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A comparative study of Ghanaian propolis extracts: Chemometric analysis of the chromatographic profile, antioxidant, and hypoglycemic potential and identification of active constituents

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ABSTRACT

Diabetes is a disease characterized by high post-prandial glucose levels, which lead to other complications such as peripheral end organ damage. The use of enzyme inhibitors in the management of Type-2 diabetes ensure the control of blood glucose levels via the control of carbohydrate metabolism. The use of standard agents such as acarbose is associated with unwanted side effects hence the need to investigate other sources of antihyperglycemic agents. Propolis, a natural substance from bees, possesses diverse biological activities including antioxidant, antimicrobial and antidiabetic properties. However, the phytochemical content of propolis and its extracts may vary depending on the geographical area, the solvent of extraction and type of bees. This study represents the first attempt to compare different extracts of propolis from the same source in sub-Saharan Africa. In this study, the effect of solvent and source of Ghanaian propolis on parameters such as the total phenolic and flavonoid contents, chromatographic profile, antioxidant and α-amylase inhibitory effects were investigated with the aim of identifying and characterizing the most promising extract, which could be of direct or indirect benefit in the management of Type-2 diabetes.

Combinations of water, ethanol-water and ethanol extracts were prepared from propolis from three regions. Phytochemical screening was performed on the extracts after which the Folin Ciocalteu method and aluminum chloride colorimetric assay were used to estimate the total phenolic and flavonoid contents respectively. Antioxidant potential of extracts was estimated using DPPH and phosphomolybdenum assays. *In-vitro* α-amylase inhibition assay was used to investigate hypoglycemic effect of the extracts. Statistical tools such as ANOVA, principal component analysis, hierarchical cluster analysis employed to determine sources of variations

Abbreviations: T2D, Type-2 diabetes mellitus; LC-MS, Liquid Chromatography-Mass spectrometry; UHPLCQ-TOFMS/MS, Ultra-High Performance Liquid Chromatography-Quadrupole-Time of Flight Mass Spectrometry; GC-MS, Gas Chromatography-Mass spectrometry; TLC, Thin Layer Chromatography; rTLC, reverse Thin Layer Chromatography; HPTLC, High Performance Thin Layer Chromatography; TPC, Total phenolic content; TFC, Total flavonoid contents; TAC, Total antioxidant activity; IC_{50}, Half-maximal inhibitory concentration; GAE, Gallic Acid Equivalence; QCE, Quercetin Equivalence; EWBE, Ethanol-water extract Bono East; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; PCA, Principal Component Analysis; HCA, Hierarchical cluster analysis.

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within the data obtained, to classify the extracts based on activity and to predict the most effective extract. This extract was then subjected to UHPLC-Q-TOF MS/MS and GC–MS techniques to characterize the constituents.

Chemometric analysis of the data obtained showed that the variations in the data could be explained by both propolis source and extraction solvent. Though ethanol extracts generally contained more constituents, the more notable activities were in the ethanol-water extracts. The ethanol-water extract of Bono East propolis (EWBE) was the most potent DPPH radical scavenger (IC_{50} of 149.37 ± 2.90 μg/mL as compared to 116.60 ± 0.93 μg/mL GAE standard). It was also one of the three extracts which were more potent than acarbose (369.89 μg/mL) in the α-amylase inhibition assay. The predominant constituents from the LC-MS dereplication of EWBE were caffeic acid and flavonoid derivatives whilst 5,5-dimethyl-1-oxa-5 silacyclononanone-9 was the most significant active constituent identified through the GC–MS analysis. The identified constituents are known to have strong antioxidant and antidiabetic properties.

The effects of source and solvent of extraction on the biological and physicochemical properties of propolis in Ghana have been quantified using statistical tools. The combined biological effects of propolis suggest a possible role in their usage in the management of type-2-diabetes and its related complications. Ethanol-water extracts were the most promising with EWBE showing the strongest antihyperglycemic activity. Such extracts represent leads towards further research into toxicity and formulation in order to develop safe and useful products for the management of type-2 diabetes.

Introduction

Background of diabetes

Diabetes mellitus (DM) is a chronic disease characterized by metabolic disorder leading to the impairment in carbohydrate, protein and fat metabolism. It is characterized by hyperglycemia in both postprandial glucose levels as well as in fasting state [1]. DM is grouped into two; insulin dependent Diabetes mellitus (Type 1) and Non-Insulin Dependent Diabetes mellitus (Type 2). In type 1 diabetes (T1D), there is absolute insulinopenia whereas insulin levels as well as insulin-like growth factor levels increases in type 2 diabetes (T2D). T2D accounts for about 90 % of cases worldwide [2].

Management of the disease using these agents imposes huge financial burden on patients and/or caregivers. The total financial cost for the management of diabetes in Ghana for example was estimated at GH₵ 420,087.67 per annum [3]. As at 2019, the estimated global direct health expenditure on diabetes was USD 760 billion, which was expected to grow to a projected USD 825 billion by 2030 and USD 845 billion by 2045 [4]

T1D is managed with insulin whereas T2D is managed by strategies to decrease postprandial hyperglycemia and lifestyle changes. Inhibitors of carbohydrate hydrolyzing enzymes such as α-glucosidase and α-amylase have been targeted to control postprandial hyperglycemia in Type-2- diabetic patients [5,6]. Orthodox inhibitors such as acarbose, are known to be associated with side effects like flatulence, diarrhea, vomiting and abdominal pains. Due to this, alternatives from natural sources which are less expensive and readily available continue to be exploited for alternative sources of inhibitors with lesser side effects. This is evidenced by the many studies on the use of natural products as antidiabetic agents. A comprehensive discussion is found in a review by Mata et al. [7]. The use of natural products for treatment of ailments is particularly common in Africa, though the trend is gradually increasing worldwide. Some novel targets for natural products treatment such as adiponectin and lipase are also discussed by Duarte et al. [8]. MiRNA expression levels and their unique potential as biomarkers have been described by Jamalpour et al. [9]. The role of antioxidants in management of metabolic disorders such as T2D cannot be over emphasized. Recent work on Algerian propolis for instance confirms the antioxidant potential of propolis and other bee products which can improve clinical outcomes for such patients [10].

Propolis in diabetes

Propolis consists of a mixture of substances put together by worker honeybees to serve as a defensive barrier to their hive. This defensive barrier comes in the form of filling cavities in the walls of the hive, mummifying dead intruders to prevent their decay and to reduce the size of the entrance to the hive during cold days [11]. The chemical characteristics of propolis are known to depend on these factors as well as plant and bee species [12]. The antioxidant and anti-hyperglycemic actions of propolis from some countries have been reported, but the results have been largely uncorrelated. Different researchers have used different extracting solvents as well as propolis from different sources.

In terms of extraction solvent, the aqueous propolis extracts have generally been less investigated with ethanol extracts being more popular. Even though the ethanol extracts have been reported to be superior in terms of phenolic content, others have suggested similar content for the ethanol and aqueous extracts [13]. Ethanolic extract of Chihuahua Mexican propolis inhibited rises in blood glucose levels in diabetic rats [14]. Propolis caused significant lowering of blood glucose after a single administration and at day 15 after daily administration in diabetic rats ($P<0.05$) [15]. Recent reports on clinical trials on Iranian propolis show beneficial effects on reducing post prandial blood glucose, serum insulin, insulin resistance, and inflammatory cytokines [16].

There are some reports on the antidiabetic potential of Nigerian propolis by Oladipo et al. [17] and Alaribe et al. [18]., but the volume of research on propolis extracts in sub-Saharan African generally lags behind other regions in the world. There is no such work on the antidiabetic properties of Ghanaian propolis extracts to date.

Objectives of the current study

Based on the above, this study aims to evaluate polar extracts (water, ethanol and aqueous ethanol) of Ghanaian propolis from different locations. The assessment focuses on chromatographic and phenolic profiles, antioxidant capacity, radical scavenging, and antiamylase activity. The study employs statistical tools to identify and quantify the contributions of propolis source and extracting solvent to the variations in the chromatographic profile and biological and physicochemical properties of propolis from Ghana. The chemometric analyses performed are novel to propolis from Africa and the combination of independent variables used are unique to this report. The work provides valuable information on correlations between phenolic profile and antioxidant capacity and enables the selection of the most suitable and consistent extracts which guarantee useful antioxidant and antidiabetic effect. The most promising extract was subsequently selected and characterized using tandem chromatographic methods such as Liquid chromatography – Mass Spectrometry (LC-MS) and Gas Chromatography – Mass Spectrometry (GC–MS). The results reveal the most useful propolis extracts and its important phytoconstituents. This provides an indication of the use of Ghanaian propolis extracts as either possible adjuncts or active ingredients for the management of T2D.

Experimental

Chemicals and reagents

Chemicals used in this study include: methanol, toluene, ethylacetate, hydrochloric acid, 95 % sulphuric acid, and formic acid were purchased from VWR International (Darmstadt, Germany), *p*-Anisaldehyde (98 %) was purchased from Sigma-Aldrich (Darmstadt, Germany), Silica gel 60 HPTLC aluminum plates (20 cm × 20 cm) was purchased from Merck KGaA (Darmstadt, Germany) and all these chemicals and reagents used were of analytical grade.

Sample collection, authentication, and preparation

Raw propolis from Bono East (Atebubu), North-East (Mamprugu Moagduri- Yagaba) and Greater Accra (Accra) regions of Ghana (Fig. 3, supplementary data) were obtained and authenticated at the herbarium section of the Department of Pharmacognosy, KNUST. Voucher specimen numbers, KNUST/HM1/21/10/06P0001, P0002 and P0003 were deposited at the herbarium for reference. The samples were pulverized using an electric blender and 25 g of each sample was weighed, cold macerated individually using 100 mL of water, ethanol, and ethanol-water (50:50, v/v) for 72 hr to form 250 mg/mL extract solutions. Each sample was filtered with a Whatmann No.10 filter paper, filtrate evaporated using a rotary evaporator (Buchi, Switzerland), concentrated using a water bath (Halo-Hollen, Denmark) and further dried in a desiccator. The concentrated extracts were kept in the refrigerator for further analysis.

Preliminary phytochemical screening

Phytochemical characterizations were performed for all the extracts using standard methods to test for the presence of alkaloids (Dragendorff reagent), glycosides (Fehling reagent), flavonoids (NaOH test), phenols (Potassium dichromate test), tannins ($FeCl_3$ test), terpenoids (Salkowski test) [19] and saponin (froth test) [20]

Total phenolic content

The total phenolic content (TPC) of all the extracts were determined using the Folin-Ciocalteu colorimetric assay, as reported by Singleton et al. [21], with slight modification. Briefly, 1.5 mL of each extract solution (1 mg/mL) and 1.5 mL of 10 % Folin-Ciocalteu reagent were mixed. After 5 min, 1.5 mL of 10 % Na_2CO_3 was added and the resulting solution vortexed. The solutions were placed in the dark for 1 hr at ambient temperature and the absorbance measured against a reagent blank at 760 nm. A standard calibration curve was prepared by using gallic acid as standard. The total phenolic content was expressed as milligram of gallic acid equivalence per gram of the extract (mg GAE/g extract).

Total flavonoid content

The total flavonoid contents (TFC) were determined by aluminum chloride colorimetric assay as reported by Chang et al. [22] with slight modifications. Briefly, 4 mL of each extract was mixed with 0.2 mL each of 10 % (w/v) aluminum chloride and 0.1 M potassium acetate solutions. The resulting solutions were vortexed and kept in the dark at ambient temperature for 30 min. The maximum absorbance of the solutions was measured against a reagent blank at 415 nm. A standard calibration curve was prepared using quercetin and the total flavonoid contents were expressed as milligram of quercetin equivalent per gram of the extract (mg QCE/g extract).

Total antioxidant capacity

The total antioxidant activity (TAC) of the extracts were estimated using phosphomolybdenum assay as reported by Prieto et al. [23] with slight modifications. An aliquot of each extract (10 mg/mL) was combined with 1 mL of phosphomolybdate reagent solution in a test tube. The reagent was prepared by combining 0.6 M H_2SO_4, 28 mM Na_2HPO_4 and 4 mM of $(NH_4)_6Mo_7O_{24}$ in equal volumes. The resulting solution was vortexed for thorough mixing and the tubes were then capped. The tubes were incubated in a water bath at a temperature of 95 °C for 90 min, cooled to ambient temperature and absorbance measured against a reagent blank at 695 nm using a microplate reader. Gallic acid (60–800 μg/mL) was used for the preparation of a calibration curve and the water-soluble antioxidant capacities of extracts were expressed as milligrams of gallic acid equivalence per gram of extracts (mg GAE/g extract).

DPPH radical scavenging activity

The antioxidant activities of the extracts were measured with the DPPH assay as reported by Molyneux et al. [24] with some modifications. Briefly, 2 mL of a freshly prepared DPPH solution (0.04 mM) was mixed with 2 mL extract at varying concentrations (200–1000 μg/mL) in separate tubes. The tubes were incubated in the dark for 30 min at ambient temperature. Gallic acid served as a positive control and was prepared in the same concentration as extracts. The reduction in absorbance was read at 517 nm using a spectrophotometer and the percentage scavenging activity was evaluated.

α-Amylase inhibition assay

The DNS reagent colorimetric assay used in the determination of the activity of α-amylase is based on the principle that, the reagent reacts with reducing sugars released as a result of hydrolyses of starch. Aldehyde functional groups in glucose undergoes oxidation in the presence of 3, 5 dinitrosalicylic acid (DNSA). In an alkaline medium, DNSA is reduced to 3-amino-5-nitrosalicylic acid (ANSA) to give an orange-red coloured complex which has maximum absorbance at 540 nm.

The α-amylase inhibition assay was performed by adopting the method as reported by Kazeem et al. [25] with some modifications. Extracts were prepared in the concentration of 200 to 1000 μg/mL. From each concentration, 200 μL was incubated at 30 °C with 200 μL of 1 mg/mL of α-amylase (1.5 U/mg) isolated from *Aspergillus oryzae* for 10 min. The substrate (1 % starch), prepared with 0.02 M potassium phosphate buffer (pH 6.9) was added to initiate the reaction for further 10 min. The reaction was stopped by the addition of 400 μL dinitrosalicylic acid (DNSA) reagent. The reagent was prepared by mixing 1 g of DNSA, 30 g of potassium sodium tartrate, 20 mL of 2 N NaOH and topping it up to 100 mL with distilled water. The reaction mixtures were then kept in a boiling water bath for 5 min and cooled afterwards to ambient temperature. The resulting solutions were further diluted with 5 mL distilled water and the absorbance measured at 540 nm using Synergy H1 Hybrid microplate reader. The values were then compared with a control which contained 200 μL of 0.02 M phosphate buffer (pH 6.9) instead of extracts. Acarbose was used as a positive control, and it was prepared in the same concentration range as extracts. A substrate blank which contained buffer instead of starch and enzyme blank which contained buffer instead of enzyme were prepared. The experiments were carried out in triplicate and the inhibitory effect stated as % inhibition.

TLC instrumentation

A CAMAG HPTLC system (Muttenz, Switzerland) consisting of Automatic TLC sampler ATS 4, TLC scanner 3 equipped with winCATS software (version: 1.4.4.6337) and TLC Visualizer 2 equipped with visionCATS software (version: 3.0) was used for the TLC analysis. 10 μL solutions of the fractions were applied on aluminium TLC plates precoated with silica gel 60 F_{254} (10 × 10 cm and 20 × 10 cm; Merck KGaA, Darmstadt, Germany) with a band length of 6 mm and developed up to 90 mm at room temperature in twin-trough glass chambers (20 × 10 cm & 10 × 10 cm) pre-saturated for 25 min. The mobile phase composition was optimized to be toluene: ethylacetate: formic acid (70:28:2, v/v/v). After development, the plates were dried and visualized under ultraviolet (UV) at 254 nm and 366 nm before and after derivatizing with Natural Product Reagent (Polyethylene 400 (NP-PEG) and Anisaldehyde-sulphuric acid reagents).

Chemometric analysis

Chemometric analysis was performed in two phases; the first was carried out to investigate the relationship among the antioxidant and anti-α-amylase activity parameters determined using the unsupervised models, principal component analysis (PCA) and hierarchical cluster analysis (HCA). Prior to the analyses, the activity parameters were standardized. Statistical significance among the parameters was tested using analysis of variance (ANOVA) at a 95 % confidence level.

The second phase of the chemometric analysis involved the fingerprint analysis to investigate the similarities and differences in the TLC profiles of the different propolis extracts developed using the rTLC web application software (Version 1.0) (http://shinyapps.ernaehrung.uni-giessen.de/rtlc/). The rTLC web application software" is a tool for analyzing TLC data. The chromatographic images obtained from the TLC analysis of the fractions were simultaneously uploaded to the software in the TIFF format and converted to a numerical data matrix for analysis. The RGB color channels, and the greyscale channel, which represents the average of the three other channels, were considered for the analysis. Data pre-processing was performed using median filtering, baseline correction, and standard normal variate algorithms. Principal component analysis (PCA) was performed to explore the similarities and differences

among the samples from different geographical locations as well as their respective extracts.

UHPLC-Q-TOF MS/MS screening of extracts

LC-MS is useful for the identification of less volatile but soluble constituents of natural product. It has very high sensitivity and can detect small quantities of compounds. The phytochemical characterization of the extracts was achieved using the UHPLC Dionex Ultimate 3000 RS Liquid Chromatography System. This consisted of a stationary phase of C18 column (2:1 × 100 mm, 2.2 μm), and a binary gradient (A: water with 0.1 % formic acid; B: acetonitrile with 0.1 % formic acid) at 0.4 mL/min with the following elution system: from 0 to 0.4 min—isocratic at 5 % B; 0.4 to 9.9 min—linear from 5 % B to 100 % B; 9.9 to 15.0 min—isocratic at 100 % B; 15.0 to 15.1 min—linear from 100 % B to 5 % B; and 15.1 to 20.0 min—isocratic at 5 % B, with an injection volume of 2 μL. The separated compounds were detected using Dionex Ultimate DAD-3000 RS within a wavelength range of 200–400 nm, and a Bruker Daltonics micrOTOF-QII time-of-flight mass spectrometer with an Apollo electrospray ionization source in a positive mode at 3 Hz within a mass range of *m/z* 50–1500 using the instrument settings: dry gas nitrogen, 9 L/min, 220 °C; nebulizer gas nitrogen, 4 bar; capillary voltage, 4500 V; end plate offset, −500 V; transfer time, 100 μs; collision gas nitrogen; collision energy; and collision RF settings were combined to each single spectrum of 1250 summations as follows: 624 summations with 80 eV collision energy and 130 Vpp + 313 summations with 16 eV collision energy and 130 Vpp + 313 summations with 16 eV collision energy and 130 Vpp. The internal dataset calibration (HPC mode) was done for each analysis with the mass spectrum of a 10 mM solution of sodium formate in 50 % isopropanol that was infused during LC re-equilibration using a diverter valve equipped with a 20 μL sample loop.

GC–MS analysis

GC–MS enables the identification of the more volatile phytoconstituents. GC–MS analysis of the samples was performed using a PerkinElmer GC Clarus 580 Gas Chromatograph interfaced to a Mass Spectrometer PerkinElmer (Clarus SQ 8 S), equipped with ZB-5HTMS (5 % diphenyl/95 % dimethyl poly siloxane) and fused to a capillary column (30 × 0.25 μm ID × 0.25 μm DF). The oven temperature was programmed from 100 °C (isothermal for 2 min), with an increase of 10 °C/min to 200 °C, then 5 °C/min to 280 °C and holding for 22 min at 280 °C. For GC–MS detection, an electron ionization system was operated in electron impact mode with an ionization energy of 70 eV. Helium gas (99.9999 %) was used as a carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 1μL. The injector temperature was maintained at 250 °C, and the ion-source temperature was 220 °C. Mass spectra were taken at 70 eV; a scan interval of 1 s and fragments from 50 to 500 Da. The solvent delay was 0 to 3 min, and the total GC/MS running time was 43 min respectively. The mass-detector used in this analysis was Turbo-Mass, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-6.1.0. Interpretation on mass-spectrum GC–MS was conducted using the database of National Institute of Standard and Technology (NIST) having more than 62,000 patterns.

Results and discussion

Phytochemical screening

Standard methods which included dragendorff's reagent (alkaloids), Salkowski test (terpenoids), foam test (saponins), Potassium dichromate test (Phenols), Fehling's test (glycosides), Ferric Chloride test (tannins) and sodium hydroxide test (flavonoids) were used in determining the phytochemical constituents of extracts. The results from the phytochemical screening of all nine extracts are shown in Table 1 below. Solvents of different polarities were used due to reason that, compounds may associate themselves differently in these solvents based on polarity. The research also focused on antioxidant properties of extracts which are mainly exerted by polarity. As expected, all propolis samples tested positive for phenols and flavonoids despite the use of different solvents for the extraction. These represent the main active constituents of propolis extracts. Tannins (8/9) and Glycosides (7/9) were also largely present. Terpenoids

Table 1
Phytochemical screening of propolis extracts.

Extract	Phytochemical						
	Tannins	Alkaloids	Terpenoids	Phenols	Flavonoids	Saponins	Glycosides
W BE	+	+	–	+	+	+	+
W NE	+	+	–	+	+	+	+
W GA	+	+	–	+	+	+	–
EW BE	+	+	+	+	+	+	+
EW NE	+	–	+	+	+	+	+
EW GA	+	+	–	+	+	+	+
E BE	+	–	+	+	+	–	+
E NE	+	–	+	+	+	–	+
E GA	–	–	+	+	+	–	–

Key: (+) Present and (-) Absent.
Note: BE, NE and GA denote Bono East, North-East and Greater Accra respectively. W, EW and E denote Water, Ethanol-Water and Ethanol propolis extracts.

were not observed in the aqueous extracts and alkaloids were absent in the ethanol extracts. Despite their absence in ethanol (organic phase), alkaloids were present in the aqueous extracts. This suggests that the alkaloids in the propolis samples were predominantly in the salt and not base form. This suggests an important role of alkaloidal salts in the biological effects of propolis. Extract EWBE was the only one that contained all phytochemicals tested. The presence of these phytochemicals, which are known to have antioxidant and antidiabetic effects. Antioxidants are important in improving diabetic status by regulating glucose metabolism, insulin secretion and decreasing insulin resistance. They also improve vascular functions, and regulate the levels of HbA1c and oxidative stress markers [26].

Phenolic and antioxidant properties

Bee propolis samples worldwide have been known to be high in phenolics and it was not surprising to see this replicated in the Ghanaian samples. Table 2 shows a summary of antioxidant and phenolic properties of all the propolis samples. Correlation analysis performed on all the parameters measured (ST1, supplementary data), showed a strong positive correlation between total phenols and total flavonoids with Pearson coefficient ($r = 0.938$). This finding agrees with Asem et al. [27], who reported a positive correlation between total flavonoids and total phenols with r being equal to 0.882, which represents a strong correlation.

However, DPPH correlated negatively and non-significantly with TPC and TFC with r being equal to −0.284 and −0.208 respectively. The result also agrees with Zhang et al. [28], who reported no significant correlation between DPPH and TFC with Pearson r equal to −0.185. The data also indicated that the contribution of the phenolics to the antioxidant activity outweighs the flavonoids, and this is an accordance with report from Mello et al. [29], who stated that, the antioxidant activity of Brazilian green propolis is mainly attributed to high levels of phenolics.

There have been confirmations as reported by Cai et al. [30] that, the radical scavenging activity of flavonoids is controlled by the number and configuration of hydroxyl groups on the aromatic ring.

Also, the configuration of other substituents as well as glycosylation have roles to play in radical scavenging activity of flavonoids. This can further be inferred that, flavonoids without hydroxyl groups such as flavones and flavanone had no radical scavenging activity as reported by Cai et al. [30]. Therefore, radical scavenging activity may not necessarily correlate with flavonoid content as was observed in this study.

α-amylase inhibition

Inhibition of α-amylase is an important predictor for hypoglycemic activity. Agents with inhibitory effect against these carbohydrate-metabolizing enzymes are known to control postprandial blood glucose levels. This is very relevant in the control of the progression of T2D. Even though all extracts showed varying levels of enzyme inhibition *in vitro*, concentration dependent effect was only observed in 5 out of the 9 extracts (Table 2). Out of the 5 extracts, WBE, EWBE and ENE were found to be more active than acarbose (369.89 μg/mL). This is indicative of the hypoglycemic potential of these propolis extracts. The lack of any observable trend in the α-amylase inhibitory activity of the extracts shows the dependence of this property on source and extracting solvent [12].

Chemometric evaluation of the activities tested

To appreciate the relationship among the determined effects of the extracts tested, including TPC, TFC, TAC, DPPH radical scavenging, and anti-amylase activities, unsupervised techniques, including PCA and HCA were used to evaluate them. Both PCA and HCA were used because they tend to reveal the inherent association existing among the determined effects of the different extracts. By so doing, it is possible to establish some form of evidence to account for the overall effects of the extracts. In the PCA, the first and second principal components (PCs) cumulatively explained about 89 % of the variabilities in the activities evaluated and accounted for the clustering of the PC scores of samples as observed in the scores plot (Fig. 1A). The absolute ethanol-based extracts (EGA, EBE, and

Table 2
TPC, TFC, TAC, DPPH scavenging activity and α-amylase inhibitory activity of extracts.

Extract	TPC (mg GAE/g)	TFC (mg QE/g)	TAC (mg GAE/g)	DPPH Assay (IC_{50} μg/mL)	α-Amylase inhibition (IC_{50} μg/mL)
W BE	88.17 ± 1.92^{a}	83.44 ± 1.31^{a}	80.07 ± 1.29^{a}	455.18 ± 4.36	238.59 ± 2.31
W NE	61.56 ± 0.54^{b}	52.56 ± 1.32^{b}	88.53 ± 0.69^{b}	ND	ND
W GA	39.53 ± 0.31^{c}	15.28 ± 0.72^{c}	36.48 ± 0.80^{c}	ND	ND
EW BE	84.12 ± 2.62^{b}	79.59 ± 4.52^{a}	74.96 ± 1.41^{d}	149.37 ± 2.90	288.25 ± 8.17
EW NE	54.70 ± 0.77^{d}	51.61 ± 0.87^{b}	109.60 ± 2.10^{e}	243.39 ± 2.27	ND
EW GA	41.70 ± 0.38^{c}	36.54 ± 0.69^{d}	58.45 ± 1.10^{f}	512.86 ± 7.60	ND
E BE	41.71 ± 2.79^{c}	6.94 ± 0.54^{e}	18.44 ± 0.65^{g}	290.98 ± 5.87	404.03 ± 9.24
E NE	54.03 ± 1.75^{d}	24.42 ± 1.08^{f}	20.61 ± 0.63^{g}	201.93 ± 3.33	231.83 ± 7.81
E GA	25.15 ± 1.15^{e}	4.22 ± 0.27^{e}	17.77 ± 0.72^{g}	566.02 ± 8.03	611.17 ± 11.23

Values in the same column followed by the same letter are not significantly different ($P<0.05$) by Tukey's multiple range test. ND: not determined. IC_{50} for positive controls gallic acid in DPPH assay = 116.60 ± 0.93 μg/mL and acarbose in α-Amylase inhibition = 369.89 ± 9.01 μg/mL TPC: Total Phenolic Content, TFC: Total Flavonoid Content and TAC: Total Antioxidant Content

ENE) were clustered in one spatial region, indicating the similarities in the bioactivities investigated. These samples were characterized by their relatively lower activities in the antioxidant-related properties, including TPC, TFC and TAC. The TAC of ethanol extracts of propolis could serve as a marker for identification and quality control purposes. Here, the influence of solvent type in the observed properties of the propolis was evident. The second cluster comprised the extracts, WGA, WNE, EWGA, and EWNE. These samples were characterized by low activities in the DPPH assay and anti-α-amylase activity.

The third cluster consisting of WBE and EWBE were also characterized by their high activities in the antioxidant-related properties (TPC, TFC and TAC), and low IC_{50}s in the anti-α-amylase assay. These observations were confirmed from the HCA which was carried out using Euclidean distance calculation and Ward Linkage. The dendrogram showing the three clusters is shown in Fig. 1B.

The results from the PCA and HCA showed general increase in the activities of the extracts when the extraction solvent for the propolis contained some water. This could be illustrated with the example of EGA, which had a TFC of 4.22 mg QE/g but EWGA and WGA had TFCs of 36.54 mg QE/g and 15.28 mg QE/g respectively. Similar observations were made for the propolis samples from other sources. In addition, the analysis showed that samples from the middle belt of the country (Supplementary data) possessed better antioxidant and anti-amylase activities than those from the other parts. For instance, both WBE and EWBE, considered to be the most effective extracts, originated from the Bono-East region.

HPTLC profiling

HPTLC represents an important quality control tool for identification and determination of adulteration in natural products. HPTLC profiles of the different extracts, which also originated from different sources, were investigated in an HPTLC fingerprint analysis. This analysis provided information necessary to establish the relationship among the phytochemical make-up of the different extracts propolis samples, thereby identifying points of similarities and differences. From simple observation of the HPTLC plates (Fig. 2) as well as the corresponding densitometric analysis of the profiles of the plates (data not shown), it was observed that irrespective of the origin of the sample, the ethanol fractions contain more compounds than the aqueous and ethanol-water extracts.

It is also worth noting that in comparing the antioxidant related parameters among the different types of extracts from similar origins, the ethanol extracts were significantly different. This phenomenon could also be partly explained by the different patterns in the HPTLC profiles developed. The PCA plot obtained from the HPTLC analysis (Fig. 3) shows obvious clustering according to propolis source. For instance, a closer look at the PCA plots for plates A, B and C reveals clustering of samples from Bono east without much distinction between extracts. However, sub-clustering according to extract type was evident in North-East region propolis samples. Analysis of profile of plate D (in contrast to A, B and C) highlighted the influence of extract type on the differences in profiles against propolis source. The absence of clustering of samples of the same extract type irrespective of source suggests that, differences in the HPTLC profiles of the samples were mainly driven by the source of the propolis extract relative to the type of extract. The effect of extract type was observable only within a particular propolis source. This has implications for quality control in the sense that similar extracts of propolis from different sources will have different TLC profiles. Hence HPTLC is a useful tool for identification of propolis samples.

Dereplication of constituents of sample EWBE

EWBE was chosen as the representative extract for dereplication because it showed remarkable consistency in all measured parameters. This extract tested positive for all phytochemical constituents (Table 1). Due to its intermediate polarity, constituents spanning a wide spectrum of polarity and phytochemical class are expected. Hence, both LC-MS and GC–MS results were used in the dereplication of the EWBE propolis extract.

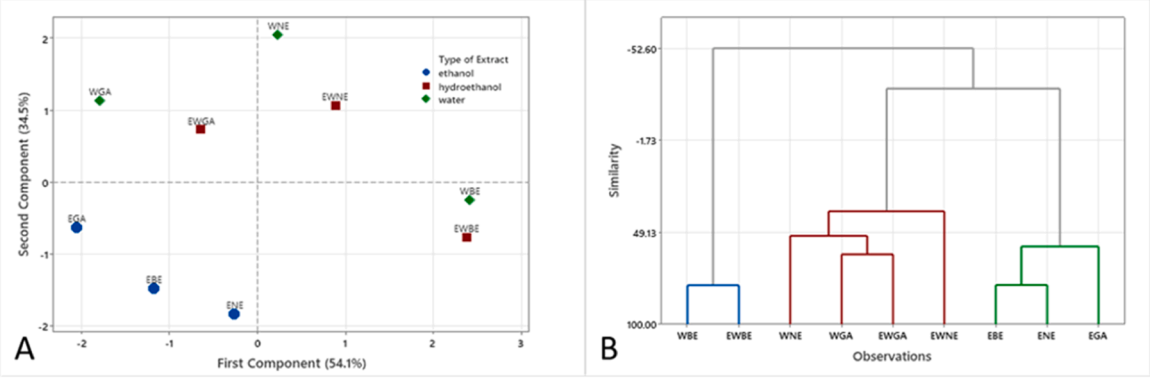

Fig. 1. Chemometric evaluation of bioactivities of the propolis extracts **(a)** Scores plot from the PCA; **(b)** Dendrogram from the HCA. Analysis was based on results in Table 2.

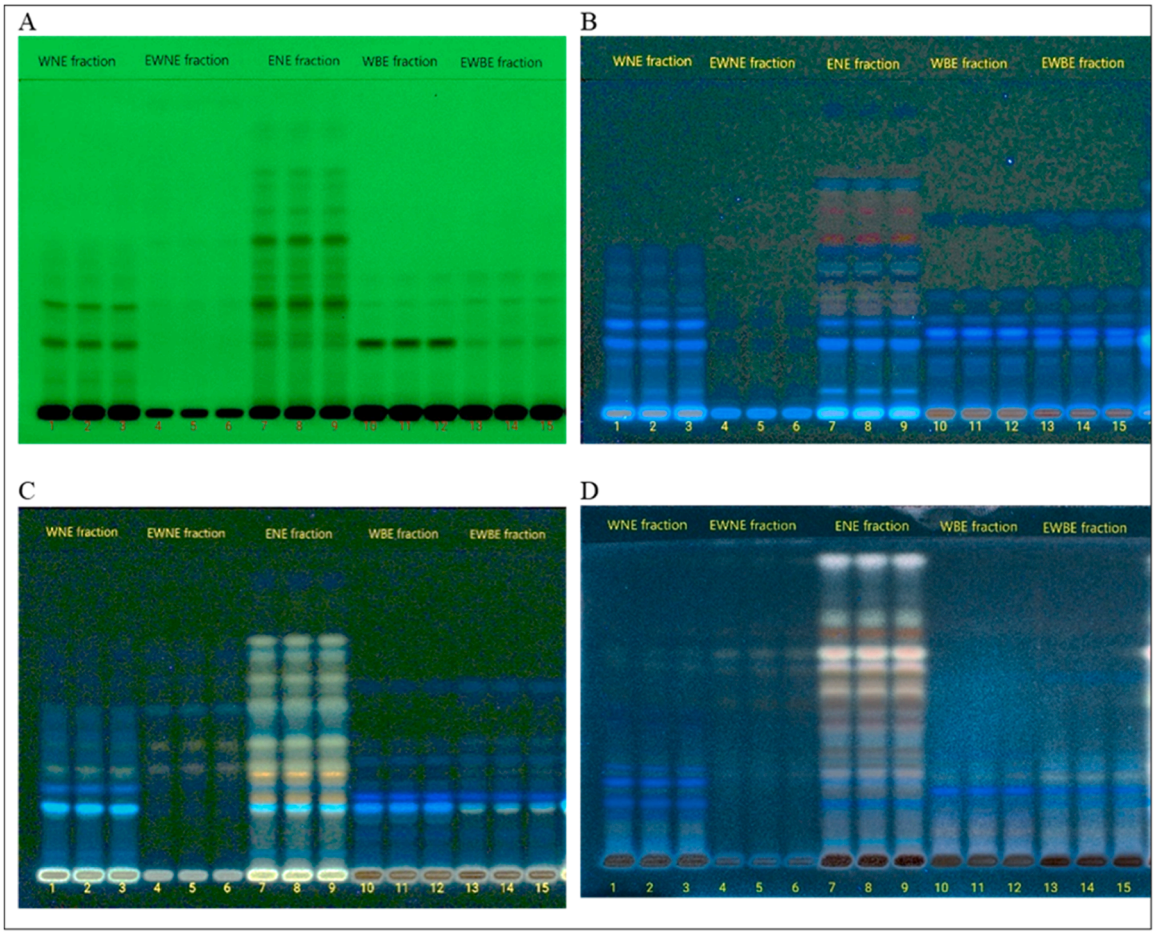

Fig. 2. HPTLC Fingerprinting Analysis of different extracts of propolis samples from three regions in Ghana. Detection of underivatized plates: **(a)** at 254 nm, **(b)** at 366 nm; Detection of derivatized plates at 366: **(c)** after NP-PEG and **(d)** after Anisaldehyde/H_2SO_4.

LC-MS characterization

The results of the LC-MS characterization can be seen in Table 3 and the Total Ion Chromatogram is shown in SF1 (Supplementary data). The dereplication was achieved via comparisons with data obtained from propolis from other sources.

The predominant constituents from the LC-MS dereplication were caffeic acid and flavonoid derivatives which are known to be responsible for the main biological effects of propolis such as anti-inflammatory and immunomodulatory effects [40]. Chlorogenic acid for instance has been reported to show good antidiabetic effect [41]. Quercetin-4′-O-glucoside, naringenin-C-glucoside and hesperidin are some of the flavonoid glycosides which were tentatively identified (Table 3). Quercetin-4′-O-glucoside (spiraeoside) is a well-documented DPPH radical scavenger [42]. The antioxidant effect of hesperidin is also well known [43]. The presence of these constituents supports the antidiabetic and antioxidant properties of the propolis samples.

GC–MS characterization

The use of GC–MS for characterization of propolis samples is quite common. This is because, propolis is known to contain volatile matter as well as small molecules, which can easily be vaporized. The GC–MS profile of EWBE and the identified compounds are found in SF2 (supplementary data) and Table 4 respectively.

GC–MS sought to identify other classes of phytochemicals such as terpenes, steroids etc. given that the polyphenolics had been identified in the LC-MS. 5,5-Dimethyl-1-oxa-5-silacyclononanone-9 was the most abundant of the identified compounds (12.8 %). Though not previously reported in propolis, it has been identified in plants with antioxidant activity such as *Sarcocephalus latifolius* [44]. Apart from that, Table 4 shows the presence of mainly steroids (14.55 %). Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl) isolated from *Cassia auriculata* which was the most abundant steroid (2.73 %) has shown hypoglycemic action when given orally in Wistar rats [45]. Lup-20(29)-en-3-ol, acetate. (3β), a triterpenoid has been previously reported to have anti-diabetic functions [46]. Triterpenes are not only implicated in reduction of hyperglycemia, but they also show strong antioxidant and are known to block formation of advanced glycation end products. This reduces incidence of diabetic nephropathy, neuropathy, or impaired wound healing [47].

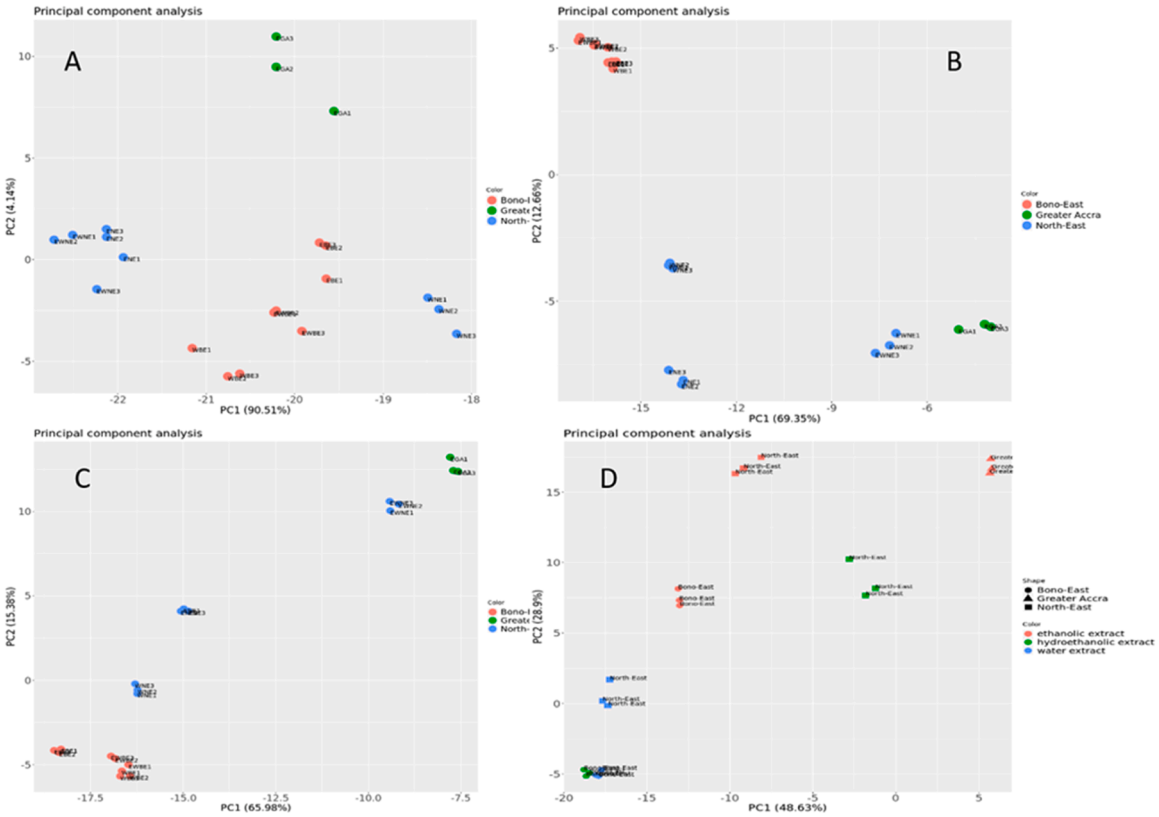

Fig. 3. PCA plot of HPTLC Fingerprinting Analysis of different extracts of propolis samples from three regions in Ghana. Detection of underivatized plates: **(a)** at 254 nm, **(a)** at 366 nm**;** Detection of derivatized plates at 366: **(c)** after NP-PEG and **(d)** after Anisaldehyde/H_2SO_4.

Table 3
Compounds tentatively identified in EWBE by LC-MS. (RT: retention time; m/z: mass to charge; Mol. Formula: Molecular formular).

RT (min)	Proposed compound	Mol. formula	Exact mass (g/mol)	Mass observed (m/z)	Reference
1.68	Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	516.4499	517.1581 $[M+H]^+$	[31]
2.46	(Neo)chlorogenic acid	$C_{16}H_{18}O_9$	354.3080	355.1032$[M+H]^+$	[31,32]
3.2	Not determined	$C_{43}H_{30}O_{10}$	706.69	707.1892$[M+H]^+$	
3.6	Methyl cinnamate	$C_{10}H_{10}O_2$	162.1848	163.04$[M+H]^+$	[33]
4.09	(Neo)Chlorogenic acid	$C_{16}H_{18}O_9$	354.3080	355.1032$[M+H]^+$	[32]
4.5	Rosmarinic acid	$C_{18}H_{16}O_8$	360.3142	361.0897$[M+H]^+$	[32]
5.2	Quercetin-4′-O-glucoside	$C_{21}H_{20}O_{12}$	464.3755	465.1047$[M+H]^+$	[34]
5.38	Not determined	$C_{12}H_{10}O_2$	186.2062	187.0393$[M+H]^+$	
7.68	3-hydroxyflavone	$C_{15}H_{10}O_3$	238.2377	239.1357$[M+H]^+$	[35]
10.82	Not determined	$C_{30}H_{30}O_{12}$	582.436	600.4698$[M+NH_4]^+$	
10.91	Not determined	$C_{26}H_{26}O_7$	450.4794	468.3895$[M+NH_4]^+$	[36]
10.95	Not determined	$C_8H_{16}O$	128.21	151.0938$[M+Na]^+$	
11.31	naringenin-C-glucoside	$C_{21}H_{22}O_{10}$	434.3925	452.3609$[M+NH_4]^+$	[31]
11.61	Caffeic acid isopropenyl ester diacetate	$C_{19}H_{40}O_4$	332.5169	333.3006$[M+H]^+$	[37]
11.73	Not determined	$C_{35}H_{72}O_{12}$	684.9361	702.5352$[M+NH_4]^+$	
11.84	Not determined	$C_{31}H_{64}O_{10}$	596.8313	614.4835$[M+NH_4]^+$	
11.91	Not determined	$C_{30}H_{52}O_6$	508.7282	526.4317$[M+NH_4]^+$	
11.97	Not determined	$C_{12}H_{24}O_3$	216.3162	239.1486$[M+Na]^+$	
12.31	Hesperidin	$C_{27}H_{30}O_{16}$	610.5163	628.4965$[M+NH_4]^+$	[38]
12.36	corymbone B	$C_{31}H_{38}O_7$	522.6277	523.4168$[M+H]^+$	[39]

Summary

The source of propolis rather than the extracting solvent is responsible for the corresponding TLC profile. The solvent for extraction, however, is important in determination of the antioxidant properties of the propolis. Ethanol extracts show remarkable consistency irrespective of source of the propolis and will be useful for quality control purposes. The chemometric analysis unraveled the

Table 4
Compounds tentatively identified in EWBE by GC-MS. (RT: retention time; Mw: molecular weight; Mol. Formula: Molecular formular).

RT (min)	Name of compound	Mol. formula	Mw (g/mol)	Compound class	Peak Area %
4.21	3,7,11,14,18-Pentaoxa-2,19-disilaeicosane, 2,2,19,19-tetramethyl-	$C_{17}H_{40}O_5Si_2$	380.7	Silyl derivative	1.286
5.46	1,3,6-Trioxa-2-silacyclooctane, 2,2,-dimethylsilyl-	$C_6H_{14}O_3Si$	162.26	Silyl derivative	2.243
8.57	5-tert-Butyl-4,6-dinitro-1,2,3-trimethylbenzene	$C_{13}H_{18}N_2O_4$	266.29	Benzene derivative	0.638
9.21	5,5-Dimethyl-1-oxa-5-silacyclononanone-9	$C_9H_{18}O_2Si$	186	Lactone	12.802
10.92	5-Ethoxy-6-methoxy-8-nitroquinoline	$C_{12}H_{12}N_2O_4$	248.23	Quinoline	1.583
15.52	Cinnamic acid, 4-methoxy-3-(trimethylsiloxy)-, trimethylsilyl ester	$C_{16}H_{26}O_4Si_2$	338.54	Silyl derivative	1.012
18.49	Epiandrosterone	$C_{19}H_{30}O_2$	290.40	Steroid	0.730
19.32	Lumisantonin	$C_{15}H_{18}O_3$	246.30	Lactone	1.122
19.79	Z-9- octadecenamide	$C_{18}H_{35}NO$	281.50	Fatty Amide	1.937
20.62	Acetic acid, 2-[(6-methoxy-4-methyl-2-quinolinyl)thio]-, hydrazide	$C_{13}H_{15}N_3O_2S$	277.34	Hydrazide	0.630
28.11	7aH-Cyclopenta[a]cyclopropa[f]cycloundecene-2,4,7,7a,10,11-hexol, 1,1a,2,3,4,4a,5,6,7,10,11,11a- dodecahydro-1,1,3,6,9-pentamethyl-, 2,4,7,10,11-pentaacetate	$C_{30}H_{44}O_{11}$	580.70	Ester	1.444
29.93	7aH-Cyclopenta[a]cyclopropa[f]cycloundecene-2,4,7,7a,10,11-hexol, 1,1a,2,3,4,4a,5,6,7,10,11,11a-dodecahydro-1,1,3,6,9-pentamethyl-, 2,4,7,10,11-pentaacetate	$C_{30}H_{44}O_{11}$	580.70	Ester	1.782
30.28	1,3-Dichloro-1,3-bis(norbomadien-2-yl)−1,3-bis(3-trimethylsilylpropyl)disiloxane	$C_{26}H_{44}Cl_2OSi_4$	555.87	Silyl derivative	0.841
30.43	9-Desoxo-9-x-acetoxy-3-desoxy-7,8,12-tri-O-acetylingol-3-one	$C_{28}H_{38}O_{10}$	534.60	Ester	0.622
30.61	Gibb-2-ene-1,10-dicarboxylic acid, 4a,7-dihydroxy-1-(hydroxymethyl)−8-methylene-, 1,4a-lactone, (1alpha,4aalpha,4bbeta,10beta)-	$C_{19}H_{22}O_6$	346.40	Dicarboxylic acid	0.628
30.76	Glycine, N-[(3à,5á,7à,12à)−24-oxo-3,7,12-tris[(trimethylsilyl)oxy]cholan-24-yl]-, methyl ester	$C_{36}H_{69}NO_6Si_3$	696.20	Steroid	1.866
31.31	Bicyclo[2.2.1}heptane,2,2,3,5,5-pentachloro-7,7-bis(chloromethyl)−1-dichoromethyl-	$C_{10}H_9Cl_9$	448.20	Haloalkane	2.443
31.53	5,7,9(11)-Androstatriene, 3-hydroxy-17-oxo-	$C_{19}H_{24}O_2$	284.40	Steroid	1.345
31.77	Pregn-5-en-20-one, 12-(acetyloxy)−3,8,14-trihydroxy-, (3á,12á,14á)-	$C_{23}H_{34}O_6$	406.50	Steroid	0.986
31.95	3-Dimethylamino-2-(4-chlorphenyl)-thioacrylic acid, thiomorpholide	$C_{15}H_{19}ClN_2S_2$	326.91	Other	1.173
32.19	9-Desoxo-9x-hydroxy-7-ketoingol 3,8,9,12-tetraacetate	$C_{28}H_{38}O_{10}$	534.60	Ester	1.007
32.46	Cholestane, 3,5-dichloro-6-nitro-, (3á,5à,6á)-	$C_{27}H_{45}Cl_2NO_2$	486.60	Steroid	0.768
32.61	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	$C_{27}H_{42}O_4$	430.60	Steroid	1.959
32.89	3-Dimethylamino-2-(4-chlorphenyl)-thioacrylic acid, thiomorpholide	$C_{15}H_{19}ClN_2S_2$	326.90	Amine	0.937
33.34	Stearic acid, 3-(octadecyloxy)propyl ester	$C_{39}H_{78}O_3$	595.00	Fatty acid Ester	0.988
33.73	7aH-Cyclopenta[a]cyclopropa[f]cycloundecene-2,4,7,7a,10,11-hexol, 1,1a,2,3,4,4a,5,6,7,10,11,11a-dodecahydro-1,1,3,6,9-pentamethyl-, 2,4,7,10,11-pentaacetate	$C_{30}H_{44}O_{11}$	580.70	Other	1.900
34.06	3,9-Epoxypregnan-14-ol-20-one, 3,11,18-triacetoxy-	$C_{27}H_{38}O_9$	506.60	Steroid	0.933
34.21	1-[2,4-Bis(trimethylsiloxy)phenyl]−2-[(4-trimethylsiloxy)phenyl]propan-1-one	$C_{24}H_{38}O_4Si_3$	474.80	Silyl derivative	0.776
34.57	Milbemycin B, 6,28-anhydro-5-O-demethyl-13-dehydro-15-hydro-25-isopropyl-15-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]−4-methyl-	$C_{40}H_{62}O_{11}$	718.90	Other	1.360
34.76	(22R)−21-Acetoxy-6à, 11á-dihydroxy-16à, 17à-propylmethylenedioxypregna-1,4-diene-3,20-dione	$C_{27}H_{36}O_8$	488.57	Steroid	1.033
35.12	2-Butenoic acid, 2-methyl-, 1,1a,1b,4,4a,5,7a,7b,8,9-decahydro-4a,7b-dihydroxy-1,1,3,6,8-pentamethyl-5-oxo-9aH-cyclopropa(3,4)benz(1,2-e)azulen-9a-yl Ester (1aR-(1aalpha, 1bbeta, 4abeta, 7aalpha, 7balpha, 8alpha, 9aalpha))	$C_{25}H_{34}O_5$	414.50	Carboxylic Ester	0.624
35.40	(14á)3,19-Epoxyandrosta-5,7-diene, 4,4-dimethyl-3-methoxy-17-methylthiomethoxy-	-	-	Steroid	0.727
35.82	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	$C_{27}H_{42}O_4$	430.60	Steroid	2.725
36.55	5-(p-Aminophenyl)−4-(O-tolyl)−2-thiazolamine	$C_{16}H_{15}N_3S$	281.40	Other	0.985
36.94	7aH-Cyclopenta[a]cyclopropa[f]cycloundecene-2,4,7,7a,10,11-hexol, 1,1a,2,3,4,4a,5,6,7,10,11,11a-dodecahydro-1,1,3,6,9-pentamethyl-, 2,4,7,10,11-pentaacetate	$C_{30}H_{44}O_{11}$	580.70	Ester	1.716
37.60	Thieno[2,3-c]furan-3-carbonitrile, 2-amino-4,6-dihydro- 4,4,6,6-tetramethyl-	$C_{11}H_{14}N_2OS$	222.31	Other	0.821
37.95	Lup-20(29)-en-3-ol, acetate. (3β)	$C_{32}H_{52}O_2$	468.80	Triterpenoid	1.385
38.18	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8- en-17-yl)-	$C_{27}H_{42}O_4$	430.60	Steroid	0.683
39.32	Acetic acid, 13-hydroxy-4,4,6a,6b,8a,11,11,14b-octamethyldocosahydropicen-3-yl ester	$C_{32}H_{54}O_3$	486.80	Triterpenoid ester	2.810
40.60	Olean-12-ene-3,15,16,21,22,28-hexol, (3á,15à,16à,21á,22à)-	$C_{30}H_{50}O_6$	506.71	Steroid	0.798

contributions of source and extraction solvent to the physicochemical and biological properties of propolis samples. The α-amylase inhibitory effect of some of the Ghanaian propolis samples compared favourably with acarbose with the most effective samples from the middle belt of the country. Ethanol-water extracts were the most promising with EWBE showing the strongest antihyperglycemic activity. Such extracts with antioxidant properties are useful as adjuncts in the management of metabolic disorder such as T2D as they preserve the pancreatic β-cell function and decrease the risk of complications. The combined antihyperglycemic and antioxidant properties of Ghanaian propolis indicates a supplementary role in the management of T2D. Thus, they could find application in traditional medicine practice in Ghana.

Conclusions

Information on phenolic, antioxidant and anti-amylase properties of Ghanaian propolis is now available to complement that which is available on sub-Saharan African samples. The study offers a thorough examination of propolis extracts, highlighting their phytochemical makeup, antioxidant capacities, and possible advantages in the treatment of T2D. The reliability of the results is increased by the use of several analytical methods. Though the results did not contain propolis from all regions in Ghana, it provides an important starting point towards the production of efficacious propolis extracts, such extracts will have to be subjected to *in vivo* tests following toxicity tests to confirm their role in management of T2D. Thereafter, development of propolis formulations into safe and efficacious products for the management of type-2 diabetes could be undertaken. These formulations may incorporate the use of current technology such as nanoparticles which improve efficacy and reduce side effects.

CRediT authorship contribution statement

Frederick Amankwaah: Investigation, Writing – original draft. **John Nii Addotey:** Conceptualization, Formal analysis, Writing – review & editing. **Emmanuel Orman:** Investigation, Formal analysis, Writing – original draft. **Reimmel Adosraku:** Conceptualization, Writing – review & editing. **Isaac Kingley Amponsah:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2023.e01956.

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