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## Phytochemical screening, determination of total polyphenols and flavonoids, and evaluation of the antibacterial activity of leaves of *Turraea heterophylla* Smith (Meliaceae)

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**Abstract**

*Turraea heterophylla* Smith (Meliaceae) is a species used in Ivorian's traditional medicine for its various properties, such as antimalarial and aphrodisiac. This study aims to determine the chemical composition of the leaves from *T. heterophylla* and their content in polyphenols, in addition the antibacterial activity of the methanolic extract. Chemical test and spectrophotometry methods were used for phytochemical study. The antibacterial activity was assessed using the agar well diffusion method against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (CIP 54127AF) and *Pseudomonas aeruginosa* (CIP 103467). Phytochemical study revealed the presence of polyterpenes, steroids, alkaloids, saponins, polyphenols and flavonoids. A very high content of flavonoids and polyphenols were observed in the ethyl acetate and aqueous extracts. The antibacterial tests indicated that the methanol extract of the leaves from *T. heterophylla* had bacteriostatic properties against the germs tested with MIC values greater than 3000 µg / mL.

**Keywords:** *Turraea heterophylla*, phytochemistry, total polyphenols, bacteriostatic

**Introduction**

The Meliaceae family is made up of dicotyledonous trees and shrubs. It includes about 51 genus and 600 species of tropical origin (APG III, 2009; ATIBT, 2016) [2, 3]. The species of this large family are widely used in traditional African and mainly Ivorian medicine; however, their chemical composition, which can guarantee their use, is not sufficiently established. Studies carried out on certain species of the family have reported the presence of saponins, alkaloids, flavonoids and limonoids (Tan *et al.*, 2011 [17]; Boua *et al.*, 2013 [6]; Yété *et al.*, 2015 [19]). In order to contribute to the knowledge of the chemical composition of the species of this family, we have chosen to study *Turraea heterophylla* Smith (Meliaceae). *T. heterophylla* is a shrub that can reach 4 m in height; found in tropical forests and savannahs. This plant commonly used by healers to treat erection problems in Côte d'Ivoire, it is attached vasorelaxation in erectile potential [BOUA Boua Benson *et al.*, 2013] [6]. Previous phytochemical studies realized on its roots led to the isolation of three compounds among which two limonoids and one diterpenoid (Akrofi *et al.*, 2011) [1]. The present study concerns the leaves of this species, and it aims to perform a phytochemical screening, to determine their total phenol content and to evaluate the antibacterial activity of the extract.

**Material and Methods****Plant material**

The leaves of *T. heterophylla* were collected in April 2016 in Gadouan, Daloa department, west of Côte d'Ivoire. After identification by the National Floristic Center of the Félix HOUPHOUËT-BOIGNY University (CNF), where a specimen is kept under the herbarium number 31235, the fresh leaves were cleaned with distilled water and then dried in an oven at 50 °C for 4 days before being reduced to powder using an electric mill (RETSCH, type SM 100).

### Biological material

The reference bacterial strains and the hospital strain used were provided by the Bacteriology-Virology Laboratory of the Pasteur Institute in Abidjan (CIP), the National Public Health Laboratory of Côte d'Ivoire and the Swiss Center for Scientific Research of Côte d'Ivoire (CSRS). The tests were carried out at the Swiss Center for Scientific Research of Côte d'Ivoire (CSRS). The reference strains are Gram-positive cocci (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* CIP 483, and Gram-negative bacilli (*Escherichia coli* CIP 54127AF and *Pseudomonas aeruginosa* CIP 103467). The strain of *Staphylococcus aureus*, of hospital origin, is sensitive to penicillin. The sprouts were stored at laboratory temperature (25 °C) in nutrient agar poured into tubes and then labeled.

### Methods

#### Preparation of methanol extract from the leaves of *T. heterophylla*

To prepare the methanol extract of the leaves of *T. heterophylla*, 200.0 g of dried and ground plant material was extracted by maceration in 600 mL of methanol for 24 hours. After filtration on Whatman n°2 paper, the Mac is extracted twice in a row with the same amount of methanol for 24 hours each. The filtrates are combined, concentrated and evaporated to dryness under reduced pressure at a temperature of 60 °C, using a rotary evaporator (BUCHI 461 type). The obtained crude methanol extract (E<sub>MeOH</sub>) was stored in a glass desiccator for further work.

#### Fractionation of the crude methanol extract

A part (7.2 g) of the crude methanol extract (E<sub>MeOH</sub>) was dissolved in distilled water than filtered. The aqueous solution (1000 mL) was extracted successively with hexane (4x 100 mL), dichloromethane (4x 100 mL) and ethyl acetate (4x 100 mL). The residual aqueous phase was dried in an oven and then extracted with methanol (3x 50 mL) to obtain the aqueous extract (Aq). The organic phases were dried over anhydrous sodium sulfate, filtered through Wattman n°2 paper, removed the solvents under reduced pressure to obtained the three extracts hexane (F<sub>HEX</sub>), dichloromethane (F<sub>DCM</sub>) and ethyl acetate (F<sub>AE</sub>).

#### Screening phytochemic of the methanol extract

##### Characterization of polyphenols by ferric chloride test

The test was carried out according to the method described by Wagner (1983) [18]. To 2 mL of extract is added a drop of 2% alcoholic ferric chloride and, the appearance of a more or less dark blue or blackish green color indicates the presence of polyphenols.

##### Characterization of sterols and polyterpens by Lieberman and Burchard test

The test was carried out according to the method described by Wagner (1983) [18]. In a capsule, 5 mL of extract is evaporated on a sand bath. Le résidu est dissous à chaud dans 1 mL d'anhydride acétique, puis transféré dans un tube à essai où 0,5 mL d'acide sulfurique concentré sont ajoutés. The appearance at the interphase of a purple ring, turning blue then green, indicates the presence of sterols and terpenes.

##### Characterization of flavonoids by cyanidin test

The method described by Wagner (1983) [18] was used for this identification. In a capsule, 2 mL of the extract is evaporated on a sand bath and the cold residue is taken up in 5 mL of

half-diluted hydrochloric alcohol and then transferred to a test tube. The formation of a pink-orange or purplish color after the addition of 2 to 3 magnesium shavings reveals the presence of flavonoids.

##### Characterization of catechetical tannins by Stiasny's reagent

The method described by Wagner (1983) [18] was used for this characterization. Dans une capsule, 5 mL d'extrait sont évaporés sur bain de sable avant d'ajouter au résidu 15 mL de réactif Stiasny. The mixture is kept in a water bath at 80 °C for 30 min, and then allowed to cool. The appearance of precipitates in the form of large flakes indicates the presence of catechetical tannins.

##### Characterization of gallic tannins

The characterization was performed according to the method described by Wagner (1983) [18]. The solution resulting from the characterization of the catechetical tannins is filtered then the filtra is saturated with sodium acetate. The addition to the saturated solution of 3 drops of 2% ferric chloride (FeCl<sub>3</sub>) causes the appearance of an intense blue-black color in the presence of gallic tannins.

##### Characterization of alkaloids

The characterization was performed according to the method described by Harbone (1998) [11]. In a capsule, 6 mL of extract are evaporated on a sand bath and then the residue is taken up in 6 mL of 96% alcohol. The alcoholic solution is dispensed into two test tubes. In the first tube n°1, are added 2 drops of Dragendorff reagents and, the appearance of a precipitate or an orange color indicates the presence of alkaloids. In the second tube n°2, are added 2 drops of Bouchardat reagent and, the appearance of a reddish-brown color confirms the presence of an alkaloid.

##### Characterization of quinone and anthraquinones

The Borntraeger reagent (half diluted ammonia) allows the detection of free quinones. The characterization was performed according to the method described by Harbone (1998) [11]. In a capsule, evaporate 2 mL of the extract on a sand bath and then triturated the residue in 5 mL of 1/5 hydrochloric acid. In a test tube, heat the solution in a boiling water bath for half an hour. After cooling, extract the hydrolyzate with 20 mL of dichloromethane in a test tube and, collect the organic phase (dichloromethane) in a test tube then add 0.5 mL of half-diluted ammonia. The appearance of a color ranging from red to purple indicates the presence of quinones.

##### Characterization of saponins

The method described by Harbone (1998) [11] was used for this characterization. In a test tube, dissolve 1 g of extract in 100 mL of distilled water, heat the mixture slightly, filter, cool and make up to 100 mL with distilled water. Place the tube vertically for 15 minutes. At the end of this period, if the foam persists, then the herbal drug contains saponins.

##### Assays of total polyphenols and total flavonoids

The measurements were carried out using the spectrophotometer 7315 JENWAY. The calibration lines obtained were drawn using gallic acid and quercetin as standards respectively for polyphenols and flavonoids. The contents of total polyphenols and total flavonoids were calculated from the following formula.

$$E = \frac{CVD}{m}$$

E: Content or concentration (mg.AG/g or mg.Qc/g of dry extract); m: Mass of the extract (g)

C: Concentration of the sample given by the spectrophotometer (mg / mL).

V: Volume of prepared solution (mL); D: Dilution factor

#### Determination by spectrophotometry of total polyphenols

The method described by Patricia *et al.*, (2020) [14] was used for the determination of total polyphenols. A volume of 2.5 mL of diluted Folin-Ciocalteu reagent (1/10) is added to 30  $\mu$ L of extract. The mixture is kept for 2 minutes in the dark at room temperature, and then 2 ml of sodium carbonate solution (75 g.L-1) are added. The mixture is then placed for 15 minutes in a water bath at 50 °C., and then cooled rapidly. L'absorbance est mesurée à 760 nm, avec de l'eau distillée comme blanc. A calibration line is carried out with gallic acid at different concentrations; each analysis is performed in triplicate and the polyphenol concentration is expressed in milligrams per milliliter of gallic acid equivalent extract (mg / mL). Gallic acid is used here as a reference standard for the quantification of total polyphenol contents; this quantity is expressed in milligram gallic acid equivalent per gram of extract (mg.eq.AG / g extract).

#### Determination by spectrophotometry of total flavonoids

The method described by Patricia *et al.*, (2020) [14] was used for the determination of total flavonoids.

In a 25 mL vial, 0.75 mL of 5% (w/v) sodium nitrite (NaNO<sub>2</sub>) was added to 2.5 mL of the extract. The mixture was added with 0.75 mL of 10% (w/v) aluminum chloride (AlCl<sub>3</sub>), and incubated for 6 minutes in the dark, and then 5 mL of sodium hydroxide (1N NaOH) was added then the volume was made up to 25 mL. The mixture was stirred vigorously before being assayed with a UV-visible spectrophotometer; the reading was then taken at 510 nm. The tests were performed in triplicate, and the flavonoid content was expressed in milligram quercetin equivalent per gram of extract (mg.eq.Qc/g of extract). Quercetin is used here as a reference standard for the quantification of the contents of total flavonoids.

#### In vitro evaluation of antibacterial activity

##### Preparation of the bacterial inoculum

A large colony well isolated from an 18 hour bacterial culture was removed using a sterile Pasteur pipette, then crushed on the wall of a tube containing 10 mL of distilled water. Using a Pasteur pipette, 5 to 6 drops of this preculture are taken and diluted in 10 mL of distilled water. This bacterial suspension produced makes it possible to have approximately 10<sup>6</sup> CFU / mL (standard condition) and constitutes the bacterial inoculum of dilution 10<sup>0</sup> (George *et al.*, 1999) [10].

##### Sensitivity test

The principle is based on the diffusion of the antimicrobial product in a solid medium in a Petri dish, with the creation of a concentration gradient after a brief moment of contact between the product and the target microorganism. The effect of the antimicrobial product on the target is assessed by measuring a zone of inhibition. Depending on the diameter of inhibition, the strain will be classified as sensitive, very sensitive, extremely sensitive or resistant. According to

Etuaful *et al.* (2005) [9], the strain is classified as non-sensitive or resistant depending on the diameter of inhibition:

- diameter less than 8 mm.: non-susceptible or resistant strain,
- diameter between 9 and 14 mm: sensitive strain,
- diameter between 15 and 19 mm: very sensitive strain,
- diameter greater than 20 mm: extremely sensitive strain.

For the procedure, the Muller-Hinton agar plate for bacteria was uniformly inoculated by flooding with a suspension of the dilution 10<sup>0</sup> microbial inoculum. The surplus was aspirated using a Pasteur pipette topped with a pro-pipette. The inoculated box was dried for 15 minutes at room temperature. Then, wells were made using a Pasteur pipette under sterile conditions and a quantity of 50  $\mu$ L of the substance to be tested (1.5 mg / mL) is deposited therein. The Petri dish was then incubated at 37 °C for 18 hours. This operation is repeated 3 times in a row. Tetracycline and gentamycin (25  $\mu$ g / mL) served as positive controls. The reading was taken by measuring the diameter (mm) of the inhibition zone around each cup using an automatic display caliper. The results are expressed by the diameter of the zone of inhibition, and only diameters of inhibition greater than 8 mm were taken into account (Aubry *et al.*, 2006) [4].

#### Determination of minimum inhibitory concentrations (MIC)

The methanol extracts which showed an inhibition diameter greater than or equal to 9 mm was selected to determine the MICs by the method of diffusion by wells on agar. After reading the MIC, the contents of the wells showing no bacterial growth visible to the naked eye are inoculated in 5 cm streaks in petri dishes on Muller-Hinton agar using a loop and then incubated at 37 °C for 18 h.

#### Culture media and antimicrobial agents

For sanitizing conditions, a laminar flow hood (CYTAIR, France) was used. Other technical equipment such as the bacteriological incubator (JOUAN type: EB 170 EL SANS, Afghanistan), the autoclave (Trade Raypa, model AE-75BRY Spain), microplates of wells with a U-bottom (12 x 8 rows), a precision electronic balance (AG 204 Delta Range), micropipettes, precision pipettes and adaptable tips were used. Dimethylsulfoxide (DMSO), gentamycin powder (Fluca; biochimika, Switzerland), tetracycline (Sigma-Aldrich; USA), Amphotericin B (Sigma-Aldrich; USA), Nystatin (Sigma-Aldrich; USA), Müller-Hinton agar (Bio-Rad®, USA) and the method of diffusion by wells on agar (HIMEDIA; Dextrose Agar; Bio-Rad®, USA) were used to study the sensitivity of microbial strains to -vis of the methanol extract of the plant.

#### Results and Discussion

##### Extraction and fractionation yield

The extraction carried out on 200.0 g of powder from the leaves of *T. heterophylla* yielded 13.5 g of crude methanol extract (E<sub>MeOH</sub>), ie a yield of 5.40%. This relatively high yield indicates that the leaves of *T. heterophylla* contain sufficient secondary metabolites extractable by methanol. The successive exhaustions of a part (7.2 g) of the E<sub>MeOH</sub> extract with hexane, dichloromethane, ethyl acetate and methanol respectively resulted in F<sub>HEX</sub> fractions (0.95 g; 13.2 %), F<sub>DCM</sub> (1.38 g; 19.2%), F<sub>AE</sub> (1.92 g; 26.7%) and F<sub>Aq</sub> (2.52 g; 35.0%). It is noted that the highest yields are obtained with the residual aqueous fraction (F<sub>Aq</sub>: 35.0%), followed by the ethyl acetate fraction (F<sub>AE</sub>: 26.7%). These two fractions contain

approximately 62%, by mass, of the metabolites contained in the crude methanol extract ( $E_{MeOH}$ ). Since methanol, ethyl acetate and water are hydrophilic solvents, it is normal for them to have the same affinity in extracting water-soluble compounds (Silverstein *et al.*, 2005) [16].

### Chemical composition of crude methanol extract

The phytochemical screening carried out on the crude methanol extract of the leaves of *T. heterophylla* ( $E_{MeOH}$ ) targeted the following families of secondary metabolites: alkaloids, polyphenols, flavonoids, tannins, sterols, polyterpenes, quinones and saponins. The results indicate that only tannins and quinones are absent (Table 1). These results are similar to those previously obtained on the stem barks of the same plant (Akrofi *et al.*, 2011 [1]; Boua *et al.*, 2013 [6]). However, the absence of tannins in the methanol crude extract of the leaves is contrary to the results of the work carried out on the root bark of the same plant by Bouquet & Debray (1974) [7]; which indicate the presence of tannins. This difference can be explained by the fact that the two organs

(leaves and roots) play different roles in the plant and also by several factors such as agro-pedological, climatic and geographical (Lee *et al.*, 2003) [12]. Phytochemical screening shows that the chemical composition of the leaves of *T. heterophylla* is similar to that of species of the genus *Turraea* (Tan *et al.*, 2011 [17]; Boua *et al.*, 2013 [6]; Yété *et al.*, 2015 [19]). Table 1 also shows that all the chemical families characterized in the crude methanol extract ( $E_{MeOH}$ ) are distributed in the  $F_{HEX}$ ,  $F_{DCM}$ ,  $F_{EA}$  and  $F_{Aq}$  fractions. The fact that polyphenols and flavonoids are found in all extracts (nonpolar and polar) confirms that there is coexistence of aglycone and glycoside in the leaves of *T. heterophylla*. Regarding sterols and polyterpenes, they are only found in nonpolar solvents: hexane and dichloromethane. This observation is normal, since these compounds are generally very soluble in lyophilic solvents such as hydrocarbons. The richness of the crude methanol extract of the leaves of *T. heterophylla* could justify the use of the decoction in many traditional treatments (N'guessan *et al.*, 2009) [13].

**Table 1:** Screening phytochemical of extract of the leaves of *T. heterophylla*

Secondary metabolites	Chemical test	Extract $E_{MeOH}$	Fractions			
			$F_{HEX}$	$F_{DCM}$	$F_{AE}$	$F_{Aq}$
Alkaloids	Dragendorff + Bouchardat	+	-	-	+	+
Polyphenols	Ferric chloride	+	+	+	+	+
Flavonoids	Magnesium coppers	+	+	+	+	+
Gallic tannins	Stiasny reagent	-	-	-	-	-
Catechetal tannins	Ferric chloride	-	-	-	-	-
Sterols and polyterpenes	Lieberman reagent	++	+	+	-	-
Quinones	Diluted ammonia	-	-	-	-	-
Saponins	Foam Test	+	-	-	+	+

NB: Abundant (++); Presence (+); Absent (-)

### Total polyphenols and total flavonoids content

The calibration line drawn, using gallic acid as the standard, gave a regression coefficient  $R^2 = 0.9935$ . The content of total polyphenols is  $75 \pm 0.00$  mg eqAG / g for the crude methanol

extract of the leaves of *T. heterophylla* ( $E_{MeOH}$ ) and  $25 \pm 0$  mg eqAG / g;  $50 \pm 0$  mg eqAG / g;  $125 \pm 7$  mg eqAG / g and  $125 \pm 7$  mg eqAG / g for the  $F_{HEX}$ ,  $F_{DCM}$ ,  $F_{AE}$  and  $F_{Aq}$  fractions respectively (Table 2).

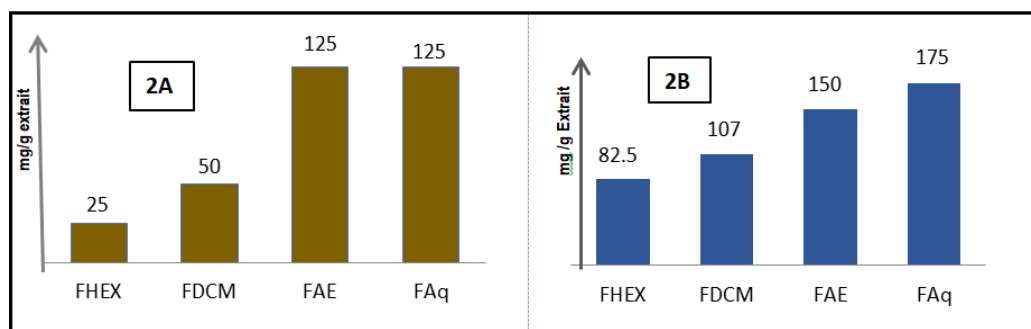
**Table 2:** Teneur en polyphénols totaux des extraits de feuilles de *T. heterophylla*

Content	Extract and fractions				
	$E_{MeOH}$	$F_{HEX}$	$F_{DCM}$	$F_{AE}$	$F_{Aq}$
Total polyphenols (mg éq.AG/g d'extrait sec)	$75 \pm 0$	$25 \pm 0$	$50 \pm 0$	$125 \pm 7$	$125 \pm 7$
Total flavonoids (mg éq.Qc/g d'extrait sec)	$150.0 \pm 0.0$	$82.5 \pm 0.0$	$107.0 \pm 0.0$	$150.0 \pm 0.0$	$175.0 \pm 0.0$

For a best comparison, these values have been translated in the form of a histogram (Figure 1A).

It is noted that the ethyl acetate ( $F_{AE}$ ) and residual aqueous ( $F_{Aq}$ ) fractions are two to three times richer in polyphenols than the hexane ( $F_{HEX}$ ) and dichloromethane ( $F_{DCM}$ ) fractions. This difference confirms that the polyphenols present in the crude methanol extract of the leaves of *T. heterophylla* are

predominantly glycosylated; and therefore more soluble in methanol, water and ethyl acetate; which better extract polyphenols (Bruneton, 1999 [8]; Azwanida 2015 [5]). Aglycones, which are poorly soluble in hydrophilic solvents, were less extracted with methanol. These results agree with the tri-phytochemical data.



**Fig 1:** Histogram of the total polyphenols (2A) and total flavonoids (2B) contents of the fractions

Regarding the total flavonoid contents, they were determined by plotting the calibration right from quercetin as a stand, and the regression coefficient obtained has the value  $R^2 = 0.9983$ . The results shows that the total flavonoid content is  $150.0 \pm 0.0$  mg eqQc / g for the crude methanol extract of the leaves of *T. heterophylla* ( $E_{MeOH}$ ), and  $82.5 \pm 0.0$  mg eqQc / g;  $107.0 \pm 0.0$  mg eqQc / g;  $150.0 \pm 0.0$  mg eqQc / g and  $175.0 \pm 0.0$  mg eqQc / g for the  $F_{HEX}$ ,  $F_{DCM}$ ,  $F_{AE}$  and  $F_{Aq}$  fractions respectively (Table 2). The histograms representing the total flavonoid contents of the fractions (Figure 1B) show increasing values when switching from less polar to more polar solvents. These results agree with those obtained with the screening phytochemic and the total polyphenol content of the fractions. Therefore, it can also be said that the flavonoids contained in the crude methanol extract of the leaves of *T. heterophylla* are predominantly glycosylated. Thus, non-glycosylated flavonoids (aglycones) are identified in the hexane ( $F_{HEX}$ ) and dichloromethane ( $F_{DCM}$ ) fractions. As for glycosylated flavonoids, it is rather in the ethyl acetate ( $F_{AE}$ ) and residual aqueous ( $F_{Aq}$ ) fractions that they are found. This

result is consistent with the principle that compounds possessing hydroxyl groups are more soluble in hydrophilic solvents such as methanol, water and ethyl acetate (Bruneton 1999<sup>[8]</sup>; Azwanida 2015<sup>[5]</sup>).

### Antibacterial activity

The antibacterial activity of the crude methanol extract of the leaves of *T. heterophylla* ( $E_{MeOH}$ ) was evaluated against five (5) bacterial strains: *P. aeruginosa* ATCC, *P. aeruginosa* CIP, *S. aureus* sensitive, *S. aureus* CIP and *E. coli* ATCC. The inhibition diameters are all greater than 9 mm (values between 9 and 9.50 mm), which means that these bacteria are sensitive to this extract (Ponce *et al.*, 2013<sup>[15]</sup>; Etuaful *et al.*, 2005<sup>[9]</sup>). Although the  $E_{MeOH}$  extract is bacteriostatic, the values of the minimum inhibitory concentrations (MIC) are all above 3000  $\mu\text{g} / \text{ml}$  (Table 3). The MICs of the  $E_{MeOH}$  extract are negligible compared to those of Tetracycline and Gentamicin, taken as positive control, whose MICs are between 0.19 and 50  $\mu\text{g} / \text{mL}$  and 1.56 and 50  $\mu\text{g} / \text{mL}$  respectively.

**Table 3:** Minimum inhibitory concentrations (MIC) ( $\mu\text{g} / \text{mL}$ ) of extract of leaves of *T. heterophylla*

		<i>P. aeruginosa</i> ATCC	<i>P. aeruginosa</i> CIP	<i>S. aureus</i> sensitive	<i>S. aureus</i> CIP	<i>E. coli</i> ATCC
Methanol extract $E_{MeOH}$		>3000	>3000	>3000	>3000	>3000
Positive control	Tetracycline	0,19	3,125	50	0,19	> 50
	Gentamicin	3,125	1,56	> 50	1,56	> 50

This result shows that the leaves of *T. heterophylla* have antibacterial properties like the roots of the same plant (Akrofi *et al.*, 2011)<sup>[1]</sup>. This activity, even low of the methanol extract, could justify the use in traditional medicine, in Ivory Coast, of the leaves of *T. heterophylla* in the treatment of various diseases such as injuries to the stomach, typhoid fever, diarrhea and hemorrhoidal crises (N'guessan *et al.*, 2009<sup>[13]</sup>; Boua *et al.*, 2013<sup>[6]</sup>).

### Conclusion

The study performed here on the leaves of *T. heterophylla* focused on tri-phytochemistry, assays and antimicrobial testing. Phytochemical screening revealed the presence of polyterpenes, sterols, alkaloids, saponins, polyphenols and flavonoids. Also, this study shows that the leaves of this species synthesize both glycosylated polyphenols and their genins (aglycones). Regarding the assays, the results show that the residual aqueous extracts and ethyl acetate have a higher content of polyphenols and flavonoids. Finally, the biological tests carried out on five (5) strains of bacteria indicate that the methanol extract of *T. heterophylla* has bacteriostatic properties.

### Notes

The authors declare no competing financial interest.

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