Synthesis of thymol glycosides under SCCO₂ conditions using amyloglucosidase from *Rhizopus* mold

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Abstract Enzymatic synthesis of water soluble thymol glycosides were carried out using amyloglucosidase from *Rhizopus* mold under supercritical carbon dioxide (SCCO₂) conditions of 120 bar pressure at 50 °C. Thymol **1** formed glycosides with D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5** and D-arabinose **6** in yields ranging from 20.6% to 54.2%. Spectral characterization studies revealed that the reaction occurred between the phenolic OH group of thymol and 1-O/2-O groups of D-fructose and C-1 group of D-galactose, D-mannose, D-ribose and D-arabinose resulting in monoglycosylated/arylated derivatives.

Keywords Amyloglucosidase · Hydrophilicity · C-1 glycosylation · Supercritical carbon dioxide · Thymol

Introduction

Thymol is an aroma compound present in the essential oil of *Nigella sativa* L. seeds. It is produced by these plant species as a chemical defence mechanism against phytopathogens (Vazquez et al. 2001). Therefore, this com-

pound has attracted much attention in food industry, as it has been used in foods such as cheese as natural preservative to prevent fungal growth. (Juven et al. 1994; Vazquez et al. 2001; Venturini et al. 2002). Thymol has also been used in medicine because of its pharmacological importance as antiseptic, antispasmodic, tonic and carminative (Didry et al. 1994). Irrespective of such biological and pharmacological activities, its use is limited, because of its poor aqueous solubility (1 g in 1 L) (The Merck index 2006), sublimation, photo reactivity and poor heat sensitivity (Ghosheh et al. 1999).

From the physiological point of view, the glycosides of thymol can be of pharmacological interest as well as in use in cosmetics and in food as additives. Several attempts have been made to produce thymol glycosides by different methods like chemical glycosylation to yield β -glucosides (Mastelic et al. 2004). Shimoda et al. (2006) reported that glucosyltransferases in the cultured cells of *Eucalyptus perriniana* have the highest specificity for thymol as substrate and are able to convert thymol into higher water soluble β -glucoside (Yield 3%) and β -gentiobioside (87%) which are accumulated in the cells.

Glycosylation occurs readily in plant cells to produce secondary metabolites like saponins and anthocyanins in the form of glycosides in higher plants (Furuya et al. 1989). Glycosylation, besides being an important versatile method for the structural modification of compounds with valuable biological activities, also allows the conversion of water-insoluble and unstable organic compounds into the corresponding water-soluble and stable ones, thereby enriching their pharmacological applications (Suzuki et al. 1996; Vijayakumar and Divakar 2005; Sivakumar and Divakar 2006).

A one-step enzymatic glycosylation is useful for the synthesis of thymol glycosides rather than chemical

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glycosylation, which requires many tedious protection-deprotection steps. Enzymatic glycosylation of thymol with different carbohydrates in supercritical fluid media has not been reported so far. The present study deals with novel synthesis of thymol glycosides with diverse carbohydrate molecules using amyloglucosidase from *Rhizopus* mold under SCCO_2 conditions (Fig. 1).

Materials and methods

Enzyme and chemicals Amyloglucosidase (EC 3.2.1.3) from *Rhizopus* mold (Cat. No. A-7255; Lot. No. 122 K1561) was purchased from M/S Sigma Chemical Company, USA and thymol from M/S Aldrich Chemical Company, USA. D-galactose and D-fructose (HiMedia Pvt. Ltd, India), D-mannose, D-ribose, D-arabinose, glacial acetic acid and sodium azide (Loba Chemie Pvt. Ltd, India); DMF (Sisco Research Laboratories Pvt. Ltd, India); Bio-Gel P-2 Gel (Bio-Rad Laboratories, Inc. USA); Silica gel G for thin layer chromatography (Sd fine-Chem Limited, India); n-butanol (Qualigens fine chemicals, India); $\text{DMSO}-d_6$ (Merck Co, India) and food grade CO_2 cylinder (99.9% purity, Kiran Corporation, Mysore, India) were employed in this present study. Amyloglucosidase activity was found to be 11.2 AU/ $\mu\text{mol}/\text{mg}/\text{min}/\text{enzyme}$ (Sumner and Sisler 1944).

Synthesis of thymol glycosides under SCCO_2 Glycosylation of thymol **1** (6.0 mmol) involved reaction with 6.0 mmol of carbohydrates (D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5** and D-arabinose **6**) in 10 ml DMF in presence of 40% (w/w carbohydrates corresponding to 680–810 μmol per min per mg of enzyme proportion in terms of hydrolytic activity units) of amyloglucosidase and 3.0 ml of phosphate buffer (0.01 M, pH 6.0) under SCCO_2 conditions of 120 bar pressure at 50 °C for 24 h. A CO_2 reactor vessel of 120 ml capacity with a magnetic stirrer, thermostatically controlled to attain a constant temperature was employed. After the reaction, CO_2 was released and the reacted products were extracted with 15–20 ml of distilled water

which was evaporated. The enzyme was denatured at 100 °C in a boiling water-bath for 5–10 min and the dried residue was subjected to chromatographic separation. Unreacted carbohydrate was separated from the product glycosides by size exclusion chromatography using Bio-gel P-2 column (100 cm $\times$ 1 cm) packed along with 0.05 mg of sodium azide to prevent bacterial contamination and eluted with distilled water at 1 ml/h rate. Column fractions were continuously monitored by Silica gel-G coated TLC plates with n-butanol: glacial acetic acid: distilled water (75:15:10 v/v) as developing solvent. Individual glycosides could not be separated well, because of similar polarity of the glycosides formed. The dried residue was subjected to HPLC analysis on an aminopropyl column (3.9 mm $\times$ 300 mm length), using acetonitrile: water (80:20 v/v) as a mobile phase at 1 ml/min flow rate and monitored with a refractive index detector. Retention times for the substrates and products are: D-galactose-9.6 min, D-mannose-8.1 min, D-fructose-8.0 min, D-ribose-5.3 min and D-arabinose-7.3 min, thymol-6-*O*-D-galactopyranoside-9.1-min, thymol-6-*O*-D-mannopyranoside-8.7 min, thymol-6-*O*-D-fructofuranoside-7.8 min, thymol-6-*O*-D-ribofuranoside-5.7 min and thymol-6-*O*-D-arabinofuranoside-7.0-min. The conversion yields were determined from HPLC peak areas of the glycosides and carbohydrate peaks and expressed as percentage glycosides formed with respect to the concentration of the carbohydrates **2–6** employed. The extent of formation of individual glycosides was determined from H-1/H-6 and C1/C6 peak areas from the ^1H and ^{13}C NMR 2D spectra (Charles et al. 2009). Error in yield measurements was ± 5 –10%. All the yields were an average from two independent experiments as the yields were within $\pm 5\%$ of error.

Spectral characterization Isolated thymol glycosides were characterized spectroscopically by UV, IR, Mass and Two-Dimensional Heteronuclear Single Quantum Coherence Transfer (2D HSQCT) NMR spectra which provided good information about the nature and type of products. Mass spectra were obtained using a Q-TOF Waters Ultima instrument (Waters Corporation, Manchester, UK) fitted

Fig. 1 Synthesis of Thymol glycosides

with electron spray ionization (ESI) source. 2D HSQCT were recorded along with ^1H and ^{13}C NMR spectra on a Bruker Avance AQS-500 MHz (Bruker Biospin, Fallanden, Switzerland) NMR spectrometer (500.13 MHz for ^1H and 125 MHz for ^{13}C) at 35 °C using 40 mg of the isolated glycosides in DMSO- d_6 solvent. In the NMR data, only resolvable signals are shown. Some of the assignments are interchangeable. The isolated glycosides, being surfactant molecules tend to aggregate in solution giving rise to broad signals, thus making it difficult to resolve the coupling constant values of some of the proton signals. Hence, in the proton part of 2D HSQCT, coupling constant values including those of few anomeric protons could not be resolved satisfactorily.

Thymol 1 Solid, mp 51.5 °C; UV (alcohol, λ_{max}): 276 nm ($\pi \rightarrow \pi^*$, ϵ_{276} –2320 M^{-1}); IR (KBr) (stretching frequency): 3610 cm^{-1} (OH), 2970 cm^{-1} (aromatic CH), 1580 cm^{-1} (C=C); MS (m/z) 150.2 [$\text{M}]^+$; 2D HSQCT (DMSO- d_6) ^1H NMR δ_{ppm} (500.13): 7.47 (H-2, 8.0 Hz), 6.72 (H-3), 7.85 (H-4, 8.0 Hz), 2.01 (H-7, 5.4 Hz), 1.37 (H-9, 5.4 Hz), 1.95 (H-10, 5.4 Hz); ^{13}C NMR δ_{ppm} (125 MHz): 127.9 (C2), 133.5 (C3), 125.8 (C4), 127.9 (C5), 154.1 (C6), 30.8 (C7), 23.5 (C9), 26.2 (C10).

Thymol-6-O-D-galactopyranoside 7 a-b Solid, Isolated yield 375 mg (20.0%); UV (λ_{max}): 283 nm ($\pi \rightarrow \pi^*$, ϵ_{283} –510 M^{-1}); IR (KBr) (stretching frequency): 3390 cm^{-1} (OH), 1250 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical), 1070 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical); MS (m/z) 311.93 [$\text{M}-1]^+$; 2D HSQCT (DMSO- d_6) **C1 α -galactoside 7a**: ^1H NMR δ_{ppm} (500.13): **Gal**: 4.97 (H-1 α), 3.88 (H-2 α), 3.68 (H-3 α), 3.63 (H-4 α), 3.32 (H-6 α); ^{13}C NMR δ_{ppm} (125 MHz): **Gal**: 95.5 (C1 α), 71.1 (C2 α), 77.6 (C3 α), 69.0 (C4 α), 73.3 (C5 α), 61.4 (C6 α). **C1 β -galactoside 7b**: ^1H NMR δ_{ppm} (500.13): **Gal**: 4.91 (H-1 β), 3.78 (H-2 β), 3.82 (H-3 β), 3.52 (H-4 β), 3.93 (H-5 β), 3.35 (H-6 β); ^{13}C NMR δ_{ppm} (125 MHz): **Gal**: 101.9 (C1 β), 70.8 (C2 β), 76.4 (C3 β), 69.1 (C4 β), 74.8 (C5 β), 62.8 (C6 β).

Thymol-6-O-D-mannopyranoside 8 a-b Solid, Isolated yield 263 mg (14.0%); UV (λ_{max}): 286 nm ($\pi \rightarrow \pi^*$, ϵ_{286} –490 M^{-1}); IR (KBr) (stretching frequency): 3340 cm^{-1} (OH), 1255 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical), 1065 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 2940 cm^{-1} (CH); MS (m/z) 311.93 [$\text{M}-1]^+$; 2D HSQCT (DMSO- d_6) **C1 α -Mannoside 8a**: ^1H NMR δ_{ppm} (500.13): **Man**: 3.0 (H-3 α), 3.40 (H-6 α); **Thymol**: 2.90 (H-7); ^{13}C NMR δ_{ppm} (125 MHz): **Man**: 78.1 (C3 α), 63.5 (C6 α); **Thymol**: 135.4 (C2), 128.0 (C3), 129.8 (C4), 154.2 (C6), 36.0 (C7), 24.6 (C8), 31.0 (C9), 31.0 (C10). **C1 β -Mannoside 8b**: ^1H NMR δ_{ppm} (500.13): **Man**: 3.58 (H-3 β), 3.35 (H-4 β), 3.01 (H-5 β), 3.40 (H-6 β); **Thymol**: 3.60 (H-3),

3.52 (H-4), 2.90 (H-7); ^{13}C NMR δ_{ppm} (125 MHz): **Man**: 101.7 (C1 β), 74.9 (C2 β), 79.0 (C3 β), 67.2 (C4 β), 77.2 (C5 β), 63.5 (C6 β).

Thymol-6-O-D-fructofuranoside 9 a-b Solid, Isolated yield 506 mg (27.0%); UV (λ_{max}): 290 nm ($\pi \rightarrow \pi^*$, ϵ_{290} –340 M^{-1}); IR (KBr) (stretching frequency): 3430 cm^{-1} (OH), 1250 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical), 1060 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 2940 cm^{-1} (CH); MS (m/z) 312.80 [$\text{M}]^+$; 2D HSQCT (DMSO- d_6) **2-O-D-fructoside 9a**: ^1H NMR δ_{ppm} (500.13): **Fru**: 3.12 (H-1), 4.30 (H-3), 3.65 (H-4), 3.78 (H-5), 3.41 (H-6); ^{13}C NMR δ_{ppm} (125 MHz): **Fru**: 64.6 (C1), 104.2 (C2), 76.1 (C3), 69.3 (C4), 83.0 (C5), 63.9 (C6); **Thymol**: 154.2 (C6), 35.9 (C7), 30.9 (C9), 30.9 (C10). **1-O-D-fructoside 9b**: ^1H NMR δ_{ppm} (500.13): **Fru**: 3.22 (H-1), 4.28 (H-3), 3.78 (H-4), 3.72 (H-5), 3.32 (H-6); **Thymol**: 2.88 (H-7); ^{13}C NMR δ_{ppm} (125 MHz): **Fru**: 61.3 (C1), 98.1 (C2), 76.0 (C3), 64.5 (C4), 81.2 (C5), 59.0 (C6); **Thymol**: 154.2 (C6), 35.9 (C7), 30.9 (C9), 30.9 (C10).

Thymol-6-O-D-ribofuranoside 10 a-b Solid, Isolated yield 190 mg (11.2%); UV (λ_{max}): 278 nm ($\pi \rightarrow \pi^*$, ϵ_{278} –380 M^{-1}); IR (KBr) (stretching frequency): 3480 cm^{-1} (OH), 1050 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 2915 cm^{-1} (CH); MS (m/z) 282.01 [$\text{M}]^+$; 2D HSQCT (DMSO- d_6) **C1 α -ribose 10a**: ^1H NMR δ_{ppm} (500.13): **Rib**: 4.70 (H-1 α), 3.40 (H-2 α); 3.50 (H-3 α), 3.90 (H-4 α), 3.65 (H-5 α); **Thymol**: 7.68 (H-3), 7.65 (H-4), 2.5 (H-7), 1.25 (H-9), 1.28 (H-10); ^{13}C NMR δ_{ppm} (125 MHz): **Rib**: 93.8 (C1 α), 69.5 (C2 α), 67.3 (C3 α), 71.0 (C4 α), 60.7 (C5 α); **Thymol**: 135.5 (C2), 128.8 (C3), 131.7 (C4), 29.9 (C7), 23.4 (C8), 29.1 (C9), 28.6 (C10). **C1 β -ribose 10b**: ^1H NMR δ_{ppm} (500.13): **Rib**: 5.05 (H-1 β), 3.20 (H-2 β), 3.80 (H-4 β), 3.30 (H-5 β); ^{13}C NMR δ_{ppm} (125 MHz): **Rib**: 96.3 (C1 β), 70.4 (C2 β), 83.3 (C4 β), 61.8 (C5 β).

Thymol-6-O-D-arabinofuranoside 11a-b Solid, Isolated yield 362 mg (21.4%); UV (λ_{max}): 307 nm ($\pi \rightarrow \pi^*$, ϵ_{307} –340 M^{-1}); IR (KBr) (stretching frequency): 3340 cm^{-1} (OH), 1260 cm^{-1} (glycosidic aryl alkyl C-O-C asymmetrical), 1050 cm^{-1} (glycosidic aryl alkyl C-O-C symmetrical), 1370 cm^{-1} (C=C), 2955 cm^{-1} (CH); MS (m/z) 305.04 [$\text{M}+\text{Na}]^+$; 2D HSQCT (DMSO- d_6) **C1 α -arabinoside 11a**: ^1H NMR δ_{ppm} (500.13): **Ara**: 5.0 (H-1 α), 3.61 (H-2 α), 3.64 (H-4 α), 3.51 (H-5 α); **Thymol**: 6.55 (H-5), 2.86 (H-7); ^{13}C NMR δ_{ppm} (125 MHz): **Ara**: 95.9 (C1 α), 69.7 (C2 α), 77.4 (C3 α); **Thymol**: 135.4 (C2), 131.4 (C4), 154.2 (C6), 35.9 (C7), 30.9 (C9), 30.9 (C10). **C1 β -arabinoside 11b**: ^1H NMR δ_{ppm} (500.13): **Ara**: 4.90 (H-1 β), 3.58 (H-2 β), 3.60 (H-3 β), 3.75 (H-4 β), 3.52 (H-5 β); ^{13}C NMR δ_{ppm} (125 MHz): **Ara**: 102.1 (C1 β), 69.5 (C2 β), 83.3 (C3 β), 75.4 (C4 β), 63.2 (C5 β).

Results and discussion

Amyloglucosidase (EC 3.2.1.3) cleaves α (1, 4)- and α (1, 6)-glycosidic linkages from the non-reducing end of starch and related maltooligosaccharides to give glucose (Ashikari et al. 1986). The present glycosylation reaction was attempted under reflux using di-isopropyl ether at 68 °C. However, the reaction mixture showed browning due to oxidation of thymol to thymoquinone. Hence, the reaction was carried out under SCCO₂ condition. In the present work, thymol **1** was reacted with carbohydrate molecules-D-glucose, D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5**, D-arabinose **6**, maltose, lactose, sucrose, D-mannitol and D-sorbitol in presence of amyloglucosidase. This reaction did not occur in the absence of the above mentioned enzyme and absence of buffer (McCarter and Withers 1994; Sivakumar and Divakar 2006). The enzymatic reaction occurred only with D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5** and D-arabinose **6** (Fig. 2).

The optimum conditions employed in this work were arrived at from several such optimization works involving a wide range of aglycon molecules in our laboratory. Optimum conditions employed for the reaction are 6.0 mmol of thymol with 6.0 mmol of carbohydrates (**2–6**), in 10 ml DMF in presence of amyloglucosidase (40% w/w carbohydrates corresponding to 680–810 μ mol per min per mg of enzyme proportion in terms of hydrolytic activity units) and 3.0 ml of phosphate buffer (0.01 M, pH 6.0) under SCCO₂ conditions of 120 bar pressure at 50 °C for 24 h (Fig. 1). The results from this glycosylation reaction are shown in Table 1. The highest conversion yield of 54.2% was achieved under SCCO₂ conditions for thymol-D-fructofuranosides.

UV spectra of the thymol glycosides, showed shifts in the $\pi \rightarrow \pi^*$ band in the 278–307 nm (276 nm for free thymol) range and IR C–O–C symmetrical stretching frequencies in the 1050–1070 cm^{−1} range and asymmetrical stretching frequencies in the 1250–1260 cm^{−1} range indicating that thymol had undergone glycosylation. In the 2D HSQCT spectra of the thymol glycosides, the following glycoside formation were confirmed from their respective chemical shift values: from D-galactose **2** to C1 α galactoside **7a**, at 95.5 ppm and H-1 α at 4.97 ppm and C-1 β -galactoside **7b** to C-1 β at 101.9 ppm and H-1 β at

4.91 ppm; from D-mannose **3** to C3 α mannoside **8a** at 78.1 ppm and H3 α at 3.0 ppm and C-1 β -mannoside **8b** to C-1 β at 101.7 ppm and H-3 β at 3.58 ppm; from D-fructose **4**, 2-*O*-D-fructofuranoside **9a** to C2 at 104.2 ppm and H-3 at 4.30 ppm and 1-*O*-D-fructofuranoside **9b** to C2 at 98.1 ppm and H-3 at 4.28 ppm; from D-ribose **5** to C1 α riboside **10a** at 93.8 ppm and H-1 α at 4.70 ppm and C-1 β riboside **10b** to C-1 β at 96.3 ppm and H-1 β at 5.05 ppm; from D-arabinose **6** to C1 α arabinoside **11a** to C1 α at 95.9 ppm and H-1 α at 5.0 ppm and C1 β arabinoside **11b** to C1 β at 102.1 ppm and H-1 β at 4.90 ppm. Mass spectra also confirmed the formation of the above mentioned glycosides.

Only mono glycosylated products were detected by 2D NMR. Thymol glycosides did not change colour on treatment with dilute alkali (1 ml of 0.1 M NaOH added to 10 mg glycoside in 1 ml of water) indicating that the phenolic OH groups is glycosylated. NMR chemical shifts of C2, C3 and C4 carbons of thymol showed reaction at the free phenolic OH of C6 of thymol. Of the glycosides synthesized in the present work, thymol-6-*O*-D-galactopyranoside **7 a-b**, thymol-6-*O*-D-mannopyranoside **8 a-b**, thymol-6-*O*-2-*O* **9a** and thymol-6-*O*-1-*O*-fructofuranoside **9b**, thymol-6-*O*-D-ribofuranoside **10 a-b** and thymol-6-*O*-D-arabinofuranoside **11a-b** are reported for the first time.

The glycosylation reaction did not occur with D-glucose. In several enzymatic reactions carried out in our laboratory, D-glucose has always reacted even under SCCO₂ conditions (Vijayakumar and Divakar 2005; Sivakumar and Divakar 2006; Charles et al. 2009). Apart from C-1 glycosylated and 2-*O* or 1-*O*-arylated products, no other secondary hydroxyl groups of the carbohydrates were found to react. Among the eleven carbohydrates employed in this work, D-glucose is the most natural carbohydrate substrate for the enzyme and the other carbohydrate molecules like disaccharides-maltose, lactose, sucrose and the two sugar alcohols-D-mannitol and D-sorbitol did not react. Earlier studies by our group on synthesis of retinol glycosides (Charles et al. 2009) under SCCO₂ conditions had shown that the more hydrophilic disaccharides and sugar alcohols did not either react or gave very low yields. Earlier studies (Vijayakumar and Divakar 2005; Sivakumar and Divakar 2006; Charles et al. 2009) had also shown that differential binding between the substrates (thymol and carbohydrates in this case) and the enzyme plays a crucial

Fig. 2 Carbohydrates reacted with Thymol

Table 1 Amyloglucosidase catalyzed synthesis of thymol glycosides

Glycosides		Product (% Proportion) ^a	Yield (%) ^b NMR/HPLC
<p>7a: Thymol-6-<i>O</i>-α-D-galactopyranoside</p>	<p>7b: Thymol-6-<i>O</i>-β-D-galactopyranoside</p>	7a (70.2) and 7b (29.8) mono galactosides	29.7/15.4
<p>8a: Thymol-6-<i>O</i>-α-D-mannopyranoside</p>	<p>8b: Thymol-6-<i>O</i>-β-D-mannopyranoside</p>	8a (49.7) and 8b (50.3) mono mannosides	23.5/27.6
<p>9a: Thymol-6-<i>O</i>-2-<i>O</i>-D-fructofuranoside</p>	<p>9b: Thymol-6-<i>O</i>-1-<i>O</i>-D-fructofuranoside</p>	9a (47.4) and 9b (52.6) mono fructosides	54.2/34.1
<p>10a: Thymol-6-<i>O</i>-α-D-ribofuranoside</p>	<p>10b: Thymol-6-<i>O</i>-β-D-ribofuranoside</p>	10a (64.7) and 10b (35.3) mono ribosides	20.6/18.2
<p>11a: Thymol-6-<i>O</i>-α-D-arabinofuranoside</p>	<p>11b: Thymol-6-<i>O</i>-β-D-arabinofuranoside</p>	11a (22.0) and 11b (78.0) mono arabinosides	42.9/39.8

^a NMR yields and product proportions were determined by 2D HSQCT NMR—H-1/C1 and or H-6a & b/C6 peak areas or their cross peaks^b HPLC yields were determined as mentioned in the text. The yields were an average from two independent experiments

role in determining whether the reaction occurs or not and extent of the reaction. Due to competition in binding between thymol and the carbohydrates for the active site of the enzyme, that tightly bound between the two, determines the transfer of the donor group to the acceptor molecule. Between thymol and D-glucose, lactose, maltose, sucrose, D-mannitol and D-sorbitol, thymol molecule could be more tightly bound than the carbohydrates and hence could not be available for accepting the carbohydrate molecules leading to no reaction in this case.

Shimoda et al. (2006) reported that glucosyltransferases in the cultured cells of *Eucalyptus perriniana* have the highest specificity for thymol as substrate and are able to convert thymol into higher water soluble β -glucoside (Yield 3%) and β -gentiobioside (87%) which are accumulated in the cells. However, the present enzymatic procedure gave better conversion of 20.6–54.2% with diverse carbohydrate molecules like D-galactose, D-mannose, D-fructose, D-ribose and D-arabinose, besides involving a simpler isolation procedure.

Even the presence of a bulky isopropyl group in this structurally complex acceptor phenol did not pose steric hindrance when the mono saccharide carbohydrate molecules are transferred to its phenolic OH group. In enzymatic reactions involving amyloglucosidase in the regular formed hydrolytic reaction, catalytic amounts of the enzyme is sufficient to affect regio-selective hydrolysis. However, in reverse glycosylation reaction, a catalytic amount of the enzyme is not sufficient to affect glycosylation. Larger amounts of the enzyme (40 % w/w of the carbohydrates corresponding to 680–810 μmol per min per mg of enzyme proportion in terms of hydrolytic activity units) are required. Such a larger amount reacts in terms of regio-selectivity in such reaction as the enzyme catalyses several non-specific reactions as well. The glycosides formed were found to be soluble in water. Thus, this study has shown that a complex phenol molecule like thymol could be glycosylated with diverse carbohydrate molecules using amyloglucosidase from *Rhizopus* mold under SCCO_2 conditions.

Conclusions

Water soluble thymol glycosides were synthesized by supercritical carbon dioxide (SCCO_2) using amyloglucosidase enzyme from *Rhizopus* mold. Glycosides were formed with D-galactose **2**, D-mannose **3**, D-fructose **4**, D-ribose **5** and D-arabinose **6**. The reaction occurred between the 1-*O*/2-*O* groups of D-fructose and C-1 group of D-galactose, D-mannose, D-ribose and D-arabinose with phenolic OH

group of thymol resulting in monoglycosylated/arylated derivatives.

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