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ORIGINAL ARTICLE

Screening of ethnic medicinal plants of South India against influenza (H1N1) and their antioxidant activity

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Abstract Antiviral activity against H1N1 influenza was studied using ethnic medicinal plants of South India. Results revealed that *Wrightia tinctoria* (2.25 μg/ml) was one of the best antidotes against H1N1 virus in terms of inhibitory concentration of 50% (IC_{50}) whereas the control drug Oseltamivir showed 6.44 μg/ml. *Strychnos minor*, *Diotacanthus albiflorus* and *Cayratia pedata* showed low cytotoxicity (>100) to the MDCK (Malin darby canine kidney) cells by cytotoxicity concentration of 50% (CC_{50}) and possessed antiviral activity suggesting that these plants can be used as herbal capsules for H1N1 virus. *W. tinctoria* and *S. minor* showed high therapeutic indexes (TI) such as 12.67 and 21.97 suggesting that those plants can be used for anti-viral drug development. The CC_{50} values of *Eugenia singampattiana* (0.3 μg/ml), *Vitex altissima* (42 μg/ml), *Salacia oblonga* (7.32 μg/ml) and *Salacia reticulata* (7.36 μg/ml) resulted in cytotoxicity of the MDCK cells, due to their high phenolic content. Findings from this study state that the plant *W. tinctoria* can be a potent source for third generation anti-viral drug development against H1N1.

1. Introduction

Viruses are one of the major infective agents causing various health problems to humans resulting in death every year (Rajasekaran et al., 2013). Among the viral diseases influenza plays a vital role in humans and animals causing serious illness and major financial strain/stress. H1N1 viruses are more contagious and were reported to have caused serious problems in many countries. In Canada, the influenza virus was identified in 1 of 1.6 admissions during 2010 influenza season

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(Schanzer et al., 2013). This influenza can be divided into various subtypes like A and B and these two types of H1N1 virus were predominantly found among the human population affecting their day-to-day life. Generally these viruses cause acute respiratory infections referred to as "flu" resulting in serious problems particularly to children. Even though vaccines are available for flu, it was reported that only 50% were effective among the elderly persons (Wang et al., 2006). Moreover this virus subtypes A and B spread globally and its mutations that create antigenic drift and shift have been reported (Stein et al., 2009). This necessitated a serious search for better antiviral drugs.

Medicinal plants are termed to be one of the easy sources to get antiviral drugs since they have a proven record for antiviral activity. Tribals living worldwide traditionally have been using most of the medicinal plants successfully for many decades (Gurib-Fakim, 2006). Transmitting the medicinal plant formulae for curing some lethal diseases orally from generation to generation resulting in the loss of various valuable medicinal plant information (Nadembega et al., 2011). Even today a majority of the developing country's population depends on herbal medicines for their primary health care (Goleniowski et al., 2006). Due to this reason studies on medicinal plants to find new anti-viral drug developments for various diseases were required, since 40% of all chemical drugs were derived from the plant source. India is where Siddha and Ayurveda medicines are common and the Traditional healers, spread all over the country have immense knowledge in curing many human diseases by using medicinal plants (Muthu et al., 2006). Medicinal plants with a potential to cure can be taken up for scientific studies to see if it can combat viral diseases, with the hope of finding next generation drugs for influenza.

Coscinium fenestratum, *Trichopus zeylanicus*, *Eugenia singampattiana*, *Vitex altissima*, *Strychnos minor*, *Diotacanthus albiflorus*, *Strychnos nux-vomica*, *Chloroxylon swietenia*, *Helicteres isora*, *Andrographis paniculata*, *Wrightia tinctoria*, *Cayratia pedata*, *Salacia oblonga* and *Salacia reticulata* are some of the medicinal plants commonly used by natives and tribals of Tamil Nadu, South India. Among them *S. minor* and *E. singampattiana* were used by Kani tribes of South India for snake bites (Ayyanar, 2008), asthma and as anti-tumour agent(Viswanathan et al., 2006; Kala et al., 2011). *Coscinium* sps., and *Salacia* sps. were reported for their anti-diabetic and anti-inflammatory activities (Nayak et al., 2013; Ravishankar et al., 2013; Yoshino et al., 2009; Ismail et al., 1997). *Chloroxylon* sps., *Cayratia* sps. and *Trichopus* sps. were reported for their antioxidant activity and metabolic content by Nilip and Gouri, (2013), Perumal et al., (2012), Tharakan et al., (2005). Duraipandiyan et al., (2006), Ponnusamy et al. (2011) studied the antimicrobial properties of *Diotacanthus* sps. and *Wrightia* sps.

A. paniculata was reported for use against flu and possesses antiviral activity (Arora et al., 2010; Coon and Ernst, 2004). Apart from *A. paniculata* all other plants were reported for antioxidant properties and not against antiviral properties. Since these plants are highly used as medicine by the natives their activity against H1N1 influenza virus will provide alternative therapeutic formulation and be helpful for the human kind. Previous studies of herbal made medicines like Shahakusan, hochuekkito, Jinchai and Lianhuaqingwen capsules are proven for its effectiveness against virus by blocking transcription and replication resulting in the reduction of the illness period (Dan et al., 2013; Hokari et al., 2012; Zhong et al., 2013; Duan et al., 2011).

Based on this background, a screening of selected south Indian medicinal plants which are mostly used by ethnic and native people are tested against H1N1 influenza virus. This study will be helpful for new therapeutic agent (third generation influenza therapeutic compounds) preparation from the medicinal plant which can be helpful for the humans to overcome influenza since the virus was reported for its high mutation ability against drugs.

2. Materials and methods

2.1. Chemicals

All the solvents used for the study were HPLC grade and the chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Plant materials

C. fenestratum (MP1), *T. zeylanicus* (MP2), *E. singampattiana* (MP3), *V. altissima* (MP4), *S. minor* (MP5), *D. albiflorus* (MP6), *S. nux-vomica* (MP7), *C. swietenia* (MP8), *H. isora* (MP9), *A. paniculata* (MP10), *W. tinctoria* (MP11), *C. pedata* (MP12, rhizome), *C. pedata* (MP13, collected from Kanyakumari Dt, Tamil Nadu, India), *C. pedata* (MP14, collected from Kanchipuram Dt, Tamil Nadu, India), *S. oblonga* (MP15) and *S. reticulata* (MP16) plants were separately collected from tropical and western Ghats region of South India. All the collected plants were identified and confirmed by an ethno-botanist from Pachaiyappa's college, Chennai, Tamil Nadu, India. The leaves were shade dried before grinding and served as plant source for the extraction.

2.3. Extraction of medicinal plants

All the plant samples (6 replicates) were taken separately and weighed (0.1 g) in an Eppendorf tube (2 ml). 1 ml of 80% methanol was added to the samples and vortexed, followed by sonication for a period of 10 min. After that the methanol was collected separately by centrifugation at 8000 rpm. This step was continued twice and all the collected supernatants were added together and evaporated to dryness using speed vac. The resulting residues were redissolved in DMSO and used for cell line studies. In the mean time for the total phenolic and flavonoid analysis, these extracts were dissolved using 100% methanol.

2.4. Cell culture

Madin Darby canine kidney (MDCK) cell and influenza AP/R/8 virus (H1N1) were used for the present study. Influenza AP/R/8 virus and MDCK cells were purchased from American Type Culture Collection (ATCC). MDCK cell was maintained at 32 °C with 5% of CO_2 in a relative humidified cell culture incubator. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of fetal Bovine Serum (FBS) and 1% of Antibiotic–Antimycotic solution (100×) was used for MDCK cell growth. DMEM, trypsin–EDTA, Antibiotic–Antimycotic Solution 100x and FBS were purchased from

Welgene (150-Seongseo Industrial complex Bukro, Dalseogu, Daegu, 704–948 Republic of Korea).

2.5. Antiviral assay

MDCK cells were seeded onto 96 well plates concentrated as 2×10^4 cells per well plate. After the first day of growth, cells seeded on the 96 well plates were washed twice with phosphate buffer saline (PBS). Then Influenza AP/R/8 virus was diluted as 5×10^3 with DMEM medium contained trypsin–EDTA and this solution was used for the infection. Then, 90 µL of virus solution and 10 µL of medicinal plant extracts were placed onto the 96 well plates in which MDCK cells were grown the previous day. Four different concentrations of the extracts (0.1, 1, 10 and 100 µL) with three replicates of individual plant extracts were used in 96 well plates for the analysis. Infected plates were incubated in CO_2 incubator for a period of 48 h. After incubation the medium was removed and washed twice with PBS. After washing the plate, the cells were fixed by adding 70% of acetone followed by incubation at −4 °C for a period of 1 h. Then the acetone was removed and the plates were dried at 60 °C under hot air oven. The completely dried plates were incubated overnight with 100 µL of SRB (0.4 mg/L). After the overnight incubation, SRB solution was removed and washed 3 times with 1% of acetic acid and dried again under hot air oven at 60 °C. Morphology of the cells was observed under microscope (reflected light microscope) under 40× magnification and images were compared for their antiviral activity. After the microscopic observation, the SRB strain was dissolved with 10 mM of Tris base and incubated overnight. Based on the spectrophotometric data measured at 510 nm 50% of inhibition concentration (IC_{50}), cytotoxic concentration (CC_{50}) and therapeutic index (TI) value were obtained and calculated.

2.6. Total flavonoid content (TFC) and total polyphenol content (TPC)

The TFC was analysed by adding 20 µL of methanol extract on 96-well plates and added with 180 µL of 90% diethylene glycol and 20 µL of 1 N NaOH. The absorbance was measured at 515 nm using a micro plate reader (Spectra max plus384, Molecular devises, USA) after 15 min of incubation (Maria John et al., 2014b).

For phenolic compound analysis, 20 µL of methanol extracts was mixed with 100 µL of 0.2 N Folin–Ciocalteu's phenol reagent and 80 µL of saturated sodium carbonate. After 1 h of incubation the samples were measured using a microplate reader 750 nm (Maria John et al., 2014b).

2.7. Radical scavenging activity

The DPPH radical scavenging potentiality of the medicinal plants was measured using 20 µL of methanol extract mixed with 180 µL of DPPH (0.5 mM) reagent and was incubated for 20 min. The absorbance was measured at 515 nm using a micro plate reader (Maria John et al., 2014b).

In case of ABTS radical scavenging activity, 20 µL of methanol extracts of all the medicinal plants was mixed with 180 µL of ABTS solution and the absorbance was measured at 750 nm using a micro-plate reader (Maria John et al., 2014c).

2.8. Metabolite analysis by HPLC

Medicinal plant extracts were finally dissolved in MeOH and was analysed under HPLC (Agilent 1100, USA) with water and acetonitrile containing 0.1% formic acid served as mobile phase A and B. The gradient flow starts from 10% B and reaches 90% at 30 min and 100% at 35 min followed by 10% at 40 min. The metabolic detection was made by using authentic standard retention time (tR) and the detector (DAD) was set at 254 nm.

2.9. Data analysis

The identified peak area was converted into log10 value and was analysed using Statistica 7 software. Based on the box-whisker plot the metabolites were compared between the samples. All data were statistically analysed using SPSS software and were compared using excel graphs.

3. Results and discussion

3.1. Free radical scavenging potential of the selected medicinal plants

DPPH and ABTS activities of the selected medicinal plants are analysed and presented in Fig 1. The plants possess variation in radical scavenging activity even between the two different radical scavenging activities to the same plant extract. Comparing the plant extracts, MP2 followed by MP3 and MP7 possesses high DPPH radical scavenging activity; whereas MP13 and 14 followed by MP12 and 9 possess lowest scavenging

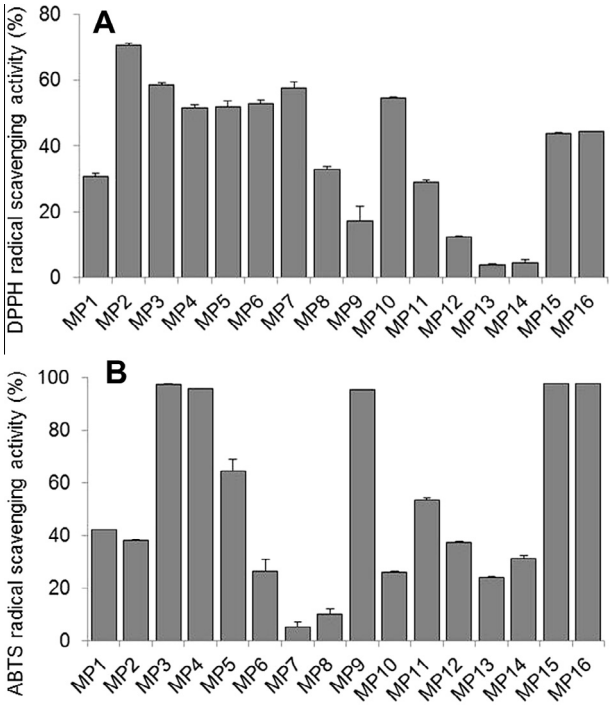

Figure 1 Free radical scavenging potential of selected medicinal plants of South India. MP-medicinal plants. (A) DPPH radical scavenging activity and (B) ABTS radical scavenging activity.

activity (Fig. 1A). In the case of ABTS, MP15, MP16, MP9, MP3 and MP4 possess high scavenging potential whereas MP7, MP8, MP13 and MP6 showed lowest activity (Fig. 1B). When comparing the two free radicals, the plant MP7 showed high DPPH activity but its ABTS scavenging potential was poor. MP9 showed high ABTS scavenging potential but its DPPH scavenging potential was found to be low than other medicinal plants analysed. MP8, MP12 and MP2 were reported for high antioxidant activity (Nilip and Gouri, 2013; Perumal et al., 2012; Tharakan et al., 2005). Antioxidant and antiproliferative properties on human hepatoma cells of *Vitex* sps. were reported by Kadir et al., (2013).

3.2. Effect of medicinal plant extracts against H1N1

The anti-viral activity of the medicinal plants at four different concentrations along with standard drug (Oseltamivir) is presented in Fig. 2. Result revealed that the plant possesses variations in their anti viral activity against H1N1. Some of the plants showed extremely higher activity against the virus and some of the plants showed cytotoxic activity to the cells. Based on the variations of IC_{50} and CC_{50} values the results were compared. Crude extract of MP11 showed amazing activity against influenza H1N1 virus where the IC_{50} was 2.25 μg/ml, CC_{50} was 28.6 μg/ml and TI was 12.7. In the control drug 'oseltamivir' it was seen that 6.44 μg/ml (IC50), > 100 μg/ml (CC50) and 15.5 (TI) values were observed. MP7 (33.4 μg/ml), MP14 (37.3 μg/ml), MP5 (46.7 μg/ml), MP6 (60.1 μg/ml) and MP13 (66 μg/ml) also showed good results against H1N1 in terms of IC_{50} and CC_{50} values (MP7–20 μg/ml, MP14- > 100 μg/ml, MP5- 1026 μg/ml, MP6- > 100 μg/ml and MP13- > 100 μg/ml, respectively). When comparing the extracts' CC_{50} values it was observed that some of the medicinal plants such as MP3 (0.33 μg/ml), MP15 (7.32 μg/ml),

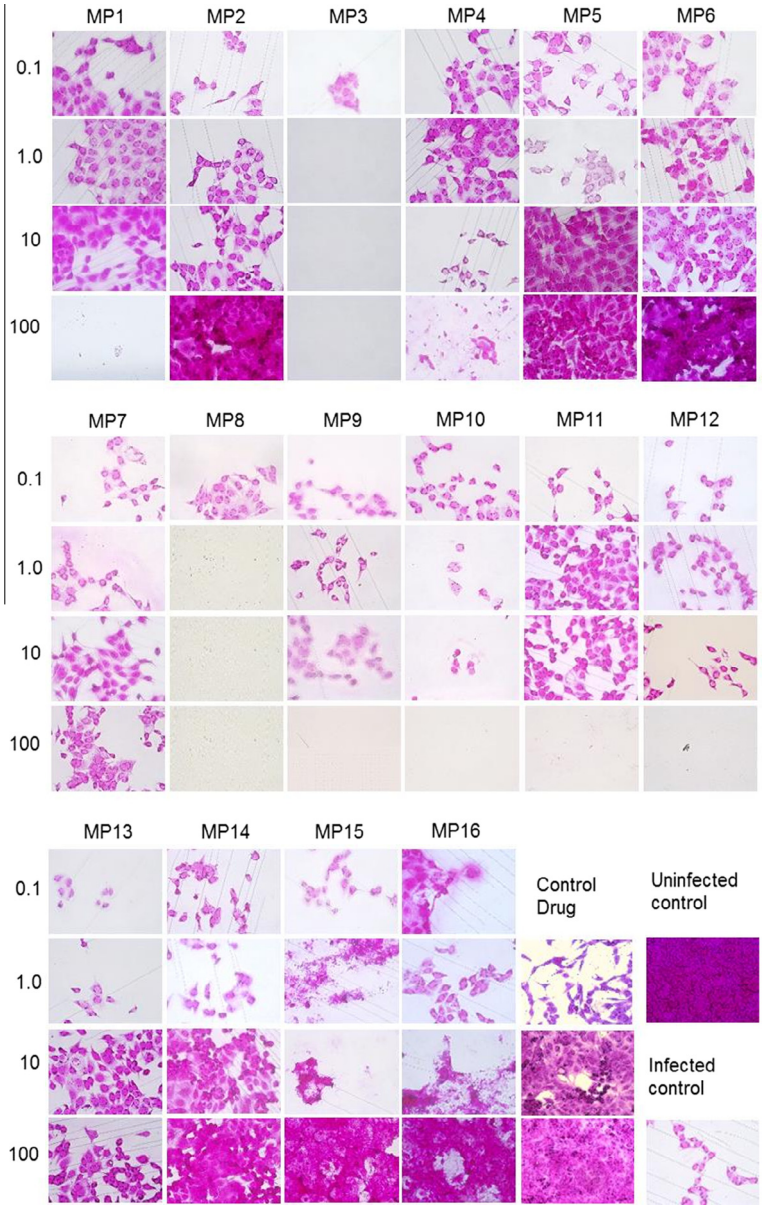

Figure 2 Anti-viral activity of selected medicinal plants of South India against H1N1 by SRB assay. MP-medicinal plants; 0.1–100 μg/ml; Control drug-Oseltamivir.

Table 1 Concentrations of the medicinal plant extracts used in the anti-viral assay and their toxicity against H1N1 virus.

Plant	IC_{50}	CC_{50}	TI
MP1	32.42 + 3.94	42.33	1.31
MP2	72.41 + 9.91	70.00	0.97
MP3	81.97 + 13.2	0.33	0.004
MP4	1145.86 + 78	42.41	0.037
MP5	46.69 + 13.7	1026.08	21.97
MP6	60.09 + 4.2	> 100	> 1.66
MP7	33.36 + 1.07	20.00	0.60
MP8	–	50	–
MP9	216.93 + 50.63	> 100	> 0.46
MP10	–	26.63	–
MP11	2.25 + 0.22	28.55	12.67
MP12	142.85 + 30.8	55.21	0.38
MP13	65.99 + 2.65	> 100	> 1.52
MP14	37.29 + 7.2	> 100	> 2.68
MP15	60.87 + 7.22	7.32	0.12
MP16	25.09 + 3.83	7.36	0.29
Control drug	6.44 + 0.32	> 100	> 15.51

Control drug- Oseltamivir; IC_{50}- Inhibitory concentration of 50%; CC_{50}- cytotoxicity concentration of 50%; TI- therapeutic index.

MP16 (7.37 μg/ml) and MP10 (26.6 μg/ml) had high cytotoxicity to the MDCK cells (Fig. 2, Table 1). In our previous study using MP3 extract showed anti viral activity against porcine reproductive and respiratory syndrome viruses (Maria John et al., 2014a). The plant MP10 was reported for its activity against flu by Coon and Ernst, (2004). But its cytotoxicity levels and its impact on normal cells are not properly reported.

3.3. Total phenolic and flavonoid content of the medicinal plants

Since the extracts showed variations in their antiviral activity, they were checked for their total phenolic and flavonoid content. The total phenolic content and flavonoid content of the selected medicinal plants are presented in Fig. 3. The phenolic content was high with MP15 (84.55 mg/g), MP16 (82.64 mg/g) followed by MP3 (63.59 mg/g) and MP4 (62.39 mg/g). In the meantime MP7 (28.69 mg/g) followed by MP1 (30.53 mg/g) registered lowest phenolic content (Fig. 3B). In the case of flavonoids it was observed that the content was high with MP9 (47.38 mg/g) followed by MP4 (46.33 mg/g). MP7 (11.88 mg/g) followed by MP2 (13.43 mg/g) registered least flavonoid content among the medicinal plants analysed (Fig. 3A).

3.4. Metabolite analysis by HPLC

In addition to these results, the individual phenolic and flavonoid content of the selected medicinal plants are profiled using HPLC against the available individual standards for identification and are presented in Fig. 4. A total of 10 metabolites were identified from the samples and all these 10 metabolites were not identified in all the plants. Result revealed that the flavonoid and phenolic acid content varied between the plants. The gallic acid and the naringin content were high with MP3 whereas caffeic acid, p-Coumaric acid and hesperidin content were high with MP4. o-Coumaric acid and vatic acid content were high with MP16. In the case of ferulic acid, rutin and quercetin were high with MP8.

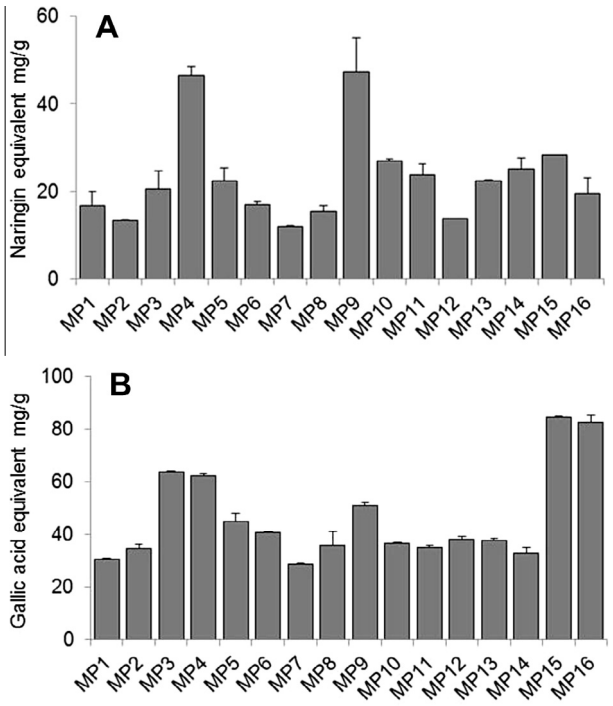

Figure 3 Total flavonoid and polyphenol content of the selected medicinal plants of South India. (A) Total flavonoid content and (B) Total polyphenol content.

Based on the result it was clear that the medicinal plant contains variations in their metabolic content and anti-viral activity. When comparing the phenolic and flavonoid content the plants possessing high flavonoid content did not show high anti-viral activity against H1N1 but the level of flavonoid ranging from 16.77 to 23.78 (mg/g) registered good antiviral activity. The medicinal plants such as MP3, MP4, MP15 and MP16 showed high total phenolic content and high cytotoxicity to the MDCK cells. In the case of MP9 even though the phenolic content was higher than MP11 its activity against virus was not good. According to Mennen et al. (2005) the high phenolic content was responsible for the cytotoxicity to the normal cells and the similar result was observed in this study. Because of high polyphenol induced pro-oxidation in the cells the concentration is important for finding the suitable medicinal plant source for anti-viral activity (Sakihama et al., 2002). Comparing the IC_{50} and TI values of MP11, it can be a potential source for new therapeutic developments even though its CC_{50} value was low when compared to the control drug. *A. paniculata* (MP10) was reported for respiratory problems and H1N1 flu preventive management in a slowdown of the cold infection time (Arora et al., 2010; Coon and Ernst, 2004). But in our study, it resulted in poor activity against H1N1 and this may be due to some toxic compounds which highly react with the cells and weaken them and hence their attachment to the walls was disturbed and because of that the IC_{50} values are not calculatable. When comparing with the control antiviral drug Oseltamivir, the plant MP11 showed activity with 2.25 μg/ml (IC_{50}) and hence this plant can be used for some active metabolite identification and new pharmaceutical preparations.

In conclusion, this study reveals various ethnic medicinal plant activities against H1N1 along with metabolic variations. *W. tinctoria* (2.25 μg/ml) is found to be more active against

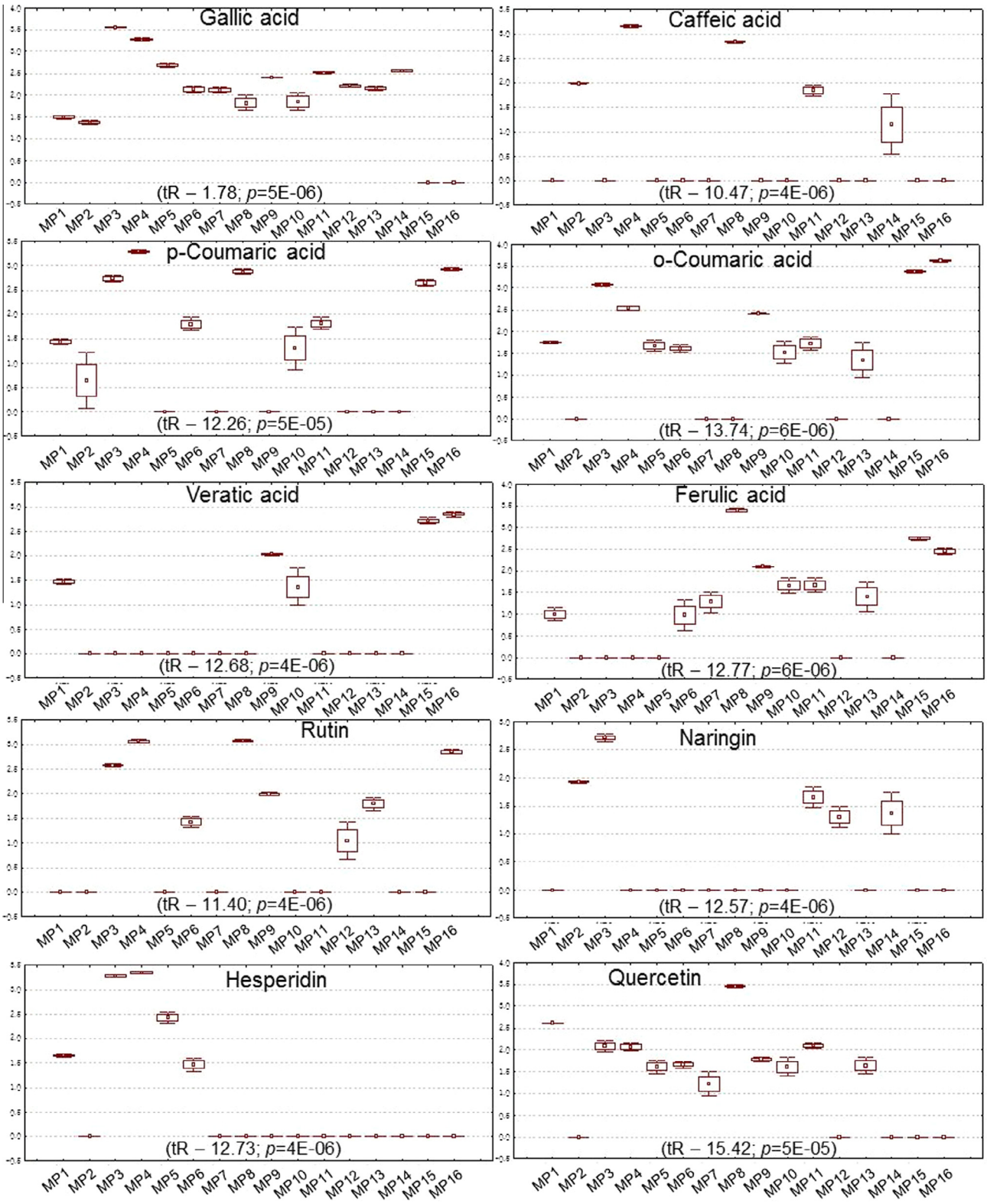

Figure 4 HPLC based metabolic profiling of the selected medicinal plants of South India. tR-retention time; $\log_{10}$ values of the peak area were plotted in *x*-axis; MP-medicinal plants.

H1N1 than the control drug Oseltamivir (6.44 μg/ml). Based on the IC_{50}, CC_{50} and TI of the ethnic medicinal plants of South India, it can be a rich source for the development of antidotes against H1N1.

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