Chemical constituents of two Ecuadorian medicinal plants, *Tagetes terniflora* Kunth and *Croton rivinifolius* Kunth

Constituyentes químicos de dos plantas medicinales Ecuatorianas, *Tagetes terniflora* Kunth y *Croton rivinifolius* Kunth

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Abstract

Phytochemical study of two medicinal plants from Ecuador, *Tagetes terniflora* Kunth, and *Croton rivinifolius* Kunth, led to the isolation and characterization of the major constituents present in the organic extracts obtained from these plants: 5-(4-acetoxy-1-butynil)-2,2′-bi-thiophene (1), 5-methyl-2,2′:5′,2″-terthiophene (2), patuletin (3) from *Tagetes terniflora*, and isocorydine (4), sweroside (5), tiliroside (6) from *Croton rivinifolius*. The structures of these compounds were established by spectroscopic analysis including two-dimensional NMR methods, MS, and comparison with published spectral data. They are recognized as secondary metabolites that represent the chemotaxonomy of *Tagetes* and *Croton* genera and could be responsible for the recognized medicinal properties attributed to these species. This paper deals with the first report that shows the presence of these compounds in these plants.

Keywords: Ecuador, *Croton rivinifolius*, *Tagetes terniflora*, metabolites, alkaloids

Resumen

El estudio fitoquímico de dos plantas medicinales de Ecuador, *Tagetes terniflora* Kunth y *Croton rivinifolius* Kunth, permitió aislar y caracterizar los principales componentes presentes en los extractos orgánicos obtenidos de estas plantas: 5- (4-acetoxi-1-butinil) -2, 2′-bitienil (1), 5-metil-2,2′, 5′, 2‴- tertiofeno (2), patuletina (3) de *Tagetes terniflora* e isocoridina (4), swerosido (5), tilirosido (6) de *Croton rivinifolius*. Las estructuras de estos compuestos se establecieron mediante análisis espectroscópico incluyendo métodos de RMN bidimensional, EM y comparación con datos espectrales publicados. Los mismos son reconocidos como metabolitos secundarios que representan la quimiotaxonomía de los géneros *Tagetes* y *Croton* y podrían ser los responsables de las reconocidas propiedades atribuidas a estas especies. Este artículo trata del primer informe fotoquímico que señala la presencia de estos compuestos en estas plantas.

Palabras clave: Ecuador, *Croton rivinifolius, Tagetes terniflora*, metabolitos, alcaloides

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Introduction

Ecuador is recognized as one of the most biodiverse countries in the world, additionally, this is accompanied by a great wealth that includes ancestral customs in medicine. Traditional medicine comprises a historical, cultural, and social rootedness in the roots of a people. It is an anonymous heritage that is transmitted from generation to generation by oral communications, contributing to the continuation of their beliefs and practices, based on empirical knowledge about the environment. According to the World Health Organization (WHO), traditional medicine is the group of diverse knowledge, practices, approaches, and health beliefs that integrate medicines based on plants, animals or minerals, manual techniques and exercises applied individually or in combination to maintain well-being, as well as to treat, diagnose and prevent diseases (WHO, 2021).

In traditional medicine, several remedies used are made from wild plants whose chemical content may vary due to genetic or environmental reasons, giving rise to the term "medicinal plant". The WHO defines a medicinal plant as any plant species that contain substances that can be used for therapeutic purposes or whose active principles can serve as precursors for the synthesis of new drugs.

In Ecuador, plants have been a primordial resource for indigenous and peasant communities; it is estimated that 80% of the population depends on traditional medicine for primary health care and wellbeing. At present, peasant communities still depend directly or indirectly on plants to meet their food, medicine, and housing needs. The direct use of medicinal plants is lower in the cities; however, in the markets of the cities and particularly in the Andean region, the use and commercialization of medicinal plants is still an active practice where around 273 species of medicinal herbs are sold (Ansaloni et al., 2010).

The genus *Tagetes* belonging to the big family Asteraceae, have species widespread around the world, many species endowed with pharmacological actions (Pryanka et al., 2013). Some authors mention that it originates from South America but has expanded from the southern United States to Argentina, and worldwide (Stefanazzi et al., 2006). This genus includes annual or perennial, erect and aromatic herbs with striated stems and pinnate leaves, and some shrubs; it is of great economic importance and includes species of edible and ornamental plants. Extracts of these species are characterized by their insecticidal and nematicidal (chemical pesticide) activity, in addition to their various pharmaceutical applications (Faizi et al., 2011; Lizarraga et al., 2017). The uses, biological activities as the major constituents present in their species are well documented (Tereschuk et al., 2003; Gupta and Vasuveda, 2012; Burlec et al., 2021). Four of the species in this genus are the most recognized and documented by researchers in different countries, *Tagetes erecta* (Gopi et al., 2012; Shetty et al., 2015; Singh et al., 2020), *Tagetes patula* (Riaz et al., 2020), *Tagetes minuta* (Schiavon et al., 2015), and *Tagetes lucida* (Lim, 2014).

Regarding the phytochemistry of these species, the nature of compounds regularly found in *Tagetes* is usually phenolic that include flavonoids, coumarins, acetylenic thiophenes, terpenes, and aromatic compounds. Many of these reported metabolites show important pharmacological actions (Takahashi et al., 2013).

Croton genus, belonging to the Euphorbiaceae family is a large genus distributed in tropical and subtropical areas of the world. Many species of *Croton* are used for medicinal purposes and proven pharmacological actions of extracts and pure compounds obtained from its species are well documented in the literature (Abega et al., 2014; Langat et al., 2020; Bezerra et al., 2020). The genus *Croton* comprises around 1200 species, being the second largest of the Euphorbiaceae family (Govaerts et al., 2000; Berry et al., 2005), and one of the most diverse, with many species endowed with medicinal properties (Salatino et al., 2007). America is considered, the continent with the major number of species, where only in Brazil more than 350 species have been documented (Van Ee et al., 2011). Regarding the number of *Croton* species in Ecuador, 39 species are recognized (Jørgensen and León-Yánez 1999), and 13 of them are classified as native (León-Yánez et al., 2011); however, several species have been documented in the last years (Smith, 2006; Riina et al., 2007, 2010, 2014, 2015). Many species under the *Croton* genus have been used in the traditional medicine of Ecuador (Bussman and Sharon, 2006; Bailon-Moscoso, et al., 2015). Studies on the biological activities, of species belonging to this genus from America, Asia, and Africa, have been proved as antimicrobial (Obey et al., 2016), antioxidant (Nath et al., 2013), anti-inflammatory (Suárez et al., 2006), anticancer (Suárez et al., 2009; Savietto et al., 2013), wound healing (Ximenes et al., 2013; Nascimento et al., 2017). Considering all the reports of new structures and proven pharmacological actions of species belonging to the genus *Croton*, this study of *Croton rivinifolius* has been considered as an opportunity for new findings.

The chemistry of *Croton* genus shows a high diversity of structures: diterpenes of different skeletons including crotofolanes (Chávez et al., 2013; Kawakami et al., 2015), clerodanes (Pizzolatti et al., 2013; Aldhaher et al., 2017; Qiu et al., 2018), kaurenes (Mora et al., 2011; Mateu et al., 2012; Pereira et al., 2012), labdanes (Bernardino et al., 2017), the more important structures in many species (Xu et al., 2018; Shi et al., 2018). Terpenes (Palmeira Júnior et al., 2006); alkaloids and flavonoids are also common in the genus (de Araújo-Júnior et al., 2004; Suárez et al., 2004; Cerqueira-Coelho et al., 2016).

Croton rivinifolius Kunth, is a native shrub widely disseminated in diverse provinces in Ecuador, the plant is used in traditional medicine to alleviate pains (Cerón Martínez, 2006). No studies with isolated compounds had been reported for this species; only one phytochemical screening indicates the presence of flavonoids as the major metabolites (Rondón et al., 2015). This is the first phytochemical study with isolation and characterization of their secondary metabolites.

Experimental

General experimental procedures

Optical rotations were measured with an Automatic Polarimeter (Jinan Hanon Instruments Co. Ltd., Jinan, China) MRC P810. ^{1}H and ^{13}C, 1D and 2D nuclear magnetic resonance (NMR) spectra were obtained on a Varian Premium Shielded-400 spectrometer (400 MHz to ^{1}H and 100 MHz to ^{13}C). Chemical shifts were reported in δ (ppm), relative to the signal of tetramethylsilane (TMS) and coupling constants (*J*) in Hz. Thin Layer Chromatography (TLC) was performed

with aluminum pre-coated Si plates with fluorescence indicator in the range of 254 nm (F254) (Merck, Darmstadt, Germany). The MS data were acquired in an equipment GC/MS Agilent Technologies 6890N. The substances were revealed by spraying with a vanillin/H_2SO_4 solution, followed by heating with a hairdryer. Column chromatography normal phase was performed using Silica gel 60 (63–200 μm) from (Merck, KGaA, Darmstadt, Germany), and RP-18 (Merck, KGaA, Darmstadt, Germany, 40–63 μm) used as stationary phase for column chromatography in reverse phase. All organic solvents were bought in Brenntag (Brengtan, Guayaquil, Ecuador), and re-distilled before use.

Plant material

The aerial parts of *C. rivinifolius* were collected in the flowering stage in Celica Canton, province of Loja, Ecuador, in April 2016, at 2255 m.a.s.l. (Meters above sea level). The plant material was identified by Dr. Nixon Cumbicus and, voucher specimens (HUTPL8027) were deposited at the Herbarium HUTPL at Universidad Técnica Particular de Loja.

The *T. terniflora* species were collected in the province of Loja; Saraguro canton at the following coordinates 3°37'11.6 "S 79°14'23.3 "W. It was identified by Bolivar Merino of the Loja Herbarium (HUNL) and, voucher specimens (PPN-as-006) were deposited at the Herbarium HUTPL at Universidad Técnica Particular de Loja.

Extraction and isolation

Tagetes terniflora

Air-dried and powdered leaves of *T. terniflora*, (526 g) were macerated with methanol at room temperature for 72h. The solvent was filtered and evaporated under reduced pressure to obtain 13.36 g of crude methanol extract This crude extract was re-dissolved in methanol: H_2O (1:1) and submitted to a partition extraction with solvents of increasing polarity to obtain the following amounts of different fractions: hexane (TTH) (2.02 g), dichloromethane (TTD) (0.67g), ethyl acetate (TTEt) (2.45g), and methanol/ H_2O (TTM) (6.05g). The TLC analyses of the fractions showed that the hexane and the dichloromethane were the richest and interesting metabolites, and there were chosen to separate by column chromatography.

From TTH partition fraction 1.039g was taken and submitted to column chromatography using silica gel (100g), eluted with hexane increasing the polarity gradient with dichloromethane to 100% of this solvent to give 25 subfractions which were joined according to the similarity of their chromatographic profile, resulting in 7 fractions (TTH-1 to TTH-7). The fractions (TTH-1) and (TTH-3) considered pure were analyzed by GC/MS and NMR to offer the compounds: 5-methyl-2,2', 5', 2'''-terthiophene (4) (Takahashi et al., 2011) and 5-(4-acetoxy- 1-butynil)-2,2'-bitienil (5) (Takahashi et al., 2011) which structures are shown Figure 1.

The (TTD) fraction was submitted to column chromatography on silica gel (50 g) eluted with dichloromethane increasing the polarity with ethyl acetate until reaching CH_2Cl_2/EtOAc (9:1). Three subfractions were obtained after TLC analysis and the subfraction (TTD2) by NMR analysis showed to be the methoxylated flavonoid patuletin (6) (Abdel-Wahhab et al., 2005).

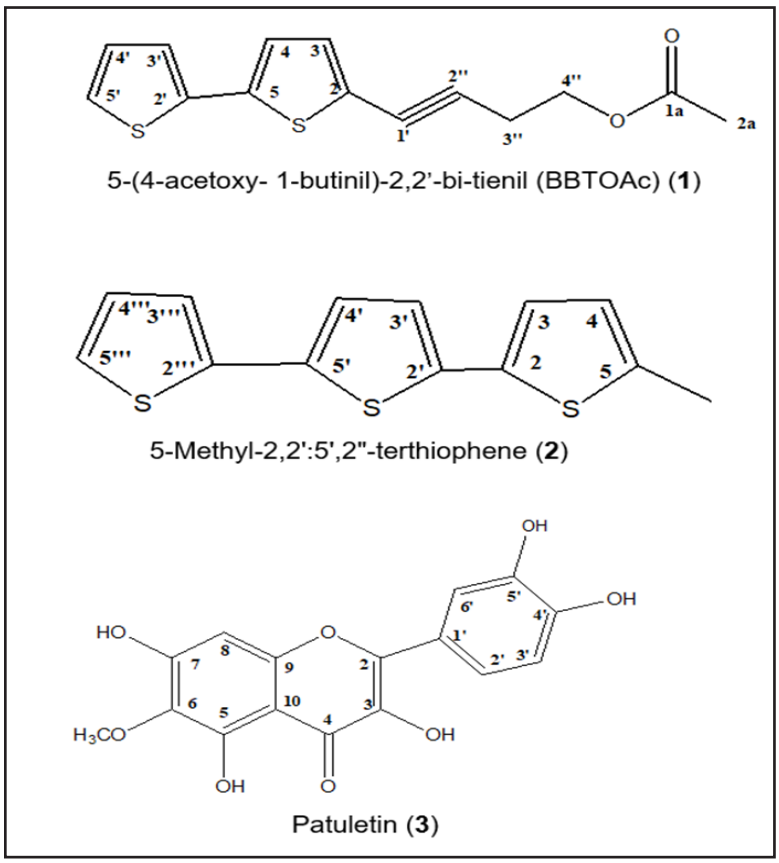

Figure 1. Isolated compounds from *Tagetes terniflora*

Croton rivinifolius

A similar procedure to the one previously described was carried out with *C. rivinifolius* using (923.38 g) of leaves. The solvent was evaporated under reduced pressure in rotary evaporator equipment; to finally obtain 101.34g of extract. From that crude (44.58g) were re-dissolved in a 1:1 water/methanol mixture and then partitioned with hexane, dichloromethane, and ethyl acetate successively, to give fractions of each solvent, hexane (CRH) (9.98 g), dichloromethane (CRD) (1.76 g) ethyl acetate (CREt) (7.54 g), and methanol/H_2O (CRM) (21.36 g). After TLC analyses of the different fractions, the CH_2Cl_2 and EtOAc fractions were considered the richer and more interesting to separate in the first place, but both, showed to have many compounds with similar Rf, making difficult the separation. For this reason, these fractions were saved for GC analysis before deciding the separation. Ethyl acetate extract (2 g) was subjected to a first gross separation in a CC with silica gel and increasing polarity of elution from (99:1) EtOAc: MeOH to finish with (90:10 EtOAc/MeOH), 77 fractions (CREtI-01/CREtI-77) were collected. After TLC analysis the fractions were pooled according to the same behavior on the TLC plate, to give 10 subfractions (CREtII-1/CREtII-10). The two subfractions with a big amount were further purified by chromatography. The less polar subfraction (CREtII-1) (235 mg) after purification with silica gel eluted by the isocratic way with EtOAc/MeOH 99:1, gave the compound sweroside (5) (65.6 mg) (Joshi, 2013). The fraction CREtII-3 (324 mg) gave after evaporation a yellow solid which was recrystallized in MeOH to give the pure tiliroside (6) (82.5 mg) (Luhata et al., 2016; Devi and Kumar, 2018).

Finally, the alkaloid isocorydine (4), was obtained from the methanolic fraction, part of the methanol extract (CRM) (0.5g), was subjected to CC over reverse phase RP-18, using H_2O/CH_3OH (6:4), to obtain as a major compound (50.3 mg) a colorless solid identified by spectroscopy analysis as the aporphine alkaloid isocorydine (Zhong et al., 2014) (Figure 2).

SPECTROSCOPIC DATA

5-(4-acetoxy- 1-butynil)-2,2′-bi-tienil (BBTOAc) (**1**). Brown oil; $C_{15}H_{14}S_2O_2$. EIMS m/z 276 $[M]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.22 (1H, dd, J = 4.0, 1.2 Hz, H-5′), 7.15

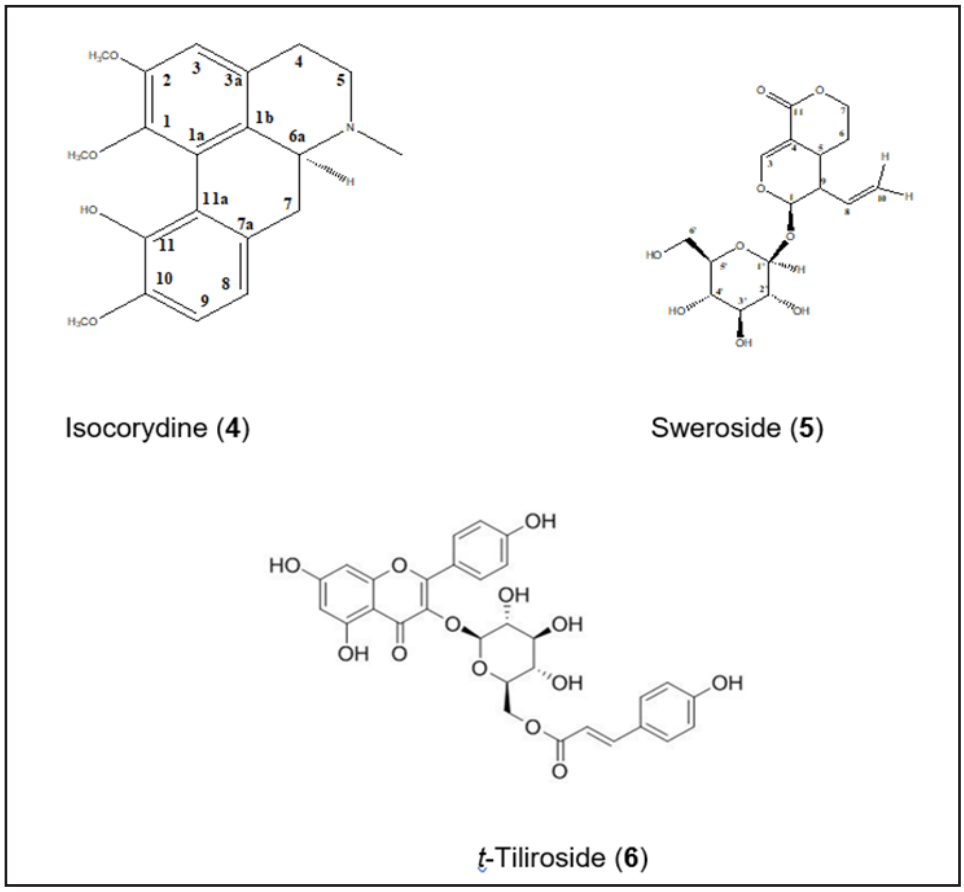

Figure 2. Isolated compounds from *Croton rivinifolius*

(1H, dd, J = 4.0, 1.2 Hz, H-4'), 7.05 (1H, d, *J* = 4.0Hz, H-3'), 7.01(1H, d, *J* = 3.6 Hz, H-3), 6.95 (1H, d, *J* = 3.6 Hz, H-4), 4.24 (2H, t, *J* = 6.8 Hz, H-4''), 2.78 (2H, t, *J* = 6.8 Hz, H-4''), 2.10 (3H, s, H-2a); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.0 (C-1a), 138.2(C-2), 136.9(C-2'), 132.6, (C-4), 128.0 (C-4'), 125.0(C-3'), 124.3 (C-5'), 123,4 (C-3), 122.2 (C-5), 90.7 (C-1''), 75.3 (C-2''), 62.2(C-4''), 21.0(C-2a), 20.4 (C-3'').

5-Methyl-2,2':5',2''-terthiophene (**2**). Brown oil; $C_{13}H_{10}S_3$, EIMS m/z 262 [M]+, ^{1}H-NMR δ: 7.20 (1H, dd, *J* = 1.2 Hz, H-5''), 7.18 (1H, d, J = 1.2 Hz, H-4''), 7.17 (1H, d, *J* = 3.2 Hz, H-4), 7.15 (1H, d, *J* = 1.2 Hz, H-4'), 7.05 (1H, d, J = 3.6 Hz, H-3''), 6.95 (1H, d, *J* = 3.6 Hz, H-3'), 6.65 (1H, dd, J = 3.6, 1.2 Hz, H-4), 2.49 (3H, s, CH_3) . ^{13}C-NMR δ: 139.5 (C-5), 137.4 (C-5'), 137.2 (C-2''), 136.4 (C-2'), 135.7 (C-2), 128.0 (C-3), 127.9 (C-4''), 126.2 (C-3''), 124.6 (C-4'), 124.5 (C-4), 123.8 (C-3'), 123.7 (C-5''), 15.6 (5-Me).

Patuletin (**3**), ^{1}H-NMR (400 MHz, CD_3OD): δ: 12.3 (1H, brs, 5-OH), 7.74 (1H, dd, J = 8.3, 2.1 Hz, H-6'), 7.63 (1H, d, *J* = 2.1 Hz, H-2'), 6.95 (1H, d, *J* = 8.3, 2.1 Hz, H-5'), 6.50 (1H, s, H-8), 3.91 (3H, s, OCH_3). ^{13}C-NMR (100 MHz, CD_3OD): δ:177.7 (C-4), 158.5 (C-9), 153.6 (C-5), 148.9 (C-2), 146.5 (C-3'), 137.1 (C-3), 132.2 (C-6), 123.9 (C-1'), 115.9 (C-2'), 115.8 (C-5'), 105.1 (C-10), 94.7 (C-8), 61.3 (OCH3).

Isocorydine (**4**). Colorless crystals, mp: 184–186 °C; $[\alpha]^{24}$ + 215.0 (c 0.1, MeOH); $C_{20}H_{24}NO_4$, EIMS: m/z [M + H] + 342. ^{1}H-NMR (400MHz,$CDCl_3$) δ (ppm): 6.70 (1H, s, H-3), 3.21 (1H, brs, H-4a), 2.72 (2H, 2d,

J = 3.6 Hz, H-4b), 3.02 (1H, 2d, *J* = 3.6 Hz, H-5b), 2.49 (1H, brs, H-5b), 2.89 (1H, d, J = 12.8 Hz, H-6a), 3.03 (1H, m, H-7a), 2.45 (1H, m, H-7b), 6.82 (1H, d, J = 8.0 Hz,H-8), 6.90 (1H, d, *J* = 8.0 Hz,H-9), 3.70 (3H, s, 1-OCH_3), 3.91 (3H, s, 2-OCH_3), 2.54 (3H, s, NCH3), 3.91(3H, s, 10-OCH3), 9.0(1H, s,11-OH). ^{13}C-NMR (100MHz, $CDCl_3$) δ (ppm): 151.3(C-2), 146.4 (C-10), 146.4 (C-11), 142.0 (C-1), 130.5 (C-3b), 130.5 (C-7a), 129.1 (C-3a), 125.9 (C-1a), 120.2 (C-1b), 120.0 (C-8), 111.4 (C-9), 111.3 (C-3), 63.0 (C-6a), 63.0 (1-OCH_3), 52.9 (C-5), 56.1 (2-OCH_3), 56.0 (10-OCH_3), 44.0 (N-CH3), 35.7 (C-7), 29.4 (C-4).

3-O-β -D-glucopyranosyl sweroside (**5**) ^{1}H-NMR (400 MHz, CD_3OD): δ: 7.59 (1H, d, J =2.4 Hz, H-3), 5.56 (2H, d, J = 1.6, H-8), 5.55 (2H, d, J = 1.6 Hz, H-1), 5.32 (1H, dd, J = 2.0, 16.8 Hz, H-10a), 5.26 (1H, dd, J = 2.0, 9.0 Hz, H-10b), 4.68 (1H, d, *J* = 8.0 Hz, H-1′), 3.89 (1H, dd, *J* = 10.0, 7.2 Hz, H6′a), 3.67 (1H, t, J = 8.8 Hz, H-3′), 3.65 (1H, dd, J = 10.0, 7.2 Hz, H-6′b), 3.43 (1H, m, H-5′), 1.71 (2H, m, H-6), 4.41 (2H, m, H-7), 3.22 (1H, dd, *J* = 8.0, 8.8 Hz, H-2′), 3.31 (1H, m, H-4′), 2.72 (1H, m, H-9), 1.71 (1H, m, H-6). ^{13}C-NMR (100 MHz, CD_3OD): δ: 168.5 (C-11), 153.9 (C-3), 133.3 (C-8), 106.0 (C-4), 120.8 (C-10), 97.9 (C-1), 99.7 (C-1′), 78.6(C-5′), 77.8 (C-3′), 74.7 (C-2′), 71.5 (C-4′), 69.7 (C-7), 62.6 (C-6′), 43.8 (C-9), 28.4 (C-5), 25.9 (C-6).

t-Tiliroside (**6**): $C_{30}H_{26}O_{13}$; amorphous yellow solid; mp 268-270 °C; MS m/z 594 [M^+]; ^{1}H-NMR (CD_3OD, 400 MHz) δ (ppm): 7.98 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 7.39 (1H, d, J = 16 Hz, H-7‴), 7.30 (2H, d, J = 8.5 Hz, H-2‴ and H-6‴), 6.81 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 6.79 (2H, d, J = 8.5 Hz, H-3‴ and H-5‴), 6.30 (1H, brs, H-8), 6.13 (1H, brs, H-6), 6.03 (1H, d, J = 16 Hz, H-8‴), 5.23 (1H, d, J = 7.6 Hz, H-1″), 4.30 (1H, dd, J = 11.8, 1.9 Hz, H-6β″), 4.18 (1H, dd, *J* = 11.6, 6.6 Hz, H-6α″), 3.55 (1H, m, H-5″), 3.46 (1H, m, H-3″), 3.45(1H, m, H-2″), 3.44 (1H, m, H-4″); ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm): 159.4 (C-2), 135.2 (C-3), 179.4 (C-4), 168.8 (C-9‴), 165.9 (C-7), 162.9 (C-9), 158.5 (C-5), 146.6 (C-7‴), 133.7 (C-6‴), 133.2 (C-2‴), 132.2 (C-2′, C-6′),131.2 (C-4‴), 127.2 (C-1‴), 122.7 (C-1′), 116.2 (C-3′, 6′), 116.8 (C-8‴), 116.0 (C-5‴), 116.0 (C-3′, C-5′), 114.7 (C-3‴), 105.6 (C-10), 103.9 (C-1″), 99.9 (C-6), 94.8 (C-8), 77.9 (C-5″), 75.8 (C-4″), 75.7 (C-2″), 71.7 (C-3″), 63.9 (C-6″).

Results and discussion

The 5-(4-acetoxy- 1-butynil)-2,2′-bitienil (BBTOAc) (**1**), 5-methyl-2,2′:5′,2″-terthiophene (**2**), patuletin (**3**), Isocorydine (**4**), 3-O-β -D-glucopyranosyl sweroside (**5**), and tiliroside (**6**), are known compounds previously described in plant species, but exposed for the first time for *Tagetes terniflora* and *Croton rivinifolius*.

These preliminary results constitute these first phytochemical studies of *Tagetes terniflora* and *Croton rivinifolius*, showing that in both cases, the secondary metabolites isolated and characterized are under the family of compounds regularly found in species of *Tagetes* and *Croton* genus. The thiophenes such as the compounds (**1**) and (**2**) are characteristic compounds found in *Tagetes* species, and they have been reported with pharmacological actions (Muzzoli et al., 2001; Takahashi et al., 2013; Vázquez-Atanacio et al., 2021). The flavonoids are a kind of compound with many biological actions and some of them, especially the methoxylated flavonoids are common in *Tagetes*, and in this case,

patuletin (**3**) represents one of these (Jabeen et al., 2016; Alvarado-Sansininea, 2018; Zarei et al., 2018).

Croton is one of the biggest genera under the Euphorbiaceae, and the richness in the chemistry of their secondary metabolites had been exposed in many reports of the phytochemistry related to it. Alkaloids of different classes are common (Suárez et al., 2004; Xu et al., 2018) and the wide spectra of pharmacological activities of these compounds are undeniable. In this case, the isocorydine (**4**) has been reported with cytotoxic (Sun et al., 2014; Zhong et al., 2014) and anti-arrhythmia effects (Wang et al., 2016). The tiliroside (**6**) is a ubiquitous flavonoid under this genus, and it is a compound with probed actions in different biological research (Luhata and Luhata, 2017; Grochowski et al., 2018). Regarding the sweroside (**5**) belonging to the iridoids, they are not common in the genus but are also metabolites with interesting results in investigations that trying to deal with biological activities (Hussain et al., 2019; Arraché-Gonçalves et al., 2021). The results here exposed shown that all the metabolites found in these two Ecuadorian medicinal plants, are considered under the chemotaxonomy of both genus, and they could be responsible for the medicinal properties attributed by the people to these species. The characterization of other compounds present in these species is undergoing in our lab, as well research that deal with the toxicity and biological actions of the compounds here reported.

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