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Phytochemical Analysis and Antioxidant Properties of Amber and Red Honeybee Propolis

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Abstract
Honeybee Propolis has been used in ethno medicine as an emollient in the treatment of measles, chicken pox, ringworm, and it was also reported that biological activities, such as antitumor, antioxidant, immunomodulatory action and anti-inflammatory has positive effect to propolis. The aim of the study is to determine the Phytochemical constituents and antioxidant properties. Preliminary phytochemical screening of the ethanol extract propolis of both samples revealed the presence of flavonoids, phenolic compounds, saponins, carbohydrate, tannins, steroids and reducing sugar. The antioxidant activity of the two samples were determined using stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay and was found to be all positive. The percentage scavenging activity of amber ethanol extract propolis is between the range of 47.07 - 53.84% which is the lowest and that of red ethanol extract propolis is between 47.52 -63.58% which is the highest. The 50% inhibitory concentration (IC_{50}) of red propolis was found to be 60µg/ml and 140µg/ml for amber propolis. The low value of IC_{50} in red propolis can be attributed to high composition of flavoniods and phenolic nucleus which make it to have significantly more antioxidant and free radical scavenger activities than the amber propolis.

Keywords: Antioxidant, 2,2-diphenyl-1-picrylhydrazyl, Honeybee, Phytochemical, Propolis,

INTRODUCTION
Natural products are broad sources for discovery of new pharmaceuticals compounds and for many years now, minerals, animals and plants were the main sources of drugs (Rates, 2001). Medicinal plants have continued to attract attention in the global search for effective antiviral, analgesic, anticancer and antimicrobial agents that can combat resistant pathogens and diseases that are rendering many conventional drugs obsolete in the treatment of

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infection (Cox, 1990). However, there is a growing interest in the use of natural products and folk medicine even in the developed countries. In developing countries like Nigeria, Ghana, Mali and Zambia the first line treatment for 60% of children with fever, resulting from malaria, is the use of herbal medicine at home (WHO, 2003).

Secondary metabolites are the classes of compounds which are known to show curative activities against several ailments in man, and therefore could explain the use of traditional medicinal plant for the treatment of some illnesses (Sabri *et al*., 2012). Plants contain bioactive chemical substances that produce a definite physiological action in the body, therefore play a significant role in traditional medicine (Sadiq *et al*., 2016). These chemical compounds (phenolic compounds, alkaloids, tannins, flavanoids, terpenoids, steroids, quinones, saponins, etc) have complex structures and with more restricted distribution than primary metabolites. They are not indispensable for the plant that contains them; at least their metabolic functions have not been discovered yet (Sabri *et al*., 2012).

Honey bees are members of genus *Apis,* and perennial insect species that can utilize nearly all habitats of the world (Bashir *et al*., 2016). There are about seven species of the honey bees with a total of 44 subspecies (Michael and Engel, 1999). Honeybees produce high-quality foods in the form of honey, building materials in the form of propolis, and chemical defenses in the form of bee venom and propolis (Bankova *et al*., 2014).

Honeybee propolis (bee glue) is a generic term used to describe a complex mixture of resinous, gummy and balsamic materials from buds, flowers and plant exudates collected by bees; salivary secretions, wax and pollen are masticated and regurgitate to form the propolis. Propolis is referred to as bee glue because it is used for construction and to seal the cracks in the bee hive (Kalia *et al*., 2013). Propolis is a lipophilic material hard and brittle when cold but soft, pliable and sticky when warm, hence the name bee glue (Hausen *et al.,* 1987). The word'*pro-polis*' is derived from the Greek *pro*- for or in defence and *polis*- the city, that is, defence of the city (or the hive) (Ghisalberti, 1979). Honey bees use this as a sealant in the hive, to fill the cracks, smoothing of internal walls and to protect the bee hive from the attack of microorganisms and other intruders like ants, flies, lizard, and snake or against wind and rain (Burdock, 1998). It also maintains sterile conditions especially near the larvae. It is usually yellow green to dark brown in colour and aromatic odour (Ghisalberti, 1979).

Propolis may differ from one place to another due to geographical differences and bee species. For example *Aroeira mansa* or *Schinus terebenthifolus* is the preferred plants source for propolis of *Tetragonisca anjustula* bees in all region of Brazil; however other species of stingless bees also collect resin from different plants depending on the vegetation of that region (Alexander *et al.,* 2009).

Propolis is a complex mixture made by bee-released and plant-derived compounds (Vijay *et al*., 2013). The chemical composition varies qualitatively and quantitatively, depending on the vegetation in the area from which it was collected (Bankova *et al.,* 2000).

The constituents of raw propolis can be divided into three parts: resin, wax and residue (Cunha *et al*., 1997). The wax comprises of beeswax, whereas the residue consists primarily of pollen grains (5% weight), heavy metals, and insoluble materials (Barth *et al*., 1997; Buenos *et al*., 1997). The resin constitutes the active compounds which give rise to the aseptic properties of propolis, and it is the resin that is usually extracted for experimentations and/or pharmaceutical purposes. The antimicrobial, antioxidant, anticancer, anti inflammatory, constituents of propolis resins and extracts are commonly phenols,

flavonoids, aromatic acids and diterpenic acids (Silici and Kutluca, 2005; Uzel *et al*., 2005; Kishore *et al*., 2013).

Propolis samples collected from Europe, South America and Asia have different chemical component (Powers, 1964). Europe and Chinese propolis have flavonoids and phenolic acid esters (Bankova *et al*., 2000), but Brazilian propolis has terpenoids and prenylated derivatives of *p*-coumaric acids (Tazawa *et al*., 1999; Tosi *et al*., 2007). Because of these differences in chemical components of propolis, its biological activities from different areas are also different (Kumazawa *et al*., 2004).Chemical components of propolis and its biological activity enable the standardization of the application of propolis (Havsteen, 2002). It was found that the differences in chemical components of propolis from different plants change the spectrum of propolis biological activity (Kumazawa *et al*., 2004).

However, raw propolis from all regions of the world, on a general note, contains 50%vegetable balsam and resin, 30% wax, 10% essential and aromatic oils, 5% pollen, and5% an assortment of additional substances such as organic debris (Pieta *et al*., 2002; Uzel *et al*., 2005; Gomez-Caravaca *et al*., 2006).

Phytochemicals are non-nutrient plant chemical compounds or bioactive components that are responsible for protecting the plant against microbial infections or infestations by pests (Doughari *et al*., 2009). Phytochemicals (Greek word '*phyto*' meaning 'plant') or phytoconstituents is the study of natural products known as phytochemistry. The bioactive components of plants are called secondary metabolites. Cowan (1999) report that propolis is rich in a wide variety of secondary metabolites. Propolis has more than 150 constituent that are biochemically active, including a mixture of polyphenols, flavonoids, phenols and ketones (Murcucci, 1995), and volatile substance in the plant extract. These metabolites such as alkaloids, tannins, steroids, terpenes, glycosides, flavonoids, carbohydrates, resin, gum are responsible for therapeutic properties of the pharmacologically active compounds which are referred to as "active constituents".

Antioxidant are substance in low concentration that inhibit oxidative processess, either by acting as free radical scavengers or converting radicals to less reactive species (Mandel *et al.,* 2009). They are used in preservation of food from deterioration, rancidity and protect both enzymatic and non-enzymatic reactions leading to oxidative damage (Halliwell and Gutteridge, 1999). Bee propolis contains many phenolic compounds as being reported by many literatures. The interest in phenolic compounds has increased due to the antioxidant and free radical scavenging activities (Dorman *et al*., 2003). Several researchers found out that polyphenols are antioxidants with redox properties which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Caldwell, 2003). Epidemiologic studies have shown a correlation between an increased consumption of phenolics antioxidants and a reduced risk of cardiovascular disease and certain types of cancer (Cook and Samman, 1996).

MATERIALS AND METHODS

Chemicals/Reagents

Chemicals used were of high quality and the reagents were of analytical grade. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma-Aldrich Company), Ascorbic acid powder (Eage Scientific Ltd. Company), ethanol 95% (Sigma-Aldrich Company) and methanol (Sigma-Aldrich Company).

Equipment
The following equipment in addition to other common laboratory materials were used for the experiment;
752W UV/visible spectrophotometer, healthequip medical England FA2104B weighing balance, rotary evaporator (Union Laboratory England, RE-52A) and incubator (DNP-9052 Eschmed Medical England).

Sample Collection
Honeybee propolis are of two types, the amber propolis was collected from honeybee seller at Shuwarin market, Kiyawa Local Government, Jigawa State, and the red propolis was collected from honeybee seller at Garko market, Garko Local Government, Kano State. Propolis was pressed to remove stored honey from it, size was reduce with scissors and spread under shade to reduce moisture.

Extraction
The raw honeybee propolis was extracted with 95% ethanol (Chia-Chi, *et al.,* 2002) using cold maceration method for 7days to exhaustion. The combined extract was filtered using Whatman filter paper and concentrated *in vacuo* to yield a brown semi solid residue referred to as honeybee ethanol extract propolis (EEP).

Preliminary Phytochemical Screening
Phytochemical screening was carryout according to the procedure adopted by Trease and Evans, (2002), Brain and Turner, (1975), Sofowora, (1982) and Evans(1996).

Test for Flavonoids
(a) ***Ferric chloride test***: About 5ml of distilled water was added to the extract and boiled on water bath for about 2min and then filtered. To 2ml of the filtrate, few drops of 10% alcoholic ferric chloride solution were added. Effervescence occurred and the dark brown solution change to green, blue to violet colouration indicate the presence of phenolic group (hydroxyl group) (Trease, and Evans, 2002).
(b) ***Lead acetate test***: A small quantity of the extract was dissolved in water and filtered. Few drop of 10% lead acetate was added to 5mls of the filtrate. A buff coloured ppt. Indicate the presence of flavonoids (Brain and Turner, 1975).
(c) ***Sodium hydroxide test***: 2ml of filtered extract was dissolved in 10% NaOH of solution to give a yellow colour. A change in colour from yellow to colourless on addition of dilute hydrochloric acid, indicate the presence of flavonoids (Trease and Evans, 2002).

Test for Carbohydrates
(a) ***General test***: A small amout of distilled water were added to the extract and later small amount of sulphuric acid was added. A dull violet precipitate indicate the presence of carbohydrate (Trease and Evans, 2002).
(b) ***Barfoed's test for sugar***: 1ml of aqueous filtrate of the extract was mixed with 1ml of Barfoed's reagent in a test tube. The test tube was then heated on water bath for few minutes. A red ppt of cuprous oxide indicated the presence of a monosacharide sugar (Brain and Turner, 1975).

Test for Reducing Sugar

(a) ***Fehling's test***: Small amount of the sample was dissolved in distilled water and allowed to extract for some time. The mixture was then filtered and filtrate was heated with 5ml of equal volume of Fehling's solutions A and B, for few minutes (5min). Formation of a

red ppt of cuprous oxide indicates the presence of free reducing sugar (Trease and Evans, 2002).

(b) ***Test for combined reducing sugar***: The extract was hydrolysed by boiling with 5ml dilute hydrochloric acid and the resulting solution neutralized with sodium hydroxide solution. Few drops of Fehling's A and B solution was added to it and heated on a water bath for 2min. Formations of reddish brown ppt of cuprous oxide indicate the presence of combined reducing sugar (Trease and Evans, 2002).

Test for Steroidal Nucleus

(a) ***Salkowskii test***: Small amount of the extract was dissolved in 2ml chloroform followed by addition of conc. H_2SO_4 to form a lower layer. A reddish brown colour at the interphase indicate the presence of a steroidal nucleus (Sofowora, 1982)

(b) ***Test for cardenolide***: Small quantity of the extract was dissolved in pyridine and a few drops of sodium nitroprusside together with a few drops of 20% sodium hydroxide solution were added. A deep red colour which fades to brownish yellow indicates the presence of cardenolides. (Sofowora, 1982).

(c) ***Keller–kiliani test for digitalis glycoside***: Small quantity of the extract was dissolved in 2ml of 3.5% ferric chloride in glacial acetic acid. This was then transferred to the surface of the 2ml conc. H_2SO_4. A reddish–brown ring obtained at the interphase of the liquid indicate the presence of a digitoxose sugar component (deoxy sugar) characteristics of cardenolide (Trease and Evans, 2002).

Test for Saponins

1ml of the extract was shaken with distilled water in a test tube, frothing which persist on warming indicates the presence of saponins (Sofowora, 1982).

Test for Tannins

Small quantity of the extract was mixed with distilled water and heated on a water bath. The mixture was filtered while conc. H_2SO_4 and 5% ferric chloride were added to the filtrate. A blue-black, green or blue-green ppt. indicates the presence of tannins (Trease and Evans, 2002).

Test for Alkaloids

3ml of the extract was stirred with 5ml of 1% aqueous hydrochloric acid on water and filtered. The filtrate was divided into 3 portion of 1ml each. To the first portion was added few drops of Dragendoff's reagent to give an orange red ppt. To the second potion was added Wagner reagent to give a reddish brown colour. To the third portion was added few drop of Mayer reagent to give a buff ppt. (Trease and Evans, 2002).

Test for Morphine Alkaloid

Thalleiquine test for quinoline alkaloids: 0.5ml of conc. hydrochloric acid and a few crystals of potassium chlorate were added to the extract. It was carefully evaporated and a drop of strong ammonia was added to it. a green colour is taken as positive (Brain and Turner, 1975).

Test for Phenolic Nucleus

Ferric chloride test: A small quantity of the extract was boiled with water and filtered. Two drops of freshly prepared ferric chloride solution was added to the filtrate, formation of a blue-black, or green precipitate was taken as evidence for the presence of phenolic nucleus (Evans, 1996).

In vitro DPPH Free Radical Scavenging Activity

The antioxidant activity of the compounds present in the ethanol propolis extract was determined by means of DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenger capacity. The propolis extract was dissolved in 500µg/ml of methanol. The solution at different concentrations such as 40, 80, 120, 160, and 200µg/ml was obtained using serial dilution from the stock solution. 5ml of methanol solution of the extract of each concentration was mixed with 0.5ml of a DPPH-methanol solution (1mM). These samples were shaken well and kept in an incubator at room temperature for 30min (Brand-Williams *et al.,* 1995).

The absorbance was measured at 517nm. The scavenging activity on the DPPH radical was calculated using the following equation

$$\text{DPPH Scavenging activity (\%)} = \frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control})} \times 100$$

Where;

Abs control = absorbance of control reaction

Abs sample = absorbance of test compound

Ascorbic acid was used as a standard or positive control. Solvent (not contain any compound) was used as the negative control. Scavenging activity was expressed as IC_{50}, which represent the concentration of the extract (µg/ml) required to inhibit 50% of free radical scavenging (Brand-Williams *et al.,* 1995).

The minimum inhibitory concentration at 50% (IC_{50}) is obtained by extrapolting the values from percentage scavenging activity against concentration.

RESULTS AND DISCUSSION

The result of preliminary phytochemical screening of amber and red ethanol extract propolis (EEP) is shown in the table below.

Table1: Preliminary phytochemical screening of amber and red ethanol extracts propolis

Group	Amber EEP	Red EEP
Flavonoids	+++	+++
Carbohydrate	+++	+++
Reducing sugar	+++	+++
Cardiac glycoside	+++	+++
Saponin	+++	+++
Tannins	++	+
Phenolic nucleus	+++	+++
Alkaloids	+	++

EEP = Ethanol Extract Propolis (-): *Negative test* (+): *Positive test*

Table2: Result of Amber EEP, Control Absorbance and Percentage Scavenging

Conc. (µg/ml)	Mean Absorbance of Amber EEP	Standard Deviation	Absorbance of Control	Percentage Scavenging (%)
40	0.236	±0.003	0.444	47.072
80	0.222	±0.003	0.427	48.009
120	0.212	±0.003	0.418	49.282
160	0.198	±0.001	0.406	51.231
200	0.181	±0.002	0.390	53.846

Control = Ascorbic Acid

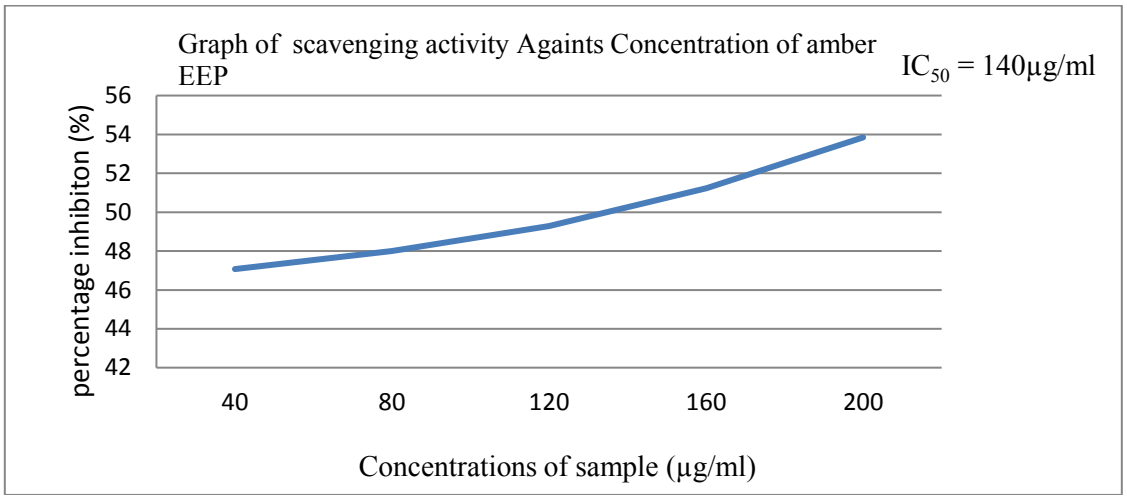

Fig. 1: Graph of scavenging activity againts concentration of amber EEP

Table3: Result of Red EEP, Control Absorbance and Percentage Scavenging

Conc. (µg/ml)	Mean Absorbance of Red EEP	Standard Deviation	Absorbance of Control	Percentage Scavenging (%)
40	0.233	±0.002	0.444	47.543
80	0.209	±0.003	0.427	51.054
120	0.185	±0.005	0.148	55.742
160	0.167	±0.002	0.406	58.866
200	0.143	±0.004	0.390	63.583

Control = Ascorbic Acid

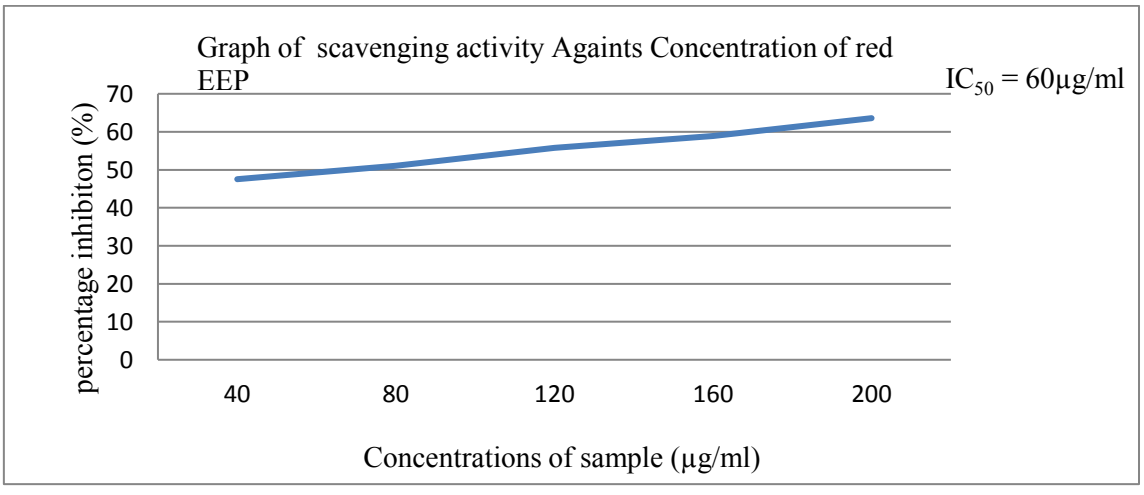

Fig.2: Graph of scavenging activity againts concentration of red EEP

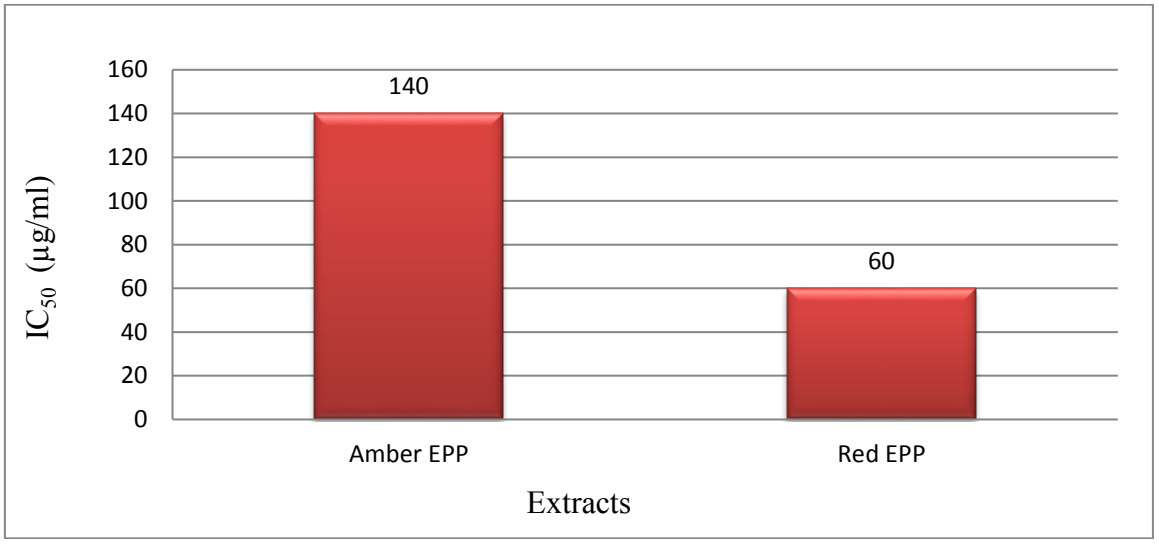

Fig.3: Graphical Presentation of IC50 of Amber and Red Ethanol Propolis Extracts

Plate 1: Amber Ethanol Extract Propolis

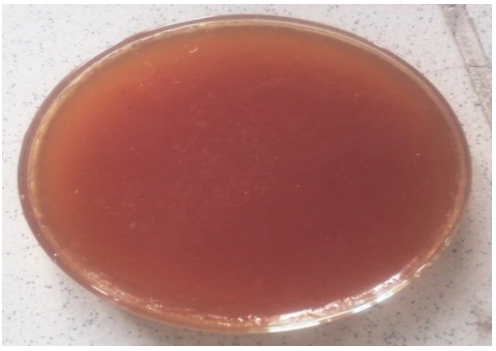

Plate 2: Red Ethanol Extract Propolis

The preliminary phytochemical screening of ethanol extract propolis (EEP) for amber and red revealed the presence of flavonoids, saponins, carbohydrate, phenolic nucleus, steroids and tannins. Both samples show a little amount of alkaloid. The presence of flavonoids and phenolic compounds account for the free radical scavengers that prevent oxidative cell damage. Their activities could be attributed to their ability to neutralize and quench free radicals (Pourmorad *et al*., 2006; Omale and Okafor, 2008; Ugwu *et al*., 2013). Nurhamizah (2016) reported the presence of terpenoids, flavonoids and essential oils in Propolis. The result is also in agreement with Preeti *et al.* (2013) that identified the presence of terpenoids, flavonoids, alkaloids, phenols, tannins and saponins. However, Ioanna *et al*. (2015) identified three triterpenes and two diprenyl-flavonoids from Congo Propolis, while thirteen triterpenes, three diprenyl-flavonoids, two monoterpenic alcohols and one fatty acid ester were also identified from Cameroon propolis.

The antioxidant activity of the two types of honeybee propolis was evaluated and found significantly potential in DPPH radical reaction system. The absorbances of samples at different concentrations are all in triplicate and the mean absorbance was recorded. The radical scavenging potential, expressed as percentage inhibition of amber and red honeybee propolis with respect to DPPH radical was between 47.07 - 53.84% and 47.52 - 63.58%

respectively (Table 2 and 3). The percentage scavenging activities of the samples were expressed in 50% minimum inhibitory concentration (IC_{50}). The lower the IC_{50} value the higher the scavenging activity. This result is in line with the findings by Nurhamizah (2016) on Malaysian stingless bee propolis that show higher antioxidant property. Mok-Ryeon *et al.* (2006) also confirm strong antioxidant activity from China propolis.

The 50% inhibitory concentration (IC_{50}) of amber propolis was found to be 140µg/ml (fig. 1) which was significantly higher than in red propolis with 60µg/ml (fig. 2). From the data in table 2 and 3 it can be suggested that red propolis contained significantly more antioxidant and free radical scavenger activity than the amber propolis. These data showed that, the percentage of free radical inhibition increased as the concentration increased in both extracts. However, the percentage of free radical inhibition was higher in red ethanol propolis extract when compared with amber ethanol propolis extract. This can be attributed to the difference in geographical location of the samples collected which account for the variation in flavonoids and phenolic content. Therefore, from the above result, it can be concluded that red propolis ethanol extract contain more flavoniods and phenolic nucleus than amber ethanol propolis extract.

CONCLUSION

Based on these findings in this work from phytochemical it can be concluded that flavonoids, saponins, carbohydrate, phenolic nucleus, steroids, and tannins are present in both samples. This makes propolis highly effective in the treatment of anti-inflammatory, anti-cancer, anti-diabetic, antifungal and other emollient diseases. While the antioxidant activities are due to the presence of flavoniods and polyphenolic compounds present in the extracts. The antioxidant activities were determined using DPPH assay and were found to be all positive with red propolis having more antioxidant than the amber propolis.

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