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## ARTICLES

*Full Length Research Paper*

# Synthesis of 2-(benzylthio)benzimidazole, 2-[(benzimidazol-2-yl)methylthio]benzimidazole and structural analogues against *Haemoncus contortus*

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The coupling of the derivatives of the 2-mercaptobenzimidazole 1 with the derivatives of the (chloromethyl)benzene 2 gives 2-(benzylthio)benzimidazole 4a-k on the one hand, and with the 2-(chlorométhyl)benzimidazole 3 on the other the 2-(benzimidazolyl methylthio) benzimidazole and analogues 5a-k. We determined the structures of all synthesized compounds by Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS). The evaluation of the anthelmintic activities of these molecules on *Haemonchus contortus* showed that the introduction of the nitro group ($NO_2$) in the structure causes a significant increase of the activity. Among the molecules evaluated *in vitro* for their anti-infectious activity, the compounds 4b, 5d, 5e, 5f and 5h revealed an activity which is comparable to that of the reference molecules (ivermectin and fenbendazole).

**Key words:** 2-mercaptobenzimidazole, (chloromethyl)benzene, 2-(chloromethyl)benzimidazole, 2-(methylthio)benzimidazole, 2-(benzylthio)benzimidazole,2-(benzimidazolyl methylthio) benzimidazole, anthelmintic, *Haemonchus contortus*.

## INTRODUCTION

Intestinal parasitic are very spread infections throughout the world and most of them are rampant in tropical areas mainly in developing countries where all favorable factors for their hatching are gathered: Hot and humid climate, lack or inadequacy of hygiene and sanitization measures and poverty. Although the consequences of the different pathologies are minor in developed countries, they are cruelly dramatic in poor or developing countries. These parasitic ailments are public health issues and are responsible for high rates of morbidity and mortality.

Gastrointestinal nematode infections are major pathologies in both human beings and animals. In sheep farming, this parasitism may be a limiting factor in the production because controlling it requires implementing medicinal measures as well as the implementation of sanitary measures. This curse causes huge economic losses in food-processing (Hussain and Dawson, 2013; Roeber et al., 2013). Several studies worldwide permitted to identify different species of nematodes and it is to be noticed that the most common nematode veterinarian

and the most dangerous is the species *Haemonchus contortus* (Achi and Zinsstag, 2003; Tehrani et al., 2012). Now, the fight against parasitic diseases rests on the use of molecules containing in their skeleton the benzimidazole. This nucleus is the pharmacophore of many drugs used in therapeutics, namely in the treatment of infectious diseases. In the chemotherapy of intestinal helminthiasis, the most determining discovery is with no doubt the one relating to the biologically active chemical class of compounds, which the most contain the benzimidazole ring in the skeleton such as the thiabendazole, the albendazole , the mebendazole and the flubendazole (Figure 1) which are common use drugs against intestinal worms. However, in veterinary medicine, the most effective means of fight remains the use of anthelmintic drugs whose representative is currently the triclabendazole (Figure 1) which is the most used (Fairweather, 2009). According to its uniqueness, several pharmacochemical investigations about its chemical profile were carried out to extend its spectrum of activity (Mahiuddin et al., 2007; Anelia and al., 2006). But, this chemotherapy shows its limits with the emergence of various strains resisting to many of these drugs (Kaplan, 2004; Fairweather, 2009; Olaechea et al., 2011; Winkelhagen et al., 2012; Van den Brom et al., 2013; Saunders and al, 2013; Ortiz and al., 2013; Brockwell et al., 2014).

In this context, it seems important for us to synthesize new agents with nematicide aims, particularly active on *H. contortus*. To reach our objectives, we carried out an arranged structural variation thanks to the substitute flexibility of positions -2 and -5 of the 2-méthylthiobenzimidazole, basic structure of the triclabendazole. Through a rational analysis of the correlations structure-activity and the perspectives of the requirements of pharmacochemistry, we applied a technique of pharmaco-modulation on the pseudo typical molecule from the basic benzimidazole skeleton by replacing the methylthio group by the methylthioaryl or methylthioheteroaryl group. This replication by molecular rearrangement allows understanding that the chemical arrangement related to the specific structural modification is able to allow improving the molecular environment in order to optimize the pharmacological action or a modulation of the biological activity.

We tested on *H. contortus* these new anthelmintic candidates drugs, stemming from these molecular reorganizations, structural analogues of the triclabendazole, in order to assess their nematocide activities.

## MATERIALS AND METHODS

### Chemistry

#### *General*

Melting points were determined using a Kofler bencheto graduating temperature (40-206°C). Purifications by column chromatography were carried out on Kieselgel 60 (230-400 mesh, Merck).$^1$H and $^{13}$C measured on a 300 MHz Bruker Avanced apparatus with tetramethylsilane (TMS) served as internal standard: The NMR spectra ($^1$H and $^{13}$C) were performed in DMSO. Mass spectra were conducted on a HP5889A spectrometer. All spectrometers analysis was realized in the CEISAM laboratory of the University of Nantes.

**General procedure for synthesis of compounds 4a-k:** Initially, we prepared 2-mercaptobenzimidazole derivatives **1** (Van Allan and Deacon, 1963) from reaction of *o*-phenylene diamine with carbon disulfide in DMF. Then, to 1 g of compound **1** dissolved in 10 mL of anhydrous ethanol added 1.2 equivalent of (chloromethyl) benzene(derivatives **2** (Scheme 1). The mixture was refluxed for 2 h. The reaction medium was then neutralized with a solution of potassium bicarbonate (5%). The resulting precipitate **4** was filtered off, washed up with cold ethanol and then purified by column chromatography on silica gel. Eluent: ethyl acetate/hexane: v/v : 30/70. Table 1 shows the physicochemical characteristics of compounds **4a-k**.

**Synthesis of 2-(benzylthio)-1H-benzimidazole 4a:** From 2-mercaptobenzimidazole (1.00 g, 6.66 mmol) and chloromethyl) benzene (1.01 g, 7.99 mmol) **4a** was obtained (1.41 g, 88%) as crystals; MP = 122-124°C.
$^1$H NMR (DMSO, 300 MHz) δ :4.57 (2H, s, S-C$\underline{H}_2$); 7.10-7.16 (2H, m, H$_{ar}$); 7.22-734 (2H, m, H$_{ar}$); 7.44-747 (3H, m, H$_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ: 35.12 (S-C$\underline{H}_2$); 114.08 (2 C$_{ar}$); 121.39 (2 C$_{ar}$); 127.27 (C$_{ar}$); 128.44 (2 C$_{ar}$); 128.80 (2 C$_{ar}$); 137.64 (C$_{ar}$); 149.66 (N=$\underline{C}$-S).
Mass (m/z) = 240. M$^+$ = 240.1 (5); M+1 = 241.1 (100); M+2 = 242.1 (17); m/z (%): 243.1 (8).

**Synthesis of 2-(3-nitrobenzylthio)-1H-benzimidazole4b:** From 2-mercaptobenzimidazole (1.00 g, 6.66 mmol) and 1-(chloromethyl)-3-nitrobenzene (1.37 g, 7.99 mmol), **4b** was obtained (1.61 g, 85%) as crystals; MP = 216-218°C.
$^1$H NMR (DMSO, 300 MHz) δ : 4.70 (2H, s, S-C$\underline{H}_2$); 7.10-7.15 (2H, m, H$_{ar}$); 7.44-7.62 (3H, m, H$_{ar}$); 7.91-7.94 (2H, m, H$_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ :33.94 (S-C$\underline{H}_2$); 113.65 (2 C$_{ar}$); 121.47 (C$_{ar}$); 122.14 (2 C$_{ar}$); 123.50 (Car); 129.85 (C$_{ar}$); 135.53 (C$_{ar}$); 139.63 (2 C$_{ar}$); 140.68 (C$_{ar}$); 147.62 (N=$\underline{C}$-S); 149.06 ($\underline{C}$-NO$_2$).
Mass (m/z) = 285. M$^+$ = 285.10 (5); M+1 = 286.1 (100); M+2 = 287.1 (20); m/z (%): 288.1 (8).

**Synthesis of 2-(4-chlorobenzylthio)-1H-benzimidazole4c:** From 2-mercaptobenzimidazole (1.00 g, 6.66 mmol) and 1-chloro-4-(chloromethyl)benzene (1.29 g, 7.99 mmol), **4c** was obtained (1.13 g, 62%) as crystals); MP = 181-182°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.60 (2H, s, S-C$\underline{H}_2$); 7.14-7.18 (2H, m, H$_{ar}$); 7.37-7.40 (2H, m, H$_{ar}$); 7.49-7.52 (4H, m, H$_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ : 35.20 (S-C$\underline{H}_2$); 114.91 (2 C$_{ar}$); 122.46 (2 C$_{ar}$); 129.30-129.62 (2 C$_{ar}$); 131.65-132.44 (2 C$_{ar}$); 132.89 (C$_{ar}$); 138.03 (C$_{ar}$); 140.34 (2 C$_{ar}$); 150.41 (N=$\underline{C}$-S).
Mass (m/z) = 274. M$^+$ = 274.94 (31.60); M+1 = 275,94 (31,60); m/z (%): 273.98 (77.84); 241.07 (24.85); 148.93 (17.16); 126.90 (30.98); 124.87 (100); 121.90 (21.31); 88.92 (23.59); 85.84 (12.52); 83.82 (15.63); 48.86 (19.66).

**Synthesis of 2-(2,4-dichlorobenzylthio)-1H-benzimidazole4d:** From 2-mercaptobenzimidazole (1.00 g, 6.66 mmol) and 2,4-dicholoro-1-(chloromethyl)benzene (1.56 g, 7.99 mmol), **4d** was obtained (1.56 g, 76%) as crystals; MP = 157-158°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.68 (2H, s, SCH$_2$); 7.16-7.18 (2H, m, H$_{ar}$); 7.37-7.39 (3H, m, H$_{ar}$); 7.50-7.52 (2H, m, H$_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ: 33.63 (S-C$\underline{H}_2$); 114.85 (C$_{ar}$); 122.43
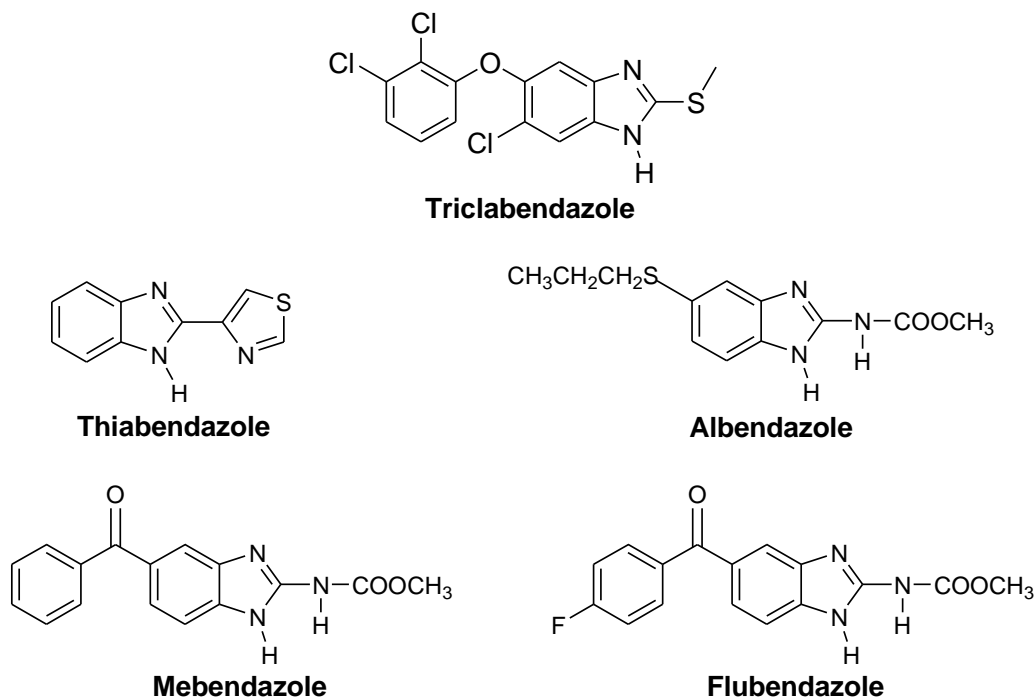
**Figure 1.** Structure of triclabendazole, thiabendazole, albendazole and flubendazole.

($C_{ar}$); 128.33 ($C_{ar}$); 129.50-129,80 ($C_{ar}$); 133.22 ($C_{ar}$); 133.91 ($C_{ar}$); 140.36 ($C_{ar}$); 149.75 (N=$\underline{C}$-S).
Mass (m/z) = 309. $M^+$ = 309.90 (27.24); m/z (%): 307.01 (41.82); 274.96 (38.40); 273 (15.67); 272.93 (100); 239.99 (14.68); 162.92 (12.04); 160.88 (41.75); 158.84 (67.05); 148.87 (15.77%); 122.93 (17.02); 121.91 (27.75); 88.93 (15.55).

**Synthesis of 2-(benzylthio)-5-nitro-1H-benzimidazole 4e:** From 5-nitro-2-mercaptobenzimidazole (1.00 g, 5.12 mmol) and (chloromethyl) benzene (0.78 g, 6.15 mmol), **4e** was obtained (1.18 g, 81%) as crystals, MP = 162-163°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.63 (2H, s, S-C$\underline{H}_2$); 7.24-7.35 (3H, m , $H_{ar}$); 7.47-7.49 (2H, m, $H_{ar}$); 7.59-7.62 (1H, m, $H_{ar}$); 8.04-8.08 (1H, m, Har); 8.32- 8.33 (1H, m, $H_{ar}$);
$^{13}$C NMR (DMSO, 75 MHz) δ: 34.96 (S-$\underline{C}H_2$); 110.34 ($C_{ar}$); 113.25 ($C_{ar}$); 117.46 ($C_{ar}$); 127.45 ($C_{ar}$); 128.50 (2 $C_{ar}$); 128.91 (2 $C_{ar}$); 137.09 (2 $C_{ar}$); 142.13 ($C_{ar}$); 155.9 (N=$\underline{C}$-S);
Mass (m/z) = 285. $M^+$ = 285.32 (100); M+1 = 286.8 (13%); m/z (%): 284.9 (35%); 283 (20%); 281.3 (10%).

**Synthesis of 5-nitro-2-(3-nitrobenzylthio)-1H-benzimidazole 4f:** From 5-nitro-2-mercaptobenzimidazole (1.00 g, 5.12 mmol) and 1-(chloromethyl)-3-nitrobenzene (1.05 g, 6.15 mmol), **4f** was obtained (1.27 g, 75%) as a crystals; MP = 210-211°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.76 (2H, s, S-C$\underline{H}_2$); 7.58-7.64 (2H, m, $H_{ar}$); 7.95-7.98 (1H, m, $H_{ar}$); 8.04-8.12 (2H, m, $H_{ar}$); 8.32 (1H, m, $H_{ar}$); 8.41 (1H, m, $H_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ: 34.06 (S-$\underline{C}H_2$); 117.53 (2 $C_{ar}$); 122.29 ($C_{ar}$); 123.62 ($C_{ar}$); 129.91 ($C_{ar}$); 135.64 ($C_{ar}$); 140.13 (2 $C_{ar}$); 142.20 ($C_{ar}$); 147.65 (N=$\underline{C}$-S); 155.37 ($C_{ar}$).
Mass (m/z) = 330. $M^+$ = 330.32 (4); M+1 = 331 (100); M+2 = 332 (15).

**Synthesis of 2-(4-chlorobenzylthio)-5-nitro-1H-benzimidale 4g:** From 5-nitro-2-mercaptobenzimidazole (1.00 g, 5.12 mmol) and 1-

chloro-4-(chloromethyl)benzene (1.00 g, 6.15 mmol), **4 g** was obtained (1.39 g, 85%) as crystals; MP = 155-156°C.
$^1$H NMR (DMSO, 300 MHz) δ:4.61 (2H, s, S-C$\underline{H}_2$); 7.31-7.37 (2H, d, $H_{ar}$); 7.49-7.31 (3H, 3m, $H_{ar}$); 8.03-8.06 (1H, dd, $H_{ar}$); 8.31 (1H, m, $H_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ: 33.60 (S-$\underline{C}H_2$); 114.4 ($C_{ar}$); 116.1 ($C_{ar}$); 118.6 ($C_{ar}$); 128.8-129.1 (4 $C_{ar}$); 137.7 ($C_{ar}$); 141.7 ($C_{ar}$); 147,1 (N=$\underline{C}$-S).
Mass (m/z) = 319. $M^+$ = 319 (5); M+1 = 320.96 (11.17); m/z (%) : 322.02 (2.08); 318.94 (28.89); 126.91 (32.10); 124.87 (100); 88.95 (16.91).

**Synthesis of 2-(2,4-dichlorobenzylthio)-5-nitro-1H-benzimidale 4h:** From 5-nitro-2-mercaptobenzimidazole (1.00 g, 5.12 mmol) and 2,4-dicholoro-1-(chloromethyl)benzene (1.20 g, 6.15 mmol), **4h** was obtained (1.45 g, 80%) as crystals; MP = 98-100°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.72 (2H, s, S-C$\underline{H}_2$); 7.38-7.40 (2H, m, $H_{ar}$); 7.63-7.64 (1H, m, $H_{ar}$); 7.70-7.72 (1H, m, $H_{ar}$); 8.07-8.10 (1H, m, $H_{ar}$); 8.36 (1H, m, $H_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ: 32.59 (S-$\underline{C}H_2$); 113.42 ($C_{ar}$); 117.51 ($C_{ar}$); 127.47 ($C_{ar}$);128.58-128.91 (2 $C_{ar}$); 131.37 ($C_{ar}$);133.51-134.25 (2 $C_{ar}$); 139.47 ($C_{ar}$); 140.32 ($C_{ar}$); 143.40 ($C_{ar}$); 155.07 (N=$\underline{C}$-S).
Mass (m/z) = 354. $M^+$ = 354.93 (21.66); m/z (%): 352.89 (29.52); 319.95 (31.20); 317.88 (84.95); 160.86 (65.31); 158.84 (100); 122.84 (16.13); 88.93 (11.21).

**Synthesis of [2-(benzylthio)-1H-benzimidazole-5-yl][phenyl] méthanone 4i:** From (2-mercaptobenzimidazol-5-yl) (phenyl) methanone (1.00 g, 3.93 mmol) and (chloromethyl)benzene (0.60 g, 4.72 mmol), **4i** was obtained (1.08 g, 90 %) as crystals; MP = 98-100°C. $^1$H NMR (DMSO, 300 MHz) δ: 4.66 (2H, s, S-C$\underline{H}_2$); 7.28-7.36 (3H, m, $H_{ar}$); 7.49-7.70 (7H, m, $H_{ar}$); 7.76-7.81 (2H, m, $H_{ar}$); 7.87 (1H, m, $H_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ: 36.00 (S-$\underline{C}H_2$); 114.42 ($C_{ar}$); 124.76
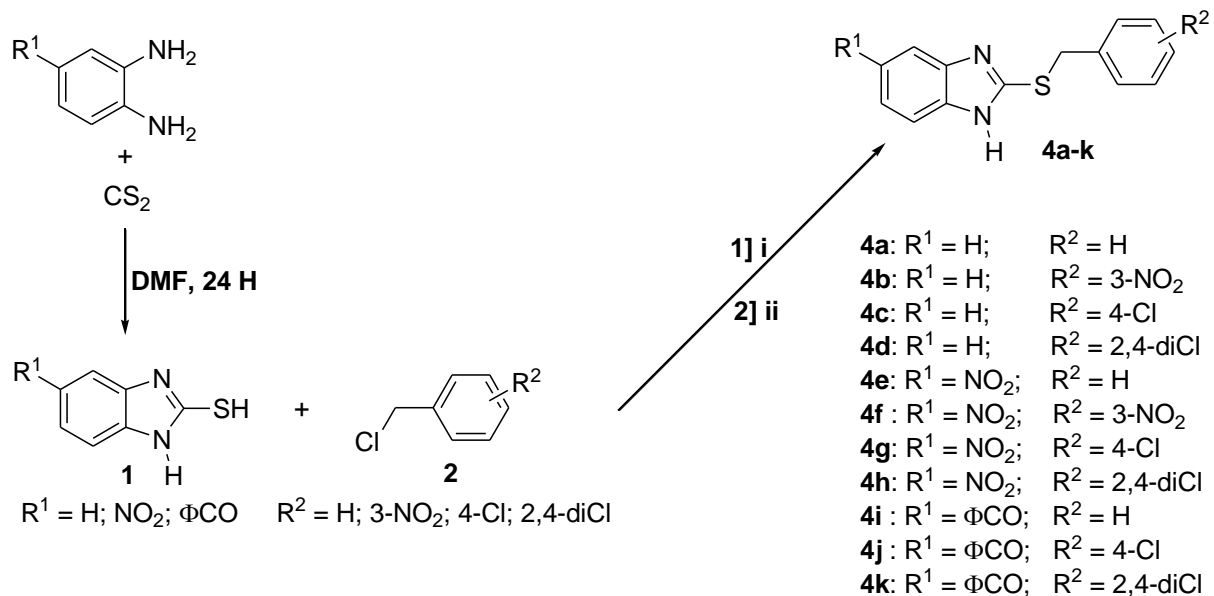
**Scheme 1.** Synthesis of 2-(benzylthio) benzimidazole 4a-k.

$(C_{ar})$, 128.31 $(C_{ar})$; 129.27-129.50 (2 $C_{ar}$); 129.80-130.27 (2 Car); 131.35-132.33 (2 $C_{ar}$); 132.62-132.88 (2 $C_{ar}$); 138.27 $(C_{ar})$; 139.06 $(C_{ar})$; 154.86 $(C_{ar})$; 167.50 (N=$\underline{C}$-S); 196.45 ($\underline{C}$=O).
Mass (m/z) = 344. $M^+$ = 344.02 (55.85); M+1 = 345.08 (12.81); m/z (%) : 311.03 (27.23); 104.94 (10.52); 90.93 (100); 76.89 (12.99); 64.92 (10.55).

**Synthesis of [2-(4-chlorobenzylthio)-1H-benzimidazol-5-yl][phényl]méthanone 4j:** From (2-mercaptobenzimidazol-5-yl)(phenyl) methanone (1.00 g, 3.93 mmol) and 1-chloro-4-(chloromethyl)benzene (0.76 g, 4.72 mmol), **4j** was obtained (1.19 g, 80%) as crystals; MP = 98-100°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.59 (2H, s, S-C$\underline{H}_2$); 7.35-7.39 (2H, m, $H_{ar}$); 7.49-7.58 (5H, m, $H_{ar}$); 7.63-7.67 (1H, m, $H_{ar}$); 7.72-7.73 (2H ,m, $H_{ar}$); 7.74-7.75 (1H, m, $H_{ar}$); 7.81 (1H, m, $H_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ: 35.03 (S-$\underline{C}H_2$); 114.59 $(C_{ar})$; 117.95 $(C_{ar})$; 123.32 $(C_{ar})$; 129.22-129.29 (2 $C_{ar}$); 130.25-131.692 (2 $C_{ar}$); 132.50-132.64 (2 $C_{ar}$); 138.71 $(C_{ar})$; 139.87 $(C_{ar})$; 143.17 $(C_{ar})$; 147.60 $(C_{ar})$; 157.88 (N=$\underline{C}$-S); 196.73 ($\underline{C}$=O).
Mass (m/z) = 378. $M^+$ = 378 (34.18); M+2 = 380 (12.09); m/z (%): 345.00 (12.52); 126.9 (34.10); 126 (11.12); 124.9 (100); 104.9 (10.16); 88.9 (22.27); 76.9 (19.78).

**Synthesis of [2-(2,4-dichlorobenzylthio)-1H-benzimidazole-5-yl][phényl]méthanone 4k:** From (2-mercaptobenzimidazol-5-yl)(phenyl) methanone (1.00 g, 3.93 mmol) and 2,4-dichloro-1-(chloromethyl)benzene (0.92 g, 4.72 mmol), **4k** was obtained (1.48 g, 91%) as crystals: MP = 110 -111°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.70 (2H, s, $H_{ar}$); 7.38-7.41 (1H, m, $H_{ar}$); 7.57-7.70 (5H, m, $H_{ar}$); 7.73-7.77 (2H, m, $H_{ar}$); 7. 86 (1H, m, $H_{ar}$).
$^{13}$C NMR (DMSO, 75 MHz) δ: 33.62 (S-$\underline{C}H_2$); 114.67 $(C_{ar})$; 117.59 $(C_{ar})$; 125.00 $(C_{ar})$; 128.54 $(C_{ar})$; 129.43 (2 $C_{ar}$); 129.00-130.44 (4 $C_{ar}$); 131.51 $(C_{ar})$; 140.11 $(C_{ar})$; 143.57 $(C_{ar})$; 153.65 $(C_{ar})$; 167.95 (N=$\underline{C}$-S); 196.58 ($\underline{C}$=O).
Mass (m/z) = 413. $M^+$ = 413 (10); M+1 = 414.17 (11.85); m/z (%) : 411.90 (23.62); 378.97 (37.21); 376158.95 (100); 344.01 (11.42);

197.98 (10.82); 160.14 (43.38); 87 (62.21); 122.88 (10.75); 105.08 (14.74); 85.90 (15.95); 83.80 (20.72); 76.87 (32.84); 48.89 (27.22).

### General procedure for synthesis of compounds 5a-k

This preparation involved three steps (Scheme 2): First step include synthesis of 2-mercaptobenzimidazole derivatives and analogues **1** from reaction of *o*-phenylene diamine derivatives or analogues with carbon disulfide in DMF according to Van Allan method (Van Allan and Deacon, 1963). The second relates the formation of 2-(méthylthio)benzimidazole **3** by condensation of *o*-phenylene diamine with chloroacetic acid in hydrochloric acidmedium (Phillips, 1928). In the third step, to 1 g of compound **1** in 10 mL of anhydrous ethanol was added 1.5 equivalent of 2-(chloromethyl)benzimidazole(derivative) **3**. The mixture was refluxed for 2 h. The reaction medium was then neutralized with a solution of sodium hydrogen carbonate (5%). The resulting precipitate **5** was filtered off, washed up with cold ethanol then purified by column chromatography on silica gel. Eluent : ethyl acetate/hexane : v/v : 80/20. Table 2 shows the physicochemical characteristics of compounds **5a-k**.

**Synthesis of 2-[(1H-benzimidazol-2-yl)méthylthio]-1H-benzimidazole 5a:** From 2-mercaptobenzimidazole (1.00 g, 6.66 mmol) and 2-(chloromethyl)-1*H*-benzimidazole (1.66 g, 10 mmol) **5a** was obtained (1.12 g, 60%) as crystals; MP = 253-256°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.80 (2H, s, S-C$\underline{H}_2$); 7.13-7.14 (4H, m, $H_{ar}$); 7.51 (4H, m, $H_{ar}$).
$^{13}$C NMR (DMSO, 75 Hz) δ: 28.86 (S-$\underline{C}H_2$); 121.56 $(C_{ar})$; 149.27 (CH$_2$-$\underline{C}$=N); 150.51 (N=$\underline{C}$-S).
Mass (m/z) = 280. $M^+$ = 280 (7); M+1 = 281 (100); M+2 = 282.1 (20); m/z : 261.1 (25); 132.9 (61); 151 (69).
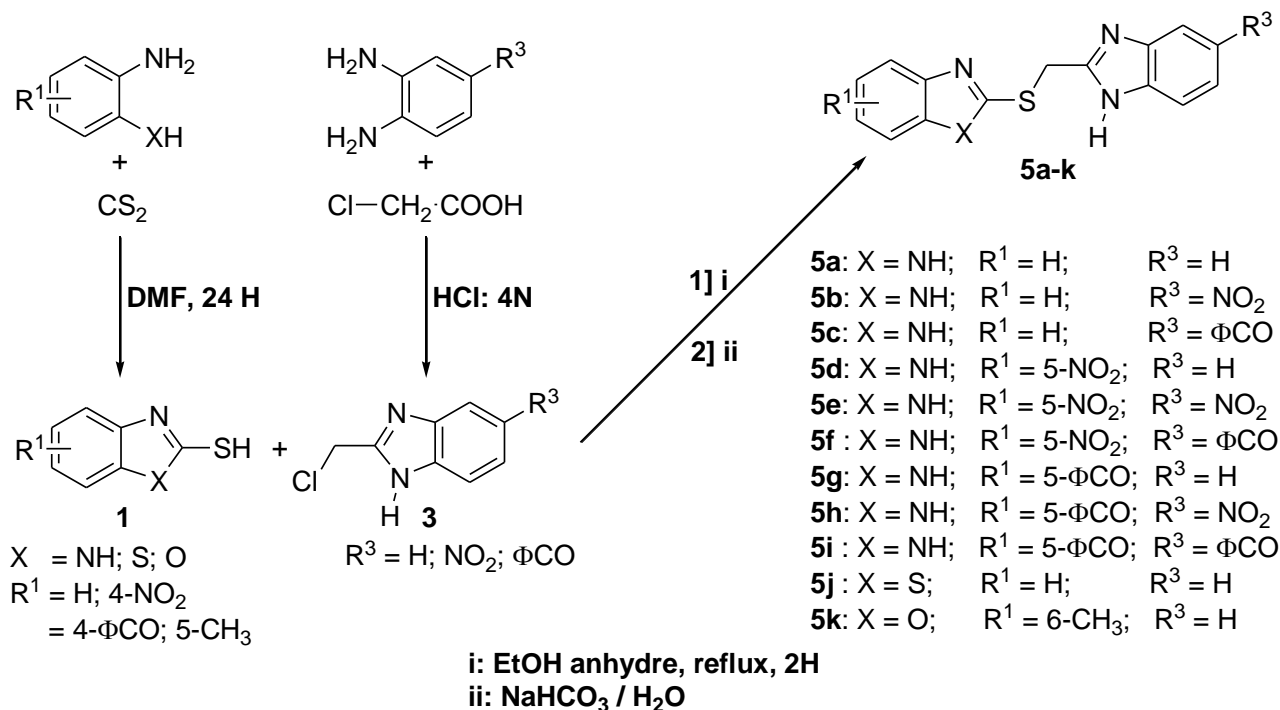
**Synthesis of 2-[(1H-benzimidazol-2-ylthio)méthyl]-5-nitro-1H-benzimidazole 5b:** From 2-mercaptobenzimidazole (1.00 g, 6.66 mmol) and 2-(chloromethyl)-5-nitro-1*H*-benzimidazole (2.11 g, 10

**Scheme 2.** Synthesis of 2-(benzimidazolyl methylthio) benzimidazole and analogues 5a-k.

mmol), **5b** was obtained (1.32 g, 61%) as a crystals; MP = 265-266°C.
^{1}H NMR (DMSO, 300 MHz) δ: 4.99 (2H, s, S-CH2); 7.24-7.27 (2H, m, Har); 7.53-7.60 (2H, m, Har); 7.67-7.70 (1H, m, Har); 8.11-8.13 (1H, m, Har); 8.47-8.48 (1H, m, Har).
^{13}C NMR (DMSO, 75 Hz) δ: 30.04 (S-CH2); 113.16 (Car); 114.89 (2 Car); 115.64 (Car); 123.52 (2 Car); 132.48-132.66 (2 Car); 143.27-143.62 (2 Car); 149.92 (CH2-C=N); 156.58 (Car); 167.92 (N=C-S).
Mass (m/z) = 325. M$^+$ = 325.35 (42.61); M+1 = 326 (12.60); m/z (%) : 149.9 (100); 148.9 (23.30); 117 (10.39); 106 (11.39); 89.9 (10.72); 62.9 (14.44).

**Synthesis of [2-((1H-benzimidazol-2-ylthio)méthyl-1H-benzimidazol-5-yl][phényl]méthanone 5c:** From 2-mercaptobenzimidazole (1.00 g, 6.66 mmol) and [2-(chloromethyl)-1H-benzimidazol-5-yl] [phenyl] methanone (2.70 g, 10 mmol), **5c** was obtained (2.00 g, 78%) as crystals; MP = 190-191°C.
^{1}H NMR (DMSO, 300 MHz) δ: 4.89 (2H, s, S-CH2); 7.17-7.19 (2H, m, Har); 7.51-7.59 (4H, m, Har); 7.66-7.75 (5H, m, Har); 7.94 (1H, m, Har).
^{13}C NMR (DMSO, 75 MHz) δ: 29.88 (S-CH2); 115.83 (Car); 119.06 (Car); 122.86 (Car); 125.18 (Car); 129.49-129.70 (2 Car); 132.17-132.55 (2 Car); 132.74 (Car); 133.21 (Car); 139.05-142.51 (3 Car); 150.06 (CH2-C=N); 154.87 (Car); 168.05 (N=C-S); 196.85 (C=O).
Mass (m/z) = 384. M$^+$ = 384.03 (100); M+1 = 385.09 (26.67); m/z (%): 351.08 (35.20); 235.01 (35.74); 149.94 (27.33); 129.90 (20.76); 76.94 (15).

**Synthesis of 2-[(1H-benzimidazol-2-yl)méthylthio]-5-nitro-1H-benzimidazole 5d:** From 5-nitro-2-mercaptobenzimidazole (1.00 g, 5.12 mmol) and 2-chloromethyl-1H-benzimidazole (1.28 g, 7.68 mmol), **5d** was obtained (1.18 g, 71%) as crystals; MP = 247-248°C.
^{1}H NMR (DMSO, 300 MHz) δ: 4.88 (2H, s, S-CH2); 7.14-7.19 (2H, dd, Har); 7.50-7.55 ( 2H, dd, Har); 7.62-7.65 (1H, d, Har); 8.06-8.09

(1H, dd, Har); 8.37-8.38 (1H, m, Har).
^{13}C NMR (DMSO, 75 MHz) δ: 28.76 (S-CH2); 113.45 (Car); 115.03 (Car); 117.55 (Car); 121.84 (Car); 139.02 (Car); 142.21 (CH2-C=N); 150.03 (Car); 155.47 (N=C-S).
Mass (m/z) = 325. M$^+$ = 325 (4); M+1 = 326 (100); M+2 = 327.2 (22); m/e (%) : 196 (10); 166 (11); 133 (37); 132 (11).

**Synthesis of 5-nitro-2-[(5-nitro-1H-benzimidazol-2-yl)méthylthio]-1H-benzimidazole 5e:** From 5-nitro-2-mercaptobenzimidazole (1.00 g, 5.12 mmol) and 2-chloromethyl-5-nitro-1H-benzimidazole (1.63 g, 7.68 mmol), **5e** was obtained (1.00 g, 53%) as crystals; MP = 180-181°C.
^{1}H NMR (DMSO, 300 MHz) δ: 4.97 (2H, s, S-CH2); 7.64-7.75 (2H, m, Har); 8.08-8.12 (2H, m, Har); 8.39-8.47 (2H, m, Har).
^{13}C NMR (DMSO, 75 MHz) δ: 28.64 (S-CH2); 117.58 (Car); 128.59 (Car); 142.21 (CH2-C=N); 142.55 (Car); 155.37 (N=C-S).
Mass (m/z) = 370. M$^+$ = 370 (19.54); m/z (%): 194.9 (100); 164.9 (37.26); 148.9 (57.42); 136.9 (21.72); 121.9 (30.48); 89.9 (46.08); 104.9 (46.15); 62.2 (61.52).

**Synthesis of [2-((5-nitro-1H-benzimidazol-2ylthio)méthyl)1H-benzimidazol-5-yl][phényl]méthanone 5f:** From 5-nitro-2-mercaptobenzimidazole (1.00 g, 5.12 mmol) and [2-(chloromethyl)-1H-benzimidazol-5-yl] [phenyl] methanone (2.08 g, 7.68 mmol), **5f** was obtained (1.58 g, 72%) as crystals; MP = 161-162°C.
^{1}H NMR (DMSO, 300 MHz) δ: 4.98 (2H, s, S-CH2); 7.55-7.59 (2H, m, Har); 7.65-7.76 (6H, m, Har); 7.95-7.96 (1H, m, Har); 8.08-8.11 (1H, dd, Har); 8.39 (1H, d, Har).
^{13}C NMR (DMSO, 75 MHz) δ: 29.59 (S-CH2); 111.59 (Car); 114.41 (Car); 115.69 (Car); 118.50 (Car); 118.92 (Car); 125.01 (Car); 129.28-129.53 (2 Car); 130.32 (2 Car); 132.62-132.97 (Car); 138.93 (Car); 140.50 (CH2-C=N); 142.34-144.46 (Car); 156.12 (Car); 167.81 (N=C-S); 196.51 (C=O).
Mass (m/z) = 429. M$^+$ = 429.87 (30.59); m/z (%) : 428.97 (100); 396.14 (21.54); 236.06 (51.34); 235.03 (96.31); 148.95 (55.25);

129.99 (44.82); 105.62 (43.28); 76.94 (49.60).

**Synthesis of [2-((1H-benzimidazol-2yl)méthylthio)-1H-benzimidazol-5-yl][phényl]méthanone 5g:** From (2-mercaptobenzimidazol-5-yl) (phenyl) methanone (1.00 g, 3.93 mmol) and 2-(chloromethyl)-1$H$-benzimidazole (0.98 g, 5.90 mmol), **5g** was obtained (1.30 g, 86%) as crystals; MP = 255-256°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.91 (2H, s, S-C$\underline{H}_2$,); 7.17-7.21 (2H, dd, H$_{ar}$); 7.54-7.61 (4H, m, H$_{ar}$); 7.64-7.7 (4H, m, H$_{ar}$); 7.73-7.78 (1H, m, H$_{ar}$); 7.91 (1H, m, H$_{ar}$).
$^{13}$C (DMSO, 75 MHz) δ: 29.77 (S-$\underline{C}$H$_2$); 116.00 (3 C$_{ar}$); 122.89 (C$_{ar}$); 124.91 (C$_{ar}$); 129.40 (C$_{ar}$); 129.64 (2 C$_{ar}$); 130.43 (2 C$_{ar}$); 131.43 (C$_{ar}$); 132.48 (C$_{ar}$); 132.66 (3 C$_{ar}$); 139.95 (C$_{ar}$); 151.32 (CH$_2$-$\underline{C}$=N); 153.97 (C$_{ar}$); 167.91 (N=$\underline{C}$-S); 196.57 ($\underline{C}$=O).
Mass (m/z) = 384. M$^+$ = 384.04 (58.75); M+1 = 385.08 (16.21); m/z (%) : 253.99 (71.46); 176.91 (62.42); 131.99 (56.79); 130.07 (100); 76.88 (52.35).

**Synthesis of [2-((5-nitro-1H-benzimidazol-2yl)méthylthio)-1H-benzimidazol-5-yl][phényl]méthanone 5h:** From (2-mercaptobenzimidazol-5-yl) (phenyl) methanone (1.00 g, 3.93 mmol) and 2-(chloromethyl)-5-nitro-1$H$-benzimidazole (1.25 g, 5.90 mmol), **5h** was obtained (1.42 g, 84%) as crystals; MP = 93-94 °C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.96 (2H, s, S-C$\underline{H}_2$,); 7.60 (2H ,m, H$_{ar}$); 7.65-7.70 (5H, m, H$_{ar}$); 7.89 (1H, m, H$_{ar}$); 8.10-8.13 (1H, dd, H$_{ar}$); 8.47-8.48 (1H, d, H$_{ar}$).
$^{13}$C (DMSO, 75 MHz) δ: 29.70 (S-$\underline{C}$H$_2$); 113.64-118.73 (3 C$_{ar}$); 124.94 (C$_{ar}$); 129.63 (C$_{ar}$); 130.42 (2 C$_{ar}$); 131.49 (2 C$_{ar}$); 132.47 (C$_{ar}$); 132.66 (C$_{ar}$); 133.06 (C$_{ar}$); 139.04 (C$_{ar}$); 143.53 (CH$_2$-$\underline{C}$=N); 153.59 (C$_{ar}$); 156.73 (C$_{ar}$); 167.92 (N=$\underline{C}$-S); 196,55 ($\underline{C}$=O).
Mass (m/z) = 429. M$^+$ = 429.01 (56.19); m/z (%) : 253.00 (78.44); 176.94 (100); 129.9 (25.66); 104.94 (26.51); 76.92 (31.40).

**[2-((5-benzoyl-1H-benzimidazol-2yl)méthylthio)-1H-benzimidazol-5-yl][phényl]méthanone 5i:** From (2-mercaptobenzimidazol-5-yl) (phenyl) methanone (1.00 g, 3.93 mmol) and [2-(chloromethyl)-1$H$-benzimidazol-5-yl] [phenyl] methanone (1.60 g, 5.90 mmol), **5i** was obtained (1.00 g, 52%) as crystals; MP = 150-154°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.95 (2H, s, S-C$\underline{H}_2$); 7.55-7.60 (4H, m, H$_{ar}$); 7.66-7.69 (6H, m, H$_{ar}$); 7.90 (2H, m, H$_{ar}$); 7.95 (2H, m, H$_{ar}$).
$^{13}$C (DMSO, 75 MHz) δ: 29.73 (S-$\underline{C}$H$_2$); 124.81 (C$_{ar}$); 129.26 (4 C$_{ar}$);130.30 (4 C$_{ar}$); 131.45 (C$_{ar}$); 131.82 (2 C$_{ar}$);132.91 (C$_{ar}$); 139.01 (C$_{ar}$); 154.46 (N=$\underline{C}$-S); 196.44-196.55 (2 $\underline{C}$=O).
Mass (m/z) = 488. M$^+$ = 488.10 (71.89); m/z (%) : 253.91 (53.38); 234.94 (72.20); 176.90 (22.37); 158.90 (33.46); 129.94 (31.15); 104.92 (85.89); 76.86 (100).

**Synthesis of 2-[(1H-benzimidazole-2yl)methylthio]benzothiazol 5j:** From 2-mercaptobenzothiazole (1.00 g, 5.98 mmol) and 2-(chloromethyl)benzimidazole (1.49 g, 8.97 mmol), **5d** was obtained (0.96 g ,54%) as crystals; MP = 176-177°C.
$^1$H NMR (DMSO, 300 MHz) δ: 4.90 (2H, s, S-C$\underline{H}_2$); 7.15-7.19 (2H, m, H$_{ar}$); 7.34-7.59 (4H, m, H); 7.59-7.91 (1H, m, H$_{ar}$); 8.01-8.04 (1H, m, H$_{ar}$).
$^{13}$C (DMSO, 75 MHz) δ: 30.31 (S-$\underline{C}$H$_2$); 121.25 (2 C$_{ar}$); 121.86 (2 C$_{ar}$); 124.56 (C$_{ar}$); 126.39 (C$_{ar}$); 134.79 (2 C$_{ar}$); 149.53 (CH$_2$-$\underline{C}$=N); 152.47 (C$_{ar}$); 165.59 (N=$\underline{C}$-S).

**Synthesis of synthesis of 2-[(1H-benzimidazole-2yl)methylthio]-6-methylbenzoxazole 5k:** From 5-methyl-2-mercaptobenzoxazole (1.00 g, 6.05 mmol) and 2-(chloromethyl)-1$H$-benzimidazole (1.51 g, 9.08 mmol), **5k** was obtained (0.91 g, 51%) as a crystals; MP = 181-182°C.
$^1$H NMR (DMSO, 300 MHz) δ: 2.42 (3H, s, C$\underline{H}_3$); 4.85 (2H, s, S-C$\underline{H}_2$); 7.15-7.19 (3H, m, H$_{ar}$); 7.48-7.54 (4H, m, H$_{ar}$).
$^{13}$C (DMSO, 75 MHz) δ: 21.07 ($\underline{C}$H$_3$); 29.40 (S-C$\underline{H}_2$); 162.42

(N=$\underline{C}$-S); 151.65 (C$_{ar}$); 149.40 (CH$_2$-$\underline{C}$=N); 139.01 (C$_{ar}$); 134.36 (C$_{ar}$); 125.6 (C$_{ar}$); 121.86 (2 C$_{ar}$); 117.76 (C$_{ar}$); 110.33 (C$_{ar}$).
Mass (m/z) = 295. M$^+$ = 295 (5); M+1 = 296.1 (100); M+2 = 297.1 (20).

## Anthelmintics activities

The nematocide test used is an enhancement of the method initially described by Diehl et al. (2004). This required prior a reasonable number of 3000 eggs of *H. contortus* obtained by experimental infection of breeding sheep. Compounds **4a-k** and **5a-k** and the anthelmintic drugs (fenbendazole and ivermectin) (7.5 mg per sample) were dissolved in 1 mL of DMSO and diluted with distilled water to obtain a dilution series in 96 well of microtiter plates. Some agar (l40 µL, 45-50°C) containing 2% of amphotericin B was added to each well with also 80 of *Haemonchus* eggs. The plates were maintained at 27°C in a humid atmosphere (90%) for 6 days. The normal development of larvae without products on trial was also carried in wells containing distilled water to serve as control to the experiment. The number of hatched eggs and the number of larvae was counted the stages of development and the mobility of larvae was recorded. For a development rate between 0 and 5%, the test molecule was considered as an active one. The tests were carried out repeatedly three times with all compounds showing a nematocide activity.

## RESULTS

## Chemical results

We synthesized and isolated 22 molecules carrying both benzimidazole and methylthio moiety in their structure. These compounds, structural analogues of triclabendazole was obtained by total synthesis with a yield varying between 51 and 91% and divided in three series (Figure 2).

The 2-(benzylthio) benzimidazole derivatives **4a-k** (Table 1), the 2-(benzimidazolyl methylthio) benzimidazole derivatives **5a-i** (Table 2) and their analogues 2-(benzimidazolyl methylthio) benzothiazole **5j** and 2-(benzimidazolyl methylthio) benzoxazole **5k** (Table 2). Moreover, benzene ring in the first two series carried various modulators such us nitro (NO$_2$), benzoyl (PhCO) and chloro (Cl).

The spectroscopic proton NMR characterization (Table 1) of all synthesized compounds showed one characteristic peak between 2.42 and 4.99 ppm corresponding to the chemical shift of the proton S-C$\underline{H}_2$-. Concerning $^{13}$C spectra, we noted two main peaks: From 28.64 to 36.00 ppm for S-$\underline{C}$H$_2$- and from 140.50 to 167.95 ppm for N=$\underline{C}$-S. The molecular peaks in mass spectrometry of these methylthio benzimidazoles (Table 1) varied between 240 and 488 depending on their substituents.

## *Anti-Haemonchus activities*

Regarding nematocidal activities of synthesized compounds **4a-k** and **5a-k** (Table 3), the antiparasitic

**Table 1.** Physicochemical characteristics of compounds4a-k.

| Compounds | Physicochemical characteristics |
|---|---|
| 4a | NMR$^1$H:4.57 (2H, s, S-C$\underline{H}_2$); 7.10-7.16 (2H, m, H$_{ar}$); 7.22-734 (2H, m, H$_{ar}$); 7.44-747 (3H, m, H$_{ar}$).NMR $^{13}$C :35.12 (S-$\underline{C}$H$_2$); 114.08 (2 C$_{ar}$); 121.39 (2 C$_{ar}$); 127.27 (C$_{ar}$); 128.44 (2 C$_{ar}$); 128.80 (2 C$_{ar}$); 137.64 (C$_{ar}$); 149.66 (N=$\underline{C}$-S). SM: 240([M+H]$^+$, 100). Yield = 88%. MP = 122-124°C. |
| 4b | NMR $^1$H: 4.70 (2H, s, S-C$\underline{H}_2$); 7.10-7.15 (2H, m, H$_{ar}$); 7.44-7.62 (3H, m, H$_{ar}$); 7.91-7.94 (2H, m, H$_{ar}$).NMR $^{13}$C: 33.94 (S-$\underline{C}$H$_2$); 113.65 (2 C$_{ar}$); 121.47 (C$_{ar}$); 122.14 (2 C$_{ar}$); 123.50 (Car); 129.85 (C$_{ar}$); 135.53 (C$_{ar}$); 139.63 (2 C$_{ar}$); 140.68 (C$_{ar}$); 147.62 (N=$\underline{C}$-S); 149.06 ($\underline{C}$-NO$_2$).SM : 285 (M+H]$^+$,100).Yield = 85%. MP = 216-218 °C. |
| 4c | NMR $^1$H:4.60 (2H, s, S-C$\underline{H}_2$); 7.14-7.18 (2H, m, H$_{ar}$); 7.37-7.40 (2H, m, H$_{ar}$); 7.49-7.52 (4H, m, H$_{ar}$). NMR $^{13}$C: 35.20 (S-$\underline{C}$H$_2$); 114.91 (2 C$_{ar}$); 122.46 (2 C$_{ar}$); 129.30-129.62 (2 C$_{ar}$); 131.65-132.44 (2 C$_{ar}$); 132.89 (C$_{ar}$); 138.03 (C$_{ar}$); 140.34 (2 C$_{ar}$); 150.41 (N=$\underline{C}$-S). SM: 274. ([M]$^+$, 32). Yield = 62%. MP = 181-182°C. |
| 4d | NMR $^1$H: 4.68 (2H, s, S-C$\underline{H}_2$); 7.16-7.18 (2H, m, H$_{ar}$); 7.37-7.39 (3H, m, H$_{ar}$); 7.50-7.52 (2H, m, H$_{ar}$).NMR $^{13}$C :33.63 (S-$\underline{C}$H$_2$); 114.85 (C$_{ar}$); 122.43 (C$_{ar}$); 128.33 (C$_{ar}$); 129.50-129,80 (C$_{ar}$); 133.22 (C$_{ar}$); 133.91 (C$_{ar}$); 140.36 (C$_{ar}$); 149.75 (N=$\underline{C}$-S).SM : 309. ([M]$^+$, 28). Yield = 76%. MP = 157-158°C. |
| 4e | NMR $^1$H: 4.63 (2H, s, S-C$\underline{H}_2$); 7.24-7.35 (3H, m, H$_{ar}$); 7.47-7.49 (2H, m, H$_{ar}$); 7.59-7.62 (1H, m, H$_{ar}$); 8.04-8.08 (1H, m, Har); 8.32- 8.33 (1H, m, H$_{ar}$). NMR $^{13}$C: 34.96 (S-$\underline{C}$H$_2$); 110.34 (C$_{ar}$); 113.25 (C$_{ar}$); 117.46 (C$_{ar}$); 127.45 (C$_{ar}$); 128.50 (2 C$_{ar}$); 128.91 (2 C$_{ar}$); 137.09 (2 C$_{ar}$); 142.13 (C$_{ar}$); 155.9 (N=$\underline{C}$-S). SM: 285. ([M]$^+$, 100). Yield = 81%. MP = 162-163°C. |
| 4f | NMR $^1$H: 4.76 (2H, s, S-C$\underline{H}_2$); 7.58-7.64 (2H, m, H$_{ar}$); 7.95-7.98 (1H, m, H$_{ar}$); 8.04-8.12 (2H, m, H$_{ar}$); 8.32 (1H, m, H$_{ar}$); 8.41 (1H, m, H$_{ar}$). NMR $^{13}$C: 34.06 (S-$\underline{C}$H$_2$); 117.53 (2 C$_{ar}$); 122.29 (C$_{ar}$); 123.62 (C$_{ar}$); 129.91 (C$_{ar}$); 135.64 (C$_{ar}$); 140.13 (2 C$_{ar}$); 142.20 (C$_{ar}$); 147.65 (N=$\underline{C}$-S); 155,37 (C$_{ar}$). SM; 330 ([M+H]$^+$, 100).Yield = 75%. MP = 210-211 °C. |
| 4g | NMR $^1$H: 4.61 (2H, s, S-C$\underline{H}_2$); 7.31-7.37 (2H, d, H$_{ar}$); 7.49-7.31 (3H, 3m, H$_{ar}$); 8.03-8.06 (1H, dd, H$_{ar}$); 8.31 (1H, m, H$_{ar}$). NMR $^{13}$C: 33.60 (S-$\underline{C}$H$_2$); 114.4 (C$_{ar}$); 116.1 (C$_{ar}$); 118.6 (C$_{ar}$); 128.8-129.1 (4 C$_{ar}$); 137.7 (C$_{ar}$); 141.70 (C$_{ar}$); 147,1 (N=$\underline{C}$-S). SM: 319. ([M]$^+$, 5).Yield = 85%. MP = 155-156°C. |
| 4h | NMR $^1$H: 4.72 (2H, s, S-C$\underline{H}_2$); 7.38-7.40 (2H, m, H$_{ar}$); 7.63-7.64 (1H, m, H$_{ar}$); 7.70-7.72 (1H, m, H$_{ar}$); 8.07-8.10 (1H, m, H$_{ar}$); 8.36 (1H, m, H$_{ar}$). NMR $^{13}$C: 32.59 (S-$\underline{C}$H$_2$); 113.42 (C$_{ar}$); 117.51 (C$_{ar}$); 127.47 (C$_{ar}$); 128.58-128.91 (2 C$_{ar}$); 131.37 (C$_{ar}$); 133.51-134.25 (2 C$_{ar}$); 139.47 (C$_{ar}$); 140.32 (C$_{ar}$); 143.40 (C$_{ar}$); 155.07 (N=$\underline{C}$-S). SM: 354 ([M]$^+$, 22). Yield = 80%. MP = 98-100°C. |
| 4i | NMR $^1$H: 4.66 (2H, s, S-C$\underline{H}_2$); 7.28-7.36 (3H, m, H$_{ar}$); 7.49-7.70 (7H, m, H$_{ar}$); 7.76-7.81 (2H, m, H$_{ar}$); 7.87 (1H, m, H$_{ar}$). NMR $^{13}$C :36.00 (S-$\underline{C}$H$_2$); 114.42 (C$_{ar}$); 124.76 (C$_{ar}$), 128.31 (C$_{ar}$); 129.27-129.50 (2 C$_{ar}$); 129.80-130.27 (2 Car); 131.35-132.33 (2 C$_{ar}$); 132.62-132.88 (2 C$_{ar}$); 138.27 (C$_{ar}$); 139.06 (C$_{ar}$); 154.86 (C$_{ar}$); 167.50 (N=$\underline{C}$-S); 196.45 ($\underline{C}$=O). SM : 344([M]$^+$, 56). Yield = 90%. MP = 98-100°C. |
| 4j | NMR $^1$H: 4.59 (2H, s, S-C$\underline{H}_2$); 7.35-7.39 (2H, m, H$_{ar}$); 7.49-7.58 (5H, m, H$_{ar}$); 7.63-7.67 (1H, m, H$_{ar}$); 7.72-7.73 (2H ,m, H$_{ar}$); 7.74-7.75 (1H, m, H$_{ar}$); 7.81 (1H, m, H$_{ar}$). NMR $^{13}$C :35.03 (S-$\underline{C}$H$_2$); 114.59 (C$_{ar}$); 117.95 (C$_{ar}$); 123.32 (C$_{ar}$); 129.22-129.29 (2 C$_{ar}$); 130.25-131.692 (2 C$_{ar}$); 132.50-132.64 (2 C$_{ar}$); 138.71 (C$_{ar}$); 139.87 (C$_{ar}$); 143.17 (C$_{ar}$); 147.60 (C$_{ar}$); 157.88 (N=$\underline{C}$-S); 196.73 ($\underline{C}$=O). SM : 378 ([M]$^+$, 34). Yield = 80%. MP = 98-100°C. |
| 4k | NMR $^1$H: 4.70 (2H, s, H$_{ar}$); 7.38-7.41 (1H, m, H$_{ar}$); 7.57-7.70 (5H, m, H$_{ar}$); 7.73-7.77 (2H, m, H$_{ar}$); 7. 86 (1H, m, H$_{ar}$). NMR $^{13}$C: 33.62 (S-$\underline{C}$H$_2$); 114.67 (C$_{ar}$); 117.59 (C$_{ar}$); 125.00 (C$_{ar}$); 128.54 (C$_{ar}$); 129.43 (2 C$_{ar}$); 129.00-130.44 (4 C$_{ar}$); 131.51 (C$_{ar}$); 140.11 (C$_{ar}$); 143.57 (C$_{ar}$); 153.65 (C$_{ar}$); 167.95 (N=$\underline{C}$-S); 196.58 ($\underline{C}$=O). SM: 413 ([M]$^+$, 10).Yield = 91%. MP = 110-111°C. |

g$\mu$ / mL) and **5d** (CL$_{100}$ = 0.002 g$\mu$ / mL) had respectively a nematicidal activity in the same magnitude order as that of the reference molecules fenbendazole (CL$_{100}$ = 0.0005 $\mu$g/mL) and ivermectin (CL$_{100}$ = 0.009 $\mu$g/mL). Compounds **5e** (CL$_{100}$ = 0.68 $\mu$g/mL), **5f** (CL$_{100}$ = 0.68 $\mu$g/mL) and **5h**(CL$_{100}$ = 0.038 $\mu$g/mL) had lower activity than that of the reference product. **4a**, **4f**, **4g**, **4h** and **4k** had very high larvicidal concentrations (CL$_{100}$ = 2.86 $\mu$g/mL) compared to the reference molecules. Larvicidal concentration (CL$_{100}$) was the lowest concentration for which the normal larval development was completely blocked (non-hatching eggs, paralysis or death of larvae).

## DISCUSSION

Analysis of the results of nematicide activities against *H. contortus* in connection with the structural changes made in the series of 2-(benzylthio) benzimidazole derivatives **4a-k** (Table 1) allowed to   make the following observations.
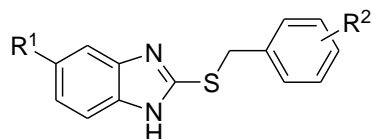
**(Methylthio) benzimidazole 4 a-k**

| | |
|---|---|
| **4a**: R$^1$ = H; | R$^2$ = H |
| **4b**: R$^1$ = H; | R$^2$ = 3-NO$_2$ |
| **4c**: R$^1$ = H; | R$^2$ = 4-Cl |
| **4d**: R$^1$ = H; | R$^2$ = 2,4-diCl |
| **4e**: R$^1$ = NO$_2$; | R$^2$ = H |
| **4f** : R$^1$ = NO$_2$; | R$^2$ = 3-NO$_2$ |
| **4g**: R$^1$ = NO$_2$; | R$^2$ = 4-Cl |
| **4h**: R$^1$ = NO$_2$; | R$^2$ = 2,4-diCl |
| **4i** : R$^1$ = ΦCO; | R$^2$ = H |
| **4j** : R$^1$ = ΦCO; | R$^2$ = 4-Cl |
| **4k**: R$^1$ = ΦCO; | R$^2$ = 2,4-diCl |
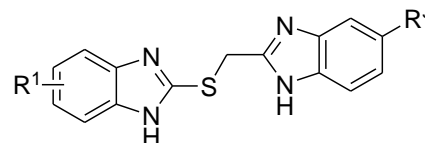
**(Methylthio) bisbenzimidazole 5a-i**

| | |
|---|---|
| **5a**: R$^1$ = H; | R$^3$ = H |
| **5b**: R$^1$ = H; | R$^3$ = NO$_2$ |
| **5c**: R$^1$ = H; | R$^3$ = ΦCO |
| **5d**: R$^1$ = 5-NO$_2$; | R$^3$ = H |
| **5e**: R$^1$ = 5-NO$_2$; | R$^3$ = NO$_2$ |
| **5f** : R$^1$ = 5-NO$_2$; | R$^3$ = ΦCO |
| **5g**: R$^1$ = 5-ΦCO; | R$^3$ = H |
| **5h**: R$^1$ = 5-ΦCO; | R$^3$ = NO$_2$ |
| **5i** : R$^1$ = 5-ΦCO; | R$^3$ = ΦCO |

X = S; O
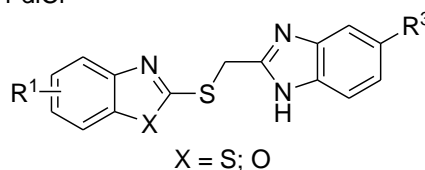
**(Methylthiobenzimidazol) benzothiazole 5j et**
**(Methylthiobenzimidazole) benzoxazole 5k**

**5j** : X = S; R$^1$ = H;        R$^3$ = H
**5k**: X = O; R$^1$ = 6-CH$_3$;  R$^3$ = H

**Figure 2.** Structure of analogues of triclabendazole synthesized.



**(Methylthio) benzimidazole 4a-k**                    **(Methylthio) bisbenzimidazole 5a-i**
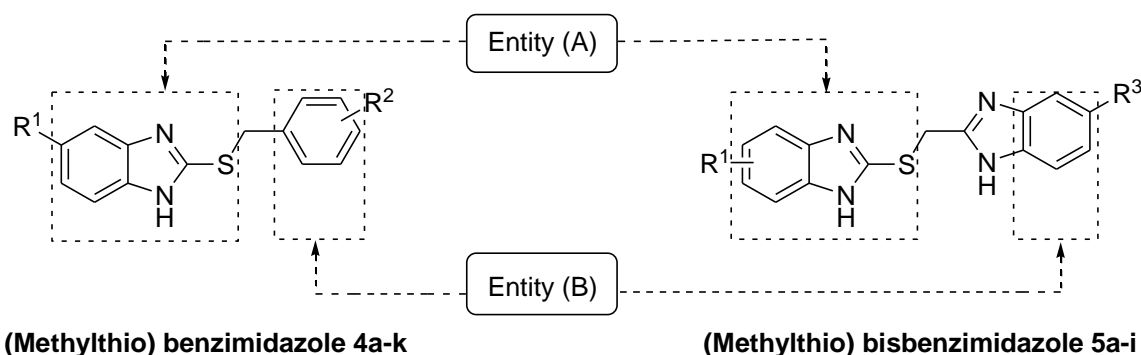
**Figure 3.** Entities (A) and (B) in compounds 4 and 5.

First, presence of benzyl group on the 2-mercapto-benzimidazole (entity (A), (Figure 3) engendered the appearance of nematicide activities. The substitutions on this nucleus caused changes of activity.

Also, when benzyl ring carried a nitro group (NO$_2$) in the isomeric position-3, an increase of helminthicide activity was observed. Indeed, the product **4b** (CL$_{100}$ = 0.0005 µg/mL) was nearly 6000 times more active than

the unsubstituted derivative **4a** (CL$_{100}$ = 2.86 µg/mL). In addition, compared to the reference nematicide molecules, the product **4b** was 18 times more effective than ivermectin (CL$_{100}$ = 0.009 µg/mL) and had an activity equivalent to that of fenbendazole (CL$_{100}$ = 0.0005 µg/mL). The presence of halogenated entities on the benzene ring namely chlorine, induced a decrease of the nematocidal activity. Thus, *para*-chloro derivative **4c** had

**Table 2.** Physicochemical characteristics of compounds 5a-k.

| Compounds | Physicochemical characteristics |
|---|---|
| 5a | NMR [1]H: 4.80 (2H, s, S-C$\underline{H}_2$); 7.13-7.14 (4H, m, H$_{ar}$); 7.51 (4H, m, H$_{ar}$). NMR [13]C : 28.86 (S-$\underline{C}H_2$); 121.56 (C$_{ar}$); 149.27 (CH$_2$-$\underline{C}$=N); 150.51 (N=$\underline{C}$-S).SM : 280 ([M+H]$^+$, 100). Yield = 60%. MP = 253-254°C. |
| 5b | NMR [1]H: 4.99 (2H, s, S-C$\underline{H}_2$); 7.24-7.27 (2H, m, H$_{ar}$); 7.53-7.60 (2H, m, H$_{ar}$); 7.67-7.70 (1H, m, H$_{ar}$); 8.11-8.13 (1H, m, H$_{ar}$); 8.47-8.48 (1H, m, H$_{ar}$). NMR [13]C : 30.04 (S-$\underline{C}H_2$); 113.16 (C$_{ar}$); 114.89 (2 C$_{ar}$); 115.64 (C$_{ar}$); 123.52 (2 C$_{ar}$); 132.48-132.66 (2 C$_{ar}$); 143.27-143.62 (2 C$_{ar}$); 149.92 (CH$_2$-$\underline{C}$=N); 156.58 (C$_{ar}$); 167.92 (N=$\underline{C}$-S). SM: 325 ([M]$^+$, 43). Yield = 61%. MP = 265-266°C. |
| 5c | NMR [1]H: 4.89 (2H, s, S-C$\underline{H}_2$); 7.17-7.19 (2H, m, H$_{ar}$); 7.51-7.59 (4H, m, H$_{ar}$); 7.66-7.75 (5H, m, H$_{ar}$); 7.94 (1H, m, H$_{ar}$).NMR [13]C : 29.88 (S-$\underline{C}H_2$); 115.83 (C$_{ar}$); 119.06 (C$_{ar}$); 122.86 (C$_{ar}$); 125.18 (C$_{ar}$); 129.49-129.70 (2 C$_{ar}$); 132.17-132.55 (2 C$_{ar}$); 132.74 (C$_{ar}$); 133.21 (C$_{ar}$); 139.05-142.51 (3 C$_{ar}$); 150.06 (CH$_2$-$\underline{C}$=N); 154.87 (C$_{ar}$); 168.05 (N=$\underline{C}$-S); 196.85 ($\underline{C}$=O). SM: 384([M]$^+$,100).Yield = 78%. MP = 190-191°C. |
| 5d | NMR [1]H: 4.88 (2H, s, S-C$\underline{H}_2$); 7.14-7.19 (2H, dd, H$_{ar}$); 7.50-7.55 (2H, dd, H$_{ar}$); 7.62-7.65 (1H, d, H$_{ar}$); 8.06-8.09 (1H, dd, H$_{ar}$); 8.37-8.38 (1H, m, H$_{ar}$). NMR [13]C : 28.76 (S-$\underline{C}H_2$); 113.45 (C$_{ar}$); 115.03 (C$_{ar}$); 117.55 (C$_{ar}$); 121.84 (C$_{ar}$); 139.02 (C$_{ar}$); 142.21 ($\underline{C}H_2$-C=N); 150.03 (C$_{ar}$); 155.47 (N=$\underline{C}$-S). SM: 325 ([M+H]$^+$, 100).Yield = 71%. MP = 247-248°C. |
| 5e | NMR [1]H: 4.97 (2H, s, S-C$\underline{H}_2$); 7.64-7.75 (2H, m, H$_{ar}$); 8.08-8.12 (2H, m, H$_{ar}$); 8.39-8.47 (2H, m, H$_{ar}$). NMR [13]C: 28.64 (S-$\underline{C}H_2$); 117.58 (C$_{ar}$); 128.59 (C$_{ar}$); 142.21 (CH$_2$-$\underline{C}$=N); 142.55 (C$_{ar}$); 155.37 (N=$\underline{C}$-S). SM: 370. ([M]$^+$, 19.54).Yield = 53%. MP = 180-181°C. |
| 5f | NMR [1]H: 4.98 (2H, s, S-C$\underline{H}_2$); 7.55-7.59 (2H, m, H$_{ar}$); 7.65-7.76 (6H, m, H$_{ar}$); 7.95-7.96 (1H, m, H$_{ar}$); 8.08-8.11 (1H, dd, H$_{ar}$); 8.39 (1H, d, H$_{ar}$). NMR [13]C : 29.59 (S-$\underline{C}H_2$); 111.59 (C$_{ar}$); 114.41 (C$_{ar}$); 115.69 (C$_{ar}$); 118.50 (C$_{ar}$); 118.92 (C$_{ar}$); 125.01 (C$_{ar}$); 129.28-129.53 (2 C$_{ar}$); 130.32 (2 C$_{ar}$); 132.62-132.97 (C$_{ar}$); 138.93 (C$_{ar}$); 140.50 (CH$_2$-$\underline{C}$=N); 142.34-144.46 (C$_{ar}$); 156.12 (C$_{ar}$); 167.81 (N=$\underline{C}$-S); 196.51 ($\underline{C}$=O). SM : 429. ([M+H]$^+$, 100). Yield = 72%. MP = 161-162°C. |
| 5g | NMR [1]H: 4.91 (2H, s, S-C$\underline{H}_2$,); 7.17-7.21 (2H, dd, H$_{ar}$); 7.54-7.61 (4H, m, H$_{ar}$); 7.64-7.7 (4H, m, H$_{ar}$); 7.73-7.78 (1H, m, H$_{ar}$); 7.91 (1H, m, H$_{ar}$). NMR [13]C: 29.77 (S-$\underline{C}H_2$); 116.00 (3 C$_{ar}$); 122.89 (C$_{ar}$); 124.91 (C$_{ar}$); 129.40 (C$_{ar}$); 129.64 (2 C$_{ar}$); 130.43 (2 C$_{ar}$); 131.43 (C$_{ar}$); 132.48 (C$_{ar}$); 132.66 (3 C$_{ar}$); 139.95 (C$_{ar}$); 151.32 (CH$_2$-$\underline{C}$=N); 153.97 (C$_{ar}$); 167.91 (N=$\underline{C}$-S); 196.57 ($\underline{C}$=O). SM: 384 ([M]$^+$, 59). Yield = 86%. MP = 255-256°C. |
| 5h | NMR [1]H: 4.96 (2H, s, S-C$\underline{H}_2$,); 7.60 (2H,m, H$_{ar}$); 7.65-7.70 (5H, m, H$_{ar}$); 7.89 (1H, m, H$_{ar}$); 8.10-8.13 (1H, dd, H$_{ar}$); 8.47-8.48 (1H, d, H$_{ar}$). NMR [13]C: 29.70 (S-$\underline{C}H_2$); 113.64-118.73 (3 C$_{ar}$); 124.94 (C$_{ar}$); 129.63 (C$_{ar}$); 130.42 (2 C$_{ar}$); 131.49 (2 C$_{ar}$); 132.47 (C$_{ar}$); 132.66 (C$_{ar}$); 133.06 (C$_{ar}$); 139.04 (C$_{ar}$); 143.53 (CH$_2$-$\underline{C}$=N); 153.59 (C$_{ar}$); 156.73 (C$_{ar}$); 167.92 (N=$\underline{C}$-S); 196.55 ($\underline{C}$=O). SM : 429 ([M]$^+$, 56). Yield = 84%. MP = 93-94°C. |
| 5i | NMR [1]H: 4.95 (2H, s, S-C$\underline{H}_2$); 7.55-7.60 (4H, m, H$_{ar}$); 7.66-7.69 (6H, m, H$_{ar}$); 7.90 (2H, m, H$_{ar}$); 7.95 (2H, m, H$_{ar}$). NMR [13]C : 29.73 (S-$\underline{C}H_2$); 124.81 (C$_{ar}$); 129.26 (4 C$_{ar}$);130.30 (4 C$_{ar}$); 131.45 (C$_{ar}$); 131.82 (2 C$_{ar}$);132.91 (C$_{ar}$); 139.01 (C$_{ar}$); 154.46 (N=$\underline{C}$-S); 196.44-196.55 (2 $\underline{C}$=O). SM: 488 ([M]$^+$, 72). Yield = 52%. MP = 150-154°C. |
| 5j | NMR [1]H: 4.90 (2H, s, S-C$\underline{H}_2$); 7.15-7.19 (2H, m, H$_{ar}$); 7.34-7.59 (4H, m, H); 7.59-7.91 (1H, m, H$_{ar}$); 8.01-8.04 (1H, m, H$_{ar}$). NMR [13]C: 30.31 (S-$\underline{C}H_2$); 121.25 (2 C$_{ar}$); 121.86 (2 C$_{ar}$); 124.56 (C$_{ar}$); 126.39 (C$_{ar}$); 134.79 (2 C$_{ar}$); 149.53 (CH$_2$-$\underline{C}$=N); 152.47 (C$_{ar}$); 165.59 (N=$\underline{C}$-S). Yield = 54%. MP = 176-177°C. |
| 5k | NMR [1]H: 2.42 (3H, s, C$\underline{H}_3$); 4.85 (2H, s, S-C$\underline{H}_2$); 7.15-7.19 (3H, m, H$_{ar}$); 7.48-7.54 (4H, m, H$_{ar}$). NMR [13]C: 21.07 ($\underline{C}H_3$); 29.40 (S-$\underline{C}H_2$); 162.42 (N=$\underline{C}$-S); 151.65 (C$_{ar}$); 149.40 (CH$_2$-$\underline{C}$=N); 139.01 (C$_{ar}$); 134.36 (C$_{ar}$); 125.6 (C$_{ar}$); 121.86 (2 C$_{ar}$); 117.76 (C$_{ar}$); 110.33 (C$_{ar}$). SM : 295 ([M+H]$^+$, 100).Yield = 51%. MP = 181-182°C. |

a larvicidal activity (CL$_{100}$ = 12.03 μg/mL) less than that of **4a** and introducing two chlorines on the benzene ring (compound **4d**) caused the disappearance of the activity (LC$_{100}$ = 424 μg/mL).

Secondly, the introduction of a substituent such as nitro on the benzimidazole ring in position-5 produced a decrease in the activity. So, compound **4f** had a larvicidal concentration (CL$_{100}$ = 2.86 μg/mL) 5720 higher than that of compound **4b**. The different substitutions made did not cause an improvement of this activity, it is the case of

chloro isomer **4g** (CL$_{100}$ = 2.86 μg/mL) and 2,4-dichloro **4h** (CL$_{100}$ = 2.86 μg/mL) which had their activity equal to that of compound **4f**; these had their activity four times greater than that of the non-nitrated derivative **4c** (CL$_{100}$ = 12.03 μg/mL). However, none of these substituted isomers at position-5 of benzimidazole by nitro group had larvicidal activity comparable to those of fenbendazole and ivermectin.

Thirdly, replacing the nitro group at position-5 by another group such as benzoyl led to the loss of

**Table 3.** Larvicidalconcentration of 4a-k, 5a-kcompounds and reference molecules.

| Compounds | $CL_{100}\mu g/mL$ | Compounds | $CL_{100}\mu g/mL$ |
|---|---|---|---|
| 4a | 2.86 | **5a** | 424 |
| 4b | 0.0005 | **5b** | 424 |
| 4c | 12.03 | **5c** | 424 |
| 4d | 424 | **5d** | 0.002 |
| 4e | - | **5e** | 0.68 |
| 4f | 2.86 | **5f** | 0.68 |
| 4g | 2.86 | **5g** | 12.03 |
| 4h | 2.86 | **5h** | 0.038 |
| 4i | 424 | **5i** | 424 |
| 4j | 424 | **5j** | 212 |
| 4k | 2.86 | **5k** | 12.03 |
| *Ivermectin* | *0.009* | ***Fenbendazole*** | *0.0005* |

nematicide activity. Nevertheless, only one compound of this series seemed to have a larvicidal activity, the **4k** ($CL_{100}$ = 2.86 µg/mL) compared to **4a**.

As for the obtained results after evaluating the nematicide activity of 2-(benzimidazolyl methylthio) benzimidazole derivatives **5a-k** (Table 2) and their analogues allowed to make some interpretations.

First, replacing benzene moiety by benzimidazolyl inhibited the nematocidal activity. The product **5a** ($CL_{100}$ = 424 µg/mL) had larvicidal concentration 18 times greater than that induced by the benzyl derivative **4b** ($CL_{100}$ = 0.0005 µg/mL). The double substitution on the benzene homocycle (B) using substituents such as nitro (**5b**: $CL_{100}$ = 424 µg/mL) and benzoyl (**5c**: $CL_{100}$ = 424 µg/mL) had no effect on the nematicide activity compared to compound **4a** ($CL_{100}$ = 2.86 µg/mL).

Secondly, introducing a nitro group at position-5 of the 2-methylthiobenzimidazole on the entity (A) of compound **5a** caused an enhancement of the activity. Thus, the larvicide product concentration **5d** ($CL_{100}$ = 0.002 µg/mL) obtained was multiplied by a factor of 212,000. It also showed an anti-*Haemonchus* which was 4 times lower respectively than those of fenbendazole and ivermectin. However, the introduction of substituents on the benzene homocyle (B) such as nitro and benzoyl decreased the ctivities.So, compounds **5e** and **5f** ($CL_{100}$ = 0.68 µg/mL) were 340 times less active than the unsubstituted derivative **5d**. Their activities however were greater than that of the unsubstituted derivatives on the entity (A). These compounds possessed nematocidal activity 623 times greater than that of **5a**.

Thirdly, the replacement of nitro on the compound **5b** of the entity (A) by a benzoyl group **5g** ($CL_{100}$ = 12.03 µg/mL) appeared to inhibit the nematocidal activity and it was 35 times more active than the unsubstituted derivative **5a**. However, this larvicidal concentration remained significantly higher than those of ivermectin ($CL_{100}$ = 0.009 µg/mL) and fenbendazole ($CL_{100}$ = 0.0005 µg/mL). When homocycle entity (B) **5g** was substituted in

its position-5 by nitro group **5h** ($CL_{100}$ = 0.038 µg/mL), there was an increase of the larvicidal activity. This was 316 times greater than **5g**. Compound **5h** showed an anti-*Haemonchus* efficiency respectively 76 times and 4 times less than that of fenbendazole and ivermectin. The presence in the same position of the benzoyl **5i** ($LC_{100}$ = 424 µg/mL) caused the greatest loss of larvicidal activity. In this series, a third substitution has been carried out. This was the replacement of the benzimidazole ring of the entity (A) by benzothiazole **5j** and benzoxazole **5k**. This homology replication resulted in slight improvement in the nematicide activity compared to compound **5a**.The larvicide concentration was divided by a factor 2 (**5d**) and 35 (**5k**). These activities remained very low compared to the reference compounds. A comparison of compounds **5j** and **5k** showed that the benzoxazole derivative exhibit anti-*Haemonchus* activity 18 times greater than its analogue benzothiazole.

**Conclusion**

The syntheses carried out around the chemical series of 2-mercaptobenzimidazole allowed to obtain new compounds, derivatives and structural analogues of 2 (methylthio) benzimidazole which have been characterized by NMR ($^1$H and$^{13}$C) and mass spectroscopy. *In vitro* nematocide assays against *H. contortus* of the synthesized compounds revealed the anthelmintic activities of compounds **4b**, **5d**, **5e**, **5f** and **5h**. Among them, **4b** and **5d** were active compared to fenbendazole or ivermectin. The structure-activity relationship study showed that coupling a benzene group with the methylthio group of 2-mercaptobenzimidazole was more advantageous to the appearance of nematocidal activities than that of a benzimidazole. In addition, the introduction of nitro ($NO_2$) group on the benzene ring was favorable to an increase of nematicide activities.

**Conflict of Interests**

The authors have not declared any conflict of interests.

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*Full Length Research Paper*

# The effectiveness of dear healthcare professional letters as a risk minimization tool in Ghana

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Dear Healthcare Professional (DHP) letter is a risk minimization tool used to inform health workers about new and emerging safety information during the marketing period of medicinal products. DHP letters in some cases may not be effective because targeted audience may not be aware of these letters or even understand them. The objectives of this study were to assess the effectiveness and relevance of DHP letters as effective risk minimization tool and to seek opinion of health workers about the most effective way of communicating safety information. A descriptive correlational study of 913 health workers selected by convenient sampling through face-to-face interviews from Apr. to June, 2014 was conducted, with a response rate of 83.15%. The data was analyzed using descriptive analysis and Pearson-chi square test ($\chi^2$) with STATA version 13. A p-value of < 0.05 was considered significant during the analysis. Of the 913 respondents only 350 (38.34%) were aware of at least one of the letters distributed in 2013 and 314 (89.71%) out of these admitted that these letters have influenced their way of prescribing, dispensing and administering the medicines involved. One hundred and ninety-two (54.82%) of the respondents rated the level of understanding of the language used as good and there was no significant difference in the health workers rating of the language used in the letters (p=0.40). Health workers suggested electronic methods such as short messaging service to their mobile phones 56.81% (438), e-mail 91 (85.85%) and posting the letters on the Food and Drugs Authority's website 26.85% (207). The results suggest that DHP letters issued by the Food and Drugs Authority (FDA) are effective in changing behavior of those who receive them but they are received late or not at all. The FDA should therefore explore other means of communicating safety information such as electronic means as suggested by the health workers.

**Key words:** Ghana, dear healthcare professional letter, risk minimization, effectiveness.

## INTRODUCTION

A Dear Healthcare Professional (DHP) letter is a correspondence usually in the form of a mass mailing from the marketing authorization holder of medicinal product or a regulatory authority addressed to doctors, pharmacists, nurses and other health workers regarding important new safety information (USFDA, 2010). DHP

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letters are intended to inform the recipients of the need to take certain action(s) or adopt certain practices to minimize particular risks and/or to reduce burden of adverse drug reactions with a medicinal product (EMA, 2014).

Throughout the life cycle of a marketed drug, new safety information emerges; some of which may lead to disability, hospitalization and even death of patients which must be communicated to health workers to ensure patient safety and to minimize the risk of adverse events. There are several risk minimization measures for communicating safety information to health workers, including press releases, black box warnings, changes in the summary of product characteristics and DHP letters. The most common and preferred method of communi-cating safety information is through the DHP letters (Piening et al., 2012; Théophile et al., 2011). Studies have revealed that about 10 to 14% of the registered medicinal products require DHP letters to inform health workers about newly identified risks within the first 3 years of their marketing authorization and also about 22% of drugs issued marketing authorization are withdrawn within the first 6 years for safety reasons which must be communicated to health workers to ensure patient safety (Arnardottir et al., 2011; Giezen et al. 2008; Qureshi et al., 2011).

The main goal of DHP letters as a risk minimization tool is to minimize the occurrence of safety issues. They are also used to communicate new or emerging safety information on medicines to health workers and the content and wording of these letters are extremely important in achieving the intended purpose. Review of the literature suggested that letters to health workers may or may not be effective in communicating safety information leading to change in behavior (Dusetzina et al., 2012; Piening et al., 2012).

The impact of four DHP letters was examined and label changes on the monitoring of serum enzymes for liver toxicity among patients on troglitazone and found that an early impact on increased monitoring behavior (from 15 to 45%) was not sustained during the six-month study period (Graham et al., 2001). The Mazor study evaluated the quality of a group of DHP letters sent by pharmaceutical companies between 2000 and 2001 which were intended to communicate important new drug safety information. The study found a correlation between the quality or perceived quality of DHP letters and the extent to which physicians perceive the new information as important. Letters that were evaluated as clearer, more concise, better organized and formatted, and focused on the most important aspects of the new safety information were also considered to be more effective in communicating the new information (Mazor et al., 2005).

Reiber et al. (2012) examined the effect of DHP letters on the prescription of specialist and non-specialist drugs and found that DHP letters had less impact on use of specialist drugs than non-specialist drugs (P < 0.05). The study concluded that the risk communication can be effective, specifically in case of well-structured information, and very serious safety issues potentially causing death or disability (Reber et al., 2012). In 2013, the Ghana Food and Drugs Authority (FDA) issued 6 DHP letters to health workers through an approved mailing list and also via the FDA's website, www.fdaghana.gov.gh on varying safety issues (Food and Drugs Authority Ghana, 2013a, b, c, d, e, f). The letters address safety issues concerning azithromycin and cardiovascular risks; the risks involved with the use of codeine for analgesia in children and adolescents; diclofenac and the risk of cardiovascular events; paracetamol and the risk of severe skin reactions; reporting incidents of therapeutic ineffectiveness and restrictions on the use of ketoconazole due to severe liver injury, adrenal gland problems and drug interactions.

Implementation and evaluation of the effectiveness of risk minimization measures is evolving and the European Medicines Agency has made provisions which require marketing authorization holders and regulatory authorities to monitor the effectiveness of risk minimization measures. Similarly, the United States, Food and Drug Administration may also require risk evaluation and mitigation strategy (REMS) for registered medicinal products to minimize or reduce the impact of adverse events (USFDA, 2009). However, the provisions in good pharmacovigilance practices (GVP) module XVI is not clear on the exact methodology for evaluating the effectiveness of the risk minimization measures (EC, 2010; EMA, 2014; Raine et al., 2011). In addition to this shortcoming, to the best of our knowledge this is the first study to evaluate the effectiveness of risk minimization measure in a developing country setting. This research therefore provides important missing information regarding the effectiveness of DHP letters as an important risk minimization tool in communicating safety information to health workers in a developing country setting and also proposes ways of improving this method.

## METHODOLOGY

### Questionnaire design

This was a descriptive correlational study using a structured questionnaire adapted from earlier study by Piening et al. (2012) and based on 3 process indicators in the Banerjee model; awareness, which measured the coverage and awareness, understanding/knowledge which assessed respondents' understanding of the information contained in the DHP letters and behavior which measures the extent of deviation from accepted behavior (Banerjee et al., 2014; Piening et al., 2012). The questionnaire was pretested using 20 respondents who were not included in the final analysis. The questionnaire was divided into 5 sections, namely; demographic characteristics, awareness/ knowledge of the of the DHP letters, quality of content/change in

behavior, source of the letters and preferences for receiving the DHP letters in the future.

## Study design and sampling

A descriptive correlation study was conducted of 1,098 health workers selected by convenient sampling through a face-to-face interview in all the 10 administrative regions of Ghana to access awareness, effectiveness, change in behavior and preferences for receiving DHP letters. Nine hundred and thirteen out of the 1,098 health workers contacted completed the questionnaire representing a response rate of 83.15%. Respondents included doctors, pharmacists, nurses and physician assistants from public and private health facilities in Ghana. The criterion for inclusion in the study was that healthcare worker must have practiced from Jan.-Dec. 2013, the period within which the DHP letters were distributed by the Food and Drugs Authority.

## Data collection and quality control

The doctors, pharmacists, nurses and physician assistants were contacted through face-to-face interview from April, 2014 to June, 2014 by the Food and Drugs Authority employees in the 10 administrative regions of Ghana. The questionnaire was reviewed by 2 pharmacovigilance experts and then pretested to evaluate its appropriateness and suitability for the study. To ensure uniformity of the process, the Food and Drugs Authority employees involved in the study were trained on the methodology of administering the questionnaire and presentation of the study objectives to the respondents. The study questionnaire had 5 sections (Sections A to E) which requested for information on the demographic characteristics of the responding health workers, awareness/knowledge of the DHP letters distributed in 2013, quality of content/change in behavior; source of the letters and preferences for receiving the DHP letters in the future. No identifying information was collected from the study participants; agreement to take part in the study was considered consent.

## Data analysis

Data collected during the study was entered into data editor in STATA version 13 developed by StataCorp, Texas 77845, USA. Pearson-chi square test ($\chi^2$) was used to determine the association between region and the profession of the healthcare worker and the awareness/understanding and the preference for receiving the DHP letters. Fischer exact test ($F^{exact}$) was used to determine the association between the categorical demographic variables when the number of counts in the contingency table was less than 5. A p-value of < 0.05 was considered significant during the analysis.

## RESULTS

Nine hundred and thirteen health workers out of 1,098 approached completed the questionnaire giving a response rate of 83.15%. The professional backgrounds of the 913 health workers were 597 (65.39%) pharmacists, 136 (14.90%) doctors, 95 (10.40%) nurses and 85 (9.31%) physician assistants. All 913 completed responses were included in the analysis and the characteristic of the respondents were given in Table 1.

## Knowledge and behavior of the health workers

Of the 913 health workers who completed the questionnaire only 350 (38.34%) had knowledge of at least one of the 6 DHP letters distributed by the FDA in 2013. On the average greater % of nurses 65(68.42 %) had knowledge of at least one of the 6 DHP letters distributed compared to other health workers ($X^2$=102.39, p<0.0005). Figure 1 provides the % of health workers' knowledge of the 6 DHP letters distributed. In total, more health workers who had knowledge of these letters, were aware of the letter relating to diclofenac and cardiovascular events 213(60.00 %) compared to the other letters, with the least being the use of codeine for analgesia in children and adolescents 132 (37.18%).

Majority of the health workers 289 (82.57%) who had knowledge of the letters remember the content while the rest did not. Nurses 60 (92.31%) are more likely to remember the safety issues presented in these letters compared to the other health workers ($F^{exact}$ = 0.040). The ability to remember the content of the letters, for other health workers varies from 37 (86.05%), 130 (81.25%) and 62 (75.61%) for physician assistants, pharmacists and doctors, respectively. Three hundred and five (87.14%) of the health workers who received the letters have used at least one of the medicines in their practice. Majority of the health workers 314 (89.71%) admitted that the content of these letters influenced the way they prescribe, dispense or administer these medicines. All physician assistants 100% (43) who received at least one of the DHP letters reported change in their prescribing behavior. The remaining health workers reported the following percentages as changes in behavior after becoming aware of the letters; 90.24% (74), 87.50% (140) and 87.69% (57) for doctors, nurses and pharmacists, respectively. There was no statistically significant relationship between change in behavior and profession ($F^{exact}$ = 0.144). Those who reported change in behavior stated that they were influenced to change prescribing practice and patient counseling. Those who stated that the DHP letters did not result in change in behavior stated reasons like the information is already known and the medicines involved were not used by them.

Apart from the 6 DHP letters sent in 2013, the health workers were also asked if they have ever received any letter from the FDA and out of the 911 who responded to this question , 497 (54.56%) has never received any letter from the FDA and the rest did.

## Quality and relevance of the information

Quality and relevance of the DHP letters were measured in terms of the used language and relevance of content. Health workers who read the letters rated the level of

**Table 1.** Background characteristics of respondents.

| Characteristics (N) | Frequency | Percent (%) |
|---|---|---|
| **Gender (913)** | | |
| Male | 354 | 41.45 |
| Female | 500 | 58.55 |
| | | |
| **Age in Years (908)** | | |
| ≤30 | 410 | 45.15 |
| 31-40 | 257 | 28.3 |
| 41-50 | 127 | 13.99 |
| ≥51 | 114 | 12.56 |
| | | |
| **Profession (913)** | | |
| Doctor | 136 | 14.9 |
| Pharmacist | 597 | 65.39 |
| Nurse | 95 | 10.51 |
| Physician Assistant | 85 | 9.31 |
| | | |
| **Number of Years practiced (853)** | | |
| ≤2 | 192 | 22.51 |
| 3-5 | 268 | 31.42 |
| 6-10 | 172 | 20.16 |
| ≥10 | 221 | 25.91 |
| | | |
| **Type of Health Facility (908)** | | |
| Government | 508 | 55.95 |
| Quasi-Government | 270 | 29.74 |
| Christian Health Association of Ghana | 96 | 10.57 |
| Private | 34 | 3.74 |
| | | |
| **Region (913)** | | |
| Ashanti | 112 | 12.27 |
| Brong-Ahafo | 112 | 12.27 |
| Central | 104 | 11.39 |
| Eastern | 96 | 10.51 |
| Greater Accra | 86 | 9.42 |
| Northern | 84 | 9.2 |
| Upper East | 83 | 9.09 |
| Upper West | 82 | 8.98 |
| Volta | 80 | 8.76 |
| Western | 74 | 8.11 |

understanding of the language used as good and satisfactory, 192 (54.82%) and 151 (43.14%), respectively and only 2 (0.57%) rated the language as poor. There was no significant difference between the health workers rating of the used language in the letters ($F^{exact} = 0.270$). Those who responded to the question on the relevance of the DHP letters rated these as good and satisfactory corresponding to 235 (65.73%) and 122 (34.2 7%),

respectively all those who read the letters stated that the content was relevant to their practice as health workers.

**Source of the DHP letters**

Majority of those who were aware of the letters 183 (53.67%) had it directly from the Food and Drugs
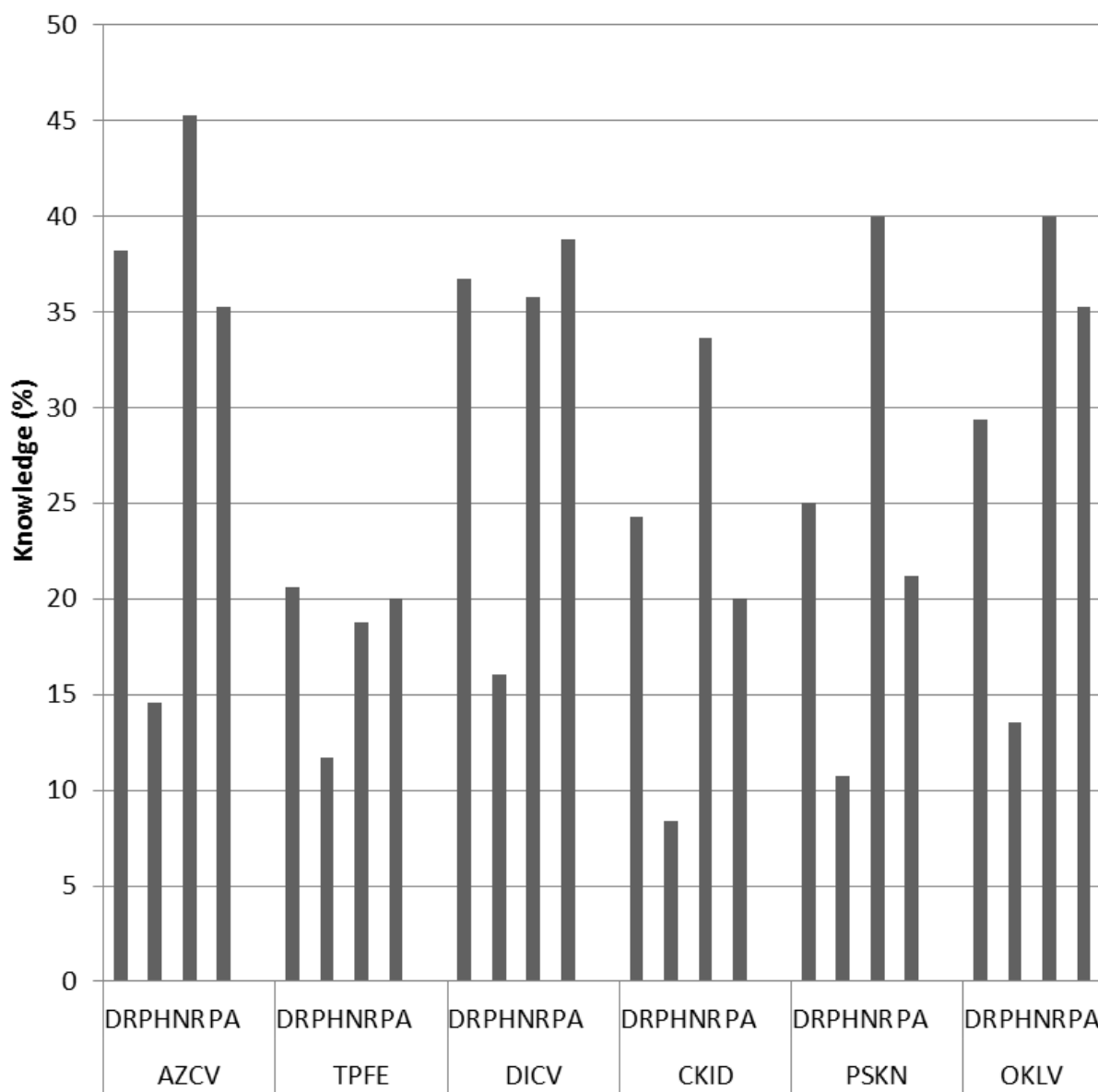
**Figure 1.** Health workers knowledge of six dear healthcare professional letters issued by the FDA in Jan-Dec. 2013. **DR**=Doctor; **PH**=Pharmacist; **NR**=Nurse; **PA**=Physician Assistant; **AZCV**= Azithromycin and cardiovascular risks; **TPFE**= Reporting incidents of therapeutic ineffectiveness; **DICV**= Diclofenac and the risk of cardiovascular events; **CKID**=Codeine for analgesia in children and adolescents; **PSKN**=Paracetamol and the risk of severe skin reactions; **OKLV**= Oral ketoconazole due to severe liver injury, adrenal gland problems and drug interactions.

Authority, followed by 136 (39.88 %) from the hospital facility where they practice. Other sources stated were from colleagues, professional associations, internet, medscape and other regulatory bodies like the Ghana Medical Council, Pharmacy Council and the Nursing and Midwifery Council. Almost all those who received the letters 318 (90.86 %) received them as hard copies and the rest as soft copies and 255 (72.86 %) considered the medium of delivery of these letters as effective.

One hundred and sixty one (46.00 %) of those who received the letters had them within 2 months with 134 (38.19%) receiving these letters 2 months after they were issued. 55 (15.71%) did not answer this question because they couldn't recall at what time to the issuance of these letters they were received. Almost all the regions received the letters within the same period, there is no differences among regions and how long it took to receive the letters ($X^2$ = 27.74; p = 0.066).

**Table 2.** Preferred source of drug safety communication [n(%)].

| Preferences | Doctor | Pharmacist | Nurse | Physician Assistant | Total | p-value |
|---|---|---|---|---|---|---|
| Text Message | 63(46.32) | 282(47.24) | 57(60.00) | 36(42.35) | 438(47.97) | 0.077 |
| Professional Journal | 53(38.97) | 103(17.25) | 39(41.05) | 22(25.88) | 217(23.77) | 0.001 |
| Professional Meetings | 45(33.09) | 191(31.99) | 32(33.68) | 37(43.53) | 305(33.41) | 0.216 |
| FDA website | 37(27.21) | 113(18.96) | 35(36.84) | 22(25.88) | 207(22.70) | 0.001 |

**Preferences for risk communication**

The most preferred means of delivering the letters to the health workers was short messaging service (SMS) to their mobile phones 438 (47.97 %) with the least preferred being the letters posted on the FDA website, 207 (22.70%). Table 2 shows health workers and the preference for risk communication. Of the 106 health workers who answered the open questions on the other preferences for risk communication 91 (85.85%) mentioned email as the preferred source, followed by social and electronic media at 10 (9.43%) and 4 (3.77%), respectively.

**DISCUSSION**

Limited number of health workers interviewed, 350 (38.34%) had awareness of at least one of the 6 DHP letters distributed by the FDA in 2013. This is surprising because the letters were distributed to all the health facilities in which the interviews were conducted. In contrast, a similar study in the Netherlands reported higher % of health workers' awareness, 49 to 67%, of the DHP letters distributed by the Dutch regulator (Théophile et al., 2011). The low awareness of health workers about the DHP letters distributed by the FDA will result in the lack of knowledge of these issues and therefore specific actions needed to minimize risk to patients from these safety issues as revealed by Nicolette et al. (Bester et al., 2016). Encouraging is the number of health workers, 314 (89.71%) who admitted that these letters have influenced their behavior relating to prescription, dispensing, and administration of the medicines involved. The finding in this study was better than what was obtained in similar studies (Cheung et al., 2008; Karpel et al., 2009).

Greater number of nurses, 65 (68.42%) were aware of the safety issues compared to other health workers, with the least being physician assistants. More pharmacists, 70 (41.92%) were aware of the safety issue regarding therapeutic ineffectiveness compared to other health workers. The fact that nurses had the greatest awareness of the letters could be explained by the fact that most of these medicines are administered by the nurses hence the interest in the safety issues regarding these medicines. The DHP letter with the best awareness

amongst all health workers was; diclofenac and the risk of cardiovascular events 213 (60.00%), followed azithromycin and cardiovascular risks 212 (59.72%), with the least being the use of codeine for analgesia in children and adolescents, 132 (37.18%). This could be explained by the fact that of the medicines, diclofenac and azithromycin are the most used analgesic and antibacterial agent, respectively; hence, the interest of the health workers in these safety issues (Van Boeckel et al., 2014; CDDEP, 2015; Desalegn, 2013; Teslim et al., 2014).

The use of codeine as analgesia in children and adolescent is not recommended in Ghana (Ghana Minstry of Health 2010a, b). This DHP letter is therefore considered not relevant to the health workers interviewed, hence their lack of interest, which is consistent with an earlier study (Piening et al., 2012). Four hundred and ninety-seven (54.56%) of the health workers interviewed have never seen or received a DHP letter from the Food and Drugs Authority. This result is high compared with those obtained in studies in the USA and Netherlands where only 18 and 15%, respectively which indicated that they had never seen or received a DHP letter before from these regulatory authorities (Lee et al., 2008; Piening et al., 2012).

The high number of health workers who confirmed that the letters have changed their behavior could be linked to the high ratings given to the DHP letters in terms of content, layout and quality of information contained in these letters which is consistent with the findings by the Mazor study (Mazor et al., 2005). Majority of health workers, 183 (53.67%) received the DHP letters from the Food and Drugs Authority as against those received by the hospital authorities 136 (39.88%), which showed that the Food and Drugs Authority remains the main source of safety communications. However, it is important the Food and Drugs Authority expand the distribution list to ensure a lot more health workers receive these safety communi-cation in view of the small number who had never seen or received a DHP letter.

It took more than 2 months for 134 (38.19%) to receive the DHP letters which is a major limitation in delivering the letters as these risk minimization information would not get to those who need these in real time to be able to take the decision required to improve the benefit-risk

assessment of the medicinal products involved. To improve this situation the health workers preferred electronic means of receiving the risk communication such as short messaging service (SMS) to their mobile phones and emails.

The strengths of this study were that data was collected from health workers in all the ten regions of Ghana which constitute a representative population of health workers in Ghana, and the period between the issuance of the DHP letters and the study is quite close therefore limiting the incidence of recall bias. Limitations associated with this study were that factors related to self-reporting studies such as accuracy of recall and personal bias may affect the study because some of the health workers might not remember receiving any of the DCHP letters for which this study is based. Secondly, the sampling method used, convenient sampling, meant it may be difficult to generalize the results to the general population. Finally, to our knowledge there is no standard for measuring the overall effectiveness of DHP letters as a risk minimization tool.

## CONCLUSION

The results suggest that although FDA's DHP letters are effective in changing the behavior of health workers, it is generally received late by the intended recipients. The FDA should therefore adopt electronic means such as emails and short messaging services for distributing the DHP letters to ensure optimum risk minimization. There is the need for future research on whether the change in behavior observed after reading the DHP letters by healthcare professionals was sustained to ensure patient safety.

## ACKNOWLEDGMENTS

## Conflict of interests

The authors have not declared any conflict of interest.

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**Appendix 1.** FDA Regional Pharmacovigilance Officers.

| Name | Region |
|------|--------|
| Abu Sumaila | Western |
| Abena Esia-Donkoh | Western |
| Martin Kusi | Central |
| Nana Ansah Adjei | Eastern |
| Vigil Prah-Eshun | Ashanti |
| Abigail Attah | Ashanti |
| Geoffrey Arthur | Volta |
| Matthew Nkum | Brong Ahafo |
| Akosua B. Adjei | Brong Ahafo |
| Sylvester Kyei | Northern |
| Gordon Akurugu | Upper West |
| Zakaria Braimah | Upper East |

*Full Length Research Paper*

# Acute toxicity and hypoglycaemic activity of the leaf extracts of *Persea americana* Mill. (Lauraceae) in Wistar rats

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*Persea americana* Mill. or avocado plant tree is well-known to people from the sub-Saharan part of Africa. Studies carried out earlier reported on the use of leaf extracts of the named plant to cure diabetes and other diseases in the south of Côte d'Ivoire. This study aimed to assess both acute toxicity and hypoglyceamic activity together with performing a comparative study. The acute toxicity was determined using the OECD 423 protocol, followed by the analysis of biochemical indicators, body weight variation and vital organs damage of healthy Wistar rats. The aqueous, ethanol and methanol extracts' hypoglycaemic properties were investigated through the hypoglycaemic activity and oral glucose tolerance test. Thereafter, the phytochemical identification of the molecular compounds' was carried out as well as polyphenols and total flavonoids quantification using Folin-ciacalteu and Neu reagent. The data analysis showed that *P. americana* leaf extracts' are well tolerated in general at a unique dose of 2000 mg/kg. Nevertheless, a slight hepatitis occurrence was observed. Additionally, all extracts exhibited significant anti-hyperglyceamic activity 2 h after glucose administration. Ethanol extract (100 mg/kg) showed a strong activity by depleting the glyceamia rate by 59.6% during 5 h after glucose uptake as compared to glibenclamide at 61.6% followed by methanol extract at 49.2%. The ethanol extract also appeared to be the most provided with phenols and total flavonoids exhibiting respective amounts of 2952.7 ± 166 µg gallic acid equivalent/g and 0.582 ± 0.012%, respectively. The current study showed that both ethanol and methanol extracts displayed a good tolerance and significant anti-hyperglyceamic activity probably due to the presence of polyphenol in the extracts.

Key words: *Persea americana* Mill., avocado, toxicity, glyceamia, diabetes, rat.

## INTRODUCTION

According to the World Health Organization (WHO), diabetes mellitus is a chronic disease that occurs when

the pancreas does not produce the required amount of insulin or when the insulin released in the body is not adequately used. As a result, an increase of glucose serum level (hyperglyceamia) occurs, bringing about complications such as diabetic neuropathy, retinopathy and cardiovascular diseases (Kumar et al., 2011; WHO, 2015).

Thus, in 2014, the estimated people living with diabetes reached 387 million and the figure is projected to reach 592 million in the coming twenty years (IDF, 2015). To illustrate, the diabetes prevalence in sub-Saharan Africa, which is currently set at 12.1 million, is expected to rise to 23.9 million by 2030 (Keter and Mutiso, 2012). From this perspective, diabetes mellitus constitutes a global health concern. Therefore, the discovery of a sustainable and adequate treatment for this disease appears to be a challenge to scientists. Studies conducted earlier reported on the use of herbal medicines and traditional remedies to cure diabetes. For example, in China and in Africa, traditional medicine accounts respectively for about 40 and 80% of the proposed treatment of the population meeting their health care needs including treatment of diabetes (WHO, 2002, 2013).

Additionally, records show that roughly 800 plants are used worldwide to treat diabetes mellitus (Alarcón et al., 1993). Among them is *Persea americana* mill. The avocado is described as a medium to large tree, 9-20 m high, and belongs to the Tracheophyta division of the Magnoliopsida class en the Lauraceae family, and endemic to Mexico (Central America). *P. americana* can also be found in most sub-tropical and tropical countries like Côte d'Ivoire. The number of species identified in the family is about 500 (Adeboye et al., 1999, Yasir et al., 2010).

Even though previous investigations have reported its biological activities, including anticancer, anti-diarrhea, analgesic and anti-inflammatory properties, little is known about its toxicity and hypoglyceamic activity (Adeyemi et al., 2002; Butt et al., 2006; Odo et al., 2014). The study aforementioned showed lower toxicity and hypoglyceamic effect. However, the mode of action was not explored in depth.

Therefore, the present study aimed at evaluating the acute toxicity from oral route uptake of three extracts of *P. americana* leaves and the comparative hypoglyceamic and anti-hyperglyceamic activities in normoglyceamic rats.

## MATERIALS AND METHODS

Leaves of *P. americana* were collected at dawn before the first sun rays at Adiopodoumé (N 5° 19' 3.49'' O 4° 8' 8.66''), a village located about 10 km from Abidjan (Côte d'Ivoire). The plant specimen was authenticated at the herbarium of the National Floristic Center of the Félix Houphouët Boigny University under the voucher number 8845 deposed at the national herbarium. It is also registered in the Integrated Taxonomic Information System under the number 18154 (ITIS, 2016).

### Extraction method

Fresh leaves of the plant were collected, washed, shade dried, cut into small pieces, and powdered in a Phillips® blender to obtain a weight of approximately 0.5 kg.

### Aqueous extract (AE) of the leaves of *P. americana*

Fifty grams (50 g) of *P. americana* leaves powder was boiled in one liter (1 L) of distilled water for 30 min, allowed to cool at room temperature (25°C) and filtered with a Fisherbrand® paper. The decoction was then lyophilized using the Martin Christ® ALPHA 2-4 LDplus, Germany and stored at 2-4°C until use for the bioassays and phytochemical analysis.

### Ethanol and methanol extracts (EE, ME)

The dried powder of *P. americana* leaves was macerated three times with absolute ethanol (10%, w/v), Prolabo®, France for 48 h at room temperature and shaken occasionally. The ethanol fraction was pooled after filtration and dried in an oven (Memmert®, Germany) at 40°C. Similar extraction procedure was repeated for methanol extract using absolute methanol (Quimicen®, Spain).

### Phytochemical analysis

### Phytochemical screening

The phytochemical screening was carried out to investigate the presence of active ingredients of extracts AE, EE and ME. The prospective compounds to be identified were generally alkaloids, flavonoids, tannins, polyphenols derivatives, triterpenoids/steroids and coumarins. These identification are performed by the appropriate extraction solvent. So, alkaloids were isolated through the Draggendorff test (Gidwani et al., 2011), flavonoids identified by the Shinida test (Vinod et al., 2010), tannins and polyphenols compounds by the $FeCL_3$ (Acros organic®, Belgium) test (Békro et al., 2007), saponins determined by foam formation and foaming index (Dohou et al., 2003), steroids and tripenoids were revealed by the Liebermann-Bürchard test (Békro et al., 2007), and coumarins were detected using NaOH (Carlo Erba®, France) reagent. The compounds aforementioned were visualized under UV-Visible light ($\lambda$=366 nm) (Rizk et al., 1986).

### Determination of total polyphenol compounds

Total polyphenol compounds' content from leaf extracts of *P. americana* were determined using the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965). The named method is

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developed as follows:

Primarily, 1 ml of plant extract (0.02 mg/ml) was mixed with 0.5 ml of the reagent. Then, 1.5 ml of $Na_2CO_3$ (17% w/v; Carlo Erba®, France) was added after shaking thoroughly with a vortex (Heidoph®, Germany). This mixture was incubated at 40°C for 10 min in a water bath. The absorbance of the total phenol compounds were read out using a Cecil® spectrophotometer (ce1021, UK). Before then, the standard calibration curve was obtained using gallic acid with a concentration range of 1 to 10 µg/ml and the figure recorded were expressed as gallic acid equivalent (GAE) (µg/g) of plant extract.

## Determination of total flavonoid compounds

Twenty milligram (20 mg) of each *P. americana* leaves extract were mixed with 10 ml of ethanol (80%, v/v). Then, after 10 min, 50 µl of the Neu reagent mixed with 12 ml of pure methanol were added to 1 ml of the crude extract. In addition, the mixture was shaken using a vortex, and the absorbance was read at a wavelength of 404 nm using the Cecil® spectrophotometer. For this experiment, quercetin solution (0.05 mg/ml) was used as positive control and the percentage of total flavonoids was expressed as quercetin equivalent according to the following relationship (Dohou et al., 2003):

$$F(\%) = \frac{0.05 \times A_{ext}}{A_q \times C_{ext}} \times 100$$

F (%): Percentage of total flavonoids in crude extract, $A_{ext}$: absorbance of crude extract, $A_q$: absorption of quercetin (standard pure compound), $C_{ext}$: plant leaves extract concentration (2 mg/ml).

## Biological material

Wistar albinos (*Rattus norvegicus var. albinus*) bred up to 2-3 months at the Animal Resources Unit of the Institute Pasteur Côte d'Ivoire, and weighing 150-250 g were used for the bioassay. Their living condition could be described as follows: the housing was cross-ventilated at mean temperature of 24-28°C with relative humidity of 60-80% and 12 h/day light. Prior to the experiments, rats were allowed to acclimatize for 2 weeks during which they were fed with standard diet and water *ad libitum* according to the international standards of animal use and care.

## Sample preparation

Extracts AE, EE, ME and reference antidiabetic drug (glibenclamide; Daonil®, Sanofi-Aventis) were prepared in a mixture of water and Tween 80% (2%, v/v) called the vehicle. Then, test samples were taken up orally by force-feeding the animals, which were under mild anaesthesia (lidocaïne oral gel; AstraZeneca®, UK). The negative control group of animals received the vehicle only orally (10 ml/kg, b.w.).

## Acute toxicity study of extracts

Acute oral toxicity was evaluated by following modified OECD test guidelines (OECD, 2001). Previously, research showed that *P. americana* exhibits low toxicity. Based on that, the "limit test" was used to assess its toxicity level (Figure 1). Thus, the experiment was designed such that, healthy female rats underwent fasting overnight, and they were divided into four groups (n=3) randomly.

Group I received vehicle (10 ml/kg; b.w.) by oral route, was considered as negative control whereas, Groups II, III and IV received 2000 mg/kg, b.w. of AE, EE and ME, respectively. The experiment was repeated 2 weeks later with another group of animals.

After extract administration, the rats were observed continuously every 1h for 4 days. Further observation was carried out every 24 h for 15 days to identify any change such as tremors, convulsions, salivation, diarrhea, lethargy, sleep, coma and decrease of the respiratory rate or any lethality. Thus, after 4 h observation, all rats were allowed to feed themselves and drink water *ad libitum*. Then, the body weight was monitored every four days. From this experiment, the median lethal oral dose ($LD_{50}$) was determined with respect to the OECD 423 standards fixed by the Globally harmonized Classification System (GHS) (Figure 1).

At day-15, animals were subjected to fasting overnight, euthanized by cervical dislocation under anaesthesia (Forene®, Abbott, USA). Blood samples were collected in red topped plastic vials and centrifuged at 1500 g for 10 min. The serum collected out of this step was used for biochemical analysis. A gross necropsy of all animals was also carried out. The heart, liver and kidney were carefully isolated and weighed individually. From the bio-analysis performed, the following parameters were determined: blood urea nitrogen (BUN), creatinine (CREA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), sodium ($Na^+$), potassium ($K^+$), chloride ions ($Cl^-$), using biochemical kits (Hitachi 704R®) and electrolyte analyzer (ISE 3000®), respectively.

## Dose selection

First, a dose of 2000 mg/kg from each extract of the plant was selected for acute toxicity evaluation. Then, both $1/10^{th}$ and $1/20^{th}$ of the dose, which do not display any behavioural alterations, were considered for anti-diabetec tests (Oliveira et al., 2008). Additionally, the $1/5^{th}$ of the dose was also selected for further identical assays.

## Acute hypoglycaemic effect on normal rats

The set of healthy males rats was organized by dividing them into 11 groups (n=5 per group), followed by, fasting blood glucose level recording, at an initial time (t=0 h), after the overnight fasting (16 h) and water uptake *ad libitum*. The negative control (Group 1) received by oral route, 10 ml/kg b.w. of vehicle aqueous Tween 80 (2%; v/v), and the positive control group (2) was administered a standard hypoglycaemic drug (glibenclamide) 10 mg/kg b.w. Groups 3 to 11 received AE, EE and ME at 100, 200, 400 mg/kg for each. Then blood was collected from tail-tip after oral administration of test samples followed by a period of observation which lasted ½, 1, 2, 3 and 5 h. Blood glucose levels were recorded using reactive strips (GOD-POD) and a glucometer (Accu-Chek® Active, Germany).

## Oral glucose tolerance test (OGTT)

Male Wistar normoglyceamic rats were subjected to overnight fasting for 16 h, followed by fasting glycaemia recording before extracts administration, which is called the ½ h glucose administration. Animals were then divided into 11 groups; each of which was made up of five rats (n=5). Group 1 taken as the negative control received the vehicle; Group 2 the positive control was treated with glibenclamide (10 mg/kg, b.w.), and Group 3 to 11 received extracts AE, EE, ME at 100, 200 and 400 mg/kg for each. Each rat was fed 2 g/kg of D(+)-Glucose monohydrate 30 min after
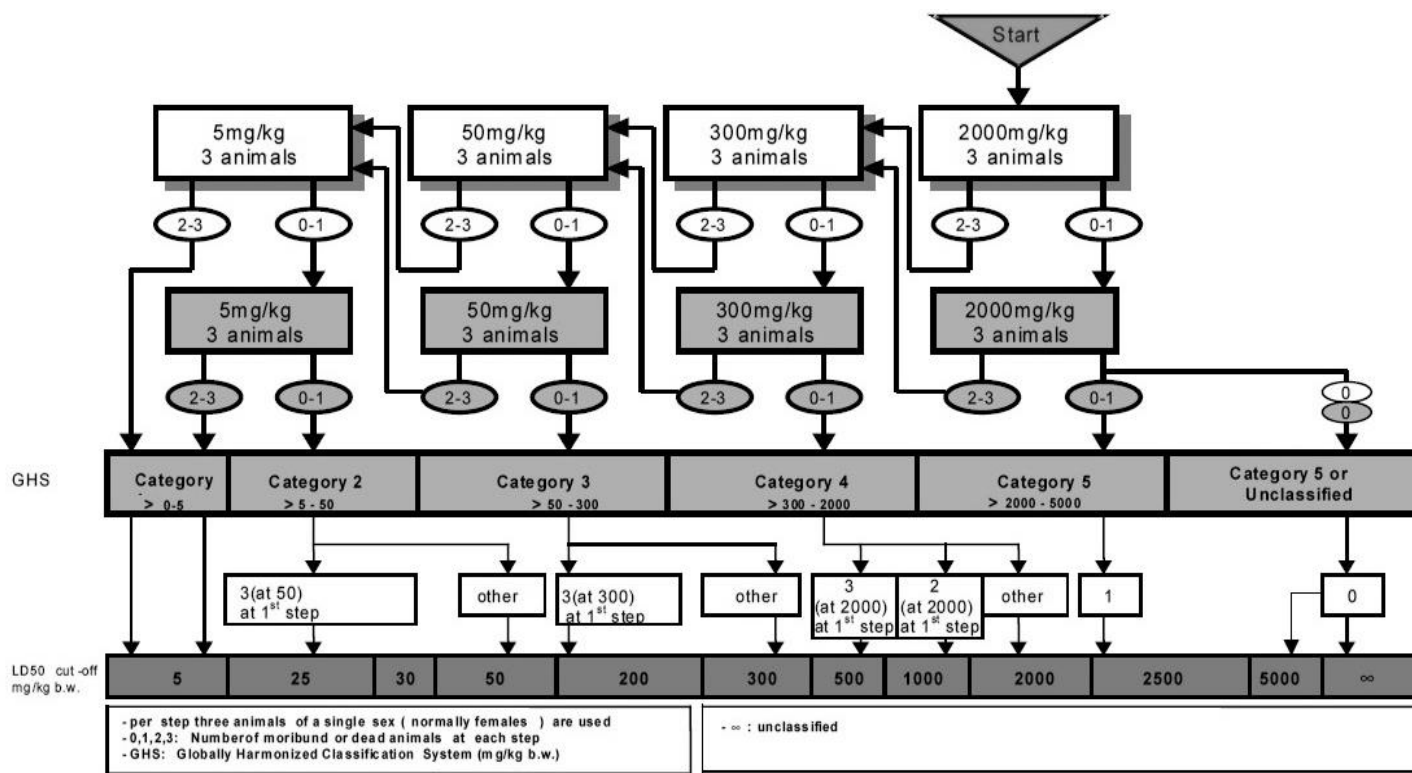
**Figure 1.** Acute oral toxicity evaluated by following modified OECD test guidelines (OECD, 2001).

administration of extract and was defined as initial time (t=0 h). Then, blood was collected from tail-tips at different time periods -½, 0, ½, 1, 2, 3 and 5 h consecutive to glucose administration. This was followed by glyceamia recording by use of a clinical glucometer (Accu-Chek Active®, Roche).

**Statistical analysis**

Results of bioassays were expressed as mean ± S.E.M using one way ANOVA, followed by the Newman-Keul test (GraphPad Prism, version 5.01) and differences were considered significant at $p \leq 0.05$. The decrease percentage of glyceamia for each group was calculated as follows:

$$Decrease\ (\%) = \frac{G_{1/2h} - G_x}{G_{1/2h}} \times 100$$

$G_{1/2h}$: Glyceamia value at ½ h, $G_x$: glyceamia at x hour; (x = 1, 2, 3 and 5).

**RESULTS**

**Phytochemical analysis**

**Yield of the crude extracts**

From the dried plant material, the yield of the crude extract AE was 20.9% (w/w), that of EE was 16.8% and

ME 18.6% (w/w).

**Phytochemical screening**

The phytochemical screening exhibited saponins, polyphenols, flavonoids, alkaloids, sterols/polyterpenoids and coumarins as the ingredients of the plant extract. Moreover, AE was identified to contain gallic tannins, whereas EE and ME contained catechic tanins. The foaming index for AE, EE and ME was respectively 33, 500 and 250.

**Determination of total polyphenol compounds**

The total polyphenols compounds identified by the Folin-Ciocalteu colorimetric method displayed absorbance values of 2707.3±155.4, 2952.7±166.0 and 1873.1±63.5 (GAE) µg/g, respectively for AE, EE and ME, using linear regression equation of gallic acid expressed as (GAE) of extract: $y = 0.0138x + 0.0651$, $r^2 = 0.8138$.

**Determination of total flavonoid compounds**

The spectrophotometric determination of total flavonoid compounds revealed a content of 0.543±0.007,
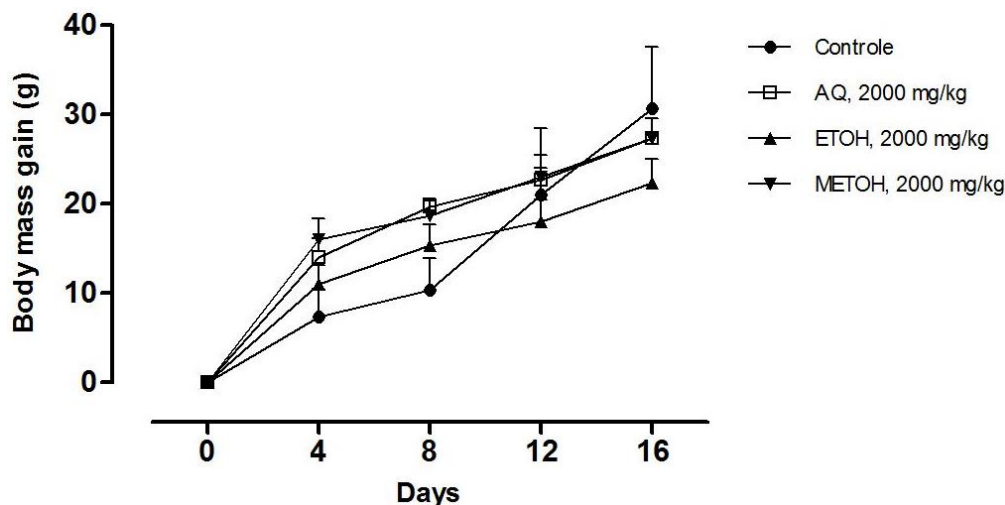
**Figure 2.** Effect on body mass gain (g) in normal healthy female rats treated with single acute oral dose of 2000 mg/kg AE, EE and ME of *P. americana* leaves. Values indicate mean±SEM (n = 3; OECD 423); ANOVA followed by Newman-Keuls, $p < 0.05$.

0.582±0.012 and 0.474±0.007% for AE, EE and ME, respectively.

## Acute toxicity determination

In this study, all *P. americana* leaf extracts did not show any toxic effects. Furthermore, neither lethality nor toxic reaction was found at a selected dose of 2000 mg/kg throughout the experiment. Mortality was also not observed when extract were used to treat the rats. With regards to the OEDC guideline, the $LD_{50}$ values offered by the extract of *P. americana* are higher than 5000 mg/kg. In addition, the gross necropsy test performed on all animals revealed neither hypertrophy, lesion, color change and appearance nor gross pathological impairment of organs at a glance. For further studies, concentrations were fixed at 100 (1/20th), 200 (1/10th) (Oliveira et al., 2008), and 400 mg/kg (1/5th) according to the study protocol.

## Changes in body weight

Throughout the 15 days observation of animals, body weight of each within a distinct group was considered for comparison. As a result, no significant difference was noticed between both control and treated groups (Figure 2). Nevertheless, at the end point, body weight gain significantly increased by 27.3% for groups fed AE and ME, and by 22.3% for groups fed with EE.

## Organ to body weight ratio

Table 1 shows the organ to body ratio of the different

groups of animals. From the data, there was no significant difference, except the liver to body weight ratio, which was higher in treated rats in comparison with the control group. Another important fact is that, the relative weights of kidney and liver were constant during the experiment.

## Biochemical analysis

Table 2 shows the serum content of electrolytic parameters ($Na^+$, $K^+$, $Cl^-$), renal and hepatic function markers (BUN, CREA, AST and ALT) in experimental rat groups as compared to the control. The biochemical parameters of animals treated with AE, EE and ME did not show significant change of level of BUN, CREA, $Na^+$, $K^+$ and $Cl^-$ in the serum even though increase of hepatic function marker levels was noticed in the serum. They appeared to be more pronounced (AST and ALT) for animals groups treated with AE and ME extract ($p < 0.01$).

## Acute hypoglycemic effect

Considering the normoglyceamic rats experiment, treatment with *P. americana* extracts at respective concentrations of 100, 200 and 400 mg/kg did not significantly reduce the blood glucose level as compared to that of the control group. Yet, glibenclamide brought about significant reduction of the blood glucose level by 40% (data not shown).

## Oral glucose tolerance test (OGTT)

Table 3 shows the blood glucose levels of experimental

Table  1. Organs to body weight ratio of the different groups of animals after 15 days of observation.

| Body weight and organs ratio (%) | Groups | | | |
|---|---|---|---|---|
| | Control | AE | EE | ME |
| Body weight (g) | 208 ± 13.50 | 182 ± 10.50 | 169 ± 14.50 | 182 ± 13.35 |
| Ratio heart/b.w.(%) | 0.39 ± 0.021 | 0.40 ± 0.037 | 0.39 ± 0.032 | 0.31 ± 0.020 |
| Ratio kidney/b.w.(%) | 0.67 ± 0.020 | 0.57 ± 0.052 | 0.68 ± 0.023 | 0.62 ± 0.038 |
| Ratio liver/b.w.(%) | 2.80 ± 0.111 | 3.45 ± 0.098 | 3.75 ± 0.113 | 3.53 ± 0.182 |

Control: Rats not treated; AE: rats treated with 2000 mg/kg of aqueous extract; EE: rats treated with 2000 mg/kg of ethanolic extract; ME: rats treated with 2000 mg/kg of methanolic extract. The values are expressed as mean ± S.E.M. (n = 3/group, OCED 423); ANOVA followed by Newman-Keuls, p < 0.05.

Table 2. Biochemical parameters of rats treated with acute oral dose of 2000 mg/kg of *P. americana* extracts after 15 days of observation.

| Biochemical parameters | Groups | | | |
|---|---|---|---|---|
| | Control | AE | EE | ME |
| BUN (mg/dl) | 20.0 ± 2.60 | 20.5 ± 1.70 | 25.0 ± 3.00 | 23.0 ± 2.60 |
| CREA (mg/dl) | 0.47 ± 0.03 | 0.50 ± 0. 06 | 0.57 ± 0. 03 | 0.43 ± 0. 03 |
| AST (U/L) | 151.67 ± 97.834 | 285.0 ± 53.799** | 224.7 ± 48.074 | 239.33 ± 159.001 |
| ALT (U/L) | 46.67 ± 5.457 | 121.3 ± 11.319 | 137.7 ± 27.865 | 185.0 ± 70.117** |
| AST/ALT | 3.25 ± 0.414 | 2.35 ± 0.398* | 1.63 ± 0.023* | 1.29 ± 0.161* |
| $Na^+$ (mM) | 139.3 ± 1.202 | 139.7 ± 0.333 | 141.0 ± 0.000 | 138.7 ± 0.882 |
| $K^+$ (mM) | 6.9 ± 0.312 | 7.0 ± 0.136 | 8.2 ± 0.704 | 7.0 ± 22.361 |
| $Cl^-$ (mM) | 104.3 ± 0.882 | 104.0 ± 1.155 | 102.3 ± 0.333 | 105.7 ± 1.333 |

BUN: Blood urea nitrogen; CREA: creatinine; AST: aspartate aminotransferase; ALT: alanine aminotransferase; $Na^+$: sodium ion; $K^+$: potassium ion; $Cl^-$: chloride ion. AE: rats treated with 2000 mg/kg of aqueous extract; EE: rats treated with 2000 mg/kg of ethanolic extract; ME: rats treated with, 2000 mg/kg of methanolic extract. The values are expressed as mean ± S.E.M. (n = 3/group, OCED 423). Significantly different from control group values: *: P < 0.05; **: P < 0.01.

rats before and after D-glucose (2.0 g/kg, b.w.) administration by oral route. It appeared that glibenclamide induced significant (p < 0.001) reduction of hyperglyceamia within a time period of ½ to 5h. At 5 h, the reduction rate was about 61.5%, whereas no significant decrease was observed in the control group. On the other side, AE induced significant (p < 0.01) glyceamia depletion within ½ h at a single dose of 100, 200 and 400 mg/kg, after D-glucose ingestion as compared to the control group and 100 mg/kg of AE brought the best reduction (5.6%). Furthermore, the EE (100 mg/kg) was responsible for a significant (p < 0.001) depletion in serum glucose level from ½ to 5 h. The reduction rates were 2.5, 36.8 and 59.6% after 1, 2 and 5 h, respectively. With regard to ME, it induced a significant decrease of hyperglyceamia between 1 and 3 h after glucose administration, except for the 200 mg/kg dose.

## DISCUSSION

*Persea americana* Mill. or avocado plant tree is well-known across both the south American and the sub-Saharan African continent (Adeboye et al., 1999; Ojewole et al., 2007). An ethnobotanical survey carried out in Côte d'Ivoire showed the use of the named plant to treat diabetes (N'guessan et al., 2009). Primarily, the present study led to a phytochemical investigation of three leaf extracts of the plant (aqueous, ethanolic and methanolic). Secondly, the study also compared the acute toxicity of the plant extracts aforementioned as well as their hypoglyceamic and anti-hyperglyceamic activities.

The chemical investigation of the crude extracts of *P. americana* revealed the presence of bioactive compounds, namely alkaloids, flavonoids, tanins, coumarine, sterols/polyterpenoids, saponins and polyphenols. These results were in accordance with those reported previously (Adeboye et al., 1999; Adeyemi et al., 2002). The amount of total flavonoids determined in a hydro-ethanolic leaf extract of *P. americana* by Lima et al. (2012) (0.730 ± 0.005%) was higher than the one obtained in the present study (0.474 - 0.582%). This may due to the solvent extraction method using the mixture water/ethanol.

From the acute toxicity evaluation performed according to the self-modified OCDE 423 protocol, on healthy

**Table 3.** Effect of *P. americana* extracts on fasting blood glucose level (mg/dl) after oral load of D-glucose (2.0 g/kg) in normoglyceamic rats.

| Groups | Dose mg/kg | Blood glucose concentration (mg/dl) ± S.E.M (% Decrease) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | -½ h (Initial) | 0 h | ½ h | 1 h | 2 h | 3 h | 5 h |
| Control | - | 63.8 ± 2.267 | 81.4 ± 2.064 | 140.8 ± 4.974 | 128.0 ± 5.206(9.1) | 92.0 ± 8.361(34.7) | 76.0 ± 2.408(46) | 59.6 ± 3.203(57.7) |
| Glibenclamide | 10 | 58.2 ± 3.056 | 58.0 ± 1.844*** | 101.0 ± 3.688** | 69.8 ± 2.035***(30.9) | 43.4 ± 2.482***(57) | 38.8 ± 2.035***(61.6) | 38.8 ± 2.596**(61.6) |
| Aqueous extract (AE) | 100 | 62.6 ± 3.341 | 74.8 ± 2.354 | 118.8 ± 3.056** | 112.2 ± 1.200*(5.6) | 81.6 ± 3.516(31.3) | 69.4 ± 0.980(41.6) | 60.6 ± 2.482(49.0) |
| | 200 | 63.4 ± 3.265 | 72.2 ± 2.800* | 117.2 ± 5.886* | 114.8 ± 2.871(2.0) | 96.6 ± 4.771(17.6) | 72.0 ± 3.098(38.6) | 63.4 ± 2.315(45.9) |
| | 400 | 55.6 ± 2.462 | 80.8 ± 2.083 | 119.4 ± 5.381** | 122.2 ± 5.911(-2.3) | 92.6 ± 9.563(22.4) | 79.6 ± 7.922(33.3) | 69.8 ± 7.513(41.5) |
| Ethanol extract (EE) | 100 | 64.8 ± 6.807 | 68.8 ± 3.513* | 105.4 ± 10.948** | 102.8 ± 5.463*(2.5) | 66.6 ± 4.118**(36.8) | 50.8 ±2.083***(51.8) | 42.6 ± 1.691***(59.6) |
| | 200 | 59.0 ± 2.408 | 75.0 ± 2.302 | 124.0 ± 4.290 | 125.8 ± 5.774(-1.5) | 97.8 ± 6.522(21.1) | 79.2 ± 3.513(36.1) | 68.4 ± 4.250(44.8) |
| | 400 | 59.2 ± 5.314 | 78.4 ± 4.675 | 78.4 ± 4.675* | 114.2 ± 5.911(-5.0) | 105.6 ± 2.542(2.9) | 86.4 ± 5.988(26.6) | 74.2 ± 2.478(31.8) |
| Methanol extract (ME) | 100 | 60.8 ± 4.684 | 71.8 ± 4.554 | 104 ± 9.891** | 82.8 ± 4.883***(21.0) | 67.0 ± 5.701*(36.1) | 58.6 ± 6.713*(44.1) | 53.2 ±7.677(49.2) |
| | 200 | 64.2 ± 3.826 | 75.2 ± 2.354 | 114.0 ± 6.641* | 94.4 ± 5.689***(17.2) | 76.0 ± 2.588(33.3) | 72.2 ± 3.555(36.7) | 63.2 ± 3.891(44.6) |
| | 400 | 58.0 ± 2.966 | 70.2 ± 1.655* | 122.8 ± 2.289* | 92.2 ± 3.121***(24.9) | 74.8 ± 5.398(39.1) | 56.6 ± 4.377*(53.9) | 46.0 ± 3.701(62.5) |

The values are expressed as mean ± S.E.M. (n = 5/group). Statistically different from control (ANOVA followed by Newman–Keuls, $p < 0.05$); *$P < 0.05$; **$P < 0.01$;*** $P < 0.001$; Values in parenthesis indicate the percentage of decrease calculated from the corresponding ½ h glyceamia value in each group.

female rats, the leaf extracts of *P. americana* were found to be well tolerated by animals at a unique oral route dose of 2000 mg/kg. Neither mortality nor compartmental trouble was observed. The protocol states that *P. americana* is part of the category 5 of the Global Harmonized classification System (GHS) with a $LD_{50}$ value greater or equal to 5000 mg/kg ($LD_{50} \geq 5000$ mg/kg), hence, non-toxic. Then, the obtained figures in this study best fit within the latter limit. Furthermore, gaining around 27g in weight by rat was observed after extracts uptake (Figure 2), even though there was no significant difference with the control group. The macroscopic autopsy of the vital organs (liver, heart and kidney) did not show any injury or color change, even though the ratio of organs to body weight index exhibited a slight increase of the treated rat's liver weight. This, according to literature, suggests that the leaf extracts used for the experiments did not show significant toxicity for the liver; consequently would do not exhibit any functional side effect on kidneys (Odo et al., 2014).

The biochemical analyses carried out however revealed a significant increase ($p < 0.01$) in the amount of either AST or ALT, respectively for group AE and ME as compared to the control group. In this study, the ratio AST/ALT, which fell respectively at 2.35 and 1.29 for groups EE and ME, indicates an abnormality as to the hepatic function of the liver; thus, confirming the outcomes of the study performed (Ezejiofor et al., 2013). On the other side, these results displayed the leaves of *P. americana* to be of lesser toxicity than that of the ripe fruit's seeds extracts. The latter parameter falls within the range of 1200.75 and 1767 mg/kg for the mice investigated (Padilla-Camberos et al., 2013; Pahua-Ramos et al., 2012). All in all, it could be stated that the unique dose of 2000 mg/kg affects in a short term, the liver's metabolism rather than that of the kidney, as confirmed by the biochemical factors (urea, creatinine and blood ionogram). However, chronical toxicity survey needs to be carried out by applying the unique dose of 2000 mg/kg to ascertain its tolerance in the long term. This could be done by assessment of the liver's synthesis function (albumin and triglycerids), cell integrity (transaminase), hepato-biliary channels permeability (alkalin phosphate and bilirubine) and its malignancy (prothrombine time period)

because the treatment of diabetes last for life. All in all, the fact that anti-hyperglyceamic effective doses are far from the $LD_{50}$ is encouraging.

The present study confirms the hypoglyceamic and antihyperglyceamic effect of the glibenclamide, which are mostly encountered with normal glyceamic rats. These effects are more pronounced than the extracts' doses administered in the course of our experiment. Previous investigation as related to hypoglyceamic and anti hyperglyceamic activity on normoglyceamic rats showed that if *P. amaricana* did not have any effect on basic glyceamia (fasting glyceamia), it would have significantly stopped the provoked hyperglyceamia. In fact, aqueous, ethanol and methanol extracts of *P. americana* exhibited an anti-hyperglyceamic activity of variable intensity and life span.

On the contrary, the three doses (100, 200 and 400 mg/kg) of the AE prevented prematurely postprandial or provoked hyperglyceamia after one hour of glucose administration. This suggests that the extracts were responsible for inhibited intestinal absorption of glucose. Consequently, the peak of hyperglyceamia was decreased in comparison with the control and glibenclamide. This phenomenon suggests that the AE could contain bioactive molecules capable of inhibiting α-amylase and α-glycosidase. Nevertheless, these results need further investigations such as absorption and inhibition mechanisms of glucose.

The EE and ME also extended the anti-hyperglyceamic activity at a low dose of 100 mg/kg after 3 to 5 h of glucose administration. Therefore, this activity seems not to be dose-dependent and could be correlated with the polyphenol's content and especially with that of flavonoids. EE having significant content of polyphenols and total flavonoids (2952.7 ± 166 µgGAE/g and 0.582%, respectively), as compared to the ME (1873.1 ± 63.5 µgGAE/g and 0.474%), brought about long lasting anti-hyperglyceamic activity. In fact, the inhibition of intestinal absorption of glucose has already been demonstrated earlier on (Tadera et al., 2006), and seems to be similar to that of hypoglyceamic sulfamids like glibenclamide.

However, extension of the anti-hyperglyceamic activity requires other mechanisms that involve the liver, the pancreas and measles. Mastery of the mechanisms mentioned, led to further studies that identified polyphenols as glucose metabolism regulators; using several mechanisms such as, protection and recovery of β cells' integrity, stimulation of insulin secretion and increase of glucose uptake by cells (Solayman et al., 2016; Vinayagam et al., 2016). Studies carried out previously, displayed an important anti-oxidizing role of polyphenols. Especially, flavonoids exhibited a protecting effect against toxicity and oxydating stress. On the other hand, saponines also demonstrated an anti-hyperglyceamic effect due to inhibition of glycogenesis (Elekofehinti, 2015; Ezejiofor et al., 2013). Finally, flavonoids, tannins and saponins also displayed

anti-hyperglyceamic properties through inhibition of the sodium-glucose transporter 1 (S-Glut 1) according to Tiwari and Rao (2002).

## Conclusion

The findings of the present study demonstrated the anti-hyperglyceamic potential of the leaf extracts (AE, EE an ME) of *P. americana* along with their lower toxicity. Consequently, it constitutes justification of the named plant as a medicinal herb. Additionally, diabetes mellitus is a chronic disease, further pharmacological investigations over a long period of time are needed to better assess plant's toxicity and elucidate mode of action.

## Conflict of interests

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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