Original Research Article

Phytochemical screening, antioxidant activity and UHPLC fingerprinting of methanolic extract of the fruits of *Quercus semiserrata* Roxb. from Meghalaya India

ABSTRACT

Quercus semiserrata Roxb. belongs to the family Fagaceae which is an Asian species of trees in the beech family. It has been found in Northeastern India, Bangladesh, Myanmar, Yunnan, Thailand and Tibet. Proteins, carbohydrates, phenols and tannins, flavonoids, steroids, terpenoids and alkaloids were detected in the fruits studied. The total phenolic content determined using Folin-ciocalteu reagent (FCR) was found to be 155.95 ± 0.18 mg gallic acid equivalents/g dry weight fruit extract and the total flavonoid content by Aluminium chloride colorimetric method was 238.78± quercetin equivalent/g dry weight fruit extract respectively. DPPH, ABTS radical scavenging activity and Reducing Power Assay were analyzed to study the antioxidant activities. Methanolic extract of *Q. semiserrata* fruit plays a significant role in antioxidant properties where the inhibition concentration (IC_{50}) for DPPH was found to be at 50.17µg/ml and 49.46 µg/ml for ABTS. β-carotene content was found to be 0.002mg/100ml and 0.0013 mg/100ml lycopene. A simplified method using reverse-phase Ultra High-performance Liquid Chromatography (UHPLC) was developed for the detection of ascorbic acid, phenolic acids and flavonoids. It was observed that *Q. semiserrata* play an important role in antioxidant activity even in low concentration.

***Keywords*:** *Quercus semiserrata*, Meghalaya, antioxidant, UHPLC

ABBREVIATIONS

FCR- Folin-ciocalteu Reagent

DPPH- 2,2-diphenyl-1-picryl-hydrazyl-hydrate

ABTS- 2,2'-azinobis-bis (3-ethylbenzothiazoline-6-sulfonic acid)

IC_{50}- Half Maximal Inhibitory Concentration

UHPLC- Ultra High Performance Liquid Chromatography

TPC- Total Phenolic Content

TFC- Total Flavonoid Content

BHT- Butylated Hydroxy Toluene

QS- *Quercus semiserrata*

Ao- Absorbance of reaction control

A_1- Absorbance of extracts or standards

$Abs_{control}$- Absorbance of control

Abs_{sample}- Absorbance of sample

1. INTRODUCTION

Phytochemicals also referred to as phytonutrients are chemical compounds produced by plants to help them resist virus infection, bacteria, and fungi and also for the consumption of insects and other animals. These plant-based foods are complex mixtures of bioactive compounds. Information on the potential health effects of individual phytochemicals is linked to information on the health effects of foods that contain those phytochemicals. Phytochemicals, as plant components with discrete bio activities towards animal biochemistry and metabolism are widely examined for their ability to provide health benefits. Such phytochemicals include terpenoids, phenolics, alkaloids and fiber etc [1]. They also act as a possible clue on the mechanism involved in the prevention of oxidative stress, inflammation and cancer.

Quercus semiserrata Roxb. is a large tree of about 16-30m tall widely distributed in Asian countries and Northeastern part of India. Fruits are about 2-3cm in diameter with soft hairs and 7-8 lines of cupular bracts. The branches of these trees spread, leaves are broader and dentated from the base, brownish green colour and greyish white beneath. It is mostly found in moist shady forest and rocky clay loam. In some places it grows on the hill slopes along with *Rhododendron* species. In Nagaland, they can be found at an altitude of about 2000-2800m. They are commonly called as sekho, phong-rong-lang-poh and sehop in Assam. This study mainly aimed to check the antioxidant activity of the fruit. There are several species of *Quercus* found in Northeastern part of India. Based on the available literature no work has been done on the phytochemical studies of this plant. The present study aimed to investigate the presence of bioactive compounds, antioxidant activity and UHPLC analysis of fruits of *Q. semiserrata*. Therefore, qualitative, quantitative analysis, antioxidant activities was performed, UHPLC fingerprinting analysis and carotenoid pigments was also carried out in the present study.

2. MATERIALS AND METHODS

2.1 Collection of plant materials

The fruits of *Q. semiserrata* were collected from Laitumkhrah, Shillong, East Khasi Hills District Meghalaya. Fruits were cleaned and washed thoroughly using tap water and distilled water. These were then cut into small pieces and shade dried until all the water molecules evaporated and become well dried for grinding using a mechanical blender. After grinding

into a fine powder, ground fruits were transferred into airtight containers with proper labeling for future use.

2.1.1 Preparation of plant extracts

Hot water extraction

1gm of dried finely powdered plant material was taken in a beaker and 40ml of distilled water was added. The mixture was heated on a hot plate with continuous stirring at 30- 40ºC for 20 minutes. Then the water extract was filtered through filter paper and the filtrate was used for the phytochemical analysis. The water extract was kept in the refrigerator when not in use.

Solvent extraction

Crude plant extract was prepared by Soxhlet extraction method. About 5 gm of powdered plant material was uniformly packed into a thimble and extracted with 250ml of solvent. The solvent used was methanol and water. The process of extraction continues for 24 hours or till the solvent in the siphon tube of an extractor becomes colorless. The extract was then transferred to the Rotary Evaporator chamber and was heated at 40-50ºC till all the solvent got evaporated. The dried extract was kept in the refrigerator at 4ºC for their future use in phytochemical analysis.

2.3 Qualitative phytochemical analysis

The extract was tested for the presence of bioactive compounds by using the following standard methods [2-4]. Test for proteins, carbohydrates, phenols & tannins, flavonoids, saponin, glycosides, steroid, terpenoid and alkaloid was carried out.

2.4 Quantitative phytochemical analysis

2.4.1 Determination of total phenolic content

Total phenolics were determined using Folin-ciocalteu reagent (FCR) [5]. 100µl of plant extract dissolved in methanol (1mg/ml) was mixed with 750µl of FCR (diluted 10-fold) and allowed to stand for 5 min at 22˚C; 750 µl of Na_2CO_3 (60g/l) solution was then added to the mixture. After 90 min, the absorbance was measured at 725 nm. The tests were performed in triplicates. Phenolic contents were determined from the standard curve and results were expressed as gallic acid equivalents.

2.4.2 Determination of total flavonol content

To determine flavonol content, Aluminium chloride colorimetric method was used. 1ml of sample plant extract was mixed with 3ml of methanol, 0.2ml of 10% aluminium chloride, 0.2ml of 1M potassium acetate and 5.6ml of distilled water and kept at room temperature for

30 minutes. Quercetin was used as standard (1mg/ml). The absorbance was measured at 420 nm. All the tests were performed in triplicates. Flavonol contents were determined from the standard curve and were expressed as quercetin equivalent (µg/mg of extracted compound) [6].

2.5 *In vitro* antioxidant activity

2.5.1 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity

DPPH radical scavenging activity was estimated by measuring the decrease in the absorbance of methanolic solution of DPPH [7]. To 5mL of DPPH solution (3.3 mg of DPPH in 100mL methanol), 1ml of each plant extracts were added and incubated for 30 min in the dark and the absorbance (A_1) was read at 517 nm. The absorbance (A_0) of a reaction control (methanol instead of plant extract) was also recorded at the same wavelength. Ascorbic acid (10-50 µg/mL) was used as a standard. Scavenging ability (%) was calculated by using the formula:

DPPH radical scavenging activity (%) = $[(A_0 - A_1)/A_0] \times 100$

where A_0 was the absorbance of reaction control and A_1 was the absorbance of extracts or standards.

2.5.2 2,2'-azinobis-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 14h at room temperature in the dark. The solution was then diluted to obtain an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (200 µl) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using a spectrophotometer [8]. The ABTS scavenging capacity of the extract was compared with ascorbic acid and percentage inhibition calculated as

ABTS radical scavenging activity (%) = $Abs_{control} - Abs_{sample}/Abs_{control} \times 100$,

where $Abs_{control}$ is the absorbance of ABTS radical in methanol; Abs_{sample} is the absorbance of ABTS radical solution mixed with sample extract/standard.

2.5.3 Reducing Power Assay

The reaction mixture contained 1.0 ml of various concentrations of extracts (200-1000µg/ml), 2.5 ml of 1% potassium ferricyanide and 2.5 ml of 0.2M sodium phosphate buffer. The mixture was incubated at 50°C for 30 min and the reaction was terminated by the addition of

2.5 ml of 10% TCA, followed by centrifugation at 3000 rpm for 10 min. 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against the blank. The reducing power ability of the sample is determined by increase in absorbance of the sample. BHT was used as the standard for comparison [9].

2.6 Estimation of Carotenoids

To determine the content of β- carotene and lycopene, 0.1g samples were taken and shaken with 10 mL of acetone-hexane mixture in the ratio of 4:6 for one minute and filtered through Whatman No. 1 filter paper. Absorbance was measured at 453,505 and 663nm using UV-VIS spectroscopy [10].

Content of β-carotene and lycopene was calculated according to the following equations:

Lycopene (mg/100mL) $= -0.0458A_{663} + 0.372A_{505} - 0.0806A_{453}$ and,

β-Carotene (mg/100ml) $= 0.216A_{663} - 0.304A_{505} + 0.452A_{453}$.

2.7 Ultra-High Performance Liquid Chromatographic (UHPLC) analysis

Standard preparation

1mg/ml of each standard was dissolved in methanol and diluted to different concentration of all the standard in methanol. The standard solutions were stored in the refrigerator at $4^{o}C$ whereas the working solution was filtered through Whatman Syringe Filter-Sterile 0.45-µm RC before UHPLC injection.

Sample preparation

Plant materials were cleaned and the edible parts were rinsed thoroughly with distilled water. This was then dried and ground into powder form. The crude plant extract was prepared by the Soxhlet extraction method. About 5gm of powdered plant material was uniformly packed into a thimble and extracted with 250ml of solvent. The solvent used was methanol. The process of extraction continues for 24 hours or till the solvent in the siphon tube of an extract becomes colorless. The extract was evaporated to dryness.

1mg/ml of the crude extract (sample) was dissolved in methanol and was filtered through Whatman Syringe Filter-Sterile 0.45-µm RC prior to UHPLC injection.

Compounds were determined by a UHPLC method using a Dionex Ultimate 3000 liquid Chromatograph (Thermo Scientific) equipped with a UV/DAD detector. Chromatographic separation was performed on a Hypersil Gold aQ (250 mm × 4.6 mm) column. The solvent system had a constant flow rate of 1.2 mL/min. The mobile phase was 100% Acetonitrile (solvent A), 0.1 % acetic acid (solvent B) and 100% HPLC water (solvent D). The gradient was as follows: 0–2 min, 1.5 %B, 92% D; 2-11min, 1.5-4% B, 80%D; 11–15 min, 4%B ,80%D; 15–20 min, 4%B, 30%,5%D; 20–30 min, 4%B, 5-92%D; 30-35 min and 35- 38 min,

1.5%B, 92% D. The injection volume was 20µL, and the temperature was kept constant at 28°C. Detection wavelengths were chosen considering the absorption maximums of UV spectra of the selected compounds [11].

3. RESULTS AND DICUSSION

3.3 Qualitative phytochemical analysis

It was observed that proteins, carbohydrates, phenols/ tannins, flavonoids, steroids, terpenoids and alkaloids were present in the fruits of *Q. semiserrata* whereas, saponins and glycosides were absent (**Table 1**). Proteins are important essential building blocks of life and carbohydrates are the main source of energy in living things. Phenols play a vital role in antioxidant activity. Flavonoids promote cellular health, antioxidant activity, normal tissue growth and renewal throughout the body [12]. Terpenoids possess antimalarial effects, anti-inflammatory, antitumor, antiviral, antibacterial, promote transdermal absorption, have hypoglycemic activities, prevent and treat cardiovascular diseases. Previous studies have also found that terpenoids have many potential applications, such as neuroprotection, insect resistance, antiaging, immunoregulation, and antioxidation [13]. Neuroactive steroids help in the control of central nervous system functions during pathological and physiological conditions that they may represent good candidates for the development of neuroprotective strategies for neurodegenerative and psychiatric disorders [14]. Alkaloids of plant origin plays a potential role as antimicrobials against Antibiotic-Resistant Infections [15] and antidiabetic properties of alkaloids of different plants was also studied [16].

Table 1. Phytochemical constituents of *Q. semiserrata* fruit

Plants	Proteins	Carbohydrates	Phenols/	Flavonoids	Saponins	Glycosides	Steroids	Terpenoids	Alkaloids
Quercus semiserrata (fruit)	+	+	+	+	-	-	+	+	+

(+) indicates present, (-) indicates absent

3.4 Quantitative phytochemical analysis

Fig 1-2 is the standard curve of total phenolic content using gallic acid as a standard and standard curve of flavonols using quercetin as standard. The total phenolic content was 155.95 ± 0.18 mg gallic acid equivalents/mg dry weight plant extract and total flavonol

content was 238.78± quercetin equivalent/mg dry weight plant extract (**Table 2**). *Q. semiserrata* shows high content of phenolics and flavonols which shows good antioxidant activity.

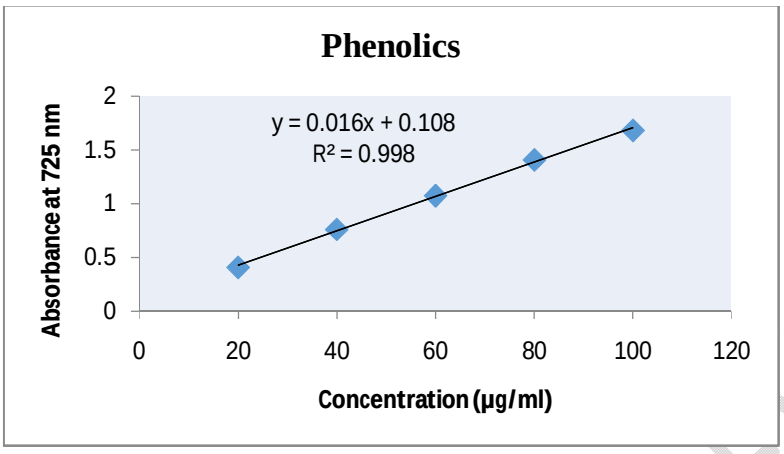

Fig. 1. Calibration graph for total phenolic content

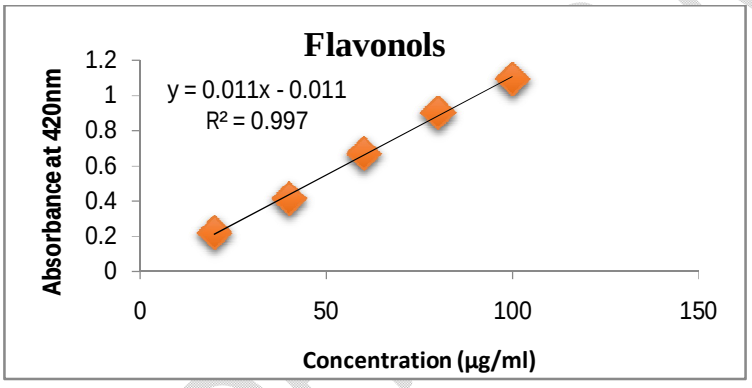

Fig. 2. Standard curve of flavonols

Table 2. Showing the results of total phenolic content (TPC), total flavonoid content (TFC), ABTS, DPPH activity and carotenoids

Plant	TPC (mg/g)	TFC (mg/g)	ABTS (Ic_{50}) µg/ml		DPPH (Ic_{50}) µg/ml	Carotenoids	
						β-carotene (mg/100ml)	Lycopene (mg/100ml)
Quercus semiserrata	155.95±0.18	238.78±0.007	49.46		50.17	0.002	0.0013

3.5 *In vitro* antioxidant activity

DPPH radical scavenging activity of ascorbic acid **(Fig. 3)** and *Q. semiserrata* (**Fig. 4**) and ABTS radical scavenging activity of ascorbic acid (**Fig. 5**) and *Q. semiserrata* **(Fig. 6)**. The IC_{50} or Inhibition concentration of Ascorbic acid for DPPH was 13.74 µg/ml and 15.88 µg/ml for ABTS (**Table 2**). Whereas, IC_{50} of *Q. semiserrata* was 50.17µg/ml for DPPH and 49.46 µg/ml for ABTS respectively (**Table 2**). There is a strong correlation between the total antioxidant capacity assayed by DPPH, ABTS, RPA methods and phenolic content which indicate that the phenolic compounds largely contribute to the antioxidant activities of *Q. semiserrata* and therefore could play an important role in the beneficial effects of these important medicinal plants. The results were in accordance with other researches, several studies have found that phenolic compounds are major antioxidant constituents in selected plants, and there are direct relationships between their antioxidant activity and total phenolic content [17-20].

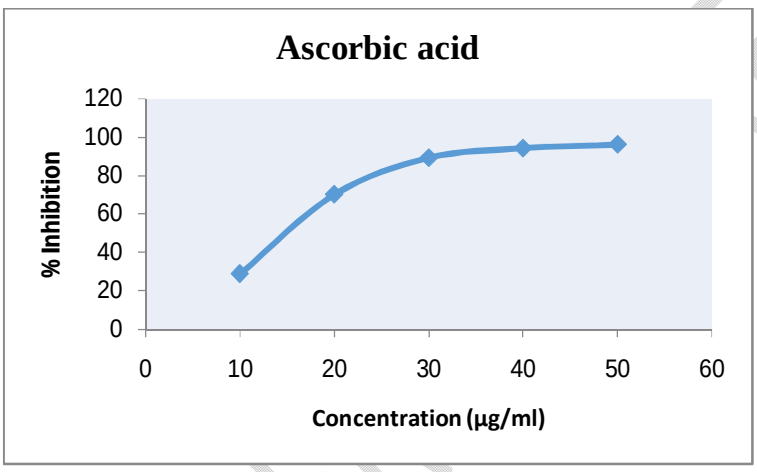

Fig. 3. DPPH percentage inhibition of standard ascorbic acid

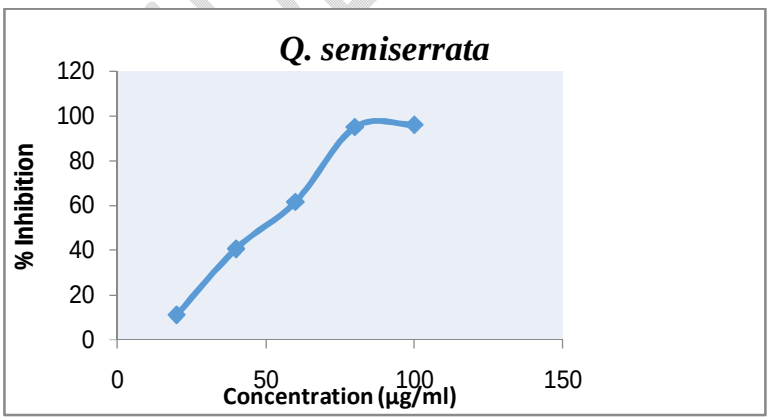

Fig. 4. DPPH radical scavenging activity of *Q. semiserrata*

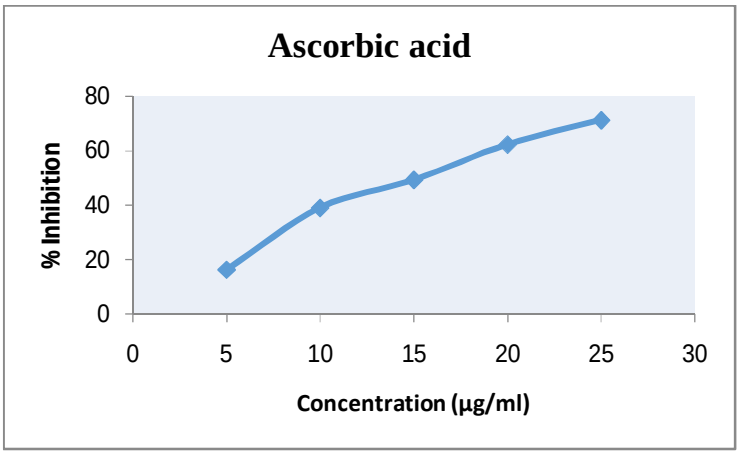

Fig. 5. ABTS radical scavenging activity of ascorbic acid

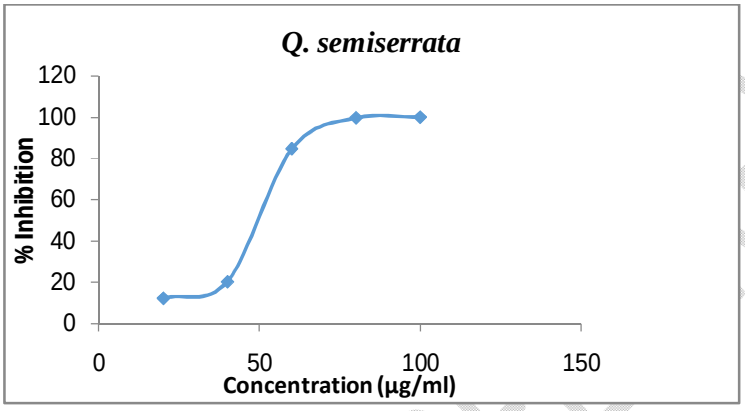

Fig. 6. ABTS radical scavenging activity of *Q. semiserrata*

Reducing Power Assay

Reducing power assay is a convenient and rapid screening method for measuring the antioxidant potential. The reduction ability (Fe^{3+} to Fe^{2+} transformation in terms of increasing absorbance) was found to increase with rising concentration. *Q. semiserrata* (0.59, 0.80, 0.97, 1.04 and 1.19) showed good reducing power capacity which can be compared with standard BHT (0.275, 0.489, 0.651, 0.729 and 0.872) in a concentration of 20-100µg/ml (**Fig. 7**). The yellow colour of the test solution changes to various shades of green. This indicates the presence of the reducers that causes conversion of Fe^{3+} or ferricyanide complex used in this method.

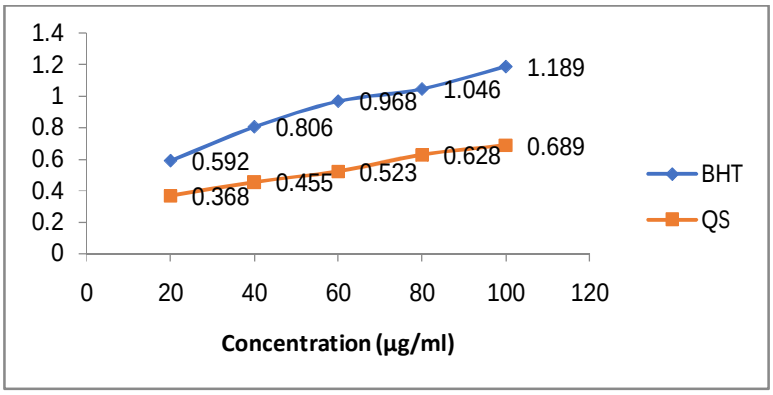

Fig. 7. Reducing Power Assay of BHT (Butylated Hydroxy Toluene) and *Q. semiserrata* (QS)

3.6 Carotenoids

It was observed that *Q. semiserrata* contain more β-carotene content than lycopene (**Table. 2**). Many epidemiological studies have shown an association between diets rich in carotenoids and a reduced incidence of many forms of cancer, and it has been suggested that the antioxidant properties [21] of these compounds are a causative factor. Attention has focused on the potential role of one specific carotenoid, β-carotene, in preventing cancer, chronic diseases, gastric cancer and numerous publications have described *in vitro* experiments and animal studies which suggest that not only can this carotenoid protect against the development of cancer, but also several other chronic diseases [22].

3.7 UHPLC Analysis

Chromatogram of ascorbic acid, phenolics and flavonoids is shown in **Fig. 8**. The chromatogram of ascorbic acid, gallic acid, catechin gallic acid, vanillin, þ-coumaric acid, rutin, quercetin, kaempferol, tannic acid and BHT was observed at a wavelength of 27.6 nm. The chromatogram of *Q. semiserrata* is shown in **Fig. 9**. Kaempferol and tannic acid was observed at a retention time of 22.12 and 24.86 minutes at a wavelength of 276nm where kaempferol was the major peak present and tannic acid as a minor peak. Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one) has been demonstrated to have beneficial effects on chronic inflammatory diseases and anti-cancer properties [23]. The antioxidant properties of tannins are of great importance in food applications due to their ability to prevent disorders related to oxidative stress, such as cancer and cardiovascular diseases [24] and are also characterized to be more environment-friendly option than those based on chemical products.

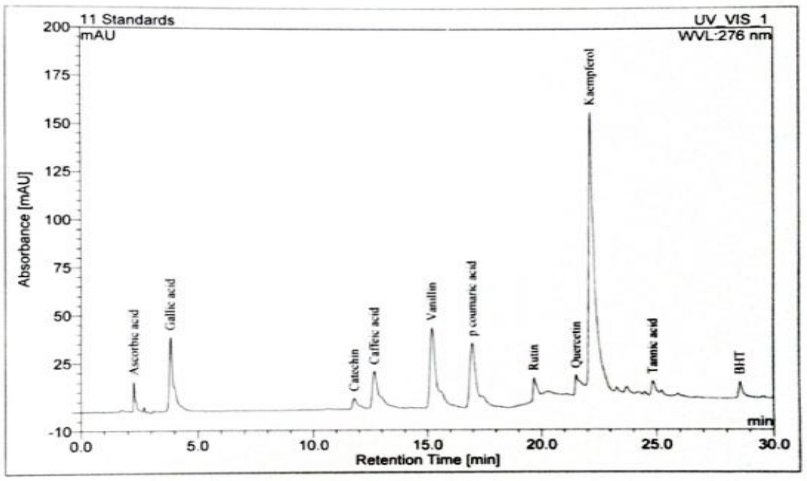

Fig. 8. Chromatogram of ascorbic acid, phenolics and flavonoids

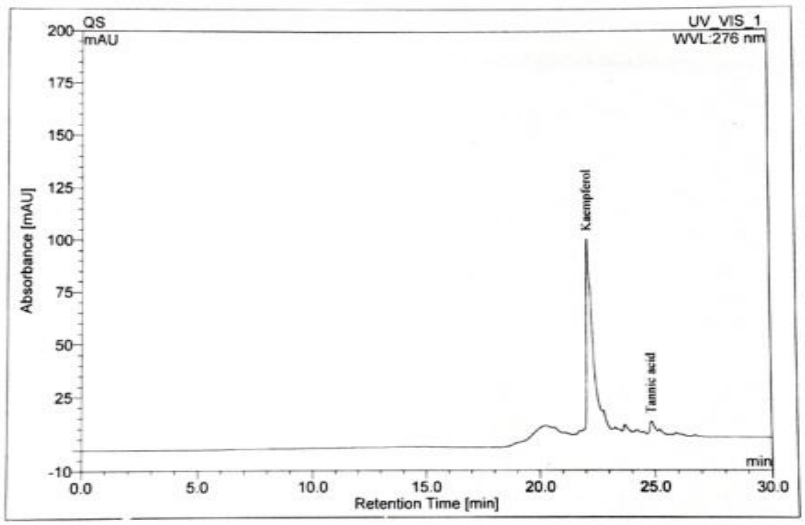

Fig. 9. Chromatogram of *Q. semiserrata* (fruit)

4. CONCLUSION

Q. semiserrata contains high amount of phenolics and flavonols and low number of carotenoids and act as a potential source of reactive oxygen species inhibiting compound which plays an important role as antioxidant activities. Kaempferol and tannic acid was also detected to be present in this fruit which was found to have anti-inflammatory effect and can prevent oxidative stress such as cancer and cardiovascular diseases. The present study focused only on qualitative, quantitative, antioxidant properties, carotenoids and UHPLC analysis. Therefore, further studies may also be carried out to investigate more on the nutritional and chemical composition of this interesting fruits.

REFERENCES

1. Dillard CJ and German JB. Phytochemicals: nutraceuticals and human health. J Sci Food Agric 2000; 80:1744±1756.
2. Sofowra A. Medicinal plants and traditional medicine in Africa. Spectrum Books Ltd., Ibadan, Nigeria, 1993; pp.191-289.
3. Trease GE, Evans WC. Pharmacognosy, 11th Edn., Bailliere Tindall, London, 1989; pp. 45-50.
4. Harborne JB. Phytochemicals Methods. Chapman and Hall Ltd., London, 1973; pp. 49-188.
5. Velioglu YS, Mazza G, Gao L, Oomah BD. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem., 1998;46: 4113–4117.
6. Aiyegroro OA, Okoh AI. Preliminary phytochemical screening and in vitro antioxidant activities of aqueous extract of *Helichrysum longifolium* DC. BMC compl. And Alt. Med., 2010; 10: 21.
7. Brand-Williams W, Cuvelier M and Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss Technology, 1995; 28: 25-30. https://doi.org/10.1016/S0023-6438(95)80008-5.
8. Arnao MB, Cano A, Acosta M. The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chem 20001; 73:239–44.
9. Oyaizu M. Studies on products of browning reaction: Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr. 1986; 44: 307-315.
10. Bhusal S, Pant DR, Joshi GP, Adhikari M, Raut JK, Pandey MR and Bhatt LR. Antioxidant Activity and Nutraceutical Potential of Selected Nepalese Wild Edible Fruits. Scientific World, 2020; Vol 13, No. 13.
11. Seal P. Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves, *Sonchus arvensis* and *Oenanthe linearis* of North-Eastern region in India. Journal of Applied Pharmaceutical Science ·February 2016.
12. Kumar S. The Importance of antioxidant and their role in pharmaceutical science. Asian Journal of Research in Chemistry and Pharmaceutical Sciences. 1(1), 2014, 27 - 44.
13. Yang W, Chen X, Li Y, Guo S, Wang Z, and Yu X. Advances in Pharmacological Activities of Terpenoids. Natural Product Communications. 2020; DOI: 10.1177/1934578X20903555
14. Melcangi RC, Panzicab G and Garcia-segura LM. Neuroactive steroids: Focus on Human Brain. Neuroscience 191 (2011) 1–5. doi:10.1016/j.neuroscience.2011.06.02.
15. Casciaro B, Mangiardi L, Cappiello F, Romeo I, Loffredo MR, Iazzetti A, Calcaterra A, Goggiamani A, Ghirga A, Mangoni ML, Botta B and Quaglio D. Naturally-Occurring Alkaloids of Plant Origin as Potential Antimicrobials against Antibiotic-Resistant Infections. Molecules 2020, 25, 3619; doi:10.3390/molecules25163619.
16. Rasouli H, Yarani R, Pociot F, Popović-Djordjević J. Anti-diabetic potential of plant alkaloids: Revisiting current findings and future perspectives. Journal Pre-proof. 2020. https://doi.org/10.1016/j.phrs.2020.104723.

17. Heim KE, Tagliaferro AR, and Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships," Journal of Nutritional Biochemistry, vol. 13, no. 10, 2002; pp. 572–584.
18. Soong YY and Barlow PJ. Antioxidant activity and phenolic content of selected fruit seeds," Food Chemistry, vol. 88, no. 3, 2004; pp. 411–417.
19. Balasundram N, Sundram K, and Samman S. Phenolic compounds in plants and agri-industrial by-products: antioxidant activity, occurrence, and potential uses," Food Chemistry, vol. 99, no. 1, 2006; pp. 191–203.
20. Loizzo MR, Tundis R, Bonesi M (2012). Radical scavenging, antioxidant and metal chelating activities of *Annona cherimola* Mill. (cherimoya) peel and pulp in relation to their total phenolic and total flavonoid contents," Journal of Food Composition and Analysis, vol. 25, no. 2, 2012; pp. 179–184.
21. Burton GW, William TM, Riley W, Nickerson JG. β-Carotene oxidation products - Function and safety. Food and Chemical Toxicology. Volume 152, June 2021, 112207.
22. Chen QH, Wu BK, Pan D, Sang LX and Chang B. Beta-carotene and its protective effect on gastric cancer. World J clin cases Aug 2021; 16; 9(23): 6591–6607.
23. Ren J, Lu Y, Qian Y, Chen B, Tao Wu, Ji G. Recent progress regarding kaempferol for the treatment of various diseases (Review). Experimental and Therapeutic Medicine 2019; 18: 2759-2776. DOI: 10.3892/etm.2019.7886.
24. Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. Dietary polyphenols and the prevention of diseases. Crit. Rev. Food Sci. Nutr, 2005; 45, 287–306.