ISSN(PRINT) : 2320 -1967
ISSN(ONLINE) : 2320 -1975

GLOBAL SCIENTIFIC INC.

ChemXpress

ORIGINAL ARTICLE

CHEMXPRESS 8(1), 48-55, (2015)

Major anthocyanin structure elucidationand radical-scavenging activities of total anthocyanins extract from *terminaliacatappa* red leaves

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Abstract : *Terminaliacatappa*red leaves contained anthocyanin pigments possessing Radical-Scavenging Activities (RSA) comparable to those of Ascorbic acid. Total Anthocyanins Extract Content (TAEC) was estimated at 6 mg of kuromanintetrafluoro acetate (KTFA) eq./g of dried material. Cyanidin 3-*O*-*β*-*D*-glucopyranoside (KTFA), was isolated and characterized using chemical, chromatographic and spectral methods. RSA of TAE were lower (10.57% Inhibition; 87.78% Inhibition) than ascorbic acid (26.72 % Inhibition; 92.30% Inhibition) respectively on both 2,2-Diphényl-1-picrylhydrazyl radical and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid radical-cation.

Keywords : *Terminaliacatappa;* Anthocyanin; Radical-scavenging activities; DPPH assay; ABTS assay; Ascorbic acid.

INTRODUCTION

Humans and animals depend on plants for their own existence. Our environment is characterized by a richly diverse plant Kingdom. Plants are an essential component of biodiversity because they play a key role in maintaining the environmental equilibrium and the ecosystem stability of the Earth. *Terminaliacatappa* L.is a Combretaceous plant, which grows commonly in tropical and subtropical

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countries. Leaves, bark and fruit of this plant have long been used in folk medicine for antidiarrheic, antipyretic, and hemostatic purposes in India, Phillippines, Malaysia, and Indonesia[1].

The rough leaves can reach more than 25 cm long. These leaves turn bright red before their fall revealing the presence of anthocyanins which are phenolic compounds widely used in the plant area and contribute significantly to the physiological processes in plants such as cell growth, germination, ripening or colouring of fruits, flowers and vegetables[2]. *Terminaliacatappa L.* red leaves are used to expel worms, applied to rheumatism, while ordinary leaves are mixed with oil and rubbed on the breast to relieve pain mammary, and finally applied to the throat against angina[3]. They were found to possess good antioxidant activity, reducing power and inhibitors of peroxidation[4,5]. The complexity of the plant extracts does not assign the beneficial effects observed to such or such group of compounds. To contribute to the identification of compounds responsible for the antioxidant activities including radical scavenging activities, it should be targeted, to separate, quantify and assess the biological properties of a particular group of compounds in a crude extract. The isolation of individual compounds by chromatographic methods allows characterizing isolated compounds usingconvenient chemical and spectral methods.

The objective of this work was to quantify Total anthocyaninsextract content from *Terminaliacatappa*red leaves, to evaluate the Radical-Scavenging Activities ofTotal anthocyanins extract and to contribute to the determination of complete structures, including that of isolated major anthocyanin.

EXPERIMENTAL

Chemicals

EtOH, MeOH, HCl, Amberlite XAD-7, ascorbic acid, Sephadex LH-20, distilled water, TLC plates (silica gel 60 F_{254}) silica, cellulose gel. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS); 2,2-Diphényl-1-picrylhydrazyl (DPPH)

Plant material: T. catappa

Red leaves were freshly harvested and dried at room temperature and then crushed. The extract was filtered and concentrated under reduced pressure at low temperature and resumed in MeOH (8 ml) for chromatographic analysis[6-8].

Extraction, purification and isolation of anthocyanins from terminaliacatappa

50 g of dried red leaves of *T. catappa*was crushed and the of powder was extracted by maceration at 5°C successively with 500ml ; then twice with 50 ml of EtOH-HCl 1% (v/v)[9]. The combined extracts were filtered and concentrated almost at 30°C in vacuo. 50 ml of HCl - H_2O (0.5: 99) system/MeOH (7: 3 ml) are added. The solution was filtered and concentrated at 25 ml. This solution was filtered and fixed on a nonionic polymeric adsorbent (Amberlite XAD-7, Aldrich Chemical Co, Milwaukee, WI) column (length 300 mm, i.d. 20 mm) which was prewashed with 0.5% HCl/H_2O; pigments were then eluted with MeOH/H_2O/HCl, 70:30:0.3) and the eluate was concentrated, filtered through a Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) column (length 300 mm, i.d. 20 mm). The final purification was achieved by preparative thin-layer chromatography (TLC) on silica gel 60 F_{254} (Merck-Clevenot Corp.) using EtOAc/HCO_2H/AcOH/H_2O, (100:11:101:26) (EFAW), as solvent system. The isolated band of adsorbent was eluted with 0.5% TFA/MeOH, and the solution of the major anthocyanin was concentrated and filtered through a RP-18 column using (0.5% TFA/H_2O)/MeOH (6:4). The eluatewas finally concentrated and freeze-dried to give about5 mg of (**1**) as TFA salts.

Chemical analysis

*Acidic hydrolysis*was performed by dissolving 1 mg of each pure compound in 4 ml in hydrochloric acid 1 N (in sealed vials)and placed in a waterbath at 100°C. Successive samplings carried out between 0 and 60 minwere analyzed by TLC with the solvent system: BAW [BuOH/AcOH/H_2O (4: 1: 5;upper phase)] on a plate of cellulose. At 60 min, cyanidinwas extracted twice by 0.5 ml of isoamyl alcohol from (**1**). These samples at 60

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minwereanalyzed by TLC on cellulose plate with the Forestal [AcOH/$HCl_{conc.}$/H_2O (30: 3: 10)] as solvent elution together with standards anthocyanidins available.

Controlled and total hydrolyses: hydrolyses are made using the pure compound isolated from the Red leaves of *T.catappa*. About 1 mg of pure compound was dissolved in 2N HCl and heated in a water bath at 100°C. Successive withdrawals between 0 and 60 min, analyzed by TLC in solvent systems: BuOH /AcOH/H_2O (BAW; 4: 1: 5:26), upper phase and EtOAc/HCO_2H/AcOH/H_2O (EFAW; 100: 11: 11) view respectively on plate of cellulose and silica plate. 60 min levy is analyzed by TLC on cellulose with AcOH/$HCl_{conc.}$/H_2O (Forestal; 30: 3: 10)see as elution solvent[10, 11].

Spectral analysis

Electronic spectrometry (UV-Visible): The spectrum was obtained using a spectrometer DES190, system with double energy (Safas). The spectrum was recorded in MeOH-HCl 0.01N from 250 to 600 nm, 2 to 3 drops of $AlCl_3$ in MeOH are added with the preceding solution to highlight the groupings orthodiphenols possibly free[10, 12].

Mass spectrometry: mass spectrometry was performed using a spectrometer Electrospray ionization (EI) in Micromass using MeOH as solvent[12, 13].

Nuclear magnetic resonance spectroscopy: the major anthocyanin compound (1) spectra have been obtained using a bruker 600 MHz Spectrometer in the solventDMSO_d_6/TFA_d1 (0.4: 0.1 v/v) system[14].

Determination of total anthocyanins extract content

TAEC was determined spectrophotometrically (CIBA CORNING 2800 Spectroscon) using a previously method developed by Abdel and Huel[14]. The anthocyanin-containing extract (1 ml) was suitably diluted with acidified EtOH and the absorbance was measured at 535 nm. TAEC in crude extract was then determined by reporting the read absorbance of the sample to a standard curve established with kuromanintetrafluoroacetate (cyanidin 3-*O*-β-*D*-glucopyranoside)in acidified EtOH.

Radical-scavenging activities using DPPH (2,2-Diphényl-1-picrylhydrazyl)assay

Radical-Scavenging Activities of Total anthocyanins extract studied was evaluated on the basis of the reduction of the free radical of 2,2-diphenyl-1-picrylhydrazyl (DPPH)[14]. To 2ml of the DPPH (2 mg/50 ml; 10^{-4} M) (A_0), 10 µL of a extract solution add(A_t). Absorbances were read every minute at 517 nm using an UV-visible spectrophotometer (CIBA-CORNING) against EtOH for 10 min. The extinction coefficient molar of DPPH radical being 12705 M^{-1} cm^{-1}, DPPH concentration is determined by:

$$[DPPH^{\bullet}] = \frac{\Delta A}{\varepsilon_{517} \times l} \text{ with } \varepsilon_{517} = 12705 M^{-1} cm^{-1}$$

and ΔA variation of absorbance versus the blank (DPPH radical in the absence of the sample solution).

Radical scavenging activity can also be expressed as a percentage (%) of radical DPPH inhibited versus the control from the following formula:

$$\%\,\text{Inhibition}(DPPH^{\bullet}) = \frac{\mathbf{A}_{\text{blank}} - \mathbf{A}_{\text{sample}}}{\mathbf{A}_{\text{blank}}} \times 100$$

Radical-Scavenging Activities using ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid]assay

$ABTS^{\bullet+}$ radical-cation was chemically generated according to the Protocol described by Re et al.[14]. 15 ml of ABTS (3.84 mg/ml; 7.01 mM) solution is prepared in distilled water with 1 ml of potassium ($K_2S_2O_8$) (10.6 mg/ml; 39.2 mM) persulphate solution. The mixture is kept to about 16 hours without light. The blue-green solution obtained $ABTS^{\bullet+}$wasconveniently diluted to obtain an absorbance of 0.7 to 734 nm. To 2ml of the radical-cation solution (3.84 mg/ml), 50 µL of the EtOH extract solution were added. Absorbanceswere red at 734 nm every 10 seconds and during 2 min (120 s) against distilled water[15]. By this method, measuring the concentration of the radical-cationreduced by the Total anthocyanins extract during a relatively shorter

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time of 120 s. Absorbance variation (ΔA) relative to the control (solution of the radical-cation in the absence of the sample) at 734 nm allowed to calculate by the following Beer-Lambert law, the concentration :

$[ABTS^{\bullet+}] = \frac{\Delta A}{\varepsilon_{734} * i}$ with $\varepsilon_{734} = 12867 M^{-1} cm^{-1}$ and ΔA variation of absorbance versus control (ABTS radical-cation in the absence of the sample solution). This Radical Scavenging Activity was also expressed as a percentage of radical-cation inhibition[14]:

$$\%\,\text{Inhibition}(ABTS^{\bullet+}) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

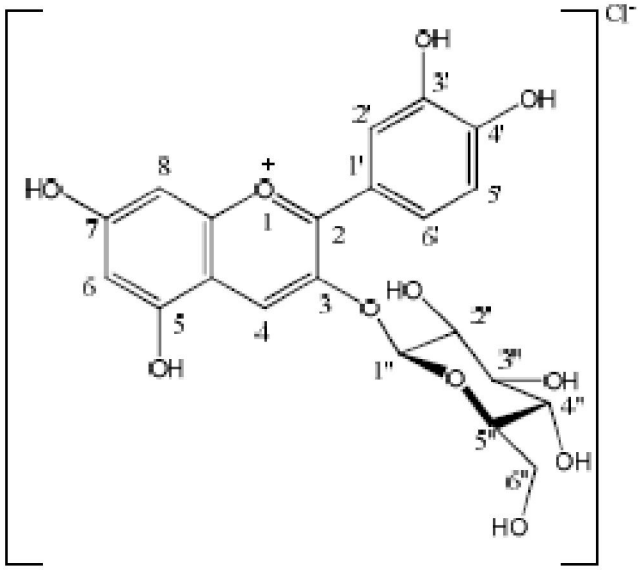

Figure 1 : Complete structure of major (1) isolated from *Terminaliacatappa* (Cyanidin 3-O-β-D-glucopyranoside)

RESULTSAND DISCUSSION

Characterization of the majoranthocyanin

Identification of the major compound the UV Visible spectrum of this major compound in the 0.01 N HCl-MeOH shows 2 bands at 280 nm and 530 nm characteristic of anthocyanin compounds[6]. Absorbance at 280 nm due to the phenolic groups does not identify this compound. However the absorption in the visible area, due to the structure of the pyryliumnucleus and the strong combination of two aromatic rings, is characteristic of the derivatives of cyanidin or the paeonidin. By addition of aluminum chloride ($AlCl_3$) 5%, (**1**)gives red shift effect (in visible area) indicating the presence of vicinal hydroxyl groups on the B ring. This indicates that (**1**) cannot be a derivative of the paeonidin witch does not give red shift effect with $AlCl_3$.

Furthermore,the ratio$A_{440}/A_{max.vis.}$ = 24% (TABLE 1) suggests that position 5 of this compound is free[16]. The TLC of totalacidic hydrolysis products of the TAEin the Forestal together with standards gave one

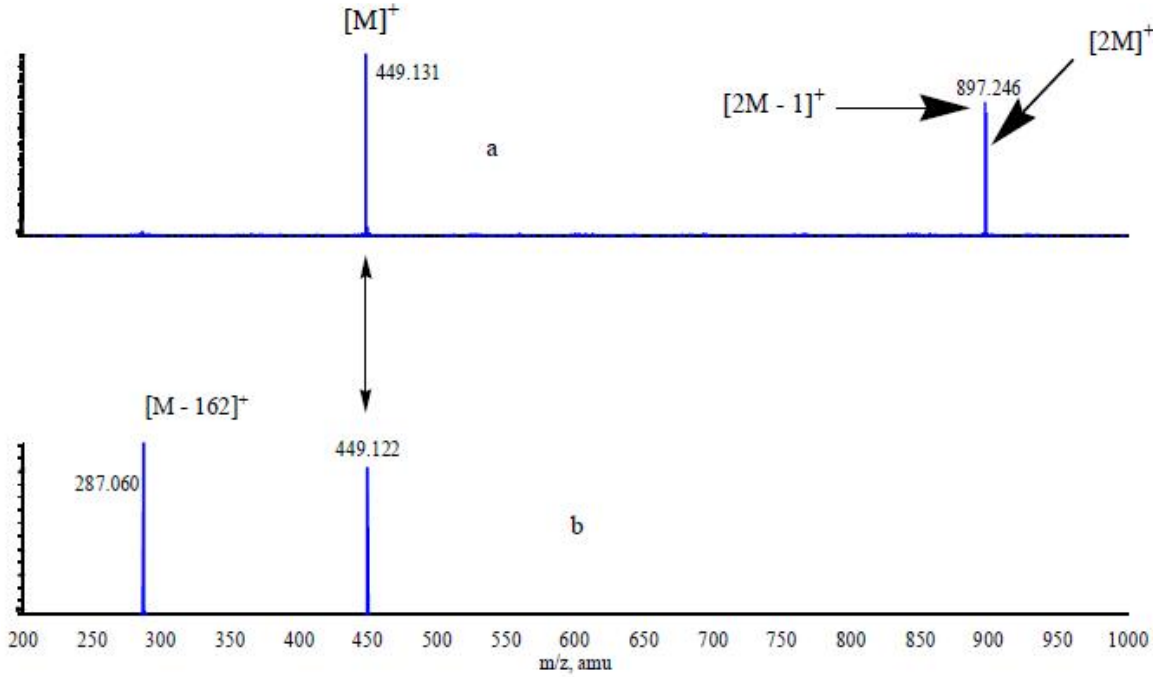

Figure 2 : Mass spectra of the major anthocyanin (1) from *T.catappa*red leaves

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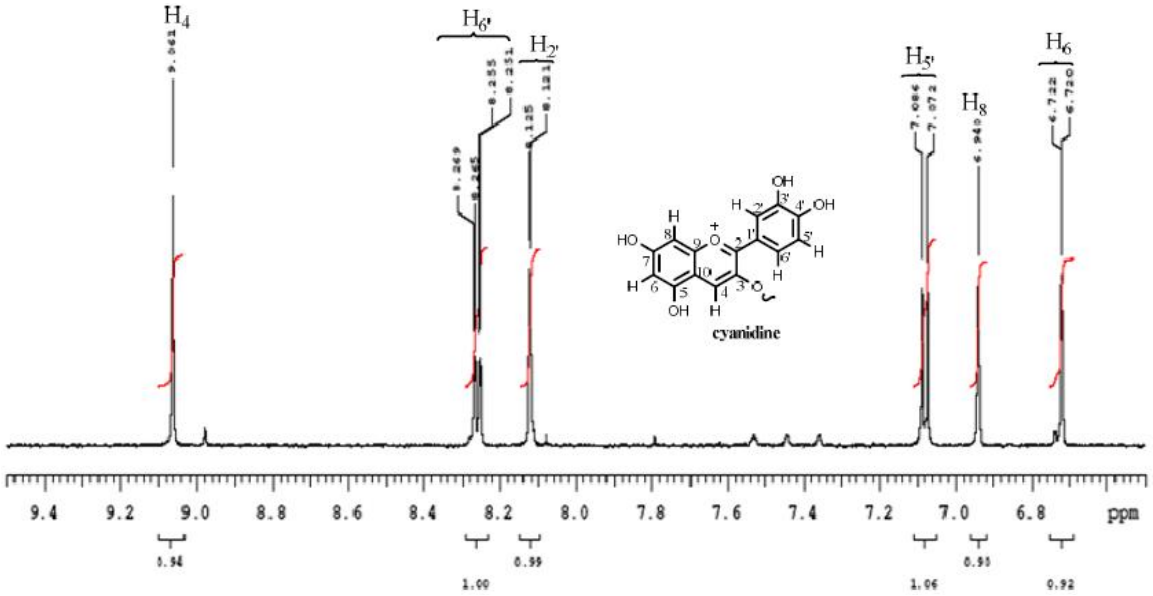

Figure 3 : Spectrum proton NMR of the aglycon of (1) in CD_3OD/TFA

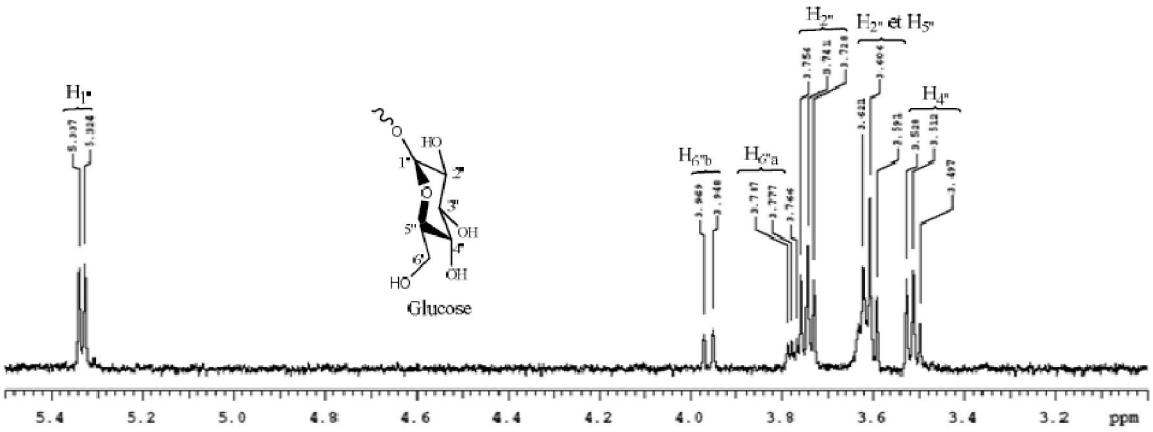

Figure 4 : TOCSY 1-D NMR spectrum: Excitation of anomericproton(5.33 ppm)

TABLE 1 : Spectral and chromatographic data of major anthocyanin (1) from *T.catappa*red leaves

$R_f$ofTLC (x100)				Spectral Data		
Aglycon		Anthocyanin		UV-Vischaracterisation (in MeOH-HCl 0,01N)		TOF ES^+
Forestal*	BAW*	BAW*	EFAW**	λ_{max}(nm)	$E_{440}/E_{max\,vis}$(%) $AlCl_3$(+)$	*m/z*
65	28	52	31	530	24 (+)	$[M]^+$ 449 $[M-162]^+$ 287

Adsorbent: *cellulose microcrystalline F_{254} (0.1 mm Merck) and **silica gel 60 F_{254} (0.1 mm); Solvent systems: BAW, BuOH/AcOH/H_2O (4:1:5 upper phase); EAFW, EtOAc/HCO_2H/AcOH/H_2O (100:11:11:26); Forestal, AcOH/$HCl_{conc.}$/H_2O (30: 3: 10)$drops of 5% $AlCl_3$ in MeOH were added: (+) = red shift; (“) = absence of red shift.

spot corresponding to cyanidin (**1**). Controlled hydrolysis of the compound gave no intermediate tasks between the aglycon and the anthocyanin. This suggests that it is a monoglycosylated compound.The sugar would be so located in position 3, because according to Raju-Garcia[2], anthocyanins are more stable if the sugar is in position 3. The mass spectrum of this major compound (Figure 2) gives amolecular ion m/z 449 (TABLE 1) in accordance with the mass calculated using the formula $C_{21}H_{21}O_{11}$. The peak at m/z 898 $[2\,M]^+$ is due to dimerization of the molecular ion in the flow of the spectrometer Chamber. In addition, secondary ion at m/z 287 was attributed to loss $[M-162]^+$anhexosyle residue (glucosyl or Galactosyl, etc.) $[C_6H_{10}O_5]$.

DQF-COSY (Figure 3) and ^{1} H NMR spectra,

cyanidin ring proton signals were detected unambiguously to δ (ppm) 9.06 (H4) 8.12 (H2'), 7.08 (H5'), 8.26 (H6'), 6.72 (H6) and 6.94 (H8). In addition, spectrum TOCSY 1-D (Figure 4) shows all signals belonging to the same system of spin as the anomeric proton. The anomeric (δ = 5.33 ppm) proton chemical shift and report $E_{440}/E_{maxvis.}$=24% (TABLES 1 and 2) are characteristic of 3-O-glycoside anthocyanidins[16]. In addition, the high value of the coupling constant observed for the signal of the anomeric proton of glycosylated (J = 7.8 Hz) suggests that it was inβ-D-glucopyranoseform[16].

According the glycosyl, the anomeric proton DQF-COSY spectrum is coupled to a proton chemical shift is δ = 3.74 ppm (H2''). The latter is coupled to one proton to 3.61 ppm (H3") which in turn is coupled to proton chemical shift 3.51 ppm (H4''). The same proton chemical shift 3.61 ppm (H5 ") is paired with a proton to 3.78 ppm (H6a"). Second methylene proton (H6b") with a wide coupling geminal ($^2J_{HH}$ = 12 Hz) with H6a" appears at 3.95 ppm.

NOESY 2-D spectrum showed proximity of the anomeric proton and H4 indicating as the substituent glycosylated on the aglycon is in position 3. Thus, these NMR data indicate that sugar in C6 can be either galactose or glucose. The proton H4"(triplet, δ = 3.51 ppm) shows a *trans*coupling with H3" and H5 "because of the high value of the coupling constant ($^3J_{H4"/H3"} = {}^3J_{H4"/H5"}$= 9 Hz). This indicates that sugar is a glucose, otherwise there would be a coupling constant ($^3J_{H4"/H3"} = {}^3J_{H4"/H5"}$ ~ 3-4 Hz) characteristic of *cis*coupling of the proton H4" with H3" and H5 "in galactose. Thus, this compound (**1**) is identified as cyanidin 3-O-β-D-glucopyranoside (Figure 1).

.Total anthocyanins extract and Radical-Scavenging Activities

The absorbance read 535 nm from the crude extract of red leaf of *Terminaliacatappa* reported on the standard curve established with kuromanintetrafluoroacetate of in the same conditions. Total anthocyanins extract content from *T.catappa*red leaves was estimated to 6 mg of eq. of kuromanintetrafluoroacetate/g dry red leaves.

DPPH radical scavengingActivity of Total anthocyanins extract was evaluated in comparison with that of Ascorbic acid taken as reference for 10 min. At the same concentration, Total anthocyanins extract of*T.catappa*red leaves presented a lower concentration of scavenged DPPH radical (14.48 µM)than Ascorbic acid (36.60 µM) (Figure 5)

At the same concentration total anthocyanins extract from *T. catappa*scavenged a concentration of radical-cation ($ABTS^{\bullet+}$) of about 51.37 µM versus a slightly higher Radical-cation scavenged concentration for Ascorbic Acid of 53.96 µM. On ABTS radical-cation, TAE had a very close value of RSA

TABLE 2 : ^{1}H-NMR Spectral data of major anthocyanin(1)[δ in CD_3OD/TFA-d_1 (5:1)] from *T.catappa*

Attribution des signaux	δ_H(ppm) J (Hz)
H_4	9,06, s
H_6	6,72, d (1,2)
H_8	6,94, d (1,2)
$H_{2'}$	8,12, d (2,4)
$H_{5'}$	7,08, d (8,4)
$H_{6'}$	8,26, dd (2,4 et 8,4)
Glucose	
$H_{1''}$	5,33, d (7,8)
$H_{2''}$	3,74, dd (7,8 et 9)
$H_{3''}$	3,61, dd (7,8 ; 9)
$H_{4''}$	3,51, t (9)
$H_{5''}$	3,61 dd (7,8 ; 12)
$H_{6a''}$	3,78 dd (12)
$H_{6b''}$	3,95, d (12)

Multiplicity : s = singulet, d = doublet, dd = double doublet

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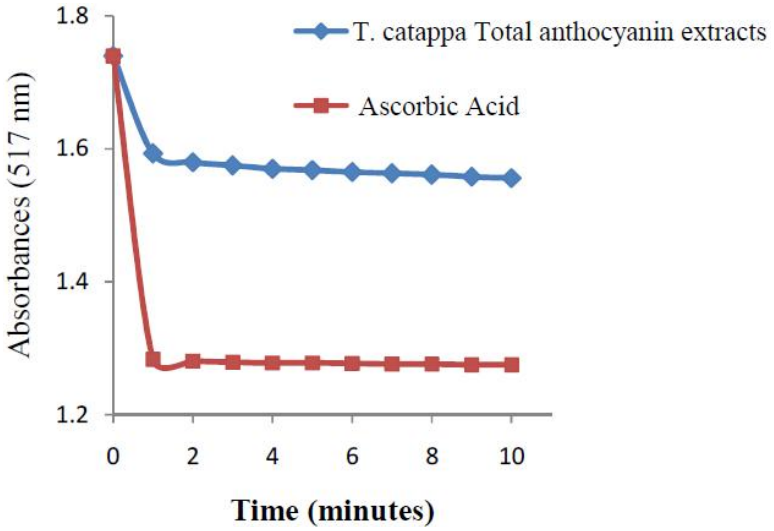

Figure 5 : curves expressing DPPH Radical-Scavenging Activitiesof acid Ascorbic and *Total anthocyanins extract* from *T. catappa*red leaves

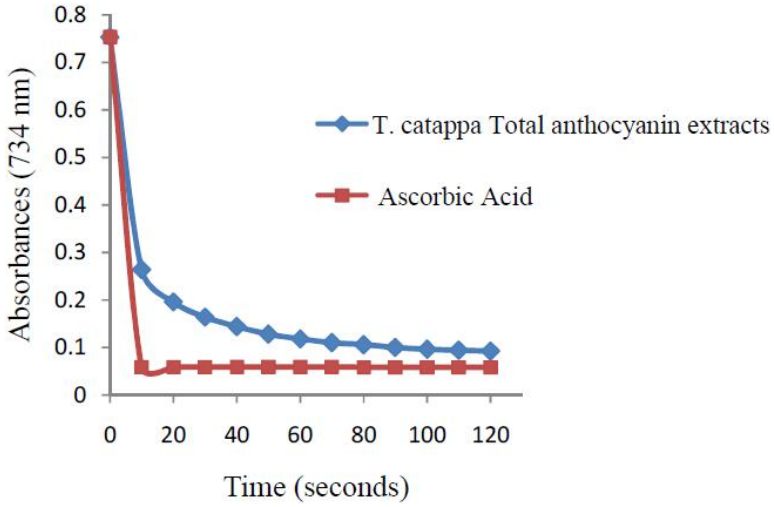

Figure 6 : Curves expressing $ABTS^{\bullet+}$Radical-Scavenging Activitiesof acid Ascorbic and *total anthocyanin of T. catappa*red leaves extracts

TABLE 3 : summary radical scavenging activitiesmeasurements

	DPPH		ABTS	
	$\%INH_{DPPH}$	$[DPPH]_{scav.}\mu M$	$\%INH_{ABTS}$	$[ABTS^{\bullet+}]_{scav.}\ \mu M$
RSA_{TAE}	10.57	14.48	87.78	51.37
RSA_{AA}	26.72	36.60	92.30	53.96

RSA_{TAE} and RSA_{AA} are respectively Radical-Scavenging Activities of Total Anthocyanins and Ascorbic Acid Extracts; $\%INH_{DPPH}$ and $\%INH_{ABTS}$ are respectively inhibition rates of DPPH radical and $ABTS^{\bullet+}$ radical-cation ; $[DPPH]_{scav.}$and$[ABTS^{\bullet+}]_{scav.}$arerespectively corresponding scavenged DPPH radical and $ABTS^{\bullet+}$ radical-cation concentrations.

(87.78%) to that of Ascorbic Acid (92.30%).

However on the DPPH radical, there was a very significant difference between Ascorbic acid and TAE. This big difference between of the reference and TAE RSA values could be explained by an observable steric from the DPPH radical site. This structure is marked by the presence of three phenyl rings surrounding the radical site on the nitrogen atom. Such structure is not easily accessible by complex molecules of the size of anthocyanins. The highest estimated RSA value of Ascorbic acid could be explained by the relative simple structure of the

molecule of vitamin C. In any case, Ascorbic Acid was more active on DPPH and ABTS radicalsthan TAEfrom *T. catappa* red leaves.(Figure 6)

ACKNOWLEDGEMENTS

The Authors thank the "UniversitéLibre de Bruxelles", Belgium and the University of Ouagadougou for their financial and technical supports.

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