Chapter 4

To purify and characterize the major phytochemicals from bhimkol blossom

4.1 Introduction

About 80% of the medicinal drugs are collected from the plant source [13]. Phytochemicals are also postulated as guardians of our health, consisting of polyphenols, terpenoids, pigments, and other antioxidants that help to treat cancer, diabetes, hyperglycemic, inflammation, cell damage, heart diseases, and many other chronic diseases [6]. Quercetin is a phytochemical belonging to the class of flavonoids, contributing various pharmacological properties [8]. The underutilized bhimkol blossom (BB) consist high content of quercetin, contributing a significant antioxidant property (81.16±0.76% DPPH inhibition) reported in previous work Muchahary and Deka [12]. Extraction and isolation of any natural compound to obtain a specific heath-beneficial effect have always been a great challenge [14]; therefore, the selection of an efficient method of extraction and isolation is very necessary. Chromatographic isolation techniques *viz*., column chromatography (using macroporous resins) [9], thin layer chromatography, paper chromatography, and reversed phase-high performance liquid chromatography (RP-HPLC) are the predominantly used techniques used in single or in combination of two or more techniques [15].

This study contributes to the isolation of quercetin rich fraction from bhimkol blossom. To our best knowledge, this is the first research paper on the isolation of quercetin from underutilized bhimkol blossom. A lack of research has been done on quercetin content in banana blossoms. The isolated quercetin rich fraction in this study will contribute great food industrial and pharmacological applications that can be incorporated directly or by preparing any delivery complex, which will possibly result in enhanced health-beneficial activities.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Chemicals and reagents used in the present study were of high purity analytical grade and the chromatographic grade analytical standards and solvents were purchased from Sigma-Aldrich (USA).

4.2.2 Isolation and purification of quercetin

4.2.2.1 Extraction of phytochemicals

Ethanol extract from bhimkol blossom (BBE) of previously prepared as Muchahary and Deka [12]. Powder of BB was mixed in an extraction solvent of 70% ethanol at a 2:30 ratio in a 100 ml beaker.Tthe sample BB powder and solvent ratio (2:30) were ultrasonicated (Q Sonica, Q700, USA) with 220 Å probe (12.7 mm diameter) at a frequency of 20 kHz, temperature (60^oC), extraction amplitude (35%), extraction time (20 min), and at 75% duty cycle (15 s pulse on and 5 s pulse off) by using extraction solvent (70% ethanol). During the extraction process, the probe was immersed in 1.5 times the diameter of the tip and without touching the bottom of the solution in a 100 ml beaker for better extraction. Then BBE was mixed with 70% ethanol in ultrapure water (Milli Q water) at a ratio of 1:10 w/v.

4.2.2.2 Collection of preparative from column chromatography

The sample solution was de-oiled with petroleum ether at a ratio of 5:1 v/v. During de-oiling, the petroleum ether and sample solution was shaken rapidly in a 50 ml centrifuged tubes. After consecutive shaking, two separate liquid phases were obtained. The liquid layer in the upper phase contained petroleum ether and the free oils present in sample, which was them removed slowly by using a micropeppete. Sample was de-oiled thrice by repeating same as earlier. Deoiled sample solution was evaporated by a hot air oven (Advantage lab, AL01-05-100, Belgium) at 40^oC and stored in glass vials at 4^oC.

Preparative from de-oiled BBE was obtained from column chromatography (Borrosil, 50 ml) (Fig. 4.1) by using pre-activated resin (Amberlite XAD 7 HP, Sigma Aldrich, USA) in a column (400 x 2.5 cm ID). Resins were activated by soaking in deionized water (Milli Q) and ethanol (chromatographic grade) overnight. After activating the resin, they were washed repeatedly to remove any free carbohydrate compounds attached on them. Column chromatography was operated where de-oiled BBE solution (0.1 mg/ml in 70% ethanol) was allowed to pass through pre-activated resin. Preparative were collected accordingly at a flow rate of 5 ml/min by following Jarial et al. [9] with slight modification. The collected preparative was evaporated by a rotary evaporator (IKA, RV10, India) at 40^oC and stored in glass vials at 4^oC.

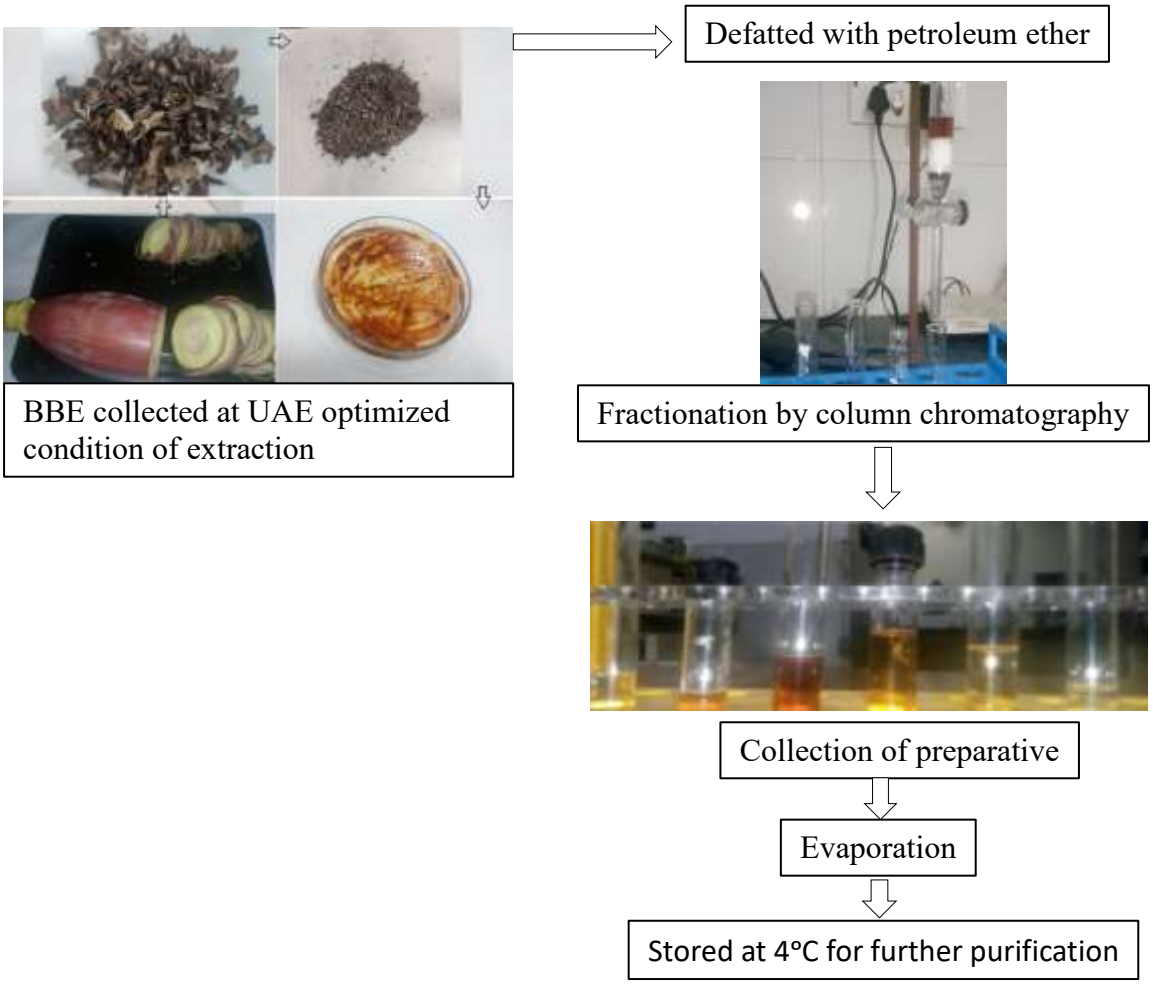

Fig. 4.1 Flowchart of collection of preparative from bhimkol blossom extract (BBE)

4.2.2.3 Isolation and purification of phytochemical rich fraction by RP-HPLC

A system of RP-HPLC (DIONEX, SR3000, US), with column C18, 5 µm particle size, 4.6 ID, 250 mm column length with a binary pump (DIONEX, LPG-3400SD) was used to separate flavonoids and collect the phytochemical rich fraction according to its retention time [13]. Mobile phases were taken in reagent glass bottles and degassed by using sonication bath for 20 min at 35^{o}C prior to the HPLC run. During degassing, bottles wre loosely capped to allow gas to go out gradually. For the run, 50 µl of evaporated preparative (3 mg/ml) was injected in mobile phase A: 3% v/v acetic acid (in acetonitrile) and B: methanol, 30:70 (A:B) % at 254 nm, 0.8 ml/min. Similarly, standard quercetin and catechin (Sigma aldrich) were run for the comparative identification of phytochemicals present in preparative. The retention time of detected quercetin by UV-visible DAD detector (DIONEX, VWD-3100) was noted. The fraction of phytochemical (BBQ-bhimol blossom quercetin) was collected in 100 ml volumetric flusks through

column void volume (4.15 ml) and volume of the outer collecting pipe (5 ml). Collected solution of phytochemical rich fraction was then evaporated to solid form by rotary evaporator at 40^{o}C. Isolated phytochemical rich fraction were quantified and calculated for its purity and yield. Antioxidant activity was also determined by DDPH inhibition assay by following Abdelaziz et al. [1], and Muchahary and Deka [12] with slight modification. After that, Isolated phytochemical rich fraction was stored at 4^{o}C in an airtight glass vials till futher analysis.

4.2.3 Characterization of isolated phytochemical rich fraction

4.2.3.1 FTIR

To detect the ultraviolet-visible (UV-VIS) spectrum profile of the BBQ, the BBQ was scanned under FTIR (Nicolet Instruments 410, Thermo Scientific, USA). FTIR detection was used to analyze predictable peaks and their functional groups at the wavenumber range of 4000 to 500 cm^{-1}.

4.2.3.2 NMR

In the NMR instrument (JEOL, Japan), ^{1}H NMR spectra of BBQ were used to identify constituents concerning the chemical shifts by molecular structures. A dissolved mixture of BBQ in DMSO (solvent) was loaded in NMR tubes (5 mm) and analyzed [18].

4.2.3.3 GC-MS

The volatile compounds profiling were performed by Gas Chromatography-Mass Spectrometry (GC-MS), and the BBE was prepared at a concentration of 1 mg/ml in ethanol [16]. The volatile phytochemical screening was done using GC-MS (Agilent Technologies, 7890A, US) with the column BR-5 MS (stationary phase: 5% diphenyl / 95% dimethyl polysiloxane and specifications 30 m x 0.25 mm ID x 0.25 µm). The carrier gas 'helium' was used at 1 ml/min flow rate at split 10:1 by the detector TQ Quadrupole MS with the software MS Workstation 8. The oven temperature was programmed at 110^{o}C hold for 3.50 min up to 200^{o}C at the rate of 10^{o}C /min. The injector temperature was 280^{o}C with a total GC running time of 40 min.

4.3 Results and discussions

4.3.1 Collection of preparative from bhimkol blossom extract (BBE)

As previously reported in Muchahary and Deka [12], the identification of phytochemicals by RP-HPLC showed various phytochemical contents in BBE. Quercetin content with significant antioxidant activity was found to be major among other phytochemicals (chlorogenic acid, syringic acid, tannic acid, saponin, ferulic acid, caffeic acid, rutin, catechin, gallic acid, sinapic acid, and coumarin) identified which were stated for its possible health-beneficial effects and wide applications in the food and drug industry [12].

Isolation and purification of any compound depend on the further application and purity requirement. Column chromatography and RP-HPLC have been widely used to separate flavonoids and other phytochemicals [3]. After administration of BBE through XAD 7HP resin in column chromatography, a slight purple preparative was collected. The percentage yield of preparative collected from the column chromatography was obtained at 9.8±0.11%. After the column chromatography, preparative collected were investigated for phytochemicals in RP-HPLC.

4.3.2 Isolation and purification of quercetin rich fraction by HPLC

The standard quercetin and catechin (Fig. 4.2a and b) detected in HPLC were used for the identification of phytochemicals in preparative. Similarly, preparative yield showed two major peaks of quercetin and catechin in the HPLC chromatogram at 3.7 and 3.81 min retention time (Fig. 4.2c), respectively. Finally, quercetin rich fraction was collected according to its RT (3.7 min) from the HPLC column outlet pipe. After the evaporation of fractions in a rotary evaporator at 45^{o}C, the yield of quercetin was estimated at 2.35±0.08 µg/ml with 53.12±0.31% purity (Fig. 4.3). Evaporated quercetin rich fraction named as bhimol blossom quercetin (BBQ). Antioxidant activity (scavenging DPPH free radical) of BBQ was found to be 68±0.12%. Further BBQ was investigated for its physicochemical characterizations.

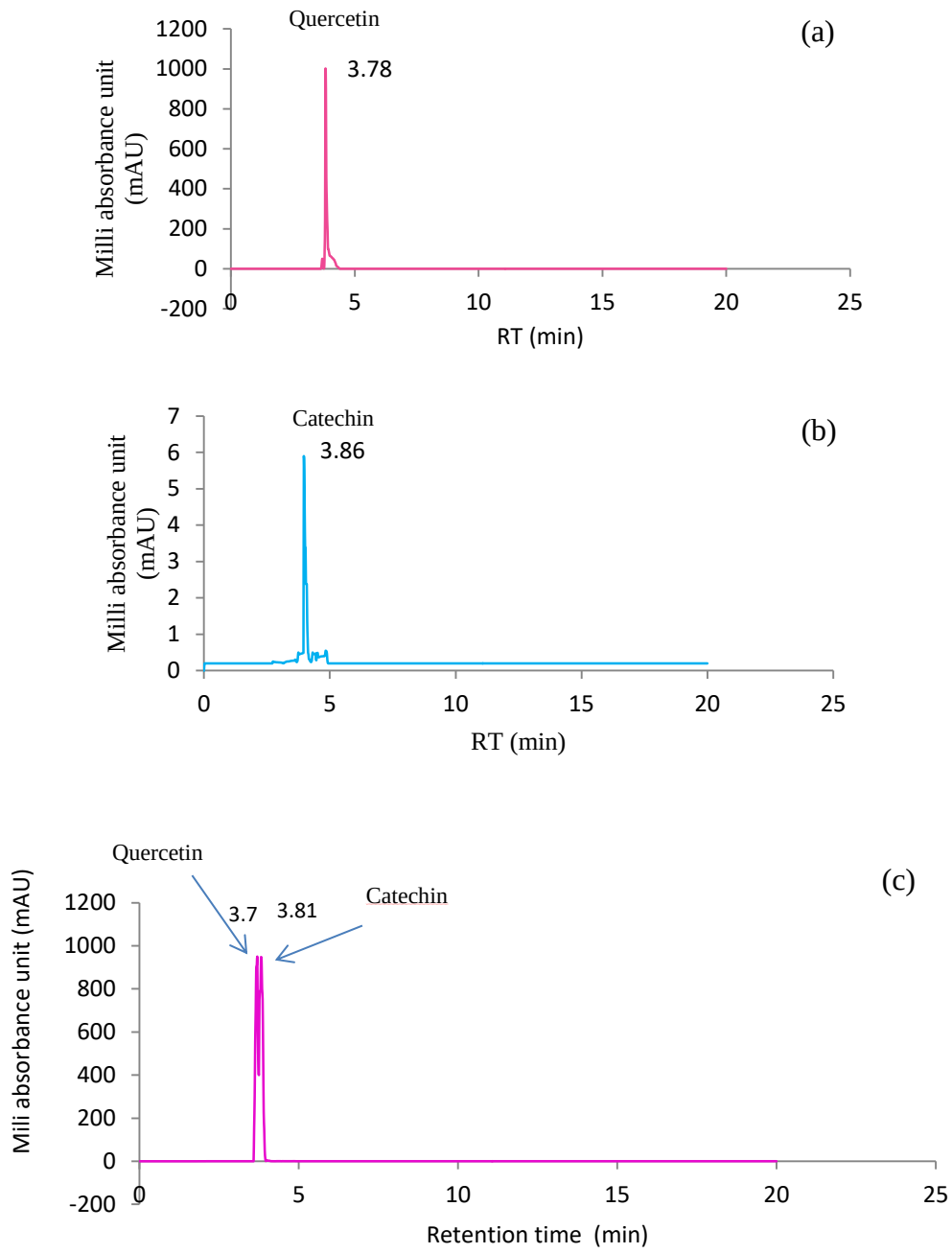

Fig. 4.2 HPLC chromatogram of standards and preparative fraction; (a) quercetin standard, (b) catechin standard, and (c) preparative fraction

Fig. 4.3 Evaporated quercetin rich fraction (BBQ) collected

4.3.3 Characterization of isolated quercetin rich fraction

4.3.3.1 NMR

The isolated compound was characterized by the comparison with ^{1}H NMR literature data which was in agreement with the proposed structure (Fig. 4.4) reported by De Souza et al. [7] and Saraswathi et al. [18]. The chemical structure of quercetin in BBQ ($C_{15}H_{20}O_7$) showed major signals of ^{1}H NMR spectra at ppm 6.38 H^6, 6.48 H^8, 6.8-6.9 $H^{5'}$, 7.64 $H^{6'}$, 7.74 $H^{2'}$, 10.1-10.31 C_{11}-OH, 10.79 C_{12}-OH, 11.66 C_{13}-OH, and 3.3-3.31 for the solvent (DMSO) used. Some characteristic peaks were observed at 6.38 and 6.48 ppm which corresponds to meta-protons H^6 and H^8 on a ring, 6.8 to7.64 ppm at the ABX system, and 7.74 corresponds to the catechol protons on the B ring. Peaks at 10.1-10.31, 10.79 and 11.66 ppm correspond to the hydroxyl groups of the quercetin structure.

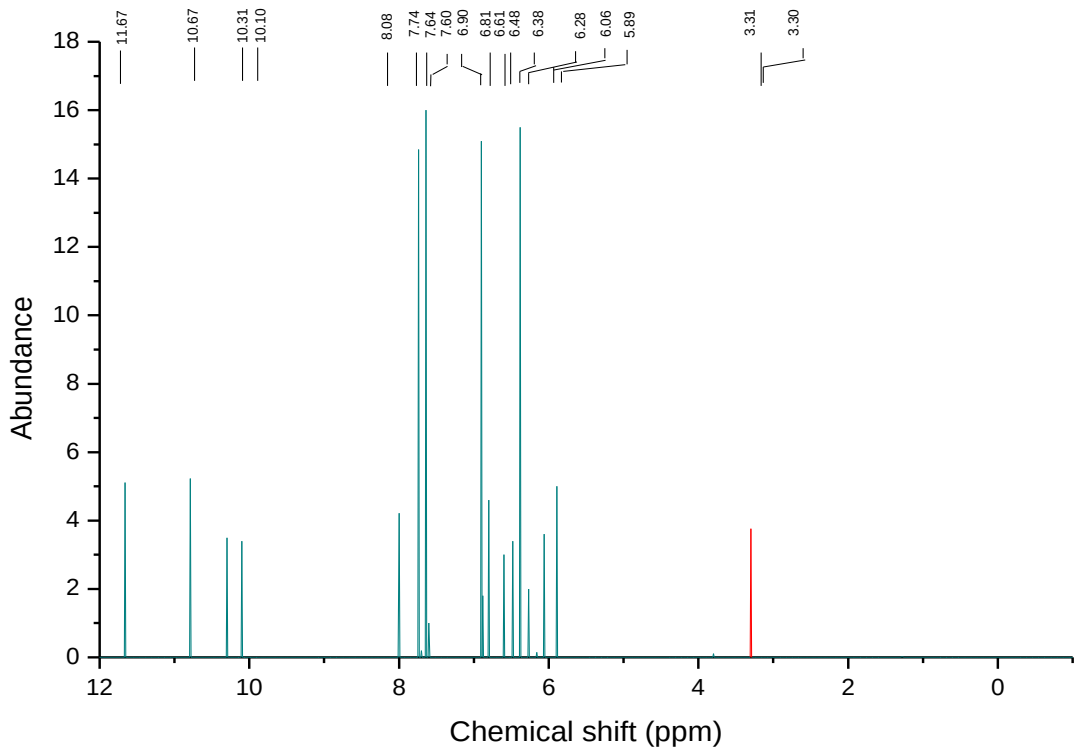

Fig. 4.4 NMR spectra of quercetin rich fraction (BBQ)

4.3.3.2 FTIR

The FTIR spectra (Fig. 4.5) of both standard quercetin (Q) and BBQ can be compared basis on their O-H, C-O, and C=O bond stretching in respective wavenumbers. Similar absorbance bands have been reported by many researchers. In the FT-IR spectrum of the standard quercetin, the broad peak at 3337 cm^{-1} corresponds to the pervasiveness of phenolic compounds containing free hydroxyl groups [19]. The absorption peak located at around 2956 cm^{-1} is due to C-H stretching in methyl and methylene groups [21]. The absorption peak at 1671 cm^{-1} is related to the stretching vibration of C=O in acryl ketones [17]. The peak at 1612 cm^{-1} can be attributed to the stretching vibration of C=C in aromatic rings [5, 22]. The peaks appeared at 1511 cm^{-1}, 1380 cm^{-1}, and 1241 cm^{-1} are attributed to the stretching vibration of C=O in aromatic rings [17], bending vibration of O-H bond in phenols [11], and stretching vibration of C-O in aryl ether [17], respectively. The peaks centered at 1166 cm^{-1} and 1093 cm^{-1}can be attributed to epoxy and alkoxy C-O stretching vibrations, respectively [5, 4]. The band located at 997 cm^{-1} is due to the deformation vibration of C-H bonds [18]. The bands centered at 933 cm^{-1}, 808

cm^{-1}, 703 cm^{-1}, and 599 cm^{-1} are due to the bending vibration of aromatic hydrocarbons [17].

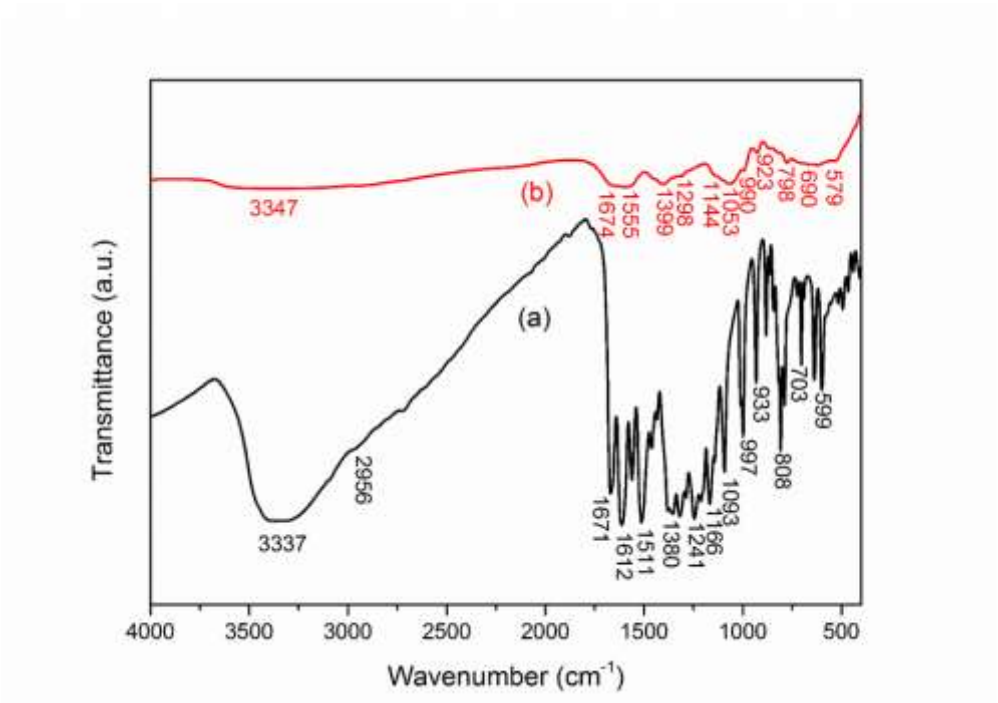

Fig. 4.5 FTIR spectra of quercetin standard and quercetin rich fraction (BBQ), (a) quercetin standard, and (b) quercetin rich fraction (BBQ)

In the FT-IR spectrum of the BBQ, the band at 3347 cm^{-1} was assigned to the stretching vibration of hydroxyl groups in the quercetin chemical structure [19]. The absorption peaks at 1674 cm^{-1} and 1555 cm^{-1} are related to the stretching vibration of C=O in acryl ketones [17] and the stretching vibration of C=C in aromatic rings [5, 22]. The peaks appeared at 1399 cm^{-1}, 1298 cm^{-1}, 1144 cm^{-1}, and 1053 cm^{-1} are attributed to the bending vibration of the O-H bond in phenols [11], stretching vibration of C-O in Aryl ether [17], and epoxy/alkoxy C-O stretching vibrations, respectively [5, 4]. The band located at 990 cm^{-1} is due to the deformation vibration of C-H bonds [18]. The bands centered at 923 cm^{-1}, 798 cm^{-1}, 690 cm^{-1}, and 579 cm^{-1} are due to the bending vibration of aromatic hydrocarbons [17]. So, the FTIR results indicated that the bonds present in the chemical structure of the isolated extract coincided with those seen for the standard quercetin.

4.3.3.3 GC-MS characterization of volatile compounds

Volatile compounds profiling of isolated fraction by GC-MS showed four major distinguished peakes at 10.07, 11.38, 12.96 and 14.99 min retention time as shown in Fig. 4.6a. The mass-to-charge ratio peaks of majorities are shown in Fig. 4.6b, c, d, and e. According to the MS- library, the compound identified the mass 253 m/z at 10.07 min, 327 m/z at 11.38 min, 135 and 197 m/z at 12.96 min, 135 m/z at 14.99 min. According to the MS-library, the compound with mass 253 m/z is monoaromatic steranes [2], 327 m/z is orientin isomer, 135 m/z is butin isomers (flavonoid isomers) [1], and 197 m/z is ethyl gallate (fragments produced when gallic acid is condensed in ethanol) [20]. Hence these were the volatine compounds present in the quercetin rich fraction (BBQ), where some might have potential antioxidant activities according to some research [1, 10]. High potential medicinal property of BBQ encourages food industries and pharmaceutical sector to further utilize the concept and administer to gain full benefits from of it.

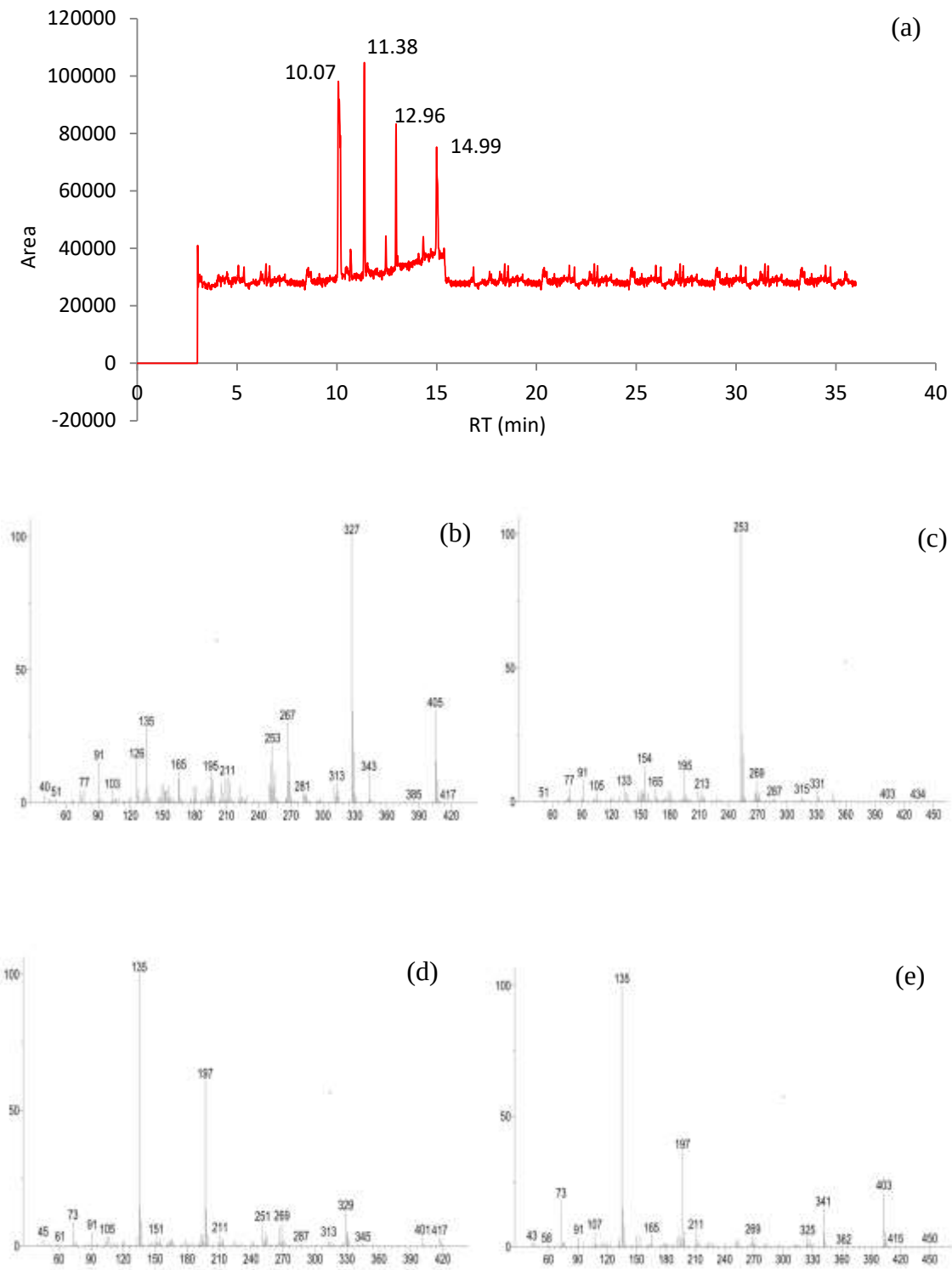

Fig. 4.6 Major volatile compounds of fraction; (a) GC-MS chromatograph of volatile compounds, (b) mass-to-charge ration at 10.07 min, (c) mass-to-charge ration at 11.38 min, (d) mass-to-charge ration at 12.96 min, and (e) mass-to-charge ration at 14.99 min

4.4 Conclusions

This study successfully conducted quercetin isolation and purification from wildly grown underutilized bhimkol blossom. Characterized isolated quercetin rich fraction was obtained using chromatographic methods had purity of isolated quercetin rich fraction 53.12±0.31% with antioxidant activity (68±0.12%). Remaining percentage of purity might be occupied by other phytochemicals and some bioactive compounds. Isolated quercetin rich fraction along with four major volatile compounds may have high possibilities of medicinal properties. This can further carry for application in numerous ways (*viz*., encapsulation, incorporation in food model, etc.).

References

1. Abdelaziz, S., Al Yousef, H. M., Al-Qahtani, A. S., Hassan, W. H., Fantoukh, O. I., and El-Sayed, M. A. Phytochemical profile, antioxidant and cytotoxic potential of *Parkinsonia aculeata* L. growing in Saudi Arabia. *Saudi Pharmaceutical Journal*, *28*(9), 1129-1137, 2020.
2. Barakat, A. O., Qian, Y., Kim, M., and Kennicutt Ii, M. C. Compositional changes of aromatic steroid hydrocarbons in naturally weathered oil residues in the Egyptian western desert. *Environmental Forensics*, *3*(3-4), 219-225, 2002.
3. Cai, W., Gu, X., and Tang, J. Extraction, purification, and characterisation of the flavonoids from *Opuntia milpa* alta skin. *Czech Journal of Food Sciences*, *28*(2), 108-116, 2010.
4. Chandran, M., Mangaleshwari, R., Udhayavani, K. R., and Murugan, K. Studies on FTIR analysis of fraction I and II of *Annona squamosa* methanol leaf extract. *World Journal of Pharmacy and Pharmaceutical Sciences*, *5*(8), 1247-1256, 2016.
5. Chandu, B., Mosali, V. S. S., Mullamuri, B., and Bollikolla, H. B. A facile green reduction of graphene oxide using *Annona squamosa* leaf extract. *Carbon Letters*, *21*, 74-80, 2017.
6. Coria-Téllez, A. V., Montalvo-Gónzalez, E., Yahia, E. M., and Obledo-Vázquez, E. N. *Annona muricata*: A comprehensive review on its traditional medicinal uses, phytochemicals, pharmacological activities, mechanisms of action and toxicity. *Arabian Journal of Chemistry*, *11*(5), 662-691, 2018.

7. De Souza, L. A., Tavares, W. M., Lopes, A. P. M., Soeiro, M. M., and De Almeida, W. B. Structural analysis of flavonoids in solution through DFT 1H NMR chemical shift calculations: Epigallocatechin, Kaempferol and Quercetin. *Chemical Physics Letters*, *676*, 46-52, 2017.
8. Ezzati, M., Yousefi, B., Velaei, K., and Safa, A. A review on anti-cancer properties of Quercetin in breast cancer. *Life Sciences*, *248*, 117463, 2020.
9. Jarial, R., Shard, A., Thakur, S., Sakinah, M., Zularisam, A. W., Rezania, S., and Singh, L. Characterization of flavonoids from fern *Cheilanthes tenuifolia* and evaluation of antioxidant, antimicrobial and anticancer activities. *Journal of King Saud University-Science*, *30*(4), 425-432, 2018.
10. Ku, S. K., Kwak, S., and Bae, J. S. Orientin inhibits high glucose-induced vascular inflammation *in vitro* and *in vivo*. *Inflammation*, *37*(6), 2164-2173, 2014.
11. Kunjiappan, S., Chowdhury, R., and Bhattacharjee, C. A green chemistry approach for the synthesis and characterization of bioactive gold nanoparticles using *Azolla microphylla* methanol extract. *Frontiers of Materials Science*, *8*(2), 123-135, 2014.
12. Muchahary, S. and Deka, S. C. Impact of supercritical fluid extraction, ultrasound-assisted extraction, and conventional method on the phytochemicals and antioxidant activity of bhimkol (*Musa balbisiana*) banana blossom. *Journal of Food Processing and Preservation*, *45*(7), e15639, 2021.
13. Pandiyan, R. and Ilango, K. Isolation, characterization, and validation of RP-HPLC method for the quantification of quercetin in *Huberantha senjiana* leaf extract. *Journal of Applied Pharmaceutical Science*, *10*(5), 110-118, 2020.
14. Pimentel-Moral, S., de la Luz Cádiz-Gurrea, M., Rodríguez-Pérez, C., and Segura-Carretero, A. Recent advances in extraction technologies of phytochemicals applied for the revaluation of agri-food by-products. *Functional and Preservative Properties of Phytochemicals*, 209-239, 2020.
15. Rasul, M. G. Extraction, isolation and characterization of natural products from medicinal plants. *International Journal of Basic Science and Applied Computer*, *2*(6), 2018.
16. Ravi, S., Shanmugam, B., Subbaiah, G. V., Prasad, S. H., and Reddy, K. S. Identification of food preservative, stress relief compounds by GC–MS and HR-

LC/Q-TOF/MS; evaluation of antioxidant activity of *Acalypha indica* leaves methanolic extract (*in vitro*) and polyphenolic fraction (in vivo). *Journal of Food Science and Technology*, *54*(6), 1585-1596, 2017.

17. Sambandam, B., Thiyagarajan, D., Ayyaswamy, A., and Raman, P. Extraction and isolation of flavonoid quercetin from the leaves of *Trigonella foenum-graecum* and their anti-oxidant activity. *International Journal of Pharmacy and Pharmaceutical Sciences*, 8, 120-124, 2016.
18. Saraswathi, V. S., Saravanan, D., and Santhakumar, K. Isolation of quercetin from the methanolic extract of *Lagerstroemia speciosa* by HPLC technique, its cytotoxicity against MCF-7 cells and photocatalytic activity. *Journal of Photochemistry and Photobiology B: Biology*, *171*, 20-26, 2017.
19. Senthamilselvi, S., Kumar, P., Prabha, A. L., and Govindaraju, M. Green simplistic biosynthesis of anti-bacterial silver nanoparticles using *Annona Squamosa* leaf extract. *Nano Biomedicine & Engineering*, *5*(2), 102-106, 2013.
20. Sun, J., Liang, F., Bin, Y., Li, P., and Duan, C. Screening non-colored phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries. *Molecules*, *12*(3), 679-693, 2007.
21. Vanitha, V., Hemalatha, S., Pushpabharathi, N., Amudha, P., and Jayalakshmi, M. Fabrication of nanoparticles using *Annona squamosa* leaf and assessment of its effect on liver (Hep G2) cancer cell line. In *IOP conference series: Materials Science and Engineering*, Vol. 191, pages 012010, Bangkok, Thailand, 2017.
22. Zahoor, M., Shafiq, S., Ullah, H., Sadiq, A., and Ullah, F. Isolation of quercetin and mandelic acid from *Aesculus indica* fruit and their biological activities. *BMC Biochemistry*, *19*(1), 1-14, 2018.