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# Antimicrobial, antimalarial and cytoxicity activities of constituents of a Bhutanese variety of Ajania nubigena

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# Antimicrobial, antimalarial and cytoxicity activities of constituents of a Bhutanese variety of Ajania nubigena

**Abstract**
An investigation of the essential oil (EO) and the crude MeOH extract of a Bhutanese variety of Ajania nubigena using GC/GC-MS and NMR found the following: a) one kg of the dried plant material contained 0.7% w/w EO; b) 44 of the 53 GC-FID peaks of the EO were identified with (3R,6R)-linalool oxide acetate (75.8 %) as the major constituent (chemotype II) and chamazulene as a new sub-chemotype; c) purification of the EO furnished (3R,6R)-linalool oxide acetate (1), chamazulene (2), (E)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4,4]non-3-ene (3), and (Z)-2-(2,4-hexadinylidene)-1,6-dioxaspiro[4,4]non-3-ene (4); d) from the crude MeOH extract, four flavonoid compounds: 1-(4-hydroxyphenyl)propan-1-one (5), oxyanin B (6), luteolin (7) (major) and the luteolin-7-O-β- D-glucoside (8) were isolated; e) among the EO and pure compounds tested for biological activities, compound 7 exhibited a broad range of moderate antiplasmodial, cytoxicity and antimicrobial activities; c) compound 8 showed significant in vitro antiplasmodial activity against P. falciparum strains TM4/8.2 and K1CB1 (multidrug resistant strain) and was identified as a potential antimalarial scaffold; and f) the in vitro antimicrobial and cytotoxicity activities were in alignment with the traditional medical uses of this plant and thus substantiate its use in Bhutanese traditional medicine.

**Keywords**
ajania, nubigena, bhutanese, variety, constituents, antimicrobial, activities, cytoxicity, antimalarial, CMMB

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# Antimicrobial, Antimalarial and Cytoxicity Activities of Constituents of a Bhutanese Variety of *Ajania nubigena*

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An investigation of the essential oil (EO) and the crude MeOH extract of a Bhutanese variety of *Ajania nubigena* using GC/GC-MS and NMR found the following: a) one kg of the dried plant material contained 0.7% w/w EO; b) 44 of the 53 GC-FID peaks of the EO were identified with (3*R*,6*R*)-linalool oxide acetate (75.8 %) as the major constituent (chemotype II) and chamazulene as a new sub-chemotype; c) purification of the EO furnished (*3R*,*6R*)-linalool oxide acetate (**1**), chamazulene (**2**), (*E*)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4,4]non-3-ene (**3**), and (*Z*)-2-(2,4-hexadinylidene)-1,6-dioxaspiro[4,4]non-3-ene (**4**); d) from the crude MeOH extract, four flavonoid compounds: 1-(4-hydroxyphenyl)propan-1-one (**5**), oxyanin B (**6**), luteolin (**7**) (major) and the luteolin-7-*O*-*β*-D-glucoside (**8**) were isolated; e) among the EO and pure compounds tested for biological activities, compound **7** exhibited a broad range of moderate antiplasmodial, cytoxicity and antimicrobial activities; c) compound **8** showed significant *in vitro* antiplasmodial activity against *P. falciparum* strains TM4/8.2 and K1CB1 (multidrug resistant strain) and was identified as a potential antimalarial scaffold; and f) the *in vitro* antimicrobial and cytotoxicity activities were in alignment with the traditional medical uses of this plant and thus substantiate its use in Bhutanese traditional medicine.

**Keywords:** *Ajania nubigena*, esential oil, flavonoid, Bhutanese traditional medicine, antimalarial, antimocrobial, cytotoxicity.

*Ajania* is a relatively small genus of the family Asteraceae with only 28-40 species which are found in Russia and Asia (Bhutan, Nepal, India, Tibet, China and Japan) [1-3]. In Bhutan, only two *Ajania* species: *A. nubigena* and *A. myriantha* have been reported [4]. *A. nubigena* is locally known as *m.khan-d.kar* and contributes to the preparation of at least four important multi-ingredient Essential Traditional Medicine Drugs (ETMDs) or polyherbal formulations [5] including a popular product called *b.dud-rtsi nga-lum* (Five Herbal Ambrosia). 'Five Herbal Ambrosia' is used in spa related health care practices. As an individual plant, it is used in the Bhutanese traditional medicine (BTM) as incense, vulnerary, expectorant, styptic and anti-epistaxis and also for treating abscesses, swelling of limbs, tumor and kidney infections [6].

*A. nubigena* has been ascribed different synonyms/basonyms as *Artemisia nubigena, Dendranthema nubigenum*, *Chrysanthemum nubigenum* and *Tanacetum nubigenum* [7]. Varieties of this plant growing in different regions of India, including Kumaon, Garwal, Uttarakhand and Uttar Pradesh, have been classified based on three main chemotypes of their essential oils (EO) [8-12]. Chemotype I contained bornyl acetate (39.7%) as a major marker constituent of the EO while chemotype II and III contained (3*R*,6*R*)-linalool oxide acetate (69.4%) and (-)-*cis*-chrysanthenol (37.0%) as their principal components, respectively [8].

While the Indian varieties of *A. nubigena* (ascribed under the basonym *Tanacetum nubigenum*) have been studied [8-12], a Bhuatanese variety growing in the extreme vegetation and climatic conditions of the Bhutan Himalaya has not been investigated for its phytochemicals or biological activities, especially for antimalarial activity and cytotoxicity. Moreover, these studies on the Indian varieties of *A. nubigena* reported the analysis of the EO of the fresh plant material, which is contrary to the manner in which this plant is used by the local people in India, Nepal and Bhutan, who use it in its dried form. Differences in the quality and the chemical constituents of these differently prepared plant samples were thus expected. Therefore, in order to scientifically validate the Bhutanese ethnopharmacological uses of this plant in its authentic manner of usage, we initially studied the antimalarial, antimicrobial and cytotoxicity activities of various crude solvent extracts of this dried plant material [6]. These preliminary studies indicated that the plant was a good source of antimalarial and antimicrobial agents. This encouraged us to investigate the chemical components of the EO and the methanol extract of the dried plant material. The EO and the isolated major phytochemicals were assessed for their antimalarial, antimicrobial and cytotoxic activities and the results are discussed here for the first time.

The hydrodistilled essential oil (0.7% w/w or 7 g/1000 g dry weight) component was analysed using GC and GC-MS which detected 53 constituent peaks of the chromatographable fraction of the total injected oil (Table 1). Of these, 44 compounds were identified with (3*R*,6*R*)-linalool oxide acetate (75.8%) as the major constituent, followed by 6-ethenyldihydro-2,2,6-trimethyl-2*H*-pyran-3(4*H*)-one (4.6%), *β*-farnesene (2.9%), epoxylinalol (2.8%), germacrene D (1.4%), bisabolol oxide A (1.2%), chamazulene (1%), (*E*)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4,4]non-3-ene (1%), and (*Z*)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4,4]non-3-ene (1%).

The percent contents of the EO were determined on the basis of their FID responses on GC. All the identified compounds are presented in Table 1.

**Table 1**: EO constituents of aerial parts of *Ajania nubigena*.

| Compound name | KI† | % oil |
|---|---|---|
| *α*-pinine | 934 | 0.1 |
| *β*-citrollene | 947 | 0.1 |
| 1-methylpentyl hydroperoxide | 949 | 0.1 |
| benzaldehyde | 957 | 0.1 |
| *cis*-linalool oxide | 1090 | 0.3 |

| | | |
|---|---|---|
| *β*-linalool | 1098 | 0.2 |
| **6-ethenyldihydro-2,2,6-trimethyl-2*H*-pyran-3(4*H*)-one** | 1108 | **4.6** |
| L-pinocarveol | 1141 | 0.1 |
| L-camphor | 1147 | t |
| pinocarvone | 1164 | 0.1 |
| (-)-borneol | 1168 | 0.3 |
| **epoxylinalol** | 1175 | **2.8** |
| homoveratrole | 1240 | 0.2 |
| **(3*R*,6*R*)-linalool oxide acetate** | 1291 | **75.8** |
| eugenol | 1359 | 0.1 |
| *α*-copaene | 1381 | 0.5 |
| aromadendrene | 1426 | 0.2 |
| ***β*-farnesene** | **1459** | **2.9** |
| unidentified | 1463 | 0.2 |
| 5-ketobornyl acetate | 1470 | 0.1 |
| seychellene | 1473 | 0.1 |
| **germacrene D** | 1482 | **1.4** |
| patchoulene | 1493 | 0.1 |
| unidentified | 1500 | 0.4 |
| *α*-muurolene | 1506 | 0.1 |
| *α*-bisabolene | 1513 | 0.1 |
| epiglobulol | 1522 | 0.4 |
| unidentified | 1529 | 0.4 |
| (*Z*)-*α*-bisabolene epoxide* | 1560 | 0.1 |
| sapathulenol | 1585 | 0.3 |
| caryophyllene oxide | 1592 | 0.1 |
| isoaromadendrene epoxide | 1595 | 0.3 |
| unidentified | 1600 | 0.1 |
| unidentified | 1613 | 0.2 |
| 6-hexadecen-4-yne | 1619 | 0.2 |
| unidentified | 1636 | t |
| cubenol | 1636 | 0.1 |
| unidentified | 1650 | 0.1 |
| 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-cycloheptane* | 1653 | 0.2 |
| eudesm-4(14)-en-11-ol | 1659 | 0.5 |
| *τ*-cadinol | 1662 | 0.3 |
| *β*-santalol | 1669 | 0.3 |
| octahydro-4a,8a-dimethyl-7-(1-methylethyl), 1(2*H*)-napthalenone | 1683 | 0.3 |
| unidentified | 1689 | t |
| 6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-napthalen-2-ol | 1694 | 0.6 |
| **chamazulene** | 1720 | **1.0** |
| **bisabolol oxide A** | 1754 | **1.2** |
| unidentified | 1766 | 0.1 |
| benzyl benzoate | 1776 | 0.2 |
| 1-octadecyne* | 1844 | 0.3 |
| **(*E*)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4,4]non-3-ene** | 1887 | **1.0** |
| **(*Z*)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4,4]non-3-ene** | 1931 | **1.0** |
| 1-phenylbicyclo[3.3.1]nonan-9-one* | 1960 | 0.1 |

Note: components larger than 1% are highlighted in bold face; t = trace components below 0.04% of oil; † = Kovats indices;
* = tentatively identified.

With reference to the Indian varieties reported, no bornyl acetate or (-)-*cis*-chrysanthenol were detected in our study thereby confirming that the major constituent of this Bhutanese variety of *A. nubigena* was chemotype II. Notably, chamazulene, with a characteristic blue color, was detected and isolated here as a new sub-chemotype for the first time from this species.

The repeated purification of the EO and the crude MeOH extract using column chromatography and preparative TLC resulted in the isolation of eight major compounds (Fig. 1).

While (*3R,6R*)-linalool oxide acetate (**1**) [12], chamazulene (**2**) [13], (*E*)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4,4]non-3-ene (**3**) [12], and (*Z*)-2-(2,4-hexadinylidene)-1,6-dioxaspiro[4,4]non-3-ene (**4**) [12] were isolated from the EO component, 1-(4-hydroxy-phenyl)propan-1-one (**5**) [14], oxyanin B (**6**) [15], luteolin (**7**) [16-17] and the luteolin-7-*O*-*β*-D-glucopyranoside (**8**) [18] were obtained from the crude MeOH extract component. They were characterized from their MS, 1D and 2D-NMR spectroscopic data and comparisons made with the data reported. Compound **6** lacked complete NMR characterization and we have updated it here in the experimental section.

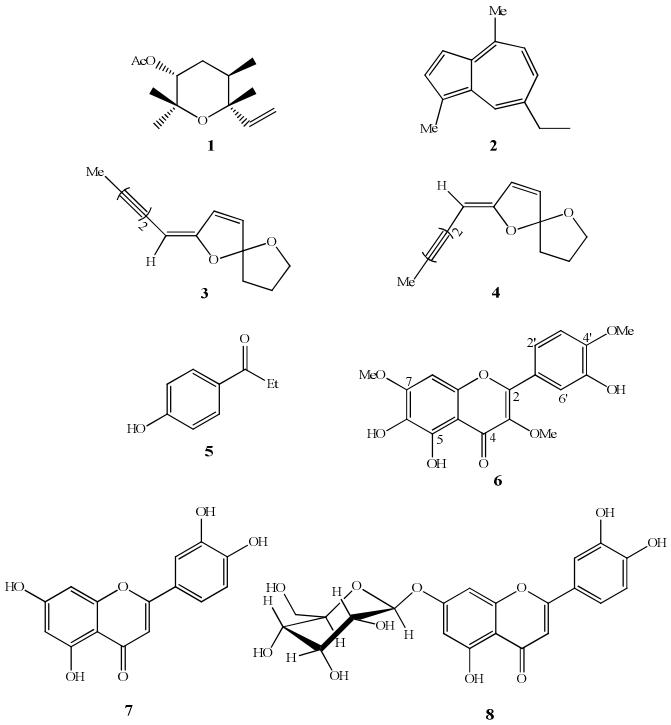


**Figure 1**: The structures of isolated compounds **1-8.**

The EO and the eight isolated compounds **1–8** were investigated for their antiplasmodial, antimicrobial and cytotoxic activities. These results, along with the positive controls and the bioassay data on the crude MeOH, hexane, $CH_2Cl_2$ and $CHCl_3$ extracts, which we previously reported [6], are shown in Table 2. The EO exhibited moderate antiplasmodial activities against *Plasmodium falciparum* strains: TM4/8.2 (a wild type chloroquine and antifolate sensitive strain) and K1CB1 (multidrug resistant strain). The antimalarial activities of the crude extracts that we previously reported [6] were almost seven fold more potent than that of EO. The EO also displayed strong antibacterial activity against *Bacillus subtilis* (in comparison to that of the standard, amoxicillin) and moderate antifungal activity against *Candida albicans* with the minimum inhibition zones (MIZ) of 13 mm and 11 mm, respectively. Interestingly, while the (*E*)-spiroether (**3**), isolated from the EO, displayed only moderate antiplasmodial activities, its isomeric form (*Z*)-spiroether (**4**) demonstrated only selective antimicrobial activities (Table 2).

Among the four flavonoid compounds tested for various bioactivities (Table 2), compound **7** showed broad spectrum activities against all subset of test organisms. It exhibited good antimalarial activities against both the TM4/8.2 and K1CB1 strains of *P. falciparum* with $IC_{50}$ values of 6.2±1 µg/mL and 6.2±1 µg/mL, respectively. It also demonstrated the highest cytoxicity against the Vero and human oral carcinoma KB cells with the $IC_{50}$ value of 14.5±7 µg/mL and 14.3±1 µg/mL, respectively.

**Table 2:** Bioactivities of the crude extracts[a], EO and the pure compounds **1–8** isolated from *A. nubegina*

| Samples | Antiplasmodial ($IC_{50}$ in µg/mL) | | Cytotoxicity ($IC_{50}$ in µg/mL) | | Antibacterial (MIZ* in mm) | | | | Antifungal (MIZ* in mm) |
|---|---|---|---|---|---|---|---|---|---|
| | TM4/8.2 | K1CB1 | VERO | KB | *S. aureus* | *B. subtilis* | *S. epidermidis* | MRSA | *C. albicans* |
| MeOH extract[a] | 9.8±1 | 8.7±1 | >10 | >10 | 5 | 5 | – | 6.5 | – |
| Hexane extract[a] | >12.5 | 6.2±3 | >10 | >10 | 5 | 5 | – | 5.5 | – |
| $CH_2Cl_2$ extract[a] | >12.5 | 9.5±1 | >10 | >10 | – | 5 | – | 5.5 | – |
| $CHCl_3$ extract[a] | 20.0±4 | 18.7±1 | >25 | >25 | – | 5.5 | – | – | – |
| EO | 66.1±2 | 70.6±5 | >100 | >100 | – | 13 | – | – | 11 |
| **1** | >100 | >100 | >100 | >100 | – | 8 | – | – | 5 |
| **2** | >20 | >20 | >20 | >20 | Nt | Nt | Nt | Nt | Nt |
| **3** | 7.3±2 | 8.8±1 | >10 | >10 | – | 7 | – | – | 5 |
| **4** | >20 | >20 | >20 | >20 | – | 11 | 8 | – | 5 |
| **5** | >10 | >10 | >10 | >10 | Nt | Nt | Nt | Nt | Nt |
| **6** | >20 | >20 | >20 | >20 | – | – | – | – | – |
| **7** | 6.2±1 | 6.2±1 | 14.5±2 | 14.3±2 | 8 | 7 | 10 | 10 | 5 |
| **8** | 2.8±1 | 2.0±1 | 33.2±4 | >45 | – | 6 | – | – | – |
| Chloroquine[b] | 0.009 | 0.08 | | | | | | | |
| Cycloguanil[b] | 0.009 | 0.8 | | | | | | | |
| Pyrimethamine[b] | 0.02 | 7.7 | | | | | | | |
| Ellipticine[c] | | | 0.09 | | | | | | |
| Doxorubicin[c] | | | | 0.5 | | | | | |
| Amoxycillin[d] | | | | | 34 | 10 | 20 | | |
| Vancomycin[d] | | | | | | | | 15 | |
| Amphotericin B[e] | | | | | | | | | 20 |

*MIZ: minimum inhibition zone with a well diameter of 4 mm. Nt: Not tested. –: Not active. [a] results reproduced from earlier report [12]. [b] Reference drugs for antiplasmodial activity. [c] Reference drugs for cytotoxicity and anticancer activities. [d] Reference drugs for antibacterial activity. [e] Reference drug for antifungal activity.

Compound 7 showed inhibitory activities against all Gram-positive bacteria (*B. subtilis*, *Staphylococcus aureus*, methicillin resistant *S. aureus* (*MRSA*), *S. epidermidis*) and fungi (*C. albicans*) with MIZ values in the range of 5-10 mm (Table 2). These inhibitions were significant when compared to that of the positive controls. However, when the MIC values of this compound (including other test samples) were determined, the best MIC-based activity observed was 125 µg/mL against MRSA and *S. epidermidis*. While the broad spectrum bioactivity of compound **7** implied that it could be a general toxin, the literature confirmed that it was in fact a common natural antioxidant flavone which had already been extensively studied and was shown to have a better safety profile [19]. Preclinical studies have shown that luetolin (**7**) possesses a variety of bioactivities, including pro-oxidant, anti-inflammatory and antimicrobial [20]. Further, its ability to inhibit angiogenesis, to induce apoptosis, to prevent carcigenosis in animal models, to reduce tumor growth *in vivo* and to sensitize tumor cells to the cytotoxic effects of some anticancer drugs suggests that this flavonoid has cancer chemopreventative and chemotherapeutic potential [20]. Luetolin (**7**) has been previously studied and found effective against leukemia, melanoma and carconomas of the pancreas, ovary, brain, kidney, lung, colon and stomach [21]. Our study demonstrated the cytoxicity of this compound against human oral carcinoma KB cells.

In contrast to compound **7**, its glycosylated analog **8** showed more selectivity, being only active against a subset of the targets (Table 2). Compound **8** exhibited the best (among test samples) and highly significant antimalarial activities with $IC_{50}$ values of 2.8±1 µg/mL and 2.0±1 µg/mL against TM4/8.2 and K1CB1 strains, respectively. Its antimalarial activity is three fold more active than the parent crude extracts (Table 2). Compound **8** also exhibited mild cytotoxicty against the Vero cells with an $IC_{50}$ value of 33.2±4 µg/mL but with no cytotoxicity to the human oral carcinoma KB cells. An earlier study showed that this compound was not toxic to RAW264.7 cells and also demonstrated that its aglycone luteolin (**7**) was more toxic than the glycosylated form [22]. The fact that cytotoxicity of compound **8** was lower than its aglycone 7, suggests that glycosylation reduces cytotoxicity. This has been substantiated by the fact that it is closely related derivatives, kaempferol and quercetin, had higher toxicities compared to their glycosides [23]. Higher lipophilicity facilitates greater penetration into the lipid membrane of organisms and therefore greater cytotoxicity which explains the higher toxicity of compound **7** compared to its glycosylated compound **8**. This compound **8,** with significant antimalarial activities and lesser cytotoxicity, would serve as a potential lead for developing an analogue with a better antimalarial therapeutic index.

In conclusion, our study found that the Bhutanese variety of *A. nubigena* contained: a) an essential oil (0.7% w/w) with (3*R*,6*R*)-linalool oxide acetate as the major constituent (75.8%) (chemotype II) and chamazulene as the new sub-chemotype; b) luteolin (**7**) as the major marker compound of the crude MeOH extract which exhibited a broad range of moderate antiplasmodial, cytoxicity and antimicrobial activities; and c) compound **8** which showed significant *in vitro* antiplasmodial activity with mild cytotoxicity which was identified as a potential antimalarial scaffold. The *in vitro* antimicrobial and cytotoxicity activities of the crude extracts, EO and compounds **1**, **3-4**, and 7- **8** were supportive of the use of this plant in BTM as a vulnerary, expectorant and for treating abcess, swelling and tumors [6].

**Experimental**

*Plant material*: *A. nubegina*, is a perennial flowering herb of 30 cm tall with slender fibrous roots and yellow flowers. It grows in alpine mountain rocky slopes and sandy grounds of Bhutan at an elevation range of 3600–4800 meters above sea level [24]. The aerial parts of wild *A. nubegina* were collected from Lingzhi in Bhutan in August 2009. The collected plant material was air-dried and a herbarium specimen with voucher number 73 was deposited at the herbarium of the PRU, Thimphu, Bhutan.

*Extraction of EO and crude methanol extract*: The pale green pleasantly aromatic EO (7 mL) was obtained by hydro-distillation (temperature at 60 °C) of 1 kg of dried plant material using a Clevenger apparatus for three hours. The EO collected was dried over anhydrous magnesium sulphate. Alternatively, air-dried plant material (2 kg) was chopped into small pieces and was repeatedly extracted with methanol (AR/HPLC grade, 5 × 3 L over 48 h). The

extract was filtered and then concentrated using a rotary evaporator to afford the crude methanol extract (58.22 g).

*Analysis of EO using GC and GC-MS*: The EO was analysed for its chemical constituents using GC and GC-MS systems. The GC analysis was performed on a Shimadzu GC-2010 Plus gas chromatograph. Hydrogen was used as carrier gas (1.5 mL/min at 40 °C in a constant total flow mode) and the separation was achieved using a Restek fused silica capillary column (Rxi-5MS: 30 m × 0.25 mm i.d., 0.25 µm film thickness). Injector and detector temperature were set at 260 °C and 300 °C, respectively. The starting oven temperature was programmed at 40 °C with an increasing temperature of 6 °C/min until it reached to 290 °C. Kovats retention indices (KI) were obtained by GC-FID analysis of an aliquot of the EO spiked with an *n*-alkane mixture (C7 to C30). The GC-MS analysis was performed using Shimadzu QP5050A GC-MS system (electron impact (EI) mode at 70 eV). The column and the GC-MS chromatographic conditions were same as that for GC but He was used as carrier gas. The EO constituents were identified by comparing mass spectra with NIST and NISTREP mass spectra library of GC-MS data system and further confirmed by comparing their Kovats indices (KI) with those reported [8, 11-12, 25]. About 53 component peaks were detected and 44 of them have been identified through MS library matching and KI comparison techniques.

*Isolation of compounds from EO and methanol extract*: A rotary evaporator was used for solvent evaporation under reduced pressure at 35 °C – 50 °C. Flash column chromatography packed with Merck Kieselgel 60 $PF_{254}$ and the pre-coated silica plates (0.2 mm silica thickness, Merck) were used for separation and purification of compounds. UV light (short wavelength of 254 nm, long wavelength of 366 nm) and ceric ammonium molybdate (CAM) were used for visualization and detection of the compounds on TLC plates. Micromass Waters Platform LCZ (single quadrupole, MeOH as solvent) was used for obtaining the LR-ESI-MS. Shimadzu GCMS-QP-5050 was used for recording the LR-EI-MS by the direct insertion technique (at 70 eV). Micromass Waters Q-ToF Ultima (quadrupole time-of-flight) mass spectrometer was used for acquiring HR-ESI-MS. A 500 MHz Varian Unity Inova, 500 MHz Varian Premium Shield (VNMRS PS 54), and 300 MHz Varian Mercury spectrometer were used for obtaining the 1D and 2D-NMR spectra using deuterated solvents depending upon the solubility of compounds**.** The known compounds were identified through MS library matching techniques (NIST and NISTREP mass spectra library) and then confirmed through comparison of their optical rotation, MS and NMR spectra with those reported in the relevant literature.

The repeated purification of the EO (5.6 ml) using column chromatography, preparative TLC and reverse phase (RP) silica-coated preparative TLC resulted in the isolation of four compounds as: (*3R*,*6R*)-linalool oxide acetate (**1**), chamazulene (**2**), (*E*)-2-(2,4-hexadiynylidene)-1,6-dioxaspiro[4,4]non-3-ene (**3**) and (*Z*)-2-(2,4-hexadinylidene)-1,6-dioxaspiro[4,4]non-3-ene (**4**).

Subsequently, the MeOH extract (70.12 g) was fractionated with hexane followed by ethyl acetate. Two fractionated extracts were concentrated resulting into hexane extract (28 g) and the ethyl acetate extract (12.5 g). The silica gel column chromatography seperation yielded 17 fractions (AN-1 to AN-17). Seperation of fraction AN-4 using silica-coated preparative TLC plates with a mobile phase of $H_2O$ (20%):MeOH (80%) yielded 1-(4-hydroxyphenyl)propan-1-one (**5**) and oxyanin B (**6**). Fractional crystallization of AN-8 with MeOH/$CHCl_3$ yielded crystal compound luteolin (**7**). Purification of fraction AN-14 using RP silica gel column chromatography with an isocratic mobile phase of 100% MeOH furnished luteolin-7-*O*-*β*-D-glucopyranoside (**8**).

**Oxyanin B (6)**

$^1$H NMR (500 MHz, DMSO-$d_6$): 6.52 (1H, s, H-8), 6.94 (1H, d, *J* = 8.5 Hz, H-3′), 7.54 (1H, d, *J* = 8.5 Hz, H-2′), 7.61 (1H, s, H-6′), 3.49 (1H, bs, C(6)-OH), 12.73 (1H, bs, C(5)-OH), 3.73 (3H, s, C(7)-OMe), 3.77 (3H, s, C(3)-OMe), 3.83 (3H, s, C(4′)-OMe).

$^{13}$C NMR (125 MHz, DMSO-$d_6$): 55.7 (C(4′)-OMe), 59.7 (C(3)-OMe), 59.8 (C(7)-OMe), 94.2 (C-8), 104.3 (C-10), 112.0 (C-6′), 115.6 (C-3′), 120.8 (C-1′), 122.1 (C-2′), 131.3 (C-7), 137.3 (C-3), 147.4 (C-4′), 149.7 (C-5′), 151.7 (C-6), 152.2 (C-5), 155.2 (C-2), 177.9 (C-4).

*Bioassays*: The EO and compounds **1**-**8** were tested *in vitro* for their antiplasmodial, antimicrobial and the cytotoxicity activities using the standard test protocols as described by us previously [12].

For antimalarial testing a multidrug resistant K1CB1 strain and a wild type chloroquine and antifolate sensitive TM4/8.2 strain of *Plasmodium falciparum* were used in the Microdilution Radioisotope Technique. Chloroquine (Sigma), pyrimethamine (Sigma) and cycloguanil were used as reference drugs for both the plasmodial strains.

For the antimicrobial assay, the test organisms including *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538), methicillin resistant *S. aureus* (*MRSA*), (DMST 20651), *Staphylococcus epidermidis* (ATCC 12228), *Vibrio cholerae* (DMST 2873) and *Candida albicans* (ATCC 10231) were used. Amphotericin B (Sigma-Aldrich, USA), vancomycin (Edicin, Slovenia) and amoxicillin (GPO, Thailand) were used as a reference drugs.

For the cytotoxicity assay, normal vero cells from kidney of African green monkey, *Cecopithecus aethiops* and the human oral carcinoma KB cells were used. Ellipticine and doxorubicin were used as reference drugs.

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