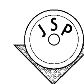
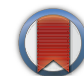
J Nat Med (2015) 69:375–386
DOI 10.1007/s11418-015-0904-x

ORIGINAL PAPER

Shoot cultures of *Hoppea fastigiata* (Griseb.) C.B. Clarke as potential source of neuroprotective xanthones

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Received: 16 February 2015 / Accepted: 23 March 2015 / Published online: 22 April 2015
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Abstract *Hoppea fastigiata*, an annual medicinal herb belonging to the Gentianaceae, is mostly found in South Asian countries, and is used by local tribes for various brain-related ailments. The genus possesses a unique class of compounds, xanthones, which are known for their potential against Alzheimer's and Parkinson's diseases. Limited availability and the potential pharmacological significance of the plants has led to the establishment of in vitro cultures of *H. fastigiata* and study of its neuroprotective principles. In vitro plantlets were established from the apical meristem of the plant in Murashige and Skoog medium with a combination of the phytohormones 6-benzylaminopurine (1 mg/L) and kinetin (0.1 mg/L), which was found to be efficacious with a growth index of 0.9 ± 0.01 after 30 days. Four different solvent extracts of in vitro cultures were evaluated for acetylcholinesterase (AChE) and monoamine oxidase A and B (MAO-A and MAO-B) inhibitory activities, amongst which the ethanolic extract showed the lowest IC_{50} value in all the assays. Three major compounds were isolated from the ethanolic extract and structurally confirmed as 1,5,7-trihydroxy-3-methoxyxanthone (**1**), 1,5-dihydroxy-3,7-dimethoxyxanthone (**2**) and 1,3,5-trihydroxy-8-methoxyxanthone (**3**). Compound **3** showed the strongest AChE inhibitory activity with mixed-type inhibition. Compounds **1** and **2** also showed promising AChE inhibitory properties with mixed and non-competitive types of inhibition, respectively. Compounds **1** and **2** showed inhibition of MAO-A (mixed and competitive, respectively) and compounds **2** and **3** showed inhibition of MAO-B (competitive and mixed, respectively). Extracts and isolated compounds showed good antioxidant capacities. The ethanolic extract and compound **2** showed the strongest antioxidant activities among the other solvent extracts and compounds, respectively.

Keywords *Hoppea fastigiata* · Acetylcholinesterase-inhibitory activity · Monoamine oxidase-inhibitory activity · Antioxidant capacity

Introduction

Hoppea fastigiata (Griseb.) C.B. Clarke (Gentianaceae) is a xanthone-producing annual medicinal plant restricted to only a few ecological niches in India. Xanthones are a class of phenolic compounds (secondary metabolites) restricted to only a few higher plant families such as Gentianaceae and Clusiaceae. Nevertheless, xanthones represent a class of natural products with interesting pharmacological properties [1]. Several xanthones from whole parts of *H. fastigiata* have been isolated and elucidated through chemical and spectroscopic analyses. These include 1,7-dihydroxy-3,5-dimethoxyxanthone [2], 1,5-dihydroxy-3,7-

Electronic supplementary material The online version of this article (doi:10.1007/s11418-015-0904-x) contains supplementary material, which is available to authorized users.

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dimethoxyxanthone [3], 1,5,7-trihydroxy-3-methoxyxanthone [4], 1,3,8-trihydroxy-7-methoxyxanthone [5] and 1,3,6-trihydroxy-8-methoxyxanthone [6]. Xanthones isolated from several Gentianaceae members in temperate regions showed broad similarity with the chemical structures reported from *H. fastigiata*; these xanthones were also shown to be potent inhibitors of the enzymes acetylcholinesterase (AChE) and monoamine oxidase (MAO) [7].

AChE is linked with Alzheimer's disease (AD), a CNS-related disorder mostly prevalent in older populations. It is responsible for impaired cognitive function and progressive memory loss [8, 9]. Due to loss of cholinergic neurons, there is a deficit of synaptic acetylcholine. In addition, the enzyme AChE degrades the neurotransmitter acetylcholine, and has been shown to accelerate AD [10]. The availability of acetylcholine can be increased by inhibiting AChE, which is responsible for the hydrolysis of acetylcholine [11]. MAO is involved in the metabolism of neurotransmitters such as dopamine, adrenaline and noradrenaline and in the inactivation of exogenous arylalkyl amines [12]. There are two sub-forms of the MAO enzyme, MAO-A and MAO-B. MAO-A is linked with depression, which is caused by the deregulation and depletion of monoamine neurotransmitters because of upregulation of MAO-A activity, and MAO-B is linked with Parkinson's disease (PD) which is caused by a loss of neurons from the substantia nigra of the brain which stops producing dopamine and affects the brain's ability to control movement [13]. MAO-A and B also play an important role as a first line of defence against the food-derived monoamines such as tyramine and 3-phenylethanolamine, absorption of which causes sympathomimetic effects such as a rise in blood pressure, which is often known as the "cheese effect" [14].

One of the plant-based drugs approved by US Food and Drug Administration (FDA) used in the treatment of AD is physostigmine, which serves as an excellent inhibitor of AChE but suffers from poor intestinal absorption in humans [15]. Likewise, the traditional irreversible MAO inhibitors show lack of selectivity for MAO sub-forms, which may lead to the cheese effect. Thus, there is increasing interest in finding better AChE and MAO inhibitors showing good brain penetration and high bioavailability and reversible selectivity [14]. Xanthone compounds isolated from *Gentiana campestris*, *Gentianella amarella* and *Garcinia mangostana* showed potent inhibitions against in vitro AChE and MAO activities [7, 16, 17].

H. fastigiata was known for its uses by several tribal peoples in various brain-related ailments. Use of this plant in the Indian systems of medicine for the treatment of haemorrhoids, cardiac dropsy and in certain mental disorders including epilepsy was also known [18]. It was therefore necessary to evaluate the extracts of *H. fastigiata* for any AChE and MAO inhibitory properties. Recently, crude extracts of wild *H. fastigiata* were shown to possess in vitro antioxidant capacities and acetylcholinesterase (AChE) inhibition activity [19]. However, no information is available on the chemical compounds of *H. fastigiata* that were responsible for such biological activities.

One of the major problems with the collection of *H. fastigiata* biomass in the field is its seasonal nature, and its life-cycle duration of only 6–8 weeks. Moreover, this tiny hygrophytic plant is very restricted in distribution and is consequently included in the IUCN Red List of Threatened Species. Therefore, to explore the full bioprospecting of *H. fastigiata*, ex situ multiplication through in vitro propagation appears to be an attractive strategy for sustainable utilization of this fragile bioresource. As xanthones were reported mostly from aerial parts of *H. fastigiata*, we anticipated that establishment of shoot cultures could be a suitable platform for production of xanthones [4]. This raison d'être is based on the expression of pathway enzymes between disorganized and organized cultures, as elucidated with *Mitragyna speciosa* shoot cultures [20].

This study aimed to establish for the first time in vitro shoot cultures of *H. fastigiata*, which could serve as a sustainable source for this species for effective utilization. The major xanthones produced in shoot cultures of *H. fastigiata* were isolated and identified. The efficacies of the isolated xanthones were evaluated for their antioxidant capacities and AChE or MAO inhibitory properties. We also report the inhibition-kinetic characteristics of isolated xanthones against eel AChE and human recombinant MAO-A and MAO-B enzymes.

Materials and methods

Chemicals

AChE from *Electrophorus electricus* (eel), human recombinant MAO-A and MAO-B, acetylthiocholine iodide (ATChI), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), kynuramine dihydrobromide and 4-quinolinol were purchased from Sigma-Aldrich India Pvt Ltd. Naphthyl acetate, fast blue B salt, 1-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), potassium persulfate, 2,4,6-tripyridyl-s-triazine (TPTZ) and Folin–Ciocalteu reagent were purchased from Himedia Laboratories Pvt Ltd, India. All the organic solvents were of analytical grade and were purchased from SRL, India.

In vitro propagation of *H. fastigiata*

Nature-grown plants were collected from the Bolpur forest (Santiniketan), West Bengal, India. The plant was identified on the basis of reproductive morphology and by the Department of Botany, Visva-Bharati University, and a voucher specimen collection number VBH-129-89 was deposited in the herbarium. *H. fastigiata* explants (apical meristem, stem, leaf and root) were washed thoroughly and kept in running water for 30 min. They were then treated with 0.1 % of Triton-X100 for 5–10 min. Afterwards, these explants were washed with double-distilled water for 5–10 min. Subsequently, explants were immersed in 70 % (v/v) ethanol for 2–3 min and washed 2–3 times with double-distilled water. Eventually, the explants were treated with an aqueous solution of 0.1 % $HgCl_2$ and rinsed 2–3 times with sterile water to remove all traces of $HgCl_2$. The sterilized explants were then inoculated on Murashige and Skoog (MS) medium with different concentrations and combinations of hormones such as NAA, IAA and BA. The growth index was calculated according to the following equation:

$$\text{Growth index} = \text{Final FW of tissue} - \text{Initial FW of tissue}/\text{initial FW of tissue}$$

where FW = fresh weight.

Extraction, isolation and characterization of compounds

Extracts were prepared using four different solvents, namely water, ethanol, ethyl acetate and petroleum ether. In vitro shoots (0.25 g) were dipped in the solvents (15 ml each) overnight and crushed using a mortar and pestle. The resulting extract was then subjected to ultrasonic extraction for 30 min. It then was centrifuged at 8000*g* for 10 min (MiniSpin^{plus}, Eppendorf). After drying the supernatant either in a vacuum rotary evaporator or vacuum concentrator (Concentrator plus, Eppendorf) as appropriate, the final extraction yields (%) were calculated using the formula $[W_2 - W_1/W_0]$, where W_0, W_1 and W_2 are the weights of initial plant material, container without extract and container with extract, respectively.

The dried ethanolic extract was re-suspended in 1 ml ethanol, filtered through a 0.45 syringe filter and analyzed by HPLC (Waters) for separation and subsequent isolation of compounds. A Symmetry^{TM} (Waters) C_{18} reverse-phase column (3.5 µm, 75 × 4.6 mm) was used and the detection of compounds was simultaneously monitored at 254 and 365 nm. The mobile phase composition of solvents A and B were 1 mM trifluoroacetic acid (TFA) in deionized water and methanol (MeOH), respectively. At time 0 min, A and B were 90 and 10 %, respectively. After 60 min, the percentages of A and B were 0:100 with a flow rate of 1 ml/min. The compounds eluted at the retention times of 30 min (compound **1**), 36 min (compound **2**) and 52 min (compound **3**) were subsequently collected. LC–MS analysis of these compounds was carried out on a QTrap 3200 system (Applied Biosystems/MDS Sciex) at Technische Universität Braunschweig as described in the literature [21]. All NMR data were collected on a Bruker Advance AMX-500 MHz FT-NMR spectrometer located at NMR Facility, Indian Institute of Technology Roorkee.

Comparative analysis of major xanthones from nature-grown and in vitro shoot cultures of *H. fastigiata*

Comparative analysis was performed by three different samples of nature-grown (7 and 30 days after germination and completely mature plant) and shoot cultures (after 7, 15 and 30 days of sub-culture) of *H. fastigiata*. The extraction was made in ethanol and the supernatant was used for HPLC analysis. The data are represented as average of compounds at different time intervals.

DPPH free radical scavenging assay

DPPH assay was performed according to a previously described method [22]. The free radical scavenging activity of the extracts was tested using DPPH free radical. Crude extracts of plant (0.1 ml) were mixed with the 0.9 ml ethanolic solution of DPPH (0.1 M). The mixture was vortexed and kept in the dark for 30 min at room temperature. The decrease in optical density was monitored at 517 nm.

ABTS free radical scavenging assay

ABTS assay was performed according to a previously described method with slight modifications [23]. ABTS was dissolved in water to 7 mM concentration. The ABTS free radical (ABTS·) was prepared by adding 2.45 mM potassium persulfate to ABTS solution and incubating in the dark for 12–16 h. For the working solution, the absorbance of the ABTS solution was adjusted by diluting it with ethanol to optical density 0.7 (±0.02) at 734 nm. The reaction mixture consisted of working solution (995 µl) and crude extract (5 µl). The mixture was then vortexed and incubated for 6 min at room temperature and the decrease in absorbance was monitored at 734 nm.

FRAP assay

The reducing power of the extract was determined by ferric reducing antioxidant power (FRAP) assay [24] with slight modifications. Stock solutions comprised TPTZ (2,4,6-tripyridyl-s-triazine) solution (10 mM) which was prepared in HCl (40 mM), $FeCl_3 \cdot 6H_2O$ (20 mM) and acetate buffer (300 mM, pH 3.6). The fresh working solution was prepared by mixing 25 ml acetate buffer, 5 ml TPTZ and 5 ml $FeCl_3 \cdot 6H_2O$. The extracts (3 µl) were allowed to react with FRAP working solution (997 µl). The mixture was then incubated for 6 min at 37 °C and absorbance was recorded at 593 nm.

Determination of total phenolic content

Estimation of total phenolic content (TPC) was done as described previously [25]. Crude extract of plant (100 µl) was mixed with 200 µl of 10 % Folin–Ciocalteu reagent and 800 µl of Na_2CO_3 (700 mM) and vortexed vigorously. The tubes were incubated for 2 h at room temperature. Increase in absorbance was then measured at 765 nm. TPC value was obtained by comparing the change in absorbance of the solution containing the extract at 765 nm with that of a calibration plot of gallic acid and was expressed as mg gallic acid equivalent/g fresh mass.

Acetylcholinesterase (AChE) inhibitory activity

The AChE inhibitory property of different solvent extracts was studied as described previously with slight modifications. The reaction mixture consisted of 500 µl of 3 mM DTNB, 46 µl of 15 mM ATChI, 356 µl of 50 mM Tris–HCl buffer (pH 8.0), sample extract and 100 µl of 0.28 U/ml AChE. The reaction mixture was monitored at 405 nm for 5 min. For IC_{50} value calculations, the absorbance was recorded after incubating reaction mixtures for 10 min. The rate of reaction before addition of the enzyme was subtracted from the reaction rate after addition of the enzyme in order to nullify the absorbance changes due to spontaneous hydrolysis of the substrate. Percentage inhibition was calculated by comparing the rate of reaction for the sample with that for the negative control [26]. To study the inhibition kinetics of the compounds, the reaction mixtures were continuously monitored at various substrate (ATChI) concentrations ranging from 100 to 700 µM and two inhibitor concentrations of 360 and 500 µM.

TLC bioautography

The AChE inhibitory activity of individual compounds was tested by the TLC bioautography method [27] with slight modifications. Samples were allowed to run onto silica gel 60 F_{254} plates which were later sprayed with the enzyme stock solution and incubated at 37 °C for 20 min. After incubation the plates were sprayed with 10 ml of naphthyl acetate (2.5 mg/ml) and 40 ml Fast Blue B salt (2.5 mg/ml). The plates were then dried and observed for white spots in comparison to the purple background. Naphthyl acetate and AChE enzyme were mixed prior to spraying onto the TLC plate to eliminate false-positive results.

Monoamine oxidase inhibition assay

MAO-A and MAO-B inhibitions were studied using recombinant human MAO enzymes as described previously [28] with slight modifications. For MAO-A inhibition, 1 ml of reaction mixture consisted of 950 µl of 0.1 M phosphate buffer containing 20.2 mM KCl, 0.7 U enzyme and 45 µl of 2 mM kynuramine dihydrobromide. For MAO-B, all reaction mixture components were the same except that 30 µl of 2 mM kynuramine was added. The reaction product was monitored at 329 nm for 5 min. Percentage inhibition was calculated by comparing the rate of reaction for the sample to that for the negative control. Finally, the reaction mixture was analyzed by HPLC to confirm the product formation by comparing their retention times with the standard 4-quinolinol obtained commercially (Sigma-Aldrich). HPLC was monitored at 329 nm at a gradient mode of 10–100 % B for 60 min and then isocratic elution at 100 % B with a flow rate of 1 ml/min. The mobile phase composition of solvents A and B was 1 mM TFA in deionized water and MeOH, respectively. To study the inhibition kinetics of the compounds, the reaction mixtures were continuously monitored at various substrate (kynuramine) concentrations ranging from 10 to 200 µM and two inhibitor concentrations of 12 and 14 µM.

Statistical analyses

All data are expressed as mean ± standard error (SE). The statistical analysis was performed using the 'Multi-Variate Statistical Package' (MVSP version 3.22). Principal component analysis (PCA) was performed to evaluate relationships between the different extraction solvents (ethanol, aqueous, ethyl acetate and petroleum ether) and the different analytical methods (DPPH, ABTS, FRAP and TPC for antioxidant capacities and AChE, MAO-A and MAO-B inhibitory activities for neuroprotective properties). Two principal components with the greatest eigenvalues were selected. Extraction solvents served as dependent variables whereas DPPH, ABTS, FRAP, TPC, AChE, MAO-A and MAO-B activities served as explanatory variables. Results are shown as the means of values and SE of independent measurements ($n = 3$) and a result

was considered statistically significant when the p value was <0.05. The means were compared using one-way ANOVA followed by Duncan's multiple range tests.

Results and discussion

In vitro propagation of *H. fastigiata*

In vitro cultures of *H. fastigiata* were established by using different combinations and concentrations of plant growth regulators in MS basal medium. Different phytohormones such as BA, kinetin, NAA, IAA and IBA were supplemented in the media either singly or in combination. Apical meristem, when used as explant, showed 100 % response in direct regeneration of shoots on the medium containing cytokinin (either BA or NAA), which initiates cell division and differentiation. Induced shoots were separated from explants and sub-cultured in the medium containing IBA (1 mg/L) which resulted in the formation of roots after 35 days of sub-culture (Supplementary Material). Subsequently, the plantlets were maintained in the medium containing a range of combinations of plant growth regulators: NAA (0.5 mg/L) and BA (0.5 mg/L), NAA (1 mg/L) and BA (0.2 mg/L), BA (0.5 mg/L) and kinetin (0.5 mg/L), BA (1 mg/L) and kinetin (0.1 mg/L), and BA (4 mg/L) and IAA (1 mg/L) which showed a promising outcome. BA (1 mg/L) and kinetin (0.1 mg/L) combinations were found to be the most efficacious of all the combinations. It was earlier reported that a positive role of synergistic effects of BA and kinetin in regulation of ethylene synthesis resulted in a high frequency of shoot organogenesis [29]. Thus it appears that BA (1 mg/L) and kinetin (0.1 mg/L) proved effective in maintaining the ethylene levels of the plantlets and faster growth of *H. fastigiata*. Using this combination, a growth index of 0.9, 15 ± 2 shoots per explant and height of 2.7 ± 0.3 cm was achieved after 30 days. The explants were incubated at 25 °C under a 16-h light photoperiod. This was the highest biomass growth achieved using BA (1 mg/L) and kinetin (0.1 mg/L), as compared to other combinations of plant growth regulators (Table 1; Supplementary Material). The cultures were sub-cultured after every 60 days, when the highest total biomass yield (25 ± 3 mg fresh weight) was recorded in the MS medium containing BA (1 mg/L) and kinetin (0.1 mg/L). A positive correlation between ethylene synthesis and tissue browning under in vitro conditions was also reported earlier [30]. Of all the selected combinations of plant growth regulators, BA (1 mg/L) and kinetin (0.1 mg/L) showed a decrease in tissue browning and subsequent enhancement of regeneration and cell differentiation of *H. fastigiata*.

Isolation and identification of compounds

The final extraction yields for aqueous, ethanol, ethyl acetate and petroleum ether solvent extracts were 15.13, 11.21, 6.92 and 6.25 %, respectively. HPLC analysis of ethanolic extract showed the presence of three major compounds (Fig. 1). Final extraction yields for compounds **1**, **2** and **3** after HPLC purification were 1.25, 0.63 and 0.59 %, respectively. Compounds **1** and **2** were structurally identified as 1,5,7-trihydroxy-3-methoxyxanthone and 1,5-dihydroxy-3,7-dimethoxyxanthone, respectively, by comparing with the standards (available at Braunschweig), and also using the ESI–MS and UV–Vis spectral information available in the literature [3, 4]. Compound **3**: yellow crystalline, m.p. 240–242 °C; ESI–MS m/z $[M + H]^+$ 273.1, 257.9, 230, 215.1, 201.1, 187; UV λ_{max} (CH_3OH) nm 247, 310; ($CH_3OH + AlCl_3$) nm 247, 324; ($CH_3OH + HCl$) nm 247, 310, (CH_3OH + NaOAc) nm 247, 315; ^{1}H-NMR (500 MHz, CD_3OD): δ 3.91 (3H, s, C_8-OCH_3), 6.33 (1H, d, $J = 8.5$ Hz, H-4), 6.61 (1H, d, $J = 8.5$ Hz, H-2), 6.9 (1H, d, $J = 8.5$ Hz, H-6), 7.6 (1H, d, $J = 8$ Hz, H-7). On the basis of TOCSY, HSQC and ROESY spectra the structural identity of the compound was confirmed as 1,3,5-trihydroxy-8-methoxyxanthone (Supplementary Material).

Table 1 Influence of different combinations of phytohormones on growth index, number of shoots and height of *H. fastigiata*

BAP (mg/L)	Kinetin (mg/L)	NAA (mg/L)	IAA (mg/L)	Growth index[a]	No. of shoots/explant[a]	Height (cm)[a]
0.5	–	0.5	–	0.364 ± 0.01 b	10 ± 1 ab	1.7 ± 0.4 a
0.2	–	1	–	0.586 ± 0.02 c	15 ± 3 c	2.3 ± 0.2 b
4	–	–	–	0.612 ± 0.03 c	09 ± 4 ab	2.7 ± 0.2 bc
–	–	–	1	0.324 ± 0.01 a	07 ± 1 a	2.9 ± 0.1 c
0.5	0.5	–	–	0.604 ± 0.03 c	12 ± 1 bc	2.8 ± 0.1 c
1	0.1	–	–	0.900 ± 0.01 d	15 ± 2 c	2.7 ± 0.3 bc

All data are expressed as mean ± SD ($n = 3$). Measurements were made after 30-day growth period

[a] Means followed by the same letter are not significantly different at 5 % level by Duncan's test

Comparative analysis of three xanthones from nature-grown and in vitro shoot cultures of *H. fastigiata*

Comparative analysis of shoots from nature-grown plants and in vitro cultures showed that the content of **1** was 5.3-fold higher in shoot cultures than in wild plants; the content of **2** was 25-fold higher in shoot cultures. The content of **3** was 1.4-fold higher in the wild plants than the shoot cultures (Fig. 2). It was interesting to note that *H. fastigiata* in vitro cultures, when grown in BA (1 mg/L) and kinetin (0.1 mg/L) medium, produced much higher contents of **1** and **2** than nature-grown plants. Cytokinins are known to enhance the production of secondary metabolites and to play an important role in cytodifferentiation and sub-cellular differentiation, such as production of anthocyanins in *Camptotheca acuminata* [31, 32]. Cytokinin and auxin are also known to modulate secondary metabolites production in vitro, as reported in *Commiphora wightii* and *Pueraria tuberosa* for terpenoids and isoflavonoids synthesis, respectively [33]. The content of **3** was higher in nature-grown plants. This could be explained by the fact that under field conditions plants are exposed to various abiotic stresses such as salinity, temperature, etc. which result in the production of certain secondary metabolites, since abiotic stresses are known to affect secondary metabolism [34].

AChE inhibitory activity from extracts of shoot culture

Extracts were prepared from in vitro cultures in four different solvents (aqueous, ethanol, ethyl acetate and petroleum ether). Testing of AChE inhibitory activity for all the different extracts was carried out followed by subsequent determination of IC_{50} values. It was observed that the ethanolic extract was most active, with the lowest IC_{50} value (157 ± 13 µg/ml), which was 97.06, 92.5 and 87.5 % lower than the aqueous, ethyl acetate and petroleum ether extracts, respectively (Table 2). Thus, the most active ethanolic fraction was subsequently tested for the presence of the active principle of AChE inhibition. Ethanolic extract of *H. fastigiata* was screened by TLC bioautography (Supplementary Material). Naphthyl acetate

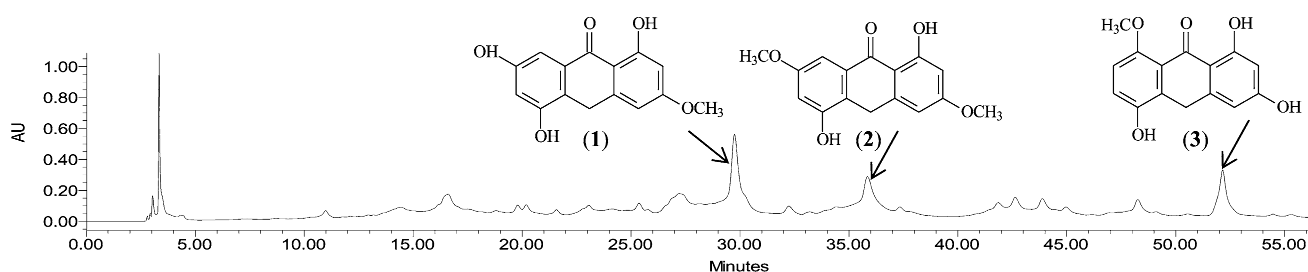

Fig. 1 Representative HPLC chromatogram showing detection of three compounds from ethanolic extract of *H. fastigiata* shoot culture. These three compounds were subsequently identified as 1,5,7-trihydroxy-3-methoxyxanthone (**1**), 1,5-dihydroxy-3,7-dimethoxyxanthone (**2**) and 1,3,5-trihydroxy-8-methoxyxanthone (**3**) on the basis of their UV–Vis spectral properties, mass spectroscopy and NMR analyses

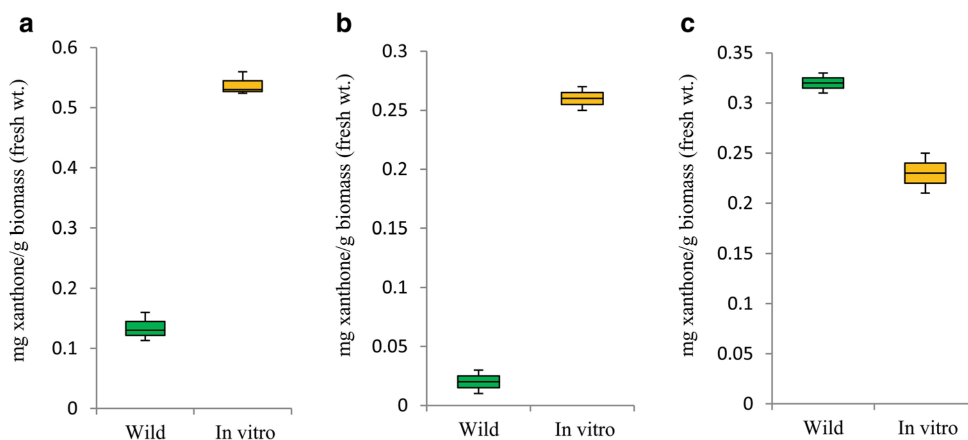

Fig. 2 *Box-and-whiskers plots* showing comparative analysis of xanthone contents in wild and shoot cultures of *H. fastigiata*. **a** 1,5,7-trihydroxy-3-methoxyxanthone (**1**) content, **b** 1,5-dihydroxy-3,7-dimethoxyxanthone (**2**) content, **c** 1,3,5-trihydroxy-8-methoxyxanthone (**3**) content

Table 2 IC_{50} values for AChE, MAO-A and MAO-B by different solvents and isolated compounds of *H. fastigiata*

Sample/extract	AChE (μg/ml)[a]	MAO-A (μg/ml)[a]	MAO-B (μg/ml)[a]
Ethanol	157 ± 13 b	25 ± 4 c	62 ± 18 c
Aqueous	5334 ± 51 f	37 ± 7 d	206 ± 34 e
Ethyl acetate	2091 ± 32 e	31.8 ± 5 c	73 ± 15 d
Petroleum ether	1251 ± 34 d	44.5 ± 3 d	324 ± 41 f
Compound **1**	282 ± 12 c	15 ± 3 b	–
Compound **2**	158 ± 15 b	12 ± 2 b	18.8 ± 3 b
Compound **3**	97 ± 11 a	–	20 ± 2 b
Standard	215.5 b	0.8 a	6.7 a

All data are expressed as mean ± SD ($n = 3$)

[a] Means followed by the same letter are not significantly different at 5 % level by Duncan's test. Standards: AChE (galanthamine), MAO-A (clorgiline) and MAO-B (deprenyl). Compound **1** is 1,5,7-trihydroxy-3-methoxyxanthone; compound **2** is 1,5-dihydroxy-3,7-dimethoxyxanthone; compound **3** is 1,3,5-trihydroxy-8-methoxyxanthone. The sign '–' indicates no inhibition

when used as substrate binds to the AChE enzyme and gives a purple-coloured complex. Upon binding with an inhibitor, the enzyme hinders enzyme–substrate interaction causing inactivation of the AChE enzyme, which fails to produce a coloured complex. Thus, the regions of the TLC plate which contain AChE inhibitors show up as white/discolored spots against the purple background, thus indicating the presence of AChE inhibitor(s) [27]. Based on TLC bioautography results, we identified three compounds as apparent inhibitors of the AChE enzyme. The AChE inhibitory property of compounds was confirmed by a colorimetric method [26]. Of the three compounds, **3** was most potent inhibitor of AChE with an IC_{50} value of 97 ± 11 μg/ml.

MAO-A and MAO-B inhibitory activity from extracts of shoot culture

Extracts were prepared from in vitro cultures in four different solvents (aqueous, ethanol, ethyl acetate, and petroleum ether). It was observed that of all the extracts the ethanolic extract was the most active for MAO-A (IC_{50} value 25 ± 4 μg/ml) and MAO-B (IC_{50} value 68 ± 18 μg/ml) when compared to the aqueous, ethyl acetate and petroleum ether extracts (Table 2). The isolated compounds were tested for their selective capability to inhibit MAO-A or MAO-B. Compounds **1** and **2** showed inhibition of MAO-A, while compounds **2** and **3** showed inhibition of MAO-B. Compounds **1** and **3** showed selective inhibition of MAO-A and MAO-B, respectively. This could provide a good opportunity for the utilization of these compounds for selective treatment of depression or PD. The reaction was confirmed by HPLC where the formation of reaction product (4-quinolinol) was monitored at 329 nm. The HPLC profile showed less 4-quinolinol formation in the MAO-A reaction than the MAO-B reaction containing ethanolic extract (Fig. 3). These results are supported by the spectrophotometric assay where the IC_{50} value for ethanolic extract against MAO-A was lower than MAO-B, as indicated earlier. Because of comparable inhibition properties of these isolated compounds with that of selective standards, inhibition kinetics studies were performed to determine the type of inhibition.

Evaluation of inhibition mechanism and kinetics of AChE, MAO-A and MAO-B

Detailed in vitro studies were carried out to understand the kinetics and mechanism of inhibition of eel AChE, recombinant human MAO-A and MAO-B by isolated compounds. Inhibitors were tested at varying concentrations depending on their IC_{50} values against AChE, MAO-A and MAO-B.

For AChE inhibition, varying concentrations of ATChI were used to study the nature of inhibition of this enzyme. The enzyme kinetics was plotted as a Lineweaver–Burk plot (Fig. 4). From the Michaelis–Menten equation based plotting, K_m, V_{max}, K_{cat}, K_{cat}/K_m and K_i values were calculated for the activity of control and compounds. For compound **1**, it was observed that the K_m value increased and V_{max} value decreased compared to control. This suggested the probability of mixed type of inhibition of AChE. For compound **2**, the K_m value of the enzyme with and without inhibitor remained the same (100 μM) whereas the V_{max} value decreased, which indicated a non-competitive inhibition of AChE. It has been observed that substitution of hydroxyl, methoxyl, allyloxy and prenylated oxyl at the C-3 position in the xanthone skeleton with a methoxyl group at the C-3 position was the most effective in inhibiting AChE [35]. This supported the inhibitory properties of both compounds **1** and **2** which possessed a methoxyl group at the C-3 position. For compound **3**, the K_m value increased and V_{max} value decreased, which also indicated the mixed type of inhibition. The K_{cat} value indicated the turn-over number of the AChE enzyme; all three compounds reduced the turn-over number but **3** was most effective. Compound **3** was most effective in reducing the catalytic efficiency (K_{cat}/K_m) of the enzyme followed by **1** and **2**, respectively (Table 3). Inhibition constant (K_i) values were also calculated for all three xanthones. When the inhibitor binds with the enzyme it is denoted as K_{ia} and when the inhibitor binds with the enzyme–substrate complex it is denoted as K_{ib}.

For MAO-A, a nonselective substrate kynuramine was used at varying concentrations to determine the type of inhibition. Based on the IC_{50} values, the inhibitor

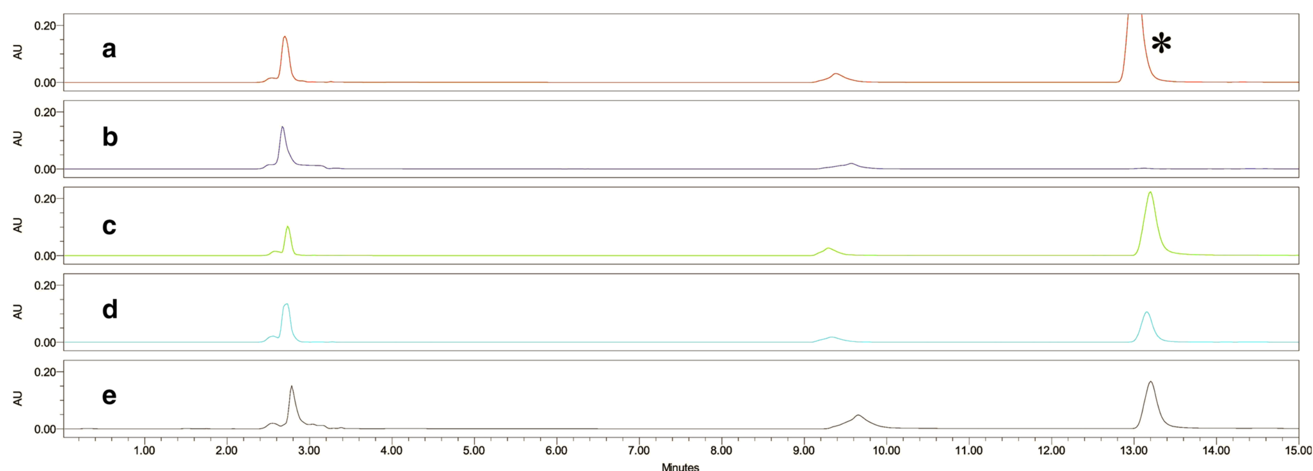

Fig. 3 Representative HPLC chromatograms for monoamine oxidase assay. *a* Standards [*4-quinolinol (product)]; *b* reaction terminated at 0 min; *c* control reaction with no inhibitor; *d* reaction showing the inhibition of MAO-A enzyme by ethanolic extract; *e* reaction showing the inhibition of MAO-B enzyme by ethanolic extract. All the reactions were performed for 60 min, except for reaction B which was terminated at 0 min

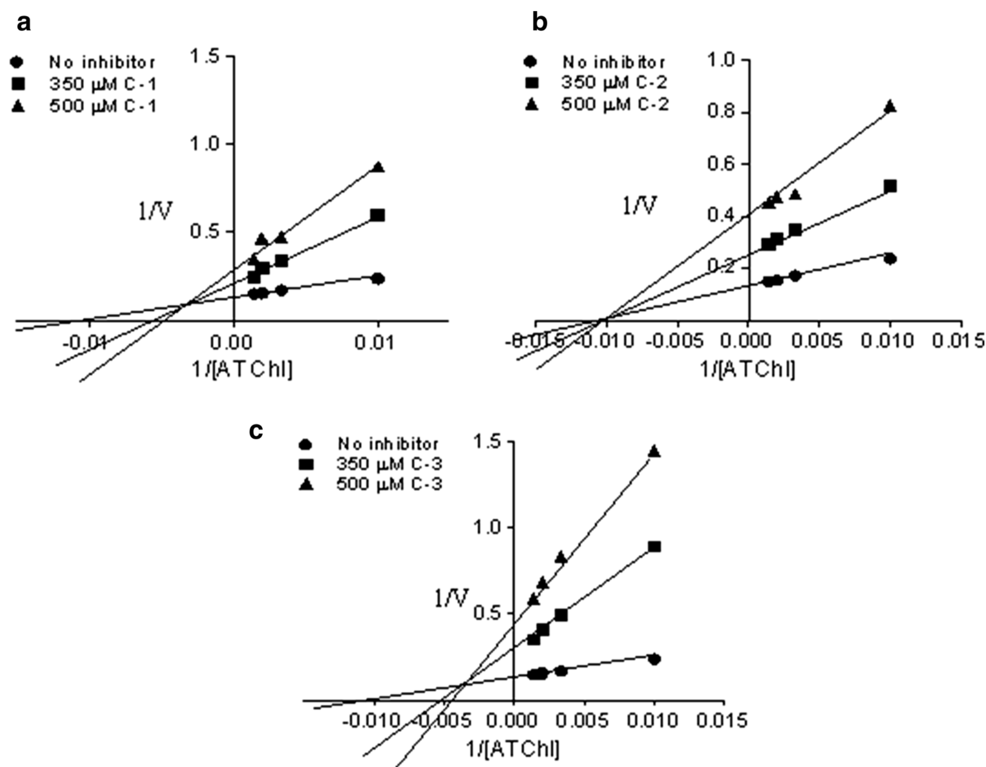

Fig. 4 Lineweaver–Burk plots for AChE dependence on substrate concentration (100–700 µM). Kinetics characteristics of inhibition of eel AChE with **a** 1,5,7-trihydroxy-3-methoxyxanthone (**1**), **b** 1,5-dihydroxy-3,7-dimethoxyxanthone (**2**), **c** 1,3,5-trihydroxy-8-methoxyxanthone (**3**). V = µmol/min and S = substrate acetylthiocholine iodide (ATChI) concentration (µM)

concentration was fixed for the three compounds. The kinetics data plotted as a double reciprocal Lineweaver–Burk plot (Fig. 5) suggested that compound **1** showed a decrease in V_{max} value along with the decrement of K_m value, which suggested the mixed reversible type of inhibition. Compounds bearing an –OH group at C-1 and $–OCH_3$ at C-3 positions showed more activity against the MAO-A enzyme [36]. Compound **2** showed the same V_{max} value with the increment of K_m value depending on the dose of inhibitor, which suggested the competitive reversible type of inhibition. For MAO-B also, kynuramine was used at varying concentrations and the data were plotted as a Lineweaver–Burk plot. The plot suggested that compound **2** showed the same V_{max} value with the increment of K_m

Table 3 Kinetic parameters of AChE, MAO-A and MAO-B (with and without inhibitor)

Assay	Inhibitor	[*I*]	V_{max}	K_m	K_{cat}	K_{cat}/K_m	K_i	Inhibition type
AChE	No inhibitor	0	7.4	100	5.92 x 10^5	6.20 x 10^3		
	Compound **1**	350	4.8	186	3.85 x 10^5	2.10 x 10^3	0.19^{Kia},0.66^{Kib}	Mixed reversible
		500	3.5	300	2.79 x 10^5	0.90 x 10^3	0.26^{Kia},0.92^{Kib}	
	Compound **2**	350	3.85	100	3.08 x 10^5	3.00 x 10^3	0.38^{Kia},0.38^{Kib}	Non-competitive reversible
		500	2.63	100	2.13 x 10^5	2.40 x 10^3	0.28^{Kia},0.28^{Kib}	
	Compound **3**	350	3.33	200	2.67 x 10^5	1.30 x 10^3	0.1^{Kia},0.29^{Kib}	Mixed reversible
		500	2.4	213	1.92 x 10^5	0.90 x 10^3	0.06^{Kia},0.16^{Kib}	
MAO-A	No inhibitor	0	56.3	72.5	43.3 x 10^5	59.7 x 10^3		
	Compound **1**	12	26.9	43.4	20.7 x 10^5	47.7 x 10^3	0.05^{Kia},0.01^{Kib}	Mixed reversible
		14	15.8	32.8	12.1 x 10^5	37.0 x 10^3	0.02^{Kia},0.005^{Kib}	
	Compound **2**	12	56.3	92.6	43.3 x 10^5	46.8 x 10^3	0.043^{Kia}	Competitive reversible
		14	56.3	202	43.3 x 10^5	21.4 x 10^3	0.007^{Kia}	
MAO-B	No inhibitor	0	0.21	14.6	42 x 10^3	2.87 x 10^3		
	Compound **2**	12	0.21	38.7	42 x 10^3	1.08 x 10^3	0.0072^{Kia}	Competitive reversible
		14	0.21	60.3	42 x 10^3	0.7 x 10^3	0.004^{Kia}	
	Compound **3**	12	0.16	40.9	32 x 10^3	0.78 x 10^3	0.004^{Kia},0.039^{Kib}	Mixed reversible
		14	0.09	51.8	19 x 10^3	0.37 x 10^3	0.002^{Kia},0.01^{Kia}	

Compound **1** is 1,5,7-trihydroxy-3-methoxyxanthone; compound **2** is 1,5-dihydroxy-3,7-dimethoxyxanthone; compound **3** is 1,3,5-trihydroxy-8-methoxyxanthone

K_{ia} ability of inhibitor to bind free enzyme, K_{ib} ability of inhibitor to bind enzyme–substrate complex. [*I*] (inhibitor) is expressed in μM, V_{max} in μmol/min, K_m in μM, K_{cat} in s^{-1}, K_{cat}/K_m in s^{-1} $μM^{-1}$, K_i in μM

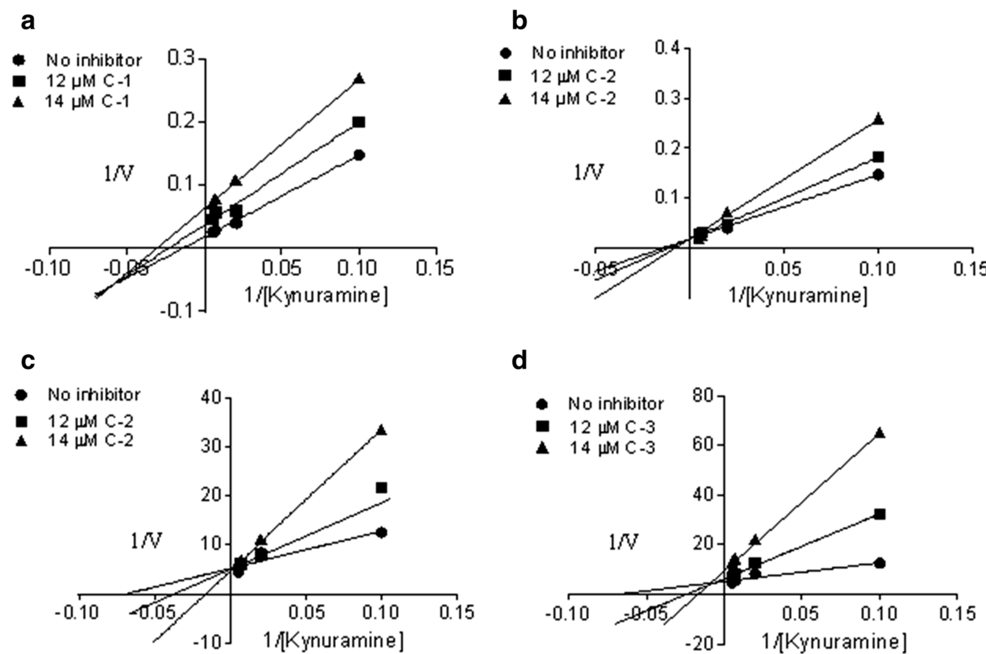

Fig. 5 Lineweaver–Burk plots for MAO-A and MAO-B dependence on substrate concentration (10–200 μM). Kinetics characteristics of inhibition of human recombinant MAO-A with **a** 1,5,7-trihydroxy-3-methoxyxanthone (**1**), **b** 1,5-dihydroxy-3,7-dimethoxyxanthone (**2**); kinetics characteristics of inhibition of human recombinant MAO-B with **c** 1,5-dihydroxy-3,7-dimethoxyxanthone (**2**), **d** 1,3,5-trihydroxy-8-methoxyxanthone (**3**). V = μmol/min and S = substrate kynuramine concentration (μM)

value, which indicated the competitive reversible type of inhibition. Compound **3** showed a decrement in V_{max} value and increment in K_m value, indicative of the mixed type of inhibition. Compounds **2** and **3** were most effective in reducing the catalytic efficiency (K_{cat}/K_m) of MAO-A and MAO-B, respectively.

Antioxidant capacities of crude extracts and compounds isolated from *H. fastigiata* shoot cultures

Antioxidant activities of *H. fastigiata* were also studied in the crude extracts of four different solvents (ethanol, aqueous, ethyl acetate and petroleum ether). The TPC of the ethanolic extract was found to be highest, followed by ethyl acetate, aqueous and petroleum ether extracts which showed the lowest values, indicating that the ethanol was most suitable solvent. DPPH activity showed the lowest IC_{50} value in the ethanolic extract, followed by ethyl acetate, aqueous and petroleum ether, thus indicated that ethanolic crude extract was most effective in scavenging the DPPH free radical. The antioxidants which are capable of donating protons to free DPPH radicals fade the violet colour which ultimately turns colorless depending upon the strength of antioxidants. Similarly, the ABTS assay was also done which showed that the ethanolic extract had a higher ABTS value, followed by ethyl acetate, aqueous and petroleum ether extracts. The ABTS free radical (ABTS·) is teal blue in colour. The antioxidants which are effective in neutralizing the free radicals by donating the lone pair of electrons to ABTS· are capable of decolorizing the ABTS solution. Thus, the less blue colour developed after reaction, the higher the potency of antioxidants to donate the free electrons. FRAP values also showed a similar trend, in which the ethanolic extract was most potent antioxidant, followed by ethyl acetate, aqueous and petroleum ether. All data suggested that the ethanolic extract has the best capacity to scavenge the free radicals. It was earlier shown that the ethanolic solvent was best in terms of extraction and antioxidant capacities in nature-grown plants. TPC in nature-grown plants was reported as 134.4 ± 3.1 mg GAE g^{-1} dry weight [19], which was very high compared to in vitro cultures. This exceptionally high level of TPC was perhaps the result of various stress factors under field conditions. Thus, a positive correlation between the high TPC and antioxidant activities was responsible for the high antioxidant capacities of ethanolic extracts [37]. The three compounds were also tested for DPPH, ABTS and FRAP assay; compound **2** (DPPH IC_{50} value = 0.0833 ± 0.003 mg/ml) showed strong antioxidant capacity followed by compound **1** (DPPH IC_{50} value = 0.4092 ± 0.002 mg/ml) and compound **3** (DPPH IC_{50} value = 0.902 ± 0.001 mg/ml) (Table 4). The antioxidant capacities of the three xanthones identified from ethanolic extract also support the strong activity of the ethanolic extract. The DPPH IC_{50} of ascorbic acid is 0.00207 mg/ml and two xanthones, cudraxanthone and macluroxanthone, isolated from *Cudrania tricuspidata* showed DPPH IC_{50} values of 10.4 and 15.4 μM, respectively [38].

Statistical analyses

Statistical analysis was done using PCA. Four different extracts were tested to detect the most suitable solvent for extraction of in vitro shoot cultures of *H. fastigiata* for antioxidant capacities (DPPH, ABTS, FRAP, TPC) and neuroprotective capacities (AChE, MAO-A and MAO-B inhibition). From the graphical representation (scatter-plot) of PCA it was clear that the first (PC 1) and second (PC 2) principal components were sufficient to explain the total variance, i.e. 99.98 % for antioxidant capacities. Three completely different groups could be clearly distinguished

Table 4 Antioxidant activities of crude extracts and compounds isolated from *H. fastigiata*

Extract/sample	DPPH[a,e]	ABTS[b,e]	FRAP[c,e]	TPC[d,e]
Ethanol	11.95 ± 0.2 a	5.77 ± 0.5 d	3.9 ± 0.2 d	1.8 ± 0.2 d
Aqueous	49.20 ± 2.3 c	0.6 ± 0.09 b	0.65 ± 0.2 b	0.5 ± 0.01 b
Ethyl acetate	20.2 ± 0.13 b	1.05 ± 0.8 c	2.61 ± 0.1 c	1.2 ± 0.1 c
Petroleum ether	124 ± 5.7 d	0.12 ± 0.002 a	0.17 ± 0.11 a	0.02 ± 0.003 a
Compound **1**	0.4092 ± 0.002	3040 ± 30	978 ± 13	–
Compound **2**	0.0833 ± 0.003	3690 ± 17	1110 ± 22	–
Compound **3**	0.902 ± 0.001	100 ± 12	200 ± 5	–
Ascorbic acid	0.00207 ± 0.0009	–	–	–

All data are expressed as mean ± SD ($n = 3$)

[a] DPPH IC_{50} value (mg/ml)

[b] ABTS value in terms of AEAC (mM/g ascorbic acid equivalent)

[c] FRAP value (mM Fe^{2+} equivalent/g)

[d] Total phenolic content (mg GAE/g dry weight). Compound **1** is 1,5,7-trihydroxy-3-methoxyxanthone; compound **2** is 1,5-dihydroxy-3,7-dimethoxyxanthone; compound **3** is 1,3,5-trihydroxy-8-methoxyxanthone

[e] Means followed by the same letter are not significantly different at 5 % level by Duncan's test

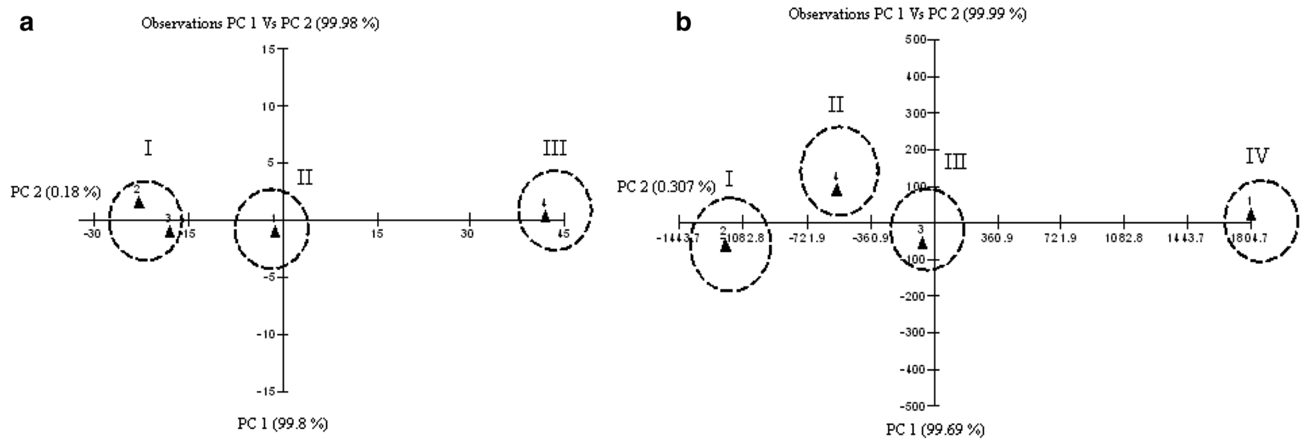

Fig. 6 **a** PCA analysis representing different extraction solvents for antioxidant capacities of *H. fastigiata*. Group *I* represents ethanolic extract (*2*) and ethyl acetate extract (*3*); Group *II* represents aqueous extract (*1*); Group *III* represents petroleum ether (*4*) extract. **b** PCA analysis representing different extraction solvents for neuroprotective capacities of *H. fastigiata*. Group *I* represents ethanolic extract (*2*) and ethyl acetate extract (*3*); Group *II* represents petroleum ether extract (*4*); Group *III* represents ethyl acetate extract (*3*); Group *IV* represents aqueous extract (*1*)

from the graph. Group I was located on the left side of the scatter-plot, and consisted of ethanol and ethyl acetate. Group II and group III included aqueous and petroleum ether, respectively. Ethanol and ethyl acetate extract can be considered to have similar potencies in free-radical scavenging capacity. Both members of group I possessed a higher amount of TPC along with higher antioxidant capacity. Similarly, for neuroprotective capacities, it was clear that the first (PC 1) and second (PC 2) principal components were sufficient to explain the total variance of 99.99 %. It was observed that no solvent systems possessed similar type of inhibitory properties. Thus all the results suggested that the ethanolic extract is the best solvent for extraction in terms of neuroprotective capacities of *H. fastigiata* extract (Fig. 6a, b).

Conclusion

Screening of different plant resources have achieved great successes in the past for finding potential neuroprotective compounds, such as the AChE inhibitor galanthamine. The present study revealed the utility of in vitro plantlets as a potential source of bioactive xanthones providing neuroprotective and antioxidant capacities. Because of the limited availability of this rare plant resource, the in vitro shoot culture system was established. The MS medium containing BA (1 mg/L) and kinetin (0.1 mg/L) was found to be most efficacious for in vitro propagation, with a growth index of 0.9, producing 15 ± 2 shoots per explant with shoot lengths of 2.7 ± 0.3 cm after 30 days of subculture. The total biomass yield achieved at the time of sub-culture was 25 ± 3 mg fresh weight (after 60 days). Three major xanthones were detected in shoot cultures of *H. fastigiata*. When compared, compound **3** showed the highest AChE inhibitory property along with inhibition of MAO-B, while compounds **1** and **3** showed selective inhibition of MAO-A and MAO-B, respectively. Compound **2** showed the strongest antioxidant capacity along with inhibition of both MAO-A and MAO-B enzymes. It was interesting to note that all the compounds exhibited the reversible type of inhibitions. The inhibition of MAO-A by compound **2** in a reversible and selective manner would be beneficial in the treatment of resistant depression, which comprises 33–57 % of all cases of depression. All the compounds showed good antioxidant properties. The significant AChE and MAO inhibitory properties of *H. fastigiata* suggest the potential of this plant for alleviation of neurological disorders associated with the depletion of acetylcholine and monoamines (AD and PD, respectively). Thus, it can be concluded that shoot cultures of *H. fastigiata* are a good source of xanthone compounds which can be interesting candidates for the design of new antidepressant drugs. This may have possible implications for herbal-based dietary supplements in alleviating these neurological disorders.

Acknowledgments Utkarsh Ravindra Moon thanks University Grant Commission, India for the award of an individual junior/senior research fellowship [F. No. 2-16/98 (SA-I), dated 4-21-2011]. Facilities created from an MHRD-sponsored research grant (4-25/2013/TS-I) were utilized in this work.

References

1. Klundt T, Bocola M, Lutge M, Beuerle T, Liu B, Beerhues L (2009) A single amino acid substitution converts benzophenone synthase into phenylpyrone synthase. J Biol Chem 284:30957–30964

2. Mukherjee KS, Chakraborty CK, Laha S, Bhattacharya D, Chatterjee TP (1991) 1,7-Trihydroxy-3-methoxyxanthone from *Hoppea fastigiata*. Pharm Biol 29:123–125. doi:10.1016/0031-9422(91)85307-L
3. Mukherjee KS, Manna TK, Laha S, Chakraborty CK (1994) A new xanthone from *Hoppea fastigiata*. Pharm Biol 32:201–203
4. Mukherjee KS, Chakraborty CK, Chatterjee TP, Bhattacharjee D, Laha S (1991) 1,5,7-Trihydroxy-3-methoxyxanthone from *Hoppea fastigiata*. Phytochemistry 30:1036–1037. doi:10.1016/0031-9422(91)85307-L
5. Mukherjee KS, Laha S, Manna TK, Roy SC (1995) Further work on *Limnophila rugosa* and *Hoppea fastigiata* (Gentianaceae). J Indian Chem Soc 72:63–64
6. Brahmachari G, Gorai D, Mondal S, Gangopadhay A, Chatterjee D (2003) A new naturally occurring xanthone bearing rare oxygenation pattern from *Hoppea fastigiata*. J Chem Res 6:362–363. doi:10.3184/030823403103174146
7. Urbain A, Marston A, Queiroz EF, Ndjoko K, Hostettmann K (2004) Xanthones from *Gentiana campestris* as new acetylcholinesterase inhibitors. Planta Med 70:1011–1014
8. Houghton PJ, Howes MJ (2005) Natural products and derivatives affecting neurotransmission relevant to Alzheimer's and Parkinson's disease. Neurosignals 14:6–22
9. Mattson MP (2004) Pathways towards and away from Alzheimer's disease. Nature 430:631–639
10. Houghton PJ, Ren Y, Howes MJ (2006) Acetylcholinesterase inhibitors from plants and fungi. Nat Prod Rep 23:181–199
11. Heinrich N, Teoh HL (2004) Galanthamine of snowdrop—the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. J Ethnopharmacol 92:147–162
12. Shih JC, Chen K, Ridd MJ (1999) Monoamine oxidase: from genes to behavior. Annu Rev Neurosci 22:197–217
13. Serrano-Dueñas M, Cardozo-Pelaez F, Sanchez-Ramos JR (2001) Effect of *Banisteriopsis caapi* extract on Parkinson's disease. Sci Rev Altern Med 5:127–132
14. Yamada M, Yasuhara H (2004) Clinical pharmacology of MAO inhibitors: safety and future. Neurotoxicology 25:215–221
15. Schulz V (2003) Ginkgo extract or cholinesterase inhibitors in patients with dementia: what clinical trial and guidelines fail to consider. Phytomedicine 10:74–79
16. Urbain A, Marston A, Sintra GL, Bravo J, Purev O, Purevsuren B, Batsuren D, Reist M, Carrupt PA, Hostettmann K (2008) Xanthones from *Gentiana amarella* ssp. acuta with acetylcholinesterase and monoamine oxidase inhibitory activities. J Nat Prod 71:895–897
17. Khaw KY, Choi SB, Tan SC, Wahab HA, Chan KL, Murugaiyah V (2014) Prenylated xanthones from mangosteen as promising cholinesterase inhibitors and their molecular docking studies. Phytomedicine 21:1303–1309
18. Ambasta SP (1999) The useful plants of India. National Institute of Science Communication, New Delhi
19. Moon UR, Sen SK, Mitra A (2014) Antioxidant capacities and acetylcholinesterase-inhibitory activity of *Hoppea fastigiata*. J Herbs Spices Med Plants 20:115–123. doi:10.1080/10496475.2013.840711
20. Wungsintaweekul J, Choo-malee J, Charoonratana T, Keawpradub N (2012) Methyl jasmonate and yeast extract stimulate mitragynine production in *Mitragyna speciosa* (Roxb.) Korth. shoot culture. Biotechnol Lett 34:1945–1950
21. Gaid MM, Sircar D, Muller A, Beurle T, Liu B, Ernst L, Hansch R, Beerhues L (2012) Cinnamate:CoA ligase initiates the biosynthesis of benzoate derived xanthone phytoalexin in *Hypericum calycinum* cell cultures. Plant Physiol 160:1267–1280
22. Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LWT Food Sci Technol 28:25–30. doi:10.1016/S0023-6438(95)80008-5
23. Re R, Pellegrinni N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237
24. Benzie IEF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 239:70–76
25. Ainsworth EA, Gillespie KM (2007) Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nat Protoc 2:875–877
26. Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88–95
27. Marston A, Kissling J, Hostettmann K (2002) A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants. Phytochem Anal 13:51–54
28. Aiyegoro OA, Van Dyk S (2011) The antioxidant properties, cytotoxicity and monoamine oxidase inhibition abilities of the crude dichloromethane extract of *Tarchonanthus camphoratus* L. leaves. Afr J Biotechnol 10:17297–17304. doi:10.5897/AJB11.2249
29. Shyamali S, Kazumi H (2007) Synergistic effects of both kinetin and both benzyl adenine improves regeneration of cotyledon explants of bottle gourd (*Lagenaria siceraria*) on ethylene production. In: Ramina A, Chang C, Giovannoni J, Klee H, Perata P, Woltering E (eds) Advances in plant ethylene research: proceedings of 7th international symposium on plant hormone ethylene. Springer, Netherlands, pp 153–155
30. Kumar PP, Reid DM, Thorpe TA (1987) The role of ethylene and carbon-dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata* in vitro. Physiol Plant 69:244–252. doi:10.1111/j.1399-3054.1987.tb04282.x
31. Suri SS, Ramawat KG (1995) In vitro hormonal regulation of laticifers differentiation in *Calotropis procera*. Ann Bot 75:477–480
32. Ramawat KG, Mathur M (2007) Factors affecting production of secondary metabolites. In: Ramawat KG, Merillon JM (eds) Biotechnology: secondary metabolites. Science Publishers Inc., Enfield, pp 59–102
33. Goyal S, Ramawat KG (2008) Synergistic effect of morphactin on cytokinin-induced production of isoflavonoids in cell cultures of *Pueraria tuberosa* (Roxb. ex. Wild.). Plant Growth Regul 55:175–181. doi:10.1007/s10725-008-9271-x
34. Ramkrishna A, Ravishankar GA (2011) Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal Behav 6:1720–1731. doi:10.4161/psb.6.11.17613
35. Qin J, Lan W, Liu Z, Huang J, Tang H, Wang H (2013) Synthesis and biological evaluation of 1,3 dihydroxyxanthone mannich base derivatives anticholinesterase agents. Chem Cent J 7:78
36. Gnerre C, Thull U, Gaillard P, Carrupt P, Testa B, Fernandes E, Silva F, Pinto M, Pinto MMM, Wolfender J, Hostettmann K, Cruciani G (2001) Natural and synthetic xanthones as monoamine oxidase inhibitors: biological assay and 3-D QSAR. Helv Chim Acta 84:552–570. doi:10.1002/1522-2675(20010321)84:3<552:AID-HLCA552>3.0.CO;2-X
37. Ghasemzadeh A, Jaafar HZE, Rahmat A (2010) Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysia young ginger (*Zingiber officinale* Roscoe). Molecules 15:4324–4333
38. Lee BW, Lee JH, Lee ST, Lee HS, Lee WS, Jeong TS, Parka KH (2005) Antioxidant and cytotoxic activities of xanthones from *Cudrania tricuspidata*. Biorgan Med Chem Lett 15:5548–5552