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RESEARCH ARTICLE

GC-MS Analysis of Main Chemical Constituents and Antioxidative Potential of Propolis from Meghalaya, India

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Abstract

Objective: Propolis or bee glue is a complex resinous honey bee product known to possess several useful pharmacological properties. Propolis is becoming increasingly popular because of its potential role in contributing to human health. The chemical composition and biological characteristics of propolis depend upon plant sources accessible to bees, bee species, geographical region and seasons. Method: In the present study, the main chemical constituents of propolis sample from Meghalaya were analyzed using gas chromatography-mass spectrometry (GC-MS) technique. The antioxidant properties of the same propolis were also determined by chemical assays such as 2, 2-diphenyl-1-picrylhydrazyl (DPPH), superoxide anion, hydroxyl radical scavenging activity, and metal chelating activity. Results: GC-MS analysis identified twenty different groups of compounds, out of which eight compounds along with their biological activity were recorded. The main constituents of propolis from Meghalaya were N-hexadecanoic acid (34.88 %), oleic acid (26.37%), tetradecanoic acid (4.90 %), 2-furancarboxaldehyde, 5-(hydroxymethyl) (3.21%) and 3,7, 11, 15-tetramethyl-2-hexadecene-1-ol (1.95%). The propolis showed significant antioxidant activity in all the assays. The antioxidant activity of the propolis may also include other chemical constituents not detected through GC-MS analysis. Conclusion: The findings demonstrate that propolis from Meghalaya is a rich source of natural antioxidants and should be very useful in the prevention of various free radicals related diseases.

Keywords: *Propolis, GC-MS, Antioxidant activity.*

Introduction

The word ‘Propolis’ comes from the Greek word, ‘Pro’ means “in front of” or “at the entrance to” and ‘polis’ means ‘community’ or ’city [1, 2] which refers to the hive defensive substance. Honeybees use propolis as a construction material for sealing openings and cracks in their beehive, as well as to stop the entry of intruders and to maintain aseptic conditions within the beehive [2].

The precise composition and constituents of propolis vary depending on the surrounding geographical vegetation and plant sources. The compounds identified in propolis resin originate from three sources i.e. plant exudates collected by bees, secreted substances from bee metabolism, and materials that are introduced during propolis elaboration [3].

In different raw propolis, a group of polyphenols, terpenoids, steroids, sugars, and amino acids have been identified as the main components [3, 4]. The most widely known pharmacologically active chemical components in propolis are flavonoids, isoflavonoids, phenolic acid, terpenes, xanthones, propolones and guttiferones [2, 3] which account for its antimicrobial, anti-inflammatory [6], antiviral [7], antibacterial [8, 9], anticancer [9, 10, 11], and wound healing activities [12].

The color-based classification is also common in propolis and varies from green to red to dark brown, and amongst this dark brown is the most common [5]. Propolis is as old as honey and generally named with its country/region such as Argentinean propolis [13], Chinese and Brazilian propolis [14],

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Netherland propolis [10], Sydney propolis [15], Portuguese propolis [16], Indian propolis [12, 17] and Meghalaya propolis [18].

India, being a vast country, with rich cultural and environmental diversity, may comprise a number of varieties of propolis differing in chemical composition and medicinal value due to different vegetation and geographical condition. Beekeeping in India is an age-old practice, which has been predominantly carried out to collect honey. However, the concept of "beekeeping as a business" has been appearing to be inculcated among the Indian population just a few decades ago [19, 20] and few reports on the propolis phytoconstituents and biological properties from India are available [17, 21].

The North-eastern region of India is unique for its ethnic and cultural diversities coupled with biological diversity. Meghalaya is one of the eight States of Northeast India, known for its clouds and the highest rainfall in the world. Meghalaya is situated between 25 ^{0} 47'-26^{0} 10' N latitude and 89^{0} 45'-92^{0} 47' E longitude comprising of 11 districts. The population of Meghalaya is predominantly tribal which mainly include Khasis, Garos and Jaintias who have their respective dialect, distinct ways of life, belief, traditions and cultural heritage [22].

The chemical composition of propolis samples has been extensively studied from different parts of the world, and interestingly, most of them were found to have some common and unique to region-specific phytochemical constituents. Recently, it was reported that the Meghalaya propolis causes a significant decrease in reduced glutathione, sialic acid and an increase in lipid peroxidation in ascites Dalton's lymphoma (DL) cells in its anticancer effect [18].

However, the chemical composition of Meghalaya propolis has not been explored. Therefore, the present study was undertaken to find out the main chemical constituents as well as the antioxidant properties of propolis from Meghalaya.

Materials and Methods

Study Area

The raw propolis was collected from the Ngunraw village of Meghalaya State. Ngunraw is a medium-sized village situated between 25°17'52.5"N latitude and 91°19'23.2"E longitude located under tehsil Mawkyrwat block of West Khasi Hills district, Meghalaya (Fig. 1). Ngunraw village is approximately 135 Km away from Shillong, the capital of Meghalaya.

The people of Ngunraw village use few animals and animal products including honey and other honeybee products for treating minor sickness/ ailments such as coughs, sore throats, wounds burns. Though honey is known traditionally to have effective healing properties, the knowledge, and effect of propolis usage in disease treatment is a newer field with limited information to these people. Agriculture and apiculture are the main sources of their livelihood and the Indian hive-bee, *Apis cerana indica* (Apidae) is the common honey bee species reared by the people in this area.

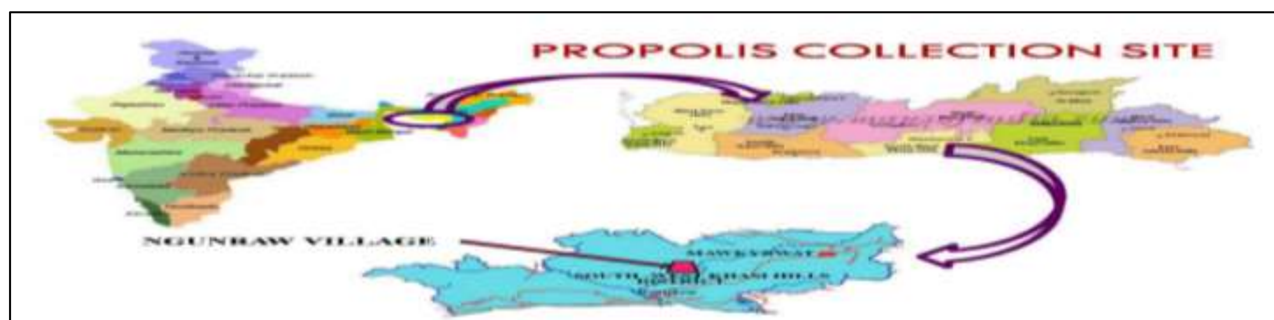

Fig. 1: Study map showing Ngunraw village (collection site) in South-West Khasi Hills district of Meghalaya, India (Maps not at scale)

Propolis Collection and Extract Preparation

Hollow tree trunks and round wooden boxes are used in traditional beekeeping (Fig. 2) while rectangular wooden boxes are also used for rearing the bees. Raw propolis was separated from the honeycombs collected from Ngunraw village, South-West Khasi Hills district of Meghalaya during the spring and winter seasons (Fig. 3). After separation of honey and wax, the remaining honeycombs

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were cleaned and raw propolis was collected, for further extraction as described earlier [9, 18]. Briefly, thirty grams of raw propolis was taken and dissolved in 300 ml of 70% methanol with continuous stirring for 48 hours. After the removal of insoluble materials by filtration with Whatman filter paper the filtrates were centrifuged at 28,000xg for 30 min, and the supernatants were collected. The supernatants were evaporated to dryness in an oven under the temperature of 50^0-55^0C. The crude extract obtained was dissolved in phosphate buffer saline (pH 7.4). Stock solutions were used to prepare the requisite dilutions. The methanolic extract of propolis has been abbreviated as MeOH-propolis in the text, Tables, and Figures.

Fig. 2: (a) and (b): Picturesque Ngunraw village (propolis collection site). (c): Local villager showing a traditional method of beekeeping usually reared in the jungle away from the village. (d): Hollow tree trunk used for traditional beekeeping

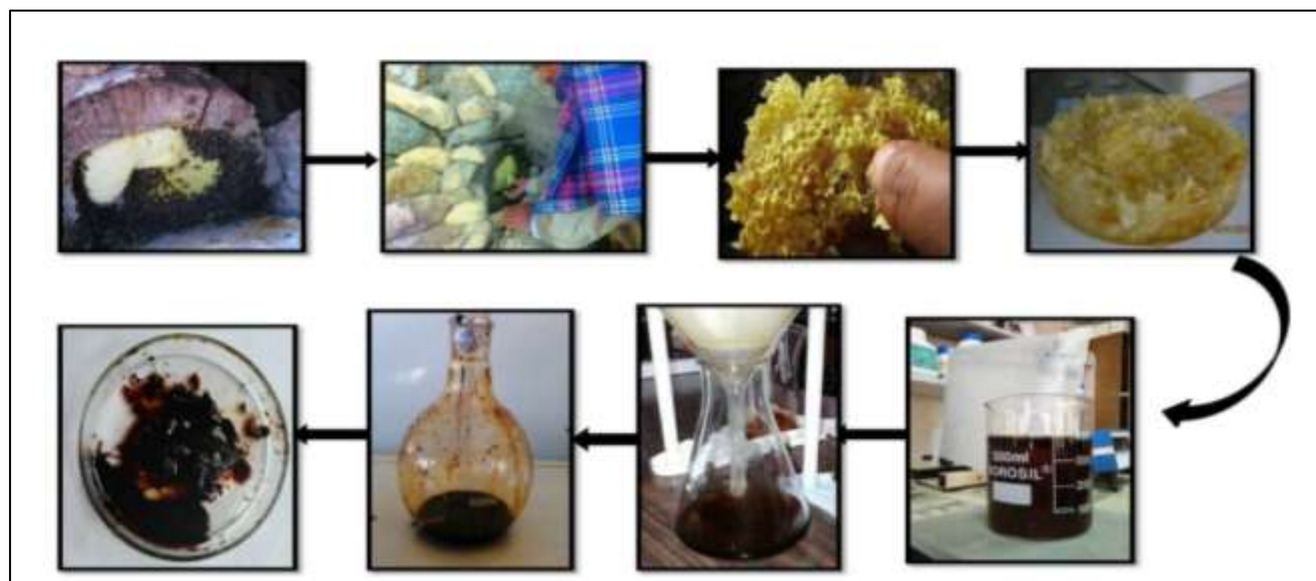

Fig. 3: Steps in collection, cleaning and methanol extraction of propolis

GC-MS Analysis of MeOH-Propolis

The GC-MS analysis of the propolis extract sample was done in Biotech Park, Indian Institute of Technology, Guwahati. The GC-MS analysis was performed with Perkin Elmer (USA) Clarus 680GC/Clarus 600 C MS, fitted with Quadrapole Prefilter Analyzer.

The chromatographic separation was performed on Elite-5MS capillary column (60m, I.D:0.25 mm; Max. program temp 350ºC), phase reference: 5% diphenyl 95 % diphenyl polysiloxane (low bleed). Elution of

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extracts was performed using carrier Helium gas 99.99 %) flowing at a rate of 1.0 ml/min. The injector mode splitless (split = 0:1) was maintained at 250 °C, and the ion source temperature was 180 °C, the initial oven temperature was programmed from 50 °C for 2 min, 5 °C/ min to 300 °C, hold 8 min. The mass detector used in this analysis was Quadrupole with pre-filter and the software adapted to handle mass spectra was Turbo mass. Chromatograms were library searched using S/W Turbo mass NIST library 2008.

Antioxidant Assay

DPPH (2, 2-diphenyl-1-picrylhydrazyl) Assay

The radical scavenging activity of propolis extracts was determined by the DPPH assay [23]. Ascorbic acid (10mg/ml DMSO) was used as a reference. Briefly, different volumes (2 - 20µl) of propolis extracts were made up to 40µl with DMSO and 2.96ml of 0.1mM DPPH was added.The reaction mixture was incubated in a dark condition at room temperature for 20 min. After the addition of an antioxidant, the decrease in the absorption of the DPPH solution was measured at 517nm. 3ml of DPPH was used as a control. The % radical scavenging activity of the extract was calculated using the formula, % RSA = Abs Control- Abs Sample/ Abs Control X 100

Superoxide Anion Radical Scavenging Activity

Superoxide anion radical scavenging activity was determined following the method of Nishikimi et al., 1972 [24]. The principle involved in this assay is the conversion of Nitroblue Tetrazolium (NBT) into NBT diformazan via superoxide radical. Briefly 1ml of NBT (156µl in 10 ml of 100 mM phosphate buffer pH 8), 1ml of NADH (468µl in 10ml 100mM phosphate buffer pH 8), 0.1 ml of propolis extract (10mg in 0.1 ml DMSO and 0.9 ml PO4 buffer) and 0.1 ml of PMS (60 µM PMS in 10 ml of 10mM PO_4 buffer pH 8) were added together and incubated at 25° C for 5 min.

After 5 min, the absorbance was read at 560nm. SOD utilizes the highly water-soluble tetrazolium salt that produces a water-soluble formazan dye upon reduction with superoxide. The rate of reduction with O_2 is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD.

Hydroxyl Radical Scavenging Activity (HRSA)

Hydroxyl radical scavenging activity was evaluated by the method described by Halliwell and Gutteridge [25]. HRSA assay is used to find the scavenging activity of free hydroxyl radicals (which damage the body cells) like hydrogen peroxide in the presence of different concentrations of sample extracts.

Various concentrations of extract (250, 500, 750 and 1000µg) were taken and 1 ml of 0.018% EDTA solution (0.018g EDTA in 100ml dist. H_2O), 1ml of DMSO and 0.5 ml of 0.22% ascorbic acid (0.22g in 100 ml dist. H_2O) was added to it.

The mixture was incubated in a boiling water bath at 80 °C for 15 min. After incubation, 1 ml of ice-cold 17.5 %TCA (17.5g in 100 ml dist. H_2O) and 3 ml of Nash reagent (prepared by adding 7.5g of ammonium acetate, 0.5 ml of glacial acetic acid and 0.2 ml of acetone to 100 ml of dist. H_2O) was added and the reaction mixture was incubated at room temperature for 15 min. The absorbance was read at 412 nm. The hydroxyl radical scavenging activity was calculated using the following formula, %HRSA = Abs Control –Abs Sample/ Abs Control X 100

Metal Chelating Activity

The metal chelating antioxidant assay was determined following the method of Soler-Rivas et al., 2000 [26]. Briefly, 100 µl of propolis extract (10mg in 1 ml DMSO) was added to 50 µl of 2mM ferrous chloride (2.5mg of FeCl2 in 10 ml of dist. H2O) and 200 µl of 5 mM ferrozine solution (24mg in 10 ml dist. H2O). The solution was mixed thoroughly and incubated in dark at room temperature for 10 min. The absorbance was read at 562 nm. 100µl of EDTA solution (10 mg in 1 ml DMSO) was used as a standard.

Statistical Analysis

All the tests were performed in triplicate and results are expressed as mean ± S.D. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine the statistical significance among groups. A *p*-value (< 0.05) was considered statistically significant.

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Results and Discussion

Propolis is a natural animal product that is obtained from apiculture and has important nutritional and medicinal properties. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds.

The large compound fragments into small compounds giving rise to the appearance of peaks at different m/z ratios. The peaks in the chromatogram were integrated and were compared with the database of the spectrum of known components stored in the GC-MS library [27]. Gas chromatography-mass spectrometric analysis of the MeOH-propolis showed the presence of various constituents with different retention times as illustrated in (Fig. 4). The details of the GC-MS analysis of the MeOH-propolis are given in Table1. Out of twenty components shown in MeOH-propolis chromatogram peak, the main constituents are N-Hexadecanoic acid (34.88%), Oleic acid (26.37%), Tetradecanoic acid (4.90 %), 2-Furancarboxaldehyde, 5-(Hydroxymethyl) (3.21%) and 3,7,11,15-Tetramethyl-2-Hexadecene-1-ol (1.95%). Among these, eight different groups of compounds along with their biological activity were characterized as shown in Table 2.

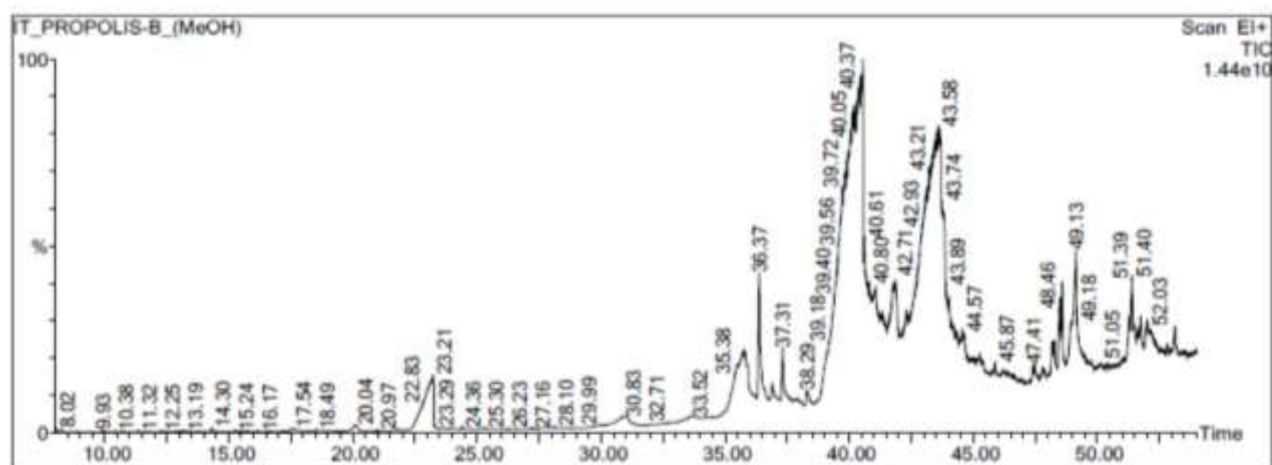

Fig. 4: GC-MS chromatogram showing different peaks of various compounds present in MeOH-propolis extract

Table 1: GC-MS based qualitative and quantitative report of chemical constituents in MeOH-propolis

S. No.	RT	Scan	Height	Area	Peak area %	Norm %
1	20.103	240	244,807,776	63,231,288.0	0.218	0.62
2	23.234	3046	2,051,223,168	932,579,392.0	3.215	9.21
3	30.998	4598	401,393,088	233,155,216.0	0.804	2.30
4	33.649	5128	178,017,024	73,594,032.0	0.254	0.73
5	35.769	5552	2,303,394,560	1,423,049,216.0	4.905	14.06
6	36.375	5673	5,056,593,408	567,742,144.0	1.957	5.61
7	36.895	5777	678,685,504	74,572,280	0.257	0.74
8	37.310	5860	2,071,755,392	204,258,064.0	0.704	2.02
9	38.285	6055	609,527,616	74,308,112.0	0.256	0.73
10	40.551	6508	11,429,265,408	10,121,506,816.0	34.88	100
11	40.606	6519	4,445,344,256.0	586,829,568.0	2.023	5.80
12	40.786	6555	2,599,243,776.0	348,253,056.0	1.200	3.44
13	41.077	66.13	2,134,544,768.0	456,145,824.0	1.527	4.51

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14	41.282	6654	876,355,008	70,899,456.0	0.244	0.70
15	41.382	6674	675,928.129	39,637,320.0	0.137	0.39
16	41.622	6722	780,604,288	51,831,468.0	0.179	4.95
17	42.307	6859	1,144,274,688	150,629,184.0	0.519	1.49
18	42.457	6889	1,126,491,136	66,197,236.0	0.228	0.65
19	43.578	7113	8,728,460,288	7,651,137,536.0	26.373	75.59
20	53.126	9022	1,178,218,752	95,626,080.0	0.330	0.94

Table 2: Chemical compounds identified in MeOH-propolis by GC-MS peak analysis

S. No.	Retention Time	Peak %	Compound Name	Molecular Weight	Molecular Formula	Groups of Compound	Homology	Biological Properties	References
1	23.234	3.215	2-Furancarbonaldehyde 5-(hydroxymethyl)	126	$C_6H_6O_3$	Furaldehyde	9.21	Inhibits the formation of sickled-cells in the blood, Anticancer, Antimicrobial	[28]
2	30.998	0.804	D-allose	180	$C_6H_{12}O_6$	Sugar	2.30	Anticancer Antioxidant	[28]
3	35.769	4.905	Tetradecanoic acid	228	$C_{14}H_{28}O_2$	Phenolic compound Fatty acids	14.06	Antimicrobial Antioxidant Antimicrobial	[29]
4	36.375	1.957	3,7,11,15-tetramethyl-2-hexadecon-1-ol	296	$C_{20}H_{42}O$	Terpene alcohol	5.61	Antimicrobial Anticancer	[30, 31]
5	37.310	0.704	3,7,11,15-tetramethyl-2-hexadecon-1-ol	296	$C_{20}H_{42}O$	Terpene alcohol	2.02	Antimicrobial Anticancer Anticancer (R26), Anti-inflammatory, Antioxidant, Diuretic Antibacterial, Antifungal, Cancer preventive, Insectifuge	[30, 31 and 32]
6	40.551	34.88	N-hexadecanoic acid	256	$C_{16}H_{32}O_2$	Fatty acids and methyl ester	100	Antitumor, Antioxidant, Anti-inflammatory	[33, 34]
7	43.578	26.373	Oleic acid	282	$C_{18}H_{34}O_2$	Aliphatic acids	75.59	Antiscorbutic, Antioxidant Antibacterial, Anti-inflammatory, Cancer preventive Dermatitigenic	[32]
8	53.126	0.330	3-hexadecanol	242	$C_{18}H_{34}O_2$	Alcohol	0.94	Antioxidant	[34]

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Chemical-constituents standards are generally used in GC-MS to decide the identity, purity, and strength of the drug source. The chemical composition of propolis is very complex and depends on the bee species, geographical season, location and the flora in the areas from where it is collected [2]. The orange plants (*Citrus sinensis*) and shrubs *Inula cappa* are very common around the propolis collection site. The bee species commonly present in the area are *Apis cerana indica.*

The GC-MS of MeOH-propolis revealed various compounds which were identified as 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 2-furancarboxyldehyde,5-(hydroxymethyl), Beta-D-glucopyranose, 16-anhydrous, tetradecanoic acid, 3,7,11,15-tetraethyl-2-hexadecene-1-ol, N-Hexadecanoic acid, 6-Octadecanoic acid, methyl ester, Oleic acid, hexadecanoic acid, 2-hydroxy-1 (hydroxymethyl) ethyl ester, palmitoyl chloride and 9-Octadecanoic acid (Z)- and 2,3-dihydroxypropoly ester which belong to eight main constituent groups as mentioned in Table 2.

The presence of these compounds in the MeOH-propolis should play an important role in its different biological activities as was observed earlier [9, 18]. The other reports on propolis have also established various biological and pharmacological properties such as antibacterial, antiviral, anti-inflammatory, anticancer and immune-modulatory activities [2, 3]. In East Asia, tea seed oil (also known as tea oil or Camellia oil) has been used as high quality culinary oil for thousands of years which are pressed from the seeds of *Camellia oleifera* and *Camellia tenuifolia*.

It was found that oleic acid is the major constituent in *C. tenuifolia* seed oil and plays a key role in the antioxidative activity of *C. tenuifolia* seed oil in *Caenorhabditis elegans* [35]. *Laminaria japonica* L. is among the most commonly consumed seaweeds in northeast Asia.

It was reported that *L. japonica* essential oil exhibits strong antibacterial, free radical scavenging, and antioxidant potential which is due to the presence of tetradeconoic acid, hexadecanoic acid, 9, 12-octadecadienoic acid, and hexadec-9-enoic acid in the oil [36]. The antioxidative activity of the rare sugar D-allose has shown that treatment suppressed mitochondrial reactive oxygen species generation in Neuro2A cells, thus, D-allose has also been found to possess antioxidant potential [37].

Antioxidant activity of the MeOH-propolis was examined with different antioxidant-measuring parameters. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is a free radical compound and has been widely used to test the free radical scavenging ability of various samples [38]. It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen- donating ability [39].

DPPH radical scavenging activity increases with increasing phenolic components such as flavonoids, phenolic acids, and phenolic diterpenes. DPPH assay of MeOH extract of propolis showed that scavenging activity increased as a function of concentration increment (Fig. 5) which suggests that propolis from Meghalaya has high antioxidant activity.

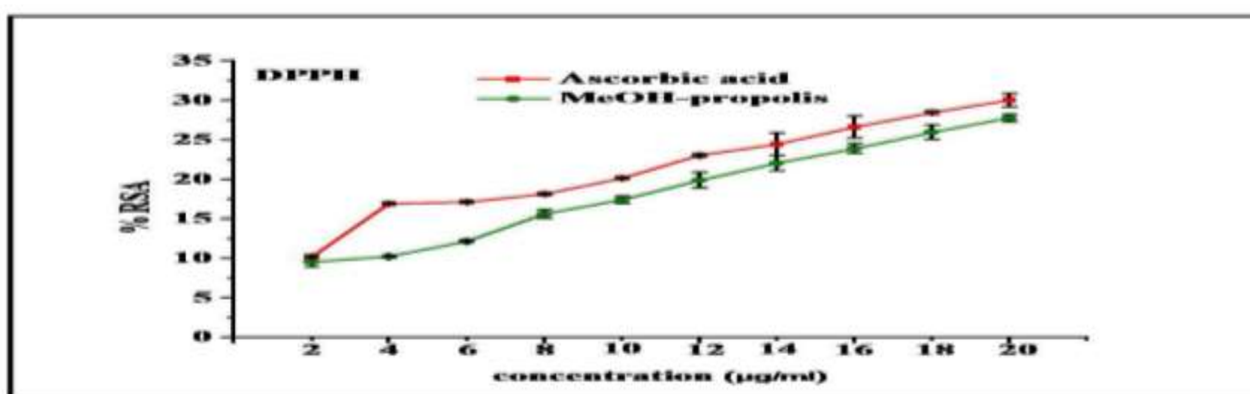

Fig. 5: Radical scavenging activity (RSA) of MeOH-propolis and ascorbic acid. The assay was done in triplicates. Results are expressed as mean ± SD, where n= 3

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The free radical scavenging effect of propolis extract from the Tamil Nadu zone also reported showing a concentration-dependent antioxidant activity [17]. The observations from the present study are in concurrence with this finding (Fig. 5). The assessment of antioxidant ability using the superoxide anion radical scavenging activity also exhibited the high antioxidant property of the MeOH-propolis. Maximum superoxide anion radical scavenging activity was observed at 30mg/ml (58%) of the MeOH-propolis extract (Fig. 6). Although the antioxidant activity of the MeOH-propolis was little lower than the standard ascorbic acid at corresponding concentration, it indicates the effective antioxidant property of propolis from Ngunraw village of Meghalaya.

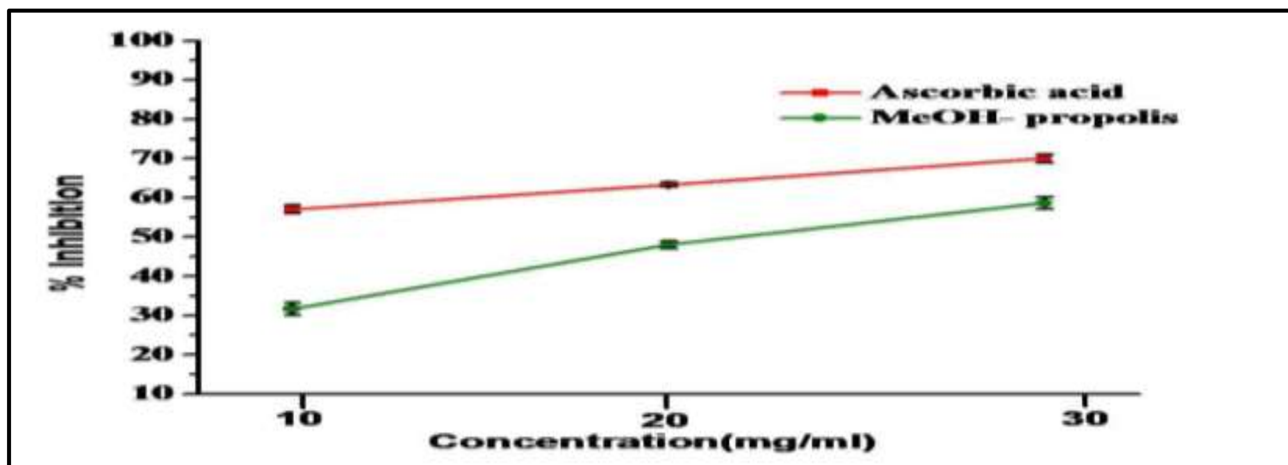

Fig. 6: Scavenging activities of MeOH-propolis on the superoxide anion radical. Ascorbic acid was used as a standard. The assay was done in triplicates. Results were expressed as mean ± SD, where n= 3

The hydroxyl radical scavenging activity (HRSA) of the MeOH-propolis showed hydroxyl radical scavenging activity and its activity was observed to increase with increasing concentration of the samples (Fig. 7) which in turn indicates its antioxidant property.

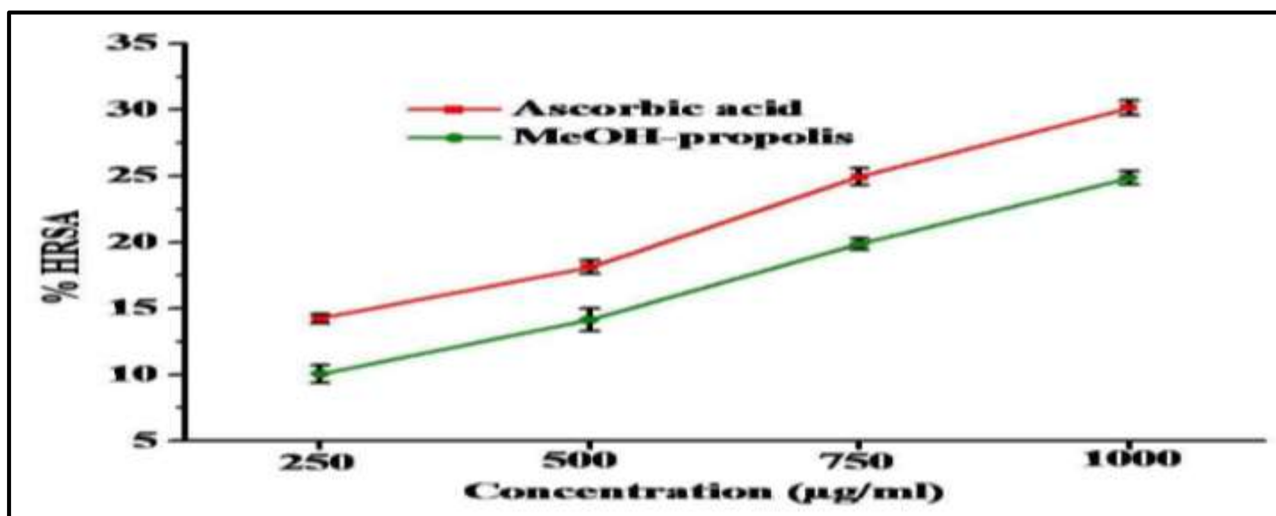

Fig. 7: Hydroxyl radical scavenging activity (HRSA) of the MeOH-propolis. Ascorbic acid was used as a standard. The assay was done in triplicates. Results were expressed as mean ± SD, where n= 3

Ferrous ions chelating activity and artificial antioxidants were determined and the dark color of the complex formed by the interaction of ferrozine with Fe 2+ ions is decreased by the action of metal chelator compounds that exist in the reaction mixtures. Thus, absorbance at 562nm is proportionally related to the chelating activity of the MeOH-propolis sample. The chelating activity of the MeOH-propolis was found to increase with increasing concentrations of the sample (Fig. 8).

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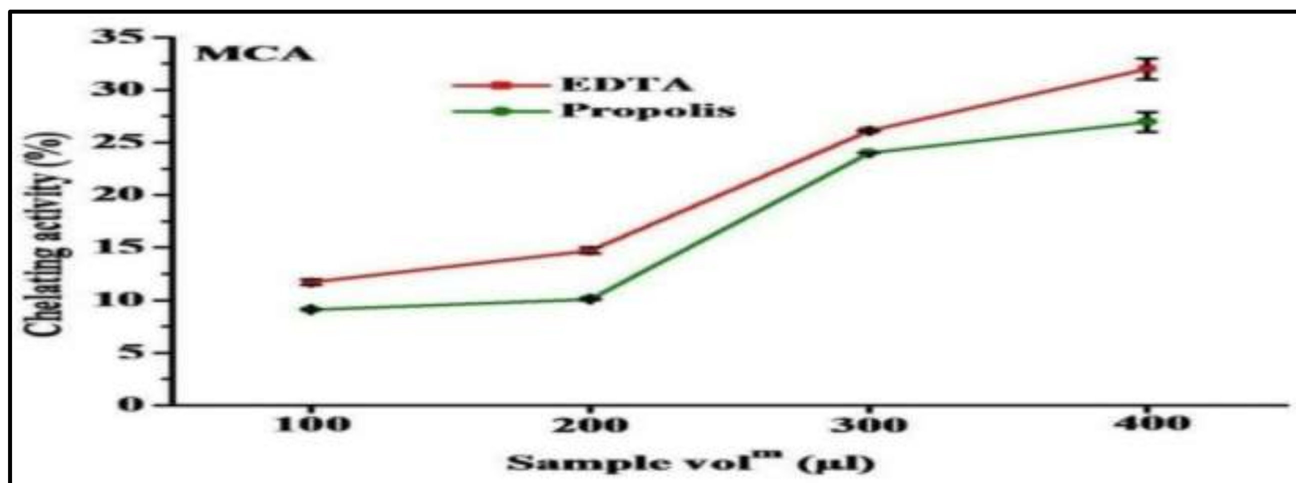

Fig. 8: Metal chelating activity of MeOH-propolis. The assay was done in triplicates. Results were expressed as mean ± SD, where n= 3

Phenolic compounds or polyphenols are presently a major axis of research because they are considered to be powerful chain-breaking antioxidants, and anti-inflammatory, antibacterial, antiviral, and anticancer agents [40]. The antioxidant effect of Brazilian red propolis has been attributed to chalcones and isoflavonoids (including 7-Omethylvestitol, medicarpin, and 3,4, 2',3'-tetrahydrochalcone) that act as electron donors [41].

Furthermore, total flavonoid content in Brazilian red propolis is correlated with antioxidant activity, suggesting that all the phenolic and flavonoid compounds present to contribute to this activity [42]. Thus, from the available earlier reports, it indicates that propolis of different origins and distinct compositions consistently exhibit antioxidant actions.

Thus, the findings from various parameters to determine antioxidant ability clearly demonstrate the antioxidant property of propolis from Meghalaya. It is, therefore, suggested that the antioxidant activity of total propolis could be because of these antioxidant compounds detected through GC-MS analysis.

Conclusion

The propolis from Meghalaya has important chemical constituents/compounds such as tetradecanoic acid, hexadecanoic acid, oleic acid, furaldehyde, hexadecanol which are known for different biological and pharmacological properties. MeOH-propolis exhibits antioxidant activity because of the presence of polyphenols such as flavonoids and phenolic acids. The antioxidant capacity of propolis should be quite important in showing its beneficial effects on human health.

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