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Quantification of phenolic content from stem-bark and root of *Hugonia mystax* Linn. using RP-HPLC

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KEYWORDS

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Total flavonoid content

Abstract *Hugonia mystax* Linn. a woody evergreen plant locally known as *Modirakanni* has been used in primary health care by tribals from Tiruvannamalai hills, Tamil Nadu, India. Ethnobotanically, the plant parts are used for rheumatism, skin diseases and inflammation. However, there is no data on active phytoconstituents in stem-bark and root mainly contributing to biological activities. In the present study an attempt has been made to quantify plant phenolics from aqueous, ethanolic and methanolic extracts of stem-bark and root of *H. mystax*. The extracts were also evaluated for their free radical scavenging potential. Quantitative determination of total phenolic content, total flavonoid content and DPPH free radical scavenging activity of plant extracts were carried out using colorimetric methods. Quantitative determination of individual phenolic compounds such as gallic acid, catechol, caffeic acid, vanillin, *p*-coumaric acid and ferulic acid in stem-bark and roots extracts were analyzed using RP-HPLC. The highest phenolic content was found in ethanol extract of root (262.2 ± 0.96 μg of gallic acid equivalent (GAE)/mg of dry plant material), whereas, the highest amount of flavonoids content was found in aqueous extract of root (18.06 ± 1.25 μg of quercetin equivalent (QE)/mg of dry plant material). Q. The highest amount of phenolic acid present was *p*-coumaric acid (3.775 mg/g of dry plant material) in methanol extract of stem-bark. All the solvent extracts of stem-bark and root have shown the presence of *p*-coumaric acid. Methanol extracts of stem-bark and root with IC_{50} values of 175.48 ± 2.14 μg/ml and 169.15 ± 1.10 μg/ml respectively, show potent free radical scavenging activity. In conclusion it can be said that, the plant is rich in phenolics and the major component *p*-coumaric acid may

Abbreviations: RP-HPLC reverse phase, high performance liquid chromatography; TPC, total phenolic content; TFC, total flavonoid content

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probably be responsible for the traditional claims of its biological activities. However, the mechanism of action of the active plant extracts needs to be investigated at the molecular level.

1. Introduction

According to the World Bank report, 80% of the South Asian population still uses traditional plant-based medicines to maintain and improve their health. The World Health Organization listed 21,000 plants used in traditional medicine around the world (WHO, 2002), whereas Schippmann and co-workers estimated this number to be 52,885 (Schippmann et al., 2002). Although there are conflicting claims on the number of plants, it is well established that medicinal plants are routinely used for primary health care in many parts of the world (Pan et al., 2014).

Plants have been integral part of herbal healing processes, which is deep rooted in the systematic classical system of Ayurveda, Siddha, and Unani, as an official health care system (Balakumar et al., 2011; Mohamed Saleem et al., 2011; Pour and Sasidharan, 2011). Folk medicine or oral tradition of health care, which exists among most tribal and rural communities also use medicinal plant knowledge of traditional healers, which is widely recognized as a valid alternative system of medicine (Al-Daihan et al., 2013; Alabri et al., 2014). In developing countries the efforts to recognize and promote the uncodified folk system of medicinal knowledge are still inadequate (Shukla and Gardner, 2006). Systematic scientific investigations are required to prove the traditional claims of effectiveness of these plants and the rationale behind their biological activities needs to be established.

Hugonia mystax Linn. a woody evergreen species, belongs to Linaceae family, which comprise about 40 species in the world; of which *H. mystax* L. was reported from India (Santapau and Henry, 1983; Pullaiah and Chennaiah, 1997). This plant locally known as Modirakanni plays a vital role in the primary health care of tribals from Tiruvannamalai hills, Tamil Nadu, India. The plant parts such as leaves, fruits, bark and roots are extensively used by traditional healers for the treatment of various ailments. Ethnobotanically, leaves and fruits have been used as an antihelmintics and for rheumatism (Sutha et al., 2009; Padel et al., 2010). Roots were used as antihelmintic and also used for dysentery, snake bite, fever, inflammation and rheumatism. Biological activities such as analgesic, anti-inflammatory and ulcerogenic were also reported (Balasubramaniam et al., 1997; Guha Bakshi et al., 2001; Rastogi et al., 2002). The decoction of bark in combination with *Curcuma aromatica*, is given with honey for inflammation in the stomach, vomiting, stomach pain, indigestion (Pushpangadan and Atal, 1984). Roots of *H. mystax* were evaluated for preliminary phytochemical screening and antimicrobial activity. Preliminary phytochemical screening showed the presence of various classes of secondary metabolites such as flavonoids, phenols, saponins, steroids, tannins and terpenoids. Antimicrobial activity of petroleum ether, chloroform, ethanol and aqueous extracts of root showed significant activity against various human pathogens (Vimalavady et al., 2012). This species was reported by Dalzell and Gibson in Bombay Flora in 1861. The species from this geographical location is yet to be explored for its phytochemical constituents and biological activity.

Considering the medicinal importance of the plant and previous findings in our laboratory, an attempt has been made to quantify plant phenolics from stem-bark and roots of the *H. mystax*, extracted with solvents of different polarity.

2. Materials and methods

2.1. Reagents and chemicals

Gallic acid, quercetin, cathechol, caffeic acid, vanillin, *p*-coumaric acid, ferulic acid and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma Chemical Co. (USA). HPLC grade ethanol (EtOH), methanol (MeOH), acetic acid, Folin Ciocalteu's Phenol reagent, sodium carbonate and aluminum chloride were purchased from Merck (Germany).

2.2. Collection of plant material

The stem-bark and root of *H. mystax* (Fig. 1) were collected from Mochemad–Vengurla, Sindhudurg district, Maharashtra. The geographical location of the collection area was 15° 48. 040′ N, 073° 39. 283′ E. The plant specimen was authenticated from the Blatter Herbarium (BLAT), St. Xavier's College, Mumbai. The submitted plant specimen matches with Blatter Herbarium specimen No. 22319 of H. Santapau.

2.3. Preparation of plant extracts

The air-dried stem-bark and roots of *H. mystax* were made into fine powder. 5 g of powder of each plant part were suspended in 50 ml of three different extracting solvent systems

Figure 1 *Hugonia mystax* plant used for the study.

like distilled water, methanol and ethanol respectively for overnight extraction. Extracts were filtered using Whatman No. 1 paper and extracts were concentrated *in vacuo* using rotary vacuum evaporator. Percentage extractive value (*w*/*w*) of each extract was calculated. For HPLC analysis, stock solutions of extracts were filtered through the syringe filter 0.45 µm pore size (Millipore).

2.4. Determination of total phenolic (TPC) and total flavonoid content (TFP) content

2.4.1. Total phenolic content (TPC)

The total phenolic content (TPC) in *H. mystax* crude extracts was determined by using the Folin–Ciocalteu method (Chang et al., 2002). Standard solutions of gallic acid of concentration 1.56–100 µg/ml were prepared in water. 50 µl of extract (1 mg/ml) or standard solution were added to 50 µl of distilled water. 50 µl of 10% Follin–Cicocalteu's (F–C) phenol reagent and 50 µl of 1 M sodium carbonate solution were added to the mixture in a 96-well plate. Distilled water was used as blank. Reactions were incubated for 60 min at room temperature and protected from light. The absorbance was measured at 750 nm with a Microplate Reader (Biotek, USA.). Total phenolic contents were expressed as µg Gallic Acid Equivalents (GAE) per mg of dry plant material.

2.4.2. Total flavonoid content

Total flavonoid content (TFC) was determined by the aluminum chloride colorimetric assay (Chatatikun and Chiabchalard, 2013). Standard solutions of quercetin of concentration 1.56–100 µg/ml were prepared in 80% ethanol. 50 µl of extracts (1 mg/ml) or standard solution was added to 10 µl of 10% the aluminum chloride solution and followed by 150 µl of 95% ethanol. 10 µl of 1 M sodium acetate was added to the mixture in a 96 well plate. 80% ethanol was used as reagent blank. All reagents were mixed and incubated for 40 min at room temperature protected from light. The absorbance was measured at 415 nm with a Microplate Reader (Biotek, USA.). Total flavonoid contents were expressed as µg Quercetin Equivalents (QE) per mg dry of plant material.

2.5. Antioxidant assay

2.5.1. Free radical scavenging activity assay

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity was performed by the reported method (Yamasaki et al., 1994). 20 µl of extracts in respective solvents was added to 180 µl of DPPH reagent prepared in methanol in a 96 well plate. Absolute methanol was used as reagent blank. Reaction mixture was incubated for 30 min at room temperature, protected from light. The absorbance was measured at 517 nm with a Microplate Reader (Biotek, USA.). The percentages of the DPPH free radical scavenging activity were calculated as follows,

$$\%\,\text{Scavenging} = [\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{control}}] \times 100$$

2.6. Quantitative analysis of phenolic acids using RP-HPLC

Quantitative analysis of individual phenolic compounds present in the different parts of plant extracts was performed on a Waters HPLC system (Model 2487), using Zorbax Eclipse ODS C18 reverse phase column (150 mm × 4.6 mm ID, 5 µm) (Agilent, Germany), 1% acetic acid in water: methanol (80:20) (*v*/*v*) used as mobile phase with 1 min/ml flow rate, UV detector set at 280 nm. Phenolic acids from each sample were identified by comparing their relative retention time with the standards of mixture chromatogram. All the chemicals and solvents used for analysis were of HPLC grade.

2.7. Statistical analysis

All the observations were taken in triplicate and the data were expressed in Mean ± Standard Error of Measurement (S.E.M). The data were analyzed using Analysis of Variance technique (ANOVA) ($p < 0.05$) and the means were separated by Bonferroni's multiple comparison tests using software GraphPad Prism 5.

3. Results and discussion

3.1. Total phenolic content, flavonoid content

Aqueous (5.56%) and methanol (4.98%) had higher extractive values for extracts of stem-bark and root respectively. Though ethanol (3.02%) was recorded for lower extractive value, it was found to be the best solvent system for the extraction of phenolic acids. Ethanol extracts of plant parts have shown highest phenolic content in comparison to methanol and distilled water (Table 1). The total phenolic content in *H. mystax* was determined by using Folin–Ciocalteu reagent. The range of phenolic acid content was from 232.5 ± 0.44 to 262.2 ± 0.96 µg of GAE/mg of dry plant material. The highest phenolic acid content was found in ethanol extract of root viz. 262.2 ± 0.96 µg of GAE/mg of dry plant material. The total flavonoid content in *H. mystax* was determined using aluminum chloride method. The range of flavonoid content was from 1.753 ± 0.71 to 18.06 ± 1.25 µg of QE/mg of dry plant material. The highest amount of flavonoid content was found in aqueous extract of root viz. 18.06 ± 1.25 µg of QE/mg of dry plant material (Table 1). Phenolic compounds are important and widely distributed secondary metabolites of plant kingdom. Plant phenolics are mainly classified into phenolic acids and flavonoids. These compounds are involved in growth and reproduction of the plant, and also play an important role in defense against ultraviolet radiation and pathogens (Manach et al., 2004; Farah and Donangelo, 2006; Elzaawely et al., 2007; Piazzon et al., 2012). Therefore, quantification of phenolic compounds has paramount importance. There have been several studies that showed F–C reagent reacts with other phytoconstituents beside phenol (Prior et al., 2005; Ikawa et al., 2003; Everette et al., 2010), which could possibly show a high amount of phenolic content. Considering the limitations of the F–C assay (Amorati and Valgimigli, 2015), there are continuous efforts from the scientific community to resolve this problem by suggesting the

Table 1 The concentration of total phenolic content (TPC) and total flavonoid content (TFC) in stem-bark and root of *Hugonia mystax*, using gallic acid and quercetin as common reference compounds respectively.

Extracts	Solvent system	% Extractive value (w/w)	TPC[a]	TFC[b]
Stem-bark	D/W	5.56	232.5 ± 0.44	9.703 ± 1.79
	Methanol	4.68	244.3 ± 5.74	17.98 ± 0.51
	Ethanol	2.86	245.1 ± 2.09	1.753 ± 0.72
Root	D/W	4.2	250.1 ± 3.32	18.06 ± 1.25
	Methanol	4.98	257.5 ± 1.37	14.19 ± 1.61
	Ethanol	3.02	262.2 ± 0.96	ND

Values represent mean ± SEM (n = 3).
ND – not detected.
[a] Total phenolic content expressed as µg of GAE/mg of dry plant material.
[b] Total flavonoid content expressed as µg of QE/mg of dry plant material.

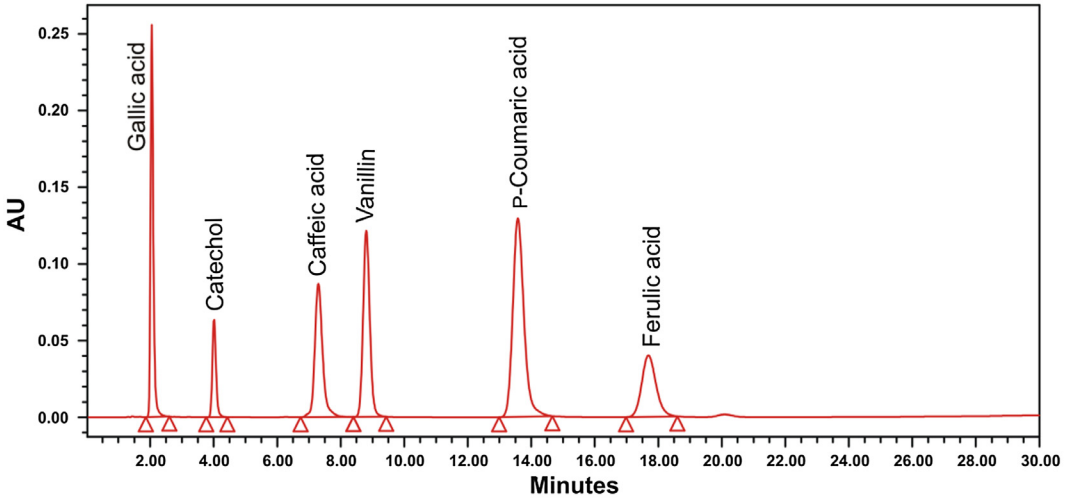

Figure 2 RP-HPLC chromatogram of standard phenolic compounds (20 ppm each): HPLC, Zorbax Eclipse ODS RP-C18 column ((150 mm × 4.6 mm ID, 5 µm), 1% (v/v) acetic acid in water:methanol (80:20) (v/v), 1 ml/min, absorbance at 280 nm (n = 2).

different approaches to improve the specificity of F–C assay (Sanchez-Rangel et al., 2013; Berker et al., 2013).

3.2. RP-HPLC analysis of individual phenolic acids and DPPH assay

Individual plant phenolics such as gallic acid, catechol, caffeic acid, vanillin, *p*-coumaric acid and ferulic acid in stem-bark and root of *H. mystax* were analyzed and quantified using RP-HPLC (Fig. 2). From the analysis it was observed that the plant parts showed the presence of all phenolic compounds except Ferulic acid. The phenolic compounds present in plant parts are, gallic acid (0.024–1.19 mg/g of dry plant material), catechol (0.067–0.292 mg/g of dry plant material), caffeic acid (0.047–0.401 mg/g of dry plant material), vanillin (0.037–0.117 mg/g of dry plant material) and *p*-coumaric acid (0.067–3.775 mg/g of dry plant material (Fig. 3). *p*-Coumaric acid was found to be the most predominant phytoconstituent present in both the plant parts selected for the study. The highest amount of phenolic acid present was *p*-coumaric acid (3.775 mg/g of dry plant material) in methanol extract of stem-bark (Fig. 4). Methanol extracts of stem-bark and root of *H. mystax* have shown potent free radical scavenging activity (Fig. 5) with the IC_{50} values, 175.48 ± 2.14 and 169.15 ± 1.10 µg/ml respectively.

Biological processes generate reactive oxygen species (ROS) as a byproduct of various metabolic processes. These free radicals are highly unstable and damage various biomolecules such as proteins, lipids and nucleic acid. ROS has been implicated in the pathogenesis of several diseases including cardiovascular diseases, cancer and degenerative diseases (Saibabu et al., 2015; Cherubini et al., 2005; Nimse and Pal, 2015). The association between chronic inflammation and rheumatoid arthritis (RA) has already been well defined. In RA patients, level of antioxidant enzymes in serum and synovial fluid is found to be less compared to healthy individual; on the other side, the non-stop generation of free radicals in inflamed joints leads to the failure of endogenous antioxidant system (Kundu et al., 2011; Hold and El-Omar, 2008; Pattison and Winyard, 2008). Phenolic acids, a major class of phytochemicals have been attributed to act as antioxidants with various physiological actions in the living system. The experimental evidences revealed that the phenolic acids can

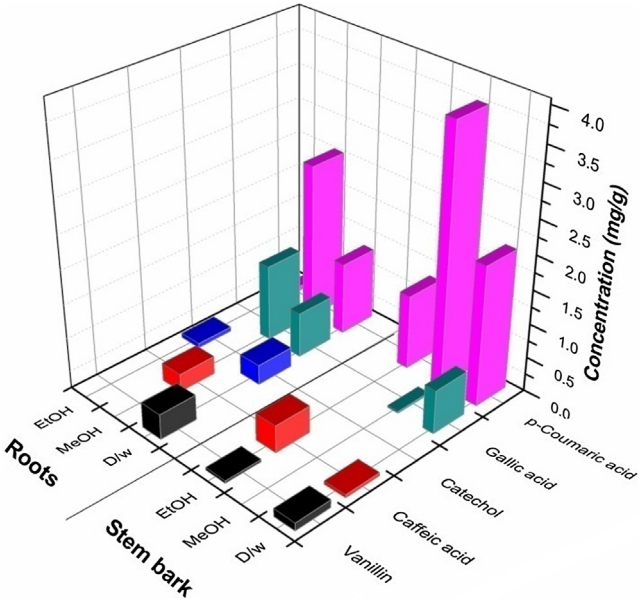

Figure 3 Quantification of individual phenolic compounds in plant parts of *Hugonia mystax* using HPLC (mg/g of dry plant material).

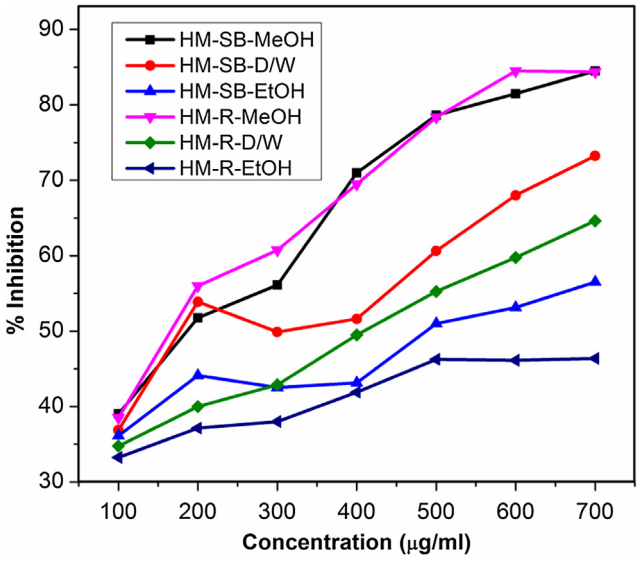

Figure 5 Free radical scavenging potential of various extracts of *H. mystax* plant parts ($n = 3$).

be utilized for preventive and therapeutic purposes in oxidative stress related diseases (Fresco et al., 2006; Razzaghi-Asl et al., 2013). Therefore it is highly imperative to study the free radical scavenging potential of herbal extracts in correlation with their biological activity. DPPH is a purple colored nitrogen centered stable free radical with an absorbance at 517 nm. Reduction of DPPH· in the presence of antioxidants produces corresponding yellow colored hydrazine DPPH-H (Cai et al., 2003; Tirumani et al., 2016). The free radical scavenging potential of phenolic acids is well known and widely studied, but they also exhibit antioxidant activity by modulating the endogenous enzymes (Roy et al., 2013). Phenolic acids such as caffeic acid, gallic acid, *p*-coumaric acids and ferulic acid showed a remarkable antioxidant and antimicrobial activity (Badhani et al., 2015). Among various hydroxycinnamic acids, caffeic acid is found to be the most potent antioxidant compared to *p*-coumaric

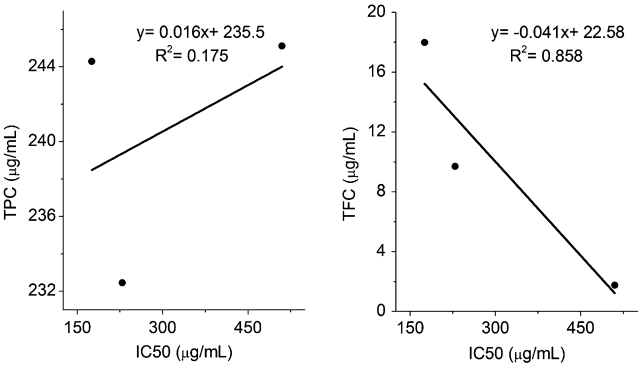

Figure 6 Correlation of total phenolic content and total flavonoid content with IC_{50} of DPPH free radical scavenging in stem-bark.

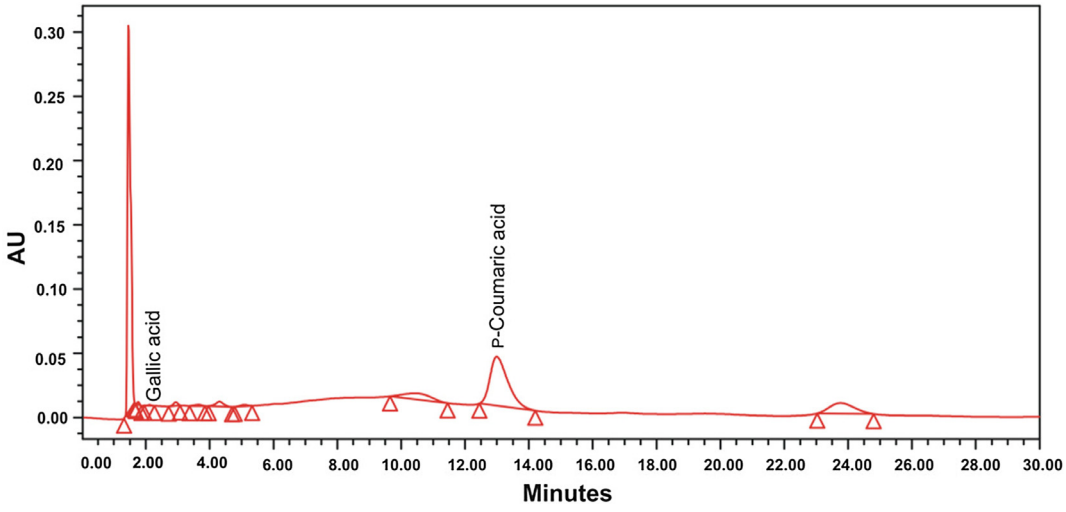

Figure 4 RP-HPLC chromatogram of methanolic extract of *H. mystax* stem-bark.

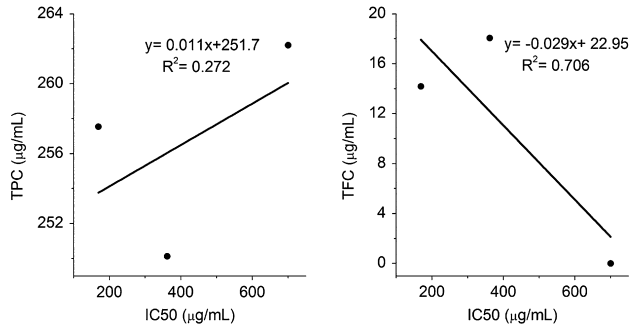

Figure 7 Correlation of total phenolic content and total flavonoid content with IC_{50} of DPPH free radical scavenging in root.

acid and ferulic acid (Gulcin, 2006). Statistical analysis revealed that, the total phenolic contents between the stem-bark and root extracts were not significantly different ($p = 0.15$). Whereas, DPPH radical scavenging activity was significantly different ($p = 0.001$). The correlation of the Total phenolic and flavonoid content with DPPH radical scavenging activity was analyzed and it was found that, the total flavonoid content of the stem-bark ($R^2 = 0.85$) as well as root ($R^2 = 0.70$) extracts exhibit stronger positive correlation with the DPPH radical scavenging activity as compared to the total phenolic content (Figs. 6 and 7). Recent observation suggests, DPPH assay is one of the most frequently used ones *in vitro* antioxidant evaluation. However, the assay needs several modifications to cover a wide range of applications (Alam et al., 2013; Kedare and Singh, 2011).

Recent studies on *H. mystax* were reviewed and it is found that, phytochemical analysis of leaf extracts (by Soxhlet method) of *H. mystax* showed the presence of secondary metabolites such as phenolic acids, flavonoids, tannins, terpenoids, steroids; whereas qualitative determination of individual phenolic compounds of extracts revealed the presence of caffeic acid, gallic acid, *p*-coumaric acid and vanillin (Pawar et al., 2014). Among the various solvent extracts tested for antioxidant potential, petroleum ether, methanol and ethanol extracts of leaf exhibit significant antioxidant activity *in vitro* (Rajeswari et al., 2014). In *vivo* anti-inflammatory activity of ethanol extracts of leaf and bark of *H. mystax* were investigated in albino rats by carrageenan induced rat paw edema method. It was observed that at the dose of 500 mg/kg of body weight of *H. mystax* extracts significantly inhibited the carrageenan induced paw edema (Rajeswari et al., 2013). However, the active phytoconstituents contributing to biological activity has not yet been identified. The molecular basis for biological effect is not yet understood. This investigation has identified *p*-coumaric acid as a major phytoconstituent predominantly present in plant parts of *H. mystax* L. It has been found that compounds rich in phenolic acids also significantly contribute to antirheumatic activity (Wang et al., 2012). In recent findings, *p*-coumaric acid significantly suppressed leukocyte infiltration and pro-inflammatory cytokine expression in ankle joints of adjuvant-induced arthritic rats model (Pragasam et al., 2013a,b). Zhao et al. (2016) revealed that *p*-coumaric acid may block NF-kB and MAPKs signaling pathways by inhibiting LPS induced inflammatory cytokines. Hence, *p*-coumaric acid has the potential of being used as immunosuppressive agent in treating autoimmune inflammatory diseases like rheumatoid arthritis.

4. Conclusion

Thus, it may be concluded from the present study that stem bark and root of *H. mystax* L. are a rich source of polyphenols, which possibly contribute to their biological activity. This is the very first study where individual phenolic compounds in stem bark and root of *H. mystax* L. have been quantified by the RP-HPLC method. Present research findings strongly support the traditional use of *H. mystax* L. plant parts by traditional healers. However, studies at molecular level are required to reveal the exact mechanism of action of active plant extracts exhibiting anti-inflammatory activity, which is under investigation.

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