



Antioxidant Content and Free Radical Scavenging Activity of Honeys of *Apis mellifera* of Obudu Cattle Ranch

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Authors' contributions

This work was carried out in collaboration between all authors. Authors CSA, OAO, GNO and CUI designed the study, performed the statistical analysis and wrote the protocol. Authors COU, CSA and KA managed the analyses of the study. Author CUI managed the literature searches while author CSA wrote the first draft of the manuscript and incorporated all corrections from co-authors. All authors read and approved the final manuscript.

Research Article

**Received 26th May 2012
Accepted 12th September 2012
Published 30th January 2013**

ABSTRACT

Honey is a naturally sweet and viscous fluid produced by honeybees (*Apis mellifera*) from the nectar of flowers. Proline, free amino acid, tannins, phenols and flavonoids content were determined in eight samples of Obudu cattle ranch honeys. Antioxidant content (ascorbic acid equivalent, quercetin equivalents, tocopherol content) and free radical scavenging activity of Obudu ranch honeys were determined by standard methods. The relationship existing between proline and antioxidant content with free radical scavenging activity was assessed by mathematical modelling using Levenberg Marquardt algorithm. Results of our study showed that Obudu ranch honey was of high quality having comparable total free amino acid, proline, phytochemical and antioxidant content with good quality honeys found elsewhere. α -Tocopherol content was $16.50 \pm 1.40 \mu\text{g}/100 \text{ g}$, quercetin equivalent antioxidant content (QEAC) was $9.43 \pm 0.9 \text{ mg}/100 \text{ g}$, ascorbic acid equivalent antioxidant content (AEAC) was $18.56 \pm 1.78 \text{ mg}/100 \text{ g}$, while the mean

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inhibitory concentration (IC_{50}) of the honey samples against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (RSA: IC_{50}) was evaluated to be 12.74 ± 0.71 mg/ml. Proline content, AEAC, QEAC and α -tocopherol were logistically related ($r^2 = 0.726, 0.971, 0.960$ and 0.888 respectively) to the radical scavenging activity. The results obtained from the present study showed that Obudu ranch honeys were of high quality and possessed antioxidant and free radical scavenging property. Since proline content of the tested honey samples were comparable to proline content of unadulterated honey samples elsewhere, the commercial samples may not be adulterated. Results showed that traditional usage of the honey for various medicinal usages may be related to its chemical content and radical scavenging property.

Keywords: Honey quality; proline content; antioxidant content; amino acids.

1. INTRODUCTION

There is a growing interest in recent years on the part of the consumers, the food industry and the research into foods and the way in which it may improve human health. The potential of a food substance to provide nutrients (carbohydrate, protein, fats, vitamins, and minerals), promote health, improve general well-being and reduce the risk of developing certain diseases makes the food substance functional. One of the most important properties of the functional food is their antioxidant capacity, which contributes to the prevention of certain illnesses, including cardiovascular diseases, cancer, and diabetes [1,2]. There exists convincing evidence that oxidative stress triggered by reactive oxygen species (ROS) plays an important role in the etiology and/or progression of a number of human diseases. Actually, the medical significance of oxidative stress has become increasingly recognized to the point that it is now considered to be implicated in virtually every disease process. Free radicals are known to be a major contributor to various chronic and degenerative diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer [3,4].

Honey is a naturally sweet and viscous fluid produced by honeybees (*Apis mellifera*) from the nectar of flowers [5]. It is a supersaturated complex natural liquid that contains about 31% glucose, 38% fructose and the remaining constituents being a wide range of materials that remarkably vary according to the floral source. In addition, there is a great variety of minor components, including phenolic acids and flavonoids, the enzymes glucose oxidase and catalase, ascorbic acid, carotenoids, organic acids, amino acids, proteins, and α -tocopherol [6]. The actual composition of honey varies, depending on many factors such as the pollen source, climate, environmental conditions, and the processing it under- goes [7,8].

Many of these substances of plant origin found in honey have been shown to have antioxidant activity [9,10,11], antimicrobial activity [12,13,14,15] and anti-inflammatory activity, antiviral property, and food preservative property [16]. Polyphenols and phenolic acids found in the honey vary according to the geographical and climatic conditions [17]. Some of them were reported as specific markers for the botanical origin of the honey. Considerable differences in both composition and content of phenolic compounds have been found in different unifloral honeys [18]. At the cellular level, flavonoids have been found to exert a variety of biological effects [19], presumably mediated by specific interaction with molecular targets. Indeed phenolic compounds and flavonoids have been shown to interact with biological macromolecules, such as nucleic acids [20], polysaccharides [21,22], proteins [23,24] and protection of nerve cell [25]. Phenolic and flavonoid content of honey samples may be responsible for many pharmacologic effects of honeys. Honey has been reported to be beneficial in wound healing [26]. Phenolic compounds and flavonoids in plants have been

shown to accelerate wound healing and protect cultured skin cells and tissues from oxidative damage [27]. Enhanced proliferation of fibroblasts and endothelial cells [28] and protection against oxidative damage in animals treated with phenolic-rich extract of the leaves of *Chromolaena odorata* have been reported [29,30] and has implication for wound healing [31]. Wu and co-workers have reported an antioxidant activity in free amino acids alone and in combination with other substances [32].

Obudu cattle ranch is located in the rain forest belt of south-eastern Nigeria. Honeys from the ranch has been packaged by commercial bee keepers and sold for profit. Adulteration of food is common among the poor traders and hawkers in the developing nations. The content of proline is an indication of the quality of honey and is also an indication of adulteration when it falls below a value of 183 mg/kg [33,34].

The proline, free amino acid, tannins, phenols, flavonoids, antioxidant content (ascorbic acid equivalent, quercetin equivalents, α -tocopherol) and free radical scavenging activity of Obudu ranch honeys have not been studied or documented. It is not known whether commercial honeys from this area are delivered pure or largely adulterated. The aim of the present study is to study and document the quality and some nutritional/antioxidant properties of Obudu cattle ranch commercial honey samples.

2. MATERIALS AND METHODS

2.1 Samples Collection/Location

Eight honey samples were collected from the Obudu cattle ranch in the Cross River State. The locations are found in the rain forest belt of south-eastern Nigeria, located on the Oshie ridge plateau of the Sankwala Mountains, approximately 65 kms from Obudu town in the north eastern region of Cross River State, Nigeria. Four honey samples (labelled 1,2,3,4) were obtained from different commercial honey hawkers in the area while honey samples (labelled 5,6,7,8) were collected from bee keepers and stored at 4.0°C prior to use.

2.2 Estimation of Proline Content

The proline content was determined by using a colour comparison after applying ninhydrin, with a proline standard. The content was expressed as a proportion to the mass of honey tested. The proline content was determined using the method of Ough as reported by Meda and co-workers [35]. Briefly, a 0.5 ml solution of honey (0.05 g/ml) was mixed with 1 ml of formic acid (80%) (BDH, Poles England), 1 ml of ninhydrin (Labosi, Paris, France) solution (3% in ethylene glycol monomethylether, from BDH, Poles England) and shaken vigorously for 15 min. The mixture was placed in a boiling water bath for 15 min and transferred to a 70°C bath for 10 min. A 5.0ml solution of 50% 2-propanol (Fluka Chemie, Switzerland) in water was then added and the mixture was left to cool and the absorbance determined (510 nm), 45 min after removal from the 70°C water bath. Water was used as the blank and 0.032 mg/ml solution of proline (BDH, Poles England) was used as the standard solution. Proline concentration in mg/g of honey was calculated as follows: Proline (mg/g) = $(E_s/E_a) \times (E_1/E_2) \times 80$, where E_s is the absorbance of the sample solution; E_a is the absorbance of the proline standard solution (average of 3 readings); E_1 is the mg of proline used for the standard solution; E_2 is the weight of honey in grams; 80 is the dilution factor. The mean of three readings was used.

2.3 Estimation of Total Phenolic Content

The Folin–Ciocalteu method Singleton et al. [36], was used to determine total phenolic content. Each honey sample (5 g) was diluted to 50 ml with distilled water and filtered through Whatman No. 1 paper. This solution (0.5 ml) was then mixed with 2.5 ml of 0.2 N Folin–Ciocalteu reagent (Sigma–Aldrich Chemie, Steinheim, Germany) for 5 min and 2 ml of 75 g/l sodium carbonate (Na_2CO_3) (Labosi, Paris, France) was then added. After incubation at room temperature for 2 h, the absorbance of the reaction mixture was measured at 760 nm against a methanol blank (Turner® 390, USA). Gallic acid (Sigma–Aldrich Chemie, Steinheim, Germany) (0–200 mg/l) was used as standard to produce the calibration curve. The mean of three readings was used and the total phenolic content was expressed in mg of gallic acid equivalents (GAE)/100g of honey.

2.4 Determination of the Flavonoid Content

The total flavonoid content of honey sample was determined according to colorimetric method as described by Zou et al. [37]. In brief, 0.5 ml of sample solution was mixed with 2ml of distilled water and subsequently with 0.15 ml of 5% NaNO_2 solution. After 6 min of incubation, 0.15 ml of 10% AlCl_3 solution was added and then allowed to stand for 6 min, followed by adding 2ml of 4% NaOH solution to the mixture. Immediately after water was added to the sample to bring the final volume to 5 ml, the mixture was thoroughly mixed and allowed to stand for another 15 min. The mixture absorbance was determined at wavelength 510 nm. The total flavonoid content was expressed in milligrams of catechin equivalents per gram of honey sample.

2.5 Determination of the Tannin Content

An aspect of the method for the estimation of tannins by AOAC was adopted [38]. 1ml of the extract (0.01%) was mixed with 0.5ml of Folin-Denis reagent and 1.0ml of 17% Na_2CO_3 . The mixture was stood at room temperature (30°C) for 3mins for colour development. The sample tested positive for tannins when they developed blue colour (Intensity varying with the concentration of tannins in the test sample).

2.6 Determination of Free Radical Scavenging Activity and Antioxidant Content

The scavenging activity of honey samples for the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described [35], Honey samples were dissolved in methanol at a concentration of 2.65–170 mg/ml and 0.75 ml of each sample was mixed with 1.5 ml of DPPH (Fluka Chemie, Switzerland) in methanol (0.02 mg/ml), with methanol serving as the blank sample. The mixtures were left for 15 min at room temperature and the absorbances then measured at 517 nm. Quercetin (0–50 mg/l) and ascorbic acid (Labosi, Paris, France) (0–40 mg/l) were used as positive controls. The radical scavenging activity was calculated as follows as: % Inhibition of DPPH radical = $[(\text{blank absorbance} - \text{sample absorbance})/\text{blank absorbance}] \times 100$. The mean of three IC_{50} (concentration causing 50% inhibition) values of each honey sample was determined graphically. The antioxidant content was evaluated as described [3], with some modifications. Honey samples were dissolved in methanol (0.02 or 0.04 g/ml) and 0.75 ml of each was mixed with 1.5 ml of a 0.02 mg/ml solution of DPPH in methanol. The mixtures were left for 15 min at room temperature and the absorbances then measured (517 nm). The blank sample consisted of 0.75 ml of a honey solution with 1.5 ml of methanol. The antioxidant content was determined using standard curves for ascorbic acid (0–10 mg/ml) and quercetin (0–6.25 mg/ml). The means of

three values were obtained, expressed as mg of ascorbic acid equivalent antioxidant content (AEAC) per 100 g of honey and mg of quercetin equivalent antioxidant content (QEAC) per 100 g of sample. The α -tocopherol content was determined using the AOAC method [39].

3. RESULTS AND DISCUSSION

Table 1 represents the proline, free amino acid, tannins, phenols, and flavonoids content of Obudu cattle ranch honeys. The content of proline is an indication of the quality of honey [35] and lower content of proline gives an indication of adulteration [33]. Seventy five percent of the honey samples in this study had good proline concentration of up to 1.83 mg/g, indicating absence of adulteration. Proline is the most abundant amino acid in honey and is used as a standard to quantify amino acid content. Using the proline index for assessment of honey quality, samples five and six seem to be apparently adulterated. It is surprising however, that sample five had antioxidant (α -tocopherol ($\mu\text{g}/100\text{g}$), QEAC (mg/100g), AEAC (mg/100g) and RSA-IC₅₀) values (Table 2) comparable to the unadulterated ones.

The Obudu cattle ranch honey samples had high total phenol and flavonoid content. The phenolic content of Obudu cattle ranch honeys are largely of non-tannins. Extracts rich in Phenolic compounds and flavonoids are known to scavenge free radicals *in-vitro* [29]. Free radical scavenging activity has been reported in free amino acid alone and in combination with other substances [32]. The Obudu cattle ranch honeys were found to contain free amino acids in substantial amounts. The ranch honeys were also found to have appreciable ascorbic acid and α -tocopherol content. Ascorbic acid and α -tocopherol are known antioxidants and free radical scavengers. Ascorbic acid is hydrophilic and functions as a most important free radical scavenger trapping free radicals in the aqueous phase thus protecting the bio-membrane from oxidative damage [40]. α -Tocopherol can act as a chain-breaking antioxidant by scavenging highly reactive lipid peroxy and alkoxy radicals, which otherwise would propagate the chain reaction of lipid peroxidation. The free radical scavenging property of honey may be due to the ascorbic acid, α -Tocopherol, free amino acid and phenolic contents.

Our results (Fig. 1) showed that free radical scavenging by honeys samples from Obudu ranch followed a logistic dose response model. The pattern of radical scavenging in all samples was similar. This observation is however not surprising, since the honey samples had the presence of different substances (Ascorbic acid, α -tocopherol, phenolic compounds and flavonoids and free amino acids etc) shown to have free radical and antioxidant properties. This pattern of relationship between honey concentration and radical scavenging activity resembles relationship common in drug-receptor interaction; though scavenging of free radicals by honey is not one. The ability to modulate quenching of free radicals may contribute to the ability of some honeys to help in resolving the state of inflammation typifying chronic wounds [41]. Radical scavenging by these honey samples may explain their usage traditionally for wound healing.

Table 1. Proline, free amino acid, tannins, phenols and flavonoids content of Obudu cattle ranch honeys

Obudu cattle ranch honeys(mg/g)	1	2	3	4	5	6	7	8	Mean	±SD
Proline	2.4	2.04	2.22	2.61	1.62	1.59	2.43	1.86	2.1	0.38
Total free amino acids	3.65	2.55	2.92	3.51	2.06	2.41	3.84	2.87	2.98	0.64
Proline/total (%)	65.8	80	76.1	74.3	78.5	66.1	63.3	64.9	71.1	6.78
Total phenols	1.30	1.28	1.28	1.06	1.12	1.17	1.14	1.18	1.19	0.09
Non tannins	1.30	1.28	1.28	1.06	1.12	1.17	1.14	1.18	1.19	0.09
Tannins	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00	0.00
Total flavonoids	0.65	0.57	0.45	0.42	0.64	0.52	0.66	0.38	0.54	0.11
Flavonoids/Phenols (%)	50.0	44.5	35.2	39.6	57.1	44.4	57.9	32.2	45.1	9.48

Values obtained/samples are means of triplicate determinations.

Table 2. Free radical scavenging activity (RSA) and antioxidant content of Obudu cattle ranch honeys

Obudu cattle ranch honeys	1	2	3	4	5	6	7	8	Mean	±SD
α-tocopherol(μg/100g)	16.0	15.4	16.6	18.4	14.8	17.2	15.5	18.4	16.50	1.40
QEAC(mg/100g)	8.9	9.3	9.0	9.1	10.4	8.5	9.0	11.2	09.43	0.90
AEAC(mg/100g)	18.1	18.3	17.9	17.8	20.5	17.0	16.9	22.0	18.56	1.78
RSA- IC ₅₀ (mg/ml)	12.50	12.30	12.64	12.80	12.10	13.70	13.90	12.00	12.74	0.71

Values obtained/samples are means of triplicate determinations.

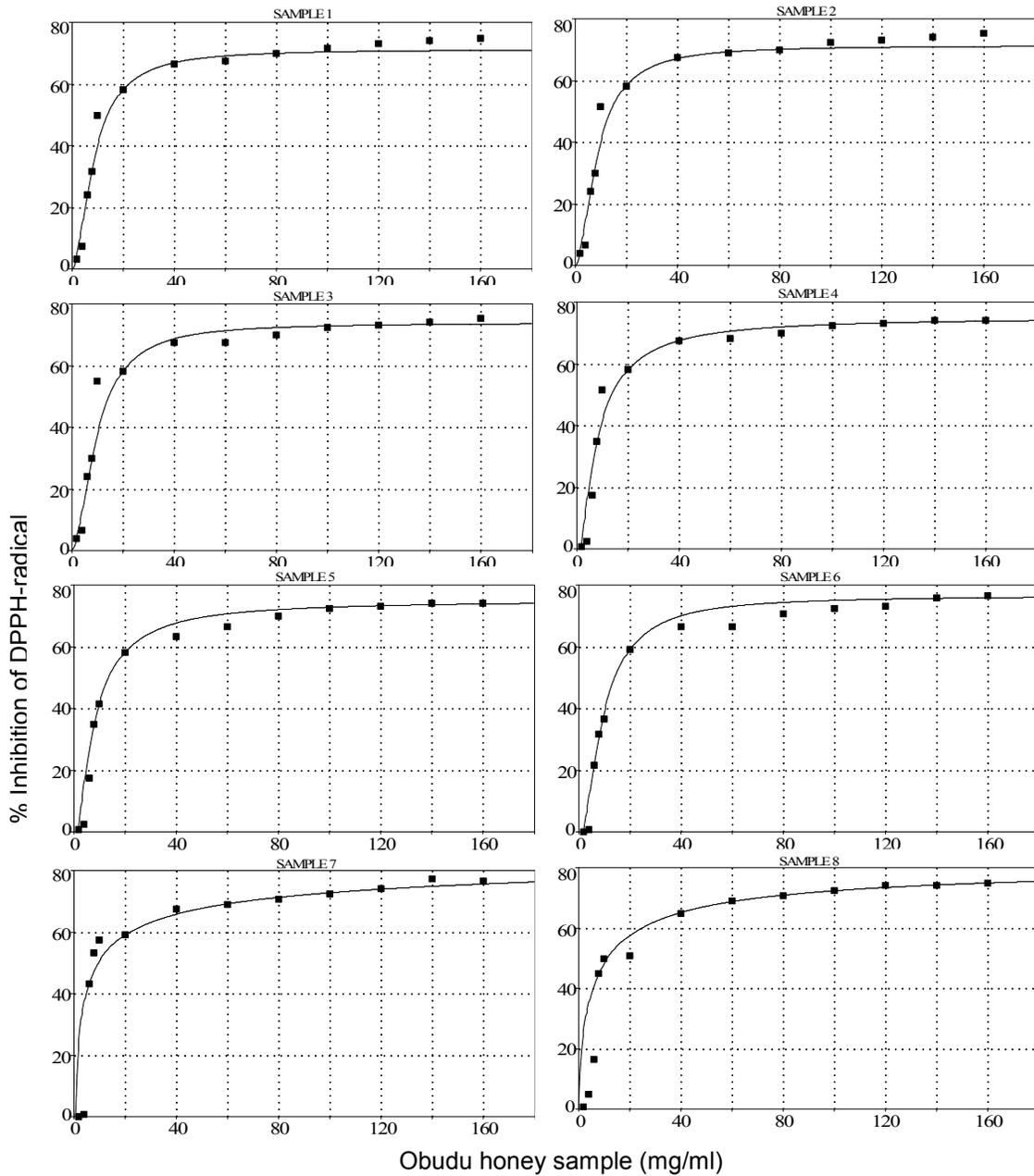


Fig. 1. Inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by Obudu cattle ranch honey

Proline concentration, antioxidant content (AEAC (mg/100g), QEAC (mg/100g), and α -tocopherol ($\mu\text{g}/100\text{g}$) related inversely with RSA-IC₅₀ (Fig. 2). The relationship was strictly logistic (equation 1) as shown (Table 3). The contribution of proline content to radical scavenging by honey sample was not significant ($p = 0.127$). The relationship between the AEAC, QEAC and α -tocopherol with RSA-IC₅₀ was significant ($p = 0.001, 0.003$ and 0.022 respectively) with high coefficient of determination (r^2 -Coef-Def = 0.971, 0.960, and 0.888

respectively. This evaluation (Table 3) indicated that radical scavenging by the honey samples were directly related to their ascorbic acid, phenol and α -tocopherol contents.

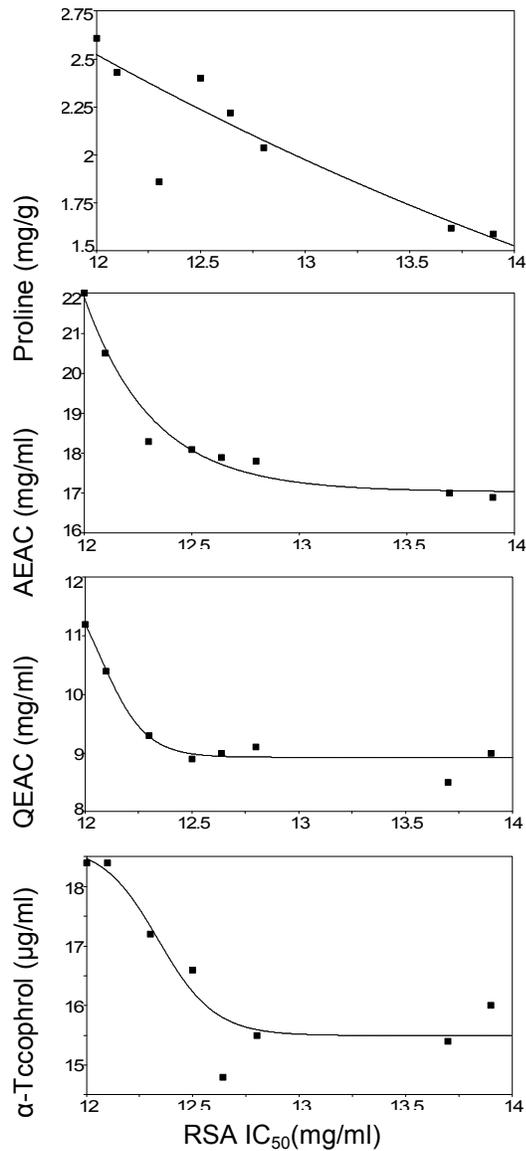


Fig. 2. Relationship of radical scavenging activity with proline and antioxidant content of Obudu cattle ranch honey sample

The summary of the results obtained in the present study showed that α -tocopherol content was $16.50 \pm 1.40 \mu\text{g}/100\text{g}$, quercetin equivalent antioxidant content (QEAC) was $9.43 \pm 0.9 \text{mg}/100\text{g}$, ascorbic acid equivalent antioxidant content (AEAC) was $18.56 \pm 1.78 \text{mg}/100\text{g}$, while the mean inhibitory concentration (IC_{50}) of the honey samples against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (RSA: IC_{50}) was evaluated to be $12.74 \pm 0.71 \text{mg}/\text{ml}$. Proline content, AEAC, QEAC and α -tocopherol were logistically related ($r^2 = 0.726, 0.971, 0.960,$ and 0.888 respectively) to the radical scavenging activity, but contribution by proline content was not significant.

Table 3. Mathematical model of relationship existing between proline and antioxidant content with free radical scavenging activity

Equations/Emperical values						Procedure	Robust Minimization	Error	
Logistic dose response (LDR abcd)						Levenberg Marquardt	PearsonVII Lim		
						r ² -Coef-Def	DF-Adj.r ²	StdEr	p-value
$y = a + \frac{b}{1 + \left(\frac{x}{c}\right)^d} \dots\dots\dots(1)$									
y	x	a	b	c	d	r²	r²		p
Proline	RSA IC ₅₀	8.26	-8.69	10.04	-3.71	0.726	0.361	0.264	0.127
AEAC	RSA IC ₅₀	17.03	491.91	10.62	37.88	0.971	0.934	0.395	0.001
QEAC	RSA IC ₅₀	8.928	3.417	12.071	115.26	0.960	0.907	0.238	0.003
α-Tocopherol	RSA IC ₅₀	15.496	3.190	12.342	93.826	0.888	0.739	0.603	0.022

4. CONCLUSION

The results obtained from the present study showed that Obudu ranch honeys were of high quality and possessed antioxidant and free radical scavenging property. Since proline content of the tested honey samples were comparable to proline content of unadulterated honey samples elsewhere, the commercial samples may not be adulterated. Results showed that traditional usage of the honey for various medicinal usages may be related to its chemical content and radical scavenging property.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Mr. Emeka Asiwe for his assistance during the course of the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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