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Polyphenol Compounds and their Benefits of *Mangifera indica* L. (Var. Kottukonam) Grow in varied Seasons and Altitude

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ABSTRACT: Secondary metabolites are bioactive chemical compounds that play a key role in defense and adaptation mechanisms, widely distributed in all parts of plants. Among them carbon-based metabolites such as polyphenols and tannins have greater ecological and economic importance. The major challenge of the present study was to fractionate and quantify the polyphenol composition through HPLC and LC-MS/MS analysis from the leaves of *Mangifera indica* L. (var. kottukonam) collected from three altitudes in four different seasons. The results of the study revealed great variation in the type and concentration of polyphenols with respect to the season and altitude. Pre-reproductive winter and reproductive summer seasons showed higher concentration of polyphenols than south west and north east monsoon seasons, coastal Vizhinjam sample revealed higher concentration of such antioxidant phytochemicals than the other altitude samples. Among different polyphenols Gallic acid concentration is highest in almost all the samples. More stressed conditions resulted to the production of more polyphenols in kottukonam variety of mango.

Keywords: HPLC, LC-MS/MS, *Mangifera indica*, Kottukonam, Polyphenols, Gallic acid.

Abbreviations: HPLC, High Performance Liquid Chromatography; LC-MS/MS, Liquid Chromatography-Mass Spectroscopy; ppm, parts per million; Rt, Retention time.

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most economically important tropical fruit crop belongs to the family Anacardiaceae. It is an evergreen tree well known for its nutritious and sweet fruit designated as `the King of Fruits' in India. Over 1500 varieties of mangoes were reported globally (Kshirsagar *et al*., 2018) and their exporting greatly influences the economy of many developing countries. Kottukonam is one of the most popular variety of *Mangifera indica* L. cultivated and restricted only to the southern parts of Kerala especially in the Thiruvananthapuram and Kollam Districts. The fruits of Kottukonam are so sweet, nutritious and more solid after ripening than other common varieties available in our state and is very rich in fibre content also.

Secondary metabolites are bioactive chemical compounds that play a key role in defense and adaptation mechanisms, widely distributed in all parts of plants. Among them carbon-based metabolites such as polyphenols and tannins have greater ecological and economic importance. Polyphenols are omnipresent secondary metabolites present in 5 to 50 gm/kg dry weight of plants and were synthesized via phenyl propanoid pathway. They provide protection to the plants against pathogens, herbivores (Skarpe and Hester 2008) and UV radiations (Tharayil *et al*., 2011). These compounds are directly related to their pharmacological properties and have the greatest potential of being valuable to human health (Podsedek and Anna 2007). Epidermological evidence supported the role of polyphenols in diet which in turn play an important role in the prevention of cancers and cardiovascular diseases (Pandey *et al*., 2009). They also have antioxidant and anti-inflammatory properties could have therapeutic and prophylactic effects for neurogenerative diseases and these health benefits makes the phenolic compounds become the most researched secondary metabolic compounds (Cory *et al*., 2018).

Polyphenols having biogenetic precursor are categorised in to; benzoic acid derivatives, hydroxycinnamic acid derivatives and depside. Ferulic, p-Caumaric, simple Coumarins, Caffeic, and Sinapic acids are simple and common phenylpropanoids and are formed through methylation, hydroxylation and dehydration reactions of cinnamic acid. The glycosides such as vanillic, p-hydroxy benzoic acid, protocatechuic etc. are benzoic acid derivatives with antibiotic, analgesic, antipyretic, sedative and cholagogic properties. Flavonoids are a class of polyphenols in plants having the general structure of a 15-carbon skeleton with two phenyl rings and a heterocyclic ring. Fisetin, quersetin, myricetin and kaempferol are the most studied flavonoids. They have a broad spectrum of biological activities in plants including cell signalling, auxin transport and pigmentation. Neutraceutical, medicinal, pharmaceutical and cosmetic applications makes the flavonoids an indispensable part in human life. The other biomolecules such as tannins, alkaloids, saponins and steroids also

plays key role in plants in the protection from predation and are having immense biological potentialities.

The availability and concentration of phytochemicals in plants are affected by seasonal variations (Uma *et al*., 2015; Mervat *et al*., 2009; Yao *et al*., 2005; Mayer *et al*., 1995). The change in amount of secondary metabolites in relation with seasonal and altitude variations in *Mangifera indica* L. (var. Kottukonam) is an underexplored area of research. In this scenario, the present study focused to fractionate and quantify the polyphenol composition through chromatographic techniques such as LC-MS/MS and HPLC analysis from the leaves of *Mangifera indica* L. (var. kottukonam) collected from three altitudes in four seasons.

MATERIALS AND METHODS

Plant and Sample Collection Area. The leaves from *Mangifera indica* L. (var. Kottukonam) with 50 to 55 years old is the study material collected from (1) Venjaramoodu (normal sea level area with Latitude: 8.68 N, Longitude: 76.91 E, Altitude: 53.00m above sea level) (2) Vizhinjam (Latitude: 8.38 N, Longitude: 77 E, 45.00m above sea level) and (3) Kulathuppuzha (Latitude: 8º 54′32.46″N Longitude: 77º 3′33.57″E Altitude: 203.6m above sea level). The first two sampling areas belongs to the Thiruvananthapuram district and last one to Kollam of Kerala state in India. Kulathuppuzha and Vizhinjam are semi high range and coastal areas respectively. The samples were collected in four seasons such as summer (February – May, average temp. of 32 to 36ºC and 135mm pptn. – sample 1), South West Monsoon (June – September, average temp. of 19 to 30ºc and 2250 to 2500mm pptn. – sample 2), North East Monsoon (October – November, average temp. of 29 to 35ºc and 450 to 500mm pptn. – sample 3) and Winter (December – beginning of February, average temp. of 18 to 28ºC and 25mm pptn. – sample 4).

Soxhlet hot continuous extraction. Finely hewed 100g of plant leaves are dried in shade at room temperature, powdered and successively extracted with 100 ml methanol for eight hours using Soxhlet hot continuous extraction method. The filtered extract was concentrated using rotary evaporator (50ºC).

Preliminary qualitative screening of polyphenols. The soxhlet extract of samples were screened for the presence of different polyphenolic compounds based on the method of Khandelwal (2007).

Test for tannins. Ferric chloride test: Add a few drops of ferric chloride solution in a small amount of extract which is diluted with distilled water in the ratio 1:4. The presence of tannin were indicated by the appearance of blue or green colour.

Test for phenols. Ferric chloride test: The extract was treated with a few drops of 5% neutral ferric chloride solution, appearance of an intense blue colour resulted the presence of phenols.

Test for flavonoids. Aqueous Sodium hydroxide test: The extract was treated with 1N aqueous NaOH, resulted yellow orange colour revealed the presence of flavonoids.

Test for alkaloids. Wagner's test: The addition of wagner's reagent (1.27g of iodine and2g of potassium iodide dissolved in 5ml of water and made up to 100ml with distilled water) to a fraction of extract, appearance of reddish-brown precipitate indicates the presence of alkaloids

Test for saponins. Foam test: A small amount of plant extract was vigorously shaken, formation of persistent foam indicates the presence of saponins.

Test for terpenoids. Liebermann-Burchard test: A few drops of acetic anhydride followed by a little drops of conc. H_2SO_4 were added to the extract (1ml) dissolved in chloroform producing dark green colour confirms the presence of terpenoids.

Test for steroids. To the extracts evaporated to dryness, add a little drops of conc, H_2SO_4 and acetic anhydride; an array of colour changes from yellow, green, brown to black detects the presence of steroids.

Quantification of phenols and flavonoids. Total phenols content were estimated by the method of Mayer *et al*., 1995. An aliquot of the extracts of four samples in 80% methanol were pipetted out separately and made up to 3 ml with the solvent. Add 0.5 ml Folin-ciocalteau reagent in to it and kept for 3 minutes. Boiling the mixture in a water bath for 1 minute after adding 2 ml of 20% Na_2CO_3. Remove the white precipitate by centrifuging it for few min and the absorbance of the clear light blue solution was recorded at 650 nm against the reagent blank containing 80% methanol (3ml), 0.5 ml Folin's reagent and 2 ml of 20% Na_2CO_3. Blue complex formation is due to the reaction between phenols and an oxidizing agent phosphomolybdate in Folin-ciocalteau reagent. A standard graph of phenols was constructed with pyro catechol by taking absorbance against concentration. The total phenols/gm tissue was determined from the standard graph.

$AlCl_3$ method with slight modification done the quantification of total flavonoid content in the methanol extract. Concisely 100 µl of extract was mixed with 100 µl of 20% $AlCl_3$ and add 2 drops of glacial acetic acid. After diluting the mixture with methanol to 3 ml incubated for 45 minutes. Then read the OD at 415 nm against the blank having extract, methanol and glacial acetic acid. Construct the standard curve by using quercetin (50-250 µg/ml) in methanol under the same condition. Total flavonoid were indicated as mg quercetin equivalent/gm of weight (Absorbance = 4.9747 mg quercetin, R2 = 0.9846).

Quantification by high performance liquid chromatography (HPLC). The methanol extract of four samples from four seasons and 13 reference compounds (1mg/mL) were filtered through 0.45µm PTFE filter; 10µL was injected into the HPLC system. The analysis was performed on a prominence UFLC system Shimadzu, Japan) containing LC-20AD system controller, phenomenex Gemini C18 column (250 × 4.6mm, 5µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 10µl volume and a diode array detector (SPD-M20A). The mobile phase used was, solvent A: methanol- acetic acid – water (10:2:88, v/v) and solvent B; methanol – acetic acid – water (90:2:8, v/v) with gradient program 0-15min 15% B, 16 -20min 50% B, 21 -35min 70% B, 36-50min 100% B and finally the column was regenerated in 10min. The

injection volume was 10µL, and the flow rate was kept at 1mL/min. The column was maintained at room temperature and eluted fractions were monitored at 280 nm, each calibration solution was analyzed. Sample peaks were identified by comparing with retention times of standard peaks. The results are represented as polyphenols in parts per million. LC Lab solution software was used for data acquisition and analysis.

Quantification using liquid chromatography and mass spectroscopy (LC-MS/MS). The extracts in 80% methanol and 28 reference compounds (catechol, catechin, quinine, naringenin, tocopherol, gallic acid, chlorogenic acid, epicatechin, syringic acid, vanillic acid, caffeic acid, epigallocatechin, ferulic acid, myricetin, quercetin, p-Coumaric acid, luteolin, apigenin, kaempferol, rutin, diadzein, hesperetin, shikimic acid, elagic acid, morin, genistein, cinnamic acid and chrysin were injected to LC-MS/MS system (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan) - HPLC (Nexera LC-30AD) equipped with an autosampler (SIL-30AC), temperature-controlled column oven (CTO-20AC) and prominence diode array detector (SPD-M20A) coupled to triple quadrupole mass spectrometer (Nexera with LCMS-8045, Shimadzu Corporation, Kyoto, Japan). Working standards were prepared by diluting the stock solution with water concentration ranging from 0.01- 1 µg/ml. The quantification of all the polyphenols was carried out on Shimadzu Shim-pack GISS C18 column (150 × 2.1 mm i.d, 1.9 µm) that used water/ formic acid (100/0.1%) mobile phase for solvent A and 100% methanol for solvent B. Polyphenols were eluted with a linear gradient system as follows: 0.5 – 1.9 min 5% of solvent B, 2.0 – 10.0 min 98% of solvent B, 10.1 – 15 min 98% of solvent B and 15.1 – 17 min 5% of solvent B, a flow rate of 0.3 mL/min, the injection volume was 10 µl and oven temperature of 40 °C. Negative and positive modes of multiple reaction-monitoring (MRM) mode were operated during LC-MS/MS with electrospray ionization (ESI). LC-MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan). An interface temperature of 400°C was conditioned for ionization, desolvation line temperature of 300°C, heat block temperature of 400°C, nebulizing gas flow (nitrogen) at 3 L/min and drying gas flow (nitrogen) at 10 L/min. Each calibration solution was analyzed. The results obtained are represented as polyphenols in parts per million.

Statistical Analysis. The data obtained were analyzed statistically by one-way analysis of variance (ANOVA) and t- test ($p < 0.05$). The results were average of five replications and represented as mean ± SD.

RESULTS AND DISCUSSION

The phytochemical extraction in methanol and their profiling through HPLC and LC-MS/ MS provides an understanding of polyphenols in *Mangifera indica* L. (var. Kottukonam) from three altitudes in four seasons. The qualitative screening of various phytochemicals in 12 samples revealed the presence of phenols, alkaloids, tannins, steroids, flavonoids, terpenoids and saponins (Table 1). The quantification of Carbon based phenolic compounds such as phenols and flavonoids showed their remarkable variations in these 12 samples (Table 2a & 2b). Phenol (sample 1 Vzm, 47.45±0.23 mg/gm tissue: sample 1 Kza, 34.87±0.27 mg/gm tissue: sample 1 Vjd, 28.439±0.30 mg/gm tissue), (sample 4 Vzm, 45.41±0.22 mg/gm tissue: sample 4 Kza, 32.23±0.11 mg/gm tissue: sample 4 Vjd, 25.82±0.26 mg/gm tissue)and flavonoid (sample 1 Vzm, 56.52±0.27 mg/gm tissue: sample 1 Kza, 47.36±0.55 mg/gm tissue: sample 1 Vjd, 41.472±0.22 mg/gm tissue), (sample 4 Vzm, 58.36±0.21 gm/gm tissue: sample 4 Kza, 48.32±0.32 mg/gm tissue: sample 4 Vjd, 38.554±0.92 mg/gm tissue)concentration were relatively much more in the samples of summer (sample 1) and winter (sample 4) seasons than that are in the south west monsoon and north east monsoon seasons. The additional stress, salinity in coastal region influenced the mango tree and the higher concentration of phenolic compounds in them is the result than the samples of other altitudes in all the four seasons. The result of the present study also an agreement to the previous work (Kale 2010). HPLC and LC-MS/MS analysis of the 12 samples gave more concreate informations about the concentration of different polyphenols in different altitude and seasons.

Thirteen standard phenolic compounds (1 mg/ml) namely (1) Gallic Acid (2) Chlorogenic Acid (3) Syringic Acid (4) p-Coumaric Acid (5) Kaempferol (6) Catechol (7) Apigenin (8) Caffeic Acid (9) Ferulic Acid (10) Cinnamic Acid (11) Quercetin (12) Myricetin and (13) Elagic Acid were analysed by HPLC, their retention time (Rt.) in minutes were 4.27, 23.31, 24.42, 25.98, 34.11, 11.74, 34.72, 23.84, 26.42, 30.39, 31.27, 28.81, and 28.35 respectively (Table 3) & (Fig. 1). Quantification and identification were done by comparing the Retention time and spectra of samples with that of standards.

Fractionation of polyphenols by HPLC in the leaves collected during summer season (sample 1) revealed the presence of gallic acid (Vzm, 5686.518: Kza, 34.294 : Vjd,4064.68 ppm), chlorogenic acid (Vzm, 658.242 ppm: Kza, 990.934 ppm: Vjd,888.382 ppm), Syringic acid (Vzm, 24.995 ppm: Kza, 143.623 ppm: Vjd,11.100 ppm), p-coumaric acid (Kza, 0.604 ppm: vjd,9.570 ppm), ferulic acid (Kza,237.534 ppm: Vjd,38.784 ppm), cinnamic acid (Vzm, 1.404 ppm: Vjd,1.404 ppm) and quercetin(Vzm, 1.585 ppm) whereas leaves from South West Monsoon (sample 2) indicated the presence of gallic acid (Vzm, 473.831 ppm: Kza, 35.432 ppm: Vjd, 4346.023 ppm), chlorogenic acid (Vzm, 832.826 ppm: Kza, 477.516 ppm: Vjd, 258.802ppm), syringic acid (Vzm, 67.671 ppm: Kza, 55.769 ppm:Vjd, 18.223 ppm), p-coumaric acid (Vzm, 84.365 ppm: Kza, 39.31 ppm) and ferulic acid (Vzm, 107.403 ppm: Kza, 65.428 ppm) (Table 4a) (Fig. 2a).

Polyphenol profiling in the leaves of *Mangifera indica* L. (var. kottukonam) in North East Monsoon (sample 3) by HPLC revealed the presence of four phenolic compounds namely gallic acid (Vzm, 736.608 ppm: Kza, 4.751 ppm: Vjd, 4499.064ppm), chlorogenic acid (Kza, 54.213 ppm: Vjd, 84.0797 ppm), syringic acid (Vzm, 266.592 ppm: Kza, 157.05 ppm) and ferulic acid(Vzm, 296.349 ppm: Kza, 186.80 ppm), but the samples

collected during Winter season (sample 4) showed gallic acid (Vzm, 5393.412 ppm: Kza, 8.443 ppm: Vjd, 4334.636ppm), chlorogenic acid (Kza, 495.188 ppm: Vjd, 66.828ppm), syringic acid (Vzm, 26.198 ppm: Kza, 78.807 ppm: Vjd, 2.156 ppm), ferulic acid (Kza, 97.246 ppm: Vjd, 1.308 ppm), cinnamic acid (Vjd, 1.956ppm) and quercetin (Vjd, 1.708ppm) (Table 4b) & (Fig. 2b)

From the 12 samples of *Mangifera indica* L. (var. kottukonam), summer (Vzm, 5: Kza, 5: Vjd, 6) and winter (Vzm, 2: Kza, 4: Vjd, 6) samples showed more forms of polyphenols. Gallic acid, a powerful antioxidant found in highest quantity almost in all the samples, followed by Chlorogenic acid (Table 4A and 4B) but which is absent in north east and winter season samples from coastal Vizhinjam region. p-Coumaric acid is totally absent in the samples from coastal area but ferulic acid present in them only in north east monsoon season. Cinnamic acid revealed in the summer samples from Vizhinjam (coastal) and Venjaramoodu (sea level), and Quercetin present only in coastal (Vizhinjam) sample in summer season and winter sample from normal sea level region (venjaramoodu). The diversity in the quantity and forms of phenolic compounds in various seasons and altitudes revealed the influence of environmental factors and is agreed with the result of previous researchers (Mpofu *et al.*, 2006; Yao *et al.*, 2005; Chinnusamy 2003).

To facilitates specific separation, identification and quantification of polyphenols in the 12 leaf samples of *Mangifera indica* L. (var. kottukonam) were subjected to the LC-MS/MS analysis against 28 standard polyphenolic compounds namely (1) Catechol, (2) Catechin (3) Quinine, (4) Naringenin, (5) Tocopherol, (6) Gallic acid, (7) Chlorogenic acid, (8) Epicatechin, (9) Syringic acid, (10) Vanilic acid, (11) Caffeic acid, (12) Epigallocatechin, (13) Ferulic acid, (14) Myricetin, (15) Quercetin, (16) p-Comaric acid, (17) Luteolin, (18) Apigenin, (19) Kaempferol, (20) Rutin, (21) Diadzein, (22) Hesperetin, (23) Shikimic acid, (24) Elagic acid, (25) Morin, (26) Genistein, (27) Cinnamic acid and (28) Chrysin. The results obtained were represented in Table 5 (Fig. 3).

Polyphenol profiling by LC-MS/MS of sample 1 (summer season) revealed the presence of 24, 20, and 22 different phenolic compounds in Vizhinjam (coastal area), Kulathuppuzha (semi high range area) and Venjaramoodu (normal sea level area) respectively whereas their number in south west monsoon samples (sample 2) are 19 (Vzm), 18 (Kza) and 21 (Vjd) (Table 6a) & (Fig. 4a). Gallic acid and apigenin showed in highest and the lowest quantities respectively in sample 1 and sample 2.

LC-MS/MS analysis of the samples in north east monsoon (sample 3) indicated 22 (vizhinjam), 17 (kulathuppuzha) and 22 (venjaramoodu) phenolic compounds. 20 (vizhinjam), 17 (kulathuppuzha) and 23 (venjaramoodu) are the number in the forms of polyphenols in the leaf sample of winter season (sample 4) (Table 6b) &(Fig. 4b). Chrysin and apigenin showed least quantities and gallic acid revealed the highest concentration in almost all the samples in these two seasons.

LC-MS/MS analysis of the methanol extracts of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes in four seasons revealed the influence of different abiotic stresses in the quantity and types of polyphenols. Gallic acid found in highest amount in almost all the samples, and apigenin and chrysin are in lowest amount. Gallic acid is a natural antioxidant helps to strengthen veins (Singh *et al.*, 2017) and to inhibit the formation of amyloid fibrils- the principal causes of Alzheimer's diseases (Mair 2000). Crysin and apigenin showed almost similar quantity in the samples (Table 6a & 6b) but other polyphenols exhibited greater variations from sample to samples. Variation in polyphenol concentration is a mechanism that helps the plants to overcome or cooperate with various stressful environmental conditions.

Catechin- a natural polyphenol and effective for the treatment of neurogenerative diseases (Pervin *et al.*, 2018) is found in all the samples except sample in north east monsoon season from coastal area. Chlorogenic acid were absent in all the semi high range area samples in all the four seasons, but present in the samples from coastal Vizhinjam region (except in winter season) and also in sea level region (Venjaramoodu) samples except in south west monsoon season have anti-oxidant and anti-inflammatory properties (Sasaki *et al.*, 2010). Caffeic acid is a well-known antioxidant which boosts immunity and controls lipid levels in blood also showed its presence in all the samples. Flavonoids are phenolic compounds isolated from plants and are valuable for their immense biological potentialities. A flavonoid namely quercetin found in all the 12 samples displayed anti-histamine, anti-cancer and anti-inflammatory activities and have also been found to reduce serum levels of low-density lipoproteins (LDL) and increase high-density lipoproteins (HDL) in addition to the reduction of triglycerides and free fatty acids, attributes to its cardiovascular disease prevention (Semwal *et al.*, 2016; Nabavi *et al.*, 2015). Myricetin another polyphenol found in all samples known to interact with a range of DNA and RNA polymerases, reverse transcriptases, telomerases, kinases and helicases due to its iron-chelating, antioxidant, anti-inflammatory and anti-carcinogenic properties. Reverse transcriptase inhibition activities exposed it as a possible antiviral drug against Rauscher murine Leukemia Virus (RLV) and Human Immunodeficiency Virus (HIV) infections (Crozier *et al.*, 1997). Syringic acid present in all samples except in the semi high range area sample in north east monsoon and winter seasons were well known for its anti-cancer, anti-proliferative, sedative, decongestant and hepato-protective actions (Ramachandran and Raja 2010) and p-coumaric acid found in S1 Vzm, S1 Vjd and S4 Vjd were well documented for its antioxidant behavior reducing the formation of carcinogenic nitrosamines in the stomach. Ferulic acid is well-known for its physiological functions such as anti-microbial, anti-inflammatory and anti-cancer activities were noticed in all samples in summer season, sea level region (venjaramoodu) sample in south west monsoon, coastal and sea level samples in north east monsoon and only in the sea level region sample in

winter season. Ferulic acid acts as a natural protector against UV radiation (Rasouli *et al*., 2017) and lowers cholesterol level in serum and increases sperm viability.

Kaempferol revealed its presence in all the 12 samples were specific to its potent pharmacological and nutraceutical activities. The consumption of plants containing kaempferol thereby conferring innumerable health benefits in the form of reducing scourge of cardio vascular diseases, cancer and arteriosclerosis due to its antioxidant properties (Saw *et al*., 2014). It also modulate crucial component in cellular signal transduction pathways associated with apoptosis, angiogenesis, metastasis and inflammation (Bradley and David 2005). Naringenin found in all the samples is anti-inflammatory, anti-oxidant flavonoids and is effective in controlling sepsis, acute hepatitis, fibrosis and cancer (Jin *et al*., 2017; Qin *et al*., 2011; Du *et al*., 2009) and another polyphenol called epigallocatechin possesses many biological activities including angiogenesis (Kondo *et al*., 2002) also present in all the samples. Tocopherol, a natural antioxidant was absent only in S1 Vjd. Epicatechin -a flavone having direct anti-oxidant property as the scavenger of free radicals or indirectly as a modulator of glutathione peroxidase and superoxidedismutase (Simos *et al*., 2012) were noticed in all the samples. Hesperetin -another flavonon present in all the twelve samples have anti-oxidant, anti-inflammatory and neuroprotective effect in different models of neurodegeneration (Ikram *et al*., 2019; Muhammad *et al*., 2019). Shikimic acid were commonly utilized as an initial material for industrial synthesis of an antiviral drug, found highest amount in S1 Vjd but absent in the samples of Kulathuppuzha in all the four seasons. Elagic acid with anti-mutagenic and anti-cancer properties are used to reduce the elevated hepatic contents of phospholipids, cholesterol, triglycerides and free fatty acids were present in all samples whereas Morin, a polyphenol found in all samples have anti-cancer (Hyun *et al*., 2015; Park *et al*., 2014; Gupta *et al*., 2013; Manna *et al*., 2007) anti-diabetic (Sendrayaperumal *et al*., 2014; Paoli *et al*., 2013) anti-inflammatory (Dilshara *et al*., 2016; Dhanasekar *et al*., 2015; Ola *et al*., 2014; Qureshi *et al*., 2012) anti-oxidant (Komirishetty *et al*., 2016; Saw *et al*., 2014; Mohammad and Elham 2013; Kim *et al*., 2010) effects. Genistein found in all samples is an isoflavons used against bladder, breast and prostate cancers (Cory *et al*., 2018; Arts *et al*., 2005; Mills *et al*., 1989) and Chrysin is an effective inhibitor of tumor cell-induced angiogenesis (Fu *et al*., 2007) were found in all samples of summer season, only in the venjaramoodu sample in south west monsoon season, coastal and sea level region samples of north east monsoon and winter seasons in very few quantities. The presence of several phenolic acids and flavonoids in *Mangifera indica* L. (var. kottukonam) having high biological and pharmacological potentialities related with their antioxidant property against seasonal and altitude stresses.

Table 1: Preliminary polyphenol analysis using methanol extracts of *Mangifera indica* L. (var. kottukonam) leaves from three altitudes [Vizhinjam (Vzm): Kulathuppuzha (Kza): Venjaramoodu (Vjd) in four seasons [sample 1, summer: sample 2, south west monsoon: sample 3, north east monsoon: sample 4, winter) [+++; Strong positive, ++; Modarately positive, +; Low positive, -; Negative].

Phytochemical	Sample 1			Sample 2			Sample 3			Sample 4		
	Vzm	Kza	Vjd	Vzm	Kza	Vjd	Vzm	Kza	Vjd	Vzm	Kza	Vjd
Phenols	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Flavonoids	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Tannins	++	++	+	++	++	+	++	++	+	++	++	+
Alkaloids	+++	++	++	+++	+++	+++	+++	++	++	+++	++	++
Terpenoids	+++	++	++	+++	++	++	+++	++	++	+++	+++	+++
Steroids	+++	++	++	+++	++	++	+++	++	++	+++	+++	+++
Saponins	+	-	++	+	+	++	+	+	++	+	-	++

Table 2a: Total Phenols and Flavonoids from methanol extract of the leaves of *Mangifera indica* L. (var. Kottukonam) in summer (sample 1) and south west monsoon (sample 2) seasons in mg/ gm tissue [Values are mean ±SD of five independent replications].

Phytochemical	Sample 1			Sample 2		
	Vzm	**Kza**	**Vjd**	**Vzm**	**Kza**	**Vjd**
Phenols	47.45±0.23	34.87±0.27	28.439±0.30	39.28±0.21	29.11±0.33	20.355±0.21
Flavonoids	56.52±0.27	47.36±0.55	41.472±0.22	49.77±0.25	46.24±0.26	20.927±0.18

Vzm: Vizhinjam (coastal area), Kza: Kulathuppuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 2b: Total Phenols and Flavonoids from methanol extract of the leaves of *Mangifera indica* L. (var. Kottukonam) in north east monsoon (sample 3) and winter (sample 4) seasons in mg/ gm tissue [Values are mean ±SD of five independent replications].

Phyto Chemical	Sample 3			Sample 4		
	Vzm	**Kza**	**Vjd**	**Vzm**	**Kza**	**Vjd**
Phenols	38.31±0.33	29.34±0.51	20.259±0.99	45.41±0.22	32.23±0.11	25.82±0.26
Flavonoids	47.24±0.32	43.42±0.32	18.897±0.91	58.36±0.21	48.32±0.32	38.554±0.92

Vzm: Vizhinjam (coastal area), Kza: Kulathuppuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 3: The Retention time (Rt) of standard mixture of polyphenols.

Sr. No.	Polyphenols	Retention Time (in min.)
1.	Gallic Acid	4.27
2.	Chlorogenic Acid	23.31
3.	Syringic Acid	24.42
4.	p-Coumaric Acid	25.98
5.	Kaempferol	34.11
6.	Catechol	11.74
7.	Apigenin	34.72
8.	Caffeic Acid	23.84
9.	Ferulic Acid	26.42
10.	Cinnamic Acid	30.39
11.	Quercetin	31.27
12.	Myricetin	28.81
13.	Elagic Acid	28.35

Table 4a: Quantification of polyphenols in six leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam, Kulathuppuzha and Venjaramoodu] in two seasons [Sample 1, summer and sample 2, south west monsoon] by HPLC analysis.

Polyphenols (in ppm)	Sample 1			Sample 2		
	Vzm	Kza	Vjd	Vzm	Kza	Vjd
Gallic acid	5686.518	34.294	4064.68	473.831	35.432	4346.023
Chlorogenic acid	658.242	990.934	888.382	832.826	477.516	258.802
Syringic acid	24.995	143.623	11.100	67.671	55.769	18.223
p-coumaric acid	-	0.604	9.570	84.365	39.31	-
Ferulic acid	-	237.534	38.784	107.403	65.428	-
Cinnamic acid	1.404	-	1.404	-	-	-
Quercetin	1.585	-	-	-	-	-

Vzm: Vizhinjam (coastal area), Kza: Kulathuppuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 4b: Quantification of polyphenols in six leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam, Kulathuppuzha and Venjaramoodu] in two seasons [Sample 3, north east monsoon and sample 4, winter] by HPLC analysis.

Polyphenols (in ppm)	Sample 3			Sample 4		
	Vzm	Kza	Vjd	Vzm	Kza	Vjd
Gallic acid	736.608	4.751	4499.064	5393.412	8.443	4334.636
Chlorogenic acid	-	54.213	84.0797	-	495.188	66.828
Syringic acid	266.592	157.05	-	26.198	78.807	2.156
p-coumaric acid	-	-	-	-	-	-
Ferulic acid	296.349	186.80	-	-	97.246	1.308
Cinnamic acid	-	-	-	-	-	1.956
Quercetin	-	-	-	-	-	1.708

Vzm: Vizhinjam (coastal area), Kza: Kulathuppuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 5: The LC-MS/MS analysis of 28 standard polyphenols.

Sr. No.	Polyphenols	Rt (min)	Parent ion (m/z)	Molecular Ion (m/z)	Ion Mode	CE (V)	r^2	Linear Range (µg/L)	DL/QL (µg/L)
1.	Catechol	1.87	111.20	78.95/6410	+ve	-9/-25	0.986	5-50	0.07/0.23
2.	Catechin	6.75	291.20	139.10/165.05	+ve	-15/-13	0.993	5-150	2.20/6.68
3.	Quinine	6.88	325.20	307.10/184.05	+ve	-24/-28	0.996	5-150	0.27/0.81
4.	Naringenin	7.28	273.2	153.05/147.15	+ve	-17/-21	0.992	5-150	0.78/2.37
5.	Tocopherol	12.87	429.50	163.15/205.05	+ve	-22/-23	0.993	5-150	2.31/7.01
6.	Gallic acid	1.91	169.20	125.05/81	-ve	17/17	0.992	5-150	3.20/9.70
7.	Chlorogenic acid	6.81	353.00	191.20/92.90	-ve	16/43	0.997	5-150	1.27/3.85
8.	Epicatechin	6.77	289.00	245.20/205.20	-ve	14/16	0.993	5-150	7.08/21.46
9.	Syringic acid	7.20	197.20	182.20/123.05	-ve	14/24	0.996	5-150	10.44/31.65
10.	Vanillic acid	6.79	167.20	152.10/108.10	-ve	17/19	0.991	5-150	14.35/43.48
11.	Caeffeic acid	6.91	179.20	135.15/134.10	-ve	16/30	0.995	5-150	0.49/1.49
12.	Epigallocatechin	2.01	456.90	169.15/125.05	-ve	17/40	0.987	5-50	2.90/8.79
13.	Ferulic acid	7.36	193.20	134.00/178.00	-ve	15/10	0.992	5-50	4.37/13.23
14.	Myricetin	7.65	317.00	151.20/179.20	-ve	25/19	0.995	5-150	0.25/0.75
15.	Quercetin	7.92	301.20	151.05/179.00	-ve	22/18	0.991	5-150	0.62/1.88
16.	p-Coumaric acid	7.34	163.00	119.15/93.10	-ve	15/33	0.992	5-150	2.13/6.46
17.	Luteolin	7.92	285.20	151.10/175.05	-ve	25/25	0.991	5-150	0.27/0.83
18.	Apigenin	8.18	269.20	149.05/151.00	-ve	23/25	0.993	5-150	0.48/1.45
19.	Kaempferol	7.83	285.20	159.15/187.05	-ve	31/27	0.993	5-150	1.44/4.36
20.	Rutin	7.34	609.20	300.00/301.15	-ve	38/34	0.995	5-150	0.08/0.23
21.	Diadzein	7.91	252.90	208.20/224.15	-ve	29/25	0.981	5-150	0.71/2.16
22.	Hesperetin	7.86	301.20	164.10/286.05	-ve	25/18	0.993	5-150	0.57/1.73
23.	Shikimic acid	1.76	172.90	111.20	-ve	11	0.969	5-150	5.79/17.55
24.	Ellagic acid	7.56	300.90	185.10/145.20	-ve	31/40	0.992	5-150	2.28/6.92
25.	Morin	7.74	301.20	151.00/149.15	-ve	20/25	0.994	5-150	0.59/1.79
26.	Genistein	7.81	269.20	133.20/132.05	-ve	31/46	0.990	5-150	0.59/1.79
27.	Cinnamic acid	7.93	147.00	103.05	-ve	13	0.993	5-150	6.60/20.01
28.	Chrysin	8.39	252.90	62.95/143.20	-ve	32/27	0.996	5-150	0.41/1.26

Table 6a: LC-MS/MS identification and quantification of phenolic compounds in *Mangifera indica* L. (Var. kottukonam) leaves collected in two seasons [Sample 1: summer and Sample 2: South West Monsoon] from three altitudes [vizhinjam (coastal), kulathuppuzha (semi high range) and venjaramoodu (sea level)].

Sr. No.	Phenolic Compound (in ppm)	Sample 1			Sample 2		
		Vzm	Kza	Vjd	Vzm	Kza	Vjd
1.	Catechin	0.473	0.036	0.468	0.043	0.035	0.314
2.	Naringenin	0.045	0.031	0.078	0.05	0.039	0.062
3.	Tocopherol	0.022	0.026	-	0.026	0.028	0.023
4.	Gallic acid	739.055	7.826	269.119	111.832	121.587	318.372
5.	Chlorogenic acid	0.0133	-	0.016	-	-	-
6.	Epicatechin	0.28	0.284	0.615	0.217	0.362	0.647
7.	Syringic acid	0.086	0.002	1.234	0.104	0.154	0.179
8.	Vanilic acid	0.095	0.479	0.749	0.174	0.273	0.152
9.	Caffeic acid	0.003	0.003	0.008	0.0035	0.008	0.014
10.	Epigallocatechin	0.123	0.017	0.07	0.056	0.034	0.036
11.	Ferulic acid	0.12	0.005	0.484	-	-	0.046
12.	Myricetin	1.043	0.06	0.994	0.061	0.032	0.956
13.	Quercetin	1.899	0.277	0.841	0.036	0.003	0.486
14.	p-Coumaric acid	0.001	-	0.023	-	-	-
15.	Luteolin	0.083	0.017	0.018	0.007	0.001	0.029
16.	Apigenin	0.008	0.005	0.006	0.005	0.005	0.005
17.	Kaempferol	0.858	0.179	0.286	0.037	0.011	0.147
18.	Hesperetin	0.202	0.039	0.06	0.012	0.008	0.045
19.	Shikimic acid	11.544	-	79.761	14.196	-	1.452
20.	Elagic acid	33.067	13.13	9.868	4.598	21.126	8.316
21.	Morin	4.523	0.994	2.051	0.126	0.029	1.097
22.	Genistein	0.047	0.013	0.02	0.005	0.006	0.035
23.	Cinnamic acid	0.102	-	-	-	-	-
24.	Chrysin	0.009	0.008	0.009	-	-	0.009

Vzm: Vizhinjam (coastal area), Kza: Kulathuppuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

Table 6b: LC-MS/MS identification and quantification of phenolic compounds in *Mangifera indica* L. (Var. kottukonam) leaves collected in two seasons [S3: north east monsoon and S4: winter] from three altitudes [vizhinjam (coastal), kulathuppuzha (semi high range) and venjaramoodu (sea level)].

Sr. No.	Phenolic Compound (in ppm)	Sample 3			Sample 4		
		VZM	KZA	VJD	VZM	KZA	VJD
1.	Catechin	-	0.027	0.362	0.042	0.028	0.445
2.	Naringenin	0.037	0.026	0.071	0.062	0.03	0.04
3.	Tocopherol	0.024	0.023	0.023	0.032	0.031	0.023
4.	Gallic acid	266.454	8.926	234.689	1971.493	9.061	437.567
5.	Chlorogenic acid	0.03	-	0.015	-	-	0.024
6.	Epicatechin	0.202	0.481	0.512	0.084	0.471	0.652
7.	Syringic acid	0.444	-	0.527	0.0087	-	1.207
8.	Vanilic acid	0.478	0.003	0.528	0.368	0.405	1.207
9.	Caffeic acid	0.014	0.007	0.014	0.005	0.006	0.021
10.	Epigallocatechin	0.352	0.02	0.079	0.058	0.014	0.074
11.	Ferulic acid	0.116	-	0.111	-	-	0.883
12.	Myricetin	0.63	0.043	0.652	0.127	0.033	1.337
13.	Quercetin	1.044	0.029	0.868	0.64	0.0328	1.298
14.	p-Coumaric acid	-	-	-	-	-	0.257
15.	Luteolin	0.013	0.003	0.023	0.018	0.009	0.027
16.	Apigenin	0.005	0.005	0.006	0.005	0.005	0.005
17.	Kaempferol	0.323	0.04	0.293	0.044	0.0408	0.554
18.	Hesperetin	0.023	0.011	0.075	0.08	0.009	0.123
19.	Shikimic acid	14.61	-	2.006	5.71	-	28.891
20.	Elagic acid	9.759	4.442	11.432	5.633	4.52	3.432
21.	Morin	2.189	0.116	2.041	2.048	0.105	2.825
22.	Genistein	0.035	0.013	0.031	0.034	0.009	0.012
23.	Cinnamic acid	0.001	-	-	-	-	-
24.	Chrysin	0.009	-	0.009	0.008	-	0.009

Vzm: Vizhinjam (coastal area), Kza: Kulathuppuzha (semi high range area), Vjd: Venjaramoodu (sea level area)

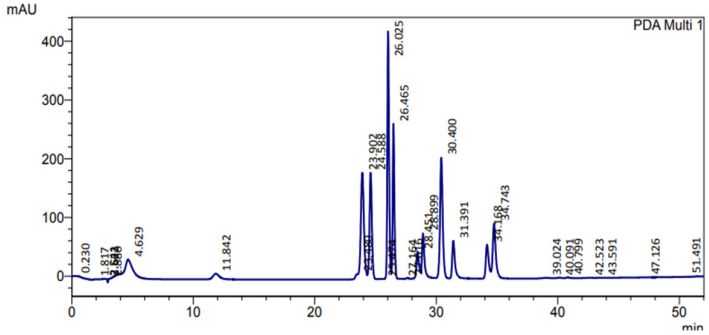


Fig. 1. HPLC chromatogram of standard mixture of polyphenols contains Gallic acid (4.27), Catechol (11.74), Chlorogenic acid (23.31), Caffeic acid (23.84), Syringic acid (24.42), p-Coumaric acid (25.98), Ferulic acid (26.42), Elagic acid (28.35), Myricetin (28.81), Cinnamic acid (30.39), Quercetin (31.27), Apigenin (34.72) with Rt.

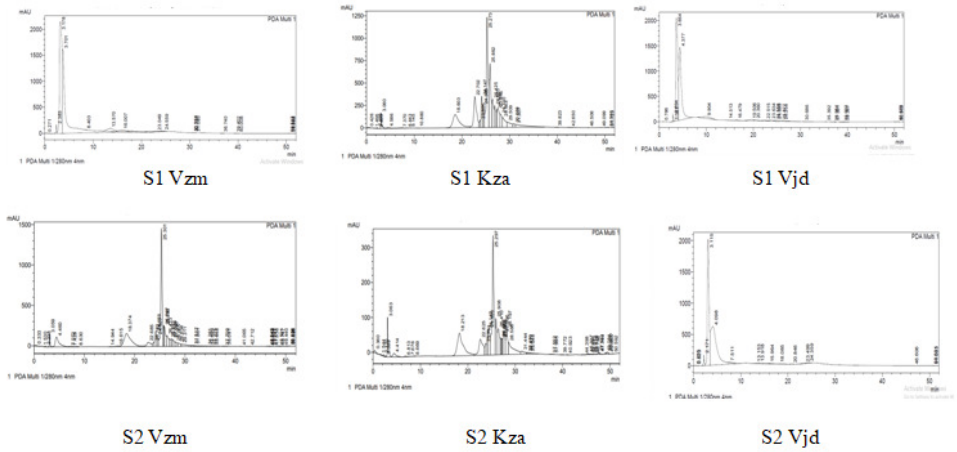


Fig. 2a. HPLC Chromatogram of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes (Vjm: Vizhinjam, Kza: Kulathuppuzha, Vjd: Venjaramoodu) in two seasons (S1: Summer, S2: South West monsoon).

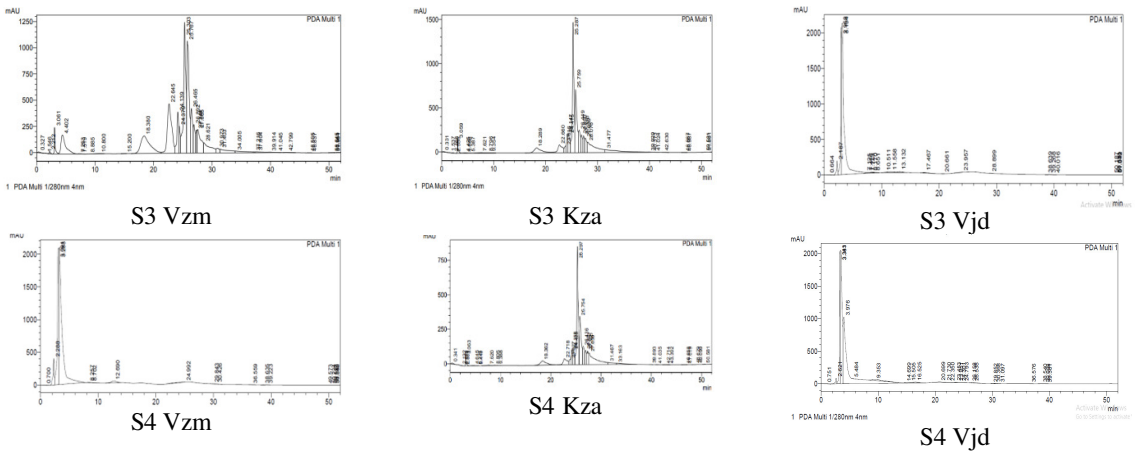
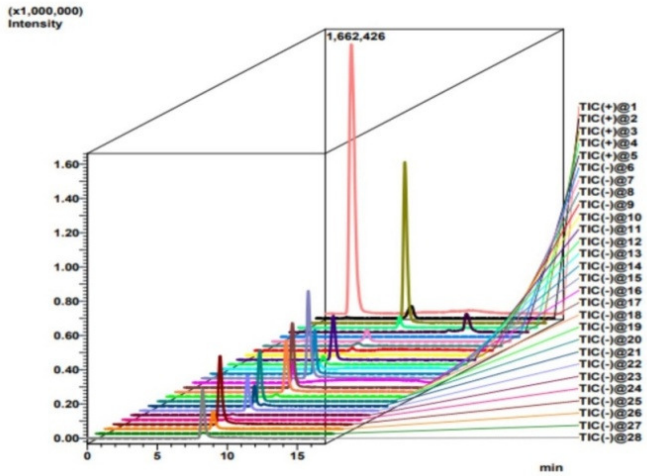


Fig. 2b. HPLC Chromatogram of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes (Vjm: Vizhinjam, Kza: Kulathuppuzha, Vjd: Venjaramoodu) in two seasons (S3: North East monsoon, S4: Winter)

Fig. 3. LC-MS/MS Chromatogram of 28 standard polyphenols.



1. Catechol, 2. Catechin, 3. Quinine, 4. Naringinin, 5. Tocopherol, 6. Gallic acid, 7. Chlorogenic acid, 8. Epicatechin, 9. Syringic acid, 10. Vannilic acid, 11. Caffeic acid, 12. Epigallocatechin, 13. Ferulic acid, 14. Myricetin, 15. Quercetin, 16. p-Coumaric acid, 17.Luteolein, 18. Apigenin, 19. Kaempferol, 20. Rutin, 21. Diadzein, 22. Hesperitin, 23.Shikkimic acid, 24. Elagic acid, 25. Morin, 26.Genistein, 27. Cinnamic acid and 28. Chrysin

Fig. 3. LC-MS/MS Chromatogram of 28 standard polyphenols.

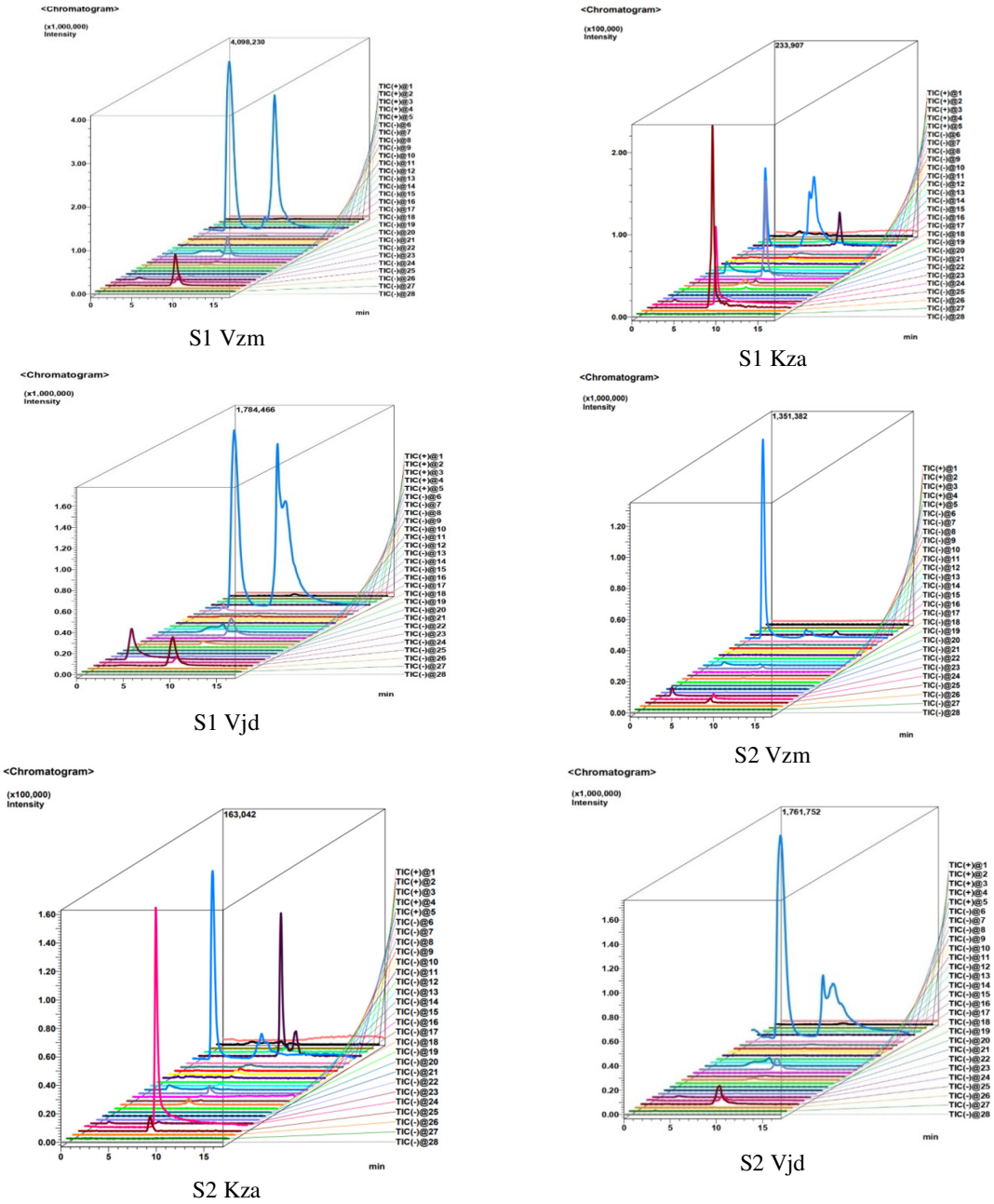
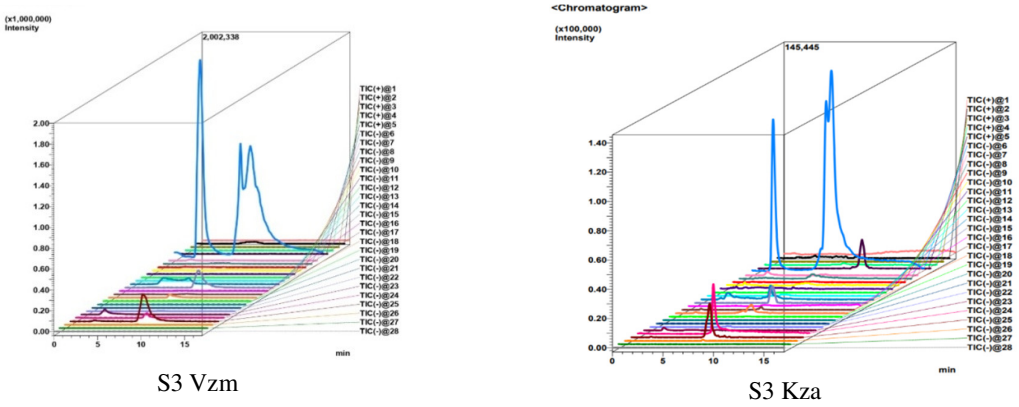


Fig. 4a. LC-MS/MS chromatogram of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam (coastal area- Vzm), Kulathuppuzha (semi high range area-Kza), Venjaramoodu (sea level area-Vjd)] in two seasons (S1, summer: S2, south west monsoon).



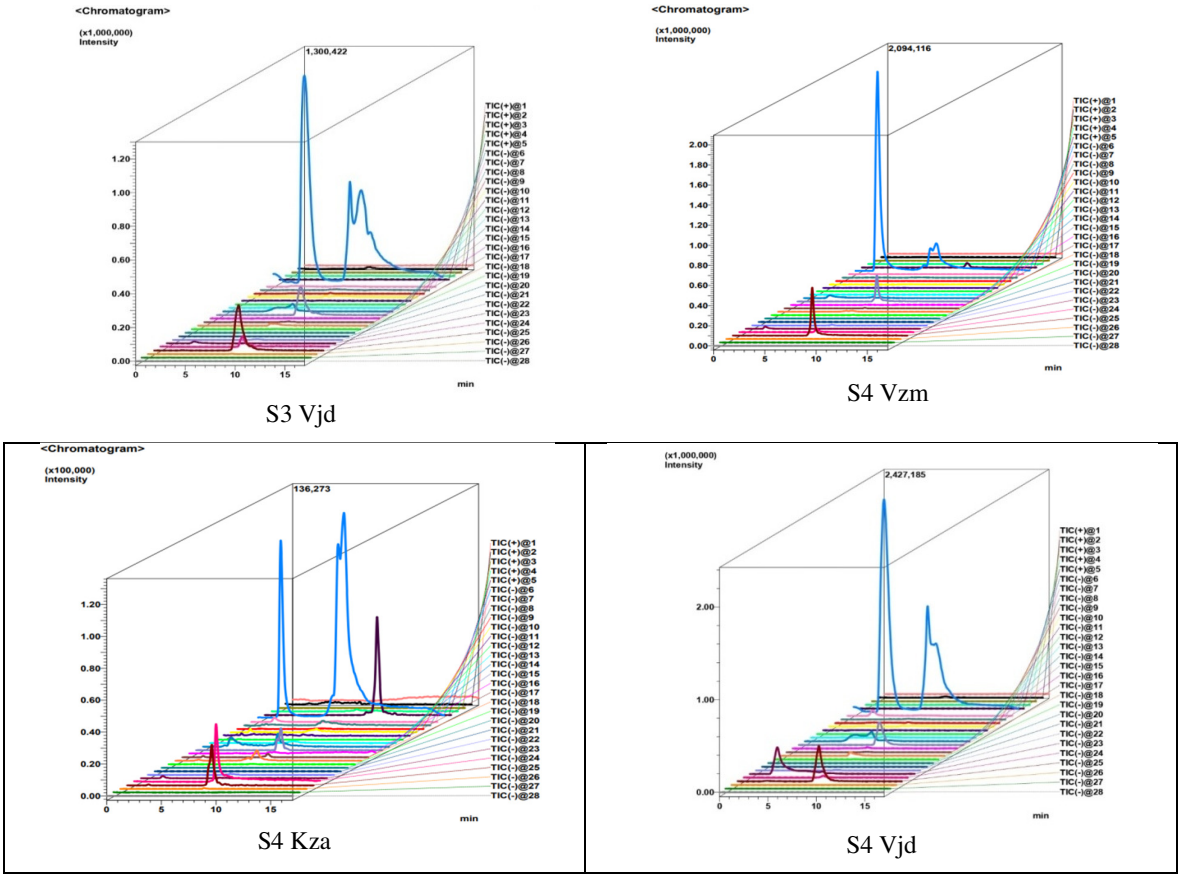


Fig. 4b. LC-MS/MS chromatogram of leaf samples of *Mangifera indica* L. (var. kottukonam) from three altitudes [Vizhinjam (coastal area- Vzm), Kulathuppuzha (semi high range area- Kza), Venjaramoodu (sea level area- Vjd)] in two seasons (S3, north east monsoon: S4, winter).

CONCLUSIONS

Efforts have been made in the identification, isolation and quantification of bioactive phytochemicals in the last few decades because of their immense therapeutical effects to human life. The results of the study disclosed the presence of an array of phytochemicals such as phenols, flavonoids, tannins, saponins, steroids, terpenoids and alkaloids in *Mangifera indica* L. (var. kottukonam). The major phytochemicals, polyphenols especially phenols and flavonoids showed noticeable level in all the twelve samples of *Mangifera indica* L. (var. kottukonam) and are identified through their specific quantitative assay methods. The LC-MS/MS and HPLC analysis of the methanol leaf extracts of the study material in four seasons from three altitudes against the standard polyphenols confirmed the presence of different phenolic compounds having antioxidant property and these results would serve as a benchmark for future comparative studies in the biochemical changes *Mangifera indica* L. (var. kottukonam) during flowering (reproductive stage) and non-flowering seasons. The quantitative analysis of the actual phenolic compounds present in the samples from summer, south west monsoon, north east monsoon and winter seasons would be facilitated by means of comparison with standard chromatogram, empowering identification and confirmation of polyphenols. The results of the study recommends that more stressed conditions were overcome by the tree by producing different polyphenols (antioxidant phytochemicals) which also will resulted to the flowering and fruiting.

FUTURE SCOPE

Polyphenols are secondary metabolites with immense biological potentialities. They are very useful to human health and has many medicinal properties. Significant variations in the concentration of different forms of phenolic compounds with respect to season and altitude was noticed in the leaves of kottukonam variety of *Mangifera indica* L. Further studies are needed to extract the phenolic compounds from the leaves of kottukonam in commercial manner and which will support the pharmaceutical industry.

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Conflict of Interest. None.

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