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Nahar, L, Talukdar, AD, Nath, D, Nath, S, Mehan, A, Ismail, FMD and Sarker, SD

**Naturally Occurring Calanolides: Occurrence, Biosynthesis, and Pharmacological Properties Including Therapeutic Potential**

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### Article

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1 Review

# 2 Naturally Occurring Calanolides: Occurrence, 3 Biosynthesis, and Pharmacological Properties 4 Including Therapeutic Potential

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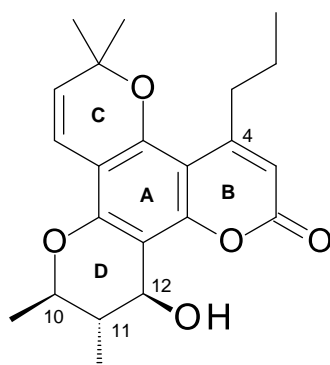
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20 **Abstract:** Calanolides are tetracyclic 4-substituted dipyrano-coumarins. Calanolide A, isolated from  
21 the leaves and twigs of *Calophyllum lanigerum* var. *austrocoriaceum* (Whitmore) P. F. Stevens, is the  
22 first member of this group of compounds with anti-HIV-1 activity mediated by reverse transcriptase  
23 inhibition. Calanolides are classified pharmacologically as non-nucleoside reverse transcriptase  
24 inhibitors (NNRTI). There are at least 15 naturally occurring calanolides distributed mainly within  
25 the genus *Calophyllum*, but some of them are also present in the genus *Clausena*. Besides significant  
26 anti-HIV properties, which have been exploited towards potential development of new NNRTIs for  
27 anti-HIV therapy, calanolides have also been found to possess anticancer, antimicrobial and  
28 antiparasitic potential. This review article provides a comprehensive update on all aspects of  
29 naturally occurring calanolides, including their chemistry, natural occurrence, biosynthesis,  
30 pharmacological and toxicological aspects including mechanism of action and structure activity  
31 relationships, pharmacokinetics, therapeutic potentials and available patents.

32 **Keywords:** calanolides; pseudocalanolides; calanolide A; *Calophyllum*; Calophyllaceae; anti-HIV,  
33 reverse transcriptase; non-nucleoside reverse transcriptase inhibitors (NNRTIs).

## 35 1. Introduction

36 Calanolides are tetracyclic 4-substituted dipyrano-coumarins, and their C-ring contains a *gem*-  
37 dimethyl group (Figure 1), e.g., (+)-calanolide A (**1**), (-)-calanolide B (costatolide) (**14**) (Figure 2). The  
38 discovery of calanolides (Table 1) from the leaves and twigs of the tree *Calophyllum lanigerum* var.  
39 *austrocoriaceum* (Whitmore) P. F. Stevens, collected from Sarawak, Malaysia in 1987 happened during  
40 one of the largest anti-HIV screening programs conducted by the National Cancer Institute (NCI)

41 during 1987-1996. In that program, over 30,000 plant extracts were screened utilizing an *in vitro* cell-  
 42 based anti-HIV screen that could determine the degree of HIV-1 replication in treated infected  
 43 lymphoblastic cells versus that in treated uninfected control cells [1,2]. Calanolide A (1) (Figure 1),  
 44 which can be described as a 11,12-dihydro-2H,6H,10H-dipyrano[2,3-f:2',3'-h]chromen-2-one  
 45 substituted by a hydroxyl (-OH) group at C-12, methyl groups at positions 6, 6, 10 and 11 and a propyl  
 46 group at C-4 (the 10*R*,11*S*,12*S* stereoisomer), was isolated as the first member of anti-HIV compounds,  
 47 calanolides, as a potential novel therapeutic option for the treatment of HIV infections. However, a  
 48 subsequent attempt to recollect this plant sample failed and the collection of other specimens of the  
 49 same species (not necessarily the same variety), afforded only a negligible amount of calanolide A  
 50 (1). In fact, calanolides are among the first plant-based compounds to demonstrate potential anti-  
 51 HIV-1 activity. Later, an extract of the latex of *C. teysmanii* showed significant anti-HIV activity in the  
 52 screening, but the major active compound was (-)-calanolide B (14, also known as costatolide),  
 53 regrettably not calanolide A (1) (Figure 2). The anti-HIV activity of (-)-calanolide B (14) was less  
 54 potent than that of calanolide A (1), possibly because of difference in stereochemistry at the chiral  
 55 centers. To date calanolides A-F and some of their methyl, acetyl and dihydro derivatives have been  
 56 reported mainly from various *Calophyllum* species (Figure 2; Table 1). Among these, the structures of  
 57 calanolides C (6) and D (7), as reported initially by Kashman et al. [1] from *C. lanigerum*, were revised  
 58 and renamed as pseudocalanolides C (8) and D (9) [3] (Figure 2). However, the true calanolides C (6)  
 59 and D (7) were later reported from *C. brasiliense* Cambess. [4-6].



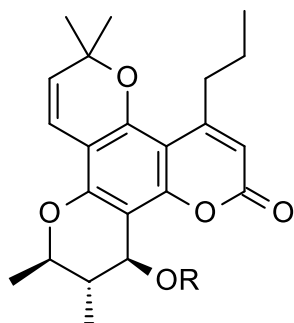
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61 **Figure 1:** Rings A, B, C and D, and carbon numbering in (+)-Calanolide A (1)

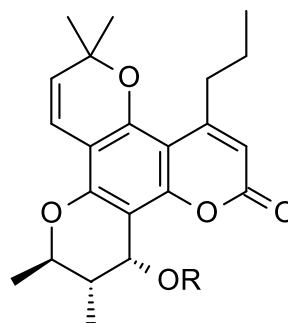
62 The first isolation process of calanolides from *C. lanigerum* var. *austrororiaceum*, involved multiple  
 63 steps, starting with the extraction of dried fruits and twigs of this plant with a 1:1 mixture of  
 64 dichloromethane and methanol, followed by a sequential solvent partitioning process involving  
 65 various solvents. The *n*-hexane and CCl<sub>4</sub> fractions emerged as the active fractions [1]. Repeated  
 66 vacuum liquid chromatography (VLC) on silica gel, eluting with a mixture of *n*-hexane and ethyl  
 67 acetate afforded crude calanolides, which were further purified by HPLC, employing normal phase  
 68 for calanolide A (1), calanolide B (4) and pseudocalanolide D (9) [reported incorrectly as calanolide  
 69 D (7)], while reversed-phase for 12-acetoxycalanolide A (2), 12-methoxycalanolide A (3), 12-  
 70 methoxycalanolide B (5), pseudocalanolide C (8) [reported as calanolide C (6)] and calanolide E (10).  
 71 The structures of these compounds were determined by a combination of UV, IR, NMR and MS  
 72 spectroscopic methods, and all spectroscopic data were published [1]. The absolute stereochemistry  
 73 of calanolides A (1) and B (4) was confirmed by a modified Mosher's method.

74 There is a review [7] and a book chapter on calanolides [8], published about six years ago, that  
 75 mainly cover anti-HIV activity, and the literature published until early 2014. This present review is  
 76 not on the genus *Calophyllum*, the family Calophyllaceae or pyranocoumarins as such, but it  
 77 exclusively focuses on various aspects of naturally occurring calanolides. This review is significantly  
 78 different from any other previous articles on calanolides in its approach and coverage, and is a  
 79 comprehensive update on naturally occurring calanolides, encompassing their chemistry, natural

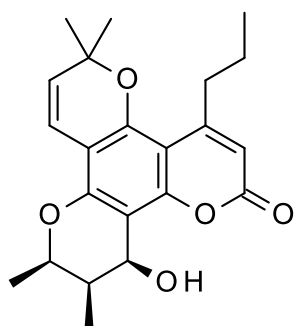
80 occurrence, biosynthesis, pharmacological and toxicological aspects including mechanism of action  
 81 and structure activity relationships, pharmacokinetics, therapeutic potentials and available patents.



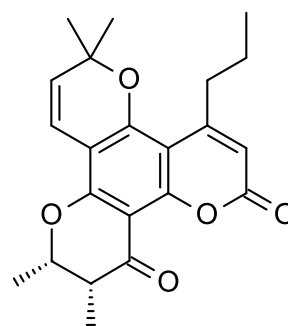
Calanolide A (1) R = H

12-*O*-Acetyl-calanolide A (2) R = Ac12-*O*-Methyl-calanolide A (3) R = Me

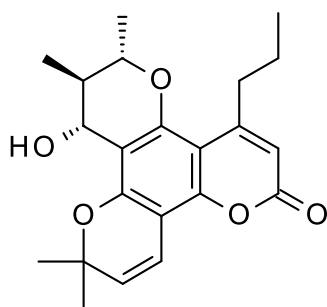
Calanolide B (4) R = H

12-*O*-Methyl-calanolide B (5) R = Me

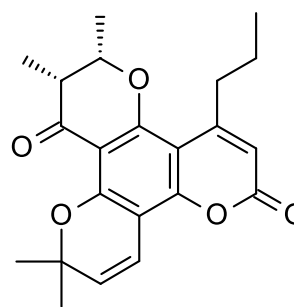
Calanolide C (6)



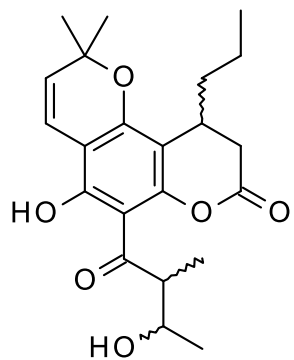
Calanolide D (7)



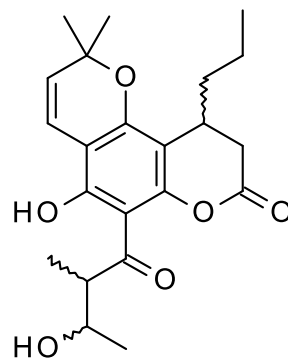
Revised to Pseudocalanolide C (8)



Revised to Pseudocalanolide D (9)

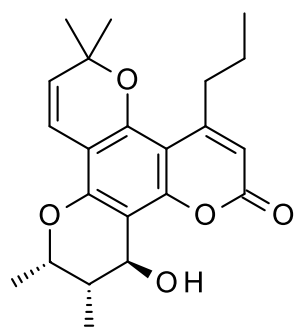
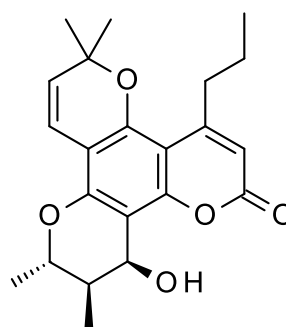
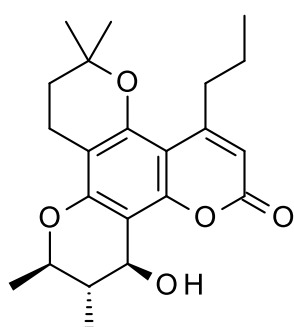


Calanolide E (10)

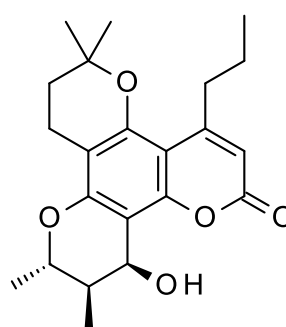


Calanolide E1 (11)

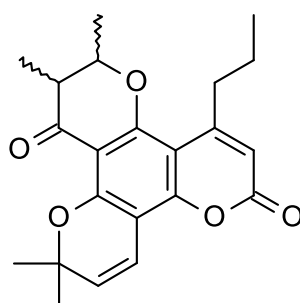
Calanolide E2 (12)

**Figure 2 (Contd.).** Naturally occurring calanolidesCalanolide F (13)  
(10-*epi*-calanolide A)Costatolide (14)  
[10,11-Di-*epi*-calanolide A or (-)-calanolide B]

7,8-Dihydrocalanolide A (15)



Dihydrocostatolide (16)



Tomentolide B (17)

82 **Figure 2 (Continued from the previous page).** Naturally occurring calanolides83 **2. Occurrence**

84 Calanolides, calanolide A (1) being the first member of these 4-substituted pyranocoumarins  
 85 isolated from *C. lanigerum* var. *austrororiaceum*, are almost exclusively distributed within the genus  
 86 *Calophyllum* L., which comprises a large group of ca. 200 species of tropical trees distributed in the  
 87 Indo-Pacific region, but was also reported from one species (*Clausena excavate* Brum. f.) of the closely  
 88 related genus *Clausena* [9-12] (Table 1). Calanolide A (1) and other calanolides were subsequently  
 89 isolated from other *Calophyllum* species, e.g., *C. brasiliense* Cambess. [4,13,14], *C. inophyllum* L. [6], *C.*  
 90 *teysmanii* Miq. [2] and *C. wallichianum* Planch. & Triana [15]. In a chemotaxonomic study on the  
 91 *Calophyllum* species, the presence of calanolides was detected in the extracts of *C. inophyllum*, *C.*  
 92 *lanigerum* var. *austrororiaceum*, *C. mole* King, *C. nodosum*, aff. *Pervillei* Vesque., *C. soulattri* Burm. f., *C.*  
 93 *tacamahaca* Willd. and *C. teysmanii* [9] (Table 1).

**Table 1.** Naturally occurring calanolides, their sources and properties

Calanolides	Sources	Physical state	Mol. formula	Mol. weight	Optical rotation [α] <sub>D</sub>	UV λ <sub>max</sub> (MeOH) nm	References
Calanolide A (1)	<i>Calophyllum lanigerum</i> var. <i>austrocoriaceum</i>	Oil	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.44	[α] <sub>D</sub> +60° (c, 0.7 in CHCl <sub>3</sub> )	228, 284 and 325	[1, 9, 11, 71]
	<i>Calophyllum brasiliense</i>						[4, 62, 81]
	<i>Calophyllum inophyllum</i>						[6, 11]
	<i>Calophyllum teysmannii</i>						[82]
	<i>Clausena excavata</i>						[10]
12-O-Acetyl-calanolide A (2)	<i>Calophyllum lanigerum</i> var. <i>austrocoriaceum</i>	Oil	C <sub>24</sub> H <sub>28</sub> O <sub>6</sub>	412.48	[α] <sub>D</sub> +20° (c, 0.5 in CHCl <sub>3</sub> )	228, 284 and 325	[1]
12-O-Methyl-calanolide A (3)	<i>Calophyllum lanigerum</i> var. <i>austrocoriaceum</i>	Oil	C <sub>23</sub> H <sub>28</sub> O <sub>5</sub>	384.47	[α] <sub>D</sub> +32° (c, 0.8 in CHCl <sub>3</sub> )	228, 284 and 325	[1]
Calanolide B (4)	<i>Calophyllum lanigerum</i> var. <i>austrocoriaceum</i>	Oil	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.44	[α] <sub>D</sub> +8° (c, 1.0 in acetone)	228, 284 and 325	[1]
	<i>Calophyllum brasiliense</i>						[5, 62]
	<i>Calophyllum teysmannii</i> var. <i>inophylloide</i>						[9]
12-O-Methyl-calanolide B (5)	<i>Calophyllum lanigerum</i> var. <i>austrocoriaceum</i>	Oil	C <sub>23</sub> H <sub>28</sub> O <sub>5</sub>	384.47	[α] <sub>D</sub> +34° (c, 0.5 in CHCl <sub>3</sub> )	228, 284 and 325	[1]
Calanolide C (6)	<i>Calophyllum brasiliense</i>	Oil	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.44	-	-	[4, 5]
Calanolide D (7)	<i>Calophyllum brasiliense</i>	Amorphous solid	C <sub>22</sub> H <sub>24</sub> O <sub>5</sub>	368.42	-	-	[6]

Calanolide E (10)	<i>Calophyllum lanigerum</i> var.	Amorphous powder	C <sub>22</sub> H <sub>28</sub> O <sub>6</sub>	388.50	[a] <sub>D</sub> +30° (c, -0.7 in acetone)	[1, 9, 16]
	<i>austrocoriaceum</i>					[83]
	<i>Calophyllum membranaceum</i>					
	<i>Calophyllum molle</i>					[9]
	<i>Calophyllum polyanthum</i>					[84]
	<i>Calophyllum teysmannii</i> var.					[16]
	<i>inophylloide</i>					
Calanolide E1 (11)	<i>Calophyllum lanigerum</i> var.	Amorphous powder	C <sub>22</sub> H <sub>28</sub> O <sub>6</sub>	388.50	-	[9, 16, 9];
	<i>austrocoriaceum</i>					
	<i>Calophyllum brasiliense</i>					[9, 14]
Calanolide E2 (12)	<i>Calophyllum molle</i>					[9]
	<i>Calophyllum lanigerum</i> var.	Amorphous powder	C <sub>22</sub> H <sub>28</sub> O <sub>6</sub>	388.50	-	[9, 16]
	<i>austrocoriaceum</i>					
	<i>Calophyllum brasiliense</i>					[14]
	Cambess.					
	<i>Calophyllum membranaceum</i>					[83]
	<i>Calophyllum molle</i>					[9]
<i>Calophyllum polyanthum</i>	[84, 85]					
Calanolide F (13)	<i>Calophyllum teysmannii</i> var.					[9, 16, 9]
	<i>inophylloide</i>					
	<i>Calophyllum lanigerum</i> var.	Amorphous powder	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.44	[a] <sub>D</sub> -51.5° (c, 227, 283, 0.3 in CHCl <sub>3</sub> )	[9, 16]
	<i>austrocoriaceum</i>					[322]
	<i>Calophyllum teysmannii</i> var.					
<i>inophylloide</i>	[9, 16]					

Costatolide (14)	<i>Calophyllum brasiliense</i>	Crystals	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.44	[a] <sub>D</sub> -19.9 (c, 228, 284 [4]
[(-)-Calanolide B]	<i>Calophyllum costatum</i>	(M.p. 181-183°)			0.42 in and 325 [17]
	<i>Calophyllum inophyllum</i> L.				CHCl <sub>3</sub> ) [17]
	<i>Calophyllum teysmannii</i> var. <i>inophylloide</i>				[9, 82, 86]
7,8-Dihydrocalanolide A (15)	<i>Calophyllum lanigerum</i> var. <i>austrocoriaceum</i>	Amorphous solid	C <sub>22</sub> H <sub>28</sub> O <sub>5</sub>	372.46	Negative optical rotation - [86]
Dihydrocostatolide (16)	<i>Calophyllum costatum</i>	Amorphous solid	C <sub>22</sub> H <sub>28</sub> O <sub>5</sub>	372.46	- - [28]
Pseudocalanolide C (8) [incorrectly named calanolide C (6)]	(8) <i>Calophyllum lanigerum</i> var. <i>austrocoriaceum</i>	Amorphous solid	C <sub>22</sub> H <sub>26</sub> O <sub>5</sub>	370.44	[a] <sub>D</sub> +68° (c, - 0.7 in CHCl <sub>3</sub> ) [1, 9, 87]
Pseudocalanolide D (9) [incorrectly named calanolide D (7)]	as <i>Calophyllum lanigerum</i> var. <i>austrocoriaceum</i>	Amorphous solid	C <sub>22</sub> H <sub>24</sub> O <sub>5</sub>	368.43	[a] <sub>D</sub> +60° (c, - 0.5 in CHCl <sub>3</sub> ) [1, 87]
Tomentolide B (17)	<i>Calophyllum tomentosa</i>	Amorphous solid (M.p. 158-160°)	C <sub>22</sub> H <sub>24</sub> O <sub>5</sub>	368.43	Racemic mixture - [1, 9, 87]



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Bernabe-Antonio et al. [5] reported the production of calanolides in a callus culture of *C. brasiliense*, where different concentrations and combinations of plant growth regulators were tested in leaf and seed explants to establish callus cultures capable of producing calanolides. Higher calanolides B (4) and C (6) production was observed in calluses from seed explants than those developed from leaves. In continuation of the search for new natural anti-HIV compounds, and at the same time to find new botanical sources of calanolides, McKee et al. [16] purified calanolide E2 (12), and calanolide F (13) from the extracts of *C. lanigerum* var. *austrocoriaceum* and *C. teysmanii* var. *inophylloide* (King.) P. F. Stevens. Later, costatolide (14), also known as (-)-calanolide B, was reported as an anti-HIV compound present in *C. cerasiferum* Vesque and *C. inophyllum* L. [17]. Calanolides A (1), and C (6), and costatolide (14) were isolated from the leaves of *C. brasiliense*, and their anti-HIV potential was evaluated [4].

### 3. Biosynthesis

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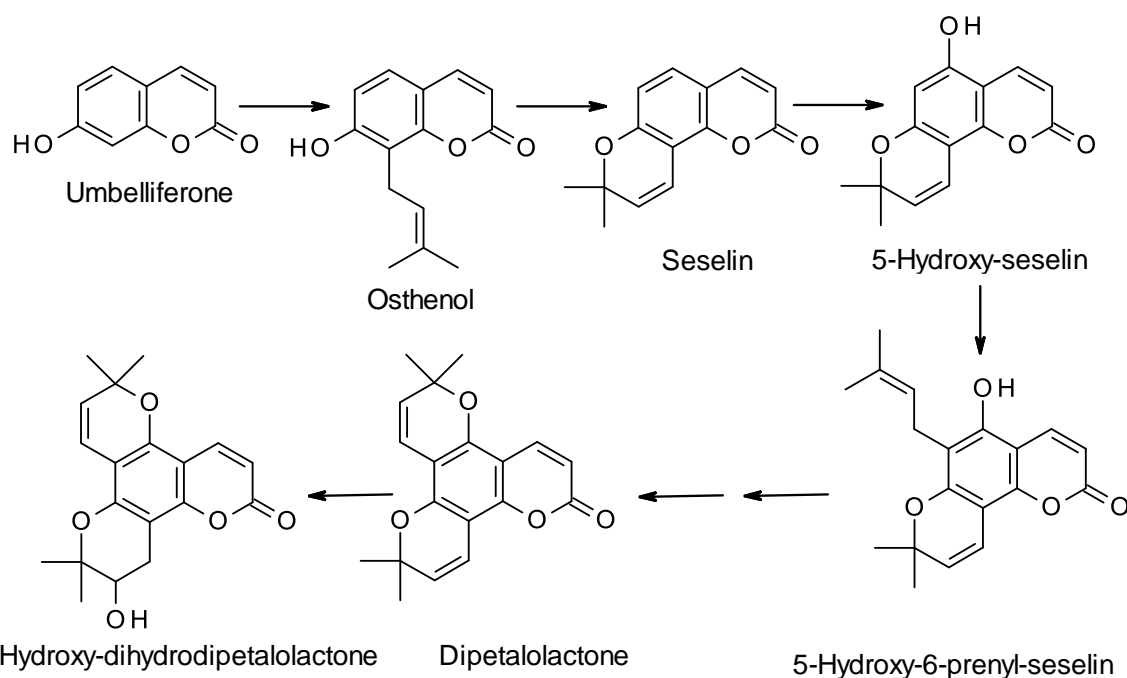
Calanolides are biosynthesized from the parent simple coumarin umbelliferone (Schemes 1-3). The biosynthesis of 7-hydroxycoumarin, also known as umbelliferon in plants starts from the amino acid L-phenylalanine, and proceeds through the formation of *trans*-cinnamic acid, *p*-coumaric acid, 2-hydroxy-*p*-coumaric acid, 2-glucosyloxy-*p*-coumaric acid, and 2-glucosyloxy-*p*-*cis*-coumaric acid with the help of various enzymes like cinnamate 4-hydroxylase, 4-coumarate-CoA ligase, 4-coumaroyl 2'-hydroxylase and so on [18]. The biosynthesis of dipetalolactone, a pyranocoumarin, and subsequent conversion to the 3-propyl-intermediate for calanolides may proceed through two routes, one through conversion of umbelliferone to osthenol (Scheme 1), and the other via formation of 5,7-dihydroxycoumarin (Scheme 2). Reactions are generally mediated by p450 monooxygenase and other non-p450 enzymes [19]. 3-Propyl-intermediate is converted to the precursor compound for calanolides A-C (1, 4 and 6), utilizing the Wagner-Meerwein rearrangement reaction, and the precursor compound is believed to be converted to calanolides with the help of p450 monooxygenase enzyme (Scheme 3). Published studies on the biosynthesis of calanolides are rather limited and only two publications are available on this topic to date [19, 20]. Therefore, detailed knowledge of specific enzymes involved in the biosynthesis of calanolides is still in its infancy.

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In a recent study, the influence of soil nutrients, e.g., Ca<sup>2+</sup> and K<sup>+</sup>, on the biosynthesis of pharmacologically active calanolides in the seedlings of *C. brasiliense* was studied [20]. It was observed that the use of K<sup>+</sup> deficient modified Hoagland solution (MHS) could induce a 15, 4.2 and 4.3-fold decrease of calanolides B (4), C (6), and apetalic acid concentrations in the leaves of the seedlings, respectively. On the other hand, Ca<sup>2+</sup> deficient MHS could lead to a decrease of 4.3 and 2.4-fold for calanolides B (4) and C (6), respectively. This study demonstrated that, like many other plant secondary metabolites, the biosynthesis of calanolides, albeit genetically controlled, may also be affected by environmental conditions, e.g., soil nutrients (minerals).

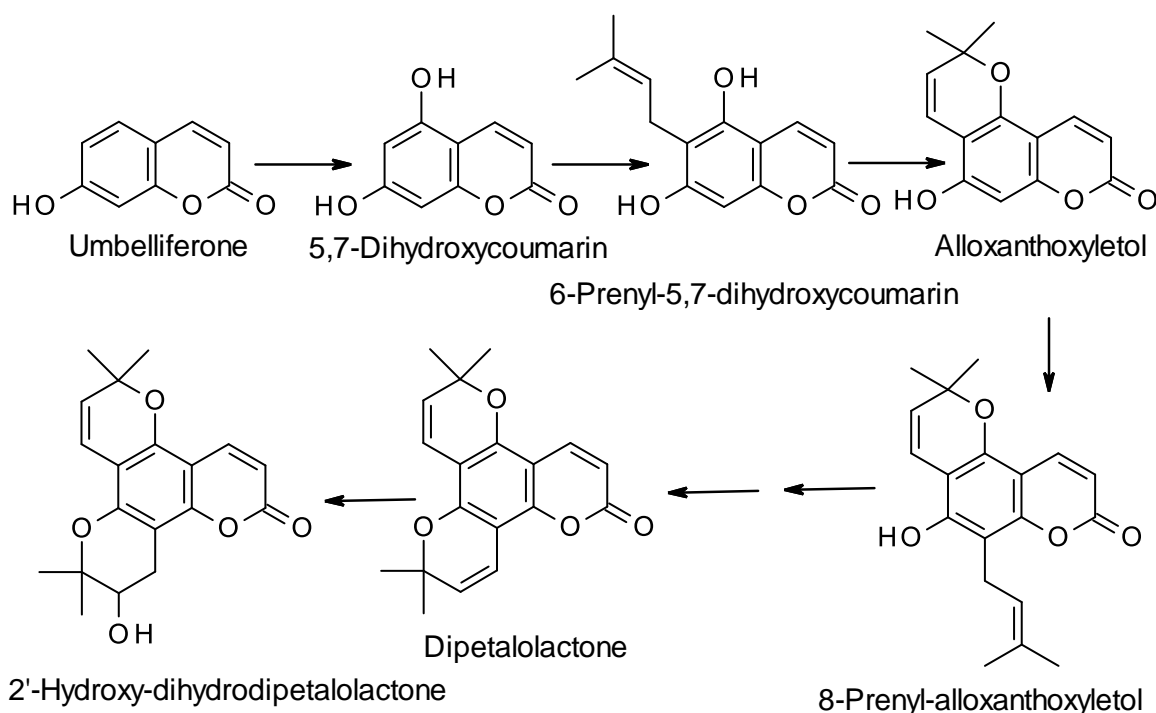
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As genes dictate biosynthesis of secondary metabolites, a study was conducted to identify candidate genes that regulate the biosynthesis of calanolides in *C. brasiliense* [19]. The unigene dataset constructed in this study could offer an insight for further molecular studies of *C. brasiliense*, particularly for characterizing candidate genes responsible for the biosynthesis of angular and linear pyranocoumarins. The candidate genes, e.g., UN36044, UN28345 and UN34582, identified in the transcriptome of the leaves, stem and roots of *C. brasiliense* might be involved in the biosynthesis of calanolides, which are essentially modified angular pyranocoumarins. Candidate unigenes in the transcriptome dataset were screened using mainly homology-based BLAST and phylogenetic analyses. It is worthy of mention that the BLAST programs are widely used for searching protein and DNA databases for optimizing sequence similarities [21]. For protein comparisons, several definitional, algorithmic and statistical refinements allow substantial decrease in the execution time of the BLAST programs and enhancement of their sensitivity to weak similarities.



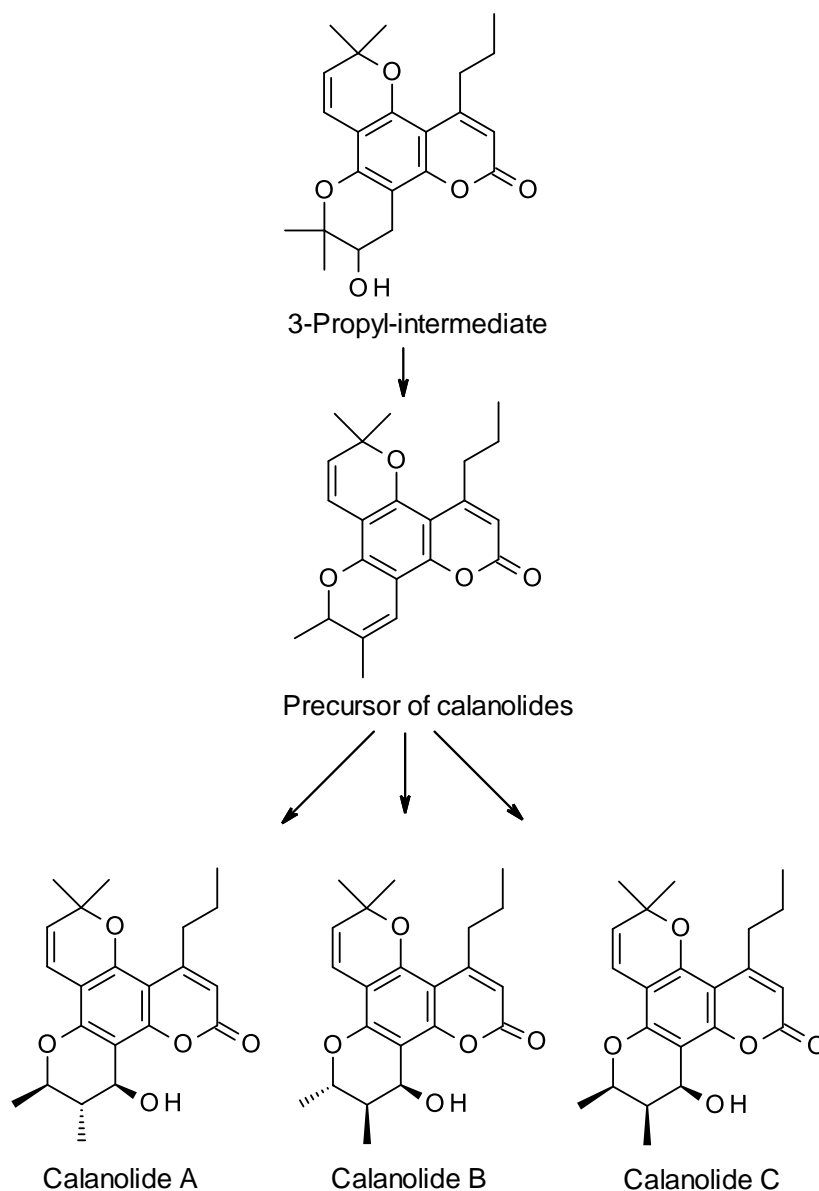
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**Scheme 1.** Plausible biosynthetic route to 2'-hydroxy-dihydrodipetalolactone from umbelliferone via formation of 5-hydroxy-6-prenyl-seselin



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**Scheme 2.** Plausible biosynthetic route to 2'-hydroxy-dihydrodipetalolactone from umbelliferone via formation of 8-prenyl-alloxanthoxyletol



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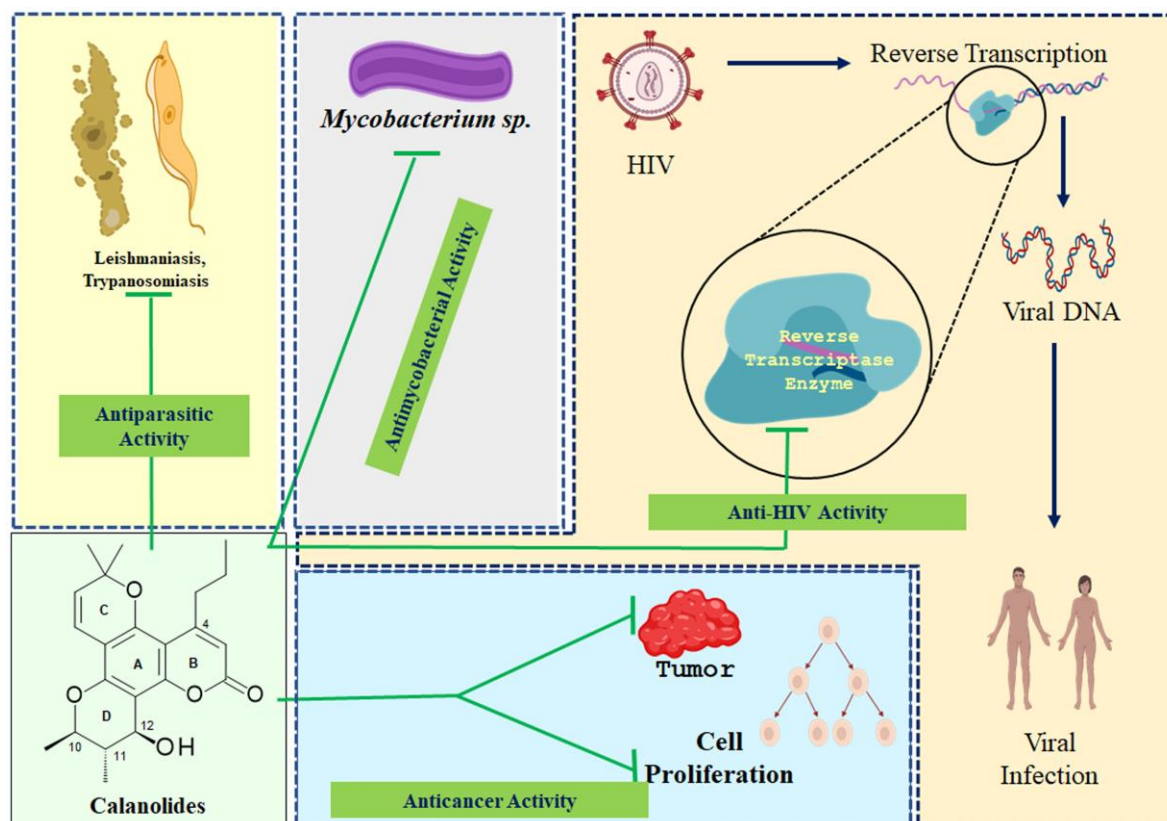
159 **Scheme 3.** Plausible biosynthetic route to calanolides A (1), B (4) and C (6) from the intermediate,  
160 2'-hydroxy-dihydrodipetalolactone

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#### 4. Pharmacological properties

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Although well-known for non-nucleoside reverse transcriptase inhibitory activity offering anti-HIV potential, calanolides have also been shown to possess various other pharmacological properties (Figure 3). The following sub-sections deal with anticancer, anti-HIV, antimycobacterial and antiparasitic activity of naturally occurring calanolides. As much of the published pharmacological studies, both *in vitro* and *in vivo* including human trials, on naturally occurring calanolides are about their anti-HIV property, over the years, significant amounts of information have become available on their mechanism of action, structure-activity-relationships, synergistic and/or additive property and their potential in anti-HIV combination therapy, which have been discussed adequately under individual headings within the anti-HIV sub-section. All other pharmacological properties of these compounds as outlined in different publications still require further investigations to establish their realistic therapeutic potential. Also, *in silico* pharmacological activity and toxicity studies on these pyranocoumarins have just begun to emerge in recent years.



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**Figure 3.** A pictorial summary of pharmacological properties of naturally occurring calanolides

#### 177 4.1 Anticancer activity

178 In the later part of 1980s, as a part of the initiative of the United States National Cancer Institute  
 179 (NCI), plant samples from the Malaysian flora were collected for routine screening for potential  
 180 cytotoxicity against a collection of cancer cell lines as well as for possible anti-HIV activity. One of  
 181 the samples, the leaves and twigs of the tree *C. lanigerum* var. *austroriciaceum*, despite not being active  
 182 against any of the cancer cell lines tested, showed inhibitory activity of viral replication when tested  
 183 against HIV-1 virus [22, 23]. However, later, calanolide A (1) and calanolide C (6) were shown to  
 184 possess antiproliferative or antitumor-promoting property through inhibition of TPA-induced EBV-  
 185 EA activation in Raji cell lines [13]. The phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (*TPA*) is  
 186 a potent stimulator of differentiation and apoptosis in myeloid leukemia cells. Calanolide A (1) was  
 187 found to be more active ( $IC_{50} = 290$  mol ratio/32 pmol *TPA*) than its 10,11-*cis*-isomer, calanolide C (6)  
 188 ( $IC_{50} = 351$  mol ratio/32 pmol *TPA*). It was inferred that 4-substituted pyranocoumarins like  
 189 calanolides might possess potential as cancer chemopreventive agents or antitumor-promoters. A  
 190 recent study with the crude ethanolic extract of the leaves of *C. inophyllum* revealed its potential as a  
 191 cytotoxic agent ( $IC_{50}$  120  $\mu$ g/mL) against the breast cancer cell line MCF-7 [24]; it was also found to  
 192 possess antiproliferative and apoptotic properties. However, no definitive proof was provided to  
 193 establish which of the secondary metabolites biosynthesized by this plant, calanolides being one  
 194 major class, were responsible for the putative anticancer activity. Although not calanolides, a few  
 195 other 4-substituted coumarins, isolated from *C. brasiliense*, were tested against human leukemia HL-  
 196 60 cells with some promising results [25], which might highlight the need for more comprehensive  
 197 studies with all major 4-substituted coumarins, including calanolides, to find antileukemia lead  
 198 compounds for new anticancer drug development. Calanolide A (1), isolated from a chloroform  
 199 extract of *Clausena excavata*, was found to induce toxicity to the cells used in a syncytium assay for  
 200 anti-HIV activity [10].

201 The efficacy of calanolide A (1) in AIDS-associated cancer was evaluated *in silico* utilizing an  
 202 integrated approach combining network-based systems biology, molecular docking and molecular

203 dynamics [26]. Molecular targets were screened and only the targets, e.g., HRAS, that are common to  
204 HIV and sarcoma, HIV and lymphoma, and HIV and cervical cancer, were utilized in this study.  
205 Calanolide A (1) was found to form a stable complex with the screened target HRAS, which is a small  
206 G protein in the RAS subfamily of the RAS superfamily of small GTPases, and is considered as a  
207 proto-oncogene; when mutated, this proto-oncogene has the potential to cause normal cells to  
208 become cancerous.

#### 209 4.2 Anti-HIV activity

210 Calanolide A (1), an anti-HIV non-nucleoside reverse transcriptase inhibitor (NNRTI), paved the  
211 way for the discovery and synthesis of a series of 4-substituted angular pyranocoumarins with  
212 potential anti-HIV property [1, 27]. NNRTIs are a class of anti-HIV drugs that prevent healthy T-cells  
213 in the body from becoming infected with HIV. Kashman et al. [1] first reported this new class of anti-  
214 HIV agents from the tropical rainforest tree, *C. lanigerum*. Calanolide A (1), 12-acetoxycalanolide A  
215 (2), 12-methoxycalanolide A (3), calanolide B (4), 12-methoxycalanolide B (5), pseudocalanolide C (8),  
216 pseudocalanolide D (9) and calanolide E (10) (Figure 2) were isolated through an anti-HIV bioassay-  
217 guided isolation. Calanolides A (1) and B (4) were found to be protective against HIV-1 replication  
218 and cytopathicity with EC<sub>50</sub> values of 0.1 µM and 0.4 µM, respectively. However, both compounds  
219 were inactive against HIV-2, which is known as less pathogenic than HIV-1 and mainly found in  
220 West African countries. The other compounds showed a low level of anti-HIV-1 activity. This study  
221 involving purified bacterial recombinant reverse transcriptases established that the calanolides are  
222 indeed HIV-1 specific reverse transcriptase inhibitors. A comparative report on the anti-HIV  
223 potentials of calanolide A (1), costatolide (14) and dihydrocostatolide (16) against a series of HIV  
224 isolates of different cellular phenotypes was published by Buckheit et al. [28], which clearly  
225 demonstrated that calanolide A (1) was the best anti-HIV candidate among the three calanolides  
226 tested.

227 Two analogs of calanolide A (1), i.e., costatolide (14) and dihydrocostatolide (16), were shown to  
228 possess anti-HIV property similar to that of calanolide A (1) [28] and could be ascribed to the class of  
229 NNRTIs. In fresh human cells, costatolide (14) and dihydrocostatolide (16) could significantly inhibit  
230 the low-passage clinical virus strains, including those representative of the various HIV-1 clade  
231 strains, syncytium-inducing and non-syncytium-inducing isolates, and T-tropic and monocyte-tropic  
232 isolates [28, 29]. In continuation of the search for new natural anti-HIV compounds, McKee et al. [16]  
233 purified calanolide E2 (12), and calanolide F (13) from extracts of *C. lanigerum* var. *austrororiaceum*  
234 and *C. teysmanii* var. *inophylloide* (King.) P. F. Stevens, and calanolide E2 (12) emerged as one of the  
235 most active anti-HIV compounds. Later, costatolide (14) was reported as an anti-HIV compounds  
236 present in *C. cerasiferum* Vesque and *C. inophyllum* L. [17], while calanolides A (1), and C (6), and  
237 costatolide (14), isolated from the leaves of *C. brasiliense*, were shown to possess anti-HIV potential  
238 [4]. Comparative anti-HIV activities of some naturally occurring calanolides, e.g., calanolide A (1),  
239 costatolide (14) and dihydrocostatolide (16), against various strains of HIV are available in the article  
240 by Buckheit, et al. [28].

##### 241 4.2.1 Activity against drug resistant strains of HIV-1

242 Interestingly, calanolide A (1) was not only found to be active against standard strains of HIV-  
243 1, but it was also active against the resistant strains, eAZT-resistant G-9106 strain of HIV-1 and  
244 pyridinone-resistant A17 strain [1, 30]. The activity against the pyridinone-resistant A17 strain was  
245 of interest as this strain is highly resistant to most of the HIV-1 specific NNRTIs, for example, TIBO,  
246 BI-RG-587 and L693,593. Later, it was established that pyranocoumarin 1 could interact with HIV-1  
247 reverse transcriptase within the previously defined common binding site for nonnucleoside  
248 inhibitors [30]. An assessment of the inhibition patterns of the chimeric reverse transcriptases  
249 containing complementary segments of HIV-1 and HIV-2 reverse transcriptases established that there  
250 was a segment between residues 94 and 157 in HIV-1 reverse transcriptase that was crucial for  
251 inhibition by calanolide A (1) [31]. However, it was assumed that there might be a second segment,

252 essential for specifying susceptibility to the drug, between amino acids 225 and 427 in HIV-1 reverse  
253 transcriptase. A couple of years later, it was noted that calanolide A (**1**) was active against virus  
254 isolates resistant to 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine and its derivative, [1-  
255 benzyloxymethyl-5-ethyl-6-(alpha-pyridylthio)uracil] [32]. Furthermore, this pyranocoumarin (**1**)  
256 showed activity against HIV with the two most common NNRTI-related mutations, K103N and  
257 Y181C, and was found to select for a mutation that does not cause cross-resistance with any other  
258 NNRTIs under investigation. It was postulated that substitution at codon Y188H of reverse  
259 transcriptase could be associated with 30-fold resistance to calanolide A (**1**) *in vitro* [33]. The  
260 compound is essentially inactive against all strains of the less common HIV type 2. It is necessary to  
261 carry out appropriate *in vivo* experimentations, either in animal models or in human clinical trials, to  
262 understand the true potential of any putative drug candidate. *In vivo* anti-HIV activity of (+)-  
263 calanolide A (**1**) was assessed in a hollow fibre mouse model [34], and it was observed that this  
264 compound could suppress virus replication in two unique, but separate physiologic compartments  
265 following oral or parenteral administration.

266 Calanolides were found to possess an enhanced antiviral activity against one of the most  
267 prevalent NNRTI-resistant viruses that is engendered by the Y181C amino acid change in reverse  
268 transcriptase as well as with reverse transcriptases that possess the Y181C change together with AZT-  
269 resistant mutations [28, 29]. Calanolides could also be active against viruses containing Y181C and  
270 K103N dual mutations, which are generally highly resistant to other known non-nucleoside reverse  
271 transcriptase inhibitors. Anti-HIV activity of naturally occurring calanolides against drug-resistant  
272 strains of HIV have made these compounds promising structural templates for new anti-HIV drug  
273 development.

#### 274 4.2.2 Calanolides in anti-HIV-1 combination therapy

275 For the treatment of HIV infections, use of combination therapy comprising several anti-HIV  
276 drugs has become a common practice in recent years. The synergistic effects of calanolide A (**1**),  
277 costatolide (**14**) and dihydrocostatolide (**16**) [28] in combination with established anti-HIV drugs, e.g.,  
278 azidothymidine (AZT), indinavir, nelfinavir and saquinavir, are available in the literature [28].  
279 Synergistic effects were observed in both cultured cells and animal models when calanolides were  
280 used in combination with other anti-HIV agents [35]. Both calanolide A (**1**) and costatolide (**14**) were  
281 found to be effective in combination therapy for HIV infections [36]; in combination with NNRTIs,  
282 costatolide (**14**) could only synergistically inhibit HIV type 1 with UC38, whilst calanolide A (**1**) in  
283 combination with one of the NNRTIs helped this drug to retain activity against virus isolates with  
284 the single Y181C mutation [28, 33, 36, 37].

285 A combination of (+)-calanolide A (**1**) and nevirapine (marketed under the trade name viramune  
286 among others for the treatment and prevention HIV-1 infection) was found to possess an additive to  
287 weakly synergistic effect in blocking replication of HIV-1 in an *in vitro* tissue culture assay [33],  
288 indicating the possibility of using (+)-calanolide A (**1**) in anti-HIV-1 combination therapy. In an *in*  
289 *vivo* study using a hollow fibre mouse model [34], the synergistic potential of (+)-calanolide A (**1**) in  
290 combination therapy with AZT, a well-known anti-retroviral medication, was further established. A  
291 more comprehensive study on the anti-HIV activity of (+)-calanolide A (**1**) and its analogs, e.g.,  
292 costatolide (**14**), dihydrocostatolide (**16**) and (+)-12-oxo-calanolide A, in combination with other  
293 inhibitors of HIV-1 replication was published about a decade ago [29, 38]. Calanolides were found to  
294 display synergistic antiviral interactions with other nucleoside and non-nucleoside reverse  
295 transcriptase inhibitors and protease inhibitors. In addition, additive interactions were also observed  
296 with calanolides when used with other anti-HIV drugs. It was concluded that the utility of convergent  
297 and divergent combination therapies using reverse transcriptase inhibitors and protease inhibitors in  
298 combination with (+)-calanolide A (**1**) or one of its analogues could be clinically relevant. Budihas et  
299 al. [39] demonstrated significant synergy between  $\beta$ -thujaplicinol and calanolide A (**1**).

## 301 4.2.3 Structure-activity-relationships (SAR)

302 Among the naturally occurring calanolides, calanolide A (**1**) is one of the most potent anti-HIV  
303 compounds and has been the focus of various studies including the study of its possible mechanism  
304 of action, structural modifications, pharmacokinetics and toxicity [9, 40-43]. The structures of  
305 naturally occurring calanolides mainly differ in their stereochemistry at various chiral centers (C-10,  
306 C-11 and C-12) on the ring D (Figures 1 and 2). McKee et al. [16] reported that calanolide-type  
307 compounds with a 12 $\beta$  hydroxyl group (as in compound **1**) generally possess anti-HIV activity. While  
308 calanolide A (**1**) and costatolide (**14**) were found to be active, (+)-calanolide C (**6**) was inactive in the  
309 *in vitro* anti-HIV assay [4, 17]. The inactivity of (+)-calanolide C (**6**) despite possessing the  
310 pharmacophoric ring D, as well as a propyl group on C-4, could be due to the  $\beta$ -*cis* orientation of  
311 methyl groups on C-10 and C-11.

312 Like any other optically active drug molecules, optical activity plays an important role in the  
313 anti-HIV activity of calanolides. It has long been established that (+)-calanolide A (**1**) and (-)-  
314 calanolide B (**14**) are potent HIV-1 inhibitors, whilst (-)-calanolide A and (+)-calanolide B (**4**) are  
315 inactive against the virus [44]. It should be mentioned here that (+)-calanolide A (**1**) is the natural  
316 product, but its enantiomer (-)-calanolide A was prepared from the naturally occurring (-)-costatolide  
317 (**14**), isolated from *C. costatum*. Similarly, to establish structure-activity-relationships of calanolides,  
318 several analogs of calanolides have been synthesized to date, and tested in anti-HIV assays [45].  
319 Although the synthesis of calanolides and the anti-HIV activity of synthetic calanolide analogs are  
320 not within the scope of this review, a few examples are given here in the context of structure-activity-  
321 relationships. One of the first attempts in this area was from Galinis et al. [45], where  $\Delta^{7,8}$  olefinic  
322 bonds within (+)-calanolide A (**1**) and (-)-calanolide B (**14**) were reduced, and C-12 hydroxyl group in  
323 (-)-calanolide B (**14**) was modified to investigate variations in anti-HIV activity compared to parent  
324 calanolides. In this study, none of the 14 derivatives was found to possess superior activity to parent  
325 calanolides but revealed some preliminary structure-activity requirements for anti-HIV potencies.  
326 Later, in order to identify the structural features of naturally occurring (+)-calanolide A (**1**) necessary  
327 for its anti-HIV activity and to prepare synthetic analogues, oxo-derivatives (+)-, (-)- and ( $\pm$ )-12-  
328 oxocalanolides, were synthesized and tested *in vitro* using a biochemical reverse transcriptase  
329 inhibition assay for determining anti-HIV activity with a promising outcome [40]. In a review article  
330 covering various aspects of anti-HIV 4-substituted coumarins with an alkyl or a phenyl group as the  
331 substituent, isolated from the genus *Calophyllum*, summarized that all *trans* configurations (10*R*, 11*S*,  
332 12 *S*), as in (+)-calanolide A (**1**) and (+)-inophyllum B (a 4-phenyl-substituted pyranocoumarin), are  
333 essential for the best anti-HIV activity [46].

334 Most of the SAR studies involving calanolides for their anti-HIV activities concentrated on the  
335 three chiral centers at C-10, C-11 and C-12 of (+)-calanolide A (**1**) [47, 48]. As the number of naturally  
336 occurring calanolides are rather limited (calanolides A-F) (Figure 2), the SAR studies were often  
337 carried out with natural calanolides as well as their synthetic analogs. Of the diastereomers,  
338 compounds containing 10,11-*trans*-methylation and 12-(*S*)-OH chirality (Figure 2) displayed the most  
339 potent activity with EC<sub>50</sub> values in between 0.18 and 2.0  $\mu$ M [47]. It was also observed that either the  
340 enantiomers (12-*R*-OH) or epimeric alcohols, *e.g.*, calanolide C (**6**) could not produce any noticeable  
341 anti-HIV effect. It could be concluded that the relative stereochemistry at C-10 and C-11 are essential  
342 structural features for potent anti-HIV activity of calanolides, and at the same time, the *S*  
343 configuration at C-12 as well as the presence of a heteroatom, *e.g.*, O, at C-12 are necessary for anti-  
344 HIV effects.

345 In order to assess the importance of the presence of 11-methyl functionality on calanolide A for  
346 its anti-HIV activity, the activity of the semi-synthetic racemic mixture of 11-demethyl-calanolide A  
347 was compared with the anti-HIV activity of its parent compound, ( $\pm$ )-calanolide A [49]. The *in vitro*  
348 HIV-1 reverse transcriptase inhibitory activity of these compounds was determined with the isotope  
349 <sup>3</sup>H assay, which is a thymidine incorporation assay that often utilizes a strategy wherein a radioactive  
350 nucleoside, <sup>3</sup>H-thymidine, is incorporated into new strands of chromosomal DNA during mitotic cell  
351 division; a scintillation beta-counter is used to measure the radioactivity in DNA recovered from the

352 cells in order to determine the extent of cell division that has occurred in response to a test agent. The  
353 cytotoxicity and inhibition of cytopathic effect of ( $\pm$ )-calanolide A and ( $\pm$ )-11-demethyl-calanolide A  
354 were studied in HIV-1 IIIB infected MT-4 cell cultures by the MTT staining method. Both compounds  
355 inhibited HIV-1 reverse transcriptase *in vitro* with IC<sub>50</sub> value of 3.028  $\mu$ M/L and 3.965  $\mu$ M/L,  
356 respectively, for ( $\pm$ )-11-demethyl-calanolide and ( $\pm$ )-calanolide A. They also inhibited cytopathic  
357 effect in HIV-1 IIIB infected MT-4 cell cultures with IC<sub>50</sub> values of 1.081 and 1.297  $\mu$ M/L, respectively.  
358 The outcome from this study indicated that ( $\pm$ )-11-demethyl-calanolide had a slightly more potent  
359 anti-HIV activity than ( $\pm$ )-calanolide A, suggesting the methyl functionality at C-11 in calanolide A  
360 (**1**) might not be an essential structural feature for anti-HIV activity. With the help of synthetic  
361 analogues a few other structural features that could impact on the anti-HIV activity of calanolides  
362 could be identified. Some of those are summarized below:

- 363 i.  $\Delta^{11,12}$  Olefination diminishes activity.
- 364 ii. A C-12 hetero atom is essential for the activity.
- 365 iii. Relative potencies of C-12 ketone, thiol, azide, amine, and acetylated derivatives suggest  
366 stringent spatial and stereochemical requirements around C-12.
- 367 iv. The enantiomers of 12-oxocalanolide A, synthetic intermediates containing one fewer  
368 chiral center, still retain anti-HIV potency in the cytopathic assays.
- 369 v. The oxygen substituent can either be in the plane of the aromatic system or possess S  
370 configuration.
- 371 vi. Optical activity is important. For example, (+)-12-oxocalanolide A and ( $\pm$ )-12-  
372 oxocalanolide A have similar (but not same) anti-HIV activity, but (-)-12-oxocalanolide  
373 A is much less active.
- 374 vii. The racemic form, for example, ( $\pm$ )-12-oxocalanolide A, is more active than its pure (+)-  
375 enantiomer, (+)-12-oxocalanolide A, which suggests a possible synergistic effect in the  
376 combination of the two enantiomers.
- 377 viii. Hydrogenation at C-7 and C-8 of calanolides has little effect on the anti-HIV activity,  
378 e.g., the dihydro derivatives of calanolides A (**1**) and B (**4**) possess the same activity as  
379 the parent calanolides.
- 380 ix. Modifications at C-4 substituent can affect the anti-HIV activity of calanolides. For  
381 example, a methyl substituent at C-4 (as in cordatolides), instead of a propyl function as  
382 in calanolides reduces the anti-HIV potency.
- 383 x. Both the surface area of the substituted group attached on C-10, S-R3, and the distance  
384 between atoms O-13 and X-14 (O, N, S), L, of the calanolide analogues play important  
385 roles in determining the inhibitory activity of HIV-1 [48].  
386

387 With the advent of various modern computational tools and mathematical models, it is now  
388 possible to study quantitative structure activity relationships (QSAR) *in silico*, and to predict the  
389 potential of any drug candidates for any therapeutic application [50]. A Caco-2 cell permeability  
390 QSAR model has recently been used to study various HIV-1 reverse transcriptase inhibitors,  
391 including (+)-calanolides A (**1**) and B (**4**), both of which showed a high degree of permeability [51].  
392 This parallel computational screening method incorporated approaches of intestinal absorption  
393 prediction, receptor affinity estimation, inhibitor shape similarity, lipophilicity, and index-based  
394 lipophilic efficiency analyses. Calanolide A (**1**), among a few other HIV-1 reverse transcriptase  
395 inhibitors, emerged as one of the prioritized hits, as a result of guided prioritization task by the better  
396 binding affinity, crystal ligand similarity, permissible log*P* value and top lipophilic ligand efficiency  
397 scores.

#### 398 4.2.4 Mechanism of action

399 The evaluation of the activity of (+)-calanolide A (**1**) against reverse transcriptase and  
400 nonnucleoside reverse transcriptase inhibitor-resistant viruses and enzyme kinetic studies for reverse  
401 transcriptase inhibition suggest that this coumarin possibly interacts with the HIV-1 reverse



transcriptase in a fashion mechanistically different from other known NNTRIs. The biochemical mechanism of inhibition of HIV-1 reverse transcriptases by calanolide A (1) was studied using two primer systems, ribosomal RNA and homopolymeric rA-dT(12-18) [52]. Calanolide A (1) was found to bind near the active site of the enzyme and interfered with dNTP binding; it inhibited HIV-1 reverse transcriptase in a synergistic fashion with nevirapine, further distinguishing it from the general class of NNRTIs. It was also observed that at certain concentrations, this compound could bind HIV-1 reverse transcriptase in a mutually exclusive manner with respect to both the pyrophosphate analog, phosphonoformic acid and the acyclic nucleoside analogue 1-ethoxymethyl-5-ethyl-6-phenylthio-2-thiouracil. It was concluded that calanolide A (1) could share some binding domains with both phosphonoformic acid and 1-ethoxymethyl-5-ethyl-6-phenylthio-2-thiouracil. It might interact with reverse transcriptase near both the pyrophosphate binding site and the active site of the enzyme. Later, the same group of researchers studied possible mechanism of action of action of calanolide A (1) against the HIV type 1 including a variety of laboratory strains, with EC<sub>50</sub> values of 0.10-0.17 μM [52]. Calanolide (1) could inhibit promonocytotropic and lymphocytotropic isolates from patients in various stages of HIV disease, and drug-resistant strains, and was found to act early in the infection process like the known HIV reverse transcriptase inhibitor 2', 3'-dideoxycytidine. It could selectively inhibit recombinant HIV type 1 reverse transcriptase but not cellular DNA polymerases or HIV type 2 reverse transcriptase. Auwerx et al. [42] studied the possible role of Thr139 in the HIV-1 reverse transcriptase sensitivity to (+)-calanolide A (1). As T139I reverse transcriptase proved to be resistant to (+)-calanolide A (1), represents a catalytically efficient enzyme, and requires only a single transition point mutation (ACA→ATA) in codon 139 could provide some explanation as to why mutant T139I reverse transcriptase virus strains, but not the other strains containing other amino acid changes at this position, predominantly emerge in cell cultures under (+)-calanolide A (1) pressure.

Calanolides are non-nucleoside reverse transcriptase inhibitors and mediate their inhibitory effect in two different template primer systems: primed ribosomal RNA template, and homopolymeric poly rA-oligoT<sub>12-18</sub> primer. Calanolide A (1) was found to inhibit reverse transcriptase by involving two binding sites, and the action is because of the bi-bi ordered mechanism of reverse transcriptase, requiring primer binding prior to polymerization [47]. Calanolide A (1) can bind HIV-1 reverse transcriptase in a mutually exclusive manner with the pyrophosphate analogues phosphoformic acid or 1-ethoxymethyl-5-ethyl-6-phenylthio-2-thiouracil. This indicates that calanolide A (1) can interact with reverse transcriptase near the pyrophosphate binding site as well as the active site. Unlike general non-nucleoside reverse transcriptase inhibitors, calanolide A (1) appears to be at least partially competitive inhibitor of dNTP binding. Clinical and laboratory assessment on viral load and CD4 count indicated that antiviral effects of calanolide A (1) appeared to be dose-dependent and maximized on day 14 or 16. Viral life-cycle studies indicated that calanolide A (1) could act early in the infection process, similar to the known HIV reverse transcriptase inhibitor 2', 3'-dideoxycytidine. In enzyme inhibition assays, calanolide A (1) could potently and selectively inhibit recombinant HIV type 1 reverse transcriptase but not cellular DNA polymerases or HIV type 2 reverse transcriptase within the concentration range tested.

#### 4.3 Antimycobacterial activity

The antibacterial (against *Bacillus cereus*, *B. pumilius*, *B. subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus* and *Vibrio cholerae*) and antifungal (against *Alternaria tenuissima*, *Aspergillus fumigatus*, *Aspergillus niger*, *Candida albicans* and *Candida tropicalis*) properties of *Calophyllum* species and their bioactive secondary metabolites, including calanolides, are already known [6, 15, 53-59]. Kudera et al. (2017) [58] reported *in vitro* growth inhibitory activity of *C. inophyllum* extract against diarrhea-causing microorganisms, e.g., *Clostridium difficile infant*, *Clostridium perfringens*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica*. The extract was particularly active against *C. perfringens* and *L. monocytogenes* (MIC = 128 μg/mL). Later, calanolide E (10) was isolated from *C. wallichianum* and tested for its anti-*Bacillus*

452 activity against *Bacillus cereus*, *B. megaterium*, *B. pumilus* and *B. subtilis* [16]. However, calanolide E  
453 (10) was not bactericidal on the tested *Bacillus* species, and at the tested concentration.

454 Based on the initial findings on promising antimicrobial properties of calanolides and  
455 *Calophyllum* extracts, efforts have recently been directed to the study on the effect of these compounds  
456 on the acid-fast bacillus *Mycobacterium tuberculosis* that causes tuberculosis [60–62]. As over the years  
457 several antibiotic resistant and multidrug-resistant *M. tuberculosis* strains have emerged, and  
458 complicated the existing treatment modalities for tuberculosis, and there has been a recent increase  
459 in incidents of tuberculosis globally observed, the need for new effective, safe and affordable  
460 antimycobacterial drugs has become paramount. *Calophyllum brasiliense* extract was reported to be  
461 active against *M. tuberculosis* (IC<sub>50</sub> 3.02–3.64 µg/mL), and a follow up HPLC analysis of the active  
462 extract provided evidence of presence of calanolides and the antimycobacterial activity induced by  
463 *C. brasiliense* was attributed mainly to calanolides A (1) and B (4) [62]. Earlier, Xu et al. [60]  
464 demonstrated that calanolide A (1), from Colombian *C. lanigerum*, was active against both drug-  
465 susceptible and drug-resistant strains of *Mycobacterium tuberculosis*, e.g., H37Ra (ATCC 25177),  
466 H37Rv (ATCC 27294), CSU 19, CSU 33, H37Rv-INH-R (ATCC 35822), CSU 36, CSU 38 and H37Rv-  
467 EMB-R (ATCC 35837). Efficacy evaluations in macrophages established that this pyranocoumarin  
468 could inhibit intracellular replication of *M. tuberculosis* at concentrations below the minimum  
469 inhibitory concentration (MIC) determined *in vitro*. It was postulated that calanolide A (1), like the  
470 antitubercular drug rifampicin, could rapidly inhibit RNA and DNA synthesis followed by an  
471 inhibition of protein synthesis, and could lead to the generation of a new class of pyranocoumarin-  
472 based antitubercular drugs. In this study, the natural calanolides A (1), B (4) and D (7), as well as their  
473 semisynthetic analogues were tested, and (+)-calanolide A (1) and the semisynthetic analogue, 7,8-  
474 dihydrocalanolide B emerged as most effective against tuberculosis with the MIC value of 3.13  
475 µg/mL. While (-)-calanolide B (14) was moderately effective, calanolide D (7) was found inactive at  
476 the highest tested concentration of 12.5 µg/mL. In fact, calanolides, especially calanolide A (1), is  
477 unique in a sense that these compounds have anti-HIV property and were found to be active against  
478 *M. tuberculosis* (MIC = 3.1 µg/mL) and an array of drug-resistant strains (MIC = 8–16 µg/mL). The  
479 antimycobacterial activity of calanolide A (1) is comparable to that of the well-known anti-tubercular  
480 drug isoniazid, and effective against rifampicin- and streptomycin-resistant *M. tuberculosis* strains. A  
481 recent patent described potent antimycobacterial property of calanolides and their analogs and  
482 provided a method of using these compounds for the treatment and prevention of mycobacterial  
483 infections [63].

#### 484 4.4 Antiparasitic activity

485 Traditionally, natural products, especially in crude forms, have long been used to treat various  
486 parasitic diseases, like babesiosis, leishmaniasis, malaria, trypanosomiasis and so on. Recently,  
487 leishmaniasis and trypanosomiasis have been in research focus of natural products researchers,  
488 aiming at discovering new drug candidates to treat these neglected diseases [64, 65]. Extracts of *C.*  
489 *brasiliense* and *C. inophyllum* and calanolides were shown effective against intracellular parasites  
490 causing American trypanosomiasis and leishmaniasis [6]. In a recent study, Silva et al. (2020) [14]  
491 demonstrated that the MeOH extract from stem bark of *C. brasiliense* was active against amastigote  
492 forms of *Trypanosoma cruzi* and *Leishmania infantum*. Bioactivity-guided purification of the extra  
493 afforded calanolides E1 (11) and E2 (12), which were found to be active against *T. cruzi* (EC<sub>50</sub> values  
494 of 12.1 and 8.2 µM, respectively) and *L. infantum*, (EC<sub>50</sub> values of 37.1 and 29.1 µM, respectively) *in*  
495 *vitro*. Calanolide E1 (11) displayed the best selectivity index (SI) with values >24.4 to *T. cruzi* and >6.9  
496 to *L. infantum* in comparison to calanolide E2 (12). It was concluded that these coumarins could be  
497 utilized as scaffolds for the design and development of novel drug candidates to treat Leishmaniasis  
498 and Chagas diseases.

499

500

## 501 5. Toxicological aspects including pharmacokinetics

502 Among the naturally occurring calanolides (Figure 2), calanolide A (**1**), a specific nonnucleoside  
503 inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, first isolated from  
504 a tropical tree *C. lanigerum* that grows abundantly in the Malaysian rain forest, is the most-studied  
505 compound in terms of its pharmacology, toxicology and synthesis. A series of animal studies [35]  
506 involving mice, rats and dogs established that calanolide A (**1**) is generally well-tolerated at oral doses  
507 of up to 150 mg/kg in rats and 100 mg/kg in dogs, and possesses a good safety profile [66, 67].  
508 Calanolides A (**1**), B (**4**) and C (**6**) were found to be nontoxic in mice ( $LD_{50} = 1.99$  g/kg), and no  
509 alternation on hepatocytes could be observed during the histological study of the mice treated with  
510 the highest dose applied [67]. During a study looking at the anti-HIV efficacy and toxicity of  
511 calanolides when used in combination with other anti-HIV drugs, no noticeable toxicity could be  
512 detected [38].

513 In the very first study on the safety and pharmacokinetics of calanolide A (**1**) in healthy HIV-  
514 negative human volunteers revealed that the toxicity of calanolide A (**1**) was minimal in the majority  
515 of subjects treated with four successive single dose, 200, 400, 600 and 800 mg. While there were no  
516 acute serious or life-threatening adverse effects were observed, among the usual minor adverse  
517 effects, dizziness, oily taste, headache, eructation, and nausea were noticed, but were of minimal  
518 clinical significance. These adverse effects were non-dose-dependent [66]. In this study, it was found  
519 that calanolide A (**1**) was rapidly absorbed following administration, with time to maximum  
520 concentration of drug in plasma ( $T_{max}$ ) values, depending on the doses, occurring between 2.4 and 5.2  
521 h. It was noted that the levels of calanolide A (**1**) in human plasma were higher than would have been  
522 predicted from animal studies, but the safety profile was benign. However, taking calanolide A (**1**)  
523 with food was found to generate significant variability in pharmacokinetics, but with no detectable  
524 interaction with food. Later, these findings were further confirmed by another similar study carried  
525 out by Eiznhamer et al. [68]. Calanolide A (**1**), the first member of the new family of NNRTIs, was  
526 found to have long elimination half-life, the benign toxicity profile, to achieve trough plasma levels  
527 approximating the  $EC_{90}$  of calanolide A (**1**) for HIV-1, to have the potential for twice daily dosing,  
528 and to offer the unique HIV-1 resistance profile could make this compound an attractive candidate  
529 for further clinical studies. It was reported that after oral administration, (+)-calanolide A (**1**) was  
530 generally well tolerated and indication of any safety concern could be observed [40]. Its plasma  
531 concentrations in humans were higher than anticipated from animal data. The AUC and  $C_{max}$  values  
532 increased with increasing dose, and it appeared that therapeutic levels could easily be achieved in  
533 humans.

534 A comparative study on the relative pharmacokinetic parameters and bioavailability of  
535 calanolide A (**1**) and its synthetic analogue dihydrocalanolide A (**15**) was reported [69]. This study  
536 compared the intravenous pharmacokinetics of the dihydro analog relative to the parent compound,  
537 calanolide A (**1**), and determined the relative oral bioavailability of each drug in CD2F1 mice. Both  
538 compounds displayed similar pharmacokinetic parameters, but the oral bioavailability of the dihydro  
539 analogue was considerably better (almost 3.5-fold) than calanolide A (**1**). Moreover, the relative  
540 ability of calanolide A (**1**) and its dihydro analog to change to their inactive epimer forms, (+)-  
541 calanolide B (**4**) and (+)-dihydrocalanolide B, respectively, was also determined; while conversion of  
542 active calanolides to inactive forms occurred *in vitro* especially under acidic conditions, no epimers  
543 of either compound were observed in plasma of mice after administration of either (+)-calanolide A  
544 (**1**) or (+)-dihydrocalanolide A (**15**). It was suggested that the selection of the dihydro derivative of  
545 calanolide A (**1**) could be a reasonable choice for further preclinical development and possible Phase  
546 I clinical evaluation as an oral drug candidate for the treatment of HIV infection. Calanolide A (**1**)  
547 was shown to be distributed readily into the brain and lymph [47]. The distribution and elimination  
548 pattern of calanolide A (**1**) and its 7,8-dihydro derivative were found to be similar, but the apparent  
549 volume of distribution ( $V_d$ ) and oral clearance of these compounds were significantly different after  
550 oral administration. It was also demonstrated that calanolide A (**1**) is generally well tolerated in doses  
551 up to 600 mg. As evident from animal studies, the gastrointestinal intolerance for this compound is  
552 not severe, but the most common adverse events as observed in human trials of calanolide A (**1**)

553 include an oily after taste and transient dizziness [47]. The calculated half-life of calanolide A (1) from  
554 800 mg dosing was reported to be 20 h [47, 66].

555 During the study directed to the evaluation of antitubercular property of calanolides and their  
556 semisynthetic analogues, the pharmacokinetic data indicated that the (+)-calanolide A (1)  
557 concentrations in plasma could be comparable to the observed *in vitro* MICs against *M. tuberculosis*  
558 [60]. Both calanolides A (1) and B (4) metabolized by cytochrome P450 CYP3A, and their blood  
559 levels could be enhanced if co-administered with ritonavir. Usach et al. [70] reported the safety,  
560 tolerability and pharmacokinetics profiles of calanolide A (1), as a result of a comprehensive Phase I  
561 clinical trial.

## 562 6. Therapeutic potential

563 Naturally occurring calanolides and their synthetic or semi-synthetic analogs have undergone  
564 several pre-clinical and clinical trials for their anti-HIV activity, aiming at novel anti-HIV drug  
565 development [2, 47, 71, 72]. In fact, calanolide A (1) was at an advanced stage of development as an  
566 anti-HIV drug about a decade ago (Singh et al., 2010) [72]. Buckheit [73] reviewed therapeutic  
567 potential of non-nucleoside reverse transcriptase inhibitors like calanolides as anti-HIV and  
568 commented on strategies for the treatment modalities for HIV infections. In fact, NNRTIs opened a  
569 new avenue of treatment of HIV infections, as previously this therapeutic area was predominantly  
570 covered by nucleoside reverse transcriptase inhibitors and protease inhibitors. Soon after the  
571 discovery of calanolides as a potential anti-HIV agents by the NCI/NIH, Sarawak Medichem  
572 Pharmaceuticals synthesized calanolide A (1) and started developing calanolide A (1) as a clinical  
573 drug for the treatment of HIV infections. It was a joint venture between the Sarawak State  
574 Government and Medichem Research Inc.

575 During 2001-2005, an interventional clinical trial was conducted on human volunteers [74],  
576 where patients were randomized to receive (+)-calanolide A (1) or placebo for 21 days. All patients  
577 could elect to receive an open-label, 3-month course of approved retroviral therapy (up to triple-drug  
578 therapy) to be selected by, and administered under the care of, the patients' physicians. If the patient  
579 had no insurance coverage or did not wish to utilize his/her insurance for anti-HIV medications,  
580 Sarawak MediChem Pharmaceuticals provided these medications at no charge for up to three  
581 months. The trial was primarily aimed at the assessment of the safety and effectiveness of (+)-  
582 calanolide A (1) in HIV-infected patients who had never taken anti-HIV drugs. In 2006, Craun  
583 Research, a company established by the Sarawak Government, acquired Sarawak MediChem, and in  
584 2016, Craun Research announced the completion of Phase I clinical trials for calanolide A (1) with  
585 doses of 200 to 800 mg, which initially started in 2013 [70]. In 2017, F18 (10-chloromethyl-11-  
586 demethyl-12-oxo-calanolide A), a synthetic structural analog of calanolide A (1) was shown to have  
587 more potent anti-HIV activity than original molecule, calanolide A (1) [75, 76]. This compound  
588 showed better druggable profile with 32.7% oral bioavailability in rat, tolerable oral single-dose  
589 toxicity in mice, and suppressed both the wild type HIV-1 and Y181C mutant HIV-1 at an EC<sub>50</sub> of 7.4  
590 nM and 0.46 nM, respectively [77]. Furthermore, it was shown that two enantiomers F18, (*R*)-F18 and  
591 (*S*)-F18, had quite similar anti-HIV property, but (*R*)-F18 was more potent than (*S*)-F18 against wild  
592 type virus, K101E mutation and P225H mutation pseudoviruses [75]. However, calanolides,  
593 particularly calanolide A (1) remains as an investigational anti-HIV drug and has not yet been  
594 approved by the FDA or any other drugs regulatory bodies for their commercial pharmaceutical  
595 production.

## 596 7. Patents

597 In 1999, calanolides and related antiviral compounds were patented by the Board of Trustees of  
598 the University of Illinois [78]. The patent covered novel antiviral compounds, calanolides, and their  
599 derivatives that could be isolated from plants of the genus *Calophyllum* in accordance with the  
600 specified method. The patent also included the uses of these compounds and their derivatives alone  
601 or in combination with other antiviral agents in compositions, such as pharmaceutical compositions,

602 to inhibit the growth or replication of a virus, such as a retrovirus, in particular a human  
603 immunodeficiency virus, specifically HIV-1 or HIV-2. Later, another patent, owned by Parker  
604 Hughes Institute, was reported, which described the novel uses of calanolides as Tec family/BTK  
605 (Bruton's tyrosine kinase) inhibitors, methods for their identification, and pharmaceutical  
606 compositions [79]. It can be mentioned here that the BTK inhibitors inhibit the enzyme BTK, which is  
607 a crucial part of the B-cell receptor signaling pathway, and these inhibitors have emerged as a new  
608 therapeutic target in a variety of malignancies, e.g. chronic lymphocytic leukemia and small  
609 lymphocytic lymphoma [80].

## 610 8. Conclusions

611 Non-nucleoside reverse transcriptase inhibitors (NNRTIs), efavirenz, nevirapine and  
612 delavirdine, have become one of the cornerstones of highly active anti-retroviral therapy for HIV  
613 infections. Calanolides, as they belong to this pharmacological class of NNRTIs, and because of their  
614 high safety margins and favorable pharmacokinetic profiles, are ideal candidates for novel anti-HIV  
615 drug development. While several analogues of the naturally occurring calanolides have been  
616 synthesized, a good number of preclinical and clinical trials have been conducted to date, and there  
617 are a few patents published, further work is still required to commercially bring any of the calanolide  
618 candidates, natural or synthetic, to anti-HIV drug market. As calanolides show an excellent  
619 synergistic and additive profile in combination with other anti-HIV drugs, it is assumed that  
620 calanolides can be considered for use in combination therapy for HIV infections.

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