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Glyphaea brevis – *In vitro* antioxidant and *in silico* biological activity of major constituents and molecular docking analyses

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ABSTRACT

Previous studies have revealed that leaf extracts of *Glyphaea brevis* possess antioxidant activity but the bioactivity and mechanisms of action of its major constituents remain unknown. This study evaluated *in vitro* antioxidant and free radical scavenging activities of *Glyphaea brevis* twigs and leaves, and probable toxicity profile, pharmacological activities and mechanisms of action of major phytoconstituents *in silico*. Phytochemical screening detected saponins, tannins, steroids, anthraquinones, flavonoids, terpenoids and phenolics in the extracts. HPLC fingerprinting revealed major compounds as ferulic, catechuic and coumaric acids. Twig extract contained more flavanols compared to the leaf extract while the leaf extract had more flavonol content. Extract of the twigs demonstrated higher ORAC, TEAC and FRAP compared to the leaf extract. *In silico* analyses predicted low acute toxicity risk and pharmacological activities which are in agreement with traditional use of the plant in the management of diseases such as dyspepsia, ulcers, chest pains, diarrhea, dysentery and sleeping sickness. The molecular docking studies revealed that coumaric acid and ferulic acid have the best binding for all proteins tested. In summary, *Glyphaea brevis* twigs possess higher antioxidant activity than the leaves and major constituents showed low toxicological potential and promising biological activities which support its ethnomedical use.

1. Introduction

Nowadays, ethical issues about use of animals for experiments are of great concern necessitating the quest for alternative procedures such as *in silico* methods which do not involve experimental animals thereby helping in reducing animal use. *In silico* methods have been developed to characterize and predict toxic outcomes in humans and environment (Raji et al., 2017; Hartung and Hoffmann, 2009).

In silico tools help to combine various up-to-date computational and experimental approaches in more intelligent ways than a battery of laboratory experimental analyses (Devi et al., 2015). In medicinal chemistry, *in silico* techniques such as virtual screening are used to evaluate pharmacological behaviour and receptor interactions of plant compounds and these methods are fast and cost-effective (Goel et al., 2011). Different online computer programs have been designed by various laboratories for *in silico* analyses. These include Prediction of Activity Spectra for Substances (PASS), ACD/Labs, admetSAR, pkCSM platform and GUSAR. These programs are used to predict comprehensive pharmacological profiles of phytoconstituents and new biological activities of such phytoconstituents. PASS is based on a robust analysis of structure activity relationships and provides more accurate predictions for phytoconstituents which belong to a new chemical class (Poroikov et al., 2003; Filimonov et al., 2014).

The results from predictions are very important and are valuable for planning experiments depending on the probability of false positives in the compound leads selected for biological activity (Dembitsky et al.,

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, Ferric reducing antioxidant power; HPLC, High-performance liquid chromatography; ORAC, Oxygen radical absorbance capacity; PDB, Protein data base; RMSD, root-mean-square deviation; TM-score, Template modelling score; TEAC, Trolox equivalent antioxidant capacity

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2005; Zotchev et al., 2006; Goel et al., 2011; Salgueiro et al., 2016). Apart from the analysis of structure-activity relationships, *in silico* methods can also be used for prediction of toxic properties of chemical structures and to derive predictions for new structures or compounds from a database with experimentally determined toxicity data. Progress in computer-supported modelling have resulted in a better understanding of the molecular mechanisms of metabolites or compounds from plants that are biologically active, and their toxicity (Boobis et al., 2002; Nigsch et al., 2009).

Medicinal plants are widely used as a potential source of primary health care in the developing world where over 3.5 billion people essentially depend on medicinal plants. In addition, majority of the world's population use medicinal plants or compounds derived from them for various medical conditions (Chhetri et al., 2008; Jamison et al., 2006; Bandaranayake, 2006; Alabri et al., 2014). According to the World Health Organization (WHO), about 80% of the world's population use traditional medicines as their first-line therapy (WHO, 2004; WHO, 2005; WHO, 2013). Medicinal plants play important pharmacological roles in the treatment of diverse medical conditions due to the presence of a number of bioactive compounds such as alkaloids, flavonoids, tannins, and other phenolic compounds (Edeoga et al., 2005; Shakya, 2016; Patel and Patel, 2017).

Previous phytochemical investigations of *Glyphaea brevis* have revealed the presence of linear long-chain aliphatic compounds, steroids, triterpenes, polyol, epicatechin, procyanidin B2 and iminosugars (Ekuadzi et al., 2014; Gossan et al., 2015; Mbosso et al., 2013). The ethnomedicinal uses of *Glyphaea brevis* have been documented including treatment of sleeping sickness, paralysis, infectious skin diseases, respiratory diseases, diarrhea, fever, stomach and lung troubles (Mshana, 2000). Some pharmacological evaluations have been carried out on the plant (Dickson et al., 2011; Ekuadzi et al., 2014). However, information on the antioxidant potential, biologically active constituents and the toxicological profile of this plant is still scanty. There is a keen interest in identifying metabolites from plants that can exert beneficial effects on human health (Sen and Samanta, 2014; Shakya, 2016; Watson et al., 2013; Vauzour et al., 2010). However, to maximally exploit the potential health benefits of medicinal plants and their active constituents, there is need to provide researchers and health practitioners with profiles of their range of potential bioactivities, and this cannot be obtained by relying on folkloric information alone. This study aimed to computationally analyse the potential biological effects of the major compounds identified in *Glyphaea brevis* extract, with a focus on antioxidant and free radical scavenging property, a property involved in many pathologies, and to estimate possible toxicity risks.

2. Materials and methods

2.1. Preparation of plant extract

Glyphaea brevis (Spreng) Monach was collected from Olodo village (7° 18′ 0″ North, 3° 37′ 0″ East) in Odeda Local Government, Nigeria. It was identified and authenticated in Forestry Research Institute of Nigeria. A voucher specimen number (FHI 110104) was deposited in the herbarium.

The fresh leaves and twigs of *Glyphaea brevis* were washed, chopped and air-dried at room temperature. The dried leaves and twigs were separately milled into powder using an electric blender (KENWOOD, Model BL490, Taiwan) and weighed. A known weight (100 g) of each of the powder was subjected to Soxhlet extraction in 500 ml of methanol for 5 h. An additional five hours was allowed until solvent in the delivery arm of the Soxhlet extractor became as colourless as the fresh methanol. The mixture was concentrated using a rotary evaporator (MODEL: RE-52A) at 40 °C, and then freeze-dried using a LABCONCO FREEZE DRIER, MODEL 64132 (Missouri, US) to give the dry extract which was used for this study.

2.2. Phytochemical screening of methanolic extract of twigs and leaves of *Glyphaea brevis*

The extracts of twigs and leaves of *Glyphaea brevis* were screened for the presence of alkaloids, saponins, tannins, anthraquinones, flavonoids, terpenoids, steroids, and phenolics using standard phytochemical methods. The qualitative results are expressed as (+) for the presence and (−) for the absence of phytochemicals.

2.2.1. Alkaloids

This was tested by dissolving 0.1 g of extracts in 5 ml of 1% hydrochloric acid (aqueous) in a steam bath and filtering the mixture. About 1 ml of Dragendorff's reagent was added to 1 ml of the filtrate. Formation of a reddish-brown colouration indicates the presence of alkaloids. (Sofowora, 1993a).

2.2.2. Saponins

The tendency of saponins to produce frothing in aqueous solution was employed as test. About 1 g of extract was dissolved in 10 ml of water inside a test-tube and the mixture shaken together. Frothing, which persisted on warming, was taken as preliminary evidence for the presence of saponins (Sofowora, 1993b).

2.2.3. Tannins

The extract (1 g) was dissolved in 5 ml of distilled water and then filtered. Thereafter, 1 ml of ferric chloride was added to the filtrate. A green precipitate indicates the presence of tannins (Trease and Evans, 1989).

2.2.4. Anthraquinones

The extract (2 g) was boiled with 10% hydrochloric acid for 3 min. The solution was filtered, cooled and extracted gently with 10 ml of benzene. The upper benzene layer was pipetted off and shaken gently in a test tube with half of its volume of 10% ammonium hydroxide solution (5 ml). The presence of pink to red colour in the ammonia layer indicated the presence of free anthraquinones (Sofowora, 1993a).

2.2.5. Flavonoids

The extract (0.5 g) was mixed with 5 ml of ethanol and the mixture was shaken and filtered after 5 min. To 1 ml of the filtrate, few drops of 80% alcoholic KOH were added. The mixture was observed for yellow colouration to confirm the presence of flavonoids (Trease and Evans, 1989; Sofowora, 1993b)

2.2.6. Terpenoids

The extract (0.2 g) was mixed with 2 ml of chloroform and allowed to evaporate. Afterwards 3 ml of concentrated H_2SO_4 was carefully added. A greyish colouration indicates positive result for the presence of terpenoids (Trease and Evans, 1989, Sofowora, 1993b).

2.2.7. Steroids

Five ml of extract was added to a mixture of 2 ml of chloroform and concentrated H_2SO_4 which then form layers. The lower layer with red coloration indicated the presence of steroids.

2.2.8. Phenolics

Extract (0.2 g) was completely detanned with acetone. The residue was extracted with warm water after evaporating the acetone on a water bath. The mixture was filtered and allowed to cool. Three drops of $FeCl_3$ solution was added, and the presence of greenish-black colour indicated the presence of phenolics (Sofowora, 1993a).

2.3. Phenolic compounds identification by HPLC profiling of Glyphaea brevis extracts

HPLC was performed using an Agilent 1200 series system (Agilent

Technologies, Santa Clara, CA, USA) consisting of a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and ultraviolet (UV) detector coupled with an analytical workstation and Phenomenex C18 column (Sigma-Aldrich, St. Louis, MO, USA).

The experiment was carried out using a reversed phase Phenomenex C18 (150 × 4.6 mm, 5 μm) column as a stationary phase at a temperature of 30 °C. The mobile phase consisted of a mixture of solution A (distilled water containing 2% acetic acid) and solution B (methanol). Solvent gradients were formed using dual pumping system (Dionex) by varying the proportion of solution A and solution B following the method described by Sabir et al., 2012 with slight modifications. The solutions were eluted at a flow rate of 1.0 ml/min with a wavelength of 320 nm. *Glyphaea brevis* concentration was 10 mg/mL and injected undiluted into the HPLC. Fifty microliters of *Glyphaea brevis* were loaded and injected unto the column. The run time for the program was 35 min.

Identification of major phenolic compounds in *Glyphaea brevis* extract was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards (Sigma-Aldrich). The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra. All chromatography operations were carried out in triplicate.

2.4. Determination of total polyphenols

Analysis of total polyphenols present in extracts of *Glyphaea brevis* twigs and leaves was carried out using the Folin-Ciocalteau reagent (F−C) (Singleton and Rossi, 1965) with gallic acid as standard. In brief, six concentrations of standard were prepared in triplicate by adding 0.1% gallic acid in distilled H_2O to final concentrations of 10, 20, 40, 60, 80 and 100 μg/mL and used to obtain a standard curve. To the gallic acid solution, *Glyphaea brevis* twig and leaf extract (1 mg/ml) and blank, 10% Folin-Ciocalteau reagent and 7.5% sodium bicarbonate were added in a ratio 1:5:4 in a microplate well and incubated for 2 h at 37 °C and read at 765 nm in a 96-well micro plate reader (Thermo Fisher Scientific, USA). The total polyphenol content of the *Glyphaea brevis* twigs and leaves was expressed as mg gallic acid equivalent (GAE)/g of *Glyphea brevis* twig and leaf extract.

2.5. Determination of flavonol content

The flavonols content was determined according to Mazza et al. (Mazza et al., 1999). A standard curve was prepared by diluting quercetin (Q) stock in 95% ethanol to final concentrations of 1, 2, 4, 8, 16 and 32 mg/mL. To 12 μl solution of blank, standard or extracts (1 mg/ml), 238 μL 0.1% HCl in 95% ethanol was added and incubated for 30 min at room temperature in a 96-well microplate. Absorbance was read at 360 nm (96-well micro plate reader (Thermo Fisher Scientific, USA) and the amount of flavonols in the extracts was derived from the quercetin standard curve. Results obtained were expressed as mg Quercetin equivalent (QE)/g of *Glyphea brevis* twig and leaf extract.

2.6. Determination of flavanol content

Flavanol content was determined with (+) catechin as standard (McMurrough and McDowell, 1978). Solutions of standard were prepared in triplicate to a final concentration of 5, 10, 20, 30 and 40 μg/mL by mixing catechin stock solution with methanol to prepare a standard curve. Briefly, 250 μl 4-(Dimethylamino) - cinnamaldehyde (DMACA) was added to 50 μl blank, standard and *Glyphaea brevis* twig and leaf extract in a 96-well microplate. After 30 min of incubation at room temperature the absorbance was read at 640 nm in a 96-well micro plate reader (Thermo Fisher Scientific, USA). The amount of catechin present in the samples was deduced from the standard curve and results obtained were expressed as mg catechin equivalents (CE)/g extract of twigs and leaves of *Glyphaea brevis*.

2.7. Determination of ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) of *Glyphaea brevis* extracts was measure according to the method of Benzie and Strain, 1996. Briefly, five concentrations of ascorbic acid standard were prepared in triplicate by weighing 0.0088 g ascorbic acid in a 50 ml tube and adding 50 ml distilled water, and mixing well until dissolved to a final concentration of 100, 150, 200, 400 and 600 μM. *Glyphaea brevis* extracts were diluted with distilled water (1:24). To 10 μl standard, *Glyphaea brevis* extracts and blank, 300 μl FRAP reagent (10:1:1 *v*/v/v sodium acetate buffer 300 mM, pH 3.6: 10 mM TPTZ in 40 mM 0.1 M HCl: 20 mM $FeCl_3{\cdot}6H_2O$) was added in a 96-well microplate. After incubation at 37 °C for 30 min the absorbance was read at a wavelength of 593 nm using a micro plate reader (Thermo Fisher Scientific, USA). FRAP value of *Glyphaea brevis* extracts were deduced using the ascorbic acid standard curve and the result obtained was expressed as μmol AAE/g extract.

2.8. Trolox equivalence antioxidant capacity (TEAC) assay

The TEAC assay is a useful assay for tracking unknown antioxidants in a complex mixture (Arts et al., 2004). $ABTS^+$ solution was prepared a day before use by mixing 5 ml ABTS salt (8 mM) with 88 μl potassium persulfate (3 mM) and then storing the solution in the dark for 24 h. The $ABTS^+$ solution was further diluted with distilled water. Briefly, 25 μl of the diluted *Glyphaea brevis* twig or leaf extracts was mixed with 275 μl ABTS solution in a 96-well clear microplate. The plate was read after 30 min incubation at room temperature in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA) at 734 nm and temperature set at 25 °C against a blank. The TEAC values were estimated using a linear equation (y = mx + c) between $ABTS^+$ scavenged (y in mM) and the Trolox concentration (x in μM). Trolox was used as the standard and results were expressed as μmol Trolox equivalents (TE)/g sample of extract.

2.9. Oxygen radical absorption capacity (ORAC) test

Briefly, 12 μl of sample was combined with 138 μl of fluorescein working solution (48 nM). The reaction was started by addition of 50 μl AAPH (150 mg/6 ml of phosphate buffer) in a black 96-well plate. The fluorescence was measured for 2 h with a Fluoroskan Spectrum microplate reader with excitation wavelength set at 485 nm and emission wavelength 530 nm at a temperature of 37 °C against a reagent blank prepared with phosphate buffer. A calibration curve of Trolox solution (83–417 μM) was prepared. ORAC values of the samples were calculated using a regression equation (y = a + bx + cx2) and expressed as micromoles of Trolox equivalents (TE) per milligram of sample (Cao et al., 1993; Wang et al., 1997).

2.10. Prediction biological activity of major compounds in Glyphaea brevis extract

2.10.1. Pharmacological activities predicted for major compounds in Glyphaea brevis

Computational screening of possible pharmacological activities of the three identified major compounds in *Glyphaea brevis* twig and leaf extracts (coumaric acid, ferulic acid and catechuic acid) were evaluated using Prediction of Activity Spectra for Substances (PASS) (http://www.pharmaexpert.ru/PASSonline/predict.php) which provides quantitative structure-activity relationships of compounds and exhibits > 3750 kinds of biological activities based on decomposition of chemical structures in 2D and/or 3D descriptors. This software estimates the predicted activity spectrum of a compound as probable activity (Pa) and probable inactivity (Pi). In this study,

Pa > 0.700 = Probable activity > 70%; Pa > Pi is considered as possible for the three major compounds; Pa > 0.7, the chance to find the pharmacological activity experimentally is extremely high (Marwaha et al., 2007).

2.10.2. Mechanism of action predicted for major compounds in Glyphaea brevis

The mechanism of action of major compounds in *Glyphaea brevis* was predicted using Prediction of Activity Spectra for Substances (PASS). This tool provides quantitative structure-activity relationships containing > 205,000 compounds based on chemical structures in 2D and/or 3D descriptors, and generation of models obtained from bioactive ligands (Drwal and Griffith, 2013). Prediction results were expressed in percentage of probable activity (Pa) and probable inactivity (Pi). Pa and Pi values vary from 0.000 to 1.000. Thus, in this study, the PASS prediction results were interpreted as follows: Pa > Pi: possible mechanism of action for the three major compounds; Pa > 0.7: the chance to find the mechanism of action experimentally is extremely high (Marwaha et al., 2007).

2.11. Prediction of toxicity studies

2.11.1. Acute toxicity predictions for Glyphaea brevis main compounds

A computational simulation study was performed to estimate possible acute toxicity risks of *Glyphaea brevis* twigs and leaves extracts three major compounds (coumaric acid, ferulic acid and catechuic acid). Two online computer programs: ACD/Labs (Toronto, Canada) and GUSAR (www.pharmaexpert.ru). The toxic risks assessed are for mouse and rats species coupled with their route of administration, results were expressed according to their reliability and applicability.

2.11.2. Chronic toxicity predictions for Glyphaea brevis main compounds

Possible chronic toxicity risks of *Glyphaea brevis* twigs and leaves extracts three major compounds (coumaric acid, ferulic acid and catechuic acid) were evaluated using computational simulation study. Three online computer programs: ACD/Labs (Toronto, Canada), admetSAR server (Cheng et al., 2012; Cheng et al., 2013), pkCSM platform (Pires et al., 2015). The toxic risks assessed were mutagenicity, carcinogenicity, cardiotoxicity, irritant and reproductive side effects. Results were interpreted and scale of toxicity risks assessment are as follows: (+) low potential, (++) medium risk, (+++) high risk and non-detected risk (ND).

2.12. Collection of major compounds in Glyphaea brevis and proteins target sequence for molecular docking studie

Coumaric acid and ferulic acid were present in abundance in the *Glyphaea brevis* extracts therefore, they were selected for the molecular docking study. The compounds were retrieved from the drugs banks (https://www.drugbank.ca/). Coumaric acid compound PDB file was extracted for the compound with the accession number DB04066 (EXPT01706). Ferulic acid PDF file was also extracted from the drugs bank with the accession number DB07767.

All selected proteins sequence were retrieved from the PubMed-NCBI database (https://www.ncbi.nlm.nih.gov/pubmed/), in which the accession number of the individual protein and these sequences were saved in a Fasta format.

2.12.1. AB Initio predictions of the proteins

The *in silico* prediction of the proteins was done by using the I-TASSER, which stands for 'Iterative Threading ASSEmbly Refinement' server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The individual proteins sequence was submitted to the online server I-TASSER, a PDB file of the result file was downloaded and the cartoon view of the predicted modelled was made and visualised by using PyMol Molecular Graphics.

2.12.2. Molecular docking interaction of proteins and selected major constituents in Glyphaea brevis

In silico molecular docking of selected antioxidants and genes involved in reactive oxygen species metabolism with the major constituents was accomplished using the online web-server, PatchDock (https://bioinfo3d.cs.tau.ac.il/PatchDock/), which allows protein-small, protein-drug molecular docking. In brief, the individual protein PDB file was uploaded first, followed by the major constituents PDB file into the server. The cluster RMSD was set to 4.0 Å as default setting and the complex type "protein-small ligand" was selected. The docking results were obtained and the interaction of the complex formation between the protein and the major constituents were analyzed and visualised using PyMol 1.3 software.

2.13. Statistical analysis

Data are expressed as means ± SD of experiments. The values were analyzed by Two-Way ANOVA, followed by Tukey's multiple comparison test using GraphPad Prism version 6.05 (GraphPad Software, La Jolla California USA). $p < .05$ was considered to be significant.

3. Results

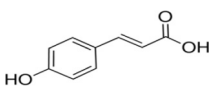
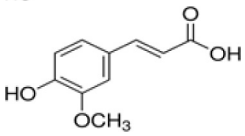
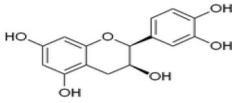
3.1. Phytochemical constituents and HPLC profiles of Glyphaea brevis leaves and twigs

Phytochemicals detected in the extracts are depicted in Table S1 which shows the presence of saponins, tannins, steroids, anthraquinones, flavonoids, terpenoids and phenolics. HPLC profiling revealed that the two extracts contain several phenolic compounds with different peaks (Figs. S1 and S2). The results obtained for the polyphenol quantification of the analyzed twig and leaf extracts are presented in Table 1. External standard method was used to obtain the polyphenolic compounds content, expressed as µg compound/g dry weight (µg/g) of plant material. However, the three identified phenolic compounds were ferulic acid (605.97 µg/g), coumaric acid (860.49 µg/g) and catechuic acid (216.30 µg/g) (Table 1).

3.2. In vitro antioxidant activity of Glyphaea brevis

The total polyphenolic content was higher in the twigs compared to the leaves ($P < .05$). However, flavonol content of the leaves was higher than that of the twigs ($P < .05$) while the flavanol content of the twigs was higher than that of the leaves ($P < .05$) (Fig. 1). Overall, the results showed that the twig extract of *Glyphaea brevis* was richer in polyphenols. The results of FRAP, TEAC and ORAC tests are depicted in Fig. 2. The twig extract showed higher FRAP, TEAC and ORAC values

Table 1
Major compounds of *Glyphaea brevis* by HPLC analysis.

S/N	Major compound	Compound structure	Content in leaves (µg/g)	Content in twigs (µg/g)
1	Coumaric acid		860.49	240.67
2	Ferulic acid		605.97	36.20
3	Catechuic acid		216.30	43.71

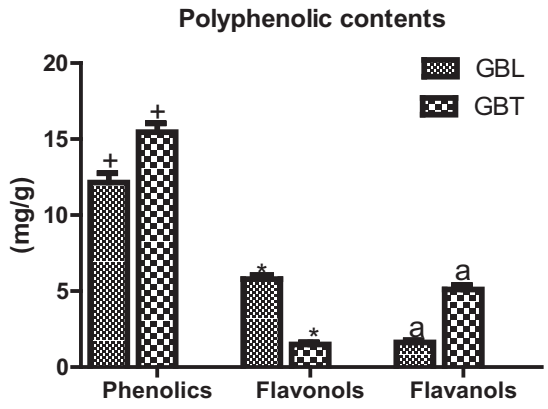


Fig. 1. Polyphenolic contents: phenolics, flavanols, and flavonols contents in the extracts of the leaves and twigs of *Glyphaea brevis*. Bars having the same alphabets/symbols are significantly different from each other. $n = 3$. Abbreviations: GBT, *Glyphaea brevis* twigs, GBL: *Glyphaea brevis* leaves.

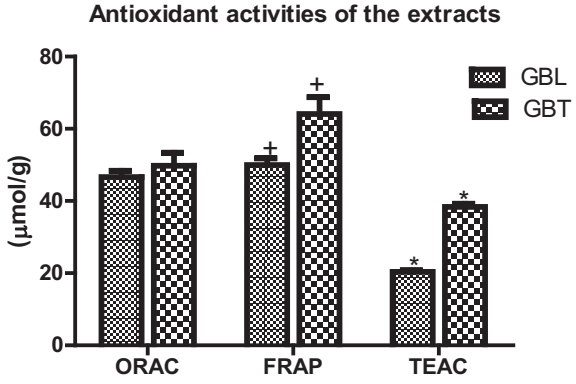


Fig. 2. Antioxidant activities of the extracts: ORAC, FRAP and TEAC activities in the extracts of the leaves and twigs of *Glyphaea brevis*. Bars having the same symbols are significantly different from each other. n = 3. Abbreviations: GBT, *Glyphaea brevis* twigs, GBL: *Glyphaea brevis* leaves.

compared to the leaf extract ($P < .05$).

3.3. Pharmacological activities and mechanisms of action predicted for *Glyphaea brevis* extracts

PASS online software was used to investigate and identify potential pharmacological activities and mechanisms of action of the main compounds in extracts of *Glyphaea brevis* twigs and leaves. The activities and mechanisms of action from PASS for each compound were selected based on the prediction of values > 0.7 (Pa > 0.07). Table S2 shows the predicted pharmacotherapeutic properties for p-coumaric acid, catechuic acid, and ferulic acid while Table S3 shows the mechanisms of action of each compound.

3.4. Theoretical acute and chronic toxicity of *Glyphaea brevis*

The theoretical LD_{50} of p-coumaric acid, ferulic acids and catechuic acids found in *Glyphaea brevis* in different species and *via* different routes of administrations is presented in Table S4. The LD_{50} of p-coumaric acid, ferulic acid and catechuic acid in rats *via* oral administration were 2904, 2754, and 2000 mg/kg, respectively. The theoretical chronic toxicity assessment of major compounds of *Glyphaea brevis* (Table S5) showed that they have no detected risks to genotoxicity, mutagenicity, hepatotoxicity and dermal toxicity; low potential risks to cardiotoxicity; and no binding to estrogen receptors. The compounds were also predicted to be non-carcinogenic and non reprotoxic.

Table 2
Predicted structure of the protein receptors and score

Proteins	C-score	Exp. TM-score	Exp. RMSD (Å)
Superoxide dismutase	1.51	0.92 ± 0.06	1.9 ± 1.6
Peroxiredoxin-2	1.63	0.94 ± 0.05	2.2 ± 1.7
Phospholipid hydroperoxide glutathione peroxidase	−2.86	0.39 ± 0.13	12.6 ± 4.3
Thioredoxin	−2.24	0.45 ± 0.15	13.0 ± 4.2
Keratin	−1.09	0.58 ± 0.14	10.4 ± 4.6
Epoxide hydrolase 2	1.71	0.95 ± 0.05	3.8 ± 2.6

3.5. Target proteins 3D structure prediction using I-TASSER

Table 2 revealed that all the proteins predicted 3-D structure gave C-score values higher than the cut-off (−1.5) except phospholipid hydroperoxide glutathione peroxidase (− 2.86) and thioredoxin (− 2.24). Furthermore, TM-scores of all the selected proteins were above 0.5 cut-off except for phospholipid hydroperoxide glutathione peroxidase and thioredoxin which have TM-scores of 0.39 and 0.45, respectively. On the other hand, the RMSD of all of the predicted structures had a value above 4 Å, except for molecule 1. Despite the fact the RMSD were not < 1 Å, the topologies of the predicted 3D structures are a consequence of it having a TM-score above 0.5, because it is proven that there is a strong correlation between the RMSD and the TM-score of the predicted protein 3D structure (Roy et al., 2010).

Besides the scoring generated by the I-TASSER server, the images of the predicted proteins visualised by PyMol are depicted in Fig. 3. The structures revealed the various secondary structure elements including α-helices, β-sheets, β-hairpins, γ-turns and β-bulges that are consistent with the structures of known proteins.

3.5.1. In silico docking of the proteins and the selected major constituents

After performing the docking of the target selected proteins and the selected major constituents, the result output provides the highest geometric score of a particular complex formed. In addition to the geometric scoring, Atom Contact Energy (ACE) which is the area covered between the two molecules, the transformation coordinates during the molecular interaction and the PDB file of the complex formed as a ball and stick structure was also retrieved (Schneidman-Duhovny et al., 2005).

As depicted in Tables 3a and b, phospholipid hydroperoxide glutathione peroxidase, thioredoxin, keratin and epoxide hydrolase 2 have the best geometric binding scores with coumaric acid and ferulic acid.

However, only the visualisation of the complex formation could give a better insight of the docking; hence, the docking complexes were visualised using PyMol software. Although the *in silico* docking of the cancer proteins showed a positive interaction with the selected drugs, we could decide on the final drugs list that warranted further validation work only if this drug bind the cancer protein, at the point where it can cause the desired activity and ability. This approach is essential because the drugs could be a source of lead compounds that would inhibit cancer proliferation, thus serving as the backbone for the development for cancer therapy. Using this principle, the visualisation showed that both coumaric acid and ferrulic acid bond at the binding site of Protein 3, 5 and 6 (Fig. 4 and Fig. 5). However, coumaric acid and ferulic acid both bind at different site of Protein 1, 2 and 4 (Fig. 4 and Fig. 5).

4. Discussion

Phytochemicals are chemical entities found in plant which often have desirable health benefits. Saponins, tannins, anthraquinones, flavonoids, terpenoids, steroids, and phenolics were detected in *G. brevis* extracts. Alkaloids, detected in the twig extract but not in the leaf extract of *G. brevis*, constitute one of the most effective class of bioactive compounds. Previous studies have reported diverse biological effects of

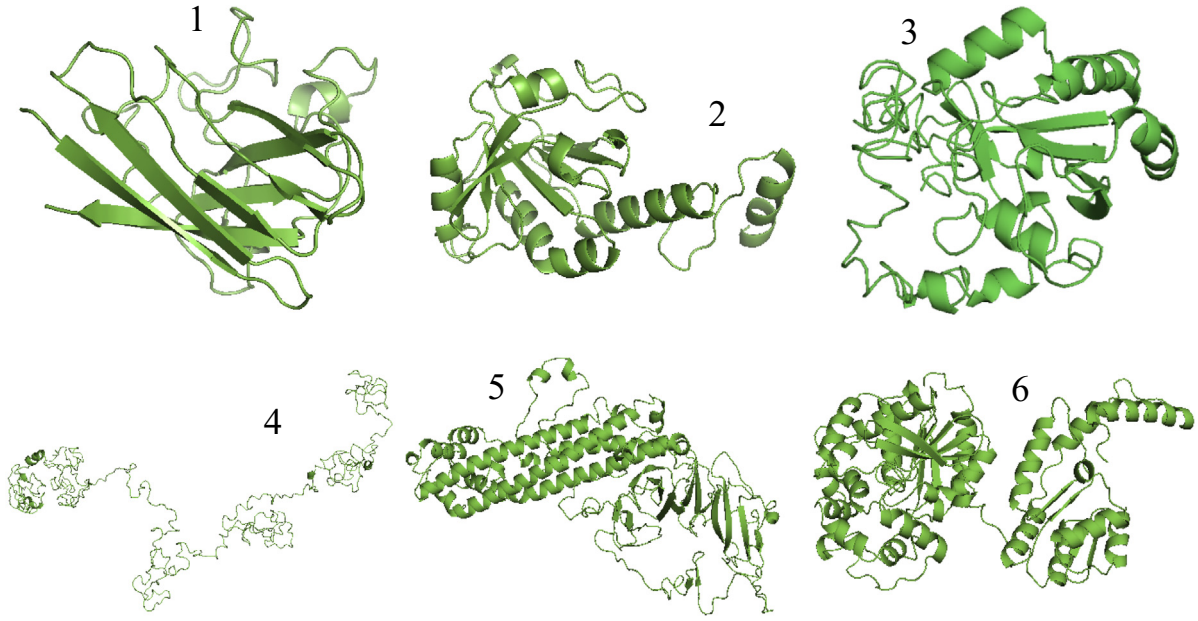


Fig. 3. Three-Dimensional structure of the predicted proteins using I-TASSER online server. Protein 1: superoxide dismutase [Cu−Zn]; Protein 2: peroxiredoxin-2; Protein 3: phospholipid hydroperoxide glutathione peroxidase; Protein 4: thioredoxin domain-containing protein 2; Protein 5: keratin; Protein 6: epoxide hydrolase 2. The AMPs showed different secondary structures including α-helices, β-sheets and extended shapes. The AMPs 3D structures were predicted using an online server I-TASSER and they were visualised using PyMol.

Table 3
Binding affinities of the protein receptors and ligand compounds after the *in silico* interaction.

Complex	Binding affinity score	Area	ACE
(a) Coumaric acid			
Protein 1 + coumaric acid	2728	297.40	- 116.88
Protein 2 + coumaric acid	2624	293.20	- 139.68
Protein 3 + coumaric acid	3022	351.40	- 32.40
Protein 4 + coumaric acid	3302	377.20	- 61.77
Protein 5 + coumaric acid	3798	433.40	- 144.11
Protein 6 + coumaric acid	3584	436.00	- 131.52
(b) Ferulic acid			
Protein 1 + ferulic acid	3112	360.90	- 91.08
Protein 2 + ferulic acid	3126	363.60	- 133.94
Protein 3 + ferulic acid	3534	404.60	- 22.34
Protein 4 + ferulic acid	3674	412.50	- 113.04
Protein 5 + ferulic acid	4150	464.40	- 154.18
Protein 6 + ferulic acid	4068	476.60	- 153.53

alkaloids such as anti-inflammatory (Souto et al., 2011) antimalarial, antimicrobial, (Dua et al., 2013; Benbott et al., 2012) cytotoxicity, antispasmodic and other pharmacological effects (Akinmoladun et al., 2007; Ameyaw and Duker-Eshun, 2009; Thite et al., 2013). Saponins are involved in plant defense system (Banso and Adeyemo, 2006). Tannins, which are also phenolics, have been reported to possess antioxidant, antibacterial, antitumor and antiviral activities (Akharaiyi, 2011; Vadlapudi and Kaladhar, 2012).

Generally, dietary polyphenols function as antioxidants by protecting the body from free radical damage, and acting as a defense mechanism against pathogens and toxins (Ganesan and Xu, 2017; Păltinean et al., 2017). Flavonoids and phenolic acids, in particular, have potent antioxidant and free radical scavenging activities and demonstrate multiple bioactivities such as anti-inflammatory, antimicrobial and anticancer properties (Hossain and Nagooru, 2011; Ganesan and Xu, 2017; Alabri et al., 2014; Thitilertdecha et al., 2008). The presence of flavonoids and other phenolics in *Glyphaea brevis* extracts are indicative of strong antioxidant capacity and ability to protect against oxidative deterioration and its consequences. Steroids, reported to possess cardiotonic, antibacterial and insecticidal properties (Bagrov et al., 2009), were also identified in the leaf and twig extracts of *G. brevis* and may be contribute to the reported the biological activities shown by the plant.

Phenolic acids and derivatives are also important in food chemistry, nutritional science, drug chemistry, and plant physiology (Birošová et al., 2007). The major compounds coumaric, ferulic and catechuic acid, identified in *G. brevis* extracts HPLC in this study have significant antioxidant radical scavenging activity (Retta et al., 2012; Roychoudhury et al., 2017). This property underlies the pharmacological effects of many natural products and the medicinal effects and ethnomedical use of *G. brevis* could stem from it. The antioxidant activity shown by *Glyphaea brevis* extracts in this study, which were assessed by carefully selected biologically relevant antioxidant tests, is a reflection of the antioxidant capacity and bioactivity of its chemical constituents. The tests assessed the reductive ability of *Glyphaea brevis* (FRAP), radical scavenging ability (TEAC) and total antioxidant capacity (ORAC) which are critical components for accurate evaluation of antioxidant potential.

The phytochemical composition, phenolic content and antioxidant and free radical scavenging activity of extracts of *G. brevis* as observed in this study, indicate that the leaves and twigs of the plant are rich sources of antioxidant compounds. Our results also suggest that the twigs are a more excellent source of these bioactive antioxidants.

In order to further investigate the biological activities, mechanism of action and possible toxicity risk of the major constituents present in extract of *G. brevis* twigs and leaves, we used computer simulations. The computer program, PASS (Prediction of Activity Spectra for Substances) was used to explore a more comprehensive pharmacological profile of the major phytoconstituents of the twigs and leaves of the plant as revealed by HPLC analysis. The biological activities of these compounds, their mechanisms of action and related side-effects were evaluated. PASS predictions is based on analysis of structure activity relationship (Poroikov et al., 2003; Jenkins et al., 2006; Filimonov et al., 2014) and its applicability to natural products has been demonstrated by previous investigators (Dembitsky et al., 2005; Zotchev et al., 2006).

Phamacotherapeutic effects predicted by PASS for major

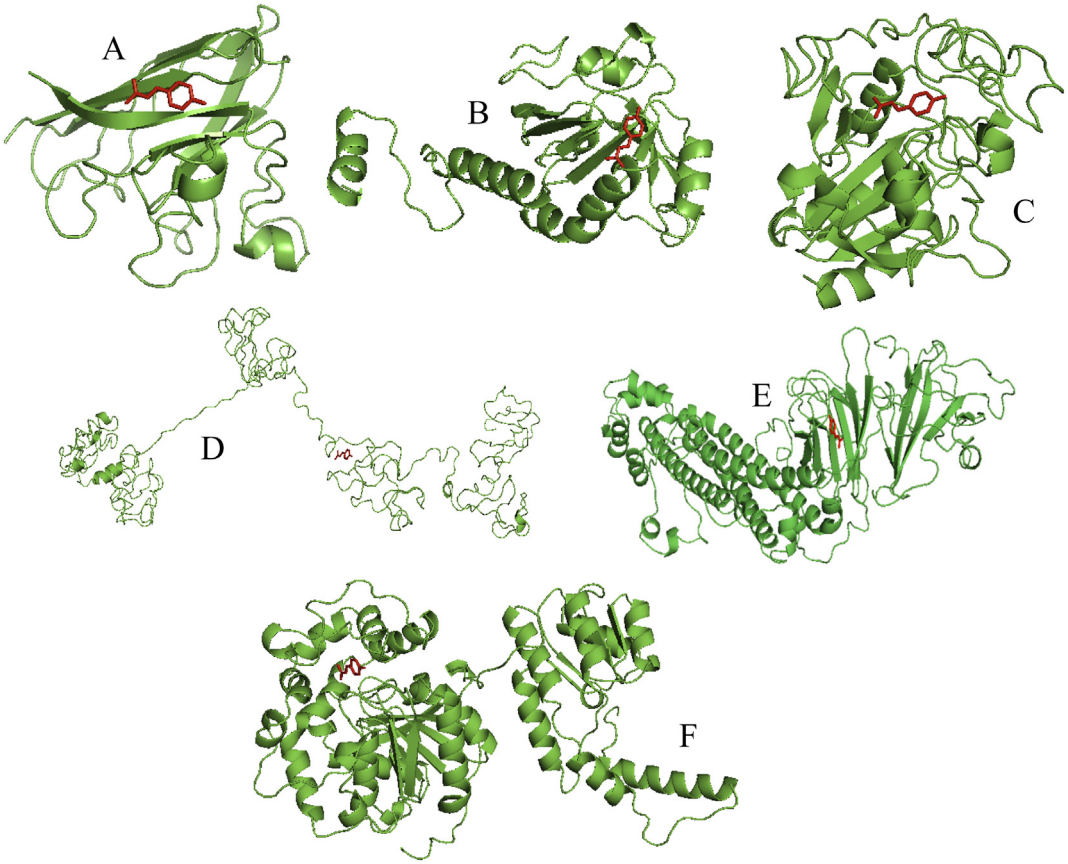


Fig. 4. Interaction between the target proteins and coumaric acid. A: superoxide dismutase + coumaric acid; B: peroxiredoxin-2 + coumaric acid; C: phospholipid hydroperoxide glutathione peroxidase, nuclear isoform B + coumaric acid; D: thioredoxin domain-containing protein 2 isoform 1 + coumaric acid; E: keratin, type II cytoskeletal 1 + coumaric acid; F: bifunctional epoxide hydrolase 2 isoform b + coumaric acid. The cartoon representation in green colour is the target protein and coumaric acid is represented in red colour. The complex were generated with the help of the visualisation software PyMol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compounds from *Glyphaea brevis* twigs and leaves identified by HPLC analysis included antioxidant, antimutagenic, free radical scavenging, antiseptic and lipid metabolism regulatory activities. This finding supports the main traditional uses of *Glyphaea brevis* such as treatment of intestinal diseases, poisoning, ulcers, skin infection and hepatitis (Mshana, 2000) and the contribution of antioxidant and free radical scavenging activity to the bioactivity of the phenolics (Ueda et al., 1996; Feng et al., 2001; Belicova et al., 2001; Birošová et al., 2007). The PASS predictions revealed various mechanisms of action of the compounds. The most prominent mechanisms of action are membrane integrity, membrane permeability, GABA agonist and GST substrate. The results from PASS in this study corroborate previous findings from Obiri et al., 2013 suggesting that *Glyphaea brevis* has possible role in stabilizing the lipid bilayer of mast cells thereby preventing degranulation (Obiri et al., 2013). In addition to this, Oshomoh and Idu in their findings established that the aqueous and ethanol extract of the stem bark of *Glyphaea brevis* showed a significant antimicrobial activity against *Staphylococcus aureus* and *Streptococcus mutans* at concentration of 3.13 mg/ml (Oshomoh and Idu, 2012) which makes the *Glyphaea brevis* stem bark suitable as potential agent for dental care and cleansing which is in line with our findings from PASS.

Owing to this information from PASS and the potential of the herb as a medicinal plant, it was necessary to investigate its possible toxic effects. The results showed that *Glyphaea brevis* has a moderate and high reliability according to ACD/i-Lab predictions and also falls in applicability domains (according to GUSAR prediction) for the species under investigation which are rats and mouse. For all routes of administration considered (oral, intraperitoneal, intravenous and subcutaneous), LD50 for all the 3 compounds in *Glyphaea brevis* indicated very low toxicity potential since the high risk is usually at a high concentrations of < 1000 μg/ml (Meyer et al., 1982). To further ascertain the potential toxicity of the plant, chronic toxicity risk predictions were determined. Results indicated that major compounds identified from of *Glyphaea brevis* exhibited low potential risk of mutagenicity. Cardiotoxicity and hepatotoxicity were not detected according to pkCSM prediction while prediction by adMETSAR server suggested that mutagenicity was not detected in the three major compounds under investigation. The major compounds of *Glyphaea brevis* demonstrated no binding to estrogen receptors according to ACD/i-Lab predictions, they are non-carcinogens and showed no toxicity to the skin according to ACD/i-Lab predictions implying that *Glyphaea brevis* has no negative effect on reproduction and the skin.

Interestingly, the *in silico* evaluation in this study indicated that there are several unknown activities, which is an eye opener providing a basis for evaluation of unknown hidden potentials of the selected phytoconstituents and related analogues in this medicinal plant. This can be further validated in *in vitro* as well as *in vivo assays* (Filimonov and Poroikov, 2008; Poroikov et al., 2009).

Phytochemical analyses and *in vitro* experiments of the antioxidant activity of plants do not provide a comprehensive molecular understanding regarding the binding orientation of bioactive major constituents to target proteins and genes involved in reactive oxygen species metabolism. Owing to the multiplicity of properties and activities of *Glyphaea brevis* from this study, we employed computational modelling namely, *in silico* molecular docking analyses to predict the interaction between major phytochemicals of this plant and selected

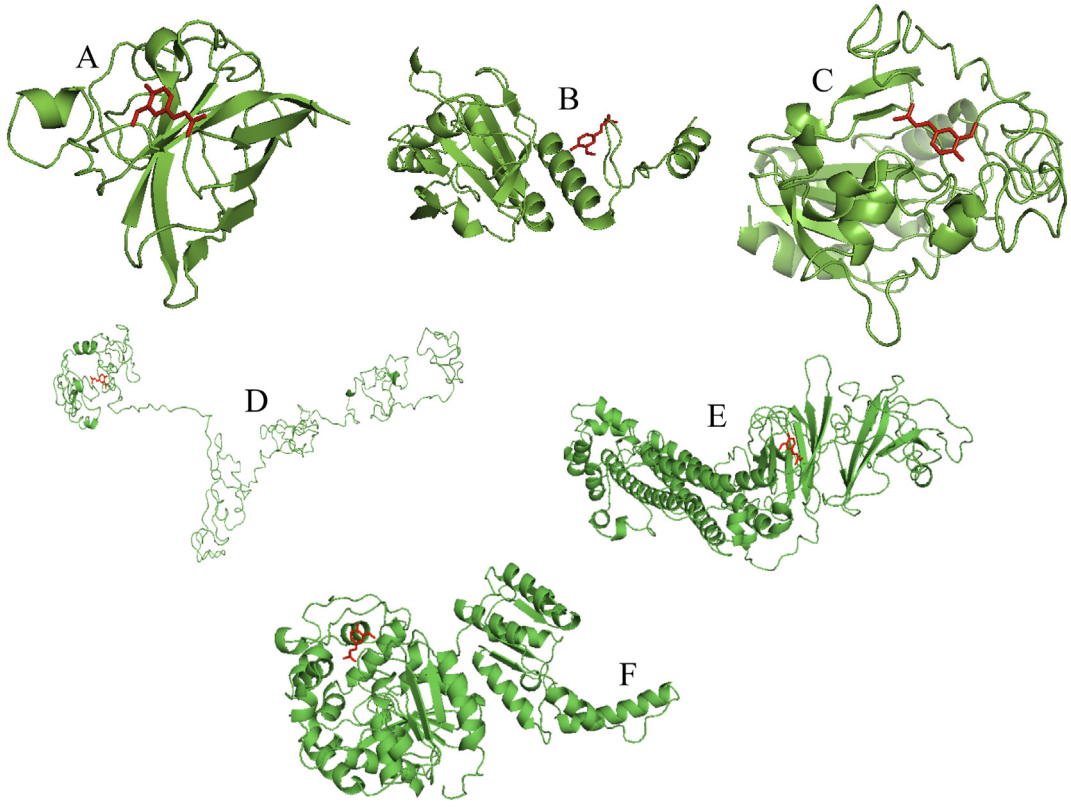


Fig. 5. Interaction between the target proteins and ferulic acid. A: Superoxide dismutase + ferulic acid; B: peroxiredoxin-2 + ferulic acid; C: phospholipid hydroperoxide glutathione peroxidase, nuclear isoform B + ferulic acid; D: thioredoxin domain-containing protein 2 isoform 1 + ferulic acid; E: keratin, type II cytoskeletal 1 + ferulic acid; F: bifunctional epoxide hydrolase 2 isoform b + ferulic acid. The cartoon representation in green colour is the target protein and the selected compound (ferulic acid) is represented in red colour. The complex was generated with PyMol software. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins. Results from the C-score and TM-score in this study, indicate that the predicted model has a correct fold and correct topology with a random similarity with the template used for the generation of this model (Thompson et al., 1994). The high RMSD obtained in this study is as the result of the TM-score and the C-score not being at the level of the cut-off values, proven the correlation between the RMSD and the TM-score (Roy et al., 2010). The geometry-based molecular docking algorithm designed to ascertain the docking transformations of the target selected proteins and the major constituents of *Glyphaea brevis* yielded good molecular shape. Moreover, the graphic observation of the docking complex shows that both coumaric acid and ferulic acid bind at the same binding site of phospholipid hydroperoxide glutathione peroxidase, keratin and epoxide hydrolase 2 suggesting that only these proteins are better targets by coumaric acid and ferulic acid, to cause the inhibition of reactive oxygen species metabolism. Nonetheless, the differential binding of both coumaric acid and ferulic acid to superoxide dismutase, peroxiredoxin-2 and thioredoxin domain-containing protein 2 do not exclude the compounds as potent antioxidants to mop up reactive oxygen species and to reduce its proliferation. Still, the molecular experiments ought to be carried out so as to confirm this finding and to draw a conclusion of the role and activity of coumaric acid and ferulic acid.

In conclusion, the results indicate that both twigs and leaves of *Glyphaea brevis* have antioxidant potential, free radical scavenging activity, low toxic risk and non cytotoxicity. These findings provide basis for the ethnomedicinal use of this plant in folk medicine, thereby establishing *Glyphaea brevis* as prospective agent for more *in vitro* and *in vivo* testing, especially evalaution for treatment of disorders involving oxidative stress or free radical generation. Also, the predicted biological activity including the pharmacological effects, mechanisms of action and specific toxicity (mutagenicity, cardiotoxicity, reprotoxicity, carcinogenicity, genotoxicity, hepatotoxicity and dermal toxicity) provides basis for further research which could unravel presently unknown medicinal benefits of the plant.

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Conflict of interest

All authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2019.04.013.

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