Endiss, Y., Escher, E., Rohr, B., Rohr, H., Weiss, N., 2005. Kato–Katz technique for helminth eggs. In: Methods in Parasitology. Swiss Tropical Institute, Basel, Available at http://www.tropeduweb.ch/Parasitology_Methods_PDF/8_Stool_Kato-Katz.pdf#search='KatoKatz technique' (accessed April 14, 2012).

Enk, M.J., Silva, G.O., Rodrigues, N.B., 2012. Diagnostic accuracy and applicability of a PCR system form an endemic area. PLoS One 7, e38947.

Fung, M.S., Xiao, N., Wang, S., Carlton, E.J., 2012. Field evaluation of a PCR test for *Schistosoma japonicum* egg detection in low-prevalence regions of China. Am. J. Trop. Med. Hyg. 87, 1053–1058.

Gal, S., Wainscoat, J.S., 2006. Detection of quantitation of circulating *Plasmodium falciparum* DNA by polymerase chain reaction. In: Lo, Y.M.D., Chiu, R.W.K., Chan, K.C.A. (Eds.), Methods in Molecular Biology 336: Clinical Applications of PCR. Humana Press Inc., New Jersey, pp. 155–162.

Gomes, L.I., Marques, L.H.S., Enk, M.J., Coelho, P.M.Z., Rabello, A., 2009. Further evaluation of an updated PCR assay for the detection of *Schistosoma mansoni* DNA in human stool samples. Mem. Inst. Oswaldo Cruz (Rio de Janeiro) 104, 1194–1196.

Han, S., Zhang, X., Wen, J., Li, Y., Shu, J., Ling, H., Zhang, F., 2012. A combination of the Kato–Katz methods and ELISA to improve the diagnosis of Clonorchiasis in an endemic area, China. PLoS One 7, e46977.

Härter, G., Frickmann, H., Zenk, S., Wichmann, D., Ammann, B., Kern, P., Fleischer, B., Tannich, E., Poppert, S., 2014. Diagnosis of neuroschistosomiasis by antibody specificity index and semi-quantitative real-time PCR from cerebrospinal fluid and serum. J. Med. Microbiol. 63, 309–312.

Kanda, Y., 2013. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 48, 452–458.

Kato-Hayashi, N., Kirinoki, M., Iwamura, Y., Kanazawa, T., Kitikoon, V., Matsuda, H., Chigusa, Y., 2010. Identification and differentiation of human schistosomes by polymerase chain reaction. Exp. Parasitol. 124, 325–329.

Kato-Hayashi, N., Yasuda, M., Yuasa, J., Isaka, S., Haruki, K., Ohmae, H., Osada, Y., Kanazawa, T., Chigusa, Y., 2013. Use of cell-free circulating schistosome DNA in serum, urine, semen, and saliva to monitor a case of refractory imported schistosomiasis haematobia. J. Clin. Microbiol. 51, 3435–3438.

Khairnar, K., Parija, S.C., 2008. Detection of *Entamoeba histolytica* DNA in the saliva of amoebic liver abscess patients who received prior treatment with metronidazole. J. Health Popul. Nutr. 26, 418–425.

Lin, D.D., Liu, J.X., Liu, Y.M., Hu, F., Zhang, Y.Y., Xu, J.M., Li, J.Y., Ji, M.J., Bergquist, R., Wu, G.L., Wu, H.W., 2008. Routine Kato–Katz technique underestimates the prevalence of *Schistosoma japonicum*: a case study in an endemic area of the People's Republic of China. Parasitol. Int. 57, 281–286.

Lodh, N., Mwansa, J.C.L., Mutengo, M.M., Shiff, C.J., 2013. Diagnosis of *Schistosoma mansoni* without the stool: comparison of three diagnostic tests to detect *Schistosoma mansoni* infection from filtered urine in Zambia. Am. J. Med. Hyg. 89, 46–50.

Matsuda, H., Tanaka, H., Blas, B.L., Nosenas, S., Tokawa, T., Ohsawa, S., 1984. Evaluation of ELISA with ABTS, 2-2′-azino-di-(3-ethylbenz-thiazoline sulfonic acid), as the substrate of peroxidase and its application to the diagnosis of schistosomiasis. Jpn. J. Exp. Med. 54, 131–138.

Mharakurwa, S., Simoloka, C., Thuma, P.E., Shiff, C.J., Sullivan, D.J., 2006. PCR detection of *Plasmodium falciparum* in human urine and saliva samples. Malaria J. 5, 103.

Nwakanma, D.C., Gomez-Escobar, N., Walther, M., Crozier, S., Dubovsky, F., Malkin, E., Locke, E., Conway, D.J., 2009. Quantitative detection of *Plasmodium falciparum* DNA in saliva, blood, and urine. J. Infect. Dis. 199, 1567–1574.

Ohmae, H., Tanaka, M., Hayashi, M., Matsuzaki, Y., Kurosaki, Y., Blas, B.L., Portillo, G.G., Sy, O.S., Irie, Y., Yasuraoka, K., 1992. Ultrasonographic and serologic abnormalities in *Schistosoma japonicum* infection in Leyte, the Philippines. Am. J. Trop. Med. Hyg. 46, 89–98.

Parija, S.C., Khairrnar, K., 2007. Detection of excretory *Entamoeba histolytica* DNA in the urine, and detection of *E. histolytica* DNA and lectin antigen in the liver abscess pus for the diagnosis of amoebic liver abscess. BMC Microbiol. 7, 41.

Pontes, L.A., Dias-Neto, E., Rabello, A., 2002. Detection by polymerase chain reaction of *Schistosoma mansoni* DNA in human serum and feces. Am. J. Trop. Med. Hyg. 66, 157–162.

Pontes, L.A., Olivera, M.C., Katz, N., Dias-Neto, E., Rabello, A., 2003. Comparison of a polymerase chain reaction and the Kato–Katz technique for diagnosing infection with *Schistosoma mansoni*. Am. J. Trop. Med. Hyg. 68, 652–656.

Sandoval, N., Siles-Lucas, M., Pérez-Arellano, J.L., Carranza, C., Puente, S., Lopez-Abán, J., Muro, A., 2006a. A new PCR-based approach for the specific amplification of DNA from different Schistosoma species applicable to human urine samples. Parasitology 133, 581–587.

Sandoval, N., Siles-Lucas, M., Lopez-Abán, J., Pérez-Arellano, J.L., Gárate, T., Muro, A., 2006b. *Schistosoma mansoni*: a diagnostic approach to detect acute schistosomiasis infection in a murine model by PCR. Exp. Parasitol. 114, 84–88.

Sinuon, M., Sayasone, S., Odermatt-Biays, S., Phompida, S., Duong, S., Odermatt, P., 2010. *Schistosoma mekongi* in Cambodia and Lao People's Democratic Republic. Adv. Parasitol. 72, 179–203.

Suzuki, T., Osada, Y., Kumagai, T., Hamada, A., Okuzawa, E., Kanazawa, T., 2006. Early detection of *Schistosome mansoni* infection by touchdown PCR in a mouse model. Parasitol. Int. 55, 213–218.

Tallo, V.L., Carabin, H., Alday, P.P., Balolong, E.J., Olveda, R.M., McGarvey, S.T., 2008. Is mass treatment the appropriate schistosomiasis elimination strategy? Bull. World Health Organ. 86, 765–771.

WHO Schistosomiasis. Available at http://www.who.int/schistosomiasis/en/index.html (accessed November 11, 2011).

Wichmann, D., Panning, M., Quack, T., Kramme, S., Burchard, G., Grevelding, C., Drosten, C., 2009. Diagnosing schistosomiasis by detection of cell-free parasite DNA in human plasma. PLoS Negl. Trop. Dis. 3, e422.

Wichmann, D., Poppert, S., Von Thien, H., Clerinx, J., Dieckmann, S., Jensenius, M., Parola, P., Richter, J., Schunk, M., Stich, A., Zanger, P., Burchard, G.D., Tannich, E., 2013. Prospective European-wide multicentre study on a blood based real-time PCR for the diagnosis of acute schistosomiasis. MBC Infect. Dis. 13, 55.

Xia, C.M., Rong, R., Lu, Z.X., Shi, C.J., Xu, J., Zang, H.Q., Gong, W., Luo, W., 2009. *Schistosoma japonicum*: a PCR assay for the early detection and evaluation of treatment in a rabbit model. Exp. Parasitol. 121, 175–179.

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Short Report: A Case of Quadruple Malaria Infection Imported from Mozambique to Japan

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Abstract. A 35-year-old Japanese man had an intermittent fever and mild headache for eight weeks after he returned to Japan from working in Mozambique. He had taken antimalarial prophylaxis (doxycycline) for 25 weeks, and stopped taking this drug two weeks after his return. Microscopic examination of a peripheral blood smear showed a mixed infection with *Plasmodium vivax*, *P. falciparum*, and *P. ovale*. In addition, a nested polymerase chain reaction and subsequent sequencing detected specific DNA sequences of four species of *Plasmodium*, including *P. malariae*. The patient was successfully treated with artemether-lumefantrine and primaquine phosphate. The present case is a rare instance of a mixed infection with four species of *Plasmodium*. Nonimmune persons in malaria-endemic areas may have a risk of mixed infection. All four species must be identified by using sensitive and specific tests, such as a nested polymerase chain reaction, in addition to conventional morphologic identification.

Although many cases of mixed malaria infection have been reported in malaria-endemic countries, coincidental infection with more than one species of *Plasmodium* spp. is rare in non-endemic countries.[1,2] In Japan, only approximately 50–80 cases of malaria are reported each year, and are all imported malaria.[3] In this report, we describe a Japanese man who was given a diagnosis of a mixed infection with four species of *Plasmodium* (*P. vivax*, *P. falciparum*, *P. ovale wallikeri*, and *P. malariae*) by using a nested polymerase chain reaction (PCR) after he returned from Mozambique.

CASE REPORT

A 35-year-old Japanese man had intermittent fever and mild headaches for eight weeks after returning from Mozambique. He returned to Japan at the end of June after working as an instructor in operating construction machinery during for two years (June 2010–June 2012). He had taken antimalarial prophylaxis (doxycycline) for 25 weeks from January through the second week of July in 2012. He had no symptoms suggestive of malaria while in Mozambique, but a slight fever developed on August 25, 2012, in Japan. A high fever developed on August 30 and he was admitted to Tokai University Hospital on September 3.

Physical examination at admission showed a body temperature of 37.9°C, a respiratory rate of 27 beats/minute, a heart rate of 100 beats/minute, a blood pressure of 108/68 mm Hg, anemia in the palpebral conjunctiva, hepatomegaly, and splenomegaly. The hemoglobin level was 11.3 g/dL, the mean corpuscular volume was 88.7 fL, and the platelet count was 54×10^9/L. Biochemical tests showed increased serum levels of aspartate aminotransferase (32 U/L, reference value < 30 U/L), lactate dehydrogenase (379 U/L, reference range = 110–219 U/L) and C-reactive protein (5.57 mg/dL, reference value < 0.3 mg/dL). A coagulation test result for the prothrombin time–international normalized ratio was slightly prolonged (1.18, reference range = 0.80–1.10). An ultrasonographic examination showed hepatomegaly (140 mm thickness along the mammary line), splenomegaly (160 × 75 mm), and reactive swelling of the portal hepatic lymph nodes.

A rapid diagnostic test (OptiMAL-IT; DiaMed, Cressier, Switzerland) for malaria with blood of the patient showed three positive bands for the control, *Plasmodium* spp., and *P. falciparum*. A thin blood smear stained with Giemsa showed *P. vivax*, *P. falciparum*, and *P. ovale* (Figure 1). Trophozoites of *P. vivax* were dominant in the microscopic examination, and the total parasitemia including other species was 1.7%.

The patient was treated with Coartem (artemether 20 mg/lumefantrine 120 mg) in a six-dose regimen for three days (total = 24 tablets), followed by primaquine, 15 mg/day for 14 days. The patient soon became afebrile and subsequently left the hospital on the seventh day of hospitalization. Since that time, *Plasmodium* has not been detected, and his symptoms did not recur.

To identify the exact *Plasmodium* species, *Plasmodium* DNA was extracted from the patient's blood by using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany)[4,5] and was subjected to a nested PCR specific for the 18S ribosomal RNA gene. *Plasmodium* genus–specific primers and four pairs of species-specific primers described in a previous study were used for the nested PCR.[6] Results showed that the patient was infected with four species of *Plasmodium*, including *P. malariae*, which was not identified in the blood smear by microscopy (Figure 2). Amplified fragments were cloned into a vector and sequenced. The fragment amplified with *P. falciparum*-specific nested primers showed 100% identity with the *P. falciparum* 18S ribosomal RNA gene (GenBank accession no. JQ627152.1, expected = 3×10^{-39}). Fragments amplified with *P. vivax*-specific, *P. ovale*-specific, and *P. malariae*-specific nested primers showed a single nucleotide substitution in the *P. vivax* 18S ribosomal RNA gene (GenBank accession no. JQ627158, expected = 1×10^{-38}), the *P. ovale* 18S ribosomal RNA gene (GenBank accession no. AB182490.1, expected = 9×10^{-44}) and the *P. malariae* 18S ribosomal RNA gene (GenBank accession no. M54897.1, expected = 9×10^{-44}), respectively.

Because two types of *P. ovale* strains (classic and variant types) are prevalent in Africa and Asia, and it has been reported that the two types are distinct species,[7] we generated specific nested-primers specific for the *P. ovale* cytochrome b

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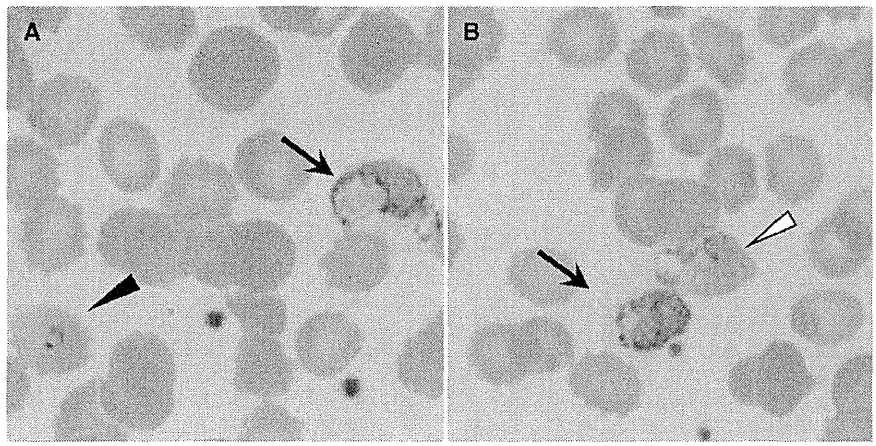

FIGURE 1. Microscopy of a Giemsa-stained peripheral blood smear of the patient. **A**, Ring form of *Plasmodium falciparum* (**arrowhead**) and a late trophozoite of *P. vivax* (**arrow**) were observed. **B**, Trophozoite of *P. vivax* (**arrow**) and trophozoite of *P. ovale* (**white arrowhead**) were observed.

gene (*cytb*) to determine the *P. ovale* species (Figure 3A). Specific bands corresponding to a 257-basepair fragment, were amplified from the sample. No specific bands were amplified from *P. vivax* genomic DNA (MRA-41, provided by the Malaria Research and Reference Reagent Resource Center) under these conditions when used as a negative control. Sequencing of the amplified fragment showed 100% identity with the *P. ovale wallikeri cytb* gene (GenBank accession no. HQ712053, expected = 1×10^{-127}), and single-nucleotide polymorphisms specific for *P. ovale wallikeri* were detected (Figure 3B). These results showed that the patient was infected with four malaria species (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale wallikeri*).

DISCUSSION

Some triple or quadruple mixed malaria infections have been reported among semi-immune residents in malaria-endemic areas of Asia[8,9] and Africa.[10,11] However, triple or quadruple mixed malarial infections have been rare among non-residents of such areas or among nonimmune persons. The present case is the first imported case of quadruple malaria in a nonimmune Japanese patient.

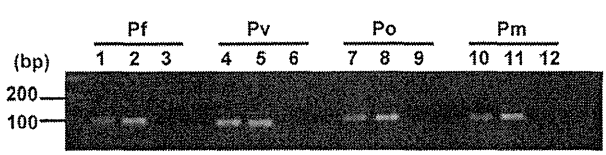

FIGURE 2. Detection of a *Plasmodium* 18S ribosomal RNA gene fragment by using nested polymerase chain reaction analysis. Amplification was performed by using a standard protocol for 35 and 20 cycles for the first and the second amplifications, respectively. Samples were first amplified with *Plasmodium* genus-specific primers and then amplified with *P. falciparum*-specific (lanes 1–3), *P. vivax*-specific (lanes 4–6), *P. ovale*-specific (lanes 7–9), and *P. malariae*-specific (lanes 10–12) oligonucleotide sets, respectively. Specific bands corresponding to *P. falciparum* (101 basepairs [bp], lanes 1 and 2), *P. vivax* (104 bp, lanes 4 and 5), *P. ovale* (115 bp, lanes 7 and 8), and *P. malariae* (115 bp, lanes 10 and 11) were amplified. Patient samples: lanes 1, 4, 7, and 10. Positive controls: lanes 2, 5, 8, and 11. Negative controls: lanes 3, 6, 9, and 12.

In mixed malaria infections, the level of parasitemia is different for each *Plasmodium* spp. In Japan, some mixed infections have been confirmed by combined use of morphologic detection and other alternative methods, including immunoassays and the molecular analyses.[12,13] The findings in this report, as well as in other reports,[14,15] indicate that molecular detection methods, such as nested PCR and real-time PCR, have superior sensitivity and specificity, which enables identification of low levels of infection and differentiation of species.

For antimalarial chemoprophylaxis, mefloquine is recommended in Japan, but it cannot be used in areas with mefloquine-resistant malaria. Doxycycline and atovaquone/proguanil can be used in all malaria-endemic areas, and these drugs are used in Japan. These drugs are effective against erythrocytic forms and also inhibit development of the normal liver stages, but they do not affect hypnozoites. Therefore, development of clinical malaria from hypnozoites of *P. vivax* or *P. ovale* has been reported more than two months after returning among travelers who continued chemoprophylaxis based on standard regimens.[16]

Daily use of doxycycline is recommended during travel to malaria-endemic areas and for four weeks after returning to a area where malaria is not endemic. However, the patient in this study stopped taking doxycycline after two weeks. Similar prophylactic failures for doxycycline against *P. falciparum* or *P. vivax* are associated with use of inadequately low doses.[17,18] Moreover, the prevalence of *P. falciparum* isolates with reduced susceptibility to mefloquine and doxycycline has been reported in Africa.[19,20]

In nonimmune persons taking chemoprophylaxis, efficacy differs for each *Plasmodium* spp. Furthermore, different levels of drug resistance to each strain and the dose of drugs used influence the clinical manifestations in cases with chemoprophylactic failure. *P. falciparum* might dominate over *P. vivax* by inducing a primary infection, as occasionally observed in patients simultaneously infected with the two species. In the present case, however, *P. vivax* predominated over *P. falciparum*. A partial effect of doxycycline was conceivably obtained because the parasitemia with *P. falciparum* and *P. malariae* was low. Detection of *P. malariae* was difficult by

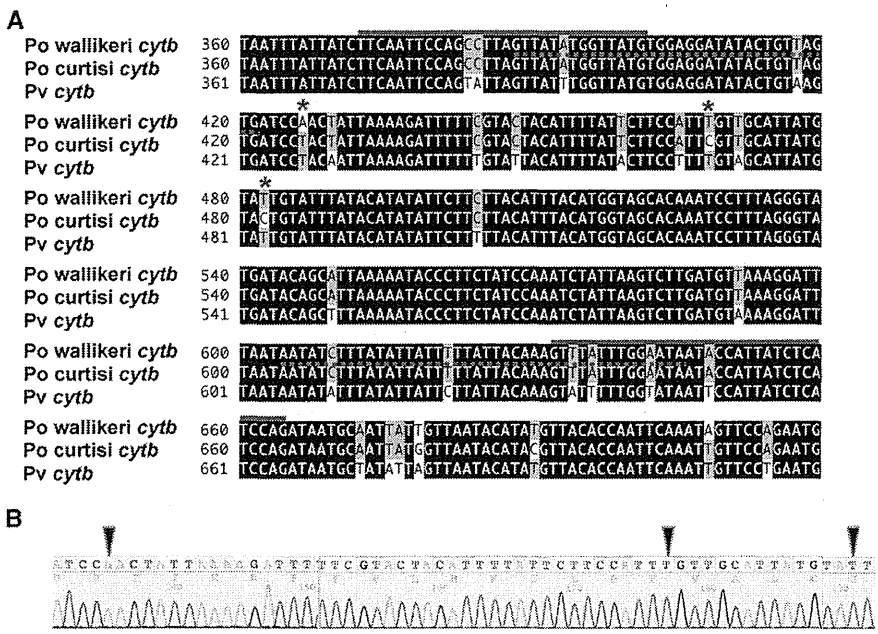

FIGURE 3. Sequence analysis of the *Plasmodium ovale* (Po) *wallikeri cytb* gene. **A**, Nucleotide alignment of the *cytb* genes from *P. ovale wallikeri* (Gen Bank accession no. GU723535), *P. ovale curtisi* (GU723514), and *P. vivax* (Pv) (AY791525). Red lines indicate regions where nested primers are annealed, respectively. Oligonucleotide sequences used to amplify polymorphic regions of *P. ovale* spp. were outer primers PoCytb-1F (5′-TTC AAT TCC AGC CTT AGT TAT ATG GTT ATG-3′) and PoCytb-2R (5′-CTG GAT GAG ATA ATG GTA TTA TTC CAA ATA AAC-3′) and inner primers PoCytb-3F (5′-GTT ATA TGG TTA TGT GGA GGA TAT ACT GTT AGT G-3′) and PoCytb-4R (5′-TAT TCC AAA TAA ACT TTG TAA TAA AAA TAA TAT AAA GAT ATT-3′). A polymerase chain reaction was performed as described above for 40 and 30 cycles for the first and second amplifications, respectively. Three single nucleotide polymorphism sites included this region specific for *P. ovale wallikeri* are indicated by red stars. **B**, Sequence analysis of a fragment amplified by using nested polymerase chain reaction identified *P. ovale wallikeri*–specific nucleotide polymorphisms, as indicated by arrowheads.

microscopy and was confirmed by using the nested PCR. A variant strain of *P. ovale* could not be detected by PCR for the classic strain, and its infection was confirmed by the more specific protocol of PCR used for the present case.

Although the Duffy-negative phenotype $FY*BE^{ES}$, which is not susceptible to infection with *P. vivax*, is dominant in sub-Saharan Africa,[21] Duffy-negative persons infected with *P. vivax* have been reported and suggested as a possible reservoir of *P. vivax* to Duffy-positive persons, such as those seen in Japanese persons.[22] As more nonimmune persons visit malaria-endemic countries in Africa, there are likely to be more cases of imported mixed infections of *Plasmodium* species, including *P. vivax*. Nonimmune persons who stay in malaria-endemic areas for long periods face an increasing risk of mixed infection, and clinical features are modified by chemoprophylaxis. When nonimmune persons show atypical clinical features for malaria or when chemoprophylaxis failure is suspected, all four species of human malaria parasites should be considered as causative agents, and the patients should be examined by using sensitive and specific tests, such as the PCR, in addition to conventional morphologic analyses.

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REFERENCES

1. Marques PX, Saúte F, Pinto VV, Cardoso S, Pinto J, Alonso PL, Rosário VE, Arez AP, 2005. *Plasmodium* species mixed infections in two areas of Manhiça District, Mozambique. *Int J Biol Sci 1:* 96–102.
2. Mayxay M, Pukrittayakanee S, Newton PN, White NJ, 2004. Mixed-species malaria infections in humans. *Trends Parasitol 20:* 233–240.
3. Infectious Disease Surveillance Center, Malaria. 2006–2009. Available at: http://idsc.nih.go.jp/disease/malaria/2010week38.html. Accessed February 2013.
4. Haranaga S, Akashi M, Yara S, Tohyama M, Toyama M, Ishimine T, Miyara T, Shinzato T, Higa F, Tateyama M,

Saito A, Toma H, 2002. Two cases of mixed infection of malaria diagnosed by PCR method. *Jpn J Infect Dis 76:* 571–575.
5. Saito-Nakano Y, Tanabe K, Mita T, 2011. Identification of pyrimethamine- and chloroquine-resistant *Plasmodium falciparum* in Africa between 1984 and 1998: genotyping of archive blood samples. *Malar J 10:* 388. doi:10.1186/1475-2875-10-388.
6. Saito-Nakano Y, Tanabe K, Kamei K, Iwagami M, Komaki-Yasuda K, Kawazu S, Kano S, Ohmae H, Endo T, 2008. Genetic evidence for *Plasmodium falciparum* resistance to chloroquine and pyrimethamine in Indochina and the Western Pacific between 1984 and 1998. *Am J Trop Med Hyg 79:* 613–619.
7. Kimura M, Kaneko O, Inoue A, Ishii A, Tanabe K, 1995. Amplification by polymerase chain reaction of *Plasmodium falciparum* DNA from Giemsa-stained thin blood smears. *Mol Biochem Parasitol 70:* 193–197.
8. Sutherland CJ, Tanomsing N, Nolder D, Oguike M, Jennison C, Pukrittayakamee S, Dolecek C, Hien TT, do Rosário VE, Arez AP, Pinto J, Michon P, Escalante AA, Nosten F, Burke M, Lee R, Blaze M, Otto TD, Barnwell JW, Pain A, Williams J, White NJ, Day NP, Snounou G, Lockhart PJ, Chiodini PL, Imwong M, Polley SD, 2010. Two nonrecombining sympatric forms of the human malaria parasite *Plasmodium ovale* occur globally. *J Infect Dis 15:* 1544–1550.
9. Snounou G, Viriyakosola S, Zhua XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN, 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol Biochem Parasitol 61:* 315–320.
10. Purnomo SA, Gomez-Saladin E, Bangs MJ, 1999. Rare quadruple malaria infection in Iran Jaya Indonesia. *J Parasitol 85:* 574–579.
11. Arez AP, Pinto J, Pålsson K, Snounou G, Jaenson TG, do Rosário VE, 2003. Transmission of mixed *Plasmodium* species and *Plasmodium falciparum* genotypes. *Am J Trop Med Hyg 68:* 161–168.
12. Miura T, Kimura M, Koibuchi T, Endo T, Nakamura H, Odawara T, Wataya Y, Nakamura T, Iwamoto A, 2005. Clinical characteristics of imported malaria in Japan: analysis at a referral hospital. *Am J Trop Med Hyg 73:* 599–603.
13. Higa F, Tateyama M, Tasato D, Karimata Y, Nakamura H, Miyagi K, Haranaga S, Hirata T, Hokama A, Cash HL, Toma H, Fujita J, 2013. Imported malaria cases in Okinawa prefecture, Japan. *Jpn J Infect Dis 66:* 32–35.
14. Boonma P, Christensen PR, Suwanarusk R, Price RN, Russell B, Lek-Uthai U, 2007. Comparison of three molecular methods for the detection and speciation of *Plasmodium vivax* and *Plasmodium falciparum*. *Malar J 6:* 124., doi: 10.1186/1475-2875-6-124.
15. Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, Zimmerman PA, del Portillo HA, Siba P, Mueller I, Felger I, 2010. Comparison of diagnostic methods for the detection and quantification of the four sympatric *Plasmodium* species in field samples from Papua New Guinea. *Malar J 9:* 361.
16. Schwartz E, Parise M, Kozarsky P, Cetron M, 2003. Delayed onset of malaria: implications for chemoprophylaxis in travelers. *N Engl J Med 349:* 1510–1516.
17. Pang L, Limsomwong N, Singharaj P, 1988. Prophylactic treatment of vivax and falciparum malaria with low-dose doxycycline. *J Infect Dis 158:* 1124–1127.
18. Shmuklarsky MJ, Boudreau EF, Pang LW, Smith JI, Schneider I, Fleckenstein L, Abdelrahim MM, Canfield CJ, Schuster B, 1994. Failure of doxycycline as a causal prophylactic agent against *Plasmodium falciparum* malaria in healthynonimmune volunteers. *Ann Intern Med 120:* 294–299.
19. Fall B, Diawara S, Sow K, Baret E, Diatta B, Fall KB, Mbaye PS, Fall F, Diémé Y, Rogier C, Wade B, Bercion R, Pradines B, 2011. Ex vivo susceptibility of *Plasmodium falciparum* isolates from Dakar, Senegal, to seven standard anti-malarial drugs. *Malar J 10:* 310.
20. Fall B, Pascual A, Sarr FD, Wurtz N, Richard V, Baret E, Diémé Y, Briolant S, Bercion R, Wade B, Tall A, Pradines B, 2013. *Plasmodium falciparum* susceptibility to anti-malarial drugs in Dakar, Senegal, in 2010: an ex vivo and drug resistance molecular markers study. *Malar J 12:* 107.
21. Howes RE, Patil AP, Piel FB, Nyangiri OA, Kabaria CW, Gething PW, Zimmerman PA, Barnadas C, Beall CM, Gebremedhin A, Ménard D, Williams TN, Weatherall DJ, Hay SI, 2011. The global distribution of the Duffy blood group. *Nat Commun. 2:* 266.
22. Mendes C, Dias F, Figueiredo J, Mora VG, Cano J, de Sousa B, do Rosario VE, Benito A, Berzosa P, Arez AP, 2011. Duffy negative antigen is no longer a barrier to *Plasmodium vivax*: molecular evidences from the African west coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis 5:* e1192.

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Anti-Trypanosomal Activity of Diarylheptanoids Isolated from the Bark *of Alnus japonica*

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Abstract: The crude extract of *Alnus japonica* bark exhibited a strong effect on the growth of *Trypanosoma brucei*. Subsequent chromatographic separation resulted in the isolation of two novel diarylheptanoids, known as alnuside C (**2**) and alnuside D (**3**), and three known compounds, 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(*R*)-*O*-β-D-glucopyranoside (**1**), oregonin (**4**) and hirsutanone (**5**). The structures of the isolates were elucidated based on the use of extensive spectroscopic and chemical methods. Among the isolated diarylheptanoids, oregonin (**4**) (a major component of plant bark) and hirsutanone (**5**) exhibited potent *in vitro* inhibitory activity against *T. brucei* growth in the bloodstream with IC_{50} values of 1.14 and 1.78 μM, respectively. We confirmed that oregonin (**4**) and hirsutanone (**5**) were not toxic to human normal skin fibroblast cells (NB1RGB) and colon cancer cells (HCT-15) at a concentration of 50 μM; however, lower levels of toxicity were observed for leukemia cells. To determine the structure activity relationships of the isolated components, we performed Conformation Search and found that the 3-oxo function of the heptane chain in the diarylheptanoid molecule is required for their trypanocidal activity.

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Introduction

Human African trypanosomiasis (HAT), commonly known as sleeping sickness, has long been a serious health and socioeconomic problem in sub-Saharan Africa (Remme *et al.*, 2002; WHO, 2013). Recently, significant progress has been made in controlling the disease. New cases have fallen to an annual rate of approximately 50,000; however, an estimated 60 million people are still at risk of contracting this disease (Barrett *et al.*, 2007; Kennedy, 2008; Mallari *et al.*, 2008a,b). The disease occurs upon infection with the protozoan parasites *Trypanosoma brucei rhodesiense* or *T. b. gambiense*, which reside in the bloodstream during the early phase of the disease but can subsequently invade the central nervous system. The later stages of the disease result in behavioral changes and disrupted sleeping patterns or coma (sleeping sickness) symptoms, which if left untreated ultimately cause death (Barrett *et al.*, 2007; Mallari *et al.*, 2008a,b).

Treatment of HAT has previously relied on the use of four drugs: suramin, pentamidine, melarsoprol and eflornithine developed in 1921, 1941, 1949 and 1990, respectively. These drugs cause severe side effects, require lengthy parenteral administration, lack efficacy and are too expensive for most patients to obtain (Docampo and Moreno, 2003). Because HAT is a disease that afflicts the poorest population in Africa, the traditional market driven pathways for drug development are not available. Furthermore, eflornithine and pentamidine are ineffective for the treatment of sleeping sickness caused by *T. b. rhodesiense*. There has also been an increase in the number of patients who fail to respond to melarsoprol due to the emergence of drug resistant forms of *T. brucei* (Pepin and Milord, 1994). The increase in drug resistant forms coupled with the use of antiquated toxic drugs to cure the disease underscores the need for new HAT treatments.

A. japonica (Betulaceae), which is a common tree found in the low mountainous areas of Northeastern China, Korea and Japan, has been used in folk oriental medicine as remedies for fever, hemorrhage, diarrhea, and alcoholism. Previous phytochemical investigations of *A. japonica* have led to the identification of various diarylheptanoids, several triterpenoids and some flavonoids (Nomura *et al.*, 1981; Wada *et al.*, 1998; Lee *et al.*, 1992; Kuroyanagi *et al.*, 2005). We also isolated several diarylheptanoids from *A. japonica* and determined their anti-oxidant and anti-influenza activities (Tung *et al.*, 2010a,b). Diarylheptanoids belong to a phenolic class of natural products that contain a 1,7-diphenylheptane skeleton, and numerous diarylheptanoids have recently been isolated from Zingiberaceae and Betulaceae plants (Nomura *et al.*, 1981; Kikuzaki *et al.*, 1991; Wada *et al.*, 1998; Gonzalez-Laredo *et al.*, 1999; Giang *et al.*, 2006; Tung *et al.*, 2010a). The well-known diarylheptanoid curcumin is the principal component of the spice turmeric (Zingiberaceae) and exhibits various biological properties such as anti-inflammatory (Xu *et al.*, 1998), anti cancer (Hossain *et al.*, 2012), anti-oxidant (Rahman and Adcock, 2006), and anti-trypanosomal (Nagajyothi *et al.*, 2012; Nose *et al.*, 1998) activities. Interest in the

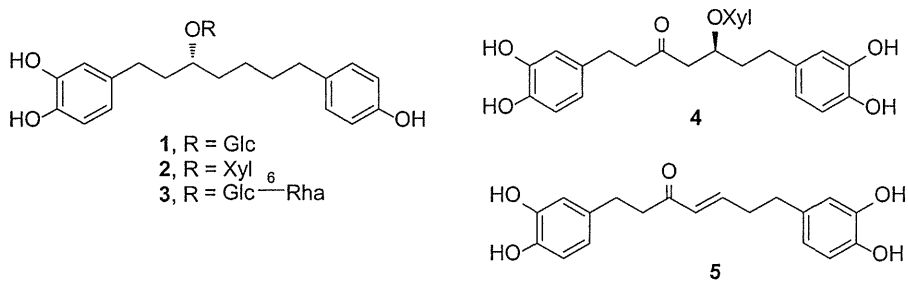


Figure 1. Structures of the diarylheptanoids (**1–5**) (Glc: β-D-glucopyranosyl, Xyl: β-D-xylopyranosyl, Rha: α-L-rhamnopyranosyl).

anti-trypanosomal activity of *A. japonica* has increased since an ethanol extract prepared from its bark was found to exhibit potent inhibitory effects on the growth of *T. brucei* during our ongoing survey to identify naturally occurring anti-trypanosomal agents. Subsequently, chromatographic purification led to the isolation of two novel diarylheptanoids, alnuside C (**2**) and alnuside D (**3**), together with three known compounds, 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(*R*)-*O*-β-D-glucopyranoside (**1**), oregonin (**4**) and hirsutanone (**5**) (Fig. 1). This paper describes the structural identification of the five isolated diarylheptanoids, their anti-trypanosomal effects and the structure activity relationships obtained by molecular modeling.

Materials and Methods

General Experimental Procedures

Optical rotations were obtained using a JASCO P-1020 automatic digital polarimeter (JASCO, Easton, USA). IR spectra were recorded on a JASCO FT/IR–4200 spectrometer. NMR spectra were recorded on a JEOL ECX 400 NMR spectrometer (JEOL, Tokyo, Japan). HR-ESI-TOFMS experiments utilized a JEOL AccuTOF^{TM} LC 1100 mass spectrometer (JEOL, Tokyo, Japan). HPLC analysis of sugars was performed on an Agilent 1100 Series HPLC system (Agilent, Santa Clara, CA, USA) equipped with a YMC-Pack NH_2 column (250 × 4.6 mm i.d., NH12S05-2546WT, YMC Co. Ltd., Kyoto, Japan) and an optical rotation detector JASCO OR-2090 (JASCO, Easton, USA). Column chromatography was performed on silica gel 60 (230–400 mesh, Nacalai Tesque Inc., Kyoto, Japan) and YMC ODS-A gel (50 μm, YMC Co. Ltd., Kyoto, Japan). TLC was performed on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) plates. Spots were visualized by spraying the plates with a 10% aqueous H_2SO_4 solution, followed by heating.

Plant Material and Purification of Compounds

The bark of *A. japonica* was donated by Prof. Takao Setsu from Kyushu University Forest in January 2011. The voucher specimen was deposited at the Herbarium of Faculty of

Pharmaceutical Science, Nagasaki International University. The air-dried sample (1.0 kg) was extracted with 50% aqueous EtOH (2.0 L × 3 times) at 40°C under sonication. After removal of the solvent, the obtained residue (115 g) was resuspended in 1.0 L of water and successively partitioned with CH_2Cl_2 and EtOAc (each 1.0 L × 3) to obtain soluble fractions of CH_2Cl_2 (35.0 g) and EtOAc (53.5 g). A part of the EtOAc fraction (40.0 g) was fractionated over a silica gel column using a gradient of $CHCl_3$–MeOH (15:1–0:1, v/v) to give six fractions (fraction 1.1–1.6). Fraction 1.2 (2.6 g) was further chromatographed using a silica gel column with $CHCl_3$–MeOH (10:1), followed by a reverse-phase C_{18} (RP) column with MeOH–H_2O (1:1) to produce compound **5** (60 mg). Fraction 1.4 (4.5 g) was subjected to a RP column with MeOH–H_2O (6:5) to obtain seven sub-fractions (fraction 2.1–2.7). Then, fraction 2.1 (1.8 g) was re-chromatographed using a silica gel column with $CHCl_3$–MeOH (6:1, v/v) to yield compound **4** (1600 mg). Fraction 2.6 (80 mg) was chromatographed using a silica gel column with EtOAc–MeOH (15:1) to produce compounds **1** (18.0 mg) and **3** (15.0 mg). Subsequently, compound **2** (11 mg) was purified from fraction 2.7 (350 mg) by silica gel column chromatography with $CHCl_3$–MeOH (5:1).

*1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3-(R)-O-ß-D-glucopyranoside (**1**)*

Colorless solid; $[\alpha]_D^{20}$–12° (*c* 0.22, MeOH); IR (film) ν_{max} 3356, 2924, 1611, 1516, 1440, 1052, 824 cm^{-1}; ^{1}HNMR (CD_3OD, 400 MHz) δ 1.32 (2H, m, H-5), 1.48 (2H, m, H-6), 1.55 (2H, m, H-4), 1.70 (2H, m, H-2), 2.44 (2H, t, J = 7.2 Hz, H-7), 2.50 (2H, t, J = 7.2 Hz, H-1), 3.12 (1H, t, J = 8.0 Hz, H-2‴), 3.24 (1H, m, H-3‴), 3.30 (1H, m, H-4‴), 3.35 (1H, m, H-5‴), 3.58 (1H, dd, J = 11.6, 5.2 Hz, H-6a‴), 3.68 (1H, m, H-3), 3.84 (1H, dd, J = 11.6, 2.0 Hz, H-6b‴), 4.26 (1H, d, J = 7.6 Hz, H-1‴), 6.46 (1H, dd, J = 8.0, 1.6 Hz, H-6′), 6.60 (2H, d, J = 8.0 Hz, H-3″, 5″), 6.62 (1H, d, J = 8.0 Hz, H-5′), 6.66 (1H, d, J = 1.6 Hz, H-2′), 6.91 (2H, d, J = 8.0 Hz, H-2″, 6″); ^{13}CNMR (CD_3OD, 100 MHz) see Table 1; (+)-HRESI-TOFMS m/z 479.2296 $[M + H]^+$ (Calcd for $C_{25}H_{35}O_9$: 479.2281).

*Alnuside C (**2**)*

Colorless solid; $[\alpha]_D^{20}$ − 19° (*c* 0.25, MeOH); IR (film) ν_{max} 3353, 2924, 1612, 1515, 1442, 1055, 825 cm^{-1}; ^{1}HNMR (CD_3OD, 400 MHz) δ 1.33 (2H, m, H-5), 1.45 (2H, m, H-6), 1.51 (2H, m, H-4), 1.68 (2H, m, H-2), 2.40 (2H, t, J = 7.2 Hz, H-7), 2.48 (2H, t, J = 7.2 Hz, H-1), 3.12 (1H, t, J = 8.0 Hz, H-2‴), 3.20 (1H, m, H-5a‴), 3.30 (1H, m, H-3‴), 3.52 (1H, m, H-4‴), 3.70 (1H, m, H-3), 3.78 (1H, dd, J = 11.6, 5.2 Hz, H-5b‴), 4.23 (1H, d, J = 7.6 Hz, H-1‴), 6.46 (1H, dd, J = 8.0, 1.6 Hz, H-6′), 6.60 (2H, d, J = 8.0 Hz, H-3″, 5″), 6.62 (1H, d, J = 8.0 Hz, H-5′), 6.66 (1H, d, J = 1.6 Hz, H-2′), 6.90 (2H, d, J = 8.0 Hz, H-2″, 6″); ^{13}CNMR (CD_3OD, 100 MHz) see Table 1; (+)-HRESITOFMS m/z 449.2186 $[M + H]^+$ (Calcd for $C_{24}H_{33}O_8$: 449.2175).

*Alnuside D (**3**)*

Colorless solid; $[\alpha]_D^{20}$ − 31° (*c* 0.21, MeOH); IR (film) ν_{max} 3350, 2925, 1614, 1516, 1445, 1050, 822 cm^{-1}; ^{1}HNMR (CD_3OD, 400 MHz) δ 1.20 (3H, d, J = 6.4 Hz, H-6⁗), 1.32

Table 1. ^{13}C NMR Data for the Three Diarylheptanoids (1–3) and the Aglycone (6) in CD_3OD

Carbon	1	2	3	6
Aglycone				
1	31.7	31.8	32.1	32.3
2	37.9	38.1	38.2	40.5
3	79.3	80.5	80.6	71.8
4	34.7	35.1	35.1	38.2
5	25.6	25.8	25.7	26.4
6	33.1	33.0	33.2	32.8
7	35.9	36.2	36.0	36.1
1′	135.6	135.7	135.7	135.4
2′	116.9	116.7	116.7	116.8
3′	144.0	144.1	144.1	144.2
4′	145.9	146.0	146.0	146.1
5′	116.2	116.5	116.3	116.2
6′	120.8	120.7	120.7	120.8
1″	134.8	132.9	134.8	134.8
2″, 6″	130.3	130.3	130.3	130.4
3″, 5″	116.0	116.2	116.0	116.1
4″	156.2	156.6	156.2	156.3
Sugar moiety				
1‴	103.2	103.7	103.7	
2‴	75.3	75.0	75.3	
3‴	78.2	77.9	78.2	
4‴	71.7	71.9	72.2	
5‴	77.8	65.5	76.7	
6‴	62.8		68.4	
1⁗			102.2	
2⁗			71.6	
3⁗			72.4	
4⁗			74.3	
5⁗			69.8	
6⁗			18.1	

(2H, m, H-5), 1.49 (2H, m, H-6), 1.55 (2H, m, H-4), 1.70 (2H, m, H-2), 2.43 (2H, t, $J = 7.2$ Hz, H-7), 2.50 (2H, t, $J = 7.2$ Hz, H-1), 3.16 (1H, t, $J = 8.0$ Hz, H-2‴), 3.25 (1H, m, H-3‴), 3.30 (1H, m, H-4‴), 3.35 (1H, m, H-5‴), 3.42 (1H, m, H-4⁗), 3.54 (1H, m, H-5⁗), 3.68 (1H, m, H-3), 3.71 (1H, dd, $J = 11.6$, 5.2 Hz, H-6a‴), 3.74 (1H, m, H-3⁗), 3.90 (1H, m, H-2⁗), 4.00 (1H, dd, $J = 11.6$, 2.0 Hz, H-6b‴), 4.26 (1H, d, $J = 7.6$ Hz, H-1‴), 4.73 (1H, br s, H-1⁗), 6.49 (1H, dd, $J = 8.0$, 1.6 Hz, H-6′), 6.61 (2H, d, $J = 8.0$ Hz, H-3″, 5″), 6.64 (1H, d, $J = 8.0$ Hz, H-5′), 6.68 (1H, d, $J = 1.6$ Hz, H-2′), 6.95 (2H, d, $J = 8.0$ Hz, H-2″, 6″); ^{13}CNMR (CD_3OD, 100 MHz) see Table 1; (+)-HRESITOFMS m/z 625.2862 $[M+H]^+$ (Calcd for $C_{31}H_{45}O_{13}$: 625.2860).

*Acid Hydrolysis of Compounds (**1–3**)*

A solution of compounds **1–3** (6.0 mg each) dissolved in 1.0 M HCl and dioxane (1:1, 5.0 mL) was heated under reflux for 3 h. After cooling, the reaction mixture was poured into ice water and neutralized with Amberlite IRA-400 (hydroxyl form), and the resin was removed by filtration. Then, the filtrate was concentrated *in vacuo* to dryness and followed by partitioning between EtOAc and H_2O. The EtOAc residue was subjected to preparative TLC with $CHCl_3$–MeOH (10:1) to produce aglycone **6**. The aqueous layers were subjected to HPLC analysis with a mobile phase of CH_3CN–H_2O (80:20, v/v) at a flow rate of 0.40 mL/min. Identification of sugar components in the aqueous layers were carried out by comparison of their retention time and optical rotation with those of authentic samples. t_R: 7.2 min (L-rhamnose, negative optical rotation), 8.3 min (D-xylose, positive optical rotation), and 9.6 min (D-glucose, positive optical rotation), respectively.

*1-(3,4-Dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(R)-ol (**6**)*

Colorless solid; $[\alpha]_D^{20}$ − 26° (*c* 0.20, MeOH); ^{1}H NMR (CD_3OD, 400 MHz) δ 1.34 (2H, m, H-5), 1.49 (2H, m, H-6), 1.53 (2H, m, H-4), 1.64 (2H, m, H-2), 2.45 (2H, t, J = 7.2 Hz, H-7), 2.52 (2H, t, J = 7.2 Hz, H-1), 3.52 (1H, m, H-3), 6.46 (1H, dd, J = 8.0, 1.6 Hz, H-6′), 6.59 (2H, d, J = 8.0 Hz, H-3″, 5″), 6.62 (1H, d, J = 8.0 Hz, H-5′), 6.65 (1H, d, J = 1.6 Hz, H-2′), 6.90 (2H, d, J = 8.0 Hz, H-2″, 6″); ^{13}C NMR (CD_3OD, 100 MHz) see Table 1; (+)-HRESITOFMS *m/z* 317.1759 $[M+H]^+$ (Calcd for $C_{19}H_{25}O_4$: 317.1753).

Anti-Trypanosomal Experiment—Sample Preparation

The purity of the isolated compounds was tested by TLC and HPLC, and caffeic acid (purity $\geq$ 98%) was purchased from Nacalai Tesque Inc., Kyoto, Japan and used without further purification. Berberine (Sigma-Aldrich, purity $\geq$ 98%) was used as a positive control.

Trypanosomes

T. brucei GUTat 3.1 (a polymorphic clone) was maintained in mice (ddY strain) weighing 20–25 g and was serially transferred at 5 d intervals.

Culture Media

Iscove's modified Dulbecco's media (IMDM, Life Technologies, Inc., New York, USA) was used to propagate *T. brucei* bloodstream forms as previously described. The culture medium was supplemented with 0.3% (w/v) sodium bicarbonate, 100 μM hypoxanthine, 30 μM thymidine (Aldrich Chemical Co. Inc., Wisconsin, USA), 40 μM adenosine (Sigma Chemical Co., Dorset UK), 1 mM sodium pyruvate (Wako Pure Chemical Industries, Osaka, Japan), 50 μM L-glutamine (Wako Pure Chemical Industries), 100 μM 2-mercaptoethanol (FlukaChemie AG, Buchs, Switzerland) and 20% (v/v) heat-inactivated

fetal bovine serum (HIFBS, HyClone Laboratories Inc., Logan, UT, USA). The medium was adjusted to pH 7.4.

Anti-Trypanosomal Assay

Cultured Trypanosoma bloodstream forms were suspended in culture medium at a concentration of 3×10^5 cells/mL. The extract, fractions or compounds were added to 96-well culture plates at appropriate concentrations and incubated at 37°C in 5% CO_2. The rates of trypanosome survival were determined by an Alamar blue assay after 24 h of incubation. 10% Alamar blue was added to the cultured medium in 96-well plates and incubated for an additional 24 hours in the dark. Data was measured using a plate reader at 470 nm.

Non-Specific Mammalian Cytotoxicity Study-Cell Culture and Treatment

NB1RGB (normal human skin fibroblast cells), HCT-15 (human colon cancer cells), Jurkat (human acute T-cell leukemia cells), U937 (human promonocytic leukemia cells), HL-60 (human promyelocytic leukemia cells), and THP-1 (human monocytic leukemia cells) were obtained from the RIKEN Bio Resource Center Cell Bank. NB1RGB cells were grown in α-MEM medium. HCT-15, Jurkat, U937, HL-60, and THP-1 were maintained in RPMI1640 medium. All cell culture mediums were supplemented with 10% FBS and 1% penicillin–streptomycin and incubated at 37°C with 5% CO_2 under fully humidified conditions (Huang *et al.*, 2012; Wu *et al.*, 2013). For the cell treatments, isolated compounds were dissolved in DMSO and stored at −20°C until use. The DMSO concentrations in the cell culture medium did not exceed 0.2% (*v/v*), and the controls were always treated with the same amount of DMSO used in the corresponding experiments.

Determination of Cytotoxicity

Cytotoxicity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. NB1RGB and HCT-15 (0.5×10^4 cells/well) and four leukemia cell lines (1×10^4 cells/well) were cultured in 96-well plates and treated with various concentrations of each of the purified compounds for 48 h. MTT solution was added to each well at the end of the treatment period, and the cells were incubated for another 4 h. The precipitated MTT-formazan product was dissolved in 0.04 N HCl–isopropanol and the amount of formazan was measured at a wavelength of 595 nm by a microplate reader (ImmunoMiniNJ-2300, Nihon InterMed, Tokyo, Japan). Cytotoxicity was calculated as the percentage of live cells relative to the control culture (Shen *et al.*, 2012; Chen *et al.*, 2013). The selectivity index (SI) was determined using the following equation: IC_{50} obtained for mammalian cells/IC_{50} obtained for Trypanosoma.

Computational Study

The software MOE (Molecular Operating Environment; Chemical Computing Group Inc.) was used for all of the calculations and analysis performed in this study. The software

version was MOE 2012.10. The computer and OS (operation system) were as follows: CPU (central processing unit) was a Intel (TM) Core (TM)2 CPU 6400 (2.13 GHz), the amount of memory was 3062 MB, and the version of the OS was Windows Vista.

Results and Discussion

The ethanol extract obtained from the bark of *A. japonica* was suspended in water and partitioned successively with CH_2Cl_2 and EtOAc followed by column chromatography to yield 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(*R*)-*O*-β-D-glucopyranoside (**1**), which was recently isolated from *A. formosana* (Lai *et al.*, 2012), alnuside C (**2**), alnuside D (**3**), along with oregonin (**4**) (Kuroyanagi *et al.*, 2005) and hirsutanone (**5**) (Kuroyanagi *et al.*, 2005). The ^{1}H and ^{13}C NMR (Table 1) spectra of compounds **1–3** suggested that they belong to the family of diarylheptanoid glycosides because they have the same aglycone structure. Further analyses of NMR data obtained from the aglycone structures revealed the existence of one 3,4-dihydroxy phenol ring and one 4-hydroxy aromatic ring in the molecules of the compounds **1–3**, similar to results observed for alnuside B (Kuroyanagi *et al.*, 2005) and (*4E*)-1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptene-3-one (Tung *et al.*, 2010a). The heptane chains in compounds **1–3** consisted of six methylene carbons and one oxymethine signal, resembling the heptane chains observed for rubranosides A–D (Gonzalez-Laredo *et al.*, 1999).

Aside from its diarylheptanoid moiety, the ^{13}C NMR spectrum of **1** showed a set of six signals (δ 103.2, 75.3, 78.2, 71.7, 77.8, and 62.8) contributing to a D-glucopyranosyl unit (Agrawal, 1992). The configuration of the anomeric position was determined to be of the β type based on the large coupling constant (J = 7.6 Hz) of the anomeric proton at δ 4.26 in the ^{1}H-NMR spectrum. Comprehensive analyses of the HMQC, HMBC, and H-H COSY spectra of compound **1** permitted complete assignments of its proton and carbon signals. The H-H COSY and selected HMBC correlations of compound **1** are illustrated in Fig. 2. Accordingly, the HMBC correlations of H-3/C-1‴ and H-1‴/C-3 confirmed the interlinkage positions at C-3 between the heptane chain and the glucose moieties. In addition, the acidic hydrolysis of compound **1** produced the aglycone, 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(*R*)-ol (**6**) (Wada *et al.*, 1998; Gonzalez-Laredo *et al.*, 1999; Lai *et al.*, 2012) and D-glucose. The HRESIMS experimental data obtained for compound **1** revealed a molecular ion peak at *m/z* $[M+H]^+$ 479.2296, thus supporting the predicted molecular formula $C_{25}H_{34}O_9$. Because the structure of compound **1** was shown to be similar to rubranosides A–D, the absolute configuration of C-1′ was assigned via application of the ^{13}C NMR glycosylation shift rule (Seo *et al.*, 1978; Wada *et al.*, 1998; Gonzalez-Laredo *et al.*, 1999). When compared with the ^{13}C NMR data of aglycone **6**, the data for compound **1** showed glycosylation shifts of −2.6 ppm for C-2 and −3.5 ppm for C-4 indicating an *R*-configuration at carbon C-3. Consequently, the structure of **1** was identified as 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(*R*)-*O*-β-D-glucopyranoside, which is first isolated from the title plant.

The NMR spectra of compound **2** differed from that of compound **1** only in the signals for the sugar moiety. The ^{13}C NMR signals at δ 103.7, 75.0, 77.9, 71.9, and 65.5 revealed

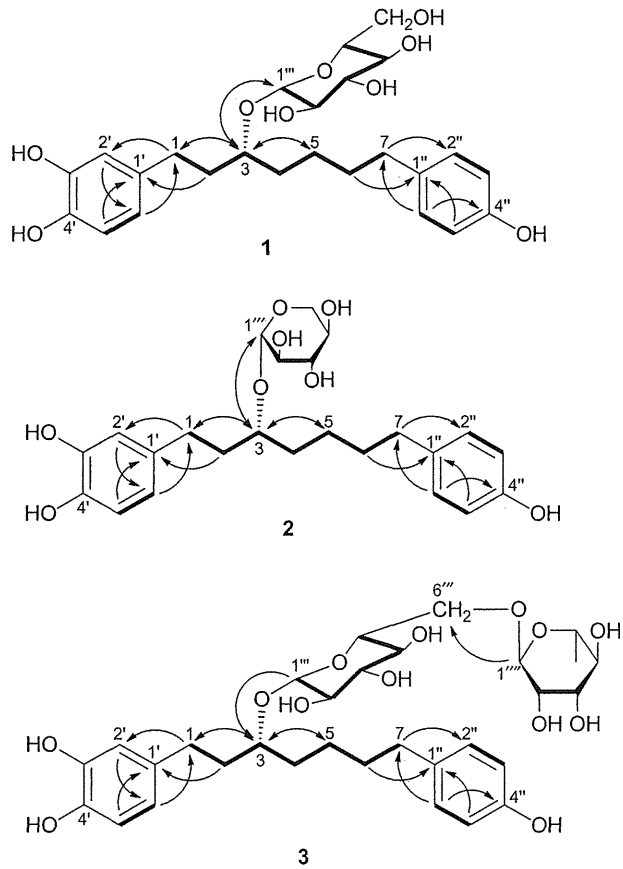


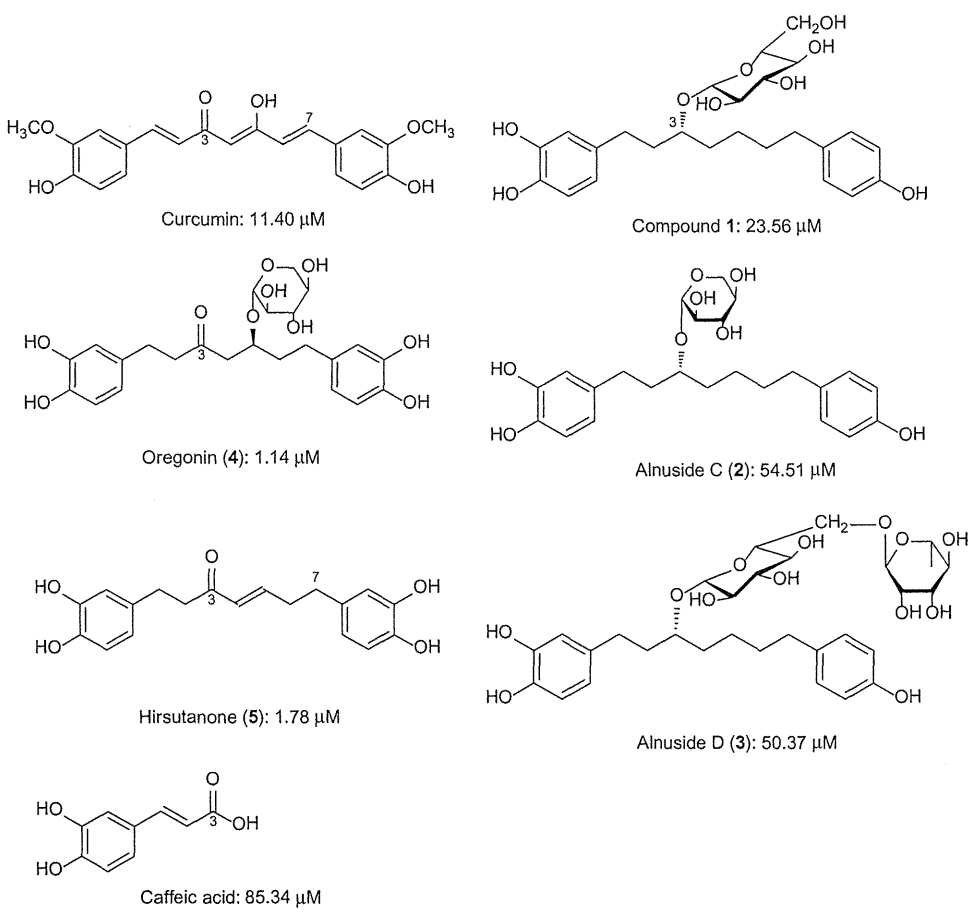
Figure 2. Selected H-H COSY (bold lines) and HMBC (arrows) correlations of compounds **1–3**.

a D-xylopyranosyl unit (Agrawal, 1992). The anomeric proton signal at δ 4.23 (d, $J = 7.6$ Hz) indicated a β conformation. Acidic hydrolysis of compound **2** produced aglycone **6** and D-xylose. A molecular ion peak at *m/z* $[M+H]^+$ 449.2186 observed in the HRMS spectrum of compound **2** was consistent with the molecular formula $C_{24}H_{32}O_8$. Similarly, the stereo chemistry of C-3 was assigned as an *R* configuration based on the glycosylation shift rule (Seo *et al.*, 1978; Gonzalez-Laredo *et al.*, 1999). Thus, the structure of alnuside C (**2**) was characterized as 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(*R*)-*O*-β-D-xylopyranoside.

Compound **3** showed two sets of ^{13}C NMR signals for the glycosyl portion suggesting a diglycosyl diarylheptanoid. Signals at δ 103.7, 75.3, 78.2, 72.2, 76.7, and 68.4 revealed a β-D-glucopyranosyl unit attached to another sugar segment at C-6. With respect to the terminal sugar unit, the remaining ^{13}C NMR signals at δ 102.2, 71.6, 72.4, 74.3, 69.8, and 18.1 were consistent with an α-L-rhamnopyranosyl unit (Agrawal, 1992). Moreover, the interlinkage between sugar units and the partial structures of compound **3** were confirmed by COSY, HMQC, and HMBC spectra, respectively (Fig. 2). Acidic hydrolysis of

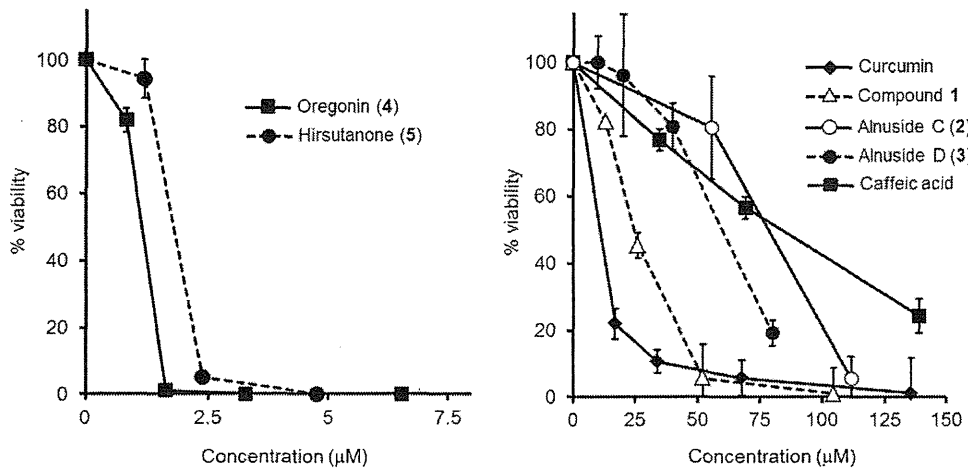
compound **3** yielded aglycone **6** and two sugar units, D-glucose and L-rhamnose. A molecular ion peak at *m/z* $[M+H]^+$ 625.2862 in the HRMS spectrum of compound **3** was compatible with the molecular formula $C_{31}H_{44}O_{13}$. Through a similar manner, the absolute configuration of C-3 was confirmed as an *R* configuration via the glycosylation shift rule (Seo *et al.*, 1978; Gonzalez-Laredo *et al.*, 1999). Based on the evidence described above, alnuside D (**3**) was structurally elucidated as 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(*R*)-*O*-[α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside] as a novel diarylheptanoid.

To investigate the trypanocidal activity of the purified compounds against forms of *T. brucei* (GUTat 3.1) found in the bloodstream, these compounds were incubated with *T. brucei* for 48 hours *in vitro*, analyzed using Alamar blue and IC_{50} values were obtained



(A)

Figure 3. Anti-trypanosomal activity and structure–activity relationships among diarylheptanoids isolated from *Alnus japonica* and caffeic acid. (A) The IC_{50} value of the tested compounds. (B) Inhibitory effect against trypanosome of the compounds at various concentrations.



(B)

Figure 3. (*Continued*)

for each component using a graph plot. As shown in Fig. 3, oregonin (**4**) and hirsutanone (**5**) exhibited more potent growth inhibitory effects (more than 50 times) than compounds **1–3,** and these compounds were more effective than the positive control berberine ($IC_{50} = 6.54\,\mu M$). Oregonin (**4**) exhibited the highest trypanocidal activity toward bloodstream forms of *T. brucei* and had the lowest IC_{50} value ($1.14\,\mu M$) among all of the compounds tested.

Next, we examined the cytotoxicity of the two most active compounds, oregonin (**4**) and hirsutanone (**5**), in the presence of mammalian cells *in vitro.* Normal skin fibroblast cells (NB1RGB), human colon cancer cells (HCT-15), and human leukemia cells (Jurkat, U937, HL-60and THP-1) were treated with oregonin (**4**) and hirsutanone (**5**) at concentrations ranging from $3.125\,\mu M$ to $50\,\mu M$ for 48 h, and the MTT assay was subsequently performed. The cytotoxic activity (IC_{50}) of each compound was correlated with the IC_{50} values obtained for trypanocidal activity to determine the corresponding selectivity index (SI = IC_{50} mammalian cells/IC_{50} Trypanosoma) (Table 2). The results demonstrated that oregonin (**4**) and hirsutanone (**5**) were not toxic at the highest assayed dose when applied to NB1RGB and HCT-15 cells. Among the four leukemia cell lines, oregonin (**4**) showed no toxicity in U937 and HL-60 cells and low SI values for Jurkat and THP-1 cells. However, hirsutanone (**5**) exhibited SI values between 6.39 and 18.87 for the four leukemia cell lines. These results indicate that oregonin (**4**) and hirsutanone (**5**) could be considered excellent anti-trypanosomal agents.

Although our compound set (**1–5**) is limited, we tried to further understand the structure activity relationships of the compounds in this series. Initially, we noticed that all of the compounds tested so far harbored two catechols or a catechol and a phenol moiety at both ends of the molecule. First, we determined whether we needed two aromatic groups in the molecule to facilitate anti-trypanosomal activity. We therefore tested the toxicity of caffeic

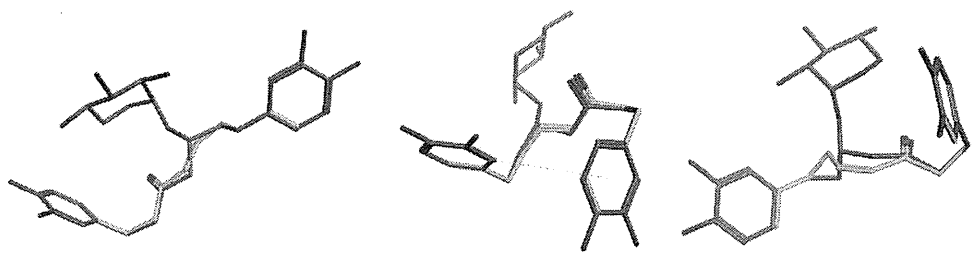
Table 2. Cytotoxicity of Compounds for Mammalian Cells (IC_{50}) and the Selectivity Index (SI) of Oregonin (4) and Hirsutanone (5)

	4		5	
Cell Lines	**IC_{50}**	**SI**	**IC_{50}**	**SI**
NB1RGB	>50	>43.85	>50	>43.85
HCT-15	>50	>43.85	>50	>43.85
Jurkat	22.16	19.44	11.37	6.39
U937	>50	>43.85	19.87	11.16
HL-60	>50	>43.85	33.59	18.87
THP-1	46.87	41.11	27.03	15.19

acid (**6**), a natural product that is similar to hirsutanone (**5**) but contains only one catechol moiety. Indeed, the activity of caffeic acid was significantly weaker when compared to hirsutanone (at least 70-fold), but it retained a marginal level of activity. Thus, one catechol is enough to exhibit the anti-trypanosomal activity, but the two aromatic groups present in oregonin (**4**) and hirsutanone (**5**) likely work synergistically to achieve higher anti-trypanosomal activity.

Keeping the importance of the two catechols in mind, next we tried to find an active conformation of both oregonin (**4**) and hirsutanone (**5**). Because the two compounds are reasonably potent (IC_{50} = *ca* 1 μM), we tried to find a consensus conformation between oregonin (**4**) and hirsutanone (**5**) with a lower energy value. Theoretically, such a conformation may be the active conformation. We carried out a Conformation Search for oregonin (**4**) using the Force Filed calculation MMFF94x and selected the three lowest energy conformations from the results. Then, we fixed the conformation of two catechol moieties by replacing the spacer between the two aromatics with the structure of hirsutanone (**5**). The resulting three structures were used as an initial conformation for the Conformation Search of hirsutanone (**5**) spacers. Figure 4 shows the three lowest energy conformations of oregonin (**4**) overlapping with hirsutanone (**5**) conformations generated by the Conformation Search. Unfortunately, these molecular modeling efforts could not reach a consensus conformation between oregonin (**4**) and hirsutanone (**5**). Therefore, we were unable to produce a 3D pharmacophore map due to the flexibility of the molecules and the limited compound set. The results could not be readily explained in terms of the structures of the diarylheptanoid derivatives. However, this preliminary biological assessment suggests that the 3-oxo function of the heptane chain in the diarylheptanoid molecules is necessary for their trypanocidal activity.

In a previous study, it was reported that curcumin, which is the principal active component of the rhizome *Curcuma longa* (turmeric) and a well-known diarylheptanoid with the highly conjugated side-chain (Bukhari *et al.*, 2013; Wang *et al.*, 2013), exhibited trypanocidal activity against both bloodstream forms and procyclic forms of *T. brucei* (GUTat 3.1) *in vitro* (Nose *et al.*, 1998). The observed effect was much higher against bloodstream forms when compared to procyclic forms with IC_{50} values of 11.40 μM and



(A) (B) (C)

Figure 4. Three lowest energy conformations of oregonin (**4**) (indicated by pink) overlapping with hirsutanone (**5**) conformations (illustrated by green) generated by molecular modeling studies.

46.52 μM (4-fold higher than procyclic forms), respectively, after a 24 h incubation (Nose *et al.*, 1998). In this study, we observed a potent IC_{50} value of 1.14 μM for oregonin (**4**) after 48 h. Furthermore, upon comparison of the structural differences between curcumin and oregonin (**4**), we concluded that one of the hydroxyl groups is blocked in curcumin. Because we have previously shown that the catechol group is necessary for the anti-trypanosomal activity of these compounds, curcumin may be less effective than oregonin (**4**) for the treatment of HAT.

Several trypanocidal natural products have been described in the literature. Some of them may be promising leads; however, their potential development as prophylactic agents is limited due to the low solubility of most of those compounds in the blood (Silveira and Rezende, 1994; Sepulveda-Boza and Cassels, 1996). Oregonin (**4**) can be isolated at high yields and is more stable than other free diarylheptanoids because the hydroxyl group of a keto–enol is conjugated to xylose in this compound. These data suggest that oregonin (**4**) may be a candidate agent for the treatment of Trypanosoma infections. Furthermore, it is well known that some natural products can exhibit their effects in a pro-drug-like manner similar to sennosides, which are metabolized by intestinal bacteria to release active anthrone which acts as a laxative (Hattori *et al.*, 1982, 1988). Thus, the isolated diarylheptanoid glycosides (**1–4**, which are more stable than the free hydroxyl groups) may also function as a pro-drug and may be a leading candidate for anti-trypanosomal treatment. Further studies regarding the mechanism of action and *in vivo* anti-trypanosomal activity are underway and will be reported in due time.

In summary, using a bioactivity-screening approach, two new diarylheptanoids (alnuside C (**2**) and alnuside D (**3**)) and three known analogs, 1-(3,4-dihydroxyphenyl)-7-(4-hydroxyphenyl)-heptan-3(*R*)-*O*-β-D-glucopyranoside (**1**), oregonin (**4**) and hirsutanone (**5**), were isolated from the bark of *A. japonica*. Of these isolates, oregonin (**4**) (a major component of the title plant) and hirsutanone (**5**) exhibited promising *in vitro* activity toward the growth forms of *T. brucei* found in the bloodstream without toxicity to mammalian cells and with IC_{50} values of 1.14 and 1.78 μM, respectively. Analysis of their structure–activity relationships revealed that the 3-oxo function of the heptanes chain in the diarylheptanoid molecules is necessary for their trypanocidal activity. Although the

trypanocidal activity of different type of diarylheptanoids isolated from three different species has been recently reported (Kamnaing *et al.*, 2003; Beniddir *et al.*, 2012; Lagnika *et al.*, 2012), this report describes the first anti-trypanosomal activity observed for diarylheptanoids isolated from an *Alnus* species.

Acknowledgments

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References

Agrawal, P.K. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry* 31: 3307–3330, 1992.

Barrett, M.P., D.W. Boykin, R. Brun and R.R. Tidwell. Human African trypanosomiasis: Pharmacological re-engagement with a neglected disease. *Br. J. Pharmacol.* 152: 1155–1171, 2007.

Beniddir, M.A., P. Grellier, P. Rasoanaivo, P.M. Loiseau, C. Bories, V. Dumontet, F. Gueritte and M. Litaudon. Diarylheptanoid glucosides from *Pyrostria major* and their antiprotozoal activities. *Eur. J. Org. Chem.* 2012: 1039–1046, 2012.

Bukhari, S.N., S.G. Franzblau, I. Jantan and M. Jasamai. Current prospects of synthetic curcumin analogs and chalcone derivatives against mycobacterium tuberculosis. *Med. Chem.* 9: 897–903, 2013.

Chen, Y.C., H.Y. Chang, J.S. Deng, J.J. Chen, S.S. Huang, I.H. Lin, W.L. Kuo, W. Chao and G.J. Huang. Hispolon from *Phellinus linteus* induces G0/G1 cell cycle arrest and apoptosis in NB4 human leukaemia cells. *Am. J. Chin. Med.* 41: 1439–1457, 2013.

Docampo, R. and S.N. Moreno. Current chemotherapy of human African trypanosomiasis. *Parasitol. Res.* 90: S10–S13, 2003.

Giang, P.M., P.T. Son, K. Matsunami and H. Otsuka. New diarylheptanoids from *Amomummuricarpum* ELMER. *Chem. Pharm. Bull.* 54: 139–141, 2006.

Gonzalez-Laredo, R.F., J. Chen, Y.M. Karchesy and J. Karchesy. Four new diarylheptanoids from *Alnusrubra* bark. *J. Nat. Prod. Res.* 13: 75–80, 1999.

Hattori, M., G. Kim, S. Motoike, K. Kobashi and T. Namba. Metabolism of sennosides by intestinal flora. *Chem. Pharm. Bull.* 30: 1338–1346, 1982.

Hattori, M., T. Namba, T. Akao and K. Kobashi. Metabolism of sennosides by human intestinal bacteria. *Pharmacology* 36: 172–179, 1988.

Hossain, D.M., S. Bhattacharyya, T. Das and G. Sa. Curcumin: The multi-targeted therapy for cancer regression. *Front. Biosci.* 4: 335–355, 2012.

Huang, M.Y., M.H. Liao, Y.K. Wang, Y.S. Huang and H.C. Wen. Effect of lavender essential oil on LPS-stimulated inflammation. *Am. J. Chin. Med.* 40: 845–859, 2012.

Kamnaing, P., A. Tsopmo, E.A. Tanifum, M.H.K. Tchuendem, P. Tane, J.F. Ayafor, O. Sterner, D. Rattendi, M.M. Iwu, B. Schuster and C. Bacchi. Trypanocidal diarylheptanoids from *Aframomum letestuianum*. *J. Nat. Prod.* 66: 364–367, 2003.

Kennedy, P.G.E. The continuing problem of human African trypanosomiasis (sleeping sickness). *Annal. Neurol.* 64: 116–126, 2008.

Kikuzaki, H., J. Usuguchi and N. Nakatani. Constituents of Zingiberaceae. I. Diarylheptanoids from the rhizomes of ginger (*Zingiber officinale* ROSCOE). *Chem. Pharm. Bull.* 39: 120–122, 1991.

Kuroyanagi, M., M. Shimomae, Y. Nagashima, N. Muto, T. Okuda, N. Kawahara, T. Nakane and T. Sano. New diarylheptanoids from *Alnus japonica* and their antioxidative activity. *Chem. Pharm. Bull.* 53: 1519–1523, 2005.

Lagnika, L., B. Weniger, B. Attioua, O. Jensen, C. Anthaume, A. Sanni, M. Kaiser, A. Lobstein and C. Vonthron-Senecheau. Trypanocidal activity of diarylheptanoids from *Schrankia leptocarpa* DC. *S. Afr. J. Bot.* 83: 92–97, 2012.

Lai, Y.C., C.K. Chen, W.W. Lin and S.S. Lee. A comprehensive investigation of anti-inflammatory diarylheptanoids from the leaves of *Alnus formosana*. *Phytochemistry* 73: 84–94, 2012.

Lee, M.W., T. Tanaka, G.I. Nonaka and I. Nishioka. Tannins and related compounds. Part 115. Dimericellagitannins from *Alnus japonica*. *Phytochemistry* 31: 2835–2839, 1992.

Mallari, J.P., A. Shelat, A. Kosinski, C.R. Caffrey, M. Connelly, F. Zhu, J.H. McKerrow and R.K. Guy. Discovery of trypanocidal thiosemicarbazone inhibitors of rhodesain and TbcatB. *Bioorg. Med. Chem. Lett.* 18: 2883–2885, 2008a.

Mallari, J.P., A.A. Shelat, T. Obrien, C.R. Caffrey, A. Kosinski, M. Connelly, M. Harbut, D. Greenbaum, J.H. McKerrow and R.K. Guy. Development of potent purine-derived nitrile inhibitors of the trypanosomal protease TbcatB. *J. Med. Chem.* 51: 545–552, 2008b.

Nagajyothi, F., D. Zhao, L.M. Weiss and H.B. Tanowitz. Curcumin treatment provides protection against *Trypanosoma cruzi* infection. *Parasitol. Res.* 110: 2491–2499, 2012.

Nomura, M., T. Tokoroyama and T. Kubota. Biarylheptanoids and other constituents from wood of *Alnus japonica*. *Phytochemistry* 20: 1097–1104, 1981.

Nose, M., T. Koide, Y. Ogihara, Y. Yabu and N. Ohta. Trypanocidal effects of curcumin *in vitro*. *Biol. Pharm. Bull.* 21: 643–645, 1998.

Pepin, J. and F. Milord. The treatment of human African trypanosomiasis. *Adv. Parasitol.* 33: 1–47, 1994.

Rahman, I. and I.M. Adcock. Oxidative stress and redox regulation of lung inflammation in COPD. *Eur. Respir. J.* 28: 219–242, 2006.

Remme, J.H., E. Blas, L. Chitsulo, P.M. Desjeux, H.D. Engers, T.P. Kanyok, J.F.K. Kayondo, D.W. Kioy, V. Kumaraswami, J.K. Lazdins, P.P. Nunn, A. Oduola, R. Ridley, Y. Toure, F. Zicker and C.M.M. Morel. Strategic emphases for tropical diseases research: A TDR perspective. *Trends Microbiol.* 10: 435–440, 2002.

Seo, S., Y. Tomita, K. Tori and Y. Yoshimura. Determination of the absolute configuration of a secondary hydroxyl group in a chiral secondary alcohol using glycosidation shifts in carbon-13 nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc.* 100: 3331–3339, 1978.

Sepulveda-Boza, S. and B.K. Cassels. Plant metabolites active against *Trypanosoma cruzi*. *Planta Med.* 62: 98–105, 1996.

Shen, K.H., Z.T. Chen and P.D. Duh. Cytotoxic effect of Eucalyptus citriodora resin on human hepatoma HepG2 cells. *Am. J. Chin. Med.* 40: 399–413, 2012.

Silveira, A.C. and D.F. Rezende. Epidemiology and control the vectorial transmission of Chagas disease in Brazil. *Rev. Bras. Med. Trop.* 27: 11–22, 1994.

Tung, N.H., S.K. Kim, J.C. Ra, Y.Z. Zhao, D.H. Sohn and Y.H. Kim. Antioxidative and hepatoprotective diarylheptanoids from the bark of *Alnus japonica*. *Planta Med.* 76: 629–632, 2010a.

Tung, N.H., H.J. Kwon, J.H. Kim, J.C. Ra, J.A. Kim and Y.H. Kim. An anti-influenza component of the bark of *Alnus japonica*. *Arch. Pharm. Res.* 33: 363–367, 2010b.

Wada, H., H. Tachibana, H. Fuchino and N. Tanaka. Three new diarylheptanoid glycosides from *Alnus japonica*. *Chem. Pharm. Bull.* 46: 1054–1055, 1998.

Wang, Y., H. Yin, L. Wang, A. Shuboy, J. Lou, B. Han, X. Zhang and J. Li. Curcumin as a potential treatment for Alzheimer's disease: A study of the effects of curcumin on hippocampal expression of glial fibrillary acidic protein. *Am. J. Chin. Med.* 41: 59–70, 2013.

World Health Organization (WHO) (2013): Human African Trypanosomiasis. http://www.who.int/trypanosomiasis_african/en/.