Journal of Institute of Science and Technology, *25*(1), 1-6 (2020)
ISSN: 2469-9062 (print), 2467-9240 (e)
Doi: https://doi.org/10.3126/jist.v25i1.29415

Research Article

ISOLATION OF FLAVONOIDS FROM *Anaphalis busua* AND THEIR ANTIBACTERIAL ACTIVITY

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(***Received:*** *February 27, 2020;* ***Revised****: April 27, 2020;* ***Re-revised****: May 6, 2020;* ***Accepted:*** *May 14, 2020)*

ABSTRACT

Anaphalis busua (Buch-Ham ex D. Don) is traditionally used to treat cuts and wounds. In the agar dilution method, the hexane extract of the aerial part of *A. busua* exhibited potent antibacterial activity with a minimal inhibitory concentration of 25 µg/ml against *Bacillus subtilis* and 500 µg/ml against *Staphylococcus aureus*. For the isolation of antibacterial compounds, the hexane extract was fractionated by silica gel and Sephadex LH 20 column chromatography. The direct bioautography method was used to determine the antibacterial activity of the fractions. The active fractions were finally purified by semi-preparative HPLC on C_{18} Phenomenex column under gradient condition. Four flavones derivatives of 3,5-dihydroxy-6,7,8-trimethoxyflavone (1), 3,5,7-trihydroxy-6-methoxyflavone (alnusin) (2), 3,5,7-trihydroxy-8-methoxyflavone (3) and pinocembrin (4) were isolated, and their structures were established by ^{1}H, ^{13}C, DEPT-135, and ESI-MS spectroscopy. These compounds were isolated for the first time from *A. busua.* The fatty acid profile of the hexane extract was analyzed by gas chromatography-mass spectrometry (GC-MS) by silylation with N-methyl-N-trimethylsilyl-trifluoro-acetamide. Pinocembrin showed antibacterial activity with the minimum inhibitory concentration of 60 µg/mL against *Bacillus subtilis* and 420 µg/mL against *Staphylococcus aureus*.

Keywords: *Anaphalis busua*, antibacterial, fatty acids, flavone derivatives, GC-MS

INTRODUCTION

The genus *Anaphalis* belongs to the Asteraceae family, which consists of 110 different species distributed mostly in central and south Asia (Nesom, 2006). *Anaphalis busua* (Buch-Ham ex D. Don) is distributed throughout Nepal at the elevation range of 1500 to 2900 m in open areas. It is an annual herb propagated by seeds. In Nepalese traditional medicine, the juice of the plant is applied to cuts and wounds. The flowers and leaves are used against cough, cold and sore throat (Manandhar, 2002). The plant is not well investigated phytochemically and pharmacologically. In our previous investigation, the extracts of *A. busua* did not show antiviral and wound healing activities (Rajbhandari *et al.*, 2009; Zimmermann-Klemd *et al.*, 2019). Phytochemical investigation of the aerial parts of *A. araneosa* indicated the presence of araneol and araneosol (Ali *et al*., 1979), helipyrone (Ali *et al.*, 1982), sterols and phthaloids (Jakupovic *et al.*, 1987). From the flowers, 5-methyl-n-hexatriacontane, anaphalisone, acyclic triterpenes, dulcioic acid and aerneosol (Sharma & Ali, 1998) have been isolated. Similarly, from the roots, three new compounds, anaphalisoleanenoic acid, anaphalisoic acid, and araneosoic acid (Sharma *et al*., 2003) have been identified.

The alcoholic extract of leaf of *A. busua* indicated the presence of tiliroside, a flavonoidal glycoside, sitigmasterol, and *β*-sitosterol (Raturi *et al.*, 2012). Antimicrobial drug resistance and hospital-acquired nosocomial infection are ever-growing problems (Struelens, 1998), and the search for more safe and effective antimicrobial agents is in high priority. Since the development of new antibiotics is a long process (Boucher *et al*., 2009), plant extracts and biologically active compounds isolated from plant species used in traditional medicine could be an alternative source of new drugs (Cowan, 1999; Giri & Rajbhandari, 2018; Joshi & Bashyal, 2018; Sharma *et al.*, 2015). The present work reported the chemical constituents and antibacterial activity of hexane extract of *A. busua*.

MATERIALS AND METHODS

General experimental procedure

The thin layer chromatography (TLC) was carried out on pre-coated silica gel GF_{254} (0.2 mm, Merck) using the solvent system hexane-ethylacetate 3:2 (v/v), and it was visualized under a UV lamp (HP-UVIS, SARSREDT-GRUPPE, DESAGA GmbH, Germany). The analytical gas chromatography was carried out on Agilent G1530N coupled with G2588A mass-spectrometer. The high performance liquid chromatographic (HPLC) purification was carried out on Shimadzu system controller SCL-10a with DAD using a semi-preparative Phenomenex C_6-Phenyl 110 column (250 x 10 mm). ^{1}HNMR spectra were recorded on the Bruker Avance 300 DPX using TMS as an internal standard. Each sample (2 mg) was dissolved in 0.5 ml $CDCl_3$ or Aceton-d_6 and transferred to a 5 mm BBO sample tube. ESI-MS was recorded on Finnigan TSQ 700 equipped with the Finnigan electrospray

(Finnigan MTH GmbH, Bremen, Germany). The sample was dissolved in acetone and sprayed into the ionization chamber of the mass spectrometer.

Plant material and extract

The aerial part of *A. busua* was collected from Kalopani area of Johmsom district in November 2009 and authenticated by comparison with the herbarium species deposited at Central Department of Botany, Tribhuvan University, Kathmandu, Nepal. A voucher specimen (#AB 463) was deposited at Research Centre for Applied Science and Technology, Tribhuvan University. The leaves and stem of dried and powdered plant materials (50 g) were successively extracted with hexane and methanol in a Soxhlet extractor. The solvent was evaporated under reduced pressure and finally dried by lyophilization to get the hexane (1.13 g) and methanol (5.79 g) extracts.

Antimicrobial assay

The antibacterial assay was performed using the agar diffusion method (Rajbhandari *et al*., 2007). Two Gram-positive bacteria: *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 6538) were used. The minimum inhibitory concentration (MIC) was determined by the agar dilution method following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2003). Muller Hinton agar was prepared according to the manufacturer's description and autoclaved at 121 °C for 15 min. The stock solution of standard antibiotics was prepared by dissolving 1 mg ampicillin in 1 mL DMSO and diluted to the total volume of 100 mL with Muller Hinton agar. The working solution was prepared by the two-fold serial dilution of the stock solution. The range of concentrations of ampicillin was from 0.1-6.4 µg/mL. Similarly, the working solutions of extracts/pure compounds were prepared by serial dilution of the stock solutions prepared in different concentrations. About 20 mL of agar in each beaker cooled to 45- 50 °C, added 1 mL of different concentrations of standard antibiotic/extracts/pure compounds prepared two-fold dilutions. After mixing properly, the content of each beaker was poured into the Petri plates. The plates were left to solidify at room temperature so that no drops of moisture remain on the surface of the agar. About 1 µL of standard inoculums of test organisms (10^4 CFU) was spotted in the agar plates with the help of micropipette, including a control plate without an antimicrobial agent. The inoculated spots were allowed to dry at room temperature and incubated in an inverted position at 37 °C for 24 hrs. The lowest concentration of the agent that completely inhibits visible growth as judged by the naked eye, disregarding a single colony or a thin haze within the area of the inoculated spot corresponds to the MIC.

In bioassay-guided fractionation of antimicrobial compounds, the direct bioautography method was applied. The developed TLC plate was sprayed with a microbial suspension. Then, the bioautogram was incubated at 25 °C for 48 h under humid condition. For visualization of the microbial growth, *p*-iodonitrotetrazolium violet was sprayed onto the bioautogram, which was re-incubated at 37 °C for 3-4 h (Begue & Kline, 1972).

GC-MS analysis of hexane extract

Hexane extract (1 mg) was mixed with methoxyamine hydrochloride in pyridine and treated with N-methyl-N-trimethylsilyl-trifluoro-acetamide by heating in a microwave (3 minutes, 240 watts). 2 µL of the solution was injected to the heated injector port (230 °C) of the gas chromatograph fitted with a capillary column (30 m DB-5MS with 0.25 mm internal diameter and 0.25 µm film thickness). Helium was used as a carrier gas with a flow rate of 1 mL/min. The column temperature was initially maintained at 70 °C for 1 min. It was gradually increased to 76 °C at a rate of 1.5 °C/min and finally increased to 330 °C at 5 °C/min. The injection split ratio was 1/25. MS was operated in the electron impact mode with ionization energy of 70 eV, and the mass fragments were detected between 35 m/z to 573 m/z. The mass transfer temperature was 250 °C. The detected compounds in the gas chromatogram were identified by processing the GC-MS data and comparing it with the mass spectral database of the National Institute of Standard and Technology, NIST.

Bioassay Directed Fractionation and Purification

The hexane extract (1 g) was subjected to silica gel column chromatography (25 cm x 2.5 cm ID, flow rate 1.5 mL/min) using a mixture of hexane-ethyl acetate (3:2 v/v). The sub-fractions were collected, each consisted of 4 mL, and they were monitored by thin-layer chromatography in solvent system hexane-ethyl acetate (3:2). Finally, the column was eluted with methanol. The sub-fractions were pooled into 15 main fractions (F_1-F_{15}).

The antimicrobial activities of the fractions were monitored by the bio-autographic method. Eleven out of fifteen fractions showed antimicrobial activity. One of the most active fraction F_8 (400 mg) was further subjected to Sephadex LH20 column chromatography (31cm x 2 cm ID, the flow rate of 1.9 mL/min) and eluted with methanol. The sub-fractions were collected and pooled into five major fractions after the TLC examination ($F_{8\text{-}1}$-$F_{8\text{-}5}$). The active fractions F_6 (40 mg), $F_{8\text{-}2}$ (95 mg), $F_{8\text{-}3}$ (80 mg) and F_9 (50 mg) were purified by semi-preparative HPLC on C_{18} Phenomenex column employing a gradient elution using 0.1 % phosphoric acid in water (A) and methanol (B). The gradient elution consisted of 80 % A to 100 % B for 30 min with a flow rate of 1.5 ml/min. HPLC purification of fraction F_9 gave compound (**1**), $F_{8\text{-}2}$ gave compound (**2**), $F_{8\text{-}3}$ gave compound (**3**), and F_6 gave compound (**4**). The phosphoric acid residue was

neutralized with ammonia and purified by passing through the C_{18} Phenomenex SPE cartridge.

RESULTS AND DISCUSSION

The hexane extract showed the potent antimicrobial activity against *B. subtilis* (inhibition zone 7 mm, 2 mg extract/disc) and *S. aureus* (inhibition zones 6 mm, 2 mg extract/disc) than the methanol extract (inhibition zone 1 mm against both *B. subtilis* and *S. aureus*, 2 mg extract/disc) in our screening experiment using the disc diffusion method. Therefore, GC-MS analysis of the hexane extract was performed which allowed the identification of some fatty acids based on retention time (Rt) and fragmentation pattern in the mass spectrum and comparing with the standard NIST mass spectral database. They are myristic acid (Rt 29.48 min), palmitic acid (Rt 29.72 min), linolic acid (Rt 29.98 min), oleic acid (Rt 30.52 min), steric acid (Rt 32.95 min), arachnic acid (Rt 35.95 min), and lignoceric acid (Rt 36.46 min). A portion of gas chromatogram with retention time between 20-40 minutes is shown in Fig 1.

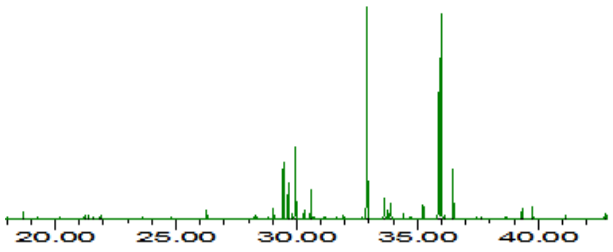

Fig. 1. Gas chromatogram of hexane extract of *A. busua*

Bio-assay directed fraction of the hexane extract led to the isolation of four flavone derivatives. The structures of these compounds were analyzed by UV, NMR, and mass spectroscopic methods. Compound (**1**) (t_R 21.65 min, 18 mg) was obtained as a yellowish-red crystalline solid. The UV spectrum showed absorption peaks at 272, 323, and 377 nm. The ESI-MS showed $[M+H]^+$ peak at m/z 345 $[M-H]^-$ peak at m/z 343 that correspond to molecular formula $C_{18}H_{16}O_7$. This indicated that it is a flavone or flavonol with two hydroxy and three methoxy groups. The ^{13}C NMR spectrum showed fifteen carbon signals. The DEPT-135 spectrum showed three methoxy groups (61.21 ppm, 61.75 ppm, and 62.11 ppm) and five methine groups (127.67 ppm doublet, 128.73 ppm doublet, and 130.50 ppm). The ^{13}C NMR spectrum confirmed an unsubstituted B-ring and a trimethoxy substituted A-ring.

The ^1H NMR profile was also consistent with that of a flavone structure. The doublet signal 8.25 ppm was assigned for aromatic protons H2' and H6', a complex multiplate signals at 7.55 ppm were due to the aromatic protons H3', and H5' and a signal at 7.25 is due to H4'. A broad singlet at 6.75 ppm was due to OH proton at C-3. The three signals at 3.97, 4.00, and 4.13 ppm, represent the three methoxy protons. Comparison of these spectral data with literature indicated that compound (**1**) is 3, 5-dihydroxy-6,7,8-trimethoxyflavone which has been isolated from *Helichrysum graveolens, Helichrysum decumbens* (Hansel & Khaliefi, 1981; Tomas-Lorente, 1989).

Compound (**2**) (t_R 20.34 min, 15 mg) was obtained as a yellow crystalline solid. The UV spectrum showed absorption peaks at 268, 326, and 356 nm. The ESI-MS showed $[M+H]^+$ peak at m/z 301 and $[M-H]^-$ peak at m/z 299 indicated that it is a monomethoxy derivative of (**1**). The NMR signal also showed a signal for one methoxy proton at 4.05 ppm. A comparison of the NMR data with the literature suggested that the structure of compound (**2**) is 3,5,7-trihydroxy-6-methoxyflavone (alnusin) or 3,5,7-trihydroxy-8-methoxyflavone. The presence of a strong fragment peak at m/z 286 in positive and 284 in negative ion mode due to the loss of methyl group [M-15] indicated the presence of the OMe group at the C-6 position. By comparison of these spectral data with literature (Asakawa, 1971), compound (**2**) was identified as alnusin.

Compound (**3**) (t_R 19.92 min, 15 mg) was obtained as a yellow crystalline solid. The ESI-MS showed $[M+H]^+$ peak at m/z 301 and $[M-H]^-$ peak at m/z 299 indicated that it is an isomer of compound (**2**) and identified as 3,5,7-trihydroxy-8-methoxyflavone by comparison of NMR data with the literature (Proksch *et al*., 1982, Karasartov *et al.*, 1992). Compound (**4**) (t_R 19.22 min, 25 mg) showed $[M+H]^+$ peak at m/z 257 and $[M-H]^-$ peak at m/z 255 in ESI-MS. The NMR showed signals at 2.85 (dd. H3a), 3.05 (dd, H3b), 6.01 (d, H6), 5.40 (dd, H2), 6.25 (d, H8), 7.30 (s, H3'/4'/5') and 7.45 (s, H2'/6') ppm. These NMR chemical shifts are identical to the chemical shift of pinocembrine (Bick *et al.*, 1972, Jung *et al.*, 1990). So compound (**4**) was identified as pinocembrin. The structures of the compounds are shown in Fig 2.

3,5-dihydroxy-6,7,8-trimethoxyflavone isolated from *Helichrysum chasmolycicum* showed moderate antifungal activity against *Candida albicans* (Suzgec-Selcuk & Birteksoz, 2011). The antimicrobial property might be associated with the lack of hydroxyl groups in ring B, as in the case of galangin (Alfolayan & Meyer, 1997). In protease inhibition activity, 3,5,7-trihydroxy-8-methoxyflavone isolated from *Helichrysum mechowianum* showed weak inhibition of protease (Malolo *et al.*, 2015). The cytotoxic activity of 3,5,7-trihydroxy-6-methoxyflavone and pinocembrin isolated from the leaf of *Chromolaena leivensis* was determined on the prostate, breast, colon, cervix, and lung cancer cells using MTT assay.

The cytotoxicity of the 3,5,7-trihydroxy-6- methoxy flavone was found to be lower than the positive control, vincristine sulfate and pinocembrin also inhibited the cell

proliferation (Torrenegra *et al.*, 2016, Mendez-Callejas *et al*., 2018). Pinocembrine showed antibacterial and antioxidant (Drewes & Vuuren, 2008), antifungal (Lago *et al*., 2004), antiviral (Lee *et al*., 2019), anti-inflammatory (Soromou *et al*., 2012) anticancer activities (Kumar *et al*., 2007, Punvittayagul *et al.*, 2012). It has neuroprotective and anti-ischemic properties, so it is used as a promising drug candidate for the prevention and therapy of Alzheimer's disease and stroke (Liu *et al*., 2008, Shi *et al.*, 2011). The presence of compounds (**1-4**) in *A. busua* demonstrate its traditional use in the treatment of various ailments.

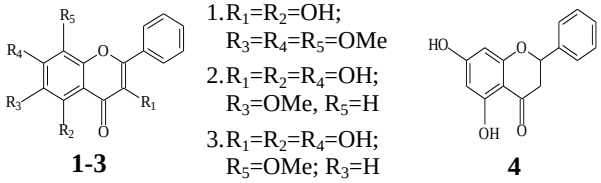

Fig. 2. Structure of the isolated compounds

Antimicrobial assay

The Agar dilution method was used to determine the MIC of the crude extracts and pinocembrin. The minimal inhibitory concentrations of extracts, pinocembrin, and ampicillin are given in Table 1.

Table 1. MIC (µg/ml) of extracts and pinocembrin

Bacteria	Hexane	Methanol	Pinocembrin	Ampicillin
B. subtilis	25.0	200.0	60.0	0.8
S. aureus	500.0	200.0	420.0	0.4

In the present investigation, the pure isolate showed only weak activity for the comparison to the extract. In such a case, the constituents in the extract collectively enhance the activity; however, on further purification, leading to the isolation of single compound(s), the relative potency of the compound of interest seems to diminish. Thus, it can assume that the combination of all the phyto-chemicals, including the fatty acids present in the extract, could have a stronger antimicrobial effect than the single compound alone.

CONCLUSION

Bioassay-guided fractionation of hexane extract of *A. busua* led to the isolation of 3,5-dihydroxy-6,7,8-trimethoxyflavone, 3,5,7-trihydroxy-6-methoxyflavone (alnusin), 3,5,7-trihydroxy-8-methoxyflavone, and pinocembrin. Literature review revealed that these compounds were not isolated before from *A. busua*. Pinocembrin showed antibacterial activity against Gram-positive bacteria *B. subtilis* and *S. aureus*. *A. busua*, which is used in ethno-medicine, could be the potential source of bioactive compounds.

ACKNOWLEDGMENTS

Authors would like to thank Dr. Stefan Kehraus, Institute for Pharmaceutical Biology, University of Bonn for recording NMR and mass spectrum and Dr. Martina Wurster, Institute of Biochemistry, University of Greifswald for antimicrobial assay.

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