ANTIOXIDANT POTENTIAL AND PHENOLIC CONTENTS OF LEAF, BARK AND FRUIT OF *AEGLE MARMELOS*

A Wali[1], M Gupta[2,*], SA Mallick[2], S Gupta[2] & S Jaglan[3]

[1]*Division of Biochemistry, Faculty of Basic Sciences, SKUAST-J, Jammu and Kashmir, India–180009*
[2]*Division of Plant Pathology, Faculty of Agriculture, SKUAST-J, Jammu and Kashmir, India–180009*
[3]*Quality Control and Quality Assurance Division, Indian Institute of Integrative Medicine, Jammu, Jammu and Kashmir, India–180001*

**moniguptaskuast@gmail.com*

Received August 2015

WALI A, GUPTA M, MALLICK SA, GUPTA S & JAGLAN S. 2016. Antioxidant potential and phenolic contents of leaf, bark and fruit of *Aegle marmelos*. Free radicals produced during cellular metabolism are responsible for various diseases. Thus, looking for natural antioxidants from medicinal plants has now become an important research. *Aegle marmelos*, commonly known as bael, is a tropical tree valued for its medicinal properties and has been used to cure many diseases. In the present study, antioxidant activity and phenolic profile of the bael leaf, bark and fruit were investigated. The methanolic extracts of bael leaf, bark and fruit were assayed for their antioxidant activities using DPPH (2,2-diphenyl,2-picrylhydrazyl) radical scavenging activity, reducing power and chelating metal ion power. The highest DPPH radical scavenging activity was observed in leaf methanolic extract (50% inhibition (IC_{50}) value = 249.3 ± 9.4 µg mL^{-1}) and least in fruit methanolic extract (IC_{50} = 1032.2 ± 7.03 µg mL^{-1}). Highest chelating power was observed in leaf methanolic extract (IC_{50} = 165.7 ± 2.3 µg mL^{-1}) and least in fruit methanolic extract (IC_{50} = 977 ± 5.7 µg mL^{-1}) whereas the IC_{50} of reducing power ranged from 0.4 to 05 µg mL^{-1}. Total phenolic content was highest in leaf methanolic extract (16.5 ± 0.3 mg gallic acid equivalents (GAE) g^{-1} dry weight) and lowest in fruit methanolic extract (10.6 ± 0.3 mg GAE g^{-1}). Reversed-phase HPLC analysis of the leaf, bark and fruit methanolic extracts, revealed the presence of different phenolic acids, viz. gallic acid, *p*-coumaric acid, vanillic acid, *p*-hydroxy benzoic acid, syringic acid, ferulic acid and chlorogenic acid.

Keywords: IC_{50}, DPPH, GAE, phenolic acid, RP–HPLC

INTRODUCTION

Aegle marmelos is a tropical tree native to South-East Asia and belongs to the Rutaceae family. It is grown throughout India, Sri Lanka, Pakistan, Bangladesh, Burma, Thailand and most of the South-East Asian countries (Singh & Roy 1984). In India, *A. marmelos* has been used as folklore medicine since ancient times to cure various human diseases. Almost every part of this tree, i.e. root, stem, bark, leaf, flower and fruit at all stages of maturity has medicinal virtues (Maity et al. 2009). *Aegle marmelos* possesses several pharmacological activities such as anti-inflammatory, antipyretic, analgesic, antioxidant and antidiabetic. The leaf of this tree is astringent, laxative, expectorant and is useful in treating ophthalmia, deafness, inflammations, cataract, diabetes and asthma (Umadevi et al. 2012). Antioxidant and therapeutic properties of *A. marmelos* fruit have been commercially exploited for production of herbal medicines and food products such as wine (Panda et al. 2014).

Free radicals and other reactive oxygen species such as superoxide ions (O_2^-), hydroxyl radicals (OH), nitric oxide radical (NO), singlet molecular oxygen (O_2), peroxynitrite radicals and hydrogen peroxide (H_2O_2) are generated by living cells during metabolism as byproducts of various physiological and biochemical processes. The most effective way to get rid of free radicals which cause oxidative stress is through the use of antioxidants. Antioxidants are substances that neutralise free radicals or their actions (Sies 1996).

Plants are source of natural or phytochemical antioxidants (Walton & Brown 1999). Recent researches have shown that antioxidants of plant origin with free radical scavenging properties have great importance as therapeutic agents for several diseases caused by oxidative stress.

Free radicals can be scavenged by synthetic antioxidants such as butylated hydroxyltoluene and butylated hydroxylanisole, but these compounds are suspected to cause side effects (Ito et al. 1983). The present study was therefore undertaken to determine the antioxidant activity and phenolic profiles of the leaf, bark and fruit of *A. marmelos*.

MATERIALS AND METHODS

Sample preparation

Leaf, fruit pulp and bark of *A. marmelos* were washed with distilled water repeatedly to remove impurities such as sand, silt, dust, pollen and insect. Samples were surface sterilised with 4% sodium hypochlorite and were air dried on filter paper at room temperature. Dried samples were powdered using mixer grinder under aseptic condition.

Methanolic extract preparation

An amount of 50 g each of *A. marmelos* leaf, bark and fruit pulp was extracted with 100 mL methanol and continuously stirred for 4 hours. The supernatant was recovered and addition of methanol was repeated until a transparent layer was formed over the extract. Each extract was pooled separately and filtered after which the extract volume was reduced at 60 ºC using rotary vacuum evaporator and was then freeze dried using a lyophiliser. Crude mass obtained was stored at 4 ºC until further use.

Estimation of antioxidant activity of extracts

DPPH radical scavenging activity in extracts of A. marmelos

In this assay, free radical scavenging activity was determined by measuring the bleaching of purple coloured methanol solution of DPPH (2,2-diphenyl,2-picrylhydrazyl) radical. Radical scavenging activity was determined according to the modified method of Abe et al. (1998). For this, 1 mL of 0.5 mM methanol solution of DPPH radical was mixed with 2 mL sample. Two milliliters of 0.1 M sodium buffer (pH 5.5) was added to the sample mixture. The mixture was shaken and kept at room temperature in the dark for 30 min and its absorbance was measured at 517 nm using double beam UV–VIS spectrophotometer. Methanol was used as negative control. Radical scavenging activity (RSA) was calculated as a percentage of DPPH radical discoloration using the equation:

$$RSA\ (\%) = (1 - A_{sample}/A_{blank}) \times 100 \qquad (1)$$

where, A_{blank} = absorbance of control (containing all reagents except the test compound) and A_{sample} = absorbance of the test sample. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition per cent of DPPH radical against extract concentration. Butylated hydroxyltoluene was used as reference compound.

Reducing power activity of extract of A. marmelos

Reducing power of *A. marmelos* leaf, bark and fruit methanolic extracts was determined according to the modified method of Oyaizu (1986). Varying concentrations of extracts were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide solution. The mixture was incubated at room temperature for 30 min. Subsequently, 2.5 mL of 10% trichloroacetic acid solution was added and the mixture was then centrifuged at 5000 rpm for 10 min. Finally, 2.5 mL of the upper layer of the solution was mixed with 2.5 mL distilled water and 2.5 mL 0.1% ferric chloride solution. Absorbance of the reaction mixture was measured at 700 nm using double beam UV–VIS spectrophotometer. Measurements were conducted in triplicate. Increased absorbance of the reaction mixture indicated increased reducing power of the sample. Extract concentration providing 0.5 of absorbance (IC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration and compared with butylated hydroxyltoluene (standard antioxidant).

Chelating power of extract of A. marmelos on metal ions

Chelating effect of *A. marmelos* extract on metal ions was estimated according to Dinis et al. (1994) with slight modifications. A total of 100 µL of varying concentrations of extracts and 2900 µL methanol were added to

60 µL of 2 mM $FeCl_2$. The reaction mixture was initiated by adding 120 µL of 5 mM ferrozine, and the mixture was then shaken vigorously and left to stand at ambient temperature for 4 min. Absorbance of the reaction mixture was measured at 562 nm. Each test sample was repeated thrice. Quercetin was used as reference compound. The ratio of inhibition of ferrozine Fe^{2+} complex formation was calculated as follows:

$$\text{Inhibition } (\%) = [A_c - A_s / A_c] \times 100 \qquad (2)$$

where, A_c = absorbance of control and A_s = absorbance of sample.

Determination of total phenolic content of A. marmelos

Total phenolic content of extract was determined according to the Folin–Ciocalteu method (Chang et al. 2001). Concentration of total phenols in extracts was measured using double beam UV–VIS spectrophotometer. Extract solution (1 mL) was mixed with 1 mL of 1 N Folin–Ciocalteu reagent. The mixture was kept at room temperature for 5 min and 2 mL of 20% Na_2CO_3 was added to it. After 10 min, absorbance of the solution was measured at 730 nm using double beam UV–VIS spectrophotometer. Results were expressed as milligram gallic acid equivalents (mg GAE) g^{-1} extract. Experiments were conducted in triplicate. Total phenolic content was calculated from the standard curve for gallic acid ($y = 0.020x - 0.099$, $r^2 = 0.999$) where concentrations of the acid ranged from 5 to 50 µg.

Quantification of phenolic compounds in methanolic extract using RP–HPLC

For determination of phenolic acids, reversed-phase high performance liquid chromatography (RP–HPLC) method was used (Tarnawski et al. 2006). Software used for data acquisition and analysis was Chrome Work Station (version 6.3.1.0504). Separation was carried out using 150 × 4.6 mm, 5 µm C18 reverse-phase column. Mobile phase comprised 2% (v/v) acetic acid in water–methanol (82:18) (v/v) and flow rate was 0.5 mL min^{-1}. Injection volume for samples was 10 µL and chromatograms were monitored at 254 nm. Identification of phenolic acids was based on retention times in comparison with standard compounds, viz. gallic acid, ferulic acid, syringic acid, caffeic acid, vanillic acid, *p*-coumaric acid, *p*-hydroxy benzoic acid and chlorogenic acid which were each prepared at concentration of 1 mg mL^{-1} in methanol. Mobile phase standards were diluted to give a concentration range of 0.5 to 5 µg mL^{-1}. The standard solutions were injected into the HPLC system and calibration curves were established for each standard compound. Concentration of the compound was calculated from peak area according to calibration curves. Results were expressed as milligrams of each compound per 100 g of dry weight of sample.

Statistical analysis

Data were analysed using one-way analysis of variance and Tukey's test was used to compare means at 5% level. Statistical analysis was done using SPSS version 14.0 (2007).

RESULTS AND DISCUSSION

DPPH radical scavenging activity

Highest DPPH radical scavenging activity was observed in leaf methanolic extract (249.3 ± 9.4 µg mL^{-1}) and lowest in fruit methanolic extract (1032.22 ± 7.03 µg mL^{-1}) (Table 1). Antioxidant activity of extracts may be due to the neutralisation of DPPH free radical either by transfer of an electron or hydrogen atom (Naik et al. 2003). The ability of extract to scavenge DPPH radical has also been related to the inhibition of lipid peroxidation (Rekka & Kourounakis 1991). Most natural antioxidant compounds often work synergistically with each other to produce a broad spectrum of antioxidant effect that creates an effective defense system against free radical attack (Madsen & Bertelsen 1995). Results of this study were relatively similar to Guleria et al. (2013) who reported DPPH radical scavenging efficiency of 80% methanolic leaf extract of *A. marmelos* to be about 203 ± 0.04 µg mL^{-1}.

Reducing power activity

In this assay, the yellow colour of the test solution changes to green depending on the

Table 1 Antioxidant capacities and total phenolic contents of methanolic extracts in different parts of *Aegle marmelos*

Methanolic extract	DPPH radical scavenging activity IC_{50} (μg mL^{-1})	Chelation power, IC_{50} (μg mL^{-1})	Reducing power, IC_{50} (μg mL^{-1})	Total phenolic content (mg GAE g^{-1} dry weight of sample)
Leaf	249.3 a ± 9.4	165.7 a ± 2.3	0.495 a ± 0.002	16.5 c ± 0.3
Bark	268.7 a ± 8.5	928.5 b ± 6.9	0.502 b ± 0.001	14.2 b ± 0.1
Fruit	1032.2 d ± 7.03	977.0 c ± 5.7	0.540 c ± 0.003	10.6 a ± 0.3

Mean values with the different letters are significantly different from each other

reducing power of test specimen. The presence of reductants in the solution causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Extracts of the three plant parts had similar reducing potential (0.4–0.5 μg mL^{-1}) (Table 1). Polyphenolic constituents of extracts may act as good electron and hydrogen atom donors and should be able to convert free radicals to stable products by terminating radical chain reaction. Antioxidant action of reductones is based on breaking of free radical chain by donation of a hydrogen atom (Gordon 1990). Reductones also react with certain precursors of peroxide, thus, preventing peroxide formation. Natural phytochemicals such as polyphenols may act in a similar way as reductones by donating the electrons and reacting with free radicals to convert them to more stable products and terminate free radical chain reaction (Pandey et al. 2010). Our results indicated that methanolic extracts possessed similar reducing power.

Chelating power

Highest metal ion chelating activity was observed in leaf methanolic extract (165.7 ± 2.3 μg mL^{-1}) followed by bark (928.5 ± 6.9 μg mL^{-1}) and fruit (977 ± 5.7 μg mL^{-1}) (Table 1). The extracts reduced iron to form inert Fe^{2+}-extract complexes. Non-flavonoids polyphenolics can reduce iron and then form Fe^{2+}- polyphenol complexes that are inert (Laughton et al. 1987). From our data, it was evident that leaf methanolic extract possessed highest Fe^{2+} ions chelating activity. Binding of iron to phenolic antioxidants can reduce interaction of iron with oxygen molecules by changing redox potential, thus, converting Fe^{2+} ion to Fe^{3+} and there by retarding oxidative damage (Singh et al. 2007).

Total phenolic content

Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid-free radicals or preventing decomposition of hydroperoxides into free radicals (Pokorny 2001). Phenolic compounds are commonly present in both edible and non-edible plants and exhibit multiple biological effects including antioxidant activity (Kahkonen et al. 1999). Total phenolic content was found to be highest in leaf extract (16.5 ± 0.3 mg GAE g^{-1}) and least in fruit extract (10.6 ± 0.3 mg GAE g^{-1}) (Table 1). Phenolic contents in the extracts correlated strongly with antioxidant activity (Chang et al. 2001). Phenolic contents in the extracts correlated strongly with antioxidant activity. Figure 1 indicates strong and positive correlation between DPPH radical scavenging activity and total phenolic content ($r^2 = 0.912$). However, poor correlation was observed between reducing power and total phenolic contents ($r^2 = 0.461$) of extracts of *A. marmelos* indicating that phenolic compounds in the present study other than those analysed were contributing to antioxidant activity. Antioxidant activities of polyphenols were mainly due to their redox properties which played important role in adsorbing neutralised free radicals. Antioxidant properties of plants can be correlated with their polyphenolic compounds (Barreria et al. 2008).

Quantification of phenolic compounds in methanolic extract using RP–HPLC

Analysis of phenolic compounds present in methanolic extracts of *A. marmelos* was carried out using RP–HPLC and nine standard phenolic compounds. The methanolic extracts showed presence of different phenolic acids, namely,

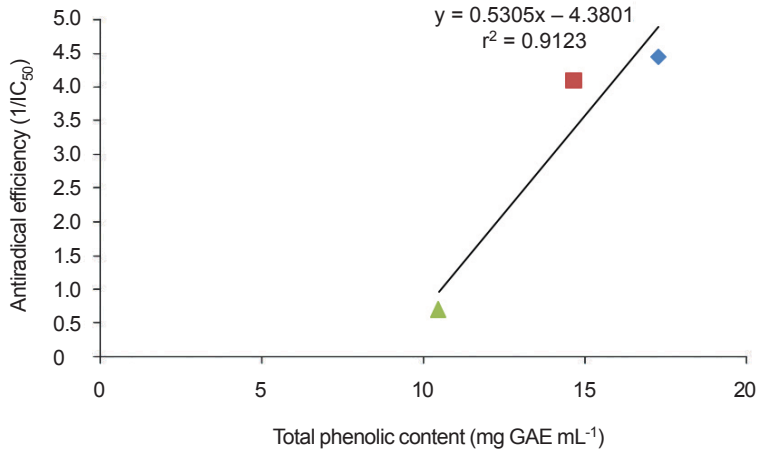

Figure 1 Correlation between DPPH radical scavenging activity expressed as antiradical efficiency ($1/IC_{50}$) and total phenol content in methanolic extracts of leaf (◆), bark (■) and fruit (▲) of *Aegle marmelos*

gallic acid, *p*-coumaric acid, vanillic acid, *p*-hydroxy benzoic acid, syringic acid, ferulic acid and chlorogenic acid. HPLC profiles of phenolics are shown in Figure 2. Gallic syringic acids were highest in leaf methanolic extract. Gallic acid and its derivatives are strong antioxidants and are able to scavenge reactive oxygen species, e.g. superoxide anions, hydrogen peroxide, hydroxyl radicals and hypochlorous acid (Kim 2007). Gallic acid derivatives are found in many phytomedicines having biological and pharmacological activities such as inducing apoptosis of cancer cells (Serrano 1998). They inhibit squalene epoxidase and interfere with the signal pathways involving Ca (II) and oxygen-free radicals (Inoue et al. 2000). Antioxidant activity in *A. marmelos* methanolic extract might be attributed to higher levels of gallic and syringic acids.

CONCLUSIONS

Crude methanolic extract of *A. marmelos* leaf possessed moderate antioxidant activity. Generally, total phenolic compounds in *A. marmelos* extracts correlated with their antiradical activities. The potential of *A. marmelos* extract as antioxidant agent may be attributed to the presence of polyphenols. This study showed that *A. marmelos* leaf could be used as potential source of antioxidant compounds for pharmaceutical industries and its fruit, as nutraceuticals.

ACKNOWLEDGEMENTS

This work was carried out under the Masters Dissertation Programme by the first author and financially supported by Sher-e-Kashmir University of Agricultural Sciences and Technology-Jammu (SKUAST-J), Jammu and Kashmir, India.

REFERENCES

Abe N, Murata T & Hirota A. 1998. Novel DPPH radical scavengers, bisorbicillinol and demethyltrichodimerol from a fungus. *Bioscience Biotechnology and Biochemistry* 62: 661–666.

Barreria CM, Ferreria CFR, Oliveira PP & Pereira A. 2008. Antioxidant activities of extracts from chestnut flower, leaves, skins and fruit. *Food Chemistry* 107: 1106–1113.

Chang ST, Wu JH, Wang SY, Kang PL, Yang NS & Shyur LF. 2001. Antioxidant activity of extracts from *Acacia confusa* bark and heart wood. *Journal of Agricultural and Food Chemistry* 49: 3420–3424.

Dinis TCP, Madeira VMC & Almeida LM. 1994. Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Archives of Biochemistry and Biophysics* 315: 161–169.

Gordon MF. 1990. The mechanism of antioxidant action in-vitro. Pp 1–18 in Hudson BJF (ed) *Food Antioxidants*. Elsevier Applied Science, London.

Guleria S, Tiku AK, Singh G, Koul A, Gupta S & Rana S. 2013. In-vitro antioxidant activity and phenolic contents in methanol extracts from medicinal

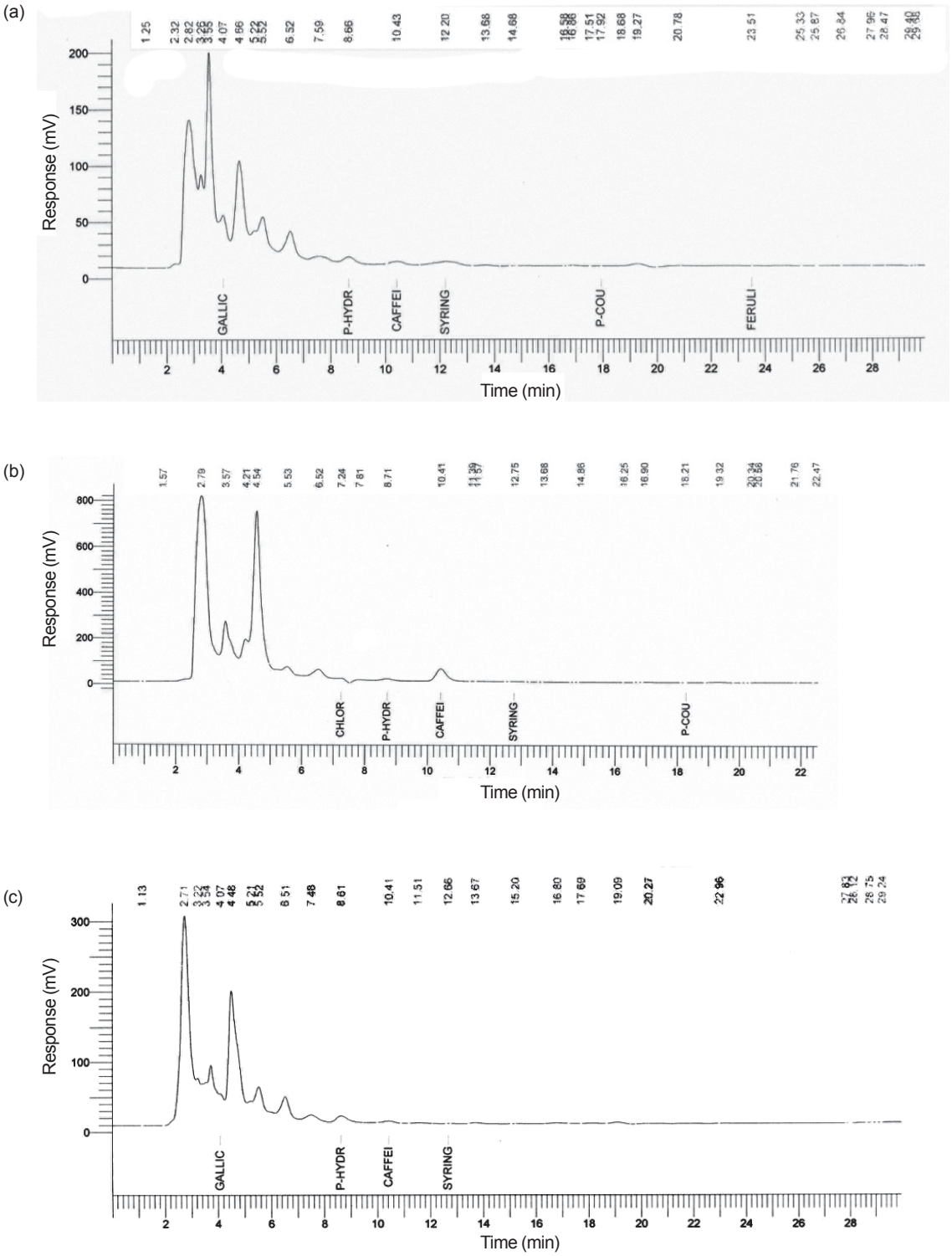

Figure 2 HPLC profiles of (a) leaf, (b) bark and (c) fruit methanolic extracts of *Aegle marmelos*

plants. *Journal of Plant Biochemistry and Biotechnology* 22: 9–15

Inoue M, Sakaguchi N, Isuzugawa K, Tani H & Ogihara Y. 2000. Role of reactive oxygen species in gallic acid-induced apoptosis. *Biological and Pharamaceutical Bulletin* 23: 1153–1157.

Ito N, Fukushima S, Hasegawa A, Shibata M & Ogiso T. 1983. Carcinogenicity of butylated hydroxylanisole in F344 rats. *Journal of the National Cancer Institute* 70: 343–347.

Kahkonen MP, Hopia AI, Vuorela HJ, Rahua JS, PIHLAJA K, KujalA TS & Heinonen M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural Food Chemistry* 47: 3954–3962.

Kim YJ. 2007. Antimilanogenic and antioxidant properties of gallic acid. *Biological and Pharmaceutical Bulletin* 30: 1052–1055.

Laughton MJ, Halliwell B, Evans PJ & Holult JRS. 1987. Antioxidant and prooxidant actions of plant phenolics quercetin gossypol and myrycetin. *Biochemical Pharmacology* 36: 717–720.

Madsen HL & Bertelsen G. 1995. Spices as antioxidant. *Trends in Food Science and Technology* 6: 271–277.

Maity P, Hansda P & Mishra D. 2009. Biological activities of crude extracts and chemical constituent of bael, *Aegle marmelos* (L) Corr. *Indian Journal of Experimental Biology* 47: 849–861.

Naik GH, Priyadarsini KI, Satav JG, Banavalikar MM, Sohoni PP & Biyani MK. 2003. Comparative antioxidant activity of individual herbal components used in ayurvedic medicine. *Phytochemistry* 63: 97–104.

Oyaizu M. 1986. Studies on product of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition* 44: 307–315.

Panda SK, Sahu UC, Behera SK & Ray RC. 2014. Bio-processing of bael (*Aegle marmelos* L.) fruits into wine with antioxidants. *Food Bioscience* 5: 34–41.

Pandey AK, Mishra A, Kumar S & Chandra A. 2010. Therapeutic potential of *C. zeylanicum* extracts: an antifungal and antioxidant perspective. *International Journal of Biological Medical Resource* 1: 228–233.

Pokorny J. 2001. Introduction. Pp 1–3 in Pokorny J, Yanishlieva N & Gordon MH (eds) *Antioxidants in Food: Practical Applications*. Woodhead Publishing Limited, Cambridge.

Rekka E & Kourounakis PN. 1991. Effect of hydroxyethyl rutosides and related compounds on lipid peroxidation and free radical scavenging activity: some structural aspects. *Journal of Pharmacy and Pharmacology* 43: 486–491.

Serrano A, Paacios C, Roy G et al. 1998. Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation. *Archives of Biochemistry and Biophysics* 350: 49–54.

Sies H. 1996. *Antioxidants in Disease, Mechanisms and Therapy*. Academic Press, New York.

Singh G, Maurya S, Marimuthu P, Murali HS & Bawa AS. 2007. Antioxidant and antibacterial investigations on essential oils and acetone extracts of some spices. *Natural Product Radiance* 6: 114–121.

Singh RN & Roy SK. 1984. *The Bael: Cultivation and Processing*. Indian Council of Agricultural Research, New Delhi.

Umadevi KJ, Varadharajan V & Krishnamurthy V. 2012. Physicochemical evaluation, phytochemical screening and chromatographic fingerprint profile of *Aegle marmelos* (L.) leaf extract. *World Journal of Pharmaceutical Research* 1: 813–817.

Tarnawski M, Depta K, Grejcium D & Szelepin B. 2006. HPLC determination of phenolic acids and antioxidant activity in concentrated peat extract—a natural immunomodulator. *Journal of Pharmeceutical and Biomedical Analysis* 41: 182–188.

Walton NJ & Brown DE. 1999. *Chemicals From Plants: Perspectives on Plant Secondary Products*. Imperial College Press, London.