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Pharmacognosic study and anti-hepatocarcinoma activity of extracts from leaves and roots of *Terminalia macroptera* Guill. & Perr. (Combretaceae)

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Abstract
Terminalia macroptera leaves and roots frequently used in Malian ethnomedicine showed some biological activities against liver diseases. The present study aimed to control the quality of powdered leaves and roots, and to evaluate the anti-hepatocarcinoma activity of their extracts. The quality control focused on the determination of the botanical and physicochemical parameters. The free radical scavenging activity of the ethanolic extracts of the leaves or roots was evaluated by the DPPH radical reduction method. The anti-hepatocarcinoma activity of the ethanolic extracts of the leaves or roots was evaluated *in vitro* on the human hepatocarcinoma (HepG2) cell line using the MTT test. The organoleptic and microscopic characters coupled physicochemical parameters for the identification of good samples of the powdered leaves and roots were established. The best free radical scavenging and anti-hepatocarcinoma activities were obtained with the ethanolic extract of the leaves with an EC_{50} of 6.1 ± 0.3 µg / mL of 15 µg / mL respectively. These findings will help more to prepare a monograph and to validate the use of *Terminalia macroptera* for liver diseases.

Keywords: *Terminalia macroptera*, Anti-free radical and anti-hepatocarcinoma activities, Liver diseases, Botanical and physicochemical parameters

Introduction
Terminalia macroptera Guill. and Perr. (Combretaceae) is widely distributed in Africa. The leaves and roots are the most frequently used parts in traditional medicine in the management of liver diseases such as hepatitis and jaundice in Mali [1, 2]. These medicinal uses are corroborated by the hepatoprotective and anti-inflammatory activities demonstrated by the ethanolic extracts of the leaves and roots respectively on a model of hepatitis induced by carbon tetrachloride in rats and on a model of inflammation induced by carrageenan [3]. These extracts have also demonstrated antiplasmodial activity [4], analgesic and antipyretic properties [3]. The decoctions of leaves and roots have demonstrated an immunodulatory property on the complement system [5]. The antiviral activity of the ethanolic extract of the roots on the herpes simplex virus has also been demonstrated [6]. Phytochemical analysis revealed that the leaves and roots contain polyphenols (flavonoids, tannins), saponononins, sterols and triterpenes. Molecules such as gallic acid, punicalagin, terfavin A, terchebuline, ellagic acid and their methoxylated derivatives have been identified by LC-CAD-MS [3, 4, 7, 8].
As part of the possible formulation of an improved traditional medicine (ITM) against liver diseases in Mali, we wanted to deepen the pharmacological studies on the leaves and roots of this species. The current study aimed to control the quality of the raw materials and to evaluate the anti-hepatocarcinoma activity of their ethanolic extracts. Indeed, herbal medicines are generally readily available, less expensive and have few or no side effects. However, one of the drawbacks is the falsification of the raw material by foreign bodies in order to increase the weight.

Material and Methods
Plant material collection and authentication
The leaves and roots of *T. macroptera* were collected in August 2015 in Siby, a village located in the Koulikoro region in Mali. A specimen of the plant, voucher number 3752/DMT, was deposited in the herbarium of the DMT/ NIRPH and authenticated by Mr. Seydou Dembele, a forestry engineer and chief of ethnobotanic service of DMT. Access and benefit sharing to

biodiversity and its associated traditional knowledge was established according to Malian national rules.

Botanical analyzes

1. Determination of organoleptic characters

The organoleptic characters of the plant powders were determined by means of the sense organs using the standard description of terms such as colour, odour, texture and taste.

2. Microscopic examination

The model of microscope used in microscopic studies was the light microscope (LEICA) fitted with a camera. A small amount of powder of the leaves or roots was mounted in the chloral hydrate solution and observed under the microscope at different magnifcations. Photomicrographs were taken with a camera (NIKON) [9].

Physicochemical analysis

Various physicochemical parameters including the moisture content and the ash values (total ash, and acid-insoluble ash) were analyzed for the leaves and roots according to standard methods [10].

Preparation of extracts

The ethanolic extracts were prepared as described previously by Haïdara and coworkers [3]. Briefly A total of 250 g dried samples was macerated in 1000 mL of 90% ethanol for 24 h and filtered using Whatman fiters N °1. This operation was repeated three times. The three filtrates were combined and evaporated under vacuo to dryness (Büchi rotary evaporator Model R-200). The crude extracts of *T. macroptera* leaves (TML) and roots (TMR) were stored in a refrigerator at 4-8 °C before use.

Determination of scavenging effect on DPPH radicals

The free radical scavenging constituents of the extracts were evaluated using the 2,2-diphenyl-1-picrylhydrazyl radical reduction test (DPPH) according to the method described by Zongo *et al.* [11]. For the determination of inhibitory concentrations 50, a concentration range of the extracts between 0.781 - 50 µg / mL was obtained by doubling dilutions. For the test, 700 µL of each dilution was mixed with 1400 µL of a DPPH solution (0.025 g / L). The samples were incubated for 30 min at room temperature, protected from light, then the absorbance was measured at 517 nm with a spectrophotometer (6705 UV / VIS Spectrophotometer JENWAY). Methanol was used as a negative control and gallic acid as a positive control. The percentage reduction of the DPPH radical was calculated according to the following formula:

$$\% \text{ Reduction} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance sample}} \times 100$$

The EC_{50} (effective concentration 50) of the extracts and gallic acid was determined using a linear regression curve using Graphpad prism software version 6.0.

Evaluation of anti-hepatocarcinoma activity

1. Culture Cells

Hepatocellular carcinoma HepG2 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 units / mL) and streptomycin (100 µg / mL).

The cells were incubated at 37 °C in a humid atmosphere containing 5% CO_2 and were subcultured twice a week.

2. Anti-hepatocarcinoma Assay

The anti-hepatocarcinoma effect of extracts of the leaves and roots of *Terminalia macroptera* was evaluated on HepG2 cells using the MTT test (3- (4, 5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) according to the method described by [12].

The HepG2 cells were cultured at the density of 1 x 10^5 cells / mL (100 µL) in 96-well plates and then incubated at 37 °C in a humidified CO_2 (5%) incubator. Twenty-four hours later, different concentrations of the extracts were added to each well containing the cells. 0.5% methanol was used as a negative control. After 48 hours, the medium was removed and then 100 µL of MTT (0.5 mg / mL in PBS) were added to each well.

The plates were incubated for 4 hours at 37 °C in a humidified CO_2 (5%) incubator. DMSO (100 µL) was added to each well to dissolve the formazan crystals trained. Absorbance was measured at 570 nm on a microplate reader (BioTek Synergy). The percentage inhibition of cell growth was calculated on the basis of a comparison with untreated cells according to the following formula:

$$\% \text{ Inhibition of cells growth} = \frac{(\text{AC} - \text{AT}) \times 100}{\text{AC}}$$

Where AC indicates the mean of the absorbance in the wells containing cells treated with 0.5% methanol and AT indicates the mean of the absorbance in the wells containing cells treated with the extracts.

The effective concentration 50 (EC_{50}) was calculated using a nonlinear regression program on the Graph Pad Prism software.

3. Cytotoxicity Assay

The cytotoxicity of the extracts was evaluated on VERO cells using the MTT test according to the method described by Mahlo and colleagues [12]. The absorbance was measured at 570 nm on a microplate reader (BioTek Synergy). Their effect was established by comparison of the absorbance of the cells treated with the extracts to those treated with 0.5% methanol used as a negative control. The cytotoxic concentration 50 (CC_{50}) was calculated using a nonlinear regression program on the Graph Pad Prism software.

4. Determination of the Selectivity Index (SI)

The selectivity index (SI) corresponding to the ratio between the cytotoxic and anti-hepatocarcinoma activities of each extract was calculated as follows:

$$\text{Selectivity index} = \frac{\text{CC50}}{\text{EC50 (HepG2)}}$$

Results

Botanical quality

Organoleptic characters

Organoleptic examination revealed that the leaf powder was green and that of the roots was beige. The two powders were fine with an uncharacteristic odour (i.e. not identifiable from odours) and a slightly bitter taste.

Microscopic features

The powder of the leaves mounted in the chloral hydrate solution showed the presence of:

- Twins of calcium oxalate (about 50-60 µm in diameter) from the parenchyma [Figure 1A] or very small and often aligned from the spinal rays [Figure 1B]
- Liberian fibers in a cluster [figure 1C] associated or not with conducting vessels [Figure 1D]
- Fragments of upper epidermis, seen from the front, with polyhedral cells and some stomata [Figure 1E] accompanied by a palisade parenchyma and fragments of lower epidermis with lobed cells carrying anomocytic type stomata [Figure 1F] to 3 to 4 annex cells.

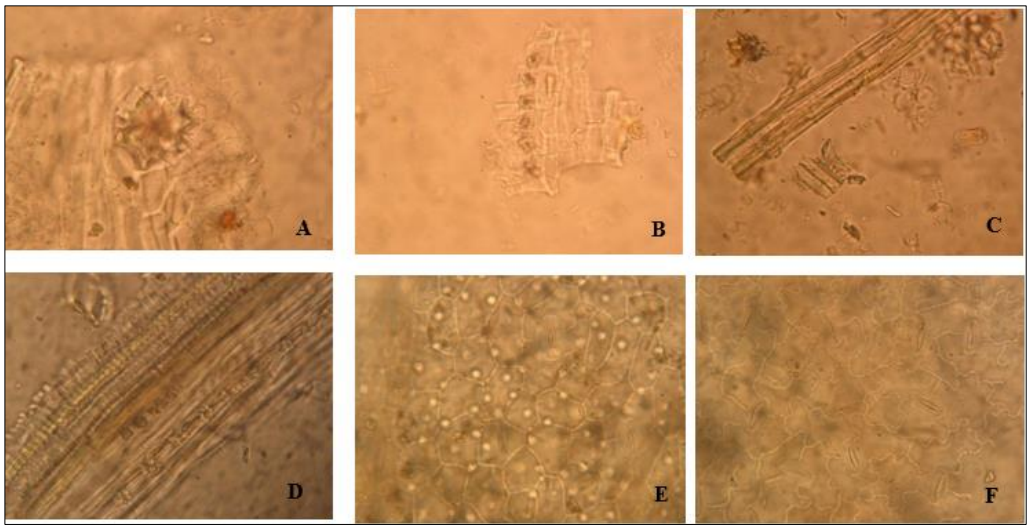

Fig 1: Microscopic elements of leaf powder (Magnification X 40)

The root powder, mounted in the chloral hydrate solution has shown the presence of

- Twins of free calcium oxalate (diameter sometimes greater than 150 µm) from the parenchyma [Figure 2A]. or in rows, of much smaller size and included in cells from the medullary rays [Figure 2B].
- Vessels, large in diameter, with punctured area [Figure 2C].
- Fragments of suber with polyhedral cells with thin walls and orange content [Figure 2D].
- Fragments of the medullary rays with more or less rectangular cells, with a slightly and regularly thickened and punctuated wall [Figure 2E].
- Many clusters of long, narrow fibers, not septate, with highly lignified wall and narrow lumen [Figure 2F].
- Many grains of ovoid starch about 10 µm long generally isolated or associated by 2 to 3 grains free or included in parenchymal cells [Figure 2G].

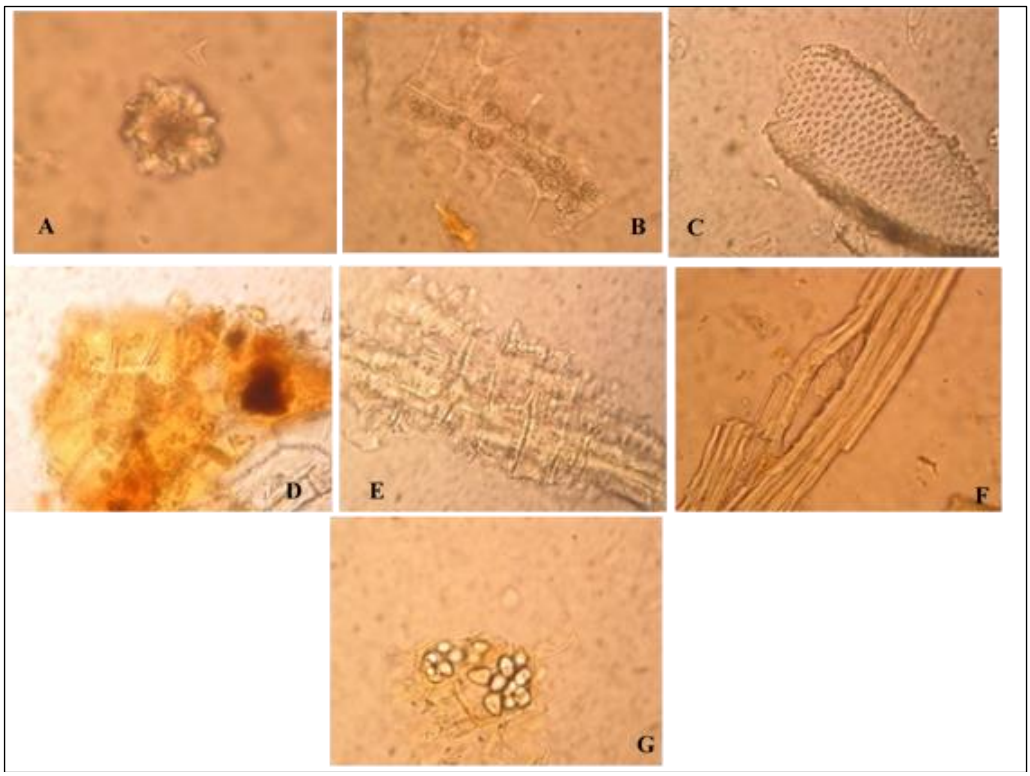

Fig 2: Microscopic elements of root powder (Magnification X 40)

Physicochemical properties

The moisture, total ash and acid insoluble ash values for the powder of the leaves were respectively 7.7 ± 0.5%; 3.44 ± 0.1% and 0.4%. Those from the powder of the roots were respectively 8.3 ± 0.3%; 7.17 ± 0.1% and 1.7%.

Extracts

Yields of leaf and root extraction were 17.6% (44 g) and 14% (35 g) respectively.

Radical scavenging activity

The ethanolic extract 96% of the leaves and roots of *T. macroptera* demonstrated an anti-radical DPPH activity with an EC_{50} respectively 6.1 ± 0.3 µg / mL and 13.4 ± 0.3 µg / mL. Gallic acid used as a positive control had an EC_{50} of 1.4 ± 0.2 µg / mL.

Anti-hepatocarcinoma activity and cytotoxicity

The extracts showed anti-hepatocarcinoma activity *in vitro* on HepG2 human hepatocarcinoma cells. The best activity was obtained with the ethanolic extract of the roots with an EC_{50} of 15 µg / mL and a selectivity index of 8.5 against HepG2 cells as shown in Table I.

Table 1: Anti-hepatocarcinoma (HepG2) activity and cytotoxicity (VERO) of ethanolic extracts from leaves and roots

Extracts (used part)	EC_{50} (µg/mL)	CC_{50} (µg/mL)	SI
Ethanolic (Leaves)	24.4	118.2	4.8
Ethanolic (Roots)	15	128	8.5

Discussion

In this study quality standards allowing the identification of good powdered samples from *Terminalia macroptera* leaves and roots were established and the anti-hepatocarcinoma properties of their ethanolic extracts have been also demonstrated.

The organoleptic and microscopic features examinations of the powdered leaves and roots of *Terminalia macroptera* have enabled the identification of the most useful characters for the botanical characterization of this medicinal plant.

We did not find any target hairs in the samples examined. On the other hand, Verhoeven and Van der Schijff, in their anatomical study of the leaves of Combretaceae from South Africa indicate that all Combretaceae have protective hairs called "hairs of Combretaceae": long unicellular with pointed ends showing thick walls and bulbous base [13]. According to Tilney, in some Terminalia species only the young leaves have protective hairs [14].

This fact should be verified on young leaves of *T. macroptera* since the leaves examined in the current work were all in adulthood. The epidermis showed a clear difference between the upper epidermis which is finely cuticularized showing cells with a rigid wall and weakly stomatiferous while the lower epidermis with cells presents a sinuous wall and bears numerous anomocytic stomata. The presence of anomocytic stomata is frequent in Terminalia, however their localization on the upper surface seems relatively rare. On the other hand, the difference between the shape of the sinuous cells on the lower face and the one for the upper face has already been reported in several species of West African Terminalia [14].

The moisture content was less than 10% in both samples. A high moisture content (generally more than 10%) promotes the growth of bacteria, yeasts or fungi during the storage of plant material [15]. These phenomena can alter the quality of the active ingredient during storage.

The acid insoluble ash content was higher with the root powder. This could be due to contamination of the plant material by sand and dust [15].

The extracts showed a free radical scavenging activity with better activity for the ethanolic extract of the leaves. Previous studies have showed the radical scavenging activity of the extracts from the leaves and roots of *T. macroptera* [5, 16, 17]. This radical scavenging activity could be beneficial in the prevention and treatment of cancers and contribute to their management. Numerous studies have shown the role of free radicals in the pathogenesis of cancers [18-20]. Indeed, they can cause either DNA and RNA damage or chromosomal instabilities, and / or alter gene expression [21].

The current investigation on *T. macroptera* revealed that ethanolic extracts of the leaves or roots exhibited anti-hepatocarcinoma activity on HepG2 cells. A previous study demonstrated the anti-proliferative activity of methanolic extract of *Terminalia macroptera* leaves harvested in Cameroon against four cell lines: NCI-H460 (lung cancer), MCF7 (breast cancer), PC3 (prostate cancer) and HeLa (cervical cancer) with an IC_{50} between 31 - 65 µg / mL [16]. However, the anti-proliferative property of root extracts has not been reported in the literature.

Ethanolic extracts from leaves and roots showed low cytotoxicity on VERO cells. According to Dzoyem and coworkers a crude extract is generally considered to have cytotoxic activity *in vitro* if the CC_{50} is < 20 µg / mL [22]. Based on this threshold the ethanolic extracts from the leaves and roots of *Terminalia macroptera* can be considered to have a weak cytotoxic activity on VERO cells.

Any sample with a selectivity index greater than 3 can be considered to have a high selectivity [23]. On the basis of this threshold the ethanolic extracts from the leaves and roots of *Terminalia macroptera* can be considered to have an anti-hepatocarcinoma properties with a high selectivity.

Conclusion

The free radical scavenging and anti-hepatocarcinoma activities of *Terminalia macroptera* extracts showed in this study could broaden its use in liver conditions. Additionally, all the botanical and physicochemical findings on the leaves or roots could be used in standardization for quality and help to prepare a monograph of this medicinal plant.

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