector (sends the signals to the software of the computer which posts a chromatogram). In this study we used the reversed-phase HPLC (stationary phase is nonpolar and the mobile phase is polar). HPLC protocol was used according to the protocol used by (HAB, 2011).

Sample: At 1 g tincture 1 mL of water and 0.5 mL ammonia are added and the mixture is passed through a chromatographic column with 14 mm internal diameter, filled with 2.5 g granulated Kieselgur. After 15 minutes it is twice eluted with 15 mL ethylic acid. The united eluates are dried. The residue is retaken with 3.0 mL mixture of 65 volumes trifluoroacetic acid and 35 volumes acetonitrile. The sample is filtered through 0.45 µm filter. As standard 0.1 g atropine and scopolamine are used, dissolved in 10 mL methanol. The injection volume was 10 µl, 15 µl, 20 µl, 25 µl, 30 µl of sample and standard.

The HPLC column was silica gel C18, 125 x 4 mm x 4 µm and the mobile phase was represented by A = acetonitrile; B = phosphate buffer pH= 3.5 (15:85 v/v). The injection debit was 0.6 mL/min. The detection is spectrophotometrically performed at 210 nm and the retention time for scopolamine is about 3.5 minutes and for atropine is about 7.6 minutes.

4. Disk paper method for testing the bacteria susceptibility to *Datura stramonium* extract. The susceptibility of the tested bacteria (*Staphylococcus aureus* ATCC 25923, Gram positive and *Escherichia coli* ATCC 25922, Gram negative) to the plant extract was determined using a paper disc diffusion assay on Nutrient Agar plates

(Atlas, 2010), following the method described by Carpa *et al.*, (2014). Bacterial suspensions were adjusted to 0.5 McFarland turbidity ($1-2 \times 10^6$ cfu mL⁻¹) and spread evenly over the entire surface of the agar plates using a sterile cotton swab. The plates were allowed to air-dry for approximately 10 minutes before the paper disc (6 mm) was placed on the agar plate. Each extract test was replicated three times. The plates were incubated at 37°C for 24 hours. For each microorganism tested, zones of inhibition of growth were examined, and the diameter of each zone was recorded.

Results and discussions

1. Qualitative analysis by thin layer chromatography for *Datura stramonium* extract. The silica gel plates were cut according to the dimensions in the protocol. In the meantime the eluent was prepared, composed of a mixture of ammonia, purified water and acetone. On the chromatograph plate 20 µl of *Datura stramonium* extract were applied. As standard a mixture of scopolamine hydrobromide and atropine sulfate was used, out of which 10 µl were applied. After the migration of compounds the plate was pulverized with Dragendorff reactive and sulfuric acid until the bands appear. The chromatogram was observed in visible light (Fig. 2).



Figure 2. Chromatogram of *Datura stramonium* extract plate in visible light

The compounds in the extract were qualitatively identified using thin layer chromatography. The standards chromatogram shows, in the superior third, an orange belt for atropine sulfate and in the median third an orange belt for scopolamine hydrobromide. The sample chromatogram shows belts off the standards, which resemble the belts from the standards chromatogram as regards position, size and color (Fig. 2). The presence of tropane atropine and scopolamine alkaloids is highlighted in the *Datura* extract while the intensity of the belts suggests that atropine is found in a larger quantity than scopolamine. By using eluent the alkaloids in the extract were successfully drawn out.

2. Quantitative analysis of alkaloids in *Datura stramonium* extract by high-performance liquid chromatography (HPLC). The quantities and areas obtained for atropine and scopolamine standards are shown in Table 1. Based on the areas obtained, one calibration curve was obtained for atropine and one for scopolamine, representing the peak area dependent on concentration.

Table 1.

Standard quantity and areas

Atropine, scopolamine quantity (mg/mL)	Atropine area	Scopolamine area
0.1	1058283	5103550
0.15	1587478	7855325
0.20	2096786	10507100
0.25	2545708	12758875
0.30	3174879	15310650

Afterwards the equation of a straight line was established, where x represents the concentration [mg/mL], while y = the area of the peak from the sample. Using the equation, the quantity of compounds in the *Datura stramonium* ethanolic extract was calculated in the moment when the area of the sample pick was reached. The area of the pick for atropine and scopolamine will be taken for the chromatogram of *Datura* extract.

In Fig. 3 is presented the chromatogram of atropine standard.

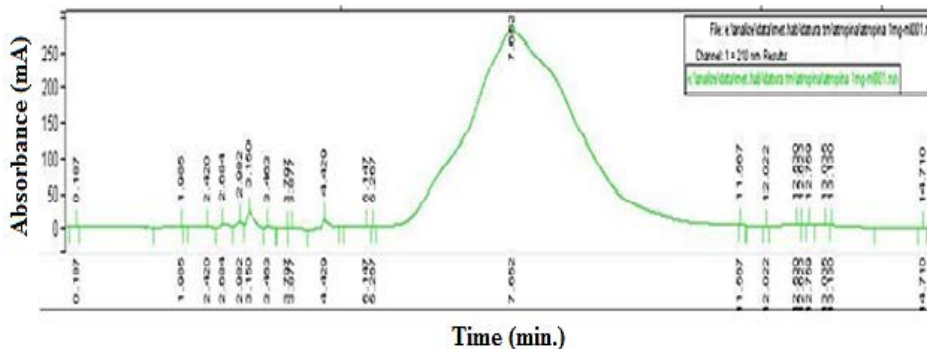


Figure 3. Chromatogram for atropine standard 1 mg/mL at 210 nm.

Fig. 4 shows the absorption spectrum of atropine standard from its chromatogram.

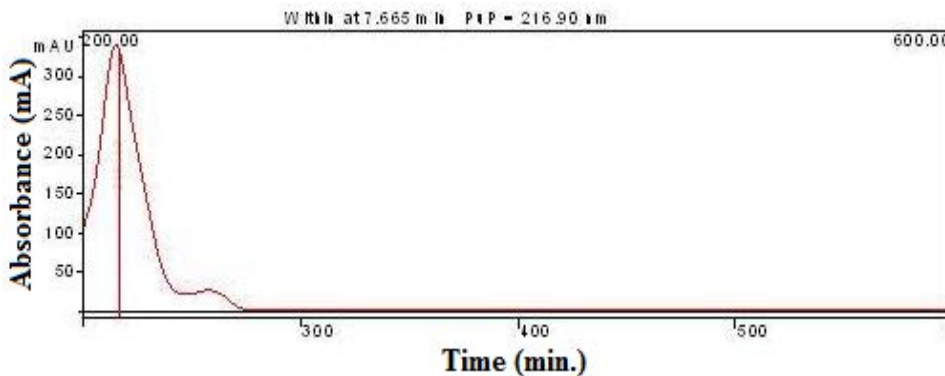


Figure 4. Atropine spectrum in atropine standard chromatogram at 210 nm

From the atropine standard chromatogram can be marked out that atropine will come out in the sample around minute 7.665. Besides atropine scopolamine was identified. The scopolamine standard chromatogram is shown in Fig. 5.

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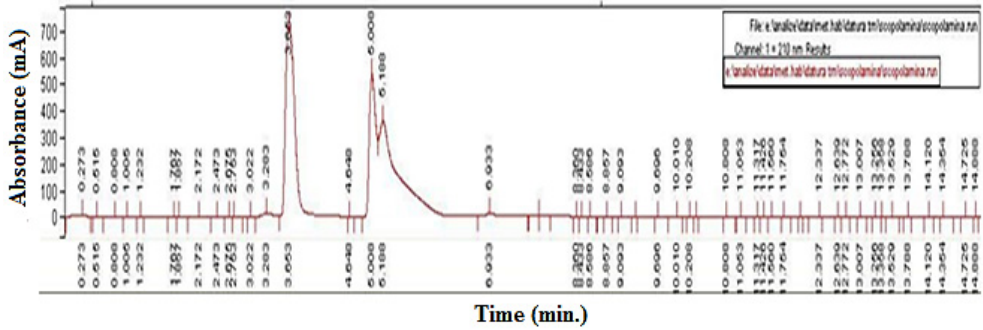


Figure 5. Scopolamine standard chromatogram 1 mg/mL at 210 nm

Based on the chromatogram was established the spectrum of scopolamine alkaloid represented in Fig. 6.

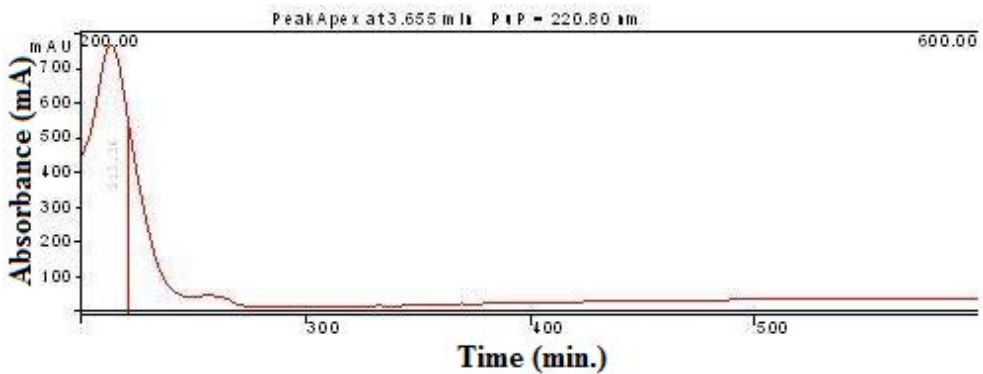


Figure 6. Scopolamine spectrum in the scopolamine standard chromatogram at 210 nm.

Based on the chromatogram we can assess that scopolamine will come out into the sample around minute 3.655. Fig. 7 shows the overlapped chromatograms of the standards (atropine and scopolamine) as well as the ethanolic extract from *Datura stramonium*.

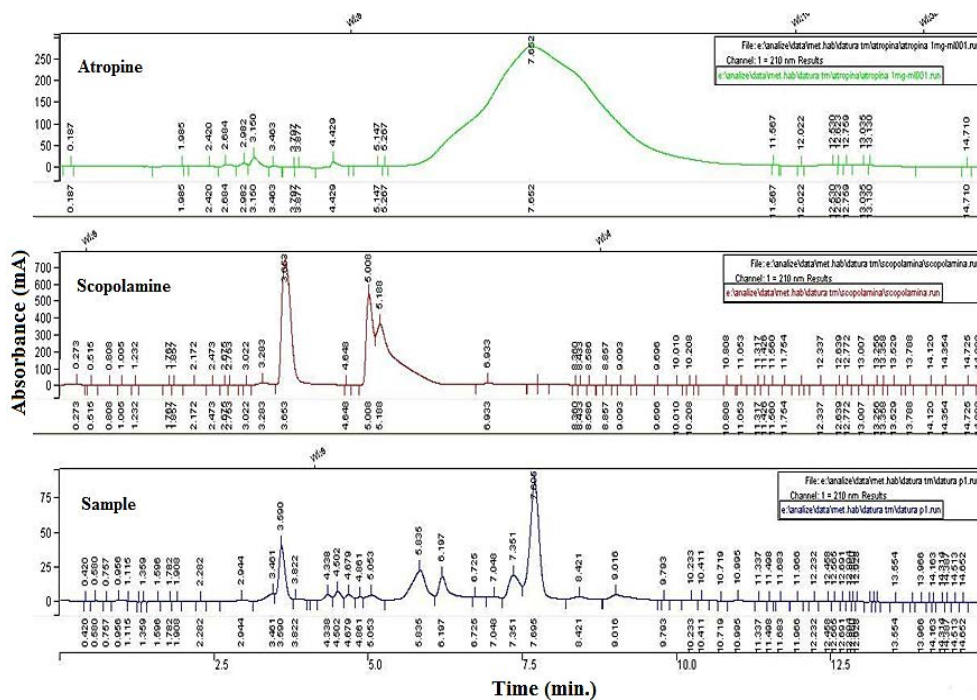


Figure 7. Standards chromatograms and chromatogram of the extract form leaves of *Datura stramonium*

The absorption spectrums of atropine and scopolamine in the sample were also recorded. In Fig. 8 is shown the absorption spectrum of atropine in the ethanolic extract of the herb.

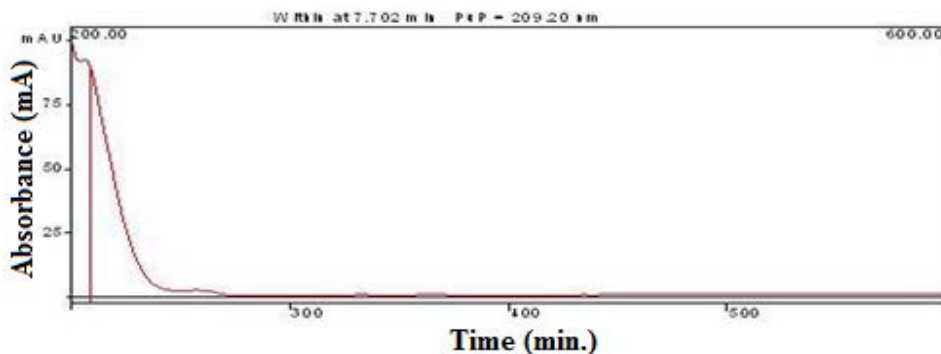


Figure 8. Atropine spectrum in the ethanolic extract of *Datura stramonium*

The absorption spectrum of scopolamine form *Datura stramonium* extract is depicted in Fig. 9.

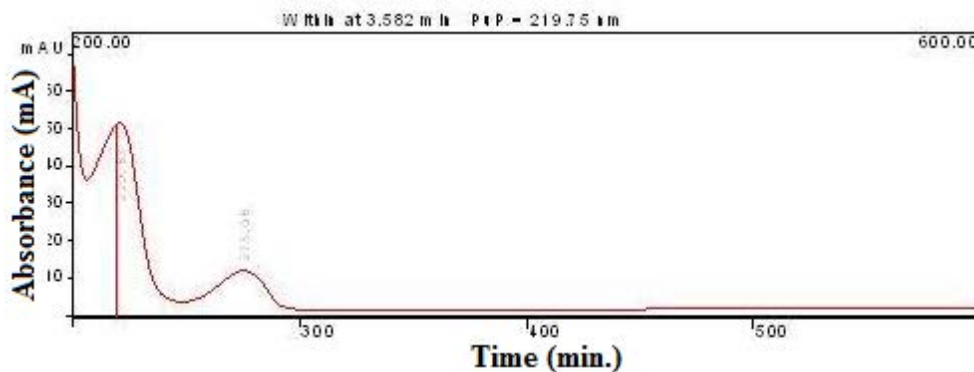


Figure 9. Scopolamine spectrum in the ethanolic extract of *Datura stramonium*

In the ethanolic extract of *Datura stramonium* can be observed an absorption pick for atropine at minute 7.659, and for scopolamine at minute 3.590. These results, compared to the absorption spectrums of standards (atropine and scopolamine) show that the substances are correctly identified. Using the equations from the standard curves of standards and according to the pink area form the extract the alkaloids quantity in an mL of sample was calculated. Based on these areas were calculated the alkaloid concentrations in *Datura stramonium* (Table 2).

Table 2.

The alkaloids quantity in the sample

90% ethanolic extract of <i>Datura stramonium</i>	Pick area in the extract at 10 nm	Quantity (mg/mL)
Atropine	11319234 (at minute 7.695)	1.13 mg/mL
Scopolamine	2906174 (at minute 3.590)	0.57 mg/mL

Both by CSS and HPLC analyses it was observed that atropine is found in a larger quantity in the ethanolic extract of *Datura stramonium*. The other peaks in the chromatogram are represented by other unidentified compounds.

The 3 compounds, hyoscyamine, atropine and scopolamine, present a special medical importance, being anticholinergic, antimuscarinic, in competition with the acetylcholine neurotransmitter present in the central and peripheral nervous system.

In case atropine or scopolamine are bound to muscarinic receptors in place of acetylcholine, the cleavage mechanism is inhibited (Akai *et al.*, 2014). Blocking of muscarinic receptors by scopolamine triggers increasing the cardiac frequency, which makes it useful in bradycardia, at urinary retention and overactive bladder. In small doses they have a sedative effect, but in large doses they have negative effects on the organism, causing agitation, disorientation, hallucinations, delirium, mental confusion and insomnia (Schmelzer and Gurib – Fakin, 2008).

3. Testing bacteria sensibility to *Datura stramonium* extract. The test strains (*Staphylococcus aureus* for Gram-positive bacteria and *Escherichia coli* for Gram-negative bacteria) were inoculated on Petri dishes with Nutrient Agar (Atlas, 2010), in aseptic conditions. At the inoculation of the test microorganism 1 mL of bacterial suspension, spread on all the surface of the medium, was used, and the surplus of culture was eliminated. After the drying of the inoculated media 6 mm paper disks were applied (Fig. 10).

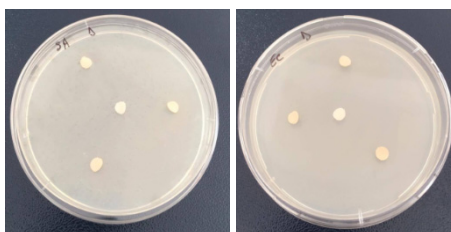


Figure 10. Applying paper disks on the NA culture medium inoculated with *Staphylococcus aureus* and *Escherichia coli* (0 moment).

The Petri dishes were incubated 18-24 h. After 24h incubation the results were assessed by measuring the emerged inhibition zone, in millimeters (Fig. 11), at *Staphylococcus aureus* and *Escherichia coli* strains. In Fig. 13 it is visible that at Gram-positive strain (*Staphylococcus aureus*) the sensibility is very high at all the tested samples, but also at Gram-negative strain (*E. coli*) the sensibility is very high at all tested samples.

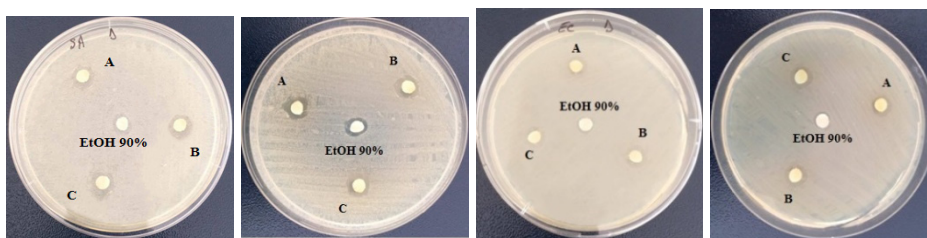


Figure 11. Assessing the sensibility *Staphylococcus aureus* and *Escherichia coli* with paper disks with *Datura stramonium* extract at 24 incubation hours.

Antibacterial and antifungal activity of plant extracts depends on the solvent used (ethanol, methanol, water, chloroform, benzene, petroleum ether). It was proved that if methanol is used as solvent a large alkaloid concentration is extracted and a small concentration of iridoids, flavonoids, saponins, sterols, tannins. Likewise, if the petroleum ether is used as extraction solvent, a very large alkaloids quantity and a smaller flavonoids concentration are obtained (Sreenivasa *et al.*, 2012). The methanolic leaves extracts showed antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Rhizopus stolonifer* (Sharma and Sharma, 2013). At a higher concentration of ethanolic stramonium extract antimicrobial activity against *Klebsiella pneumoniae*, *Fusarium sp.* and *Aspergillus niger* was observed (Reddy, 2009). The aqueous extracts from raw *Datura stramonium* leaves showed inhibition on *Bacillus*, *E. coli* and *Klebsiella* and a high sensibility on *S. aureus* and *Sarcina* (Shobha *et al.*, 2014). The raw extract of *Datura* leaves, using ethyl acetate as solvent displayed a good inhibition against *S. aureus* resistant to methicillin, the strain isolated from festering samples of skin infections. (Venkanna *et al.*, 2013).

Conclusions

Datura stramonium is a plant which belongs to *Solanaceae* family and contains alkaloids as atropine, hyoscyamine and scopolamine, considered anticholinergics, which are biosynthesized in root, transported through xylem to the aerial part and stored in vacuoles. In order to obtain ethanolic *Datura* extract fresh herb is used and a 90% ethanol concentration.

By CSS alkaloids as atropine and scopolamine were qualitatively identified at the *Datura* extract.

By HPLC the alkaloids quantity in the extract was assessed: 1.7 mg/mL (out of which 1.13 for atropine and 0.57 for scopolamine).

For testing the sensibility of *E. coli* and *S. aureus* the paper disks method, with 6 mm paper disks impregnated with 40 µl extract, was used. Both tested strains showed resistance but at *E. coli* a higher inhibition was observed at all the samples containing *Datura* extract. Not only the identified compounds (atropine and scopolamine) are responsible of the antimicrobial activity, but all the secondary metabolites present in the extract.

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