ANTIOXIDANT PROPERTIES OF POLYPHENOL OF *Moringa oleifera* LEAVES AND POLYPHENOL GLYCOSIDE CATALYZED BY TRANSGLYCOSYLATION OF *Trichoderma viride*

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ABSTRACT. *Purpose of this study was to synthesize polyphenol glycoside as transfer products that may have some biological activities, by application of transglycosylation reaction in the present of polyphenolic compound which was extracted from Moringa oleifera leaves as its acceptor and different flours as its substrate for the transglycosylation. The reaction was catalyzed by glucosyltransferase derived from fungal culture of Trichoderma viridae as a source of crude enzyme. The formation of transfer products was determined using TLC and HPLC which exhibited that polyphenol glycoside could be synthesized through the enzymatic reaction. The study had shown that substrates such as starch, maltodexrin, corn flour, wheat flour, rice flour and cassava flour were also as potential substrates to synthesis the polyphenol glycoside in the presence of polyphenolic extract as acceptor. The result of HPLC analysis presented that the isolated glycosides had retention times and concentration of 1.446 (0.0017 mg/ml), 1.431 (0.14mg/ml), and 1.474 (0.012mg/ml), respectively, as compared to the retention time of arbutin (1.474) that was applied as authentic standard. Observation using ^{1}H NMR as well as ^{13}C NMR showed that structures of the transfer products were identified as gallic acid-4-O-β-glucopyranoside, ellagic acid-4-O-β-glucopyranoside, and catechin-4'-O-gluco pyranoside, respectively. IC_{50} value of EAGP for DPPH was 46.12µg/ml as compared to ascorbic acid (35.96µg/ml), BHT (39.73µg/ml) and α-tocopherol (42.62µg/ml) respectively, while IC_{50} value of EAGP for ABTS was 64.01µg/ml as compared to ascorbic acid (30.13µg/ml), BHT (38.09µg/ml) and α-tocopherol (54.84µg/ml) respectively, whilst IC_{50} value of EAGP for H_2O_2 was found to be 51.90µg/ml while for BHT, ascorbic acid and α-tocopherol were 44.18µg/ml, 47.18µg/ml, and 49.57µg/ml, respectively.*

KEYWORDS. *Trichoderma viridae*, transglycosylation, *Moringa oleifera*, polyphenol

INTRODUCTION

Moringa oleifera Lam. is a tree that grows widely throughout Asia and Southeast Asia including Sabah in Malaysia. It is known as the drumstick tree based on the appearance of its immature seed pods, the horse-radish tree based on the taste of ground root preparations, and the ben oil tree from seed-derived oils. In some areas, immature seed pods are eaten, while

the leaves are widely used as a basic food because of their high nutrition content (Thurber and Fahey, 2009; Mbikay, 2012; Razis *et al.,* 2014). Moringa leaves have been characterized to contain a desirable nutritional balance, containing vitamins, minerals, amino acids, and fatty acids (Moyo *et al*., 2011; Teixeira *et al*., 2014; Razis *et al*., 2014). Additionally, the leaves are reported to contain various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics, and carotenoids (Alhakmani *et al*., 2013; Vongsak *et al*., 2014). Razis *et al*. (2014) summarized the potential health benefits of *M. oleifera*, focusing on their nutritional content as well as antioxidant and antimicrobial characteristics.

Phenolic compounds, however exhibit instability due to oxidation, light and biochemical changes, which was indicated by occurence of browning reaction, thus its properties and the use as biological active compound was decreased or even vanished. As compared to polyphenol glucosides that were enzymatically synthesized, they were able to demonstrated quite high stability toward the chemical changes (Sulistyo *et al.,* 2008). Enzymatic glycosylation of polyphenols into its glucosides has a number of advantages in contrast to chemical synthesis, which include low cost production, easiness on handling microbial strains and its enzyme for the purpose of synthesis of bioactive compounds. In contrast to conventional chemical synthesis that usually requires tedious protection/de-protection manipulations in order to achieve regio- and stereo-selectivity, enzymatic glycosylation usually provides perfect control of the anomeric configuration and high regio-selectivity without the need of any protecting groups (Crout and Vic, 1998).

Enzymic transglycosylation permits insoluble and less stable bioactive compounds to be changed into the resultant soluble and more stable compounds during suitable single-step biological glucosylation (Shimoda *et al.,* 2006). Additionally, glucosides of physiologically bioactive substances, i.e. vitamin glucosides, have been suggested to be functional anti-allergic agents (Satoh *et al.,* 2001). Cyclodextrin glucano transferase [CGTase, 1,4-□-D glucan 4-□-D-(1,4-glucano)-transferase, EC 2.4.1.19] catalyzes a reversible conversion of polysaccharides and polyphenol compounds to polyphenol glycosides. Through transglycosylation reaction, polyphenol glycosides as the main transfer product, was found to be higher resistant than that of polyphenol aglycone to light irradiation (Sulistyo *et al.,* 2008). CGTase is a starch degrading extracellular enzyme which relates to α-amylase family of glucoside hydrolases (Van der Maarel *et al.,* 2002). It is a distinctive enzyme which is able to produce cyclodextrins (CDs) via intra-molecular transglucosylation called cyclization and transferring glucose residues to an acceptor which has an OH-group. CGTase also has the capability to degrade starch and CDs into simpler compounds (Kometani *et al*., 1996; Ibrahim *et al.,* 2005). Through the cyclization process, CGTase is also capable of catalyzing coupling and disproportionation reactions and apparently it possesses weak hydrolytic activity (Alcalde *et al.,* 2003).

In this study, we evaluated the enzymatic transglycosylation catalyzed by CGTase of fungal strain *T. viridae* in the presence of polyphenolic extract derived from *M. oleifera* leaves that was applied as acceptor for the enzymatic reaction. Chemical structures of the synthesized products were elucidated through NMR spectroscopy technique and their antioxidant activity was furthermore examined.

MATERIALS & METHODS

Media for production of CGTase

Fungal *T. viridae* was grown on a liquid medium, containing 30g glucose, 0.5g yeast extract, tryptone 1.0g, 1.8g NH_4Cl, 2g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.1g $CaCl_2$. $7H_2O$, 0.035g $FeSO_4.7H_2O$, 0.007g $MnSO_4$. $7H_2O$, 0.011g $ZnSO_4.7H_2O$, 0.005g $CuSO_4.7H_2O$, 0.002g $CoCl_2.5H_2O$, 0.0013g $Na_2MoO_4.2H_2O$, 0.002 g H_3BO_4, 0.0005g $Al_2(SO_4)_3$, and 20g of soluble starch dissolved in one liter of phosphate buffer pH 7.0 (Hashmi *et al.*, 2014). The culture of *T. viridae* was incubated for 72-120h, in a shaker incubator at 150 rpm and 40°C. After inoculation then the medium was centrifuged at 8000x*g* for 20 min at 4°C to obtain a supernatant and used as a source of crude CGTase (Sulistyo *et al.*, 2001; 2002).

Enzymatic assay for CGTase

The crude CGTase was assayed for cyclization activity performed by following the method as suggested by Shim *et al.,* (2004). The procedure is based on the reduction in colour intensity due to inclusion complex formation of β-cyclodextrin (β-CD) with phenolphthalein (Alcalde *et al.,* 1999). Soluble starch (2%, w/v) was used as substrate in 50 mM sodium acetate buffer pH 6.0. Approximately 0.1 ml of crude CGTase was added onto 1 ml of soluble starch solution, and then incubated at 80°C for 10 min. The amount of β-CD was determined by measuring the reduction in absorbance at 550 nm. One unit of cyclization activity was defined as the amount of enzyme producing 1μmol of β-CD per minute. Hydrolytic activity was determined by incubating 0.1 mL of CGTase with 0.3% (w/v) soluble starch in 50 mM acetate buffer pH 6.0 for 20 min at 80°C, and its absorbance was measured at 550nm. The increase in reducing sugar content was calculated according to standard curve of glucose (Forouchi and Gunn, 1983). One unit of hydrolytic activity was described as the amount of enzyme releasing 1 μmol of glucose per min under assay conditions.

Extraction of polyphenolic compound

Extraction of polyphenol was done by following the method of Charoensin (2014) with a slight modification. The leaves of *M. oleifera* was purchased from local market at Kota Kinabalu, Sabah, Malaysia. The samples were dried in a hot air oven at 50°C for 72 h and then ground into powder and stored at 4°C prior to extraction *M. oleifera* powder (15 g) was extracted with 350 ml of methanol, and then the liquid extract was filtered through Whatman No. 1 filter paper. The residual solvent was removed by using rotary evaporator and all extracts were freeze dried to obtain the polyphenolic constituents.

Assay for transglycosylation capacity

Approximately 0.5 ml of CGTase solution was added into 2.0ml of reaction mixtures containing 0.1g of wheat flour as substrates in sodium phosphate buffer (pH 7.0), and 0.04g of polyphenols extracted from *M. oleifera* leaves as its acceptors and were incubated at 40°C for 24h. The enzyme reaction was stopped by boiling at 100ºC for 10 min. The reaction products were analyzed using TLC and developed using ethyl acetate, acetic acid and distilled water (3: 1: 1, v/v). The TLC plate was heated at 80-90ºC for 1 h prior to spray with a reagent of 20% H_2SO_4 in methanol, and then heated at 150ºC for 5-10 min. The spot of the reaction product that showed Rf value parallel to the spot of arbutin as the authentic standard for polyphenolic glucoside was referred as the spot of transfer product (Sulistyo *et al*., 2014).

Isolation and purification of transfer product

A reaction mixture (200 ml) containing polyphenolic constituents of *M. oleifera* used as substrate-acceptor and wheat flour as substrate-donor were incubated with CGTase at 40ºC for 24h as the same procedure mentioned above prior to extraction with diethyl ether to remove excess of polyphenols residue that might allegedly prefer to dissolve into solvent phase, where the constituents of glycosides as transfer product might allegedly be remained in water phase was concentrated and furthermore charged onto column chromatography containing octa-dodecyl-silica gel (ODS). The column chromatography was then eluted with gradient solvent of methanol in 1% formic acid (v/v). Fraction solutions resulted by flushing the column that is exhibited single spots on TLC plate within their RF values were parallel to the spot of arbutin were then collected and concentrated (Hashmi *et al.*, 2014).

HPLC/UV-VIS analysis

The purified transfer products were also analyzed by using HPLC. The HPLC/UV-VIS system was comprised of Agilent HPLC system provided with a pump, an automatic injector, a UV-VIS detector and a degasser. Separations were carried out using Apollo C18 reversed-phase column at a room temperature. Acetonitrile (A) and 0.1% aqueous H_3PO_4 (B) was used as a mobile phase with a gradient elution of 24% (A) at 0-12 min, 24-50% (A) at 12-22 min, 50-24% (A) at 22-40 min and 24% (A) at 40-50 min. The separation was monitored through absorbance at 254 nm at flow rate of 0.5 ml/min (Chiang *et al.*, 2012).

Structure identification by using NMR spectroscopy

The purified transfer products were also analyzed by using HPLC. The HPLC/UVVIS system was comprised of Agilent HPLC system provided with a pump, an automatic injector, a UV-VIS detector and a degasser. Separations were carried out using Apollo C18 reversed-phase column at a room temperature. Acetonitrile (A) and 0.1% aqueous H_3PO_4 (B) was used as a mobile phase with a gradient elution of 24% (A) at 0-12 min, 24-50% (A) at 12-22 min, 50-24% (A) at 22-40 min and 24% (A) at 40-50 min. The separation was monitored through absorbance at 254 nm at flow rate of 0.5 ml/min (Chiang *et al*., 2012). The ^{1}H-NMR, ^{13}C-NMR spectra were measured using a Varian XL-400spectrometer in DMSO solution (Shimoda and Hamada, 2010).

Antioxidant activity against DPPH, ABTS and H_2O_2

Radical scavenging activity of selected enzymatically synthesized polyphenol glycoside as ellagic acid-4-O-β-glucopyranoside (EAGP) was furthermore assayed as antioxidant activity against 1,1- diphenyl-2-picryl hydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and hydrogen peroxide (H_2O_2), respectively (Shekhar and Anju, 2014; Dimitrova *et al.,* 2010; Oyedemi *et al.,* 2010). Different concentrations of EAGP (10 to 100 µg/ml) were permitted to react with the respective oxidator and absorbances were determined at 517nm, 734nm and 230nm for DPPH, ABTS and H2O2, respectively against the control solution comprising the sample of EAGP without DPPH, ABTS and H_2O_2. Ascorbic acid, butylated hydroxytoluene (BHT) and α-tocopherol as commercial antioxidant agents were utilized to perform the same activity. The IC50 value of the respective compounds needed to inhibit 50% of the DPPH ABTS and $H_2O_2$was determined.

RESULTS AND DISCUSSION

Transglycosylation activity assay of CGTase

Reaction mixtures containing wheat flour as its substrates, polyphenol extracted from *M. oleifera* leaves as its acceptor and crude CGTase derived from *T. viridae* were incubated to synthesize polyphenolic glycosides as transglycosylation (transfer) products. They were synthesized enzymatically via intermolecular transglycosylation reactions, where it is a process to transfer glucose residues to any acceptor having OH- group. Arbutin (4-hydroxyphenyl-D-glucanopyranoside) was applied as an authentic standard component for determination of existing transfer product on the TLC chromatogram. Results of experiment demonstrated that the substrate of soluble starch, corn flour, rice flour, wheat flour and cassava flour might be all potentially utilized as an alternate for soluble starch as a substrate. The results also displayed that was in the presence of wheat flour as its substrate, we were able to synthesize three types of polyphenolic glycosides (**Fig. 1**).

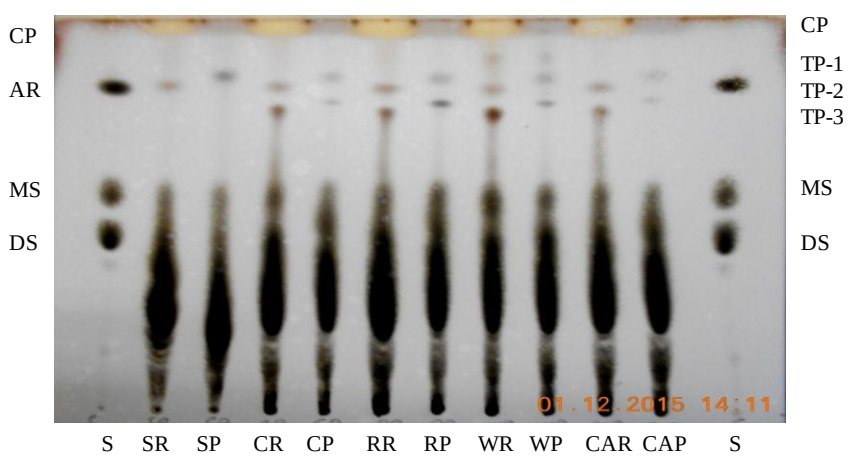

Figure 1: Chromatogram of reaction products of *T. viridae* CGTase in the presence of different acceptors (R, resorcinol; P, pyrocatechol) and substrates (S, soluble starch; C, corn flour; R, rice flour; W, wheat flour; CA, cassava flour) S (standard solution containing arbutin, maltose and glucose).

T. viridae CGTase showed the capability to synthesize polyphenol glycosides through transglycosylation reaction in the presence of different substrates and acceptors. The spots of transfer products those were parallelly corresponding to RF value of the spot of arbutin were considered as the enzymatically synthesized polyphenol glycosides. On the other results of the TLC analysis, it showed that CGTase of *T. viridae* could transfer glucosyl group resulting from hydrolysis in the present of polyphenolic extract originated from *M. oleifera.* The result showed that *T. viridae* CGT*ase* had shown high activity in transferring glucose units to the acceptor of polyphenolic compounds derived from *M. oleifera* (*data not shown*).

The result showed that the transfer products could be separated and isolated from other unwanted components after some fractionation processes were furthermore analyzed by using TLC. All the excessive polyphenolic residues and sugars were removed by continuous fractionation according to the gradient concentration of the solvent. After conducting ODS chromatography followed by HPLC purification, we obtained the following yields for TP-1, TP-2, and TP-3. Purified polyphenolic glycosides referred as TP-1, TP-2, and TP-3 were recovered successfully by using solvent at the ratio of methanol to formic acid (1%) was 60:40 (v/v). The presence of transfer products could be determined on the TLC plate showing the Rf values of their spots were approximately parallel to the spot of arbutin.

HPLC analysis of purified transfer products

Approximately 20µl of a fractioned solution containing expected polyphenol glycoside was directly applied onto HPLC under the optimum conditions as described earlier. The peaks performed on chromatogram were analyzed by comparing the retention time (RT) of arbutin to that of RT of the detected peaks of the respective fractioned solutions (**Fig. 2**). The RT for arbutin as authentic standard was recorded at 1.474. whereas, the RT for TP-1, TP-2 and TP-3 were recorded at 1.446, 1.431 and 1.474, respectively as shown in Fig. 2. According to the standard curve of arbutin prepared as authentic standard by plotting a graph regarding with the concentrations of arbutin against peak area obtained from the HPLC chromatogram, it showed that the concentration of these TP-1, TP-2 and TP-3 were 0.0017 mg/ml, 0.14mg/ml and 0.012mg/ml, respectively.

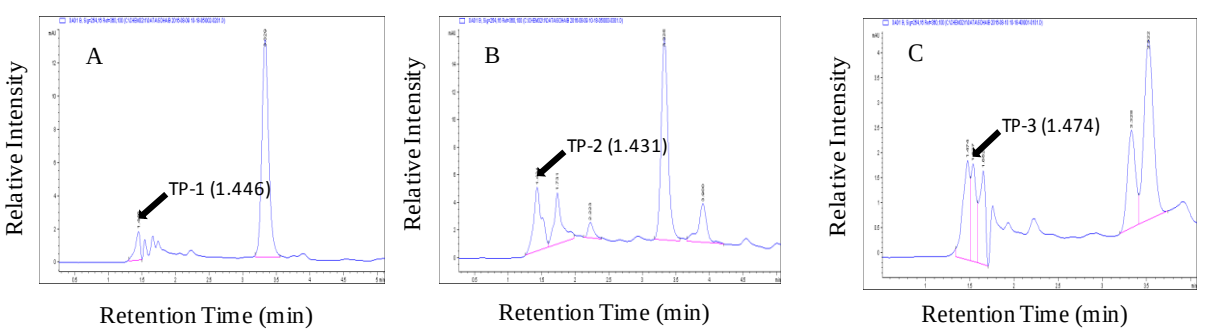

Figure 2: HPLC chromatogram of fractioned solution containing TP-1(A), TP-2 (B) and TP-3 (C) following elucidation through ODS column chromatography.

Structural determination of polyphenolic glycosides.

All the structures of these purified polyphenol glycoside constituents were those that were enzymatically synthesized by using *T. viridae* CGTase in the presence of wheat flour as its donor of glycosyl group, and polyphenols were extracted from *M. oleifera* leaves as its acceptor were furthermore established on the basis of NMR spectroscopic techniques as referred as gallic acid-4-O-β-glucopyranoside (TP-1), ellagic acid-4-O-β-glucopyranoside (TP-2) and catechin-4'-O-β-glucopyranoside (TP-3). **Fig. 3A** shows that the TP-1 was identified as Gallic acid-4-O-β-glucopyranoside based on ^{1}H-NMR spectrum (500 MHz; CD_3OD-d_4/ppm) δ was 7.03 (1H, bs, H-3), 7.03 (1H, bs, H-7), 4.35 (1H, d, J 7.8 Hz, H-1′), 4.26 (1H, dd, J 8.3, 7.8 Hz, H-2′), 3.26 (1H, dd, J 9.3, 8.3 Hz, H-3′), 4.33 (1H, dd, J 9.3, 9.3 Hz, H-4'), 3.37 (1H,m, H-5′), 4.06 (1H, dd, J 11.3, 5.6 Hz, H-6′α), 4.37 (1H, bd, J11.3 Hz, H-6′β), while the result of ^{13}C-NMR spectrum (125MHz; DMSO-d_6/ppm) was 166.2 (C-1),123.6 (C-2),108.8 (C-3),144.96 (C-4), 138.9 (C-5), 144.9 (C-6), 108.8 (C-7), 101.9 (C-1′), 73.8 (C-2′), 78.4 (C-3′), 71.1 (C-4′), 79.9 (C-5′), 63.9 (C-6′).

The TP-2 was identified as Ellagic acid-4-O-β-glucopyranoside based on ^{1}H NMR spectrum (500 MHz; DMSO-d_6/ppm) was δ 7.80 (1H, s, H-5), δ 6.39 (1H, s, H-5′), δ 6.05 (1H, m, H-1"), δ 4.08 (1H, m, H-2"), δ 4.30 (1H, m, H-3"), δ 4.19 (1H, m, H-4"), δ 4.06 (1H, m, H-5"), δ 4.18 (1H, m H-6"$_a$), δ 4.33 (1H, m H-6"$_b$), while the result of ^{13}C NMR spectrum (125 MHz; DMSO-d_6/ppm) was δ 126.4 (C-1), 140.6 (C-2),132.7 (C-3), 140.2 (C-4), 101.6 (C-5), 120.2 (C-6), 161.2 (C-7), 111.4 (C-1′), 158.7 (C-2′), 160.9 (C-3′), 146.4 (C-4′),120.5 (C-5′), 108.5 (C-6′), 165.0 (C-7′), 101.7 (C-1″), 74.9 (C-2″), 77.4 (C-3″), 70.1 (C-4″), 78.2 (C-5″), 63.2 (C-6″) (**Fig. 3B)**.

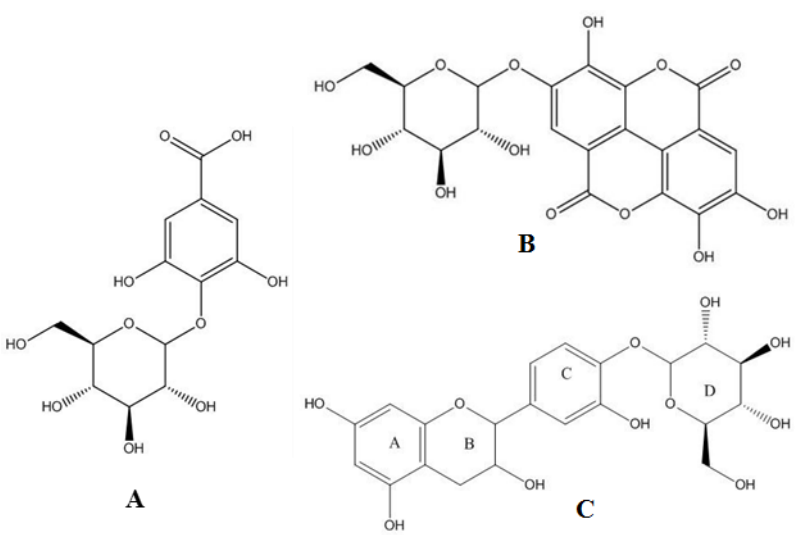

Figure 3: Chemical structure of TP-1, gallic acid-4-O-β-D-glucopyranoside (A); TP-2, ellagic acid-4-O-β-D-glucopyranoside (B); TP-3, catechin-4'-O-β-D-glucopyranoside (C).

The TP-3 was identified as Catechin-4'-O-β-glucopyranoside based on ^{1}H-NMR (500 MHz; CD_3OD-d_4/ppm) δ was 7.24 (1H,d,H-2), 6.84 (1H, d, H-5), 7.01 (1H, d, H-6), 4.60 (1H, d, H-1′), 3.99 (1H, dd, H-2′), 2.52-2.94 (2H, m, H-3′), 5.84 (1H, d, H-5′), 5.93 (1H, d, H-7′). δ 4.05 (1H, m, H-1"), δ 3.48 (1H, m, H-2"), δ 3.38 (1H, m, H-3"), δ 4.13 (1H, m, H-4"), δ 3.40 (1H, m, H-5"), δ 3.68 (1H, m H-6"$_a$), δ 3.89 (1H, m H-6"$_b$), while the result of ^{13}C NMR spectrum (125 MHz; DMSO-d_6/ppm) was 130.2 (C-1),108.6 (C-2),144.8 (C-3),147.96 (C-4), 116.9 (C-5), 119.9 (C-6),82.4 (C-1′), 67.7 (C-2′), 29.6 (C-3′), 156.4 (C-4′), 96.5 (C-5′), 157.5 (C-6′), 95.9.0 (C-7′), 93.3(C-8′),100.7 (C-1″), 73.9 (C-2″), 80.4 (C-3″), 71.3 (C-4″), 78.9 (C-5″), 64.2 (C-6″) (**Fig. 3C)**.

Antioxidant activity against DPPH, ABTS and hydrogen peroxide

Free radical scavenging activity or antioxidant ability of EAGP was determined using DPPH, ABTS and H_2O_2 as assayed oxidant. Ascorbic acid, α-tocopherol and BHT, and were used as a standard to compare with polyphenol glycoside. The current study showed that EAGP exhibited strong antioxidant capacity as compared to the standard α-tocopherol, BHT and ascorbic acid. EAGP exhibited strong radical scavenging activity of DPPH with a value of IC_{50} 46.12 µg/ml as compared to the ascorbic acid (35.96 µg/ml), α-tocopherol (42.62 µg/ml) and BHT (39.73 µg/ml) respectively (**Fig. 4**). The recorded IC_{50} value of EAGP was lower as compared to the standard but still indicating strong antioxidant potential near to the standard. The radical scavenging activity of EAGP was compared with those of ascorbic acid, BHT and α-tocopherol at the same concentration and presented as % of inhibition against ABTS. EAGP significantly quenched ABTS expressing IC_{50} value of 64.01 µg/ml as compared to BHT (38.09µg/ml), α-tocopherol (54.84 µg/ml) and ascorbic acid (30.13 µg/ml) respectively (**Fig. 4**). Moreover, IC_{50} value of EAGP against H_2O_2 free radicals was recorded as 51.90 µg/ml compared to the ascorbic acid, BHT and α-tocopherol those were 44.18 µg/ml, 47.19µg/ml and 49.57µg/ml, respectively (**Fig. 4**). This scavenging activity may be due to the existence of hydroxyl groups devoted to the aromatic ring structures and therefore help to reduce the free radicals.

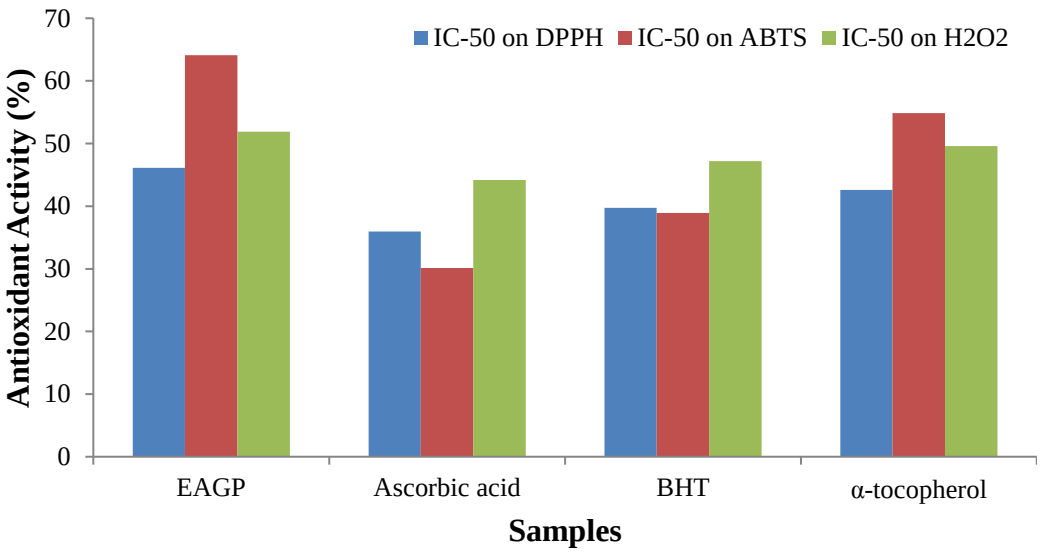

Figure 4: Free radical scavenging activity of EAGP against DPPH ABTS H_2O_2 compared to other samples of antioxidant agents.

CONCLUSION

This paper reports on the synthesis and stabilized polyphenolic constituent that was extracted from *M. oleifera* leaves as source of bioactive compounds by application of transglycosylation reaction CGTase derived from fungal culture of *T. viride*. The enzymatically synthesized products were qualitatively determined using TLC and quantitatively estimated using HPLC, and furthermore determined as transfer products as referred to arbutin as authentic standard. The transglycosylation products were furthermore identified as recognized as novel structures of polyphenol glycosides according to their respective chemical structures through ^{1}HNMR and ^{13}CNMR spectroscopy as gallic acid-4-O-β-D-glucopyranoside, ellagic acid-4-O-β-D-glucopyranoside and catechin-4'-O-β-D-glucopyranoside, respectively. The ellagic acid-4-O-β-glucopyranoside (EAGP) was then selected and subjected to assess its antioxidant potential through *in vitro* analyses. IC_{50} value of EAGP against DPPH ABTS and H_2O_2 free radical scavenging activities was considerably higher as compared to ascorbic acid, BHT and α-tocopherol, respectively that were applied as samples of commercial antioxidant agents.

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