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Isolation and identification of phenolic compounds by HPLC and electrospray ionization mass spectrometry and their free radical scavenging activity of *Shorea tumbuggaia* Roxb.

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Abstract

The impetus for developing analytical methods for phenolic compounds in natural products has proved to be multifaceted. The traditional usage of *Shorea tumbuggaia* as a folklore medicine, the plant parts are administered to counteract different ailments. Hence the present study intended to isolate phenolic compounds from stem bark of *Shorea tumbuggaia* by using 70% acetone and poly vinyl poly pyrrolidone; and characterized by U.V. Visible spectrometry, High performance liquid chromatography/ electrospray ionization mass spectrometry. And an assessment of the ability of the extracted phenols to scavenge the hydrozen perioxide; sueproxide, DPPH was determined with reference to ascorbic acid by spectrophotometric methods. Total 90 phenolic compounds were obtained at both positive and negative ion modes of LCMS. Among the isolated phenols, 20 phenolic compounds have been identified based on their retention time and m/z values. Namely, Benzoic acid, caffeoyl-5-ferryol quinic acid, thymol, homovanillic acid, eudesmic acid, caffeic acid, 9 CoA, chrysin, pinocembrin, luteolin, quercetin-3-rutinoside, ellagic acid, cohumulone, caffeic acid hexoside, lupulone, deoxy hexoside of ellagic acid, 3'-4-Di hydroxyl 5-6 dimethoxy 7-0-glucoside flavones, oleuropein, pelargonidin-3-rutinoside and heptameric procyanidin. These phenols has showed good antioxidant activity the highest hydrogen peroxide (H_2O_2) radical scavenging effect of the isolated phenolic compounds has been recorded at 81.8% when compared to the DPPH and superoxide ion activities with reference to ascorbic acid. This study illustrate the rich array of phenolic compounds and their free radical scavenging activity of stem bark of *Shorea tumbuggaia* could be utility as health beneficial bioactive compounds.

Keywords: *Shorea tumbuggia*, stembark, phenolic compounds, liquid chromatography, Electro Spray Ionization mass spectrometry, hydrozen peroxide

Introduction

Plants are sessile organisms that cannot evade their environment and have thus evolved all sorts of plastic mechanisms to deal with a wide range of potential threats. Among those mechanisms, the expansion of secondary metabolites with defence, communication and protection roles is now considered to be particularly important. The range of secondary metabolites including phenols, amines, indoles, alkaloids and sulphonates may act as reductant substrates of peroxidases [1]. Researchers have become increasingly interested in dietary phenolic compounds because of free radical scavenging activity and other potential beneficial effects on human health associated with their consumption [2]. Many activities have been reported for most of the phenolic compounds from plants they act as anti-oxidant, anti-inflammatory, anti-viral and anti carcinogenic agents [3].

Phenolic compounds are one of the most diverse groups of phytochemicals that are universally distributed in fruits, vegetable and herbs. Approximately 8000 phenolic compounds have been isolated from natural resources [4]. Polyphenols in nature generally occur as conjugates of sugar, usually o-glycosides, phenolic acids contain two distinctive carbon frame works, the hydroxyl cinnamic and hydroxyl benzoic structures [5, 6]. Although the basic skeleton remains the same, the numbers and positions of the hydroxyl groups on the aromatic ring make the difference and establish the variety [7].

Natural antioxidants such as phenols, flavonoids and tannins are increasingly attracting attention because they are natural disease preventing, health promoting and anti-ageing substances [8]. Antioxidants may serve the task of reducing oxidative damage in humans induced by free radicals and reactive oxygen species under oxidative stress conditions. These conditions can cause DNA and protein damage, lipid peroxidation, cancer, ageing and inflammatory activity [9]. Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical induced tissue injury [10].

Besides well known and traditionally used natural antioxidants from tea, wine, fruits, vegetables and spices are already exploited commercially either as antioxidant additives or a nutritional supplements [11]. Number of plant species have been investigated in the search for novel antioxidants [12-15]. Still there is a demand to find more information concerning the antioxidant potential of plant species.

Shorea tumbuggaia is a globally threatened endemic, endangered tree taxon with economic and medicinal values. Leaf juice is used as ear drops for children [16]. The stembark having anti ulcer activity [17]. Stem bark is good source for secondary metabolites [18] maximum total phenolic content has been reported in stem bark after flower buds of *Shorea* [19]. The traditional usage of *Shorea tumbuggaia* as folklore medicine, the plant parts are administered to counteract heavy sweating. The gum is used in indigenous medicine as an external stimulant and a substitute for arbutus [20]. The present study aimed to expand the compositional database of on phenolic compounds by isolation, characterization and free radical scavenging of *Shorea tumbuggaia* stem bark.

Materials and Methods

Extraction of polyphenols from stembark

The fully matured healthy stembark of *Shorea tumbuggaia* were collected from Tirumala hills, Chittoor District of Andhra Pradesh, India during December 2010. The materials were washed thoroughly and shade dried.

30 g of stem bark powder reduced in a mortar and consequently extracted with 500 mL of dichloromethane by ultra-sonication for about 30 min and shaken by vortex for 30 min to remove hydrophilic compounds. Dilapidated powder were extracted with 500 mL of acetone/water (70:30, v/v) by sonication for 15 min and shaking for more 15 min to extract polyphenols. 150 mL of each stembark water extract (pH adjusted to 4.0) was mixed with 5 g of PVPP (30 mg/mL) for 15 min of shaking for adsorption of phenolic compounds to PVPP. The remaining PVPP was re-extracted twice again with 200 mL of fresh extraction solvent for the same period time that was used before. The combined extracts were evaporated at room temperature by rotary evaporation to remove the organic solvent (acetone).

HPLC-ESI-MS/MS analysis

The qualitative study of the phenolic compounds in all samples was performed by HPLC coupled on-line with electrospray ionization (ESI) mass spectrometry. The HPLC system (Agilent 1100 series) consisted of a low-pressure quaternary pump (Agilent 1100 series) and an auto-sampler. A quadropole ion trap mass spectrometer (Agilent 1100) equipped with an ESI source in the positive and negative ion mode and Xcalibur software Version 1.4 (Finningan) were used for data acquision and processing.

Free Radical Scavenging Activity

Hydrogen Peroxide scavenging activity of *Shorea tumbuggaia* stembark extract was determined using a modification of the method of Ruch *et al* [21]. Superoxide Scavenging Activity [22], 1,1-diphenyl-2-picrylhydrazyl Radical activity (DPPH) Hatano *et al.* [23].

Results and Discussion

In this study over 90 phenolic compounds were extracted from lyophilized samples of *Shorea tumbuggaia* stem bark. Among these 20 phenolic compounds were identified by comparing rentention times, UV VIS, HPLC and MS spectral data with those of literature data. The tentative identification of these compounds were summarized in Table-1 and Fig.4. U.V. Visible spectrometry was a valuable tool for identifying the class of phenolic compounds. The extracted sample solution has given maximum height of the peak at 262 nm from the range of 254 nm to 300 nm of peak (Fig.1). Similar results obtained the Aaby *et al.,* [24, 7]. Among the eluted peaks of HPLC 3rd peak showed more height (512 mv) with large area (24,539 mvs) (Fig.2). Acidification of the mobile phase allows the best separation, because the hydroxyl groups are kept in their acidic form there by increasing their retention on the column and decreasing peak broadening caused by formation of deprotonated form [25]. Liquid chromatography, electrospray ionization – Tandem mass spectrometry (LC-ESI-MS) has been given MS fragmentation data for structural characterization (Fig.3).

The fragment ions m/z 113 is unique to caffeic acid was observed. So this ions were identified as deprotonated caffeic acid hexoside. Thymol was identified as it showed identical LC MS characteristics as that of the standards m/z 149. It will produce the fragments at m/z 131 and m/z 120 by the loss of water and an ethyl (C_2H_2-CH_3) group. Similar results has been obtained from Lamiaceae spices by Hossain [26]. Pinocembrin [M-H$^-$] ion of m/z 255 and chrysin m/z 253 identified by MS fragments. These two compounds may be produced from m/z 579 fragmentation with loss of 152 amu. These compounds have been found in most European honey samples and rose mary honey Roman [27]. Identification of caffeic acid was achieved by comparing the ESI-MS spectra with those of pure standard. While has generated in negative ion mode gave the deprotonated molecule at m/z 135 [M-H$^-$] due to the loss of CO_2, reported from *Olea europara* by Savarese *et al.,* [25].

Negative mode ion at m/z value at 491 is similar to that of quercetin glycoside. But these compound seems to be a derivative of Quercetin glucoside. The corresponding kaempferol derivative was also observed in Black berry [28]. The identification of oleuropein was corroborated by detection of the molecular ion at m/z 539 and its aglycone fragment at m/z 377. Similar results were reported from olive pulp. The mass spectrum of m/z 539 was formed by the lose of 162 Da and another intense peak at m/z 377 indicative of the elimination of another hexose unit [29]. These two main fragments correspond to oleuropein and its aglycone respectively and together they support the hypothesis of a hexose derivative of the oleuropein structure. Among the eluted phenolic compounds,

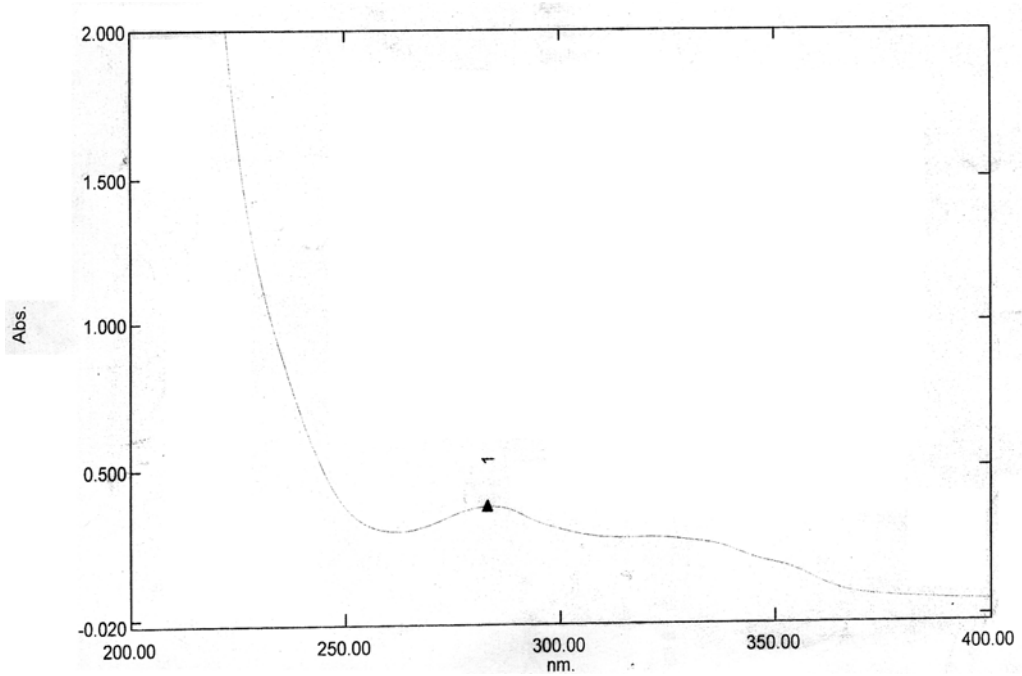

Figure-1 U.V. Visible Spectra of Phenolic Extract of *Shorea tumbuggaia*

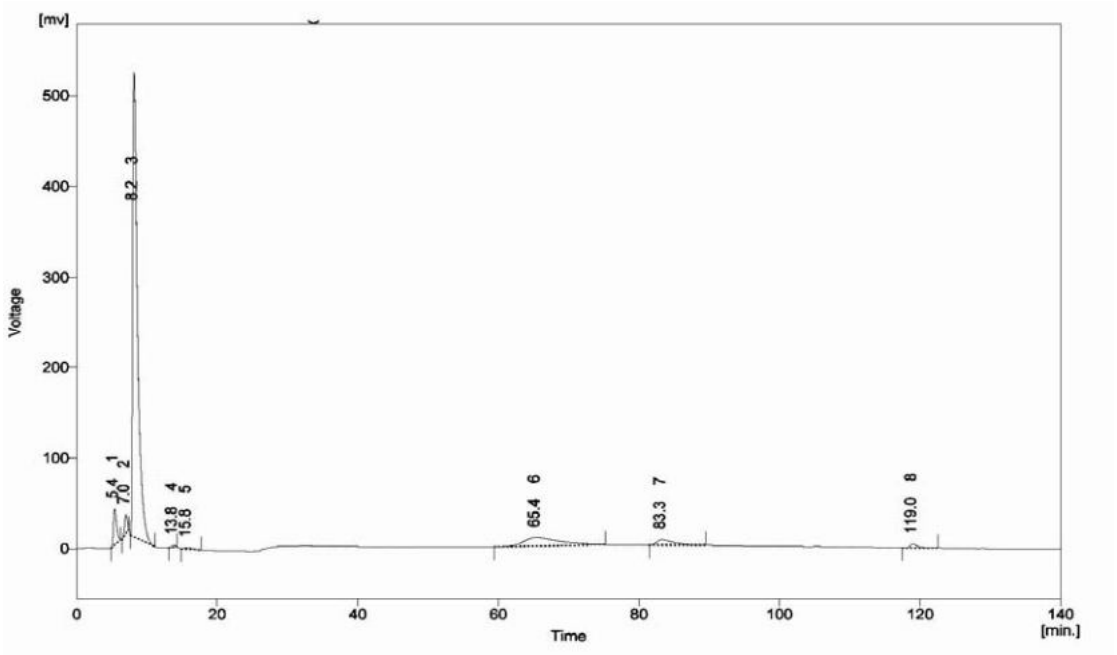

Figure-2 HPLC Chromatogram (monitored at 270 nm)of phenolic extract of *Shorea tumbuggaia*

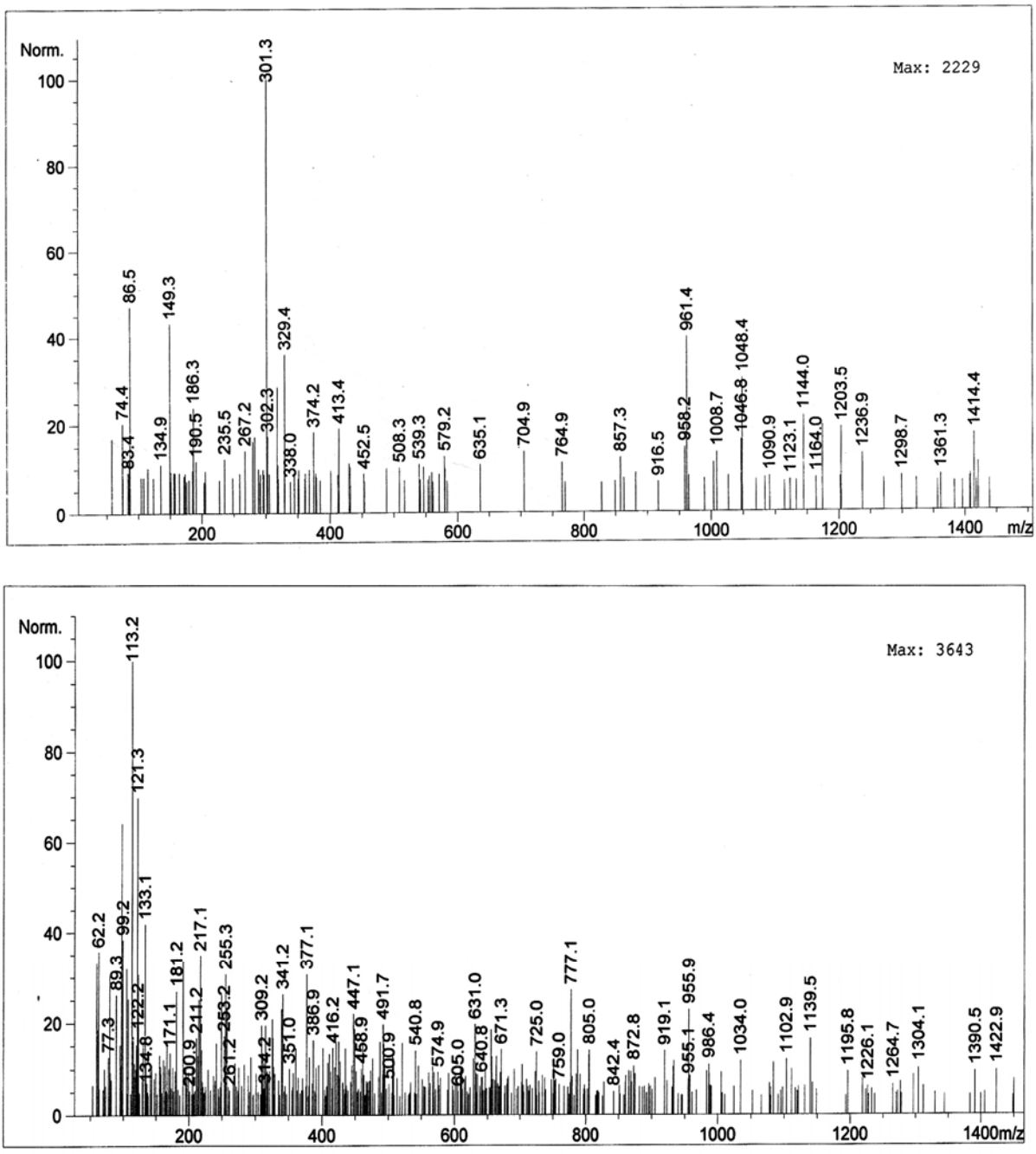

Figure-3 Mass spectra of phenolic extract of *Shorea tumbuggaia*
a) spectra obtained in positive ion mode fragmentation
b) spectra obtained in nagative ion mode fragmentation

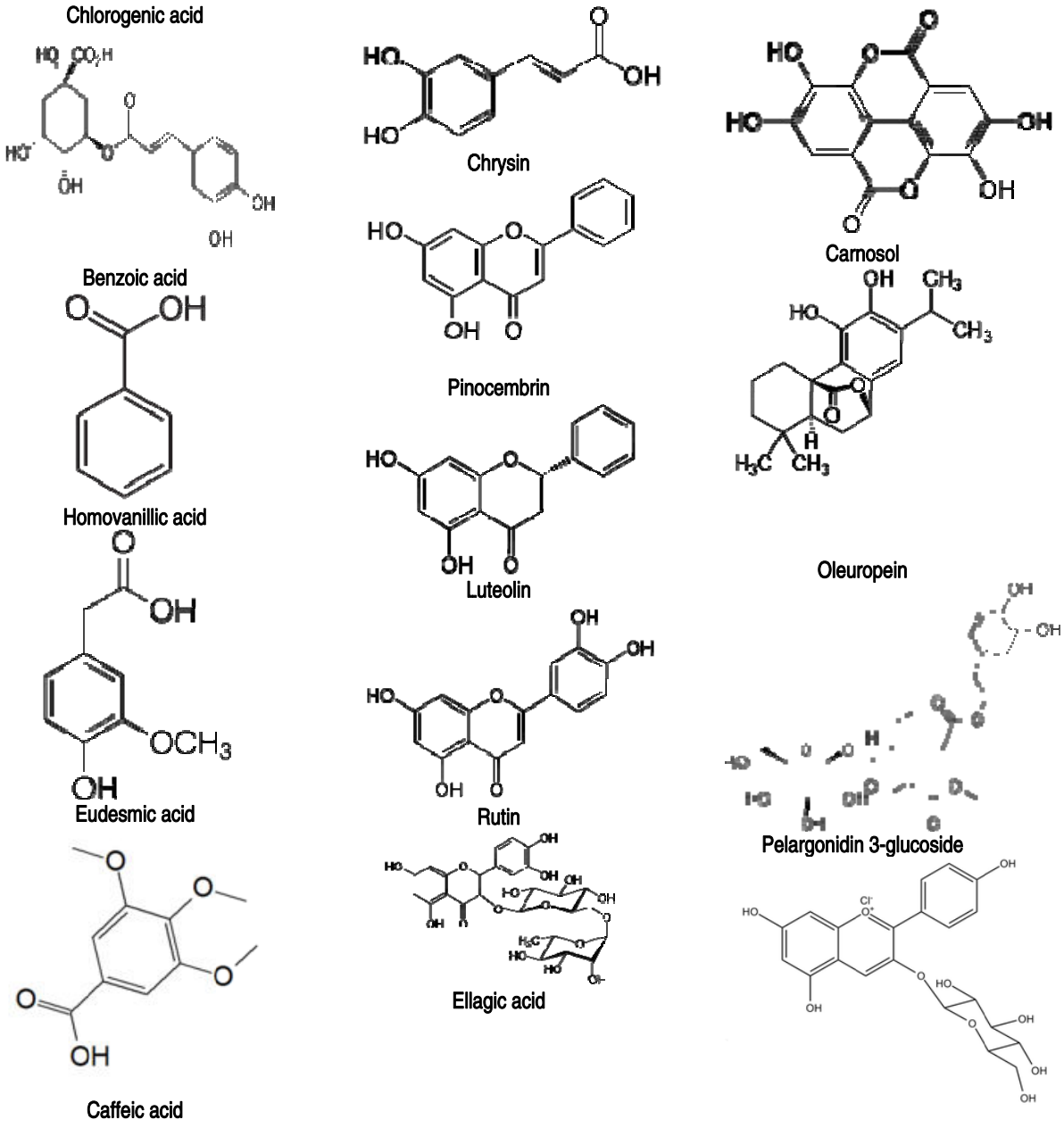

Figure-4 Chemical structures of phenolic compound detected in *Shorea tumbuggaia*

Table-1: Phenolic Compounds Identified in *Shorea*

S.No.	Pseudomolecular Ion m/z values	Empirical molecular formula	compound
1.	113	$C_9H_8O_4$	Coffeic acid
2.	121	$C_7H_6O_2$	Benzoic acid
3.	149	$C_{10}H_{13}O^-$	Thymol
4.	181	$C_9H_{10}O_4$	Homovanillic acid
5.	211	$C_{10}H_{12}O_5$	Eudesmic acid
6.	235		9-COA
7.	253	$C_{15}H_{10}O_4$	Chrysin
8.	255	$C_{15}H_{12}O_4$	Pinocemibrin
9.	267	$C_{15}H_{10}O_6$	Luteolin
10.	301	$C_{15}H_9O_7$	Quercetin-3-rutimoside (rutin)
11.	302	$C_{14}H_6O_8$	Ellagic acid
12.	329	$C_{20}H_{26}O_4$	Cohumulone / Carmosol
13.	341	$C_{15}H_{17}O_9$	Caffeic acid hexoside
14.	377		Drviative Oleuropein aglycone
15.	413		Lupulone
16.	447		Deoxylexoside of Ellagiacid
17.	491		DDGF
18.	539	$C_{25}H_{32}O_{13}$	Oleuropein
19.	579		Pelargonidin
20.	1008		Heptameric procyanidin

Table-2: Free radical scavenging activity of phenolic compounds of *Shorea*

	8µl	15µl	30µl	Mean
Hydrogen Peroxide Scavenging activity (%)	40.25	60.28	81.83	81.33
Free radical scavenging activity (%)	25.24	38.24	58.85	58.85
Superoxide scavenging activity (%)	12.58	24.67	46.4	46.35

benzoic acid (m/z 121 and 122); homo vanillic acid (m/z 181); and eudesmic acid (m/z 211) are derivatives of the benzoic acid. These are also reported from Oak barrels by Regalado *et al.,* [30]. The molecular ion at m/z 447 can yields fragments at m/z 315 and m/z 301. This peak could be a methyl ellagic acid pentose conjugate as described by Mullen *et al.* [31], Soong *et al.* [32] and Zhang *et al.* [33]. The presence of free ellagic acid was confirmed by its retention time and MS data with m/z 302. Spectrum acquired in full scan mode displayed intense molecular ion at m/z 301 while is a diagnostic of quercetin derivatives. These fragment showed in higher mass region with a relatively high intensity.

Cohumulone (m/z 329) Lupulone (m/z 413) was identified. These were also reported from hop (*Humulus lupulus*) extracts by Magalhaes *et al.* [34]. But m/z 329 has been reported as carnosol from *Origanum majorana* and Lamiace spices [26]. According to Hossain *et al.* [26] m/z 329 is major fragment of m/z 359 known as methoxy carnosol by subsequent loss of carbondioxide molecule. Positive mode ion at m/z 579 identified as Pelargonidin-3-rutinoside, it is also reported by Aaby *et al.* [24] from Strawberry the main product ions of m/z 235 were m/z 115, 113, 103, 85 and 75 which are generated by cleavage of the six membered ring of octulopyrano sonic acids part and it was identified as (9 CoA) 9-Caffeyl – 2, 7-anmydro 2-ctulopyranosonic acid which was recorded from *Erigeron breviscapus* by Zhang *et al.* [33]. The ion peak at m/z 1008 was attributed to the doubly charged species of heptameric procyanidin no clear multiply charged species beyond the doubly charged ones were detected, because of the lower concentration of larger tannin molecules [35]. Results of accurate mass measurement are another diagnostic feature of these compounds. Nevertheless, with no other information available; it was not possible to identify the structures and natures of other compounds.

Polyphenols are a group of secondary metabolites involved in the H_2O_2 scavenging in plant cells. They posses ideal structural chemistry for radical scavenging activity and more effective than tocopherol and ascorbate [36]. The highest free radical scavenging activity of the isolated phenolic compound has been recorded at 81.8%. When compared to the DPPH and superoxide ion activities by comparing with Ascorbic acid (Table.2). Whereas 94% has been reported from *Mellilotus officinalis* by Pourmorad [10], *Hyptis suaveolens* by Sandhyarani [37] and *Catheranthus roseus* by Ferrers [38].

Conclusions

Application of LCMS in the current study provide useful information to characterize 20 phenolic compounds from phenolic profile of *Shorea tumbuggaia*. And the antioxidant activity of phenols showed good effect on H_2O_2 activity than superoxide and DPPH scavenging activity. Moreover these phenols could be used as natural antioxidants substituting the synthetic antioxidants in food, cosmetic and pharmaceutical industries.

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