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Natural anti-inflammatory terpenoids in *Camellia japonica* leaf and probable biosynthesis pathways of the metabolome

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Abstract

Background: Metabolomics of *Camellia japonica* leaf has been studied to identify the terpenoids present in it and their interrelations regarding biosynthesis as most of their pathways are closely situated. *Camellia japonica* is famous for its anti-inflammatory activity in the field of medicines and ethno-botany. In this research, we intended to study the metabolomics of *Camellia japonica* leaf by using gas chromatography-mass spectroscopy technique.

Results: A total of twenty-nine anti-inflammatory compounds, occupying 83.96% of total area, came out in the result. Most of the metabolites are terpenoids leading with triterpenoids like squalene, lupeol, and vitamin E. In this study, the candidate molecules responsible for anti-inflammatory activity were spotted out in the leaf extract and biosynthetic relation or interactions between those components were also established.

Conclusion: Finding novel anticancer and anti-inflammatory medicinal compounds like lupeol in a large amount in *Camellia japonica* leaf is the most remarkable outcome of this gas chromatography-mass spectroscopy analysis. Developing probable pathway for biosynthesis of methyl commate B is also noteworthy.

Keywords: *Camellia japonica*, Metabolomics, GC-MS, Anti-inflammatory compounds, Lupeol

Background

Inflammation in the body is a result of a natural response to injury which induces pain, fever, and swelling. Both corticosteroids and non-steroidal anti-inflammatory drugs are used to reduce this pain by acting on the anti-inflammatory pathways. But these drugs have undesirable side effects as gastric ulceration, infrequently myocardial infarction, and stroke (Maroon et al. 2010). For centuries, many plant and animal-derived natural compounds have been used to treat inflammation. Those compounds as dietary supplements and herbal remedies are becoming increasingly popular because of their relatively few side effects unlike steroidal and nonsteroidal anti-inflammatory drugs.

Camellia japonica is an ornamental flowering plant belongs to the family Theaceae. The wild plants of the genus *Camellia* originated in China (Shandong, east Zhejiang), Taiwan, southern Korea, and southern Japan (Konemann 2004). *Camellia japonica* is reported as a bioactive plant in folk medicine of South Korea, Japan, and China. Antioxidant and anti-inflammatory activities of *Camellia japonica* leaves are already reported and this plant is proved to be a source of triterpenes, flavonoids, tannin, and fatty acids having antiviral, antioxidant, and anti-inflammatory activities (Lee et al. 2017). Seed from this plant is used as a traditional medicine and in folk remedies for the treatment of bleeding and inflammation. Compounds from seed oil and stem bark extract of *Camellia japonica* are known to have anti-inflammatory activities (Lim 2014). Leaf extract of *Camellia japonica* is reported to have a high concentration of vitamin E (25.35%), n-eicosane (10.2%) with other six active components (neophytadiene; all trans-squalene; n-octacosane; 6,9-pentadecadien-1-ol, α-linolenic acid, and n-hexadecanoic acid) related to hyperuricemia (Yoon et al. 2017). However,

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the seed oil of this plant is already established as a medicinal ingredient in the pharmaceutical and food industry (Akihisa et al. 1997). Camellianoside is a quercetin O-glucoside isolated from the leaves of *Camellia japonica* and exhibits antioxidant activity. It has a role as a metabolite and a radical scavenger (Onodera et al. 2006). Our present investigation aimed to make a GC-MS analysis of *Camellia japonica* leaf extract to find out the compounds involved in exhibiting anti-inflammatory activity and study the pathways involved in their synthesis. The application of pathway study in this phytochemical analysis is an innovative strategy for targeting active compounds from this plant extract.

Methods

Sample collection and preparation

Healthy leaves were collected from an organically cultivated *Camellia japonica* plant, which was collected from Darjeeling Himalaya. Leaves of *Camellia japonica* were crushed in liquid nitrogen and mixed in methanol (widely used solvent for extraction) to make *Camellia japonica* leaf extract (CJLE). The extract was left overnight on a shaker at 25 °C. The extract was centrifuged and the supernatant was collected for GC-MS analysis.

Gas chromatography-mass spectrometry analysis

Methanolic extract (CJLE) with a concentration of 25 mg/ml was used for GC-MS. This method was adopted from Das et al. (2014) and Labar et al. (2019) with a slight modification. One microliter of CJLE was injected in split mode in the instrument (GCMS-QP2010 Plus). Injection temperature was 260 °C and the interface temperature was set to 270 °C. Ion source temperature was adjusted to 230 °C. Helium was used as carrier gas. Total flow rate was 16.3 ml min^{-1} and the column flow rate was 1.21 ml min^{-1}. Mass spectra were recorded at 5 scan s^{-1} with a scanning range of 40-650 m/z. Quantification of compounds was done on the bases of their peak areas. The data obtained from GCMS analysis were further analyzed from available literature.

Studies on biosynthesis pathways of different terpenoids found in CJLE

Studies on biosynthesis pathways of different compounds detected in GC-MS were done by reviewing several literatures and databases (The Kyoto Encyclopedia of Genes and Genomes database 2020 and The PubChem 2020).

Results

Gas chromatography-mass spectrometry analysis

Total of fifty peaks were found in the chromatogram (Table 1; Fig. 1) showing forty-seven different compounds where twenty-nine compounds are reported natural anti-inflammatory agents with a summative value of 83.96% in total area. The major anti-inflammatory molecules detected in the extract were squalene (27.25%), lupeol (17.26%), diethyl phthalate (5.11%), vitamin E (5.01%), and patchouli alcohol (3.49%) along with other twenty-four anti-inflammatory compounds contributing a total share over eighty-three percentage of peak area (shown in Table 2; Fig. 2) where some of them are reported as pain relievers and as anti-arthritic.

Compounds are arranged on the bases of their chemical groups regarding their biosynthesis pathways, which are mainly sesquiterpenes, triterpenes, diterpene, monoterpenes, tocopherols, phthalate esters, cannabinoid, and others (Table 2; Fig. 3). Interestingly, more than seventy-four percentage of the extract belongs to terpenoids and their derivatives. Total of twenty compounds (patchoulol, alpha-, and gamma-patchoulene, alpha-gurjunene, caryophyllene, isoledene, etc.) with 19.58% percentage of total peak area belong to the class sesquiterpene while triterpenoids like squalene, oxidosqualene, methyl commate B, and lupeol have occupied 50.02% of peak area (shown in Fig. 3). Furthermore, five compounds are present from the group monoterpenes (including their derivatives as linalyl acetate) while sclareol (diterpene), CB-86 (cannabinoid), and vitamin E (tocopherol) are lone representatives of their chemical groups having rich bioactivities. A total of 7.52% area belongs to phthalate esters where two of them are reported bioactive but their biosynthesis or biodegradation pathway in this particular plant is not clear due to absence of previous reports and precursors or intermediates in our result.

Studies on biosynthesis pathways of different terpenoids found in CJLE

Triterpenes, sesquiterpenes, monoterpenes, and tocopherol are major groups of bioactive components found in plant bodies where their pathways are closely related with each other as isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) play the role of precursors in their biosynthesis (Fig. 4). The biosynthesis pathway of terpenoids in plants involves and regulates a number of pathways, where bioactive compounds like squalene, lupeol, vitamin E, patchouli alcohol, eucalyptol, and linalyl acetate are synthesized which have been found in CJLE.

Triterpenoids

Triterpenoid (50.02%) is the major group of compounds reported in this GC-MS analysis of CJLE. Squalene, one of the triterpenes found in CJLE, is involved as a common precursor for synthesis of various hormones in animals and sterols in plants. Moreover, squalene, itself is a major bioactive compound having anti-inflammatory property (Table 2). Oxidosqualene, also named as epoxysqualene and (3S)-2,3-epoxy-2,3-dihydrosqualene, is another triterpene compound found in our GC-MS as

Table 1 GC-MC result of *Camellia japonica* leaf extract

Peak index	R. time	Area	Area%	Name
1	7.368	493233	1.18	Eucalyptol
2	10.733	274998	0.66	Linalyl acetate
3	11.342	182020	0.43	1,7,7-TRIMETHYLBICYCLO[2.2.1]HEPT-2-YL ACETA
4	12.157	751582	1.79	3-CYCLOHEXENE-1-METHANOL, .ALPHA.,.ALPHA.,
5	12.753	129266	0.31	EPIGLOBULOL
6	13.026	794979	1.89	1H-CYCLOPROP[E]AZULENE, 1A,2,3,4,4A,5,6,7B-OC
7	13.207	555776	1.32	Caryophyllene
8	13.360	551887	1.32	Isoledene
9	13.567	93953	0.22	3-Chloropropane-1,2-diol, bis(tert-butyldimethylsilyl) ethe
10	13.639	93299	0.22	Seychellene
11	13.737	59132	0.14	NEOALLOOCIMENE
12	13.792	65085	0.16	1H-3a,7-Methanoazulene, 2,3,6,7,8,8a-hexahydro-1,4,9,9-t
13	13.881	65370	0.16	1H-3a,7-Methanoazulene, octahydro-1,9,9-trimethyl-4-met
14	14.116	140615	0.34	BICYCLO[7.2.0]UNDEC-4-ENE, 4,11,11-TRIMETHYL-
15	14.439	118763	0.28	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undec
16	15.137	329737	0.79	(1aR,3aS,7S,7aS,7bR)-1,1,3a,7-Tetramethyldecahydro-1H-
17	15.224	255742	0.61	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-
18	15.286	2142686	5.11	1,2-BENZENEDICARBOXYLIC ACID, DIETHYL ESTE
19	15.447	757801	1.81	Epicurzerenone
20	15.531	308410	0.74	Chenodiol
21	15.617	15884	0.04	Unidentified compound
22	15.673	412507	0.98	1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e]azulene-4,
23	15.787	716851	1.71	TRIDEUTERIOMETHYL 10-EPOXY-7-ETHYL-3,11-DI
24	15.988	768032	1.83	METHYL (3-OXO-2-PENTYLCYCLOPENTYL)ACETA
25	16.159	1287600	3.07	1-(4-ISOPROPYLPHENYL)-2-METHYLPROPYL ACET
26	16.248	79006	0.19	1-(4-ISOPROPYLPHENYL)-2-METHYLPROPYL ACET
27	16.345	544685	1.30	2-PENTEN-1-OL, 5-(2,3-DIMETHYLTRICYCLO[2.2.1.0
28	16.394	1466093	3.49	Patchouli alcohol
29	16.590	257277	0.61	1-Naphthalenepropanol, .alpha.-ethenyldecahydro-2-hydro
30	16.651	187453	0.45	(3aR,4R,7R)-1,4,9,9-Tetramethyl-3,4,5,6,7,8-hexahydro-2
31	16.822	938511	2.24	Santalol, E-cis,epi-.beta.-
32	16.915	104923	0.25	2-FURANMETHANOL, 5-ETHENYLTETRAHYDRO-.A
33	17.169	498732	1.19	4,8-DIMETHYL-3,8-NONADIEN-2-ONE
34	17.367	245089	0.58	ACETYL CEDRENE
35	17.609	152712	0.36	1H-Benzocycloheptene-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1
36	17.941	346720	0.83	Neophytadiene
37	18.129	184873	0.44	4,6,6,7,8,8-HEXAMETHYL-1,3,4,6,7,8-HEXAHYDROC
38	18.212	185266	0.44	7-ACETYL-1,1,3,4,4,6-HEXAMETHYL TETRALIN
39	19.882	666930	1.59	Ethylene brassylate
40	20.105	66589	0.16	CYCLODODECASILOXANE, TETRACOSAMETHYL-
41	22.495	86863	0.21	SILICONE OIL
42	23.572	75245	0.18	SILICONE OIL
43	24.252	1011139	2.41	Bis(2-ethylhexyl) phthalate
44	26.522	11434137	27.25	Squalene

Table 1 GC-MC result of *Camellia japonica* leaf extract *(Continued)*

Peak index	R. time	Area	Area%	Name
45	27.528	178555	0.43	CB-86
46	27.664	230045	0.55	SOLANESOL
47	27.796	523171	1.25	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,
48	30.688	2100440	5.01	Vitamin E
49	36.935	1787264	4.26	METHYL COMMATE B
50	45.960	7241879	17.26	Lupeol

oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11, 15,19-heneicosapentaenyl)-, (all-E)-. It is actually an intermediate in triterpenoid biosynthesis pathway which is produced from squalene by the enzyme squalene mono-oxygenase (E.C. 1.14.14.17) (Fig. 5). Another major triterpene and anti-inflammatory compound detected by our GC-MS analysis is lupeol, which is present in high amount (17.26 %) and famous for its wide range of bioactivities. Methyl commate B ($C_{31}H_{50}O_3$) is also an anti-inflammatory triterpene (Table 2) present in CJLE, which is actually a methyl ester of comic acid B. Methyl esterification may occur due to exposure of sample to methyl alcohol during extraction. Both lupeol and methyl commate B are biosynthesized in the same pathway where degradation of squalene occurs. Furthermore, step by step enzymatic reactions involved in the formation of

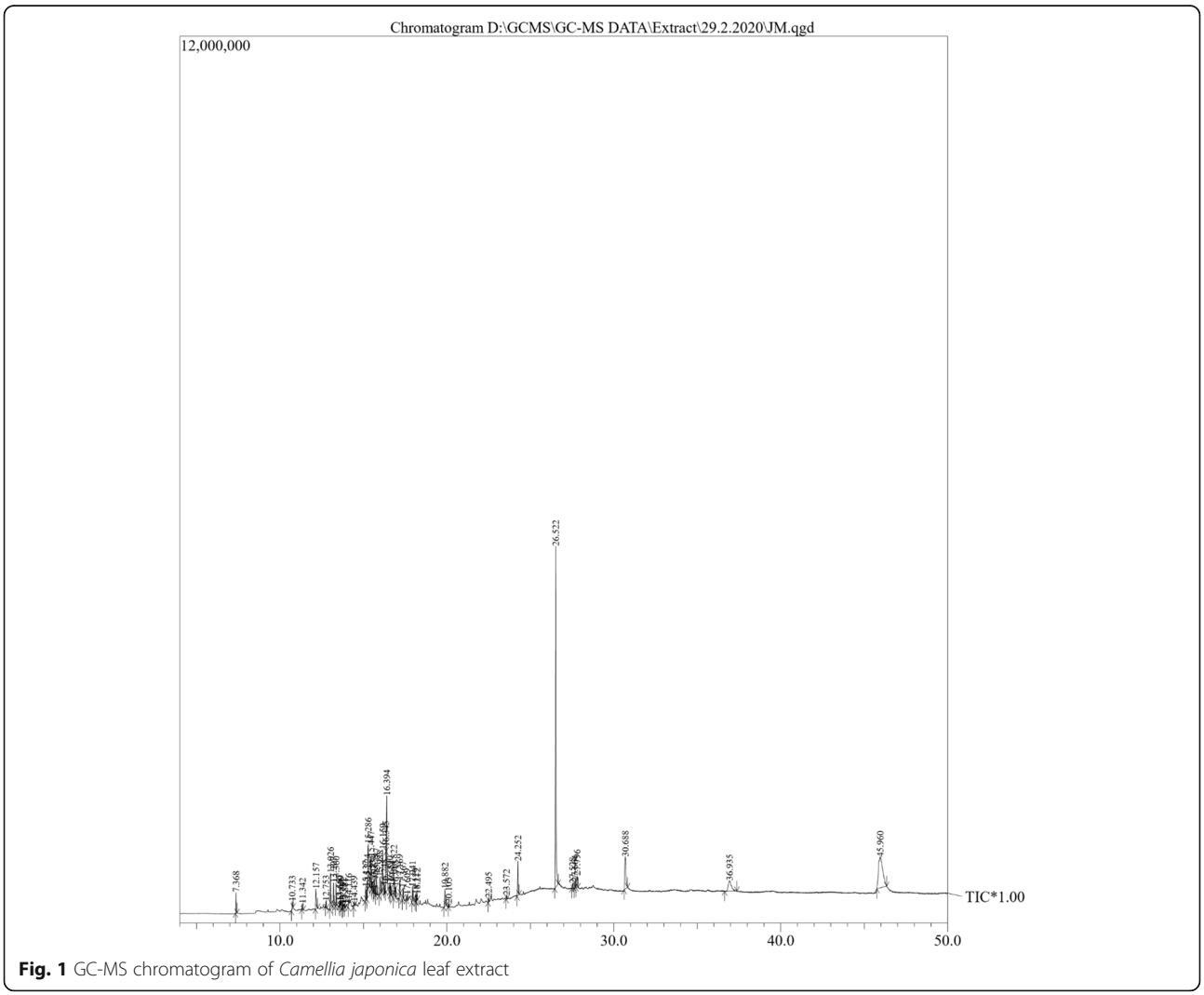

Fig. 1 GC-MS chromatogram of *Camellia japonica* leaf extract

Table 2 Type of compounds present in *Camellia japonica* leaf extract and anti-inflammatory compounds in it

Peak index	*Camellia japonica* leaf compounds	Type	Area%
1	**Eucalyptol** (Juergens et al. 2003)	Monoterpene	1.18
2	**Linalyl acetate** (Peana et al. 2002)	Monoterpene	0.66
3	**1,7,7-trimethylbicyclo[2.2.1]hept-2-yl acetate** (Yang et al. 2014)	Monoterpene	0.43
4	**3-cyclohexene-1-methanol, .alpha.,.alpha.,4…** (Held et al. 2007)	Monoterpene	1.79
5	**Epiglobulol** (Jayaprakash et al. 2019)	Sesquiterpene	0.31
6	**1 h-cycloprop[e]azulene, 1A,2,3,4,4A,5,6,7B-oct…** (Rajput et al. 2018)	Sesquiterpene	1.89
7	**Caryophyllene** (Fernandes et al. 2007)	Sesquiterpene	1.32
8	Isoledene	Sesquiterpene	1.32
10	**Seychellene** (Raharjo et al. 2017)	Sesquiterpene	0.22
11	**Neoalloocimene** (Pravdich-Neminskaya and Kachkov 1978)	Sesquiterpene	0.14
12	1H-3a,7-Methanoazulene, 2,3,6,7,8,8a-hexahydro-1,4,9,9-t	Sesquiterpene	0.16
13	1H-3a,7-Methanoazulene, octahydro-1,9,9-trimethyl-4-met	Sesquiterpene	0.16
14	**Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8** (Tambe et al. 1996)	Sesquiterpene	0.34
15	(1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca	Sesquiterpene	0.28
16	**(1AR,3AS,7S,7AS,7BR)-1,1,3a,7-Tetramethyldecahydro-1H- or maaliol** (Sah et al. 2012)	Sesquiterpene	0.79
17	**1H-Cycloprop[E]azulen-7-ol, decahydro-1,1,7-trimethyl-4-m or (+)-spathulenol** (do Nascimento et al. 2018)	Sesquiterpene	0.61
18	**1,2-benzenedicarboxylic acid, diethyl ester** (Singh et al. 2012)	Phthalate ester	5.11
19	**Epicurzerenone** (Makabe et al. 2006)	Sesquiterpene	1.81
22	1,1,4,7-Tetramethyldecahydro-1H-cyclopropa[e]azulene-4,7	Sesquiterpene	0.98
23	Trideuteriomethyl 10-epoxy-7-ethyl-3,11-dim		1.17
24	**Methyl (3-oxo-2-pentylcyclopentyl)acetat…** (Dang et al. 2008)		1.83
27	**2-penten-1-ol, 5-(2,3-dimethyltricyclo[2.2.1.02…** (Bommareddy et al. 2019)	Sesquiterpene	1.3
28	**Patchouli alcohol** (Li et al. 2011)	Sesquiterpene	3.49
29	**1-Naphthalenepropanol, .alpha.-ethenyldecahydro-2-hydrox…** (Tsai et al. 2018)	Diterpene	0.61
30	**(3AR,4R,7R)-1,4,9,9-Tetramethyl-3,4,5,6,7,8-hexahydro-2H… or cyperenone** (Gupta and Shaw 2009)	Sesquiterpene	0.45
31	Santalol, E-cis,epi-.beta.-	Sesquiterpene	2.24
32	2-furanmethanol, 5-ethenyltetrahydro-.a	Monoterpene	0.25
34	Acetyl cedrene	Sesquiterpene	0.58
35	**1H-Benzocyclohepten-7-ol, 2,3,4,4A,5,6,7,8-octahydro-1,1, … or widdrol** (Jin et al. 2015)	Sesquiterpene	0.36
36	**Neophytadiene** (Bhardwaj et al. 2020)	Sesquiterpene	0.83
37	4,6,6,7,8,8-hexamethyl-1,3,4,6,7,8-hexahydrocy		0.44
38	7-acetyl-1,1,3,4,4,6-hexamethyl tetralin		0.44
39	**Ethylene brassylate** (Kim et al. 2006)		1.59
40	**Cyclododecasiloxane, tetracosamethyl-** (Kumar et al. 2018)		0.16
43	**Bis(2-ethylhexyl) phthalate** (Mohammed et al. 2014)	Phthalate ester	2.41
44	**Squalene** (Fernando et al. 2018)	Triterpene	27.25
45	Cb-86	Cannabinoid	0.43
46	**Solanesol** (Yan et al. 2015)	Terpene-alcohol	0.55
47	Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,1	Triterpene	1.25
48	**Vitamin E** (Jiang 2014)	Tocopherols	5.01
49	**Methyl commate B** (Arora and Kumar 2018)	Triterpene	4.26
50	**Lupeol** (Saleem 2009)	Triterpene	17.26

Anti-inflammatory compounds are written in bold. References against each anti-inflammatory compound are given in round brackets

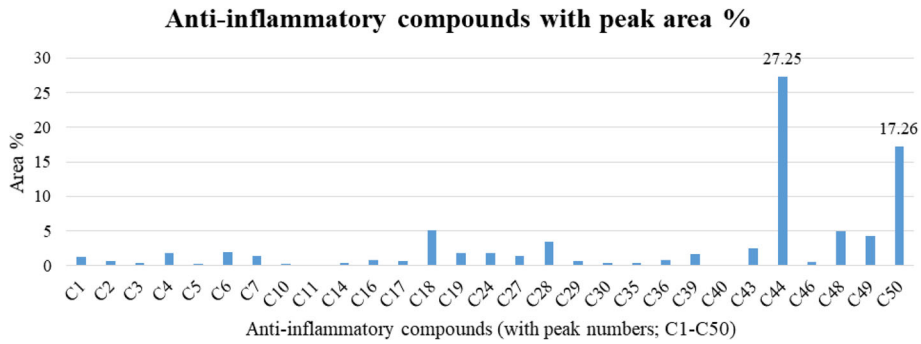

Fig. 2 Anti-inflammatory compounds of *Camellia japonica* leaf extract. C1-C50 = GC-MS detected compounds' peak numbers in order (Table 1)

lupeol and methyl commate B (Figs. 6 and 7) are described in the "Discussion" section.

Sesquiterpenoids

Patchouli alcohol (3.49%), the major sesquiterpene present in this leaf extract, is a germacrene and synthesized from FPP by the enzyme patchoulol synthase (E.C. 4.2.3.70) (Fig. 8) (https://www.genome.jp/kegg/pathway.html). In the same pathway, nineteen other sesquiterpenes of the plant are also produced from FPP by different enzymes.

Monoterpenoids

Total of four monoterpene compounds have been found in our GC-MS result. Among them, the higher amount of monoterpene is terpinyl acetate or 3-cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, acetate (1.79%) which is the acetate ester of alpha-terpineol synthesized from GPP by the enzyme alpha-terpineol synthase (E.C. 4.2.3.112). Another major monoterpene eucalyptol (1.18%) follows the same pathway which is synthesized from GPP by the enzyme 1,8-cineole synthase (E.C. 4.2.3.108). Linalyl acetate (0.66%) is also a monoterpene which is the acetate ester of linalool. Biosynthesis of linalool by linalool synthase enzyme (E.C. 4.2.3.26) is also established in monoterpenoid biosynthesis pathway which is shown in Fig. 9.

Vitamin E, sclareol, and solanesol

According to our GC-MS result, vitamin E (5.01%), more specifically alpha-tocopherol, is one of the major anti-inflammatory compound which is synthesized in ubiquinone and other terpenoid quinone biosynthesis pathway. Sclareol (0.61%; a diterpene) and solanesol (0.55%; a nonaprenol), two anti-inflammatory compounds (Table 2), are present in minute amounts in CJLE, but being lone representatives of their respective chemical groups and having relations with earlier mentioned terpenoids regarding biosynthesis pathways, they are included in this study. Like other terpenoids, both

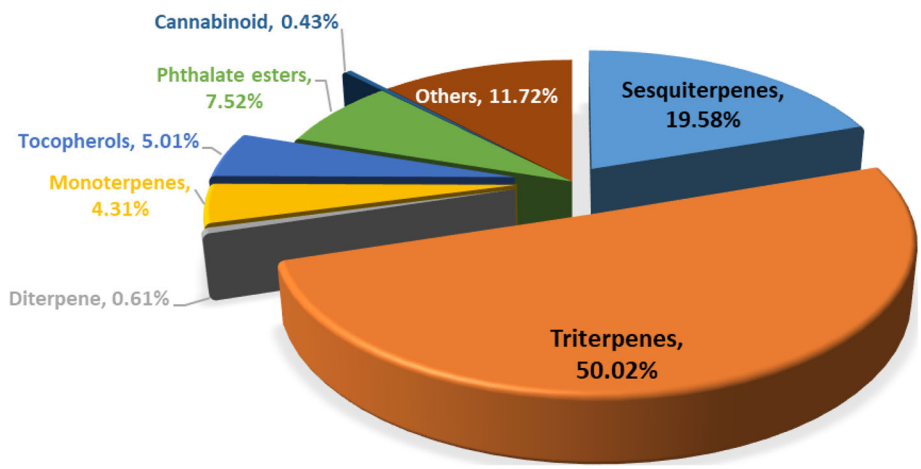

Fig. 3 Types of compounds on the basis of their biosynthesis in *Camellia japonica* leaf extract; majority of triterpenoids in *Camellia japonica* leaf

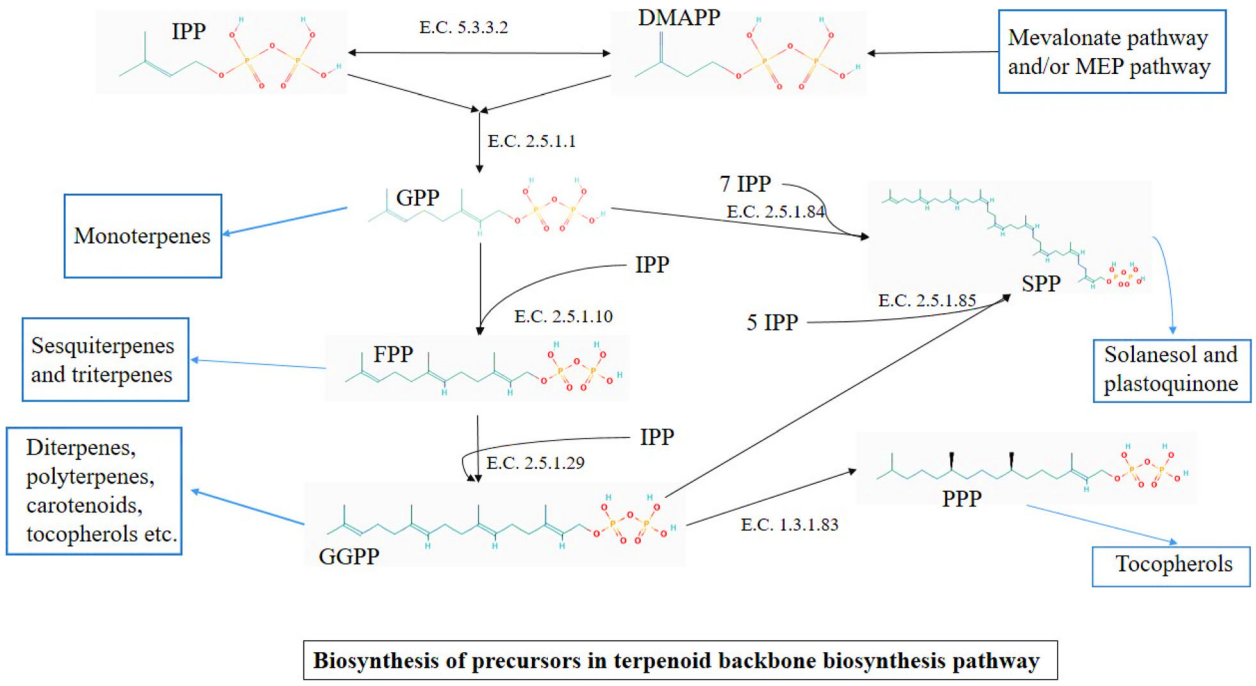

Fig. 4 Biosynthesis of different direct precursors in the terpenoid backbone biosynthesis pathway. IPP, isopentyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranyl-geranyl diphosphate; GPP, geranyl diphosphate; SPP, solanesyl diphosphate; PPP, phytyl diphosphate (compound structures source: https://pubchem.ncbi.nlm.nih.gov)

alpha-tocopherol (Fig. 10), sclareol, and solanesol (Fig. 11) are biosynthesized from IPP and DMAPP derived precursors in plastids through MEP metabolic pathway which is described in the "Discussion" section.

Figure 12 is designed as a brief diagram on biosynthesis pathway of all the abovementioned CJLE compounds found in the GC-MS result.

Discussion

Biosynthesis pathways are studied to investigate origin, precursors, intermediates, and breakdown products of different compounds, probable pathways of newly reported compounds having similar established structures, reactions, and relations between several compounds in individual plant bodies. In our research, we have used GC-

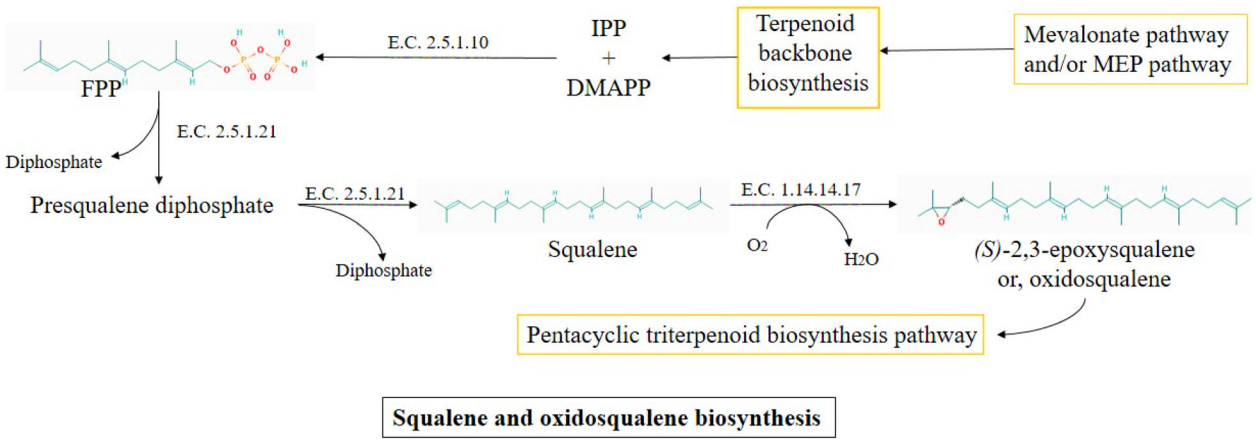

Fig. 5 Squalene and oxidosqualene biosynthesis; first stage of triterpenoid biosynthesis pathway. IPP, isopentyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate (compound structures source: https://pubchem.ncbi.nlm.nih.gov)

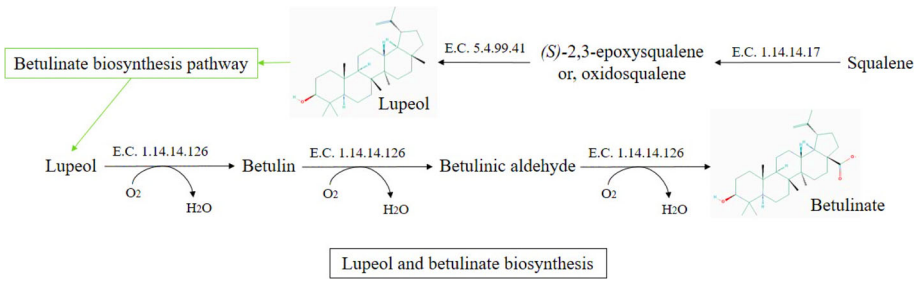

Fig. 6 Lupeol biosynthesis and degradation (common biosynthesis pathway for any pentacyclic triterpene) (compound structures source: https://pubchem.ncbi.nlm.nih.gov)

MS, one of the preliminary and first-step technique of metabolomics, to analyze the terpenoids present in *Camellia japonica.* Then studies on their biosynthesis pathways were carried out where several interactions and interrelations came out along with designs of probable biosynthesis pathways of unestablished metabolomes.

Wang et al. (2013), in their studies on biosynthesis pathway of terpenoid, proposed involvement of three different stages; firstly, the generation of C5 isopentyl diphosphate (IPP) precursor and its double bond isomer dimethylallyl diphosphate (DMAPP); secondly, the generation of direct precursors like geranyl diphosphate or GPP (for monoterpenes), farnesyl diphosphate or FPP (for triterpenes and sesquiterpenes), and geranylgeranyl diphosphate or GGPP (for diterpenes and tocopherols) (Fig. 4); and thirdly, biosynthesis and modification of terpenes via oxidation-reduction, acylation, glycosylation, and other reactions. In general, biosynthesis of sesquiterpene and triterpene takes place in the cytosol part of the cell while monoterpenes, tocopherols, and solanesol are synthesized inside the plastid just like chlorophylls and carotenoids in higher plants.

Triterpenes, in general, are produced from the precursor FPP which is derived from two IPP molecules and one DMAPP (McGarvey and Croteau 1995) by farnesyl diphosphate synthase through mevalonate pathway as shown in Fig. 4. Both squalene (27.25%) and lupeol (17.26%) are two abundant most compounds in this leaf extract which imparts anti-inflammatory activity to it. Squalene is produced from FPP by the enzyme squalene synthase (E.C. 2.5.1.21) (Fig. 5). In plants, squalene is metabolized by the enzyme squalene mono-oxygenase (E.C. 1.14.14.17) to produce (3S)-2,3-epoxy-2,3-dihydrosqualene or oxidosqualene (Wang et al. 2010) which is also present in our result. It then enters into pentacyclic triterpenoid biosynthesis pathway. Oxidosqualene can be converted into pentacyclic triterpenes like lupeol, alpha-amyrin, beta-amyrin, and several potentially bioactive phytosterols, an amazing number of structurally diverse backbones, including over hundred identified in plants (Wang et al. 2010) by oxidosqualene cyclase enzymes. Lupeol is a pharmacologically active pentacyclic and lupine type of triterpene. According to Saleem (2009), lupeol exhibits various pharmacological activities

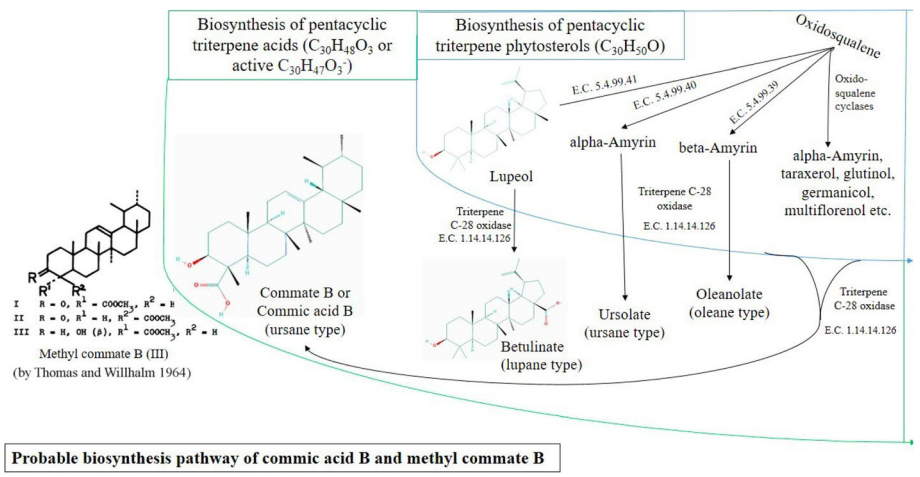

Fig. 7 Probable biosynthesis pathway of commic acid B and methyl commate B (compound structures source: https://pubchem.ncbi.nlm.nih.gov)

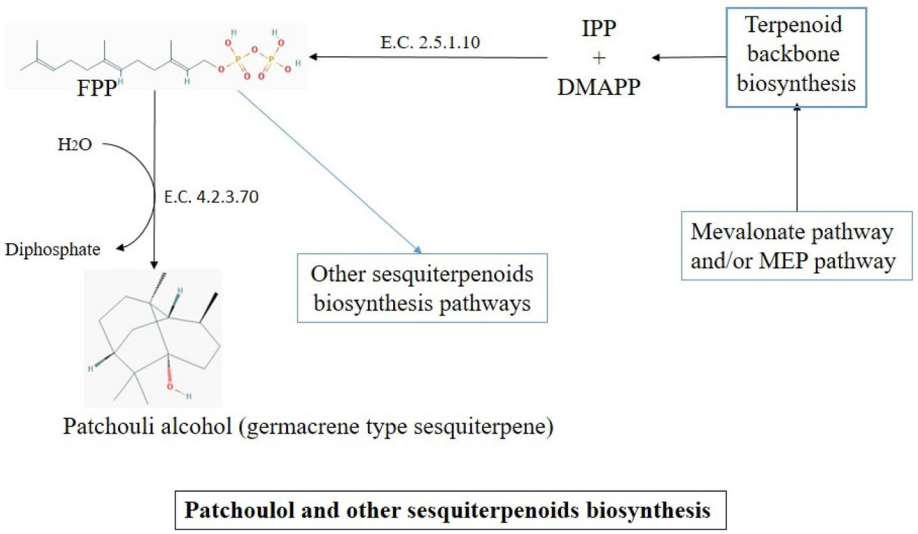

Fig. 8 Sesquiterpenoid biosynthesis pathway from FPP; i.e., patchouli alcohol. IPP, isopentyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate (compound structures source: https://pubchem.ncbi.nlm.nih.gov)

against inflammation (Table 2), cancer, arthritis, diabetes, heart diseases, renal toxicity, and hepatic toxicity. Lupeol has been extensively studied for its inhibitory effects on inflammation under in vitro and in animal models where it showed positive responses (Saleem 2009). Lupeol synthase (E.C. 5.4.99.41), an oxidosqualene cyclase, is a multifunctional enzyme that forms lupeol (Fig. 6) and other triterpene alcohols (like beta-amyrin). Sequence analysis suggests that lupeol synthase diverged from cycloartenol synthase after plants diverged from fungi and animals for which fungi and animals do not synthesize lupeol (Herrera et al. 1998), and presence of

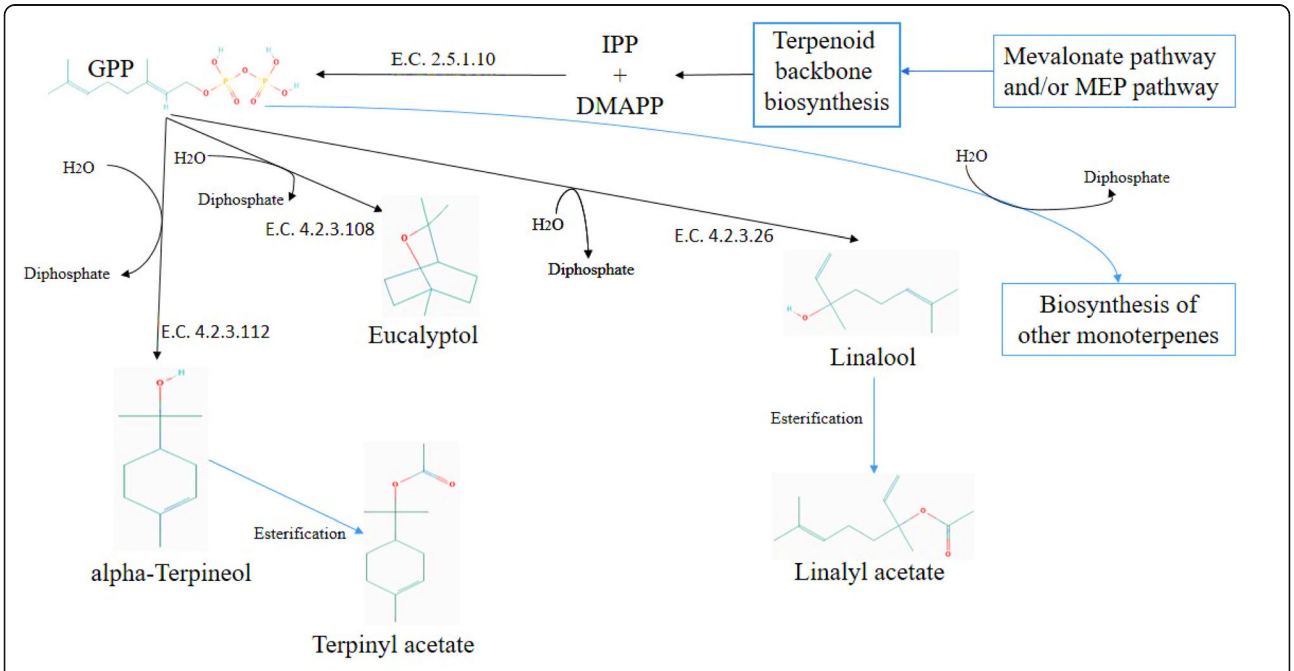

Fig. 9 Monoterpene biosynthesis pathway; formation of terpinyl acetate, eucalyptol, linalyl acetate, and other monoterpenes. IPP, isopentyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate (compound structures source: https://pubchem.ncbi.nlm.nih.gov)

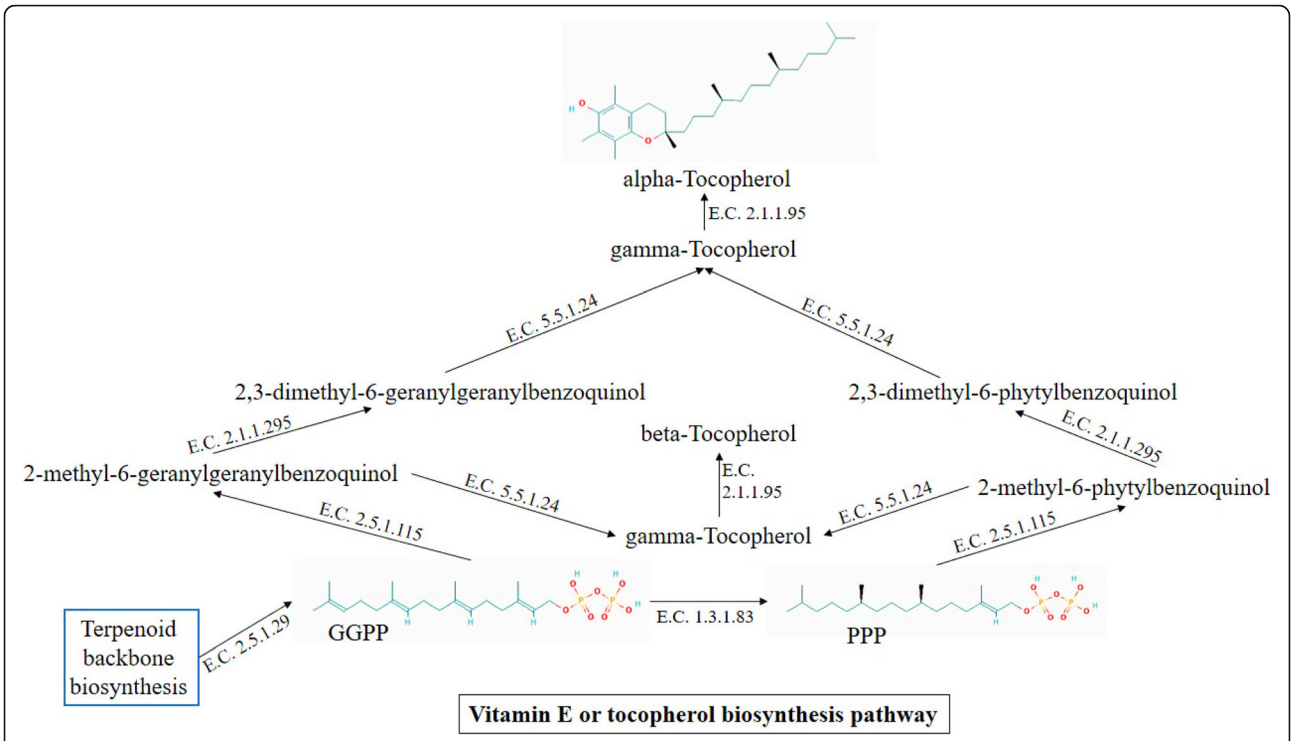

Fig. 10 Vitamin E or tocopherol biosynthesis pathway. GGPP, geranyl-geranyl diphosphate; PPP, phytyl diphosphate (compound structures source: https://pubchem.ncbi.nlm.nih.gov)

squalene, oxidosqualene, and lupeol together as major compounds has already proved the presence of lupeol synthase in this plant. According to Huang et al. (2012), lupeol then enters into betulinate biosynthesis pathway where after a series of oxidation process it converts into betulinate; $C_{30}H_{47}O_3^-$ (parent compound betulinic acid; $C_{30}H_{48}O_3$) by the enzyme lupeol-28-monooxygenase (E.C. 1.14.14.126). According to Saleem (2009), different fruits (olive, mango, and Japanese pear), aloe leaves, elm plant, and ginseng oil are the major source of lupeol. Lupeol was reported in *Camellia* seed oil (Akihisa et al. 1997) but there is no previous report that discloses the presence of lupeol in the leaves of *Camellia japonica.* Methyl commate B is another major triterpene found in CJLE. Unfortunately, there is no report on biosynthesis pathway for methyl commate B or comic acid B but the structure of comic acid B ($C_{30}H_{48}O_3$) is very much close to other pentacyclic triterpene acids like betulinic acid (lupeol derived), ursolic acid (alpha-amyrin derived), and oleanolic acid (beta-amyrin derived) which have the same chemical formula, that is $C_{30}H_{48}O_3$, and most of them are seen potentially medicinal in nature. According to the terpenoid biosynthesis pathway, these $C_{30}H_{48}O_3$ pentacyclic triterpene acids or active $C_{30}H_{47}O_3^-$ (ionized) are usually produced from squalene derived pentacyclic triterpene phytosterols or secondary alcohol ($C_{30}H_{50}O$) by oxidoreductase enzymes which are functionally same and termed as triterpene C-28 oxidase (E.C. 1.14.14.126). According to our interpretation, the same reactions should be followed in comic acid B formation from its specific phytosterol precursor. However, methyl commate B is not derived from lupeol as it is not a lupine type of triterpene but an ursane (Thomas and Willhalm 1964). Established triterpene biosynthesis pathways and their interactions have helped us to design a probable pathway for comic acid B synthesis (Fig. 7) where a squalene derived $C_{30}H_{50}O$ phytosterol (the precursor comic acid B) must be present as a precursor just like other triterpenes. But, in our GC-MS result, it is either absent or not detected as a precursor, thus, *Camellia japonica* must be subjected for further research to find responsible genes and enzymes behind biosynthesis of this compound.

After triterpene, the second most major chemical group in this plant extract is sesquiterpene including twenty different compounds, which are again produced from the precursor FPP following the mevalonate pathway. After terpenoid backbone biosynthesis and before squalene synthesis, FPP gets involved in the sesquiterpenoid biosynthesis pathway where it converts into a number of anti-inflammatory sesquiterpenes (Table 2) including patchouli alcohol.

Four monoterpene compounds have also been found in our GC-MS result as described earlier. Monoterpenes in

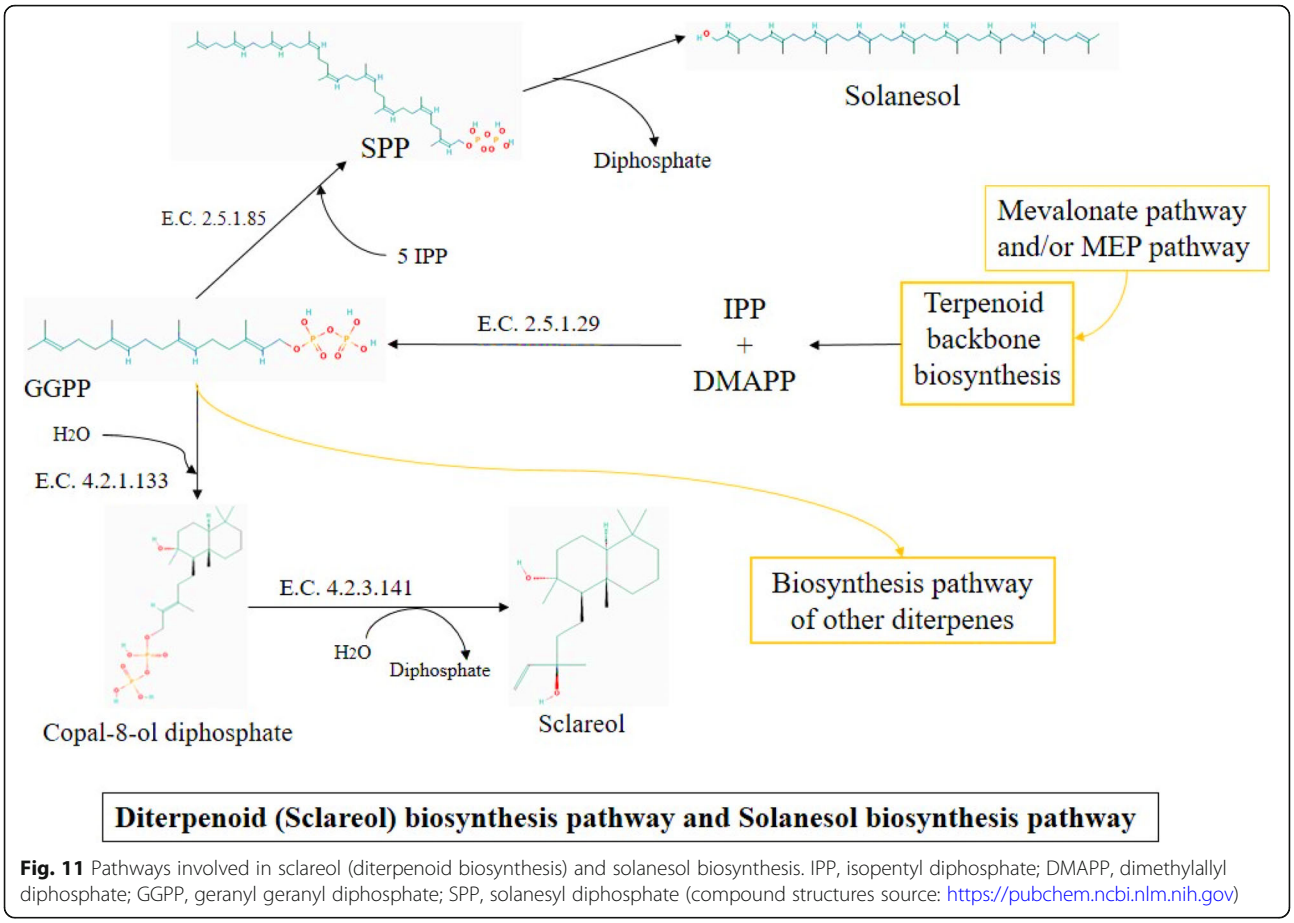

Fig. 11 Pathways involved in sclareol (diterpenoid biosynthesis) and solanesol biosynthesis. IPP, isopentyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranyl geranyl diphosphate; SPP, solanesyl diphosphate (compound structures source: https://pubchem.ncbi.nlm.nih.gov)

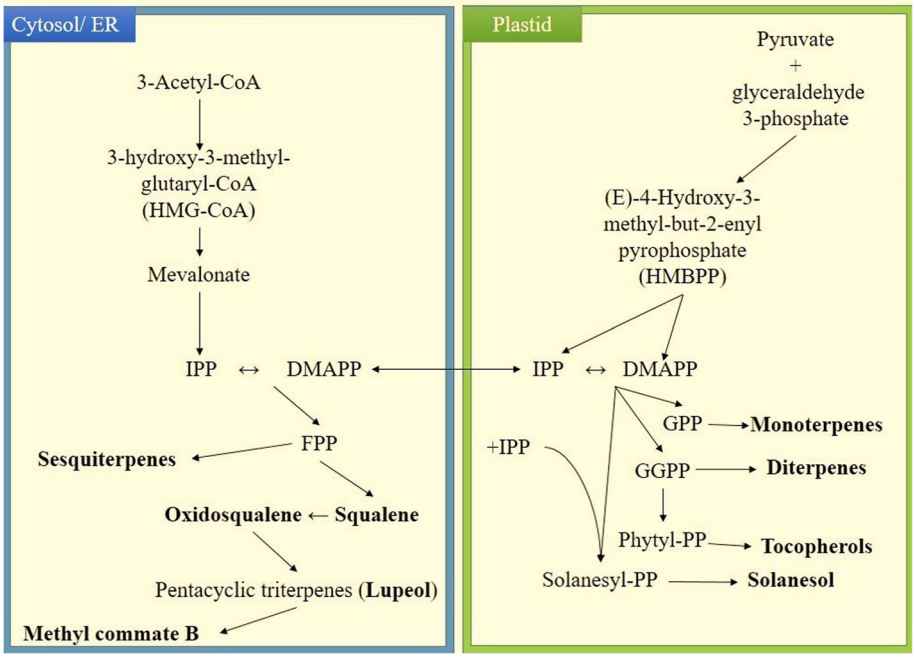

Fig. 12 Single diagram showing biosynthesis of major CJLE compounds. IPP, isopentyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranyl-geranyl diphosphate; GPP, geranyl diphosphate; PP, diphosphate

plants are biosynthesized from GPP which is produced by the enzyme geranyl pyrophosphate synthase (E.C. 2.5.1.1) from one IPP and one DMAPP molecule which further produces monoterpenes by GPP diphosphate-lyases (Fig. 4) inside the plastid (Davis and Croteau 2000).

After FPP and GPP, GGPP is the main precursor in this plant as it converts into vitamin E, another major compound detected in CJLE. This precursor, itself is synthesized from IPP and DMAPP (Wang et al. 2013) like other terpenoid precursors which proves IPP and DMAPP as important factors that help us to understand interrelations between different terpenes over their biosynthesis. Moreover, enzyme geranyl diphosphate synthase (E.C. 2.5.1.29) forms GGPP which further converts into phytyl diphosphate or PPP (Fig. 4) by enzyme geranyl reductase (E.C. 1.3.1.83) to synthesize vitamin E. After completing a series of reactions, tocopherol or vitamin E is synthesized where 2-methyl-6-phytylbenzoquinol (E.C. 2.5.1.115) and 2,3-dimethyl-6-phytylbenzoquinol (E.C. 2.1.1.295) take part in order as intermediates (Fig. 10). Another pathway of tocopherol biosynthesis is reported in plants where the same enzymatic reactions occur and GGPP converts directly into vitamin E without forming PPP (https://www.genome.jp/kegg/pathway.html). Biosynthesis of chlorophyll, carotenoids, plastoquinone, ubiquinone, solanesol, and diterpenoid like sclareol is also initiated by the same precursor GGPP. GGPP converts into copal-8-ol diphosphate (E.C. 4.2.1.133) which further produces sclareol (E.C. 4.2.3.141) as shown in Fig. 11. Solanesol, another terpenoid (a non-cyclic terpene alcohol) detected in CJLE, is synthesized from GGPP following the same three-stage terpenoid biosynthesis pathway of Wang et al. (2013). According to Yan et al. 2017, solanesyl diphosphate (SPP), the precursor of solanesol and plastoquinone, is synthesized from IPP, DMAPP, GPP, FPP, and GGPP by solanesyl diphosphate synthase (Fig. 4).

Some phthalate esters were also detected but they are already reported as chemical contaminants from laboratorial plastic tools (Reid et al. 2007), thus, further research is needed to establish their origin in this extract.

Moreover, natural products are still the most successful source of biologically active lead compounds in drug discovery (Atanasov et al. 2015) and there are some cases where the crude extract is more active than the isolated pure compound, e.g., the extract of *Artemisia annua* has more potent antimalarial properties than its pure natural product, artemisinin (De Donno et al. 2012) and our CJLE can be an example of this hypothesis too as we have found many anti-inflammatory compounds from different chemical groups where not only a few of triterpenes in higher amounts are responsible but also a number of compounds from sesquiterpene, monoterpene, tocopherol, diterpene biosynthesis pathways are there which also exhibits the same property.

Conclusion

Ongoing anti-inflammatory assays, other experiments, and gene sequencing studies are needed to confirm this metabolite-based pathway study and to find out the key enzymatic genes behind the synthesis of those anti-inflammatory terpenoids found in CJLE. Herbal medications are becoming popular day by day because of their relatively few side effects, and our GC-MS analysis suggests not only as an ornamental plant but also this plant should be cultivated vigorously in this region to use the leaves as anti-inflammatory herbal formulations. Moreover, twenty-nine anti-inflammatory compounds with a share of more than eighty-three percentage area in *Camellia japonica* leaf extract; large peaks of metabolites leading by bioactive triterpenoids like squalene and lupeol (which can be isolated to use in pharmaceutical industries as this research has shown their abundance in *Camellia japonica* leaf); abundance of novel anticancer and anti-inflammatory medicinal compound lupeol with 17.26% peak area; and design of unexplored biosynthesis pathway of common plant component methyl commate B through metabolomics; are the key outcomes and highlights of the study.

Abbreviations

CJLE: *Camellia japonica* leaf extract; DMAPP: Dimethylallyl diphosphate; FPP: Farnesyl diphosphate; GC-MS: Gas chromatography-mass spectrometry; GGPP: Geranylgeranyl diphosphate; GPP: Geranyl diphosphate; IPP: Isopentyl diphosphate; SPP: Solanesyl diphosphate; PPP: Phytyl diphosphate

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by SM, AG, and MB. The first draft of the manuscript was written by SM and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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