

# Hispanamide: An Antioxidant Phenolic Propanamide from the Leaves of *Acalypha hispida* (Burn.F)

Ganiyat Kehinde Oloyede<sup>1</sup> Oluwakayode Olubunmi Odeja<sup>2</sup> Olawale Abiodun Bankole<sup>1</sup>  
Waheed Adekunle Akanmu<sup>1</sup> Patricia Akpomedaye Onocha<sup>1\*</sup>

1. Natural products/Medicinal Chemistry Unit, Department of Chemistry, University of Ibadan, Ibadan, Nigeria

2. Chemistry Department, Federal University of Petroleum Resources, Effurun, Delta State, Nigeria

## Abstract

*Acalypha hispida* (family Euphorbiaceae) is widely known for its medicinal applications in ethno-medicine. The most active antioxidant fraction of the leaf methanol extract was investigated with the aim of isolating and elucidating the active constituents. Bioassay guided isolation and purification was achieved using standard chromatographic techniques and invitro evaluation of ability of fractions, isolates and standards to scavenge 2, 2-Diphenyl-1-picrylhydrazyl radical (DPPH). The concentration of the pure isolate and standards required to scavenge 50% of the hydroxyl radical (IC<sub>50</sub>) was carried out to assess their antioxidant effectiveness. The lower the IC<sub>50</sub> value, the greater the antioxidant effectiveness of the compound. Various spectroscopic techniques were used in the structural elucidation. The active isolate was identified as 4-hydroxy-N-[1-(hydroxymethyl)-2-phenylethyl-benzenepropanamide and named Hispanamide. Hispanamide exhibited a higher antioxidant effectiveness (IC<sub>50</sub> of 0.339 mg/ml) than the antioxidant standards Ascorbic acid (IC<sub>50</sub> of 0.373 mg/ml) and  $\alpha$ -Tocopherol (IC<sub>50</sub> of 0.482 mg/ml) used for the assay. The order of decreasing antioxidant effectiveness is hispanamide > Ascorbic acid >  $\alpha$ -Tocopherol. This is the first report of the isolation of this phenyl propanamide from plant source and its occurrence in the plant validates the ethnomedicinal uses of *Acalypha hispida*.

**Keywords:** Hispanamide, Phenolic propanamide, Antioxidant effectiveness, hydroxyl radical, *Acalypha hispida*.

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## 1. Introduction

The role of free radicals in many diseased conditions has been established. Various biochemical reactions in our body generate reactive oxygen species which are capable of damaging important bio-molecules and if not effectively scavenged by cellular constituents, constitute serious health hazards to man since they lead to degenerate diseases such as cancer, premature aging, diabetes, tumors, cirrhosis, cardiovascular, nervous, rheumatic and pulmonary disorders, cardiovascular diseases, cataract and macular degeneration (Halliwell & Gutteridge, 1990; Halliwell et al, 1992). Continuous intake of antioxidants is said to be necessary and important in reducing cellular damage and ageing process (Potterat, 1997).

Different parts of the plant is used in ethno-medicine for the treatment of kidney ailments, leprosy, gonorrhea, asthma, as a laxative, expectorant and diuretic (Iwu et al, 1999; Kafaru, 2000; Sofowora, 2008). Due to the detection of many bioactive compounds in plants with possible antioxidant activity, there has been increased interest in the relationship between antioxidants and disease risks (Nilsson, 2006). There is therefore need for discovery of more plants and compounds with antioxidant activities.

The presence of phenolics, flavonoids, glycosides, steroids, saponins, phlobatannins, and hydroxyanthraquinones had been detected in a previous phytochemical screening of the aqueous and leaf methanolic extracts of *A. hispida* (Iniaghe et al, 2010; Okorundu et al, 2009). Antifungal properties of extracts of leaves of *A. hispida* have also been reported (Ejechi and Soucey, 1999). In addition, alcoholic extracts of *A. hispida* have been reported to be biologically active against *P. aeruginosa*, *E. coli* as well as *S. aureus* and *S. typhi* (Okorundu et al, 2009). Gallic acid, corilagin, cycloartane-type triterpenoids, flavonoids like quercetin and kaempferol derivatives have been isolated from the plant (Adesina et al, 2000; Bergitte et al, 2003; Gutierrez-Lugo et al, 2002). We had earlier reported the toxicity and antioxidant activity of fractions obtained from chromatographic separation of leaf methanolic extract of *A. hispida* (Onocha et al, 2011).

This present research report is a bioassay guided isolation, purification and characterization of the pure isolate from the most active antioxidant fraction obtained from the previous work (Onocha et al, 2011). In continuation of our research work on bioactive components and derivatives from Nigerian medicinal Euphorbiaceae plants and search for source of new antioxidants and therapeutic drugs from plant source (Onocha et al, 2004; Oloyede et al, 2010a,b; Onocha and Ali, 2010; Onocha and Olusanya, 2010; Onocha et al., 2010; 2011; Oloyede et al., 2011a,b), we now report for the first time from plant source, the isolation of Hispanamide: an Antioxidant Phenolic Propanamide from the leaves of *Acalypha hispida*. It had earlier been synthesized from Phenyl Alaninol, 4-hydroxy hydrocinnamic acid and triethylamine as one of the N-substituted benzenepropanamides invented, synthesized and patented by Kaplan and Tiqwa (2014) as nonsteroidal drugs for use in the treatment of pain and inflammation.

## 2. Materials and methods

### 2.1 Chemicals and reagents

Hexane (Hex), ethyl acetate (EtOAc), methanol (MeOH), butanol, Dichloro methane (DCM), chloroform, hydrochloric acid, ammonia solution, naphthol, bismuth nitrate, potassium iodide, sodium hydroxide, sodium chloride, copper sulphate pentahydrate, sodium potassium tartarate, potassium chloride, glacial acetic acid, disodium hydrogen phosphate, and dihydrogen potassium phosphate were all BDH general purpose chemicals and distilled prior to use. Dimethylsulphoxide (M&B, England), hydrogen peroxide, silica gel 60 - 120 microns (Merck, Germany), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid and  $\alpha$ -tocopherol were obtained from Sigma Chemical Co (St Louis, MO).

### 2.2 Equipment and Apparatus

Soxhlet apparatus, Mettler analytical balance H80 (UK), Water Bath (Gallenkamp), Rotavapor RII0 (Buchi, England), silica gel 60 F254 (precoated aluminium sheets - Merck Germany), pH meter (Jenway model), UV-Visible spectrophotometer (Unico1200 & Perkin Elmer lambda 25 models), Glass Column chromatographic materials and fraction collectors.

### 2.3 Fractionation Procedure

In the previous study (Onocha et al, 2011), of the 236 fractions collected (with 14 pooled fractions B1 – B14), fraction B9 (139-150 – eluted with 30% MeOH in EtOAc) interestingly, was found to exhibit a higher significant activity (99.7 %) at the concentration of 0.1 mg/ml than all the fractions and standards used in scavenging hydroxyl radical. It was also found to be the most active of all the fractions at scavenging free radicals at concentrations ranging from 0.0625 mg/ml to 1.0 mg/ml with the following percentage inhibition: 91.6, 92.9, 93.8, 94.1 and 94.9 (at 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml, respectively). At the same concentration ranges, it exhibited higher activity than the standards Ascorbic Acid (with percentage inhibition: 44.3, 65.4, 67.8, 68.7 and 90.9) and  $\alpha$ -Tocopherol (with percentage inhibition: 10.4, 12.1, 12.4, 12.4 and 15.4) while it was comparable in activity to that of the standard butylated hydroxyanisole (with percentage inhibition: 91.9, 93.9, 94.0, 94.3 and 95.4) used for the assay.

Thus fraction B9 (0.845g) was pre-adsorbed with 0.5g of silica gel prior to column chromatographic separation. 20g of silica gel was packed into the column and the adsorbed fraction was added on top of the column in the dry state. The mobile phase was gradiently introduced to elute the material. Solvent system used was hexane, ethyl acetate and methanol while silica gel 60-120 microns was used as adsorbent. The proportion of the more polar solvent in the nonpolar one (Hex: EtOAc) was increased by the following percentages: 25%, 33%, 50%, 75%, then 100% of EtOAc and finally 5% of MeOH in EtOAc was used. 10ml each of the effluent were collected in the fraction collectors and monitored by TLC. Different solvent systems employed for TLC included EtOAc/Hex (2:1), EtOAc/Hex (1:2), EtOAc/Hex (1:1), 100% EtOAc, EtOAc/Hex (4:1), EtOAc/Hex (3:1) EtOAc/Hex (4:1), EtOAc/Hex (5:1), Dichloro methane (DCM)/MeOH (3:1) DCM/MeOH (4:1), DCM/MeOH (5:1), MeOH/DCM (8:1), MeOH/DCM (2:8), MeOH/DCM (3:1), MeOH/DCM (2:1), MeOH/DCM (1:1) and adsorbent for TLC was silica gel 60 F254 precoated aluminium plates. The retention factor  $R_f$  calculated from TLC analysis of the 100 fractions obtained was used as the basis for bulking the fractions accordingly, thus: 1-15 (Y1), 16-34 (Y2), 35-65 (Y3), 66-77 (Y4), 78-92 (Y5 – eluted with 100% EtOAc), 93-100 (Y6). Thereafter, free radical scavenging activity test were carried out using the scavenging effect on DPPH. Only the active fraction Y5 which exhibited free radical scavenging activity with DPPH was subjected to further chromatographic separation. Thus fractions Y5 was further purified by isocratic elution on a micro column packed and eluted with EtOAc/Hex (1:1). Thereafter, elution was completed using EtOAc/Hex (3:1) and subsequently 100% EtOAc. 25 fractions were collected from the micro column. Similar fractions were again pooled together based on the TLC analysis. Subsequently, free radical scavenging activity test were carried out on the pooled fractions 1-13 (B9YD), 14-17 (B9YC), 18-20 (B9YB) and 21-25 (B9YA). The active fraction B9YB was recrystallized with methanol to give a white solid, Mp-149-150 °C.

### 2.4 Scavenging Effect on 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The ability of the extracts to scavenge DPPH radical was determined according to Oloyede et al., 2010b; Onocha et al., 2011. 2, 2-diphenyl-1-picrylhydrazyl radical (3.94 mg), a stable radical was dissolved in methanol (100ml) to give a 100  $\mu$ m solution. To 3.0 ml of the methanolic solutions of DPPH was added 0.5 ml of each of the bulked fractions with doses ranging from 0.0625 mg/ml to 1.0mg/ml. The decrease in absorption at 517 nm of DPPH using UV-Visible spectrophotometer was measured 10 minutes later. The actual decrease in absorption was measured against that of the control (Table 1) and the percentage inhibition was also calculated using the formula:  $I (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$ . The same experiment was carried out on ascorbic acid and  $\alpha$ -tocopherol which are known antioxidants. All test and analysis were run in triplicates and the results obtained were averaged.

### 3. Results and Discussion

#### 3.1 Spectral data

White solid, mp-149-150 °C. IR (KBr)  $\nu_{\max}$   $\text{cm}^{-1}$ : 3440-3380 (OH, NH), 1640 (-CON), 1450 (N-H).  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ ),  $\delta$ (ppm): 7.47 (2H, d,  $J=8.0$  Hz, H-2,6), 7.44 (2H, dd,  $J=8.0, 6.8$  Hz, H-3',5'), 7.23 (1H, dd,  $J=6.8, 6.4$  Hz, H-4'), 6.99 (2H, d,  $J=8.0$  Hz, H-3,5), 6.96 (2H, d,  $J=8.0$  Hz, H-2',6'), 4.07 (H, dd,  $J=6.8, 6.4$  Hz, H-9'), 2.83 (2H, d,  $J=7.2$  Hz, H-7'), 2.81 (2H, t,  $J=7.6$  Hz, H-7), 2.58 (1H, dt,  $J=7.2, 6.8$  Hz, H-8') 2.56 (2H, t,  $J=7.6$  Hz, H-8).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CD}_3\text{OD}$ ),  $\delta$  (ppm): 131.3 (C-1), 118.6(C-2/6), 123.6 (C-3/5), 148.3 (C-4), 30.8 (C-7), 36.3 (C-8), 175.1 (C-9), 124.1 (C-1'), 124.5 (C-2'/6'), 124.4 (C-3'/5'), 123.7(C-4'), 30.9 (C-7'), 35.6 (C-8'), 64.4 (C-9'). HRESIMS [M-H]:  $m/z$  299.1681 (calcd. 299.2241), TOF MS ES+:  $m/z$  149 ( $\text{OHC}_6\text{H}_4\text{CH}_2\text{CH}_2\text{CO}$ )<sup>+</sup> is indicative of the presence of p- hydroxyl hydrocinnamic acid moiety.

The  $^1\text{H}/^{13}\text{C}$  spectra revealed the presence of 21 protons and 18 carbons atoms and the HRESIMS [M-H] confirmed the molecular formula of Compound B9YB as  $\text{C}_{18}\text{H}_{21}\text{O}_3$  with  $m/z$  299.1681 (calcd. 299.2241). The IR spectrum revealed a broad signal at  $\nu_{\max}$  3440-3380, 1660 and 1450  $\text{cm}^{-1}$  which suggested the presence of hydroxyl and amide groups in the molecule. The  $^1\text{H}$  NMR spectrum exhibited resonances in the low-field region for two sets of AAB'B' type signals at  $\delta_{\text{H}}$  7.47 (2H, d,  $J=8.0$  Hz, H-2,6), 6.96 (2H, d,  $J=8.0$  Hz, H-2',6'), 6.99 (2H, d,  $J=8.0$  Hz, H-3,5) and 7.44 (2H, dd,  $J=8.0, 6.8$  Hz, H-3',5'). The fourth signal was coupled to a fifth signal at  $\delta$  7.23 (1H, dd,  $J=6.8, 6.4$  Hz, H-4'). In the high-field region, two coupled triplets of methylene protons appeared at  $\delta$  2.81 (2H, t,  $J=7.6$  Hz) and 2.56 (2H, t,  $J=7.6$  Hz) attributable to H-7 and H-8 respectively, suggesting that the 1, 4 disubstituted aromatic ring fragment was of the p- hydroxyl hydrocinnamic acid moiety (Molbase encyclopedia). This was strongly supported by the presence of the fragment ion at  $m/z$  149 ( $\text{OHC}_6\text{H}_4\text{CH}_2\text{CH}_2\text{CO}$ )<sup>+</sup>. A third coupled doublet methylene was observed at  $\delta$  2.83 (2H, d,  $J=7.2$  Hz) attributable to H-7'. In addition, a methine proton which appeared at  $\delta$  2.58 (1H, dt,  $J=7.2, 6.8$  Hz) coupled to both the methylene at H-7' and a hydroxymethyl at  $\delta$  4.07 (H, dd,  $J=6.8, 6.4$  Hz) was assigned to H-8' while the hydroxyl methyl was assigned to H-9'. The structure of B9YB was therefore deduced to be an amide of p- hydroxyl hydrocinnamic acid and 2-hydroxymethyl- 3-phenyl-ethylamine (Figure 1).. The one bond proton-carbon connectivities were confirmed by the heteronuclear single quantum correlation spectroscopy (HSQC) experiments while the  $^2\text{J}$  and  $^3\text{J}$  long range proton-carbon couplings were indicated through the heteronuclear multiple bond correlation spectroscopy (HMBC) experiments.

The HMBC spectrum exhibited a number of cross peaks representing long range heteronuclear interactions between the quaternary carbons and the proton (Figure 2). The carbonyl carbon at  $\delta$  175.1 which exhibited long range couplings with the hydroxyl methyl protons resonating at  $\delta$  4.07 (2H, dd,  $J = 6.8, 6.4$ , Hs-9') also caused cross peaks with the methine proton at  $\delta$  2.58 (1H, dt,  $J = 7.2, 6.8$  Hz, H-8') and the methylene protons at  $\delta$  2.56 (2H, t,  $J = 7.6$  Hz, H-8) and  $\delta$  2.81(2H, t,  $J = 7.6$  Hz, H-7). Similarly, the downfield oxygen-bearing carbons at  $\delta$  148.3 was identified as the oxygen-bearing aromatic carbons, i.e., C-4 and it exhibited HMBC interactions with the protons resonating at  $\delta$  6.99 (2H, d,  $J = 8.0$  Hz, H-3/5) and  $\delta$  7.47 (2H, d,  $J = 8.0$  Hz, H-2/6), thus indicating that the aromatic methine carbons C-3/C-5 was bound to the oxygen-bearing aromatic quaternary carbon C-4. This was further supported by the cross peak the aromatic quaternary carbons at  $\delta$  131.3 (C-1) caused with the aromatic protons at  $\delta$  7.47 (2H, d,  $J = 8.0$  Hz, H-2/6). Further cross peaks observed between quaternary aromatic C-1 ( $\delta$  131.3) and the methylene protons at  $\delta$  2.56 (2H, t,  $J = 7.6$  Hz, H-8) and  $\delta$  2.81(2H, t,  $J = 7.6$  Hz, H-7) confirmed the p- hydroxyl hydrocinnamic acid moiety and established that the carbonyl group and amino group of the amide moiety were at positions 9 and 10 respectively.

The quaternary aromatic C-1' ( $\delta$  124.1) exhibited a long range couplings with the aromatic methine proton at  $\delta$  7.23 (1H, dd,  $J = 6.8, 6.4$  Hz, H-4') and cross peaks with the methylene protons at  $\delta$  2.83 (2H, d,  $J = 7.2$  Hz, H-7') and aromatic protons at  $\delta$  6.96 (2H, d,  $J = 8.0$  Hz, H-2'/6') and  $\delta$  7.44 (2H, dd,  $J = 8.0, 6.8$  Hz, H-3'/5'). This confirmed the 2-hydroxymethyl- 3-phenyl-ethylamine moiety.

The COSY spectrum exhibited cross peaks which represent coupling between protons H-7 ( $\delta$  2.81, 2H, t,  $J = 7.6$  Hz) and H-8 ( $\delta$  2.56, 2H, t,  $J = 7.6$  Hz); H-7' ( $\delta$  2.83, 2H, d,  $J = 7.2$  Hz) and H-8' ( $\delta$  2.58, 1H, dt,  $J=7.2, 6.8$  Hz); H-3'/5' ( $\delta$  7.44, 2H, dd,  $J = 8.0, 6.8$  Hz) and H-4' ( $\delta$  7.23, 1H, dd,  $J=6.8, 6.4$  Hz,).

Thus, with the HMBC and HSQC connectivities together with the HREIMS and COSY 45°, the chemical shifts of all the protons and carbons could be assigned unambiguously.

The structure was thus identified as 4-hydroxy-N-[1-(hydroxymethyl)-2-phenylethyl]-benzenepropanamide (Fig 1) by its NMR data and comparison with the published data in literature (Kaplan and Tiqwa, 2014; Molbase encyclopedia). To the best of our knowledge, this is the first time this phenyl propanamide is isolated from plant source and its occurrence in the plant justifies the ethnomedicinal uses of *Acalypha hispida*.

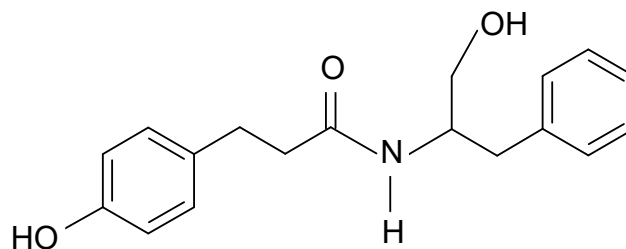


Figure 1: The structure of Hispanamide - 4-hydroxy-N-[1-(hydroxymethyl)-2-phenylethyl]benzenepropanamide.

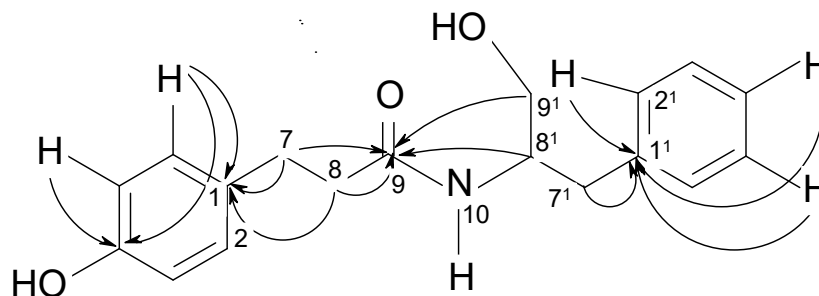


Figure 2: HMBC correlations of Hispanamide

### 3.2 Antioxidant activity of Hispanamide (B9YB)

In the DPPH scavenging radical method, the percentage inhibition of the B9YB isolate was the highest at all concentrations ((Figure 3). The  $IC_{50}$  (the concentration of the samples required to scavenge 50 % of the DPPH radicals) was used to examine the antioxidant effectiveness of sample. The lower the  $IC_{50}$ , the greater the overall effectiveness of the suspected antioxidant sample. The results obtained revealed that B9YB had the best antioxidant effectiveness with  $IC_{50}$  of 0.339mg/ml followed by Ascorbic acid with  $IC_{50}$  of 0.373mg/ml and the synthetic antioxidant standard  $\alpha$ -Tocopherol at  $IC_{50}$  of 0.482 mg/ml.

The order of decreasing antioxidant effectiveness is B9YB > Ascorbic Acid >  $\alpha$ -Tocopherol.

CONC.(mg/ml)	Absorbance values from scavenging effects on DPPH at 517nm		
	B9YB	Ascorbic Acid	$\alpha$ -Tocopherol
1.0	0.120 $\pm$ 0.000	0.131 $\pm$ 0.000	0.452 $\pm$ 0.001
0.5	0.067 $\pm$ 0.001	0.103 $\pm$ 0.001	0.449 $\pm$ 0.002
0.25	0.190 $\pm$ 0.000	0.204 $\pm$ 0.001	0.444 $\pm$ 0.002
0.125	0.247 $\pm$ 0.000	0.335 $\pm$ 0.001	0.417 $\pm$ 0.002
0.0625	0.186 $\pm$ 0.002	0.220 $\pm$ 0.002	0.369 $\pm$ 0.032
$IC_{50}$	0.339	0.373	0.482

Table 1: Scavenging Effect and percentage inhibition of Hispanamide and standards on 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

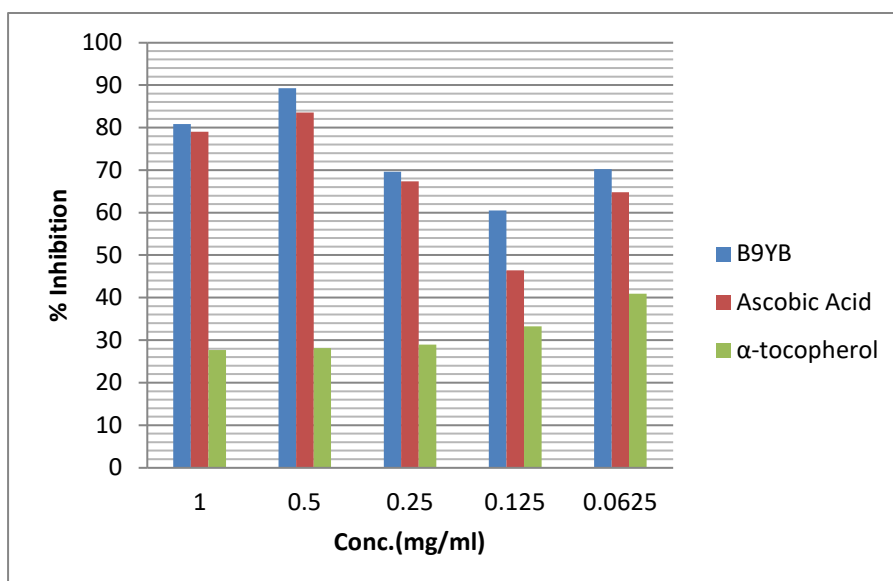


Figure 3: Percentage inhibition of Hispanamide and Standards on 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

#### 4. Conclusion

A number of N-substituted benzenepropanamides have been invented, synthesized and used alone as nonsteroidal drugs in the treatment of pain and inflammation or in combination with other analgesic. The isolation and elucidation of Hispanamide, one of such derivatives for the first time from natural sources (plants) and the establishment of its antioxidant effectiveness enhances its anti-inflammatory properties and could be useful for the treatment of ailments resulting from oxidative stress. Furthermore, its presence in the plant justifies the ethnomedicinal uses of *Acalypha hispida*.

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#### Conflict of interest

The authors declare that there is no conflict of interest.

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