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Antimicrobial activity and brine shrimp toxicity of propolis collected from various regions of Tanzania

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ABSTRACT

Background: Propolis has been traditionally used in many countries for management of various health conditions and many previous studies have shown that it has many biological activities, including antimicrobial activity and cytotoxicity. However, not much has been studied with regard to Tanzanian propolis; hence this study reports biological activity of propolis collected from 9 regions of Tanzania. **Objectives:** The purpose of this study was to evaluate the antimicrobial activity and potential cytotoxicity of propolis samples collected from various regions of Tanzania. **Methods:** Ethanolic extracts of 28 propolis samples collected from 9 regions in Tanzania were evaluated for antimicrobial activity against Gram $^{+}$ve bacteria, Gram -ve bacteria and fungi, using the broth microdilution method, through which minimum inhibitory concentrations were determined. Brine shrimp lethality test was done using *Artemia salina* nauplii, as a preliminary indication of potential anticancer activity. **Results:** Out of 28 tested propolis samples, 21 exhibited antimicrobial activity on one or more microorganisms at the tested concentrations, with MICs of 1.25 – 5.0 mg/ml. Fourteen out of 28 samples were found to be toxic on brine shrimps, with a sample from Singida region being the most toxic. **Conclusion:** Propolis samples from various regions of Tanzania demonstrated antimicrobial activity on some microorganisms, indicating that they are a potential source of antimicrobial agents. Some samples also exhibited brine shrimp toxicity; implying that they may also be toxic on cancer cells and thus, may be a potential source of anticancer agents. They may also be a source of compounds with other biological activities.

Keywords: Tanzanian Propolis, Antimicrobial Activity, Brine Shrimp Toxicity.

INTRODUCTION

Propolis (bee glue) is a colored resinous sticky substance that is produced from bee secretions, beeswax, plant exudates and pollen collected from the surrounding vegetation [1,2]. The bees use it to smooth out internal walls, seal cracks and maintain an aseptic environment, stable moisture and temperature in the hives throughout the year. Therefore, it defends the bee hive and strengthens the honey comb, protecting the bee colony from diseases [2-4]. Propolis has been used in traditional medicine in various parts of the world for many years for the treatment of a variety of ailments such as colds, wounds, ulcers, rheumatism, sprains, cardiovascular diseases, diabetes, respiratory infections, cancer, dental caries and many others [3-7]. These medicinal uses have been attributed to various biological activities, such as anti-inflammatory, antimicrobial, antiviral, antioxidant, antitumor, antiulcer, antidiabetic and immunomodulatory activities [3,4,8,9].

Propolis has a variety of chemical constituents which are responsible for the observed biological activities; these constituents include fatty acids, phenolic acids and esters, flavonoids, monoterpenes, sesquiterpenes, diterpenes, triterpenes, steroids, aromatic aldehydes and alcohols, and naphthalene and stilbene derivatives. The chemical composition of propolis varies, depending on the geographical location and types of plants from which propolis is collected by the bees [10]. For example, propolis samples from temperate regions, which are derived mainly from poplar trees, contains mainly flavonoids, aromatic acids, and their esters [10]. while that from tropical regions contains mainly prenylated phenyl propanoids and diterpenes [11,12]. This variation could be responsible for the variation in biological activities of propolis [12].

There are many diseases some of which have no cure or are resistant to current treatment and new diseases continue to emerge and need effective treatments. Since propolis is reasonably safe [5]. with a variety of chemical constituents and biological activities, it has the potential of being a source of effective and safe drugs for the management of various ailments.

Tanzania is a country with a rich biodiversity and is renowned for production of honey bee products. Honey and beeswax are well known and widely used for nutritional and medicinal purposes. Propolis is

also produced to a certain extent in Tanzania, but its uses are not well known. Moreover, not much research has been conducted on this bee product originating from Tanzania. Previously we conducted a study on two samples of propolis from Iringa and Tabora regions; the results of which were promising, since both samples exhibited antimicrobial activity on the microorganisms tested [13]. Therefore, the current study aimed at further investigating the antimicrobial potential of more samples from various regions. Furthermore, considering the fact that some diseases, such as cancer, still need effective drugs for their management, it was important that a preliminary study be conducted to evaluate the potential of Tanzanian propolis samples.

MATERIALS AND METHODS

Propolis samples

Propolis samples were purchased from various beekeepers in nine regions of Tanzania, which are the main producers of honey and other beehive products. The regions included Iringa, Rukwa, Katavi, Tabora, Singida, Kigoma, Dodoma, Tanga and Kilimanjaro. Samples were kept in clean tightly closed containers and stored in a deep freezer at -20°C, prior to extraction.

Preparation of propolis extracts

Frozen propolis samples (13.7 – 100 g) were reduced to small pieces using a mortar and pestle; then subjected to maceration with ethanol (96%) at a ratio of 1:5 of sample and ethanol, respectively. Maceration was done at room temperature, for 5 days with occasional agitation; after which the extract was decanted, followed by filtration through Whatman No. 1 filter paper. To ensure exhaustive extraction, maceration was repeated two more times, for five days each and the extracts were pooled and the filtered extracts were eventually dried at 50°C, *in vacuo* using a rotary evaporator. Dried extracts were kept in a refrigerator, at 4°C, until when required for biological activity tests.

Screening for antimicrobial activity

Antimicrobial activity of propolis extracts was tested against various standard microorganisms obtained from the Microbiology laboratory of Muhimbili National Hospital, Dar es Salaam, Tanzania. These included Gram positive bacteria: *Staphylococcus aureus* (ATCC 25923); Gram negative bacteria: *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC708903), *Salmonella typhi* (ATCC 8385) and *Pseudomonas aeruginosa* (ATCC 27853); and fungi: *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (ATCC 90112).

Tests were done using the broth microdilution method utilizing a 96 well microtiter plate, through which minimum inhibitory concentrations (MICs) were determined. Briefly, the propolis extract was dissolved in dimethyl sulphoxide (DMSO; Sigma®, Poole, Dorset, UK) to make a solution of 100 mg /ml. The prepared solution was further diluted with sterile distilled water to make a working solution with a concentration of 20mg/ml. Double strength Mueller-Hinton broth (50 µl; Carl Roth GmbH + Co. KG, Germany) was introduced into each well of the first row of the microtiter plate and to each of the other wells 50 µl of normal strength broth was introduced. The working solution (50 µl) of each test sample was introduced into the respective first row wells of the plate, mixed well and two-fold serial dilutions were prepared along the column. Test microorganisms (50 µl) were added to each well with the final density being equivalent to 0.5 MacFarland. Final propolis test sample concentrations ranged from 5 to 0.039 mg/ml. DMSO (5%) was used as a negative control, while Ciprofloxacin (2 – 0.016 µg/ml; Sigma Aldrich, Germany and Fluconazole (15.6 – 0.122 µg/ml; Sigma Aldrich, Germany) were used as positive controls for bacteria and fungi, respectively. The microtiter plates were incubated for 24 hrs., at 37°C for bacteria and 28°C for fungi, after which 40 µl of p-Iodonitrotetrazolium (INT) (Sigma Aldrich, Germany) chloride (0.2 mg/ml) solution was added to the wells and incubated further for 30 minutes. The lowest concentration at which no purple coloration appeared was taken as the minimum inhibitory concentration (MIC).

Test for brine shrimp toxicity

Brine shrimp toxicity testing was done as a preliminary test to predict the potential of propolis samples to have cytotoxic effect on cancer cells [14]. Brine Shrimps eggs were purchased from Aquaculture Innovations (Graham's town, South Africa). The sea salt used for preparation of artificial sea water was obtained by evaporation of water collected from the Indian Ocean, along the Dar es Salaam Coast. Artificial seawater was prepared by dissolving sea salt in distilled water to make a concentration of 3.8g/L. After filtration, the artificial sea water was placed into a sterilized tank which was divided into two unequal compartments by perforated polythene wall, and the pH was adjusted to 7.0. One part of the container was covered with an aluminum foil (by 80%) while the other part was left open and was illuminated with a lamp. Brine shrimp eggs (500 mg) were sprinkled into the covered part of the tank and allowed to hatch. Mature nauplii for use in the brine shrimp toxicity test were collected after 24 to 36 hours of hatching.

Propolis extracts were dissolved in DMSO to make a stock solution of 40 mg/ml each, which was further diluted to make final test concentrations ranging from 8 µg/ml to 240 µg/ml. Ten nauplii were introduced into each test concentration of propolis samples as well as the positive and negative controls. The number of surviving larvae was counted after 24 hours of incubation under the illumination. DMSO (0.6% v/v) in artificial sea water was used as a negative control and cyclophosphamide was used as a positive control. Tests were done in duplicate and the mean percentage mortality was plotted against the logarithm of concentrations using the Fig P computer program (Bio soft Inc., USA). Regression equations from the graphs were used to calculate LC_{50} and the 95% CI values.

RESULTS AND DISCUSSION

Twenty eight samples of propolis collected from various regions are as listed in Table 1. Out of these seven were collected from hives of stingless bees and the rest were obtained from hives of stinging bees. Most extracts were brown in color; however, two samples (No. 14 and 16) which were collected from Kisaki, Singida region, were of a whitish tint. Yields of extracts ranged from 6.8% to 65.3% w/w; with the highest yields being from samples No. 14 and 16 (61.4% and 65.3% w/w, respectively), and the lowest yield (6.8 %w/w) was from sample No. 15 which was also collected from Singida. The differences in both the yields and colors could be due to variation in the types of the chemical constituents present in the collected samples. Those giving the highest yields could be composed mainly of compounds with low to intermediate polarity and the propolis samples with low yield may be having more polar components which could not be easily extracted with absolute ethanol, a solvent with intermediate polarity.

Table 1: Yields of extracts from various propolis samples

Sample No.	Place of collection (Region)	Weight of Propolis extracted (g)	Extract yield (% w/w)	Color of extract
1	Sao Hill, Mufindi (Iringa)	100.0	51.2	Dark brown
2	Kalambo (Rukwa)	23.1	33.8	Dark brown
3	Mpanda (Katavi)	100.0	27.7	Dark brown
4	Ilagala (Kigoma)	100.0	24.4	Dark brown
5	Uvinza (Kigoma)	100.0	24.6	Dark brown
6	Sambala (Kigoma)	100.0	22.4	Dark brown
7	Kagera-Nkanda, Kasulu (Kigoma)	100.0	24.8	Dark brown
8	Mgela Buhigi Lole, Kasulu (Kigoma)	100.0	28.9	Dark brown
9[a]	Uvinza (Kigoma)	100.0	37.0	Dark brown
10	Sikonge (Tabora)	100.0	15.1	Brown
11	Utyatya, Sikonge (Tabora)	36.0	23.7	Brown
12	Iswagala Forest Reserve, Sikonge, (Tabora)	71.8	24.7	Brown
13[a]	Tabora (Tabora)	100.0	15.1	Brown
14	Kisaki (Singida)	13.7	61.4	Whitish tint
15	Kisaki (Singida)	48.4	6.8	Brown
16[a]	Kisaki (Singida)	82.0	65.3	Whitish tint
17[a]	Kisaki (Singida)	52.8	29.8	Brown
18	Kisaki (Dodoma)	31.0	39.3	Brown
19	Kisaki (Dodoma)	100.0	38.1	Brown
20	Taula, Handeni (Tanga),	100.0	50.7	Dark brown
21	Taula, Handeni (Tanga)	69.0	31.1	Dark brown
22	Lushoto (Tanga)	100.0	47.1	Dark brown
23	Lushoto (Tanga)	100.0	42.7	Dark brown
24[a]	Lushoto (Tanga)	59.4	21.2	Dark brown
25	Makanya, Same (Kilimanjaro)	100.0	34.2	Dark brown
26	Makanya, Kitongoji E, Same (Kilimanjaro)	100.0	40.7	Dark brown
27[a]	Vudee, Milimani, Same (Kilimanjaro)	100.0	27.8	Dark brown
28[a]	Siha (Kilimanjaro)	100.0	37.6	Dark brown

[a] Propolis samples from stingless bees' hives

Antimicrobial activity

Out of 28 propolis samples tested, 21 (75%) exhibited antimicrobial activity (Table 2), with MIC ranging from 1.25 to 5 mg/ml) with six samples (No. 5, 7, 12, 14, 16 and 18) being active against four or more test microorganisms at the used test concentrations. Sample No.16 was the most active with MIC of 1.25 mg/ml on *E. coli* and *K. pneumoniae* and 2.5 mg/ml on *S. aureus* and *C. albicans*, indicating that it may be a promising source of antimicrobial agents for various microorganisms.

Among the test microorganisms, *E. coli* was the most sensitive microorganism to the tested extracts, being sensitive to 18 out of 21 active extracts at the tested concentrations, while *Pseudomonas aeruginosa* and *S. typhi* were the least sensitive; showing sensitivity to only four extracts, with MIC values of MIC 2.5 to 5 mg/ml and 5 mg/ml, respectively. These findings differ from findings in some other studies, for example studies on Cuban and Brazilian propolis extracts were found not active against *E. coli*, but exhibited activity on other bacteria [15,16]. However, in other studies, for example the one done on Iranian propolis, *E. coli* was found to be sensitive to propolis extracts [17]. These differences could be due to the fact that the studied propolis samples were from different countries/geographical locations and possibly, were also collected during different seasons, which may have led to variations in the chemical composition and eventually, the antimicrobial activity.

In this study it was observed that a sample which was collected from Iringa (No. 1) was among those which were not active at the tested concentrations. However, from a previously reported study a sample that was obtained from the same region demonstrated activity against certain microorganisms, which included *E. coli, C. albicans C. neoformans*, with MIC values of 1.67 to 6.67 mg/ml, and was inactive on *S. aureus*, *S. typhi* and *P. aeruginosa* [13]. Furthermore, samples from Tabora in the current study also exhibited weaker activity (MIC 2.5 to >5 mg/ml) than that of a sample from the same region reported previously (MIC 0.42 – 1.67 mg/ml). These differences could be due to seasonal variation of propolis chemical composition; the sample from Iringa which was used in this study was collected in the month of August, during the dry season and it is possible that the previously studied samples were collected during a different season; it is not known during which month or season it was collected. Other studies in other countries also demonstrated that the chemical composition as well as antimicrobial effect of propolis extracts varied with the season of collection [18-20].

In another study which involved propolis samples from several countries, a sample from Tanzania, exhibited antimicrobial activity with a MIC 15.62 µg/ml, against standard strains of Gram positive bacteria including, *S. aureus, S. epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecalis* and *B. subtilis* [21]. However, it was not specified as to which region of the country and when it was collected.

Table 2: Antimicrobial activity (MIC; mg/ml) of various propolis samples

Sample No.	S. aureus	E. coli	K. pneumoniae	P. aeruginosa	S. typhi	C. albicans	C. neoformans
1	>5	>5	>5	>5	>5	>5	>5
2	>5	2.5	2.5	5	>5	>5	>5
3	2.5	>5	>5	>5	>5	>5	5
4	>5	5	>5	>5	>5	>5	>5
5	1.25	2.5	2.5	2.5	>5	>5	2.5
6	2.5	5	5	>5	>5	>5	>5
7	2.5	2.5	5	>5	>5	>5	5
8	>5	>5	>5	>5	>5	>5	>5
9	>5	>5	>5	>5	>5	>5	>5
10	>5	>5	>5	>5	>5	>5	5
11	>5	5	>5	>5	>5	>5	>5
12	5	5	2.5	5	5	>5	5
13	>5	5	>5	>5	>5	5	5
14	5	1.25	>5	>5	5	5	5
15	>5	1.25	5	>5	>5	5	>5
16	2.5	1.25	1.25	>5	5	2.5	>5
17	>5	5	>5	>5	>5	>5	>5
18	>5	5	>5	5	5	>5	5
19	>5	>5	>5	>5	>5	>5	>5
20	>5	5	>5	>5	>5	1.25	>5
21	>5	5	>5	>5	>5	2.5	>5
22	>5	>5	>5	>5	>5	>5	>5
23	>5	>5	>5	>5	>5	2.5	>5
24	>5	5	>5	>5	>5	>5	>5
25	>5	5	>5	>5	>5	2.5	>5
26	>5	>5	>5	>5	>5	>5	>5
27	>5	>5	>5	>5	>5	>5	>5
28	>5	5	>5	>5	>5	5	>5
Ciprofloxacin	$0.5x10^{-3}$	$0.016x10^{-3}$	$0.016x10^{-3}$	$0.25x10^{-3}$	$0.5\ x10^{-3}$	NA[a]	NA
Fluconazole	NA	NA	NA	NA	NA	$0.49x10^{-3}$	$0.49x10^{-3}$

[a]NA = Not Applicable

Brine shrimp toxicity

When tested for brine shrimp toxicity 14 (50%) out of 28 propolis extracts demonstrated varying levels of toxicity ($LC_{50} \leq 100$ µg/ml) to the brine shrimp larvae, with LC_{50} values ranging from 7.75 to 96.87µg/ml (Table 3).

The most toxic was the sample with a whitish tint (No.16) collected from Singida, with LC_{50} 7.75 µg/ml; followed by moderately toxic samples No. 1 (Iringa), 3 (Katavi) and 14 (Singida) with LC_{50} values of 14.90 µg/ml, 13.13 µg/ml and 17.56 µg/ml, respectively. Furthermore, three samples (No. 1, 3 and 16) were more toxic than the positive control, cyclophosphamide which exhibited a LC_{50} value of 16.36 µg/ml. Ten samples were mildly toxic (LC_{50}> 30 < 100 µg/ml) while 14 samples, which had LC_{50}>100 µg/ml, were considered not toxic [22]. The brine shrimp test in this study was used to identify propolis samples with a potential of having anticancer activity [14]. it is, therefore, possible that the samples that exhibited toxicity to brine shrimps may also be toxic to cancer cells; but also possibly, may have other biological activities [14]. Furthermore, since brine shrimp lethality test is also a preliminary test for potentially toxic samples, it is possible that samples which were highly toxic to brine shrimps may also be toxic and hence may not be safe for use [14].

CONCLUSION

From this study some propolis samples from various regions of Tanzania have exhibited antimicrobial activity against some microorganisms; hence may be a source of effective antimicrobial agents. Some samples have also been shown to have cytotoxic activity on brine shrimp larvae, and thus may, possibly, also be cytotoxic on cancer cells or possess other biological activities. Further studies are recommended to identify the active compounds and also evaluate the biological activity of Tanzanian propolis collected during various seasons to determine which will be the best season for sample collection.

Table 3: Brine shrimp toxicity of various propolis samples

Sample No.	LC_{50} (µg/ml)	95% CI; (µg/ml)
1	14.90	11.95 – 18.59
2	49.46	35.05 – 69.79
3	13.13	8.16 – 21.13
4	57.66	44.44 – 74.81
5	110.97	78.40 – 157.16
6	103.49	79.50 – 134.73
7	70.38	55.72 – 88.90
8	100.19	72.92 – 137.66
9	116.42	78.94 – 171.69
10	128.27	97.63 – 168.53
11	53.76	45.68 – 67.73
12	96.87	73.29 – 128.51
13	126.71	94.02 – 170.77
14	17.56	13.98 – 22.06
15	57.24	48.76 – 67.19
16	7.75	5.94 – 10.12
17	387.44	248.18 – 604.85
18	80.36	66.07 – 97.74
19	1209.32	607.71 – 2406.51
20	>1000	NT[a]
21	1244.64	558.30 – 2774.74
22	88.70	71.60 – 109.90
23	85.10	68.26 – 106.10
24	465.38	286.94 – 754.78
25	1244.64	646.79 – 2395.14
26	158.31	124.80 – 200.81
27	59.63	50.48 – 70.43
28	276.16	191.95 – 397.30
Cyclophosphamide	16.37	12.01 - 22.31

[a] Not tested

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Conflict of Interest

None declared.

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