**ABSTRACT**

The methanolic extract of leaves from *Artemisia annua* L. (Asteraceae) was investigated for the study of its bioactive substances, such as antibacterial activity and their acute toxicity in rats. HPLC/MS\(^2\) method was employed to identify the chemical constituents of this methanolic extract. Two coumarin derivatives including, esculetin (1) and scopoletin (2), were characterized. Antibacterial activity of this extract was evaluated on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* at the final concentration of 1.5 mg/mL. This extract was bacteriostatic on all the bacteria strains with MIC value greater than 30 mg/mL. Acute toxicity study with this plant was done on wistar albino rats. During acute toxicity studies, there were no any clinical signs found in general behavior and mortality at any dose level used (50 mg-2000 mg/kg body weight). These results suggest that the plant extract possesses compounds that are not acutely toxic with antibacterial properties.

**KEYWORDS:** *Artemisia annua*, Asteraceae, antibacterial activity, acute toxicity

**1. INTRODUCTION**

Plants are a major source of medicine. They have been throughout human history. Herbal remedies are widely available for the prevention and treatment of various diseases. Many plants synthesize substances that are useful for the maintenance of health in animals and humans.\(^{[1]}\) In several countries of Africa, remedies made from plants play an important role in the health of many people. Africa is an impressive source of medicinal plants.\(^{[2]}\) Many of these plants are used in traditional medicine as antimalarial or antimicrobial agents but only a few are documented. Furthermore, 80 \% of the world’s population use plants as their primary source of medication since antibiotics are sometimes associated with adverse side effects to the host including immunosuppressive, hypersensitivity and allergic reactions. It would be interesting to develop alternative antimicrobial drugs based on natural substances from medicinal plants to treat infectious diseases.

*Artemisia annua* is an aromatic plant that has been used by chinese medicine.\(^{[3,4,5,6,7,8,9]}\) This species was chosen for this study because it has a potent remedy for malaria, which, despite official statistics, remains the most devastating on the planet.\(^{[10,11,12,13,14]}\) Artemisinin is the active compound of this plant.\(^{[15,16,17,18]}\) Artemisinin and its semi-synthetic derivatives are a group of drugs used against *Plasmodium falciparum* malaria.\(^{[19,20,21,22]}\) This plant has been domesticated in Korhogo. The objective of this work was, first, to evaluate the chemical
composition of the one grown in Korhogo and secondly evaluate his cytotoxic and his antibacterial activities.

2. MATERIAL AND METHODS
2.1. Plant material
The leaves of Artemisia annua L. (Asteraceae) were collected in August 2016 in Korhogo (north of Côte d’Ivoire). They were identified by Pr. Ipou Ipou Joseph (Centre National de Floristique- Université Félix Houphouët-Boigny de Cocody-Abidjan). A voucher specimen (Silué Souleymane et Assi Jean n° 464) is deposited in the Herbarium of the Botanic Laboratory of the University Félix Houphouët-Boigny.

2.2. Preparation of the extract
Three hundred (300 g) of the dried and powdered leaves of Artemisia annua was subjected to 3 successive macerations of 24 h in 3 L of distilled methanol to yield, after evaporation under reduced pressure, 79 g of methanolic extract.

2.3. HPLC/MS\(\text{a}\) analyses of methanolic extract (Mass Spectrometry Analysis)
Samples were analyzed using an Agilent LC-MS system comprising an Agilent 1260 Infinity HPLC coupled to an Agilent 6530 Q-TOF-MS equipped with an ESI source operating with a positive polarity. A Sunfire analytical C18 column (150 x 2.1 mm; i.d. 3.5 µm, Waters) was used, with a flow rate of 250 µL/min and a linear gradient from 5 % B (A: H_2O + 0.1 % formic acid, B: ACN) to 100 % B over 30 min. ESI conditions were set with the capillary temperature at 320 °C, source voltage at 3.5 kV, and a sheath gas flow rate of 10 L/min. The divert valve was set to waste for the first 3 min. There were four scan events: positive MS, window from m/z 100–1200, then three data-dependent MS/MS scans of the first second, and third most intense ions from the first scan event. MS/MS settings were one fixed collision energies default charge of 1, minimum intensity of 5000 counts, and isolation width of m/z 2. In the positive-ion mode, pirin C_4H_8N_4 [M+H]^+ ion (m/z 121.050873) and the hexakis (1H, 1H, 3H-tetrafluoropropoxy)-phosphazene C_13H_10F_22N_4O_6P_3 [M+H]^+ ion (m/z 922.009798) were used as internal lock masses. Full scans were acquired at a resolution of 11 000 (at m/z 922). A permanent MS/MS exclusion list criterion was set to prevent oversampling of the internal calibrant.

For the on-line HPLC/MS\(\text{a}\) analyses, an Agilent 1100 Series liquid chromatography system was utilized, which was coupled to the ion trap mass spectrometer. The positive ion ESI-MS\(\text{a}\) experiments were conducted using the same conditions as those for the pure compounds described above. HPLC separation was carried on an Agilent XDB-C18 column (2.1_150 mm, 5 mm) (0.3 mL/min). The mobile phase and concentration for each fraction were the same as those used in HPLC/HRMS analyses.

2.4. Biological Activity
The methods used in this study for the toxicity and antibacterial tests are similar to those used by Touré et al., 2018.[23]

2.4.1. Antibacterial Assays
The antibacterial activity was assessed according to the protocol used by Ahoua et al., 2015.[24] Gentamicin and tetracycline were used as positive control.

This activity was evaluated on Staphylococcus aureus ATCC 25923, Staphylococcus aureus (CIP) 4.83, Pseudomonas aeruginosa (CIP) 103467, Escherichia coli (CIP) 54127AF and Staphylococcus aureus sensitive to penicillin strains provided by the Microbiology Laboratory of Centre Suisse de Recherches Scientifiques en Côte d’Ivoire.

2.4.1.1. Sensitivity test
Mueller-Hinton agar in Petri dishes (thickness = 4 mm) were soaked with an inoculum equivalent to 0.5 of McFarland. After drying, wells (diameter = 6 mm) were made in the agar using sterile Pasteur pipette. Fifty microliters (50 µL) of extract (1500 µg/ml in DMSO) or antibiotic (25 µg/ml in distilled water) was poured in the wells. Plates were left at ambient laboratory temperature for 15 to 30 min for a pre-diffusion of the solutions, and then incubated at 37 °C for 18 h. After incubation, the diameters (mm) of inhibition zones were measured. These tests were carried out twice.

2.4.1.2. Determination of Minimum Inhibitory Concentration (MIC)
The minimum inhibitory concentrations (MICs) were determined by using broth microdilution method in 96-wells microplates. The MIC is the lowest concentration at which the visible growth of a strain was completely inhibited (no visible turbidity in wells). The plant extracts were solubilized in DMSO (30 mg/mL) and serially diluted in Mueller-Hinton medium, from 1500 to 1.5 µg/mL. The final concentrations were 50 to 0.05 µg/mL for antibiotics. All the tested bacteria were used with an initial inoculum of 3 x 10^6 bacteria/mL. The microplates were incubated at 37 °C for 18 h.

2.4.2. Acute toxicity
The acute oral toxicity test was performed by using the Organization for Economic Co-operation and Development (OECD) guidelines 423 (OECD, 2001).[25]
2.4.2.1. Experimental animals
Experiments were performed using healthy non-pregnant young adult female rat (Wistar) weighing 106-113 g. Female rats were chosen because of their greater sensitivity to treatment. The animals were randomly divided into five groups each containing three rats. They were identified by the markings using a yellow stain. They were marked on head, body, tail and body, head and body, body and tail, to ease the observation. [26, 27]

The animals were housed in polypropylene cages (55 x 32.7 x 19 cm), with sawdust litter in a temperature controlled environment (23 ± 2º C). Lighting was controlled to supply 12 h of light and 12 h of dark for each 24 h period. Each cage was identified by a card. This card stated the cage number, number and weight of the animals it contained, test substance code, administration route and dose level. They have been fed from granules of the company IVOGRAIN with tap water in baby bottles. [28, 29]

2.4.2.2. Administration of test substance
The test substance was administered in a single dose by gavage using specially designed rat oral needle. Animals were fasted all night.

Following the period of fasting, animals were weighed and test substance was administered orally at a dose of 5, 50, 300 and 2000 mg/kg. After this, food for the rat was withheld for 3 to 4 hours.

Control rats are subjected to intra-gastric gavage of physiological serum (NaCl) at the rate of 10 mL / kg body weight of the animal. Based on the body weight of the animal on the day of treatment, the quantity of the test substance was calculated.

2.4.2.3. Signs recorded during acute toxicity studies
Animals were observed individually after at least once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total of 14 days. All the rats were observed at least twice daily with the purpose of recording any symptoms of ill-health or behavioral changes.

Direct observation parameters include tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern are the other parameters observed. The time of death, if any, was recorded. After administration of the test substance, food was withheld for further 1-2 h. The number of survivors was noted after 24 h and then these were maintained for a further 14 days with a daily observation.

2.4.3. Statistical analysis
Data were expressed as mean values ± SD (standard deviations). All the data were analyzed by one-way ANOVA and differences between the means were assessed with Dunnet/Turkey’s multiple comparison tests. Differences were considered significant at p < 0.05. All analyses were carried out using Graph Pad software (USA).

3. RESULTS AND DISCUSSION
3.1. Results
3.1.1. Phytochemical
HPLC method was used to separate and analyze bioactive constituents of methanolic extract from Artemisia annua L. Thereby; two known compounds (1 and 2) were isolated and identified, thanks to LC-ESI-MS/MS coupling method. The LC-ESI-MS/MS coupling is a method commonly used to identify natural products, such as phenolic compounds, in plants. [30, 31, 32] The principle is based onto the comparison of sample peaks with those of available reference compounds analyzed under the same LC-ESI-MS/MS conditions (retention
time, UV and mass data). Both compounds were detected at UV detector set 254 and 280.0 nm.

Esculetin (1): The retention time of this compound was 14.599 min. The MS/MS analyze exhibited the peak of the pseudo-molecular ion [M+H]⁺ at m/z 179.0388, that corresponds to the molecular formula C₁₅H₁₈O₇ (Cal. 315.3236 g/mol). This compound was identified as Esculetin (Figure 2), a coumarin derivative.

Scopoletin (2): The retention time of this compound was 12.121 min. The MS/MS analyze exhibited the peak of the pseudo-molecular ion [M+H]⁺ at m/z 193.0494, that corresponds to the molecular formula C₁₅H₁₂O₇ (Cal. 327.3302 g/mol). This compound was identified as Scopoletin (Figure 2), an over coumarin derivative.

These results are in agreement with those reported by literature, which shown that esculetin and scopoletin were previously isolated in Artemisia genus and Asteraceae family. Coumarins have intensively been investigated for a wide range of therapeutic purposes because of reported anti-cancer, anti-neurodegenerative or anti-inflammatory activities to name a few. Esculetin and scopoletin have antioxidant and antimicrobial activities, respectively. In plants scopoletin has been characterized as a phytoalexin.

3.1.2. Antibacterial activity

In the current investigation, the antibacterial activity of methanolic extracts from Artemisia annua L. (Asteraceae) domesticated in Korhogo was evaluated against gram-positive and gram-negative bacteria. The diameters of the inhibition zones were measured.

Table 1 shows the inhibition diameters of the methanolic extract on the tested bacteria. The results showed that the extract was active against the tested bacteria with inhibitory diameters ranging from 8 to 10 mm. Pseudomonas aeruginosa ATCC (10 mm), Staphylococcus aureus CIP (8.50 mm), P. aeruginosa CIP (8.50 mm), S. aureus sensitive to penicillin (9 mm) and Escherichia coli ATCC (9 mm) were sensitive to the methanolic extract of the leaves from Artemisia annua L. (Asteraceae) during domestication in Korhogo.

This extract was bacteriostatic on all the tested bacteria and the MIC values were greater than 300 µg/mL (Table 2). The MIC values of the standards ranged from 0.19 to up to 50 µg/mL for tetracycline and from 1.56 to up to 50 µg/mL for gentamicin.

Table 1: Inhibition diameters (mm) of methanolic extract from Artemisia annua on the tested bacteria.

<table>
<thead>
<tr>
<th>Extracts/Positive control</th>
<th>Bacteria</th>
<th>P. aeruginosa ATCC</th>
<th>P. aeruginosa CIP</th>
<th>S. aureus Sensitive</th>
<th>S. aureus CIP</th>
<th>E. coli ATCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisia annua</td>
<td>10.00 ± 0.58⁺</td>
<td>8.50 ± 0.58⁺</td>
<td>9.00 ± 1.73⁺</td>
<td>8.50 ± 0.29⁺</td>
<td>9.00 ± 0.00⁺</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>27.00 ± 0.00⁺</td>
<td>27.00 ± 0.58⁺</td>
<td>23.00 ± 0.00⁺</td>
<td>26.00 ± 0.58⁺</td>
<td>23.00 ± 0.58⁺</td>
<td></td>
</tr>
<tr>
<td>Gentamycine</td>
<td>26.50 ± 0.78⁺</td>
<td>28.00 ± 0.00⁺</td>
<td>25.00 ± 0.00⁺</td>
<td>27.00 ± 0.58⁺</td>
<td>26.00 ± 0.00⁺</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>366.00</td>
<td>456.00</td>
<td>83.803</td>
<td>541.33</td>
<td>686.40</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Table 2: Minimal Inhibitory Concentration (MIC) (µg/mL) values of the methanolic extract from Artemisia annua.

<table>
<thead>
<tr>
<th>Extracts/Positive control</th>
<th>Bacteria</th>
<th>P. aeruginosa ATCC</th>
<th>P. aeruginosa CIP</th>
<th>S. aureus Sensitive</th>
<th>S. aureus CIP</th>
<th>E. coli ATCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisia annua</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td>&gt;3000</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.19</td>
<td>3.125</td>
<td>50</td>
<td>0.19</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>Gentamycine</td>
<td>3.125</td>
<td>1.56</td>
<td>&gt;50</td>
<td>1.56</td>
<td>&gt;50</td>
<td></td>
</tr>
</tbody>
</table>


3.1.3. Acute toxicity

The present study conducted as per the OECD guidelines 423, revealed that methanolic extract from Artemisia annua L. (Asteraceae) domesticated in Korhogo did not produce any mortality throughout the study period of 14 days even when the limit dose was maintained at 2000 mg/kg body weight. The oral LD₅₀ was indeterminable being in excess of 2000 mg/kg body weight. So, testing
the extracts at a higher dose may not be necessary and the extracts were practically non-toxic. Table 3 indicates the parameters observed before and after the administration of the test substance for the three extracts. All parameters observed were normal even at the highest dosage of 2000 mg/kg body weight of the test animal. This clearly indicated that the above extracts do not produce oral toxicity. The medium lethal dose (LD50) of the extracts is higher than 2000 mg/kg body weight and hence, in a single dose administration, the plant extracts had no adverse effect.

Table 3: Effect of the methanolic extracts of Artemisia annua on acute oral toxicity activity (N: Normal; A: Absent; P: Present).

<table>
<thead>
<tr>
<th>Response</th>
<th>Unmarked</th>
<th>Head</th>
<th>Body</th>
<th>Tail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Alertness</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Grooming</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Pain response</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Torch response</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Tremors</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Convulsion</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Gripping strength</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Corneal reflex</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Pupils</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Urination</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Salivation</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Skin colour</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lacrimation</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Hyper activity</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

Table 4: Body weight of different animals (control and treated) for extract from Artemisia annua.

<table>
<thead>
<tr>
<th>Weekly weather</th>
<th>First day</th>
<th>Third day</th>
<th>Sixth day</th>
<th>Eighth day</th>
<th>Twelfth day</th>
<th>Fourteenth day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight in (g) of untreated control animals</td>
<td>106±2</td>
<td>107±3</td>
<td>109±2</td>
<td>112±2</td>
<td>113±4</td>
<td>115±3</td>
</tr>
<tr>
<td>Weight in (g) of animals treated at the dose of 5 mg/kg of body weight</td>
<td>107±3</td>
<td>110±2</td>
<td>112±3</td>
<td>113±3</td>
<td>116±2</td>
<td>119±2</td>
</tr>
<tr>
<td>Weight in (g) of animals treated at the dose of 50 mg/kg of body weight</td>
<td>106±2</td>
<td>109±3</td>
<td>112±2</td>
<td>113±2</td>
<td>116±3</td>
<td>119±2</td>
</tr>
<tr>
<td>Weight in (g) of animals treated at the dose of 300 mg/kg of body weight</td>
<td>109±3</td>
<td>112±4</td>
<td>115±3</td>
<td>119±3</td>
<td>123±3</td>
<td>125±2</td>
</tr>
<tr>
<td>Weight in (g) of animals treated at the dose of 2000 mg/kg of body weight</td>
<td>108±2</td>
<td>110±3</td>
<td>113±2</td>
<td>115±3</td>
<td>121±3</td>
<td>124±3</td>
</tr>
</tbody>
</table>

Table 5: Organ weight (g) of female rats in acute toxicity test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment and dose</th>
<th>Organs weights (gms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Control</td>
<td>Weight in (g) of untreated control animals</td>
<td>3.79 ± 0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.80 ± 0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.81 ± 0.29</td>
</tr>
</tbody>
</table>

3.2. DISCUSSION
Antibacterial activity of leaves from methanolic extract from Artemisia annua L. (Asteraceae) domesticated in Korhogo had not been the subject of preliminary study. This plant possesses significantly (p < 0.05) antibacterial activity against tested organisms. According to the scale of diameter of inhibition of Ponce et al. (2013), the methanolic extract of Artemisia annua L. at 1500 and 250 µg/mL showed the highest zone of inhibition which varied from 8 to 10 mm for P. aeruginosa ATTC, S. aureus CIP and E. coli ATTC. The zone of inhibition varied, suggesting the varying degree of efficacy and different phytoconstituents of plant on the target organism. The antibacterial activity of the plants may be due to the presence of various active principles in their leaves.
The different activities observed are weak compared to that of the reference molecule.

In addition, the acute oral toxicity test is used for evaluating any adverse effects appearing within a short time after a single large oral dose of the test substance or after multiple doses given within 24 h. The methanolic extracts from Artemisia annua L. at a dose of 2000 mg/kg did not cause any observable signs or symptoms of toxicity. The normal behaviour of the test animals during a period of 14 days suggests the non-toxic nature of the above extracts. The results showed that the leaves from Artemisia annua L. did not cause death or result in any other signs of toxicity. To our knowledge, this is the first time that the study of the acute toxicity of Artemisia annua during domestication in Korhogo was done. This study has shown that this plant can be used by the population without risk.

4. CONCLUSION
This study has shown that methanol extract from the leaves of Artemisia annua L. was non-toxic and possesses antibacterial activity even if they remain relatively weak. Although, the antibacterial study of leaves extract is found less, the antimicrobial potential of these leaves extract can be useful to study biocontrol activity. The non-toxic nature of this extract is evident from the acute oral toxicity conducted as per OECD guidelines. Further studies are needed to isolate and characterize the bioactive principles to develop new antibacterial drugs.

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