



ORIGINAL ARTICLE

# Anti-influenza (H1N1) potential of leaf and stem bark extracts of selected medicinal plants of South India



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## KEYWORDS

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**Abstract** Variations in antioxidant and anti-viral activities (against Influenza AP/R/8 (H1N1) virus) between the leaves and stem bark of selected medicinal plants were studied. Malin Darby canine kidney (MDCK) cells were used for the viral infection and the antiviral activity of the extracts was studied using sulphorhodamine B (SRB) assay. The stem bark of the plants including *Strychnos minor*, *Diotacanthus albiflorus*, *Strychnos nux-vomica* and *Chloroxylon swietenia* showed higher flavonoid contents as well as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) scavenging activity when compared with their leaves. In case of 1,1-diphenyl-2-picrylhydrazyl (DPPH) activity, the stem bark of *S. nux-vomica* and leaf extract of *C. swietenia* showed the highest activity. Based on the IC<sub>50</sub> values, the stem bark extracts of *Cayratia pedata* (20.5 µg/mL) and *S. minor* (22.4 µg/mL) showed high antiviral activity. In the mean-time *S. nux-vomica*, *C. swietenia* and *C. swietenia* bark extracts showed cytotoxicity to the MDCK cells. When comparing the stem bark and leaves the content of gallic acid, ferulic acid, o-coumaric acid, total flavonoids (TFC) and total phenols (TPC) was higher in stem bark and hence their anti-viral activity was high. Further study based on the metabolites against H1N1 can reveal the potential of therapeutic compounds against the viral disease.

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## 1. Introduction

Plants are the major source of discoveries of novel therapeutic compounds because of their high secondary metabolite content. These metabolites can be classified into various groups such as flavonoid, phenolic, alkaloids etc. Based on the content and type the metabolites influence the antioxidant activity

against various diseases. Biosynthesis of secondary metabolites in plants is highly regulated by environmental conditions (Mariya et al., 2003). South India is one of the major resources for a plethora of diverse medicinal plants and is reported for traditional use against various diseases. In fact the origin and diversity of Siddha medicine which was followed and practiced by Siddhas was found to be evidence for the diversity of the medicinal plant in South India. Even today many people are using plant sources for their primary medical needs not only in South India but all over the world (Goleniowski et al., 2006; Gurib-Fakim, 2006). Because the valuable information on medicinal plants was passed on to children and mostly orally their medicinal properties have become scattered (Nadembega et al., 2011). From the little knowledge that we have about the uses of such plants for treating various diseases it will be possible to garner information on new therapeutic production which could be very useful in the long run.

Humans are prone to infection by various viral diseases and H1N1 is found to be an important problem. Its morbidity and mortality are rather high. These viral diseases spread all over the country and lead to severe health problems in children and the elderly. Even though vaccines are available for flu, their success rate on the elderly is less (Wang et al., 2006). These viruses are reported for their immunity against various drugs because of mutation (Stein et al., 2009). In this context, the discovery of newer medicines is required and hope rests on plants as the major source. Chinese and Japanese formulated herbal medicines such as Shahakusan, hochuekkito, Jinchai and Lianhuaqingwen capsules and reported for high anti-viral activity (Dan et al., 2013; Hokari et al., 2012; Zhong et al., 2013; Duan et al., 2011). Fifty medicinal plant extracts of rainforests like Borneo, Sarawak, and Malaysia, were studied and reported for H1N1 (Rajasekaran et al., 2013).

*Strychnos minor*, *Diotacanthus albiflorus*, *Strychnos nux-vomica*, *Chloroxylon swietenia*, and *Cayratia pedata* are some of the important medicinal plants of South India in which the leaves and stem bark were used against various diseases. Available literature on these traditional medicinal plants illustrates that different parts of *C. swietenia* are used for scorpion bites and the stem is used against cold and cough (Ratnam and Raju, 2008; Reddy, 2008). Roots and leaves were also reported for healing headache (Nilip and Gouri, 2013). In the case of *C. pedata* the anti-inflammatory activity with water and alcohol extracts was well studied (Rajendran et al., 2013). *D. albiflorus* showed anti-microbial activity with leaf extracts (Duraipandiyan et al., 2006). *S. minor* was used against poison bites by tribals of South India (Ayyanar, 2008). The genus *Strychnos* that was extensively reviewed for its activity against various diseases is discussed by Rajesh et al., 2011. The root bark of *S. nux-vomica* was reported for its anti-diarrhoeal potential by Shoba and Thomas (2001). The anti-inflammatory properties of the metabolites like strychnine, brucine, strychnine N-oxide isolated from the seeds were reported by Yin et al. (2003). Even though these medicinal plant parts like leaves and stem bark were studied for various diseases including cold, their activity against virus was not studied.

The main objective of the present study was to find the antiviral activity of the medicinal plants against H1N1 and to find the variations in activity among the leaves and stem bark. Findings of the results might be helpful for antiviral drug formulations.

## 2. Materials and methods

### 2.1. Chemicals

All the solvents used for the study were of HPLC grade and the chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). The cell culture medium and chemicals were purchased from Welgene, 150-Seongseo Industrial Complex Bukro, Dalseogu, Daegu, 704-948 Republic of Korea.

### 2.2. Plant materials

The leaves (L) and stem bark (s) of the medicinal plants such as *S. minor* (MP-L1, MPs1), *D. albiflorus* (MP-L2, MPs2), *S. nux-vomica* (MP-L3, MPs3), *C. swietenia* (MP-L4, MPs4), and *C. pedata* (MP-L5, MPs5) were separately collected from Tamil Nadu, South India and were shade dried. The plants were identified and confirmed for the genus and species by an ethno-botanist from Pachaiyappa's college, Chennai, Tamil Nadu, India during the collection of samples.

### 2.3. Extraction of medicinal plants

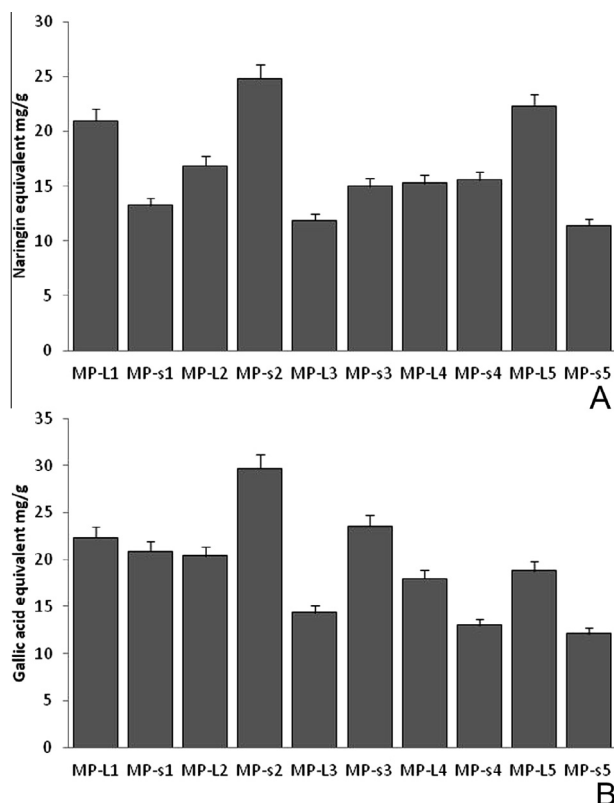
Shade dried samples (0.1 g) were separately weighed and extracted with 1 ml of methanol. After sonication for a period of 10 min, the samples were centrifuged at 8000 rpm for 10 min. The supernatant was collected and the extraction procedure was repeated. All the collected supernatant was pored together and evaporated under speed vac. The resulting pellet was redissolved with DMSO for cell culture studies and with methanol for the analysis of polyphenols, flavonoid content, antioxidant studies and for HPLC analysis.

### 2.4. Cell culture

Influenza AP/R/8 virus (H1N1) and Malin Darby canine kidney (MDCK) cells were purchased from American type culture collection (ATCC) and used for the present study. The MDCK cells were grown by using Dulbecco's modified eagle's medium (DMEM) added with 10% of foetal bovine serum (FBS) and 1% of antibiotic-antimycotic solution (100×). MDCK cells were maintained at 32 °C with 5% of CO<sub>2</sub> in a relative humidified cell culture incubator.

### 2.5. Antiviral assay

In a 96 well plate the MDCK cells ( $2 \times 10^4$ ) were seeded and allowed to grow for a period of 24 h. After that the cells were washed twice with phosphate buffered saline (PBS) and the influenza AP/R/8 virus (diluted as  $5 \times 10^3$  with DMEM medium contained trypsin-EDTA) was introduced for the infection. Virus solution (90 µL) and medicinal plant extracts (10 µL) of different dilutions (0.1, 1, 10 and 100 µL) were placed onto the 96 well plates with three replicates. These plates are incubated for a period of 48 h under CO<sub>2</sub> incubator. After incubation (48 h) the medium was removed and washed twice with PBS before fixing the cells. The cells were fixed by following a sequence of steps such as incubating the cells with 70% of acetone for 1 h at -4 °C followed by removing the solvent and dried the cells at 60 °C under hot air oven.



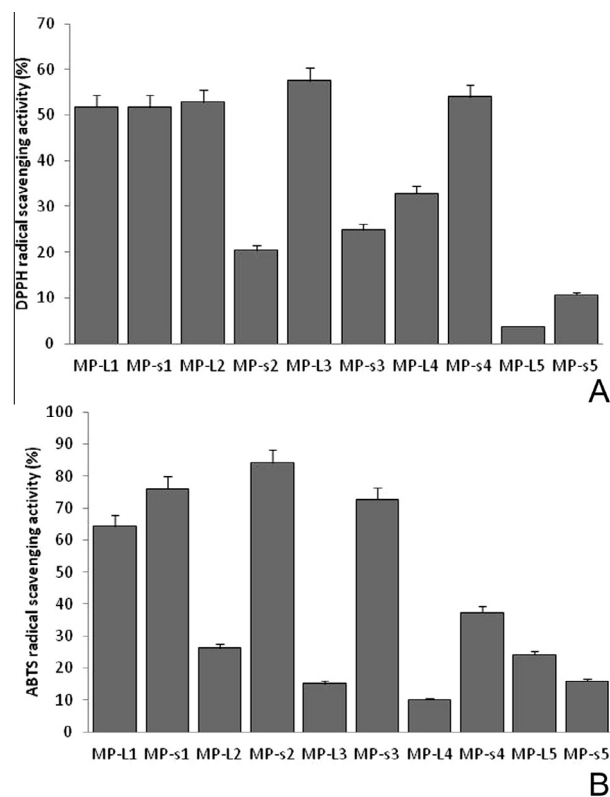
**Figure 1** Comparative analysis between leaves and stem bark extracts of selected medicinal plants for their total flavonoid (A) and total phenolic (B) content.

#### 2.6. SRB assay

The SRB assay was performed by adding 100  $\mu$ L of SRB (0.4 mg/L) reagent to the dried 96 wells and incubated overnight. After incubation the SRB reagent was decanted and washed thrice with 1% of acetic acid. The plates were dried at 60  $^{\circ}$ C and the cell morphology was observed under microscope (reflected light microscope) at 40 $\times$  magnification. The images were taken and compared for the antiviral activity. The 96 well plates containing the cells were treated with 10 mM of Tris base and incubated overnight. SRB strains in the cells were completely dissolved in the buffer and were read under a 96 well plate reader at 510 nm to calculate the inhibition concentration of 50% ( $IC_{50}$ ), cytotoxic concentration of 50% ( $CC_{50}$ ) and therapeutic index (TI).

#### 2.7. Total flavonoid content (TFC)

The total TFC content of the plant samples was measured by adding 180  $\mu$ L of 90% diethylene glycol and 20  $\mu$ L of 1 N NaOH in a 96 well plate containing 20  $\mu$ L of methanol extract. The optical density of the samples was measured at 515 nm using a micro plate reader (Spectra max plus384, Molecular devices, USA) after 15 min of incubation. Calculations were made based on the naringin concentration and are expressed in mg/g of sample.



**Figure 2** Comparative analysis between leaves and stem bark extracts of selected medicinal plants for their anti-oxidant activity against DPPH (A) and ABTS (B).

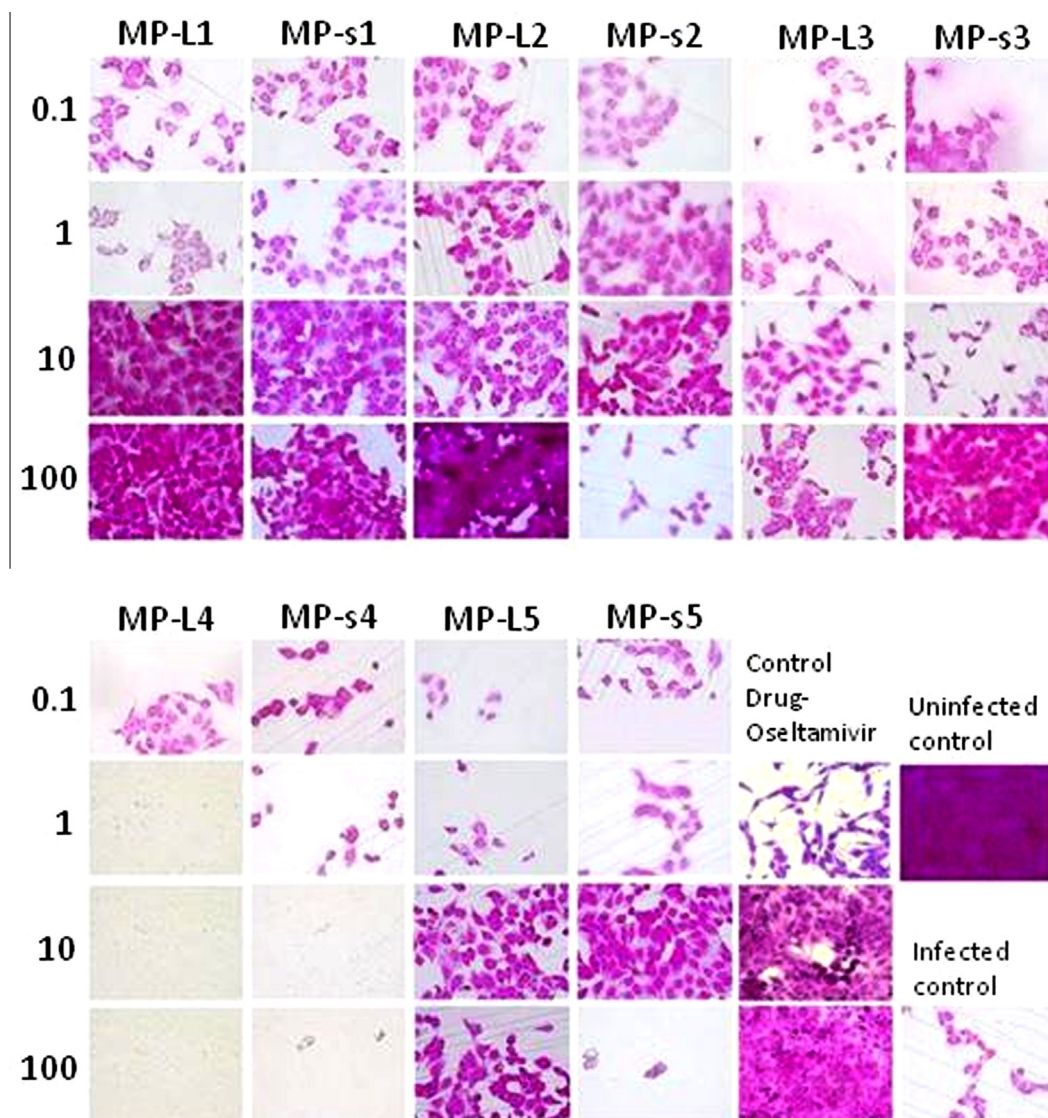
#### 2.8. Total polyphenol content (TPC)

The total TPC content of the plant samples was measured by adding 100  $\mu$ L of 0.2 N Folin-Ciocalteu's phenol reagent and

**Table 1** Cytotoxicity of the medicinal plant extracts against H1N1 virus.

S. No.	Name	Toxicity of the extracts		
		$IC_{50}$	$CC_{50}$	TI
1	MP-L1	46.69	1026.07	21.97
2	MP-s1	22.43	100	4.45
3	MP-L2	60.09	100	1.66
4	MP-s2	33.98	50	1.47
5	MP-L3	33.36	20	0.59
6	MP-s3	23.60	40	0.59
7	MP-L4	ND	50	ND
8	MP-s4	ND	3.95	ND
9	MP-L5	65.99	100	0.60
10	MP-s5	20.50	18.30	0.89
11	Oseltamivir	6.44	100	15.51

Control drug – Oseltamivir;  $IC_{50}$  – inhibitory concentration of 50%;  $CC_{50}$  – cytotoxicity concentration of 50%; TI – therapeutic index.



**Figure 3** Comparative analysis between leaves and stem bark extracts of selected medicinal plants for their anti-viral activity against H1N1 by SRB assay. MP – medicinal plants; control drug – Oseltamivir.

80  $\mu$ L of saturated sodium carbonate in a 96 well plate containing 20  $\mu$ L of methanol extract. The optical density of the samples was measured after 1 h of incubation at 750 nm. The calculations were made based on the values obtained for gallic acid at different concentrations and expressed in mg/g of sample.

### 2.9. Free radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity of the samples was measured by following the procedures of Maria John et al. (2014, 2015). 20  $\mu$ L of methanol extract was mixed with 180  $\mu$ L of DPPH (0.5 mM) and ABTS reagents were incubated for 20 min and 7 min. The absorbance was measured at 515 nm and 750 nm for the respective analysis using a micro plate reader.

### 2.10. Secondary metabolite analysis by HPLC

The methanol extracts were analysed by HPLC (Agilent 1100, USA) to find the metabolic variations. Water and acetonitrile containing 0.1% formic acid served as mobile phases A and B with the flow rate of 1 ml/min. The samples were analysed using  $C_{18}$  column with a diode array detector (DAD) at 254 nm. Total run time of the sample was 40 min and based on the individual standards retention time (tR) the metabolite identification was made.

### 2.11. Data analysis

Data analysis was performed using different softwares such as Statistica 7 for metabolite comparison, heat map for metabolite correlation studies and SPSS for statistical analysis of the biochemical analysis. For the individual metabolite analysis,

$\log_{10}$  values were used for their comparison between the samples.

### 3. Results and discussion

#### 3.1. Total phenolic and flavonoid contents of the medicinal plants

Total flavonoid and polyphenol contents of the medicinal plant extracts were compared and are presented in Fig. 1. Results revealed that the flavonoid content was the highest for MP-s2 (24.82 mg/g) followed by MP-L5 (22.30 mg/g). MP-s5 (11.36 mg/g) followed by MP-L3 (11.88 mg/g) had the lowest flavonoid content among the samples analysed. Among the five plants tested, the stem bark showed a high flavonoid content with MP-s2, MP-s3 and MP-s4 when compared to their leaves (MP-L2, MP-L3 and MP-L4). In the case of total polyphenol content, MP-s2 registered a high (29.73 mg/g) phenolic content followed by MP-L1. MP-s5 and MP-s4 registered the lowest content in terms of polyphenols. Here the stem bark of MP-s2 and s3 showed a high phenolic content than that of their leaves (Fig. 1B).

#### 3.2. Free radical scavenging potential of the selected medicinal plants

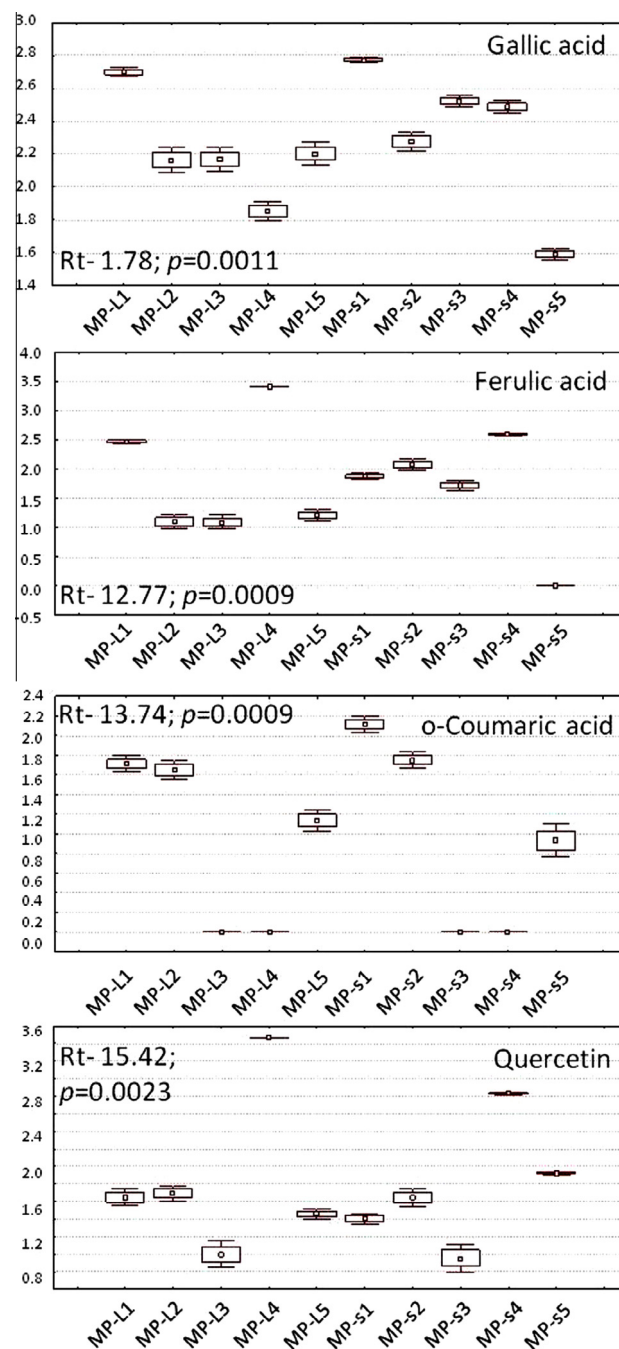
The leaves and stem bark of the selected medicinal plants were compared for their antioxidant potential using DPPH and ABTS assay (Fig. 2). The plants and their parts such as leaves and bark showed variation in terms of DPPH and ABTS activities. MP-L3 (57.51%) followed by MP-s4 (53.93%) resulted in high DPPH scavenging potential whereas MP-L5 followed by MP-s5 had the lowest activity. In the case of MP-L1, MP-s1 and MP-L2 possess the same activity in terms of statistical analysis (Fig. 2A). In terms of ABTS activity, the stem bark extract of the medicinal plants MP-s1, MP-s2, MP-s3 and MP-s4 showed high antioxidant activity. Among all the samples analysed MP-s2 (84.17%) followed by MP-s1 (76.05%) registered high scavenging potential. Here also MP-L5 and MP-s5 registered lowest scavenging activity (Fig. 2B).

#### 3.3. Antiviral activity against H1N1

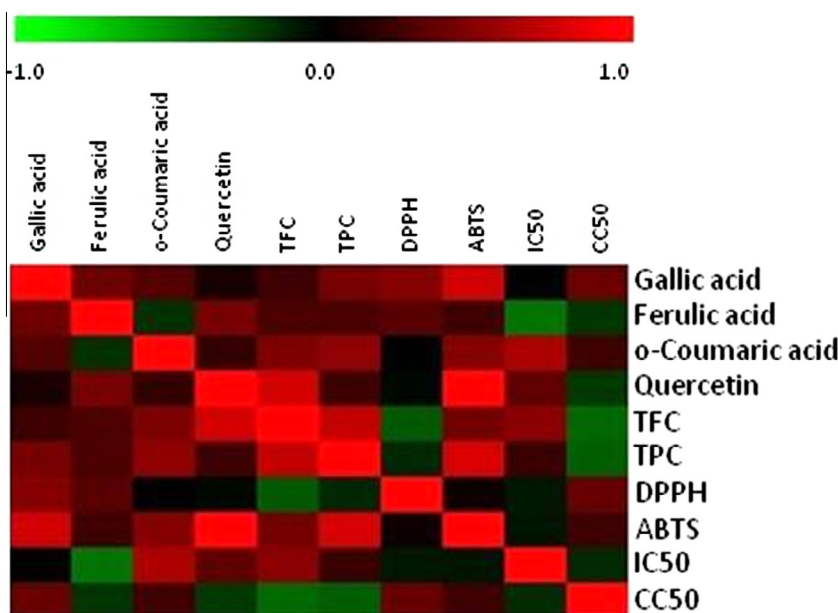
For anti-viral activity assay, the extract of 0.1, 1, 10 and 100  $\mu\text{L}$  was used (Table 1). Results of the cells treated with different concentrations of medicinal plant extracts and the standard drug (Oseltamivir) are presented in Fig. 3. The leaves and stem bark showed variations in their antiviral activity. Based on the  $\text{IC}_{50}$  and  $\text{CC}_{50}$  values the antiviral activities of the extracts were compared. MP-s5 and MP-s1 showed high antiviral activity in terms of  $\text{IC}_{50}$  values (20.5  $\mu\text{g}/\text{mL}$  and 22.4  $\mu\text{g}/\text{mL}$ ) whereas the  $\text{CC}_{50}$  values of the samples are 18.3  $\mu\text{g}/\text{mL}$  and  $>100 \mu\text{g}/\text{mL}$ . These two samples are stem bark and showed high activity against H1N1 virus. MP-s3, MP-L3, MP-s2, MP-L1, MP-L2 and MP-L5 also showed good antiviral activity ( $\text{IC}_{50}$  value of 23.60  $\mu\text{g}/\text{mL}$ , 33.3  $\mu\text{g}/\text{mL}$ , 33.9  $\mu\text{g}/\text{mL}$ , 46.7  $\mu\text{g}/\text{mL}$ , 60.1  $\mu\text{g}/\text{mL}$  and 65.2  $\mu\text{g}/\text{mL}$ ). These samples showed  $\text{CC}_{50}$  value of 20  $\mu\text{g}/\text{mL}$ , 50  $\mu\text{g}/\text{mL}$ , 1026  $\mu\text{g}/\text{mL}$ ,  $>100 \mu\text{g}/\text{mL}$  and  $>100 \mu\text{g}/\text{mL}$ , respectively. We observed that MP-L4 and MP-s4 did not show antiviral activity because their cytotoxicity level was high with the MDCK cells being observed (Table 1).

#### 3.4. Metabolite analysis by HPLC

The extracts were analysed for their metabolic variations and compared with their antioxidant and antiviral activities. The metabolites such as gallic acid, o-coumaric acid, ferulic acid and quercetin contents were predominantly changed among the samples. When comparing the plant extracts gallic acid and o-coumaric acid levels are high in MP-s1 followed by MP-L1 being observed. Whereas ferulic acid and quercetin contents were high with MP-L4 followed by MP-s4 (Fig. 4).



**Figure 4** Metabolic variations of leaves and stem bark of medicinal plants analysed by HPLC. tR – retention time;  $\log_{10}$  values of the peak area was plotted in the x-axis; MP – medicinal plants.



**Figure 5** Correlation study between metabolites and cytotoxicity against H1N1.

The metabolic correlation with antioxidant and antimicrobial activities is presented in Fig. 5. Results revealed that gallic acid, ferulic acid, o-coumaric acid, and TFC and TPC contents were positively correlated with antioxidants and antiviral activity ( $IC_{50}$ ). Based on the results it was clear that the antiviral activity was not only dependent on their total phenolic or flavonoid content but also their individual metabolic levels.

The phenolic compounds and flavonoids serve as potent antioxidant source (Mariya John et al., 2009) and used against various human diseases. The flavonoids are reported for antiviral activity including HIV, hepatitis B and influenza (Jassim and Najji, 2003; Li et al., 2008; Liu et al., 2008; Mori et al., 2008). The medicinal plants particularly the bark of the plants showed high phenolic and flavonoid contents and resulted in their high activity against ABTS. MP-s4 extracts reported against cold and influenza infection also show cold as common symptom (Ratnam and Raju, 2008), but the leaves and stem extracts showed cytotoxicity to the MDCK cells. When comparing the leaves and stem bark of the medicinal plants, the stem bark of MP-s2, MP-s3 and MP-s4 showed high flavonoid contents and hence these stem barks were used traditionally against various diseases. Even though the phenolic content level of MP-5 (*C. pedata*) was low, their antiviral activity against H1N1 was high. This plant was traditionally used for anti-inflammatory activity as their activity against virus was high (Rajendran et al., 2013; Maria John et al., 2014, 2015).

Based on the studies of the phenolic and flavonoid contents, it was clear that the high phenolic content resulted in cell toxicity in spite of their high antioxidant activity. The metabolic correlation with antiviral activity suggests that coumaric acid, quercetin, TFC and TPC show positive correlation whereas ferulic acid shows negative correlation. Previous reports of quercetin resulted in inhibitory properties of several viruses and the present study also confirms the activity by positive correlation (Mucsi and Pragai, 1985). As stated by Hwa Jung Choi et al. (2009), the quercetin 3-rhamnoside was strongly active against influenza virus (Choi et al., 2009). Quercetin, TFC and TPC were positively correlated with  $IC_{50}$  value

suggesting the possibility of those plant extracts against H1N1 in the mean time these metabolites showed negative correlation with  $CC_{50}$ . Coumaric acid showed positive correlation with  $IC_{50}$  as well as  $CC_{50}$ , hence this phenolic acid contains antiviral activity along with cytotoxicity to the MDCK cells. This may be the reason for the cytotoxicity of the cells by high phenolic content containing plants. According to the above results the stem bark with high flavonoid contents showed low cytotoxicity with high antiviral activity. MP-s5, MP-s1, MP-s3, MP-L3, MP-s2, MP-L1, MP-L2 and MP-L5 showed good antiviral activity and can be an alternative source for the development of new therapeutic compounds.

#### 4. Conclusions

Based on the above it was clear that the extracts from stem bark and leaves showed variations in their metabolic contents resulting in changes in antiviral activity against H1N1. The metabolite correlation study revealed that the flavonoids directly correlated with anti-viral activity but high phenolic content resulted with cytotoxicity to the MDCK cells. The metabolic variations and antiviral activity of the plant extracts suggest that it can be used as a source for new therapeutic compound development against H1N1 since this viral disease was spread worldwide.

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