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Short communication

Nitric oxide inhibitory and anti-*Bacillus* activity of phenolic compounds and plant extracts from *Mesua* species

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ABSTRACT

Species from the genus *Mesua*, Calophyllaceae, are rich source for phenolic compounds such as coumarin xanthone, and benzophenone derivatives. An investigation on the potential biologically active phenolic compounds **1–5** and crude extracts from the stem bark of *Mesua hexapetala* (Hook. f.) P.S. Ashton and *Mesua beccariana* (Baill.) Kosterm. for nitric oxide inhibitory activity on RAW 264.7 macrophage as well as anti-*Bacillus* activity on selected *Bacillus* were carried out. Hexapetarin (**1**), which we reported as a new compound isolated from *M. hexapetala* showed very good nitric oxide inhibitory activity with an IC_{50} value of 30.79 ± 2.68 μM. This compound also gave very significant activities towards *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 33019, *Bacillus megaterium* ATCC 14581 and *Bacillus pumilus* ATCC 14884 in disc diffusion and minimum inhibitory concentrations assay. Moreover, 1,3,7-trihydroxy-2,4-di (3-methyl-2-butenyl)xanthone (**2**) isolated from *M. hexapetala* showed very significant nitric oxide inhibitory activity with an IC_{50} value of 12.41 ± 0.89 μM and does not exhibit anti-*Bacillus* activity on four types of *Bacillus*. Meanwhile, compounds **3–5** were inactive in the nitric oxide activity test and anti-*Bacillus* assay.

Introduction

Medicinal plants are an importance source of lead compounds for the drug discovery process in which it provides new leads against various pharmacological targets that includes cancer, inflammatory related diseases, diabetes, hypertension, fever, cough and cold. Natural product drug discovery offers great hope in the discovery of bioactive leads that are safer with less side effects. The genus *Mesua*, Calophyllaceae, found distributed mostly in Peninsular Malaysia and East Malaysia especially in the state of Sarawak. Some *Mesua* species such as *M. ferrea* L. and *M. beccariana* (Baill.) Kosterm. possess very good ethnopharmacological values towards many biological activities especially cytotoxicity towards some cancer cell lines (Karunakaran et al., 2016). Most research on the genus *Mesua* concentrate on its biological activities and are focused on *M. ferrea*, *M. racemosa*, *M. elegans*, *M. beccariana*, *M. congestiflora*, *M. lepidota* and *M. daphnifolia*. Furthermore, the genus *Mesua* has been reported to be ethno-medicinal and a prolific source of benzophenone, coumarin and xanthone derivatives which show potent biological activities towards cytotoxic, antibacterial, anti-inflammatory and acetylcholinesterase inhibitory activities (Mazumder et al., 2004; Ee et al., 2005a,b, 2012; Awang et al., 2010; Teh et al., 2010; Roy et al., 2013; Rouger et al., 2015; Teh et al., 2011; Karunakaran et al., 2015).

Inflammation is the main factor contributing to diseases like arthritis, autoimmune disorders, cancer, cardiovascular disease, diabetes and neurological disease. Moreover, inflammation is a natural host defense that reacts towards invading pathogens and tissue damage with involvement of the phagocytic cells such as macrophages, mast cells, and the innate lymphocytes (Syam et al., 2014; Yousefi et al., 2015). Nitric oxide plays a vital role in the key molecular signaling constituent involved in inflammatory processes. Mass production of NO is one of the main factors that contribute towards chronic degenerative diseases such as cancer, arthritis, neurodegenerative and cardiovascular disorder (Leong et al., 2014, 2015).

Bacterial infection is also considered to be one important factor that can lead to inflammatory related diseases such as Inflammatory Bowel Disease, arthritis, gastroenteritis, and anthrax and cutaneous infection. These diseases are all mainly caused by the positive gram bacterium *Bacillus* such as *B. cereus*, *B. anthracis* and *B. pumilus* (Ahmed et al., 1995; Chan et al., 2003; Tena et al.,

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2007; Kimouli et al., 2012; Tan et al., 2015; Shivamurthy et al., 2016). Previous research mentioned about the significant antibacterial activity shown by the genus *Mesua* and phenolic compounds such as coumarin and xanthone derivatives (Mazumder et al., 2004; Karunakaran et al., 2015). Biological interference with NO production and bacterial inhibition are necessities in developing potent leads for anti-inflammatory and anti-bacterial related diseases. This paper reports findings on inhibition of the extracted plant crude extracts and isolated phenolic compounds **1–5** from *Mesua hexapetala* and *M. beccariana* on LPS induced RAW 264.7 macrophages and four type of *Bacillus* bacterium which are *B. subtilis* ATCC 6633, *B. cereus* ATCC 33019, *B. megaterium* ATCC 14581 and *B. pumilus* ATCC 14884.

Results and discussion

Biological assay

Cytotoxicity and NO inhibitory activity

NO inhibitory tests were determined against eight plant extracts and pure compounds **1–5** isolated from *M. hexapetala* and *M. beccariana*, respectively. Hexapetarin (**1**) came from the chloroform and ethyl acetate extracts while 1,3,7-trihydroxy-2,4-di-(3-methyl-2-butenyl)xanthone (**2**) was from the hexane extract of *M. hexapetala.* Beccarixanthone T, (**3**) beccamarin T (**4**) and mesuasinone (**5**) were isolated from the hexane extract of *M. beccariana*. The structural elucidations of compounds **1–5** were achieved with the aid of 1D and 2D NMR, MS and IR and the data of compounds **1**, **3**, **4** were published in previous research (Karunakaran et al., 2015, 2016) articles while compounds **2** and **5** are reported here and compared with previous literature (Iinuma et al., 1996; Teh et al., 2010). Before proceeding to the Griess assay (NO), the samples were tested for their toxicity on RAW 264.7 macrophages. The toxicity test (MTT) is important in determining the cell viability of the RAW 264.7 macrophages towards the sample as the percentage of cell viability influences the nitric oxide (NO) production. More than 80% of cell viability will exhibit stable NO production in IFN-γ/LPS induced RAW 264.7 macrophage. Moreover, the toxicity test can determine the minimum concentration required to inhibit the NO production as well as the concentration of the sample which does not exhibit any toxicity towards the RAW 264.7 macrophage. The toxicity test conducted was based on protocols from previous research (Tan et al., 2015) with slight modifications. From the toxicity data of *M. hexapetala* and *M. beccariana* plant extracts [hexane (MHH, MBH), chloroform (MHC, MBC), EA (MHE, MBE) and acetone (MHA, MBA)] displayed in Figs. S1 and S2, the cell viability of *M. hexapetala* and *M. beccariana* treated cells were more than 80% even at a higher concentration of 500 μg/ml, thereby indicating negligible toxicities towards the RAW 264.7 macrophage cells.

The tested phytochemicals, hexapetarin (**1**) and 1,3,7-trihydroxy-2,4-di-(3-methyl-2-butenyl)xanthone (**2**) exhibited toxicities at concentrations between 125 μg/ml and 500 μg/ml. Compound **1** did not exhibit substantial toxicity at concentrations ranging below 62.5 μg/ml. At concentrations ranging below 31.25 μg/ml compound **2** does not exhibit substantial toxicity towards RAW 264.7 macrophages. Hexapetarin (**1**) showed 80% cell viability from a concentration range of 62.5 μg/ml and below. For compound **2** the non-toxic concentration was from 31.25 μg/ml and below. Beccarixanthone T (**3**), beccamarin T (**4**) and mesuasione (**5**) exhibited more than 80% cell viability even at the highest concentration of 500 μg/ml, thereby exhibiting negligible toxicity. The toxicity data of selected pure compounds are shown in Fig. S3. The purpose of conducting the toxicity test before the Griess assay (NO) was to screen a suitable potential hit or herbal candidate that could inhibit the NO production without impairing the RAW 264.7 macrophage cells.

The NO inhibitory assay were conducted on all the plant extracts, which are hexane (MHH, MBH), chloroform (MHC, MBC), EA (MHE, MBE) and acetone (MHA, MBA) of the *M. hexapetala* and *M. beccariana* as well as for pure compounds **1–5**. The starting concentrations in NO inhibition assays conducted were based on the non-toxic concentration range in the MTT assay. The concentration of LPS used to stimulate the RAW 264.7 macrophage cells was 5 mg/ml which was higher than the concentration used by most researchers which is 1 mg/ml. The purpose of using higher LPS concentration is to inspect the sensitivity of induced cells under high stimulation of LPS towards the samples tested. Nitric oxide is an important mediator involved in inflammatory process whereby it is vital for the body defence mechanism against invading pathogens and abnormalities. Overproduction of NO can lead to serious damage of tissues through lipid peroxidation and DNA mutation which promote to chronic degenerative diseases such arthritis, neurodegenerative disorders and others (Leong et al., 2015). If the sample can treat induced cells and inhibit the over production of NO it can be a good lead candidate for anti-inflammatory drug discovery. This assay was conducted based on a previous report protocol (Leong et al., 2014) with slight alterations. The results obtained from the assay performed, indicated that compound **2** exhibited substantial NO inhibitory activity towards the IFN-γ/LPS stimulated RAW 264.7 macrophages with IC_{50} values of 4.72 ± 0.34 μg/ml (12.41 ± 0.89 μM).

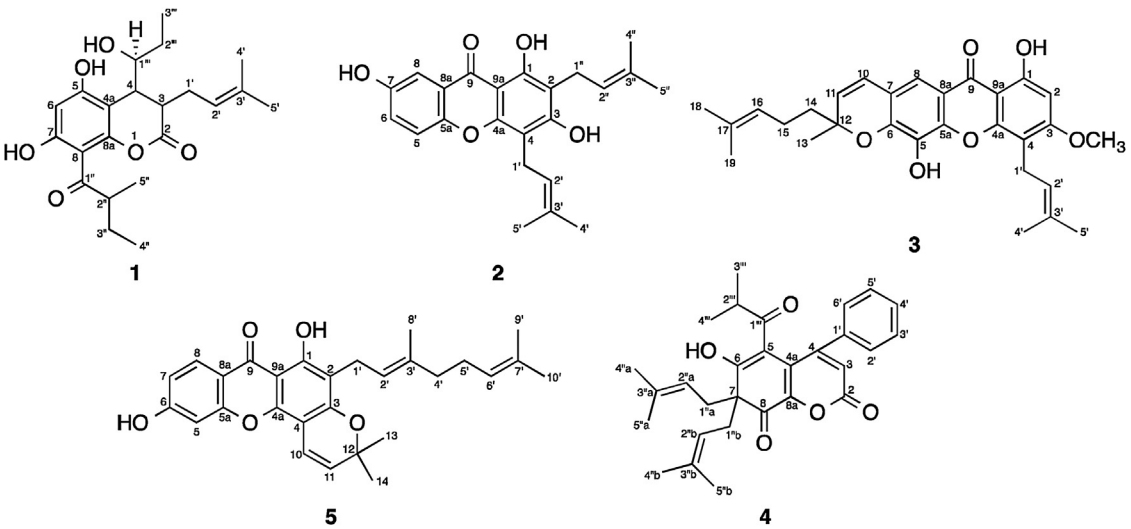

Table 1
Inhibitory activities of plant extracts and compounds **1–5** on LPS/IFN-γ induced NO production in RAW 264.7 macrophages.

Plant extracts/pure compounds	IC_{50} (μg/ml) ± S.E.M.	IC_{50} (μM) ± S.E.M.
MHH	28.09 ± 2.48	–
MHC	58.86 ± 2.87	–
MHE	56.77 ± 2.32	–
MHA	>100	–
MBH	58.36 ± 2.43	–
MBC	>100	–
MBE	>100	–
MBA	41.26 ± 2.22	–
1	11.95 ± 1.04	30.79 ± 2.68
2	4.72 ± 0.34	12.41 ± 0.89
3	>50	>50
4	>50	>50
5	>50	>50
Curcumin[a]	7.36 ± 0.00	20 ± 0.00

Note: Each value of IC_{50} represented mean ± S.E.M. of three independent experiments; a, positive control substance.

Meanwhile, hexapetarin, a new compound we isolated earlier from *M. hexapetala* (Karunakaran et al., 2015) also showed a good inhibitory activity with an IC_{50} value of 11.95 ± 1.04 μg/ml (30.79 ± 2.68 μM). The other pure compounds **3–5** did not exhibit anti-inflammatory activities and their IC_{50} values were more than 50 μg/ml.

For the plant extracts activity, the hexane extract of *M. hexapetala* (MHH) displayed very potent activity towards the NO inhibition from the IFN-γ/LPS stimulated RAW 264.7 macrophage with an IC_{50} value of 28.09 ± 2.47 μg/ml. Since, the NO inhibitory active compound 1,3,7-trihydroxy-2,4-di-(3-methyl-2-butenyl)xanthone (**2**) was isolated from the MHH extract, it can be assumed that compound **2** may be one of the crucial contributor towards the MHH's NO inhibitory activity on LPS/IFN-γ induced RAW 264.7 macrophage. The other plant extracts, MHC and MHE from *M. hexapetala* as well as MBH and MBA from *M. beccariana* showed good NO inhibitory activities with IC_{50} values of 58.86 ± 2.87 μg/ml, 56.77 ± 2.32 μg/ml, 58.36 ± 2.43 μg/ml and 41.26 ± 2.22 μg/ml respectively. Meanwhile, MHA, MBC and MBE did not display any NO inhibitory activities as their IC_{50} values were more than 100 μg/ml. The NO production of LPS/IFN-γ stimulated RAW 264.7 macrophage against various concentration of bioactive MHH plant extract as well as the bioactive pure compounds **1** and **2** are summarized in Figs. S4–S6. The IC_{50} values of the respective samples are shown in Table 1.

Anti-Bacillus *activity*

The anti-*Bacillus* activity of the plant extracts and pure compounds **1–5** were evaluated against four types of *Bacillus* bacterium, *B. subtilis*, *B. cereus*, *B. megaterium* and *B. pumilus*. These bacteria are food borne pathogens which can cause food poisoning and inflammatory related diseases such as gastroenteritis and cutaneous infection (Ahmed et al., 1995; Chan et al., 2003; Tena et al., 2007; Kimouli et al., 2012; Shivamurthy et al., 2016). Previous studies have established the significant antibacterial activity shown by the genus *Mesua* and phenolic compounds such as coumarin and xanthone derivatives (Mazumder et al., 2004). Biological interference with NO production and bacterial inhibition are necessities in developing potent leads for anti-inflammatory and anti-bacterial related diseases.

In this anti-*Bacillus* study, all the samples (plant extracts and pure compounds) were evaluated using four types of *Bacillus* bacteria with the preliminary screening using disc diffusion method. Chlorhexidine with a concentration of 1 mg/ml was used as positive control. The disc diffusion tests revealed that hexapetarin (**1**) showed amazing results for the four *Bacillus* bacteria in which it gave very significant activity towards *B. cereus* with an inhibition zone of 15.00 ± 0.29 mm, followed by *B. subtilis* and *B. pumilus* with inhibition zones of 14.33 ± 0.17 mm. The same compound also displayed good activity on *B. megaterium* with an inhibition zone of 12.33 ± 0.17 mm. Compounds **2–5** did not show any activity towards the tested *Bacillus* bacteria. Meanwhile, the MHH extract showed potent anti-*Bacillus* activities on *B. subtilis*, *B. cereus*, *B. megaterium* and *B. pumilus* with inhibition zones of 10.33 ± 0.44 mm, 11.33 ± 0.17 mm, 14.83 ± 0.17 mm and 12.33 ± 0.17 mm respectively. The summary of the results of the disc diffusion tests is in Table S1.

MIC and MBC susceptibility tests were also conducted on hexapetarin (**1**) as well as the bioactive plant extracts, MHH, MHC and MBH. Surprisingly, hexapetarin (**1**) exhibited excellent MIC activities at very low concentrations of 3.91 μg/ml, 7.81 μg/ml and 15.62 μg/ml for *B. pumilus*, *B. subtilis* and *B. megaterium* but gave good activity for *B. cereus* at 62.50 μg/ml. However, hexapetarin (**1**) did not show any MBC activity towards the *Bacillus* bacteria. The MHH crude extract displayed very potent MIC activity towards *B. megaterium*, *B. cereus* and *B. pumilus* at concentrations of 7.81 μg/ml and 31.25 μg/ml both respectively. For *B. subtilis* it showed an MIC activity of 125 μg/ml. Moreover, MHH portrayed MBC activity for *B. cereus* at a concentration of 500 μg/ml. The MHC extracts also exhibited very good MIC activities for *B. pumilus*, *B. megaterium* and *B. subtilis* with concentrations of 7.81 μg/ml, 15.62 μg/ml and 62.50 μg/ml respectively while showing activity towards *B. cereus* at a concentration of 125 μg/ml. MHC however, did not display any MBC activity. All the MIC and MBC activities are summarized in Table S2.

Discussion

Based on the results obtained from the NO inhibitory assay, the presence of hydroxyl group which are more than two moieties might be responsible for the NO inhibition activity with a significant IC_{50} value less than 50 μM. Moreover, the position of OH in the parent structure of coumarin and xanthone could also play a vital role in enhancing the bioactivity. It was observed that hexapetarin (**1**), with its OH at position C-5 and the hydroxylpropyl moiety at position 4 gave good NO inhibition activity with an IC_{50} value of 11.95 ± 1.04 μg/ml (30.79 ± 2.68 μM). The pattern of substitution of the hydroxyl moiety in a xanthone derivative might play a role in the NO inhibition activity. For natural occurring xanthones, it was observed that when the OH was at position C-1 and at position C-3 the compounds were less bioactive but when the OH were bonded to the carbons at positions C-5, C-6 and C-7 the NO inhibition activity was enhanced (Isakovic et al., 2008). Moreover, if the OH moieties present in position C-1, C-3 and C-5 or C-6 or C-7 or in all mentioned position, the compound gives remarkable bioactivity results. Compound **2** gave amazing activity on NO inhibition activity. This compound has an OH moiety at position 7. For the anti-microbial activity, it is very well known that most coumarin derivatives exhibit good activity towards positive gram bacteria. For hexapetarin (**1**), the remarkable anti-bacterial activity towards the four selected *Bacillus* type bacterium is due to the presence of the hydroxylpropyl moiety as well as the other hydroxyl groups.

Conclusion

In conclusion, the newly discovered natural product hexapetarin (**1**) exhibited highly significant NO inhibitory activity on LPS/IFN-γ induced RAW 264.7 macrophage as well as potent anti-*Bacillus* activity towards four types of *Bacillus* bacteria. Moreover, compound **2**, 1,3,7-trihydroxy-2,4-di(3-methyl-2-butenyl)xanthone showed excellent NO inhibitory activity on LPS/IFN-γ induced RAW 264.7 macrophage with an IC_{50} value

of 4.72 ± 0.34 μg/ml. Compounds **1** and **2** can be potential lead compounds for anti-inflammatory drug candidates for future drug discovery. Compound **1** can also be a potential lead compound for anti-bacterials against the four *Bacillus* bacterium. The MHH plant extract displayed a very significant activity in the NO inhibitory activity and anti-*Bacillus* assays, hence, can be proposed for use in potential herbal extract formulations.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.01.007.

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