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Study of the Chemical Composition and Amtimicrobial Action of *Dillenica Indica* Peel, Fruit and Leaves Extracts

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***Abstract*—** *The use of medicinal plants has grown over the years, this is due to the popular culture that already exists and to the increase in people's knowledge about the benefits of these plants. Dillenia indica popularly known as elephant apple or april flower is considered a medicinal plant that, according to studies, has antidepressant, anti-leukemic, anti-inflammatory, antioxidant, anti-diabetic, anti-hyperlipidemic, antimicrobial, cytotoxic and anxiolytic properties. This factor aroused interest in obtaining extracts of this to evaluate the antimicrobial action of these extracts. To obtain the extracts, separate samples of the leaves, bark and seeds were kept in contact with ethyl acetate for 3 days with daily agitation. After this period, the extract was filtered and dried by rotary evaporation. The analysis of the chemical composition of the extracts was performed by a Gas Chromatography coupled with Mass Spectrometry. The antimicrobial effect of the extracts was verified by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The values of MBC and MIC of the extracts of leaves, bark and seeds against the microorganisms in question were 0.1% v/v. Employing chromatography it was possible to identify several organic acids in the three extracts of D. indica. These acids are probably the compounds responsible for the antibacterial activity shown by the studied extracts.*

I. INTRODUCTION

According to Maciel, Pinto and Veiga (2002), the popular culture of using medicinal plants and the efficiency of their use, that is, the beneficial effects that their use provides, collaborate in a significant way for the practice of consumption of medicines plants. As a result, this popular culture arouses the curiosity and interest of researchers in developing this natural resource for medicinal purposes. According to Jawla et al. (2009), medicinal plants have provided many clues to fighting diseases since the emergence of civilization.

India is one of the 12 biodiversity centers in the world, with more than 45.000 different plant species (Jawla et al., 2009). There are many species of plants that have been used by tribal communities and in various regions of India, but their pharmacological and phyto-pharmacological importance are still unknown as these plants are rarely available. Among these plants there are several belonging to the family *Dilleniaceae*, which are not very well known, but have considerable medicinal value (Gandhi & Mehta, 2013). According to Bhagyasri et al. (2017), several studies report the potential of *D. indica* (Figure 1) to assist

in wound healing, diabetes, bone fracture, cuts, burns and abdominal pain.

A B C

Fig.1: Dillenica indica: A- fruit, B- cross section of the fruit, C- fruit pulp.

D. indica is known for its antidepressant, anti-leukemic, anti-inflammatory, antioxidant, anti-diabetic, anti-hyperlipidemic, anti-microbial, cytotoxic and anxiolytic properties (Kumar, Kumar and Prakash, 2011). The genus Dillenia has 60 species, but only the plants *D. indica* and *D. pentagyna* are considered to have significant medicinal value. The leaf, bark and fruit of these plants are used as traditional medicine and have therapeutic effects (Gandhi & Mehta, 2013). The plant is a small to medium sized tree growing up to 15 m in height. Its leaves are 15 to 36 centimeters long, with a visibly wavy surface with printed veins (Bhagyasri et al., 2017) and the flowers are large, 15 to 20 centimeters in diameter with five white petals and numerous yellow stamens. The fruits are 10 to 15 cm in diameter, with undefined and persistent sepals, fleshy and slightly swollen. The seed contains 5 or more carpels, soaked in compressed, glutinous pulp, with hairy margins. Fruit production occurs from July to August and ripens in November and December. The flowers occur in May and June (Gandhi & Mehta, 2013).

According to Kumar, Kumar and Prakash (2011), the methanolic extract of fruits of *D. indica* L. display significant antileukemic activity in human leukemic cell lines. This finding led to the chromatographic fractionation of the methanolic extract and from this fractionation, the ethyl acetate fraction displayed the greatest anti-leukemic activity. Bhagyasri et al. (2017) comment that the main compound was betulinic acid, and that betulinic acid could explain the anti-leukemic activity of the methanolic extract and the ethyl acetate fraction. Apu et al. (2010) reported the antimicrobial properties of *D. indica* ethyl acetate leaf extract against Gram-positive bacteria (*Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus and Scarina lutea*) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella paratyphi, Salmonella typhi, Shigella boydii, Shigella dysenteriae, Vibrio mimicus* and *Vibrio mimicus parahemolyticus).* Haque et al. (2008) commented that the ethyl acetate leaf extract had antifungal properties against *Candida albicans, Aspergillus niger* and *Saccharomyces cerevisiae*.

Considering the above, the present study aimed to evaluate the chemical composition and antimicrobial activity of the extracts of the *D. indica*.

II. METHOD

Sampling: The fruits of the *D. indica* plant were collected in the months of April, May and October of 2019 in Guará, São Paulo state - Brazil, located at latitude 20º25'42 "south and at longitude 47º49'27" west, where there are a considerable number of trees of this species.

Obtaining the extracts: For the extraction of *D. indica*, ethyl acetate solvent and three parts of the plant were used: leaves, seeds, and fruit peel. The fruits were separated and cut into small pieces and immediately afterwards were placed in flasks together with each of the solvents separately and sealed so that the volatilization of the solvents would not occur. The leaves were placed in separate flasks with for each of the solvents and sealed. The three parts of the plants were kept immersed in the solvent separately for seven days. After that, the acetate extract was filtered and concentrated. This extraction process was carried out in triplicate, and the extracts were stored in amber sealed amber flasks, under refrigeration until analysis.

Microorganisms tested: The reference strains used were *E. coli* (ATCC 35218), *S. aureus* (ATCC 29213) and *B. cereus* (ATCC 11778). The bacterial strain was reactivated using Müeller Hinton agar and incubated at 37°C for 24 hours. For this study, bacterial suspensions prepared using the direct suspension method were used as inoculum, in which four colonies were suspended in sterile saline solution and adjusted to the standard 0.5 of the McFarland scale in a spectrophotometer at 625 nm. This procedure ensures that each milliliter of the inoculum has approximately $1.5x10^8$ Colony Forming Units (CFU) ((NCCLS, 2003a). For the extract, saline solution was used as a positive control and chlorhexidine was used as negative control.

Minimum Inhibitory Concentration (MIC): The determination of the minimum inhibitory concentration (MIC) of the extracts obtained in this study was performed in microdilution plates with 96 wells arranged in 12 columns and 8 rows. First, an initial standard solution of the extract with a concentration of 8% was prepared using

0.4 ml of the extract, 0.05 ml of Tween 80 and 4.2 ml of sterile distilled water. In the microdilution plate, 6 dilutions were tested for each microorganism. In each of the wells of the plate, 100 µL of the Müeller Hinton broth were added. Then 100 µL of the initial standard solution of each extract was added to the second line (B) and the subsequent concentrations were obtained through serial dilution, resulting in concentrations of 4% to 0.1%. In the end, 100 µL of the contents were dispensed in the wells of the last line, so that the volume would be equal to the others. The wells of the 1st row were used as growth control, the extract was not added and the wells of the 8th row as negative control (Chlorhexidine 2%). At the end, 10 µL of the bacterial suspension were added to all wells and the plates with the bacteria were incubated at 37°C for 48 hours. MIC was the lowest concentration that completely inhibited growth, that is, in which no turbidity was observed in the medium. The tests were performed in triplicate (Cavalcanti, Almeida and Padilha, 2011, NCCLS, 2003b)

Minimum bactericidal concentration (MBC): To determine the antibacterial activity of the *D. indica* extract, the MBC was determined. The analysis consists of adding extract concentrations equal to or greater than that of the MIC to tubes containing BHI broth. Subsequently, bacteria were inoculated into the tubes, which are intended to analyze the antibacterial effect. For control, tubes were produced only with BHI and extract. The tubes were incubated for 16 hours, at a temperature of 37º under agitation. After that, they passed through a centrifuge where the supernatant was discarded, and the bacterial cells were resuspended in BHI broth and inoculated in the plates containing the appropriate culture medium. The plates were incubated at the appropriate temperature and time for the growth of each bacterium, after which the plates were analyzed visually (Santurio et al., 2007).

Gas chromatography coupled to Mass Spectrometry (GC-MS): GC-MS analyzes were carried out in collaboration with Ourofino AgroCiência in Uberaba-MG-Brazil. For GC-MS analysis, samples were prepared by weighing 1g of extract in a 10 mL volumetric flask. Then, 5 ml of HPLC grade acetone were added and the system was ultrasound for 10 minutes. The volume of the volumetric flask was measured, the solution homogenized and filtered through a 0.45 µm RC filter. The analyzes by GC-MS were performed on a High-Resolution Gas Chromatograph, Shimadzu, model 2010 with Mass Spectrometry Detector. Column: Agilent DB-5MS (30 m x 0.25 mm - 0.25 µm). The operating conditions were: Injector temperature: 220° C, Injection Mode: Splitless, Sampling time: 2 minutes, Flow control mode: Linear speed (45.0 cm.seg-1), Pressure: 15.7 psi, Total flow: 19.4 mL min-1, Column flow: 1.49 mL min-1, Column temperature: Gradient mode, as shown in the table 1:

Table 1 - Data used in the temperature gradient for the analysis of GC-MS.

Ratio (°C/min)	Final Temperature (°C)	Residence time (min)
-	80	2
40	140	-
10	280	-

The Parameters of the Mass Spectrometry Detector were: Ion source temperature: 200° C, Interface temperature: 280° C, Solvent cutting time: 3 minutes, Detector voltage: Relating to the result of the Tuning, Initial detection time: 3.0 minutes, Final detection time: 17.0 minutes, Acquisition mode: SCAN, Acquisition time: 0.25 seconds, SCAN mass / charge ratio (m / z): 40 to 600, Injection volume: 1 µl.

III. RESULTS AND DISCUSSION

MIC and MBC

The MIC values of *D. indica* extracts from leaves, bark and seeds against *E. coli*, *S. aureus* and *B. cereus* were 0.1% v/v for the three analyzed extracts. This result was more efficient than the work of Zauli et al. (2004) which analyzed MIC and MBC for *D. indica* against *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538), *S. typhimurium* (ATCC 14028), *P. aeruginosa* (ATCC 25619) *Streptococcus mutans* (ATCC 25175) *S. salivarius* (CDC 262) and obtained in the MIC 95.8 mg/mL for *E. coli* and *S. typhimurium* and 47.9 mg/mL for *S. aureus*, *P. aeruginosa*, *S. mutans* and *S. salivarius* and in the MBC concentration of 71.85 mg/mL for *S. aureus*, *P. aeruginosa*, *S. mutans* and *S. salivarius*, and greater than 95.8 mg/mL for *E. coli* and *S. typhimurium*.

Apu et al. (2010) investigated the leaves of the methanolic crude extract of *D. indica* Linn. (*Dilleniaceae*) for the evaluation of antimicrobial activities. Antimicrobial activity was determined using the disk diffusion method. The mean zone of inhibition ranged from 6 to 8 mm at a concentration of 400 µg/disc. Alam, Chowdhury and Mazumder (2011), tested the methanolic extract of the bark of *D. indica* against four Gram positive and seven Gram negative bacteria and remarkable activities against all the tested bacteria were observed. The lowest minimum inhibitory concentration (MIC) value was observed in *Staphylococcus aureus* and was 0.312 %. Reddy et al. (2009), commented that the hexane extract from the seed powder of *D. indica* was evaluated for antimicrobial and antioxidant activities and exhibited a broad spectrum of

antimicrobial activity. MIC values for different bacterial and fungal strains ranged in concentration from 1.0 to 2.0 mg/ml.

GC-MS of bark extracts

After the separation by gas chromatography of the extract of the *D. indica* bark, 9 peaks were obtained, as given in Figure 2, which were analyzed by Mass Spectrometry for their structural determination.

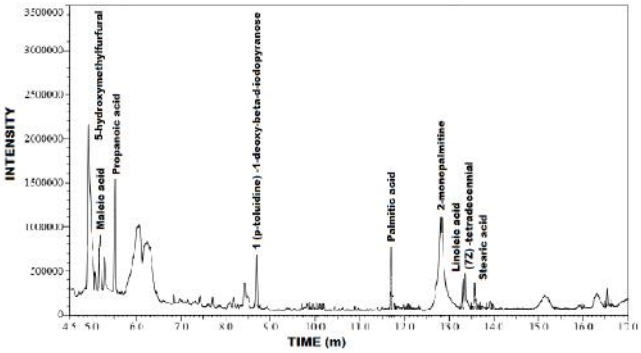


Fig.2: Chromatogram of separation of the D. indica bark extract by GC-MS.

GC-MS of pulp extract (seed)

After the gas chromatographic separation of the pulp extract of the *D. indica* seed, 9 peaks were also obtained (Figure 3), which were analyzed by Mass Spectrometry for their structural determination.

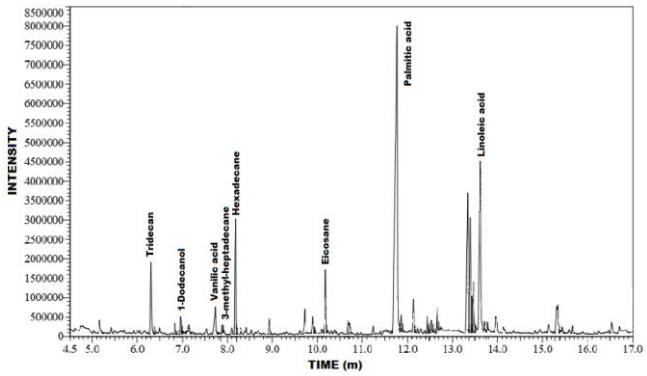


Fig.3: Chromatogram of separation of the D. indica seed extract by GC-MS.

1) GC-MS of leave extract

After gas chromatographic separation of the *D. indica* leaf extract, 4 peaks were also obtained, as shown in Figure 4, which were analyzed by Mass Spectrometry for their structural determination.

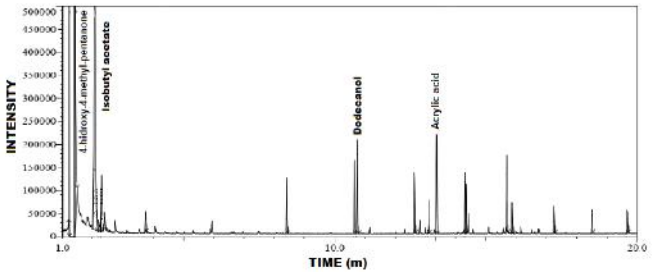


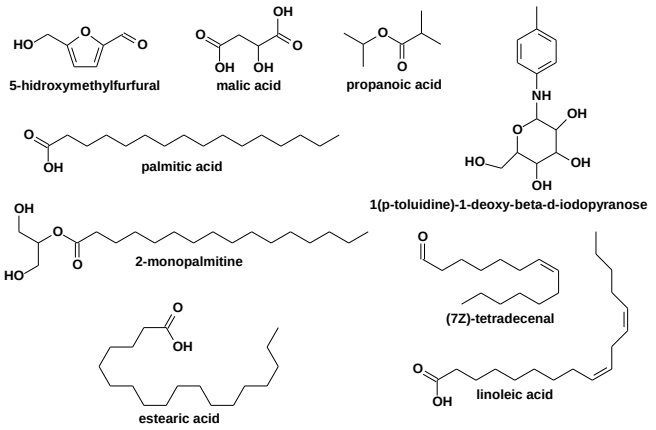
Fig.4: Chromatogram of separation of the extract of the leaves of D. indica by GC-MS.

Table 2 summarizes the chemical compounds found in the studied extracts and figure 5 summarizes the chemical structures of the compounds found in the studied extracts.

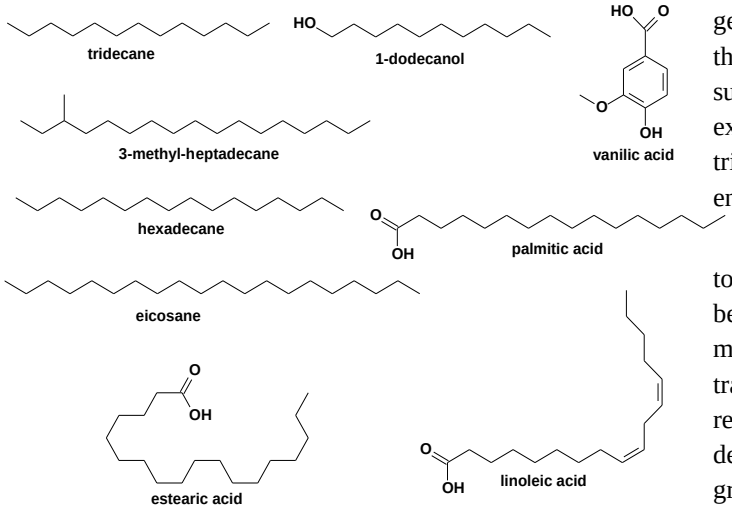
Table 2. Compounds present in the studied extracts of D. indica.

Bark Extract	Pulp extract (seed)	Leave extract
• 5-hidroxymethylfurfural • Malic acid • Propanoic acid • 1(p-toluidine)-1-deoxy-beta-d-iodopyranose • Palmitic acid • 2-monopalmitine • Linoleic acid • (7Z)-tetradecenal • Estearic acid	• Tridecane • 1-dodecanol • Vanilic acid • 3-methyl-heptadecane • Hexadecane • Eicosane • Palmitic acid • Linoleic acid • Estearic acid	• 4-hidroxy-4-methyl-pentanone • Isobutyl acetate • dodecanol • Acrylic acid

BARK EXTRACT



PULP EXTRACT (SEED)



LEAF EXTRACT

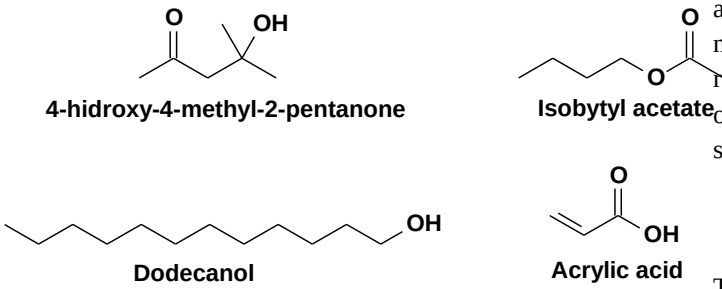


Fig.5: Chemical structure of the compounds presents in the extract of the bark of D. indica.

Analyzing Table 2 and Figure 5, it is possible to observe the presence of several organic acids in the three extracts of *D. indica*. The organic acids have been used as food additives and preservatives to prevent food spoilage and prolong the shelf life of perishable foods. As a group, these compounds mainly include straight chain saturated monocarboxylic acids and their derivatives (unsaturated, hydroxyl, phenolic and multicarboxylic versions) and are known as fatty acids[15]. Thus, probably, the organic acids present in the studied extracts are the compounds responsible for the antibacterial activity presented by the studied extracts.

According to Ricke (2003) the potential bacterial targets of biocidal compounds include the cell wall and the cytoplasmic membrane. Although the antibacterial mechanisms for organic acids are not fully understood, there are some proposals for the mode of action of these compounds. Given the weak acidic nature of most of these compounds, pH is considered a determinant of effectiveness, because it affects the concentration of undissolved acid formed. Non-dissociated forms of organic acids can penetrate the lipid membrane of the bacterial cell and, once at the neutral pH of the cell cytoplasm, dissociate into anions and protons. The generation of both species causes problems for the bacteria that must maintain a cytoplasm with a pH close to 7 to support the functional macromolecules. Excess proton exports require consumption of cellular adenosine triphosphate (ATP) and can result in depletion of cellular energy.

As stated by Ricke (2003), organic acids are able to decouple the cytoplasmic membrane. Organic acids are believed to interfere with the structure of the cytoplasmic membrane and membrane proteins, so that electron transport is decoupled, and subsequent ATP production is reduced. Another hypothesis is that organic acids serve as decouplers that generally dissipate pH and electrical gradients across cell membranes.

Less direct antibacterial activities have also been attributed to organic acids and include interference with nutrient transport, damage to the cytoplasmic membrane resulting in leakage, disruption of the permeability of the outer membrane and influence of macromolecular synthesis (Ricke, 2003).

IV. CONCLUSIONS

Through this study it can be concluded that the extracts of *D. indica* (leaves, fruits and seeds) showed antimicrobial activity, with antimicrobial efficiency of the three extracts against all tested microorganisms. From the chemical evaluation made with the extracts, there are many organic acids in their constitution and these compounds are possibly responsible for the antimicrobial activity observed in this study. The studied extracts have promising characteristics for use as natural antimicrobial species in both the food, pharmaceutical and cosmetic industries in order to replace synthetic antimicrobials.

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