

Article

Antidiarrheal Thymol Derivatives from *Ageratina glabrata*. Structure and Absolute Configuration of 10-Benzoyloxy-8,9-epoxy-6-hydroxythymol Isobutyrate

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Abstract: Chemical investigation of the leaves from *Ageratina glabrata* yielded four new thymol derivatives, namely: 10-Benzoyloxy-8,9-dehydro-6-hydroxythymol isobutyrate (**4**), 10-benzoyloxy-8,9-dehydro thymol (**5**), 10-benzoyloxythymol (**6**) and 10-benzoyloxy-6,8-dihydroxy-9-isobutyryloxythymol (**7**). In addition, (8S)-10-benzoyloxy-8,9-epoxy-6-hydroxythymol isobutyrate (**1**), together with other two already known thymol derivatives identified as: 10-benzoyloxy-8,9-epoxy-6-methoxythymol isobutyrate (**2**) and 10-benzoyloxy-8,9-epoxythymol isobutyrate (**3**). In this paper, we wish to publish the structures and complete assignments of ¹H and ¹³C NMR data of compounds **1** – **7**, and the absolute configuration for compound **1**, unambiguously established by single crystal X-ray diffraction, and evaluation of the Flack parameter. The in vitro antiprotozoal assay showed that compound **1** and its derivative **1a** were the most potent antiamebic and anti-giardial compounds. Both compounds showed selectivity and good antiamebic activity like emetine and metronidazole, respectively, two antiprotozoal drugs used as positive controls. In relation of anti-propulsive effect, compound **1** and **1a** showed moderate inhibitory activity, their activities were comparable to quercetin and compound **9**, respectively, two natural antiprotozoal compounds used as positive controls. These data suggest that compound **1** may play an important role in antidiarrheal properties of *Ageratina glabrata*.

Keywords: *Ageratina glabrata*; thymol derivatives; antiprotozoal and antidiarrheal properties

1. Introduction

Ageratina glabrata (Kunth) R.M. King & H. Rob., Asteraceae, is a shrub endemic to Mexico widely distributed along the country. The importance of *A. glabrata* in Mexican traditional medicine is given by its use for treating pain and gastrointestinal disorders associated with bacterial infections. However, chemical studies of the species are scarce. Previous phytochemical studies of *A. glabrata* show that less polar extracts are composed mainly by thymol and eudesmane derivatives [1,2,3] while the essential oil is constituted mainly by monoterpenes[4]. On the other hand,

evaluation for antibacterial activity of extracts of *A. glabrata* against antibiotic resistant pathogenic bacteria show that less polar extract is the most active[5]. Preliminary studies on analgesic effect of extracts of *A. glabrata* showed a moderate effect, in the hot plate test[6].

As part of our search for antiprotozoal and antidiarrheal compounds in *Ageratina* species, we have previously published our results on *A. cylindrica*[7,8]. The aim of this paper is to report on the isolation, structural characterization, and the antiprotozoal and antipropulsive evaluation of the thymol derivatives **1** - **4** and **7**. Compounds **4** - **7** are new thymol derivatives, while compounds **1** - **3**, have been previously reported from the same species, but their data were poorly described[3]. The chemical structures of the isolated compounds were established by spectroscopic methods, mainly MS and 1D, 2D NMR experiments (DEPT, COSY, NOESY, HSQC, HMBC), while the structure and absolute configuration of **1**, were confirmed by single crystal X-ray diffraction.

2. Results and Discussion

Detailed investigation of dichloromethane extract of the leaves of *A. glabrata* resulted in the isolation of seven thymol derivatives together with the known flavonoid pectolinarigenin[9] and benzoic acid.

Compound **1** (Figure 1) was isolated as colorless crystals, whose molecular composition was determined as $C_{21}H_{22}O_6$ on the basis of its HRDARTMS molecular ion at m/z 371.15003 $[M + H]^+$ (calculated for $C_{21}H_{22}O_6$ 371.14946), indicating nine degrees of unsaturation in the molecule. Its IR spectrum showed characteristic absorptions for hydroxyl (3599 cm^{-1}) and ester groups ($1757, 1723\text{ cm}^{-1}$).

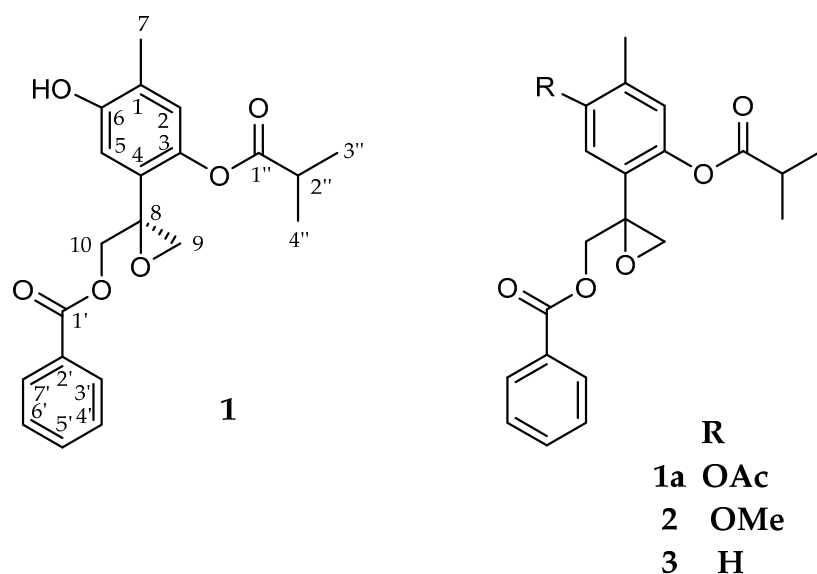


Figure 1. Chemical structure of **1** - **3**.

The ^{13}C NMR spectrum of **1** showed 19 resonances representing 21 carbon atoms due to three CH_3 , two CH_2 , eight CH groups (included two symmetric ones), and eight quaternary C atoms, according to DEPT and HSQC experiments. The 1H NMR spectrum exhibited in the aromatic region two one-proton signals as singlets at δ 6.79 and 6.94 assigned to H-2 and H-5, respectively, indicating a tetra-substitution of the thymol ring. Characteristic resonances in the aromatic region at δ 7.97 (2H), 7.55 (1H) and 7.40 (2H), indicated the presence of a benzoate moiety, while a heptet δ 2.83 (1H, $J = 7.2$ Hz) and two doublets at δ 1.312 and 1.307 (3H, $J = 7.2$ Hz) revealed the presence of an isobutyrate group in the molecule. Two AB spin systems with doublets at δ 3.12, 2.86 ($J = 5.2$ Hz) and 4.77, 4.48 ($J = 12.3$ Hz) due to C-9 and C-10 methylene protons supported the presence of the oxirane and the benzoate ester functionalities, respectively.

The relative position of the ester groups was established via correlations observed in the HMBC experiment, indicating that the benzoate was attached at C-10. Accordingly the C-10 methylene

protons at δ 4.77 and 4.48 showed a 3J correlation peak with the carbonyl carbon at δ 166.3, which in turn showed correlation peaks with the aromatic protons signal at δ 7.97 (H-3'/7'). Further long range couplings of the C-10 methylene protons with the quaternary carbon at δ 57.3 (C-8) and the methylene carbon at δ 51.3 (C-9), suggested the presence of the oxirane group at C8-C9. Therefore, the isobutyrate group must be attached at C-3. Acetylation of **1** with pyridine and acetic anhydride led to the acetate derivative **1a** (Figure 1). The ^1H and ^{13}C NMR spectra (Table 1) of **1a** displayed features similar to those of **1**, except for the presence of a sharp methyl singlet at δ 2.31 in the ^1H NMR spectrum, and two extra carbon resonances (δ 168.9 and 20.8) in the ^{13}C NMR spectrum, associated with the presence of the acetate group. Based on all above data, the structure of compound **1**, was established as 10-benzoyloxy-8,9-epoxy-6-hydroxy thymol isobutyrate.

Table 1. NMR Spectroscopic Data (^1H 400 MHz, ^{13}C 100 MHz, CDCl_3) of **1** and **1a**

Position	1			1a	
	δ_{C} , Type	δ_{H} (J in Hz)	HMBC	δ_{C} , Type	δ_{H} (J in Hz)
1	126.2, C			132.2, C	
2	124.8, CH	6.79, d (0.8)	1, 3, 4, 6, 7	125.0, CH	6.96, brs
3	141.7, C			146.2, C	
4	127.2, C			127.9, C	
5	114.8, CH	6.94, s	1, 3, 6, 8	122.7, CH	7.23, s
6	152.1, C			147.0, C	
7	15.8, CH_3	2.18, s	1, 2, 6	16.3, CH_3	2.17, s
8	57.3, C			56.9, C	
9a	51.3, CH_2	3.12, d (5.2)	4, 8, 10	51.2, CH_2	3.11, d (5.2)
9b		2.86, d (5.2)	4, 8, 10		2.87, d (5.2)
10a	65.6, CH_2	4.77, d (12.3)	1', 4, 8, 9	65.9, CH_2	4.76, d (12.2)
10b		4.48, d (12.3)	1', 4, 8, 9		4.47, d (12.2)
1'	166.3, C			166.1, C	
2'	129.7, C			129.0, C	
3'	129.8, CH	7.97, m	1', 5'	129.9, CH	7.97, m
4'	128.5, CH	7.40, m	3', 5', 7'	128.5, CH	7.42, m
5'	133.4, CH	7.55, tt (7.5, 1.3)	3', 7'	133.3, CH	7.55, tt (7.4, 1.2)
6'	128.5, CH	7.40, m	3', 5', 7'	128.5, CH	7.42, m
7'	129.8, CH	7.97, m	1', 5'	129.9, CH	7.97, m
1''	176.2, C			175.3, C	
2''	34.3, CH	2.83, hep (7.2)	1'', 3'', 4''	34.3, CH	2.84, hep (7.2)
3'', 4''	19.1, 19.2, CH_3	1.312, 1.307 d (7.2)	1'', 2''	19.0, 19.1, CH_3	1.31, 1.32, d (7.2)
6-OH		5.78, brs			
COCH_3				20.8, CH_3	2.31
C=OCH_3				168.9, C	

Concerning the absolute configuration, a single crystal of **1** was mounted on an X-ray diffractometer equipped with $\text{CuK}\alpha$ monochromated radiation and collected at 150 K, compound (**1**) crystallize with two molecules crystallographically independent, in the monoclinic system, space group P2(1). The structure was solved by direct methods using a full-matrix least-squares and refined to a discrepancy index of 4.23 %. The absolute configuration of **1** was determined using an anomalous dispersion effects in diffraction measurements on the crystal, the Flack parameter[10],

which for the enantiomer shown in Figure 2 was $x = -0.03(1)$, thus permitting confirmation of the proposed structure, and definition of the (8S) (Figure 2). In the structure the oxyrane ring is making a dihedral angle with the phenol group of $56.3(2)^\circ$ and $55.7(2)^\circ$ in both structure.

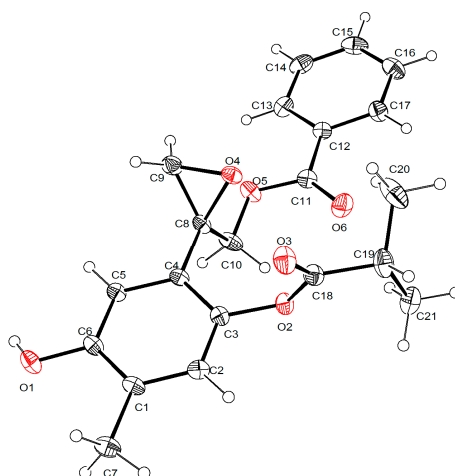


Figure 2. X-ray crystal structure of (+)-(8S)-10-benzoil-oxy-8,9-epoxy-6-hydroxy thymol isobutyrate (**1**).

It is necessary to point out that IR and ^1H NMR data of compound **1**, were similar to those published for eupaglabrin, a thymol derivative with an odd structure isolated from the same species [1]. Its structure was established based mainly on chemical degradation and low resolution 60 MHz ^1H NMR data. Years later, a compound with the structure **1** and similar spectroscopic data, was also published. Nonetheless, spectroscopic data were limited to incomplete ^1H NMR and some interchanged assignments[3]. In conclusion, the chemical structure and the absolute configuration of the thymol derivative previously reported from *A. glabrata*[1,3], must be represented by the stereoisomer **1**.

Compound **2** (Figure 1), was isolated in minute quantities, and identified as the methyl ether derivative of **1**. Its HRDARTMS showed a *pseudo*-molecular ion peak at m/z 385.16429 $[\text{M}+\text{H}]^+$, in agreement with a molecular formula $\text{C}_{22}\text{H}_{24}\text{O}_6$. (calculated for $\text{C}_{22}\text{H}_{25}\text{O}_6$, 385.16511). The NMR spectral data of compound **2** (Table 2) were similar to those of **1**, except for the presence of the methoxy group with signals at δ_{H} 3.82 and δ_{C} 55.9 in its ^1H and ^{13}C NMR spectra, respectively, in addition to the downfield shift of the C6 signal from 152.1 to 155.6 ppm, indicative of the methoxy group at that position. Thus, compound **2** was identified as the methyl ether derivative of compound **1**. Methylation of **1**, using iodomethane afforded compound **2**, spectroscopically identical with the isolated compound, allowing the complete NMR assignments.

Spectroscopic data indicated that compound **3** (Figure 1) correspond to the 6-deoxy derivative of **1**. Its molecular composition $\text{C}_{21}\text{H}_{22}\text{O}_5$ was deduced on the basis of its HRDARTMS *pseudo*-molecular ion peak at m/z 355.15516 $[\text{M}+\text{H}]^+$, (calculated for $\text{C}_{21}\text{H}_{23}\text{O}_5$, 355.15455), and ^{13}C NMR, DEPT, and HSQC experiments. The ^1H NMR spectrum (Table 2), showed in the aromatic region three one-proton resonances at δ (brd, $J = 0.7$ Hz), 7.43 (d, $J = 7.8$) and 7.07 (ddd, $J = 7.8, 1.6, 0.7$ Hz) assigned to H-2, H-5 and H-6 of the thymol moiety, respectively, indicating a tri-substitution pattern of the thymol ring. Careful analysis of the spectrum indicated long range $^1\text{H}/^1\text{H}$ couplings of H-2 and H-6 with the benzylic protons of the methyl group C-7 ($^3J = 1.6, ^4J = 0.7$ Hz). The presence of the benzoate and isobutyrate esters, were evident by the presence of characteristic benzoate signals ($\delta_{\text{H/C}}$ 7.98/129.7, 7.42/128.4 and 7.55/133.1), and the methine septet ($\delta_{\text{H/C}}$ 2.85/34.2), and methyl doublets ($\delta_{\text{H/C}}$ 1.32/18.9, 1.33/19.0) of the isobutyrate. The relative position of the ester moieties, was confirmed via HMBC 2J and 3J long range couplings.

Table 2. NMR Spectroscopic Data (¹H 400 MHz, ¹³C 100 MHz, CDCl₃) of **2** and **3**

Position	2		3	
	δ_C , Type	δ_H (J in Hz)	δ_C , Type	δ_H (J in Hz)
1	128.6, C		140.0, C	
2	124.6, CH	6.83, brs	123.1, CH	6.88, brd (0.7)
3	141.6, C		148.7, C	
4	126.9, C		126.1, C	
5	109.9, CH	6.97, s	128.9, CH	7.43, d (7.8)
6	155.6, C		126.8, CH	7.07, ddd (7.8, 1.6, 0.7)
7	16.2, CH ₃	2.19, brd (0.4)	21.1, CH ₃	2.35, s
8	57.3, C		57.0, C	
9a	51.3, CH ₂	3.13, d (5.3)	50.9, CH ₂	3.12, d (5.3)
9b		2.87, d (5.3)		2.85, d (5.3)
10a	65.8, CH ₂	4.79, d (12.2)	65.9, CH ₂	4.76, d (12.2)
10b		4.48, d (12.2)		4.47, d (12.2)
1'	166.1, C		165.9, C	
2'	129.9, C		129.72, C	
3'	129.8, CH	7.99, m	129.67, CH	7.98, m
4'	128.5, CH	7.43, m	128.4, CH	7.42, m
5'	133.3, CH	7.55, m	133.1, CH	7.55, tt (7.6, 1.3)
6'	128.5, CH	7.42, m	128.4, CH	7.42, m
7'	129.8, CH	7.99, m	129.67, CH	7.98, m
1''	175.9, C		175.3, C	
2''	34.3, CH	2.83, hep (7.2)	34.2, CH	2.85, hep (7.0)
3'', 4''	19.1, 19.2, CH ₃	1.31, 1.32 d(7.2)	18.9, 19.0, CH ₃	1.32, 1.33, d(7.0)
OCH ₃	55.9, CH ₃	3.82, s		

Compound **4** (Figure 3) was isolated as colorless oil, whose molecular formula C₂₁H₂₂O₅ was established according with its HRDARTMS, which shows an [M+H]⁺ exact mass peak at *m/z* 355.15360 (calculated for C₂₁H₂₃O₅, 355.15455). The ¹H NMR of compound **4** (Table 3) displayed characteristic signals for the benzoate ($\delta_{H/C}$ 8.03/130.2, 7.43/128.6 and 7.55/133.2) and the isobutyrate ($\delta_{H/C}$ 2.75/34.2, 1.27/19.1) esters. Two one-proton aromatic signals, which appeared as broadened singlets, at δ 6.81, 6.74, suggested a tetra-substituted thymol ring. The presence of two vinylic proton signals which appear as apparent quartets at δ 5.48 (*J* = 1.6 Hz, H-9a) and 5.25 (*J* = 1.2 Hz, H-9b), indicated that the common epoxy group at C8-C9 in compounds **1-3**, was replaced by an olefinic terminal methylene. The observed small *J* values, are due to allylic couplings with the methylene protons at C-10, which appeared as a broadened doublet of doublets at δ 5.0 (*J* = 1.6, 1.2 Hz). The above data, allowed the identification of compound **4** as 10-benzoyloxy-8,9-dehydro-6-hydroxythymol isobutyrate (**4**), which could be considered as the precursor of compound **1**.

A compound with the same structure **4**, was reported isolated from *A. glabrata* by Bohlman *et al.* (1977), but the limited published ¹H NMR data completely differ from those of compound **4**, and are in disagreement with the structure.

The described compounds **1 – 3**, have been published as isolated before from *A. glabrata*[1,3,11], but as mentioned before, only limited NMR data were available. In this paper the structure and absolute configuration of compound **1**, have been unambiguously established by NMR and single

crystal X-ray diffraction, as well as, the complete assignment of the ^1H and ^{13}C NMR data for compounds 1 – 3.

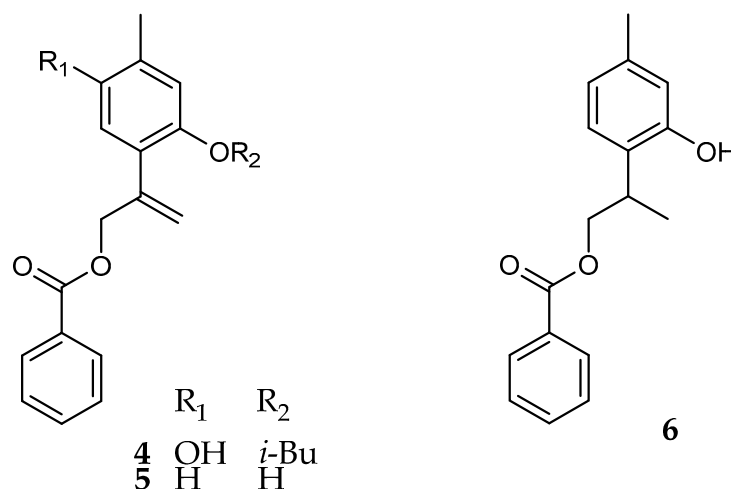


Figure 3. Chemical structures of 4 – 6.

Compound 5 and 6 (Figure 3) are new natural thymol derivatives; they were obtained in minute amounts after successive chromatography columns and purified by thin layer chromatography. The ^1H NMR spectrum of 5 (Table 3) displayed similar features than those of compound 4, except for the lack of signals ascribed to the isobutyrate moiety and the -OH group at C-6. The presence of an olefinic terminal methylene was evident from the two one-proton signals at δ 5.56 (q, $J = 1.6$, H-9a) and 5.31 (q, $J = 1.2$, H-9b), both coupled with the allylic methylene protons at C-10. Thus, the structure of compound 5 was established as 10-benzoyloxy-8,9-dehydrothymol (5).

Compound 6, was identified as the dihydroderivative of 5. Therefore, the ^1H NMR spectrum (Table 3), did not show the olefinic methylene signals, instead of that, the spectrum displayed a methyl doublet signal at δ 1.42 (d, $J = 6.8$ Hz, 3H) as the part X_3 , of the spin system ABMX_3 , due to H₂-10, H-8 and H₃-9 protons. One methine proton signal at δ 3.50 appeared as a doublet of quartets of doublets ($J = 8.0, 6.8, 4.8$, Hz, 1H), coupled with the methyl doublet and the methylene protons H₂-10, according with the HSQC and HMBC experiments. The protons of the C-10 methylene bearing the benzoyloxy group appeared as the AB part of the spin system with signals at δ 4.19 (dd, $J = 10.8, 8.0$ Hz) and 4.53 (dd, $J = 10.8, 4.8$ Hz). Consequently, the structure of compound 6, was established as the 10-benzoyloxy thymol (6), and represents a new natural thymol derivative.

Compound 7 was obtained as colorless oil, whose ^1H and ^{13}C NMR spectra (Table 4) displayed similar spectral features as those of compound 1. Two one-proton aromatic singlets at δ 6.66 and 6.60 indicating the tetra-substitution of the thymol ring. The presence of a benzoate and an isobutyrate was evident from their characteristic signals ($\delta_{\text{H/C}}$ 7.98/129.9, 7.41/128.7, 7.55/133.6), for the benzoate and ($\delta_{\text{H/C}}$ 2.53/34.1, 1.05, 1.08/18.9, 19.0) due to the isobutyrate. The main differences between 1 and 7 were the multiplicity and chemical shifts of signals due to the methylene groups bearing the oxygen functionalities. The ^1H NMR spectrum displayed two AB spin systems with signals centered at δ_{H} 4.51, 4.58 (d, $J = 11.6$, C-9), and 4.64, 4.68 (d, $J = 12.0$, C-10), coupled with the methylene carbon signals at δ_{C} 67.5 and 68.1, according with the HSQC spectrum, respectively. In the HMBC experiment, the proton signals of both AB methylene groups, showed 2J and 3J long range couplings with the tertiary carbon signals at δ_{C} 78.3 (C-8) and 120.3 (C-4). On the other hand the AB system centered at δ 4.55 showed to be coupled with the carbonyl at δ 177.9, which in turn, is coupled with the methine heptet and methyl doublets of the isobutyrate ($\delta_{\text{H/C}}$ 2.53/34.1, 1.05, 1.08/19.0), while the one, centered at δ 4.66 showed couplings with the carbonyl at δ 167.1, that in turn is, coupled with the benzoate protons H-3'/7' ($\delta_{\text{H/C}}$ 7.98/129.9). Thus, the isobutyrate is attached to C-9, and the benzoate at C-10. According with the above data, the structure of compound 7 was established as 10-benzoyloxy-6,8-dihydroxy-9-isobutyryloxythymol (7).

Table 3. NMR Spectroscopic Data (^1H 400 MHz, ^{13}C 100 MHz, CDCl_3) of 4 – 6

Position	4		5		6	
	δ_{C} , Type	δ_{H} (J in Hz)	δ_{C} , Type	δ_{H} (J in Hz)	δ_{C} , Type	δ_{H} (J in Hz)
1	125.0, C		140.2, C		138.0, C	
2	124.9, CH	6.80, brs	116.9, CH	6.78, d (0.7)	116.8, CH	6.68, d (0.8)
3	141.4, C		153.5, C		154.0, C	
4	130.9, C		122.6, C		125.3, C	
5	115.9, CH	6.74, s	129.3, CH	7.03, d (7.7)	127.2, CH	7.10, d (7.8)
6	151.6, C		121.2, CH	6.71, ddd (7.7, 1.7, 0.7)	121.6, C	6.74, ddd (7.6, 0.8, 0.4)
7	15.8, CH_3	2.22, brs	21.4, CH_3	2.32, s	21.1, CH_3	2.28, s
8	140.2, C	-	141.9, C		32.4, C	3.50, dqd (8.0, 6.8, 4.8)
9a	117.3, CH_2	5.48, q (1.6)	116.7, CH_2	5.56, q (1.6)	16.7, CH_3	1.42, d (6.8)
9b		5.25, q (1.2)		5.31, q (1.2)		
10a	66.7, CH_2	5.0, dd (1.6, 1.2)	66.6, CH_2	4.99, dd (1.6, 1.2)	70.2, CH_2	4.53, d (10.9, 4.8)
10b						4.19 dd (10.9, 8.0)
1'	166.3, C		167.2, C		167.5, C	
2'	130.2, C		129.8, C		130.3, C	
3'	129.8, CH	8.03, m	129.9, CH	8.09, m	129.8, CH	8.04, m
4'	128.6, CH	7.43, m	128.7, CH	7.47, m	128.5, CH	7.56, tt (7.2, 1.6)
5'	133.2, CH	7.55, tt (7.4, 1.4)	133.6, CH	7.60, tt (7.2, 1.3)	133.2, CH	7.44, m
6'	128.6, CH	7.43, m	128.7, CH	7.47, m	128.5, CH	7.56, tt (7.2, 1.6)
7'	129.8, CH	8.03, m	129.9, CH	8.09, m	129.8, CH	8.04, m
1''	176.1, C					
2''	34.2, CH	2.75, hep (7.0)				
3'', 4''	19.1, CH_3	1.27, d (7.0)				

The HRDARTMS of **7**, did not show the $\text{C}_{21}\text{H}_{25}\text{O}_7$ $[\text{M}+\text{H}]^+$ ion at m/z 389, instead of that, it displayed the $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$, ion peak at m/z 371.14833 in agreement with a molecular formula $\text{C}_{21}\text{H}_{23}\text{O}_6$ (calculated 371.14946), due to the loss of water from the molecular ion.

It is documented that some of these compounds are artifacts originated by the hydrolytic opening of oxirane-ring followed by trans-esterification from O-3 to O-9 during the isolation process, or after purification[12,13]. In case of compound **7**, it is indeed an artifact, since it was obtained from compound **1**, after storing at room temperature for some time.

Recently a thymol derivative isolated from *A. glabrata* has been described[14]. Its proposed structure **8**, was established on the basis of MS, ^1H and ^{13}C NMR data. Comparison of the published data with those of compound **7**, showed to be identical. Therefore, the structure **8** must be revised, and changed to the structure **7**.

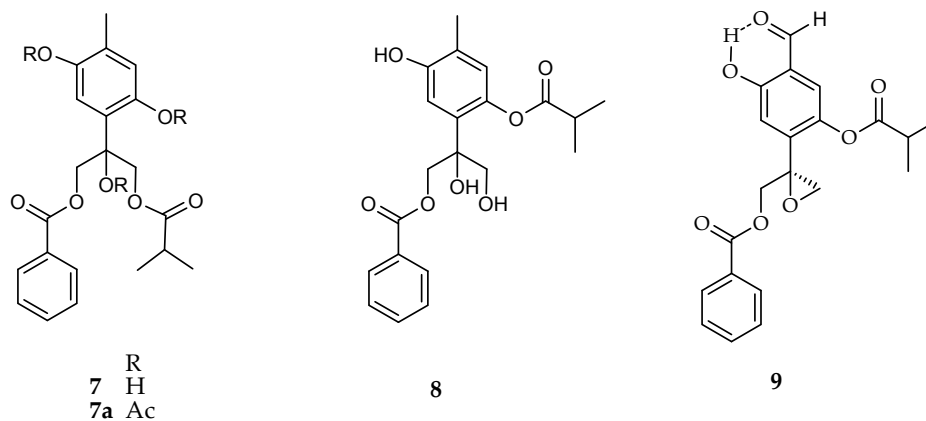


Figure 4. Chemical structures of 7 – 9.

Table 4. NMR Spectroscopic Data (^1H 400 MHz, ^{13}C 100 MHz, CDCl_3) of 7 and 7a

Position	7			7a	
	δ_{C} , Type	δ_{H} (J in Hz)	HMBC	δ_{C} , Type	δ_{H} (J in Hz)
1	126.2, C			132.3, C	
2	120.2, CH	6.66, brs	3, 4, 6, 7	126.7, CH	6.96, d (0.7)
3	149.8, C			145.3, C	
4	120.3, C			127.7, C	
5	113.0, CH	6.60, s	1, 3, 6, 8	121.8, CH	7.16, s
6	147.2, C			147.1, C	
7	15.7, CH_3	2.17, brs	1, 2	16.1, CH_3	1.63, brs
8	78.3, C			80.8, C	
9a	67.5, CH_2	4.58, d(11.9)	4, 8, 10, 1''	62.9, CH_2	4.96, d (11.4)
9b		4.51, d(11.9)	4, 8, 10, 1''		4.81, d (11.4)
10a	68.1, CH_2	4.68 d(12.0)	1', 4, 8, 9	63.6, CH_2	5.12, d (11.4)
10b		4.65, d(12.0)	1', 4, 8, 9		4.91, d (11.4)
1'	167.0, C			165.8, C	
2'	129.1, C			129.6, C	
3'	129.9, CH	7.98, m	1', 5', 7'	129.8, CH	7.95, m
4'	128.7, CH	7.41, m	3', 5', 7'	128.6, CH	7.43, m
5'	133.6, CH	7.55, tt (7.4, 1.3)	3', 7'	133.4, CH	7.55, tt (7.5, 1.3)
6'	128.7, CH	7.41, m	3', 5', 7'	128.6, CH	7.43, m
7'	129.9, CH	7.98, m	1', 5', 7'	129.8, CH	7.95, m
1''	177.9, C			176.3, C	
2''	34.1, CH	2.53, hep (7.0)	1'', 3'', 4''	34.0, CH	2.52, hep (7.0)
3'', 4''	18.9, 19.0, CH_3	1.05, 1.08, d (7.0)	1'', 2''	18.8, 18.9, CH_3	1.08, 1.10, d (7.0)
3-OCO CH_3				21.3, 169.4, CH_3 , CO	2.38, s
6-OCO CH_3				20.9, 168.8, CH_3 , CO	2.31, s
8-OCO CH_3				21.4, 168.8, CH_3 , CO	2.00, s

Acetylation of **7** with pyridine and acetic anhydride yielded compound **7a**. The ^1H and ^{13}C NMR spectra (Table 4) of **7a** displayed features similar to those of **7**, except for the presence of three sharp methyl singlet at δ 2.00, 2.31, 2.38 in the ^1H NMR spectrum, which showed couplings with the methyl carbon signals at δ 21.4, 20.9 and 21.3 in the HSQC experiment, and with the carbonyl carbons at 168.8, 168.8 and 169.4, in the HMBC experiment, respectively. In addition, downfield shifts of the aromatic protons H-2, H-5 and the methylene protons of CH₂-9 and CH₂-10, inherent to the presence of the acetyl groups, were observed.

Table 5. In vitro Antiprotozoal Activity and Inhibition of Hyperperistalsis of Thymol derivatives

Compound	IC ₅₀ μM (CI) ^a		ID ₅₀ $\mu\text{mol/kg} \pm \text{SD}$ ^b
	<i>Entamoeba histolytica</i>	<i>Giardia lamblia</i>	Inhibition of hyperperistalsis
1	1.6 (1.8-1.57)	36.9 (38.3-5.9)*	2.000 \pm 0.003**
1a	0.84 (0.87-0.80)	24.2 (24.7-24.0)*	0.810 \pm 0.021**
2	169.6 (171.3-168.9)*	191.2 (192.1-190.8)*	0.457 \pm 0.004**
3	25.9 (26.2-25.7)*	48.3 (48.4-45.4)*	0.740 \pm 0.003**
4	61.2 (62.3-59.8)*	68.0 (69.7-67.4)*	1.430 \pm 0.006**
7	45.6 (46.9-44.3)*	60.7 (62.3-56.9)*	0.380 \pm 0.003**
pectolinarigenin	43.6 (44.9-41.9)*	68.7 (70.3-67.4)*	0.598 \pm 0.001**
9^c	184.9 (186.9-180.7)*	167.4 (168.7-165.8)*	0.85 \pm 0.005
Emetine ^d	2.18 (2.2-2.14)	0.83 (0.87-0.82)	
Metronidazole ^d	0.23 (0.58-0.17)	1.22 (1.57-0.81)	
Loperamide hydrochloride ^d	-	-	0.2 \pm 0.001

^a) Results are expressed as mean (n = 6), CI = 95% confidence intervals; *P < 0.05 compared to emetine and metronidazole. ^b) Results are expressed as mean (n = 6) \pm SD; **P < 0.05 compared to loperamide hydrochloride. ^c) See ref 7; ^d) Positive controls.

Compounds **1** – **4**, **7** and **1a** were investigated for antiprotozoal activity (Table 5) against *Entamoeba histolytica* and *Giardia lamblia*. Compound **1** and its derivative **1a** showed selectivity and good antiprotozoal activity on *Entamoeba histolytica* trophozoites, their effects were similar to emetine and metronidazole, two antiprotozoal drugs used as controls, respectively. In contrast, to the case of *Giardia lamblia* both compounds showed moderate anti-giardial activity. The remaining compounds **2** – **4**, **7**, and pectolinarigenin showed moderate antiprotozoal activity on both protozoa.

Compounds **1** – **4**, **7** and **1a** were also tested on the charcoal-gum acacia-induced hyperperistalsis model in rats. Compounds **1** and **1a** showed moderate inhibitory activity on hyperpropulsive movement of the small intestine in rats; their activities were comparable to quercetin and compound **9**, but no exceeded that of loperamide hydrochloride (Table 5) antidiarrheal drug used as positive control. In addition, the remaining compounds **2** – **4**, **7**, and pectolinarigenin showed moderate inhibitory activity. It is important to point out that the presence of an acetate group at the C (6) position in thymol derivative **1a** seems to be important for the inhibition of hyperperistalsis and antiprotozoal activity. Finally, the antidiarrheic properties reputed for *Ageratina glabrata* in Mexican traditional medicine may be due to the presence of thymol derivatives **1** – **4**, **7** and of flavonoid, pectolinarigenin.

4. Materials and Methods

4.1. General Experimental Procedures

Melting points were measured on a Fisher-Johns apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 323 polarimeter. Ultraviolet absorptions were recorded on a Shimadzu UV 160U spectrophotometer. IR spectra were obtained on a Bruker Tensor 27 spectrometer. The 1D and 2D NMR experiments were performed on a Bruker Advance III spectrometer at 400 MHz for ¹H and 100 MHz for ¹³C. Chemical shifts were referenced to TMS and J values are given in Hz. The HRDARTMS data were recorded on a Jeol AccuTOF JMS-T100LC mass spectrometer. Prep TLC was carried out on precoated Macherey Nagel Sil G/UV254 plates of 1.0 mm thickness. Silica gel 230-400 mesh (Macherey-Nagel), Sephadex LH-20 (Pharmacia Biotech) and octadecyl functionalized silica gel (Sigma Aldrich) were used for column chromatography. The X-ray data were collected on a Bruker D8 Venture κ -geometry diffractometer.

4.2. Plant Material

Ageratina glabrata was collected at Cuernavaca, Morelos, Mexico, in February 2015. Plant material was identified by Dr. Laura Calvillo Canadell, and a voucher specimen (MEXU-1 333 471) was deposited at the National Herbarium (MEXU) of the Instituto de Biología, UNAM.

4.3. Extraction, Isolation and Characterization

The air-dried and powdered leaves of *A. glabrata* (690g) were extracted CH₂Cl₂ (2 L x 3) at room temperature for 48 h. The extract was concentrated at reduced pressure to yield 18 g of residue. The crude extract was subjected to CC on silica gel using gradient elution with EtOAc/hexanes (80 : 20) to obtain 29 eluates, 150 mL each, which were combined in 17 major fractions (A – Q) by TLC evaluation. Pectolinarigenin (50.8 mg) crystallized from fraction N. Fraction B (2.78 g) was subjected to CC on silica gel using gradient elution with EtOAc/hexanes (90 : 10) to obtain 55 eluates, 150 mL each, which were combined in 25 major fractions (BA – BX). Compound **1** (450 mg) crystallized from fractions BG to BJ. Fraction BD (725 mg) was further fractionated over silica gel using dichloromethane as eluent to obtain 6 major fractions (BDA-BDF), fraction BDD (64.5 mg) was subjected to silica gel TLC eluting with Hexanes: EtOAc (70 : 30) to give compound **2** (17.9 mg). Fraction BG (261.6 mg) was subjected to silica gel CC eluting with dichloromethane : acetone (95 : 5) to give 6 major fractions (BGA-BGF). Fraction BGB was subjected to silica gel TLC eluting with dichloromethane : acetone (99 : 1) to give compound **3** (4.8 mg). Fraction C (120 mg) was separated by CC on silica gel eluted with dichloromethane : acetone (0:100 – 100:0) to obtain seven fractions (CA-CG). Compound **5** was identified in fraction CA. Fraction B was subjected to silica gel TLC eluting with dichloromethane to obtain compound **6**. Pure **4** (13.3 mg) was obtained from fraction CE. Fraction G (889 mg) was subfractionated over a Sephadex LH-20 column, using MeOH as eluent to give five fractions (GA – GE). Benzyl benzoate crystallized from fraction GA. Ilicic acid was obtained as pure compound from fraction GB. Fraction GC was subjected to silica gel CC using CH₂Cl₂ as eluent to obtain seven fractions (GCA-GCG). Pure compound **7** was obtained from fraction GCD.

(8S)-10-benzoiloxy-8,9-epoxy-6-hydroxythymol isobutyrate, **1**: Colorless crystals (CH₂Cl₂-hexane); mp 110-112 °C; [α]₅₈₉ +14.5 (c 0.001, MeOH); UV (MeOH) λ_{\max} (log ϵ) 204 (4.71), 225 (4.52), 280 (3.30) nm; IR (CHCl₃) ν_{\max} 3599, 2980, 1752, 1722, 1273 cm⁻¹; ¹H and ¹³C NMR (CDCl₃) see Table 1; HRDARTMS *m/z* 371.15003 (calculated for C₂₁H₂₂O₆+H, 471.14946).

10-benzoiloxy-8,9-epoxy-6-acetyloxythymol isobutyrate, **1a**: Colorless oil; [α]₅₈₉ +14.7 (c 0.002, MeOH); UV (MeOH) λ_{\max} (log ϵ) 203 (4.47), 252 (4.52), 273 (4.51) nm; IR (CHCl₃) ν_{\max} 2979, 2937, 1757, 1721, 1270 cm⁻¹; ¹H and ¹³C NMR (CDCl₃) see Table 1; HRDARTMS *m/z* 413.16040 (calculated for C₂₃H₂₄O₇+H, 413.16003).

10-benzoiloxo-8,9-epoxy-6-methoxythymol isobutyrate, **2**: Colorless oil; $[\alpha]_{589} +14.6$ (c 0.0015, CHCl₃); UV (MeOH) λ_{\max} (log ϵ) 204 (4.54), 225 (4.34), 276 (3.70) nm; IR (CHCl₃) ν_{\max} 2979, 2938, 1751, 1722, 1272 cm⁻¹; ¹H and ¹³C NMR (CDCl₃) see Table 2; HRDARTMS m/z 385.16429 (calculated for C₂₂H₂₄O₆+H, 385.16511).

10-benzoiloxo-8,9-epoxythymol isobutyrate, **3**: Colorless oil; $[\alpha]_{589} -3.9$ (c 0.002, CHCl₃); UV (MeOH) λ_{\max} (log ϵ) 203 (4.27), 221 (4.16), nm; IR (CHCl₃) ν_{\max} 2976, 2938, 1757, 1723, 1271 cm⁻¹; ¹H and ¹³C NMR (CDCl₃) see Table 2; HRDARTMS m/z 355.15516 (calculated for C₂₁H₂₂O₅+H, 355.15455).

10-Benzoyloxy-8,9-dehydro-6-hydroxythymol isobutyrate, **4**: Colorless oil; UV (MeOH) λ_{\max} (log ϵ) 204 (4.31), 225 (4.23), 276 (3.58) nm; IR (CHCl₃) ν_{\max} 3522, 2953, 2927, 1757, 1713, 1291 cm⁻¹; ¹H and ¹³C NMR (CDCl₃) see Table 3; HRDARTMS m/z 355.15360 (calculated for C₂₁H₂₂O₅+H, 355.15455).

10-Benzoyloxy-8,9-dehydrothymol isobutyrate, **5**: Colorless oil; ¹H and ¹³C NMR (CDCl₃) see Table 3.

10-benzoyloxythymol isobutyrate, **6**: white powder; mp 94-97 °C $[\alpha]_{589} +12.27$ (c 0.002, CHCl₃); UV (MeOH) λ_{\max} (log ϵ) 205 (3.87), 224 (3.80), 275 (3.21) nm; IR (CHCl₃) ν_{\max} 3599, 3400, 2970, 2928, 1752, 1714, 1279 cm⁻¹; ¹H and ¹³C NMR (CDCl₃) see Table 3.

10-benzoyloxy-6,8-dihydroxy-9-isobutyryloxythymol, **7**: Colorless oil; $[\alpha]_{589} 0.00$ (c 0.001, CHCl₃); UV (MeOH) λ_{\max} (log ϵ) 205 (4.96), 228 (4.89), 293 (4.40) nm; IR (CHCl₃) ν_{\max} 3603, 3398, 2979, 2936, 1724, 1291 cm⁻¹; ¹H and ¹³C NMR (CDCl₃) see Table 4; HRDARTMS m/z 371.14933 [M-H₂O]⁺ (calculated for C₂₁H₂₃O₆, 371.149646). ESIMS m/z 411.

10-benzoyloxy-6,8-diacetyloxy-9-isobutyryloxythymol acetate, **7a**: Colorless oil; $[\alpha]_{589} + 3.600$ (c 0.001, CHCl₃); UV (MeOH) λ_{\max} (log ϵ) 203 (6.32), 223 (6.16), 274 (5.27) nm; IR (CHCl₃) ν_{\max} 2978, 2936, 1759, 1370, 1271 cm⁻¹; ¹H and ¹³C NMR (CDCl₃) see Table 4; DARTMS m/z 546 [M-C₄H₈O₂]⁺.

4.4. Antiprotozoal Assays

Entamoeba histolytica strain HM1-IMSS used in all experiments was grown axenically at 37°C in TYI-S-33 medium supplemented with 10% heat inactivated bovine serum. In the case of *Giardia lamblia*, strain IMSS: 8909:1 was grown in TYI-S-33 modified medium supplemented with 10% calf serum and bovine bile. The trophozoites were axenically maintained and for assays were employed in the log phase of growth. In vitro susceptibility tests were performed using a subculture method previously described [15]. Briefly, *E. histolytica* (6 × 10³) or *G. lamblia* (5 × 10⁴) trophozoites were incubated for 48 h at 37 °C in the presence of different concentrations (2.5 – 200 µg/mL) of the crude extract or pure compounds in dimethyl sulfoxide (DMSO). Each test included metronidazole (Sigma) as standard amoebicidal and giardicidal drugs, a control (culture medium plus trophozoites and DMSO), and a blank (culture medium). After incubation, the trophozoites were detached by chilling and 50 µL samples of each tube were subcultured in fresh medium for another 48 h, without antiprotozoal samples. The final number of parasites was determined with a haemocytometer and the percentages of trophozoites growth inhibition were calculated by comparison with the control culture. The results were confirmed by a colorimetric method: the trophozoites, were washed and incubated for 45 min at 37 °C in phosphate buffer saline with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and phenazine methosulfate. The dye produced (formazan) was extracted and the absorbance was determined at 570 nm. The experiments were performed in duplicate for each protozoan and repeated at least three times. The *in vitro* results were classified as follows: if the samples displayed an IC₅₀ less than 20 µM, the antiprotozoal activity was considered good, from 21 to 160 µM the antiprotozoal activity was considered moderate, from 161 to 200 µM the antiprotozoal activity was considered weak and over 200 µM/mL the samples were considered inactive. Data were analyzed using probit analysis. The percentage of trophozoites surviving was calculated by comparison with the growth in the control group. The plot of probit against log concentration was made; the best straight line was determined by regression analysis and the 50%

inhibitory concentration (IC₅₀) values were calculated. The regression coefficient, its level of significance ($P < 0.05$ indicates significant difference between group) and correlation coefficient were calculated and 95% CI values determined.

4.5. Animals

Male Sprague–Dawley rats (200–250 g) were obtained from the animal house of the IMSS. These studies were conducted with the approval of the Specialty Hospital Bio-Ethical Committee of the National Medical Center “Siglo XXI” from IMSS (Approval No: R-2012-3601-182). Investigation using experimental animals was conducted in accordance with the official Mexican norm NOM 0062-ZOO-1999 entitled Technical specifications for the production, care and use of laboratory animals[16]. They were fasted overnight but tap water was available ad libitum until the start of the experiments.

4.6. Effect on Charcoal–Gum Acacia-Induced Hyperperistalsis

The method, described by Williamson *et. al.*[17] was adopted to study the effect of the compounds on hyperperistalsis in rats. The test animals were divided into control group and test groups containing six rats in each group. Rats were treated orally with each compounds [0.01, 0.1, 1.0, 10, 20, 40 mg/kg in 1 mL of a 2% dimethyl sulfoxide (DMSO) solution in water], or vehicle (1 mL of a 2% DMSO solution in water) or loperamide hydrochloride (Sigma) (0.1, 1.0, 10, 20, 40 mg/kg in 1 mL of a 2% DMSO solution in water). After 20 min, each of these animals was given 1 mL of charcoal meal [10% charcoal suspension in 5% aqueous arabic gum] by oral route. All animals were sacrificed after 30 min, the stomach and small intestine were removed and extended on a clean glass surface. The distance moved by the charcoal meal from the pylorus was measured and then expressed as a percentage of the distance from the pylorus to the caecum. After, the plot of percentage of inhibition against concentration was made; the best straight line was determined by regression analysis and the 50% inhibitory concentration (IC₅₀) values were calculated. The regression coefficient, its level of significance (P) and correlation coefficient were calculated. The experiments were performed six times for each concentration. IC₅₀ values are mean \pm S.E.M. $P < 0.05$ (1 – 5way ANOVA followed by Dunnett’s post hoc test), GraphPad Prism Version 5.03 was used.

4.7. X-ray Crystallography of Compound 1

Colorless crystals of 0.376 x 0.188 x 0.098 mm³, with empirical formula C₂₁H₂₂O₆, and Mr = 370.39, crystallized in a Monoclinic crystal system, P2₁, with cell parameters $a = 10.0148(8)$ Å, $b = 7.6733(6)$ Å, and $c = 24.814(2)$ Å. $V = 1872.2(3)$ Å³, $Z = 4$, $D_{\text{calcd}} = 1.314$ Mg/m³, $\mu = 0.796$ mm⁻¹, $F(000) = 784.0$. Compound 1 was irradiated with Cu K α radiation ($\lambda = 1.54178$ Å) on the Bruker D8 Venture κ -geometry diffractometer with microfocus X-ray source and Helios multilayer mirror as monochromator, using an APEX 3 program[18] at 150(2) K. Data reduction was achieved using the SAINT program[18] Totals of 23784 reflections were collected, from which 6748 ($R_{\text{int}} = 0.0767$) reflections were independent. Structure was solved using direct methods and then refined with the SHELXS and SHELXL programs[19] with full-matrix least-squares on F₂, respectively. ORTEP-3 software was used for the figures[20].

The final values $S = 1.064$, $R1 = 0.0479$, and $wR2 = 0.1092$ were based on 6748 reflections observed, 499 parameters. The largest different peak and hole for 1 was 0.196 and -0.161 eÅ⁻³. Crystallographic data (excluding structure factors) have been deposited at the Cambridge Crystallographic Data Centre under the reference number CCDC 1490853. Copies of the data can be obtained free of charge on application to the CCDC, 12 Union Road, Cambridge CB2 IEZ, UK. Fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: The ¹H and ¹³C spectra for all compounds.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds **1**, **1a**, **2**, **3** and **7** are available from the authors.



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