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IN-VITRO CYTOTOXICITY OF INDIAN BEE PROPOLIS ON CANCER CELL LINES

R SHUBHARANI*[1], V SIVARAM[1] AND B R KISHORE[2]

[1] Laboratory of Biodiversity and Apiculture, Department of Botany, Bangalore University, Bangalore-560056, India
[2] V Sivaram Research Foundation, R R Layout, Jnana bharathi, Bangalore- 560056, India,

ABSTRACT

Propolis is a natural resinous hive product collected by honeybees from exudates of plant sources. It has been used in folk medicine due to its various chemical compounds with biological properties since ancient times. The aim of this study was to compare the chemical constituents and to evaluate the in-vitro cytotoxicity and hemolytic activity of Indian propolis against human breast cancer (MCF 7), colon cancer (HCT 116), and prostate cancer (PC 3) cell lines by MTT assay. The propolis extracts were incubated with cancer cell lines for 24 hours, cytotoxicity was measured colorimetrically and the IC_{50} value was calculated. Propolis samples were analyzed by GC-MS and 44 compounds were identified. The results indicated that inspite of the differences in the chemical composition of propolis collected from different geographic locations all the samples exhibited significant cytotoxic activity and did not affect normal cells. It is concluded that the Indian propolis is a potential natural source of chemopreventive agent.

KEY WORDS: **Breast cancer, Colon cancer, Cytotoxicity, Hemolysis, Bee Propolis, Prostate cancer**

R SHUBHARANI
Laboratory of Biodiversity and Apiculture, Department of Botany,
Bangalore University, Bangalore-560056, India

INTRODUCTION

Cancer can be regarded as a group of diseases characterized by an abnormal growth of cells. Cancer may occur in any type of cells or tissues of the body and approximately 11 million people are diagnosed with cancer and more than 6 million die from this disease every year[1]. The epidemiological findings strongly suggest that the cancer rates are the highest in affluent societies mainly due to lifestyle issues. Nearly 180,000 women are diagnosed every year with breast cancer in North America[2]. Mortality rates of cancer have increased during the past 60 years in every country, especially in developed countries and becoming more frequent in developing countries as well[3]. According to International agency for research on cancer, it is estimated that cancer is a major health problem in India. They have reported that about 555,000 people have died of cancer in 2010[4]. It is reported that the incidence of breast cancer is rising rapidly in India as a result of changes in reproductive risk factors, dietary habits and increasing life expectancy[5]. Therefore, there is a need to develop mechanism based approach for management of cancer, besides the present major treatment modalities which may include surgery, medication, radiotherapy, and immunotherapy. Chemoprevention is one of the most promising upcoming modality in oncology research which focuses on prevention of cancer using naturally occurring or synthetic agents which inhibit, delay, or reverse carcinogenesis[6]. The National Cancer Institute, United States, routinely measures the growth inhibitory properties of chemopreventive drugs against human tumor cell lines. Propolis, a resinous sticky substance collected from plants by honeybees is one of the richest sources of phenols and phenolic compounds, which are widely recognized as chemopreventive compounds[7-8]. The phenolic compounds display a wide range of pharmacological properties including antimicrobial, anti-inflammatory, anti-diabetic, antioxidant, hepatoprotective and cytostatic activity[9]. These bioactive compounds vary based on the geographical location and floral diversity. This has led to an increasing emphasis on cancer prevention strategies in which propolis is used as dietary supplement being the richest source of plant phenolic compounds. Several in-vitro studies of propolis in human cancers involving mechanisms like inhibition of cell cycle progression, cell proliferation, tumor growth arrest, prevention of metastasis, and apoptosis has been frequently reported in literature[10-15]. The present study was carried out to evaluate the Propolis properties of *Apis mellifera* collected from different parts of India by determining the in-vitro cytotoxicity using some cancer cell lines, such as breast cancer (MCF 7) cell lines, colon cancer (HCT 116) cell lines and prostate cancer (PC 3) cell lines with hemolytic activity. The chemical compositions were analyzed using GC-MS. The comparative study of biological properties of Propolis from the different geographic locations with different chemical composition is the most interesting trend in recent Propolis research[16]. There are very few studies on the chemical composition of Indian Propolis and its application in human health care.

MATERIALS AND METHODS

The Propolis samples were collected from different locations in India like Karnal from Haryana, (K [HA]), Dehradun from Uttarkand (D [UK]), Coimbatore from Tamil Nadu (C [TN]) and Bijapur from Karnataka (B [KA]) States of India, during January- March 2014.

(i) Preparation of ethanol extract of Propolis

The Propolis extract was prepared by mixing 5 grams of each Propolis sample with 50 ml of 70% ethanol and extracted at room temperature. The 70% ethanol used is a semi polar solvent which can extract all active components with different polarity in the propolis[17]. The ethanol extract was evaporated under vacuum till dryness. The extractive value (extracted matter) was recorded as 24.6% of K [HA] sample, 30.4% from D [UK] Propolis sample, 29.8% of C [TN] Propolis sample and 31.2% from B [KA] Propolis. The resulting extracts obtained used for the GC-MS analysis and determination of

cytotoxic effects on different human cancer cell lines.

(ii) GC-MS Analysis

The propolis samples were analyzed in GC-MS Clarus 500 (Perkin Elmer) instrument equipped with Restek Rtx^R – 5 (30 m X 0.25 mm and 0.25µm of film thickness) column and flow rate of Helium as mobile phase was set to 0.7mL/min. In gas chromatography, the oven temperature initially was programmed at 40^{o}C for 5 minutes, then rise to 200^{o}C at a rate of 6^{o}C/min and finally holds at 280^{o}C for 1 hour.

(iii) Cell culture

Cancer cell lines were obtained from American type culture collection centre, United States. The stock culture of these cell lines were cultured in Dulbecco's modified eagle's medium supplemented with 10% FBS and 1% Streptomycin. The cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere and the confluent cells were sub cultured with 0.25% trypsin and the cells were suspended in DMEM (MCF 7) and RPMI (HCT 116 and PC 3) media.

(iv)Determination of cytotoxicity of propolis

The cytotoxicity of propolis extracts were tested by quantitative colorimetric MTT assay[18]. The cells were plated into 96-well plates and incubated at 37°C in 5% CO_2 incubator for 24 hours. After incubation, each concentration of propolis extract (5-320µg/mL) was added. The plates were incubated at 37°C in 5% CO_2 incubator for 24 hours. Later, MTT solution (100µL/well) was added and incubated for 3-4 hours. Finally, the blue formazan crystal was dissolved with 100 µL of Dimethyl Sulfoxide and absorbance was measured at 590 nm. The percentage of inhibition was calculated comparing with the cell control. The median inhibitory concentration (IC_{50}) values were determined.

(v) Hemolytic assay

This method is based on the release of hemoglobin which can be measured by spectrophotometer and is suited to evaluate the haemocompatibility of biomaterial on Red Blood Cells (RBCs). Blood was collected from healthy human beings and centrifuged at 1000 rpm for 10 minutes at 4^{o}C. Plasma was removed and erythrocyte was washed thrice with PBS (pH 7.4). Propolis extract of different concentrations (5, 10,20,40,80,160 and 320µg/mL) was added to erythrocyte suspension and incubated at 37°C for 1 hr. Later, the volume was adjusted to 1mL with PBS, centrifuged at 300 rpm for 3 minutes and hemoglobin concentration was measured by means of a spectrophotometer at 540 nm[19]. Propolis samples were compared with reference material (1% SDS) to evaluate hemolytic activity.

RESULTS AND DISCUSSION

The propolis extracts were subjected to GC-MS analysis, the most often used technique for chemical analysis and the total separated peaks are shown in Figure 1-4. The chromatograms of all major peaks were matched with National Institute Standard and Technology (NIST) database for identification of chemical compounds present in the sample. The compounds that belong to different chemical groups were identified in each propolis samples. According to GC-MS analysis, it is concluded that the four propolis samples showed qualitative similarities in 18 compounds like Oxalic Acid, Propanoic acid, Formaldehyde oxime trimer, Hydrazine, Hydroxyethylhydrazine, Hydroxypropanoic acid , 1,2-Ethanedio, Methylglyoxal, Butanone, Acetic acid, Propanedioic acid, Methanecarbothiolic acid , 1 Pentene 2 methyl, 1-Hexene, (3-Methyl-oxiran-2-yl)-methanol, Cyclopropane, Cyclohexane, Cyclopentane methyl and 2 amino-oxazole. According to the difference in plant sources, each propolis sample is characterized by certain specific compounds. Sample K [HA] is the only sample containing Cyclobutane, 2 Azetidinone and Boranamine. Sample D [UK] was characterized by the presence of the compounds like Formic acid, Methanamine and Butanenitrile. Sample C [TN] was characterized by the presence of Hydrogen azide and Sample B[KA] was characterized by the presence of o-Methylisourea hydrogen sulfate, 1-Nitro-á-d-arabinofuranose, tetraacetate, Tetraacetyl-d-xylonic nitrile, 14-Oxa-1,11-diazatetracyclo[7.4.1.0(2,7).0(10,12)]tetradeca-2,4,6-triene, 11-acetyl-6,9-bis (acetyloxy) -4-

formyl-8-[(aminocarbonyloxy)methyl], O-Methylisourea, 2,6-dibromo-4-(4-morpholinylthiocarbonyl) phenyl ester, N,N-[Dimethyl]-N'-methyl-N'-[2-[2-naphthalenyl]-6-trichloromethylpyrimidin-4-ylethylenediamine, Bufa-4,20,22-trienolide, 6-(acetyloxy)-3-(á-D-glucopyranosyloxy)-8,14-dihydroxyl, 1,2,4-Triazino[5,6-E][1,2,4]-triazine-3,6-dione, Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl and Diacetyl sulphide are new to propolis. Isopropyl Alcohol present in samples B[KA] and K[HA], 1,2,4,5-Tetroxane, 3,3,6,6-tetramethyl is present in samples B[KA] and C[TN], Methyltartronic acid has been present in samples C[TN] and D[UK] and (3-Methyl-oxiran-2-yl)-methanol has been characterized in samples K[HA] and D[UK]. In the present study, 3 cancer cell lines (MCF 7, HCT 116, and PC 3) were treated with 4 propolis extracts at various concentrations ranged from 5-320µg/mL for 24 hours in vitro. The cell viability was evaluated by colorimetric MTT assay, which depends on the reduction of MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by mitochondria of living cells to form blue formazan product[20] According to the result, the cell line MCF 7 inhibition was observed maximum in B [KA] and K [HA] with IC_{50} value 26.88µg/mL and 43.47µg/mL compared to C [TN] and D [UK], with IC_{50} values were 66.53µg/mL and 104µg/mL respectively. The similar result has been observed in cell line HCT116, where the highest induced cytotoxicity in B[KA] and K[HA] with IC_{50} value 75.33µg/mL and 90.47µg/mL whereas C[TN] and D[UK] resulted the IC_{50} value of 93.72µg/mL and 101.40µg/mL. In cell line PC3, B [KA] exhibited pronounced cytotoxic effect (41.80µg/mL) while IC50 of propolis from C [TN], K [HA] and D [UK] were 63.86µg/mL, 65.50µg/mL and 134.5µg/mL respectively (Table 1). The hemolytic activity of crude propolis extracts was performed by spectroscopic method at different concentrations (5, 10, 20, 40, 80,160 and 320µg/mL) using fresh human blood sample. Based on the result, all the studied samples showed very less toxic effect on human RBCs (< 10% hemolysis) at different dilutions (Figure 5). The tested propolis can be considered as safe to human erythrocytes. Hemolytic activity is a requirement to be tested for any blood contacting medical device where the interactions between blood and biomaterials may induce lysis of erythrocytes, particularly during prolonged contact or during contact of blood with large surfaces. Since many cancer drugs used presently may cause adverse side effects by being cytotoxic to normal cells, hence it is necessary to find new compounds that will not cause any adverse side effects of being cytotoxic to normal cells.

Figure 1
GC- MS Chromatogram of K [HA]

Figure 2
GC- MS Chromatogram of D [UK]

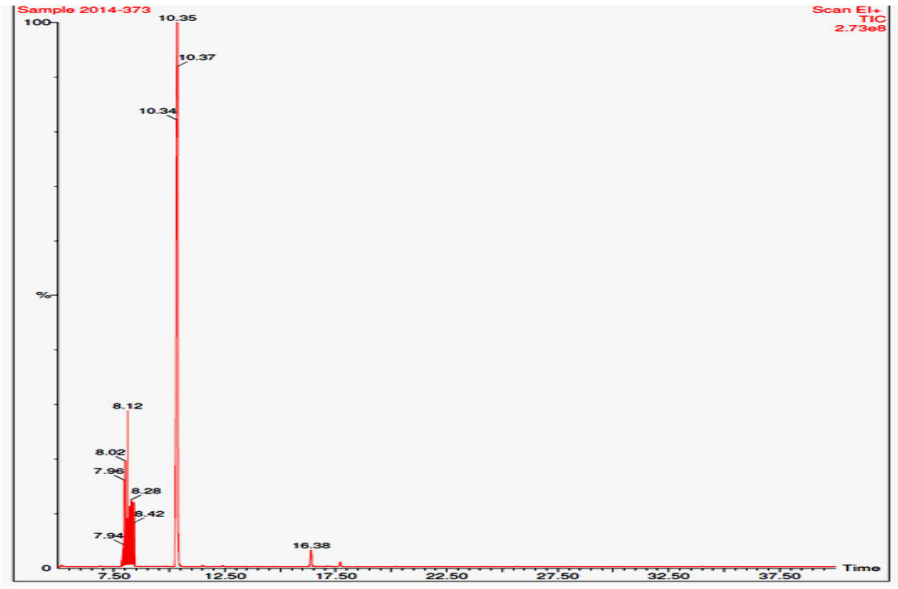


Figure 3
GC- MS Chromatogram of C [TN]

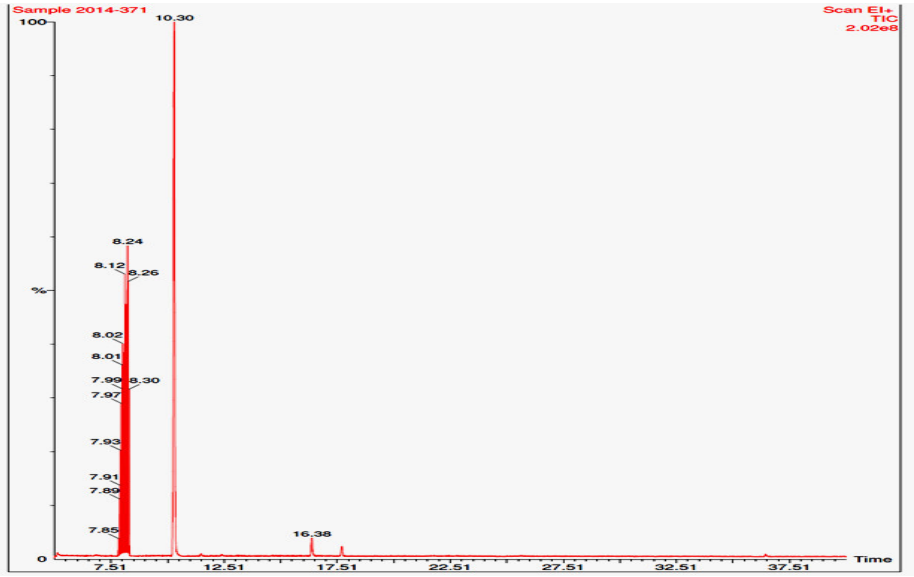


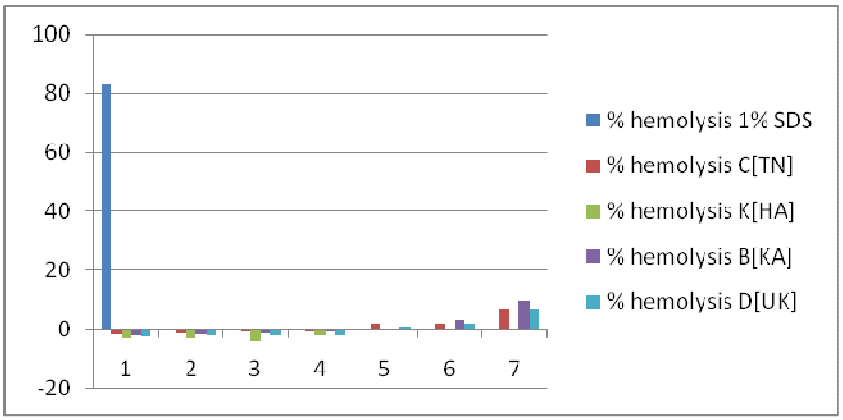
Figure 4
GC- MS Chromatogram of B [KA]

Table 1
Percentage of cytotoxicity and IC_{50} value of Indian propolis extracts on MCF 7, HCT116 and PC 3 cell lines

Samples	Conc. µg/mL	% Inhibition MCF 7	% Inhibition HCT 116	% Inhibition PC 3
Control	1% DMSO	0.00	0.00	0.00
C[TN]	5	1.07	0.53	2.40
	10	4.28	3.83	8.65
	20	15.10	12.66	18.98
	40	30.49	22.16	34.94
	80	45.29	36.15	62.90
	160	60.98	59.96	74.51
	320	72.89	65.96	90.04
IC_{50}		**66.53µg/mL**	**93.72µg/mL**	**93.86µg/mL**
K[HA]	5	1.70	1.98	3.47
	10	8.23	4.49	8.07
	20	17.94	19.39	24.24
	40	39.54	26.39	38.61
	80	51.98	44.66	54.55
	160	67.39	57.19	66.12
	320	74.48	75.99	88.23
IC_{50}		**43.47µg/mL**	**90.47µg/mL**	**90.47µg/mL**
B[KA]	5	9.56	5.94	2.90
	10	17.77	13.03	7.76
	20	36.04	19.66	16.63
	40	51.90	34.56	45.29
	80	67.29	51.32	61.65
	160	73.37	66.09	77.52
	320	85.10	79.82	82.22
IC_{50}		**26.88µg/mL**	**75.33µg/mL**	**41.80µg/mL**
D[UK]	5	2.51	2.90	0.36
	10	7.90	8.44	6.38
	20	14.22	16.62	12.32
	40	25.47	28.36	22.09
	80	46.61	39.58	48.39
	160	61.17	63.59	63.67
	320	78.49	74.08	87.13
IC_{50}		**104µg/mL**	**101.40µg/mL**	**134.50µg/mL**

Note: C [TN]- Coimbatore [Tamil Nadu], K[HA]-Karnal [Haryana], B[KA]- Bijapur [Karnataka], D[UK]-Dehradun [Uttarkand]

Figure 5
Percentage of hemolytic activity from the different concentrations (5, 10,20,40,80,160 &320µg/mL) of propolis extracts on Human RBCs



According to MTT cytotoxic assay, all propolis samples showed cytotoxicity against the examined cell lines, but the percent of inhibition varied depending on propolis origin. It was noted that propolis collected from Bijapur show the highest cytotoxic activity against HCF 7, HCT 116 and PC 3, but propolis collected from Coimbatore and Karnal had moderate activity against all tested cell lines. Propolis collected from Dehradun exhibited lower cytotoxicity when compared with propolis collected from other regions. The variation in cytotoxic activity seems to be due to the differences in the chemical composition of different propolis samples. The higher cytotoxicity was demonstrated by Bijapur propolis, probably attributed to the presence of some compounds such as o-Methylisourea hydrogen sulfate, 1-Nitro-á-d-arabinofuranose, tetraacetate, Tetraacetyl-d-xylonic nitrile, 14-Oxa-1,11-diazatetracyclo[7.4.1.0(2,7).0(10,12)]tetradeca-2,4,6-triene, 11-acetyl-6,9-bis(acetyloxy)-4-formyl-8-[(aminocarbonyloxy)methyl], O-Methylisourea, 2,6-dibromo-4-(4-morpholinylthiocarbonyl) phenyl ester, N,N-[Dimethyl]-N'-methyl-N'-[2-[2-naphthalenyl]-6-trichloromethylpyrimidin-4-ylethylenediamine, Bufa-4,20,22-trienolide, 6-(acetyloxy)-3-(á-D-glucopyranosyloxy)-8,14-dihydroxyl, 1,2,4-Triazino[5,6-E][1,2,4]-triazine-3,6-dione and Pregan-20-one, 2-hydroxy-5,6-epoxy-15-methyl as per our investigation by GC-MS. The chemical composition of these propolis samples indicated that they are a potential source of naturally occurring bioactive compounds with biological and pharmacological applications. Several authors have studied chemical content of some Indian propolis [21-23]. but still there is a need to study the detailed chemical constituents of propolis from different ecological zones of India. Kartal et al., (2003)[24] has provided comprehensive report on the use of propolis to treat several human diseases. The chemical composition and their bioactivity vary depending on geographical and botanical origin [25-27]. The ethanolic extract of Brazilian propolis showed potent cytotoxicity against prostate cancer cell line[28]. The ethyl acetate extract of Indonesian propolis induced sufficiently strong cytotoxicity on MCF 7 with IC_{50} 47.45µg/mL, similarly the ethanolic extract of *Trigona* spp. from Indonesia induced 81.43% of cytotoxicity on human breast cancer cell lines [29-30] which support the present result. The significant anticancerous activity of propolis is due to the presence of phenolic compounds and its ability to induce cytotoxicity at low concentration against carcinoma cell lines besides safeguarding normal cells even at high concentration [31].

CONCLUSION

The present findings suggest that the ethanolic extract of propolis indicated it is the most effective and promising inhibition of human breast cancer, colon cancer, and prostate cancer cell lines. These effects are attributed to the chemical composition of propolis, which is highly dependent on the

geographical location of the flora. The GC-MS analysis of these 4 samples showed the presence of 44 compounds which was identified by comparing of their mass spectra with the NIST library data and literature. The identification of chemical compounds present in propolis is extremely valuable and promising with respect to standardization and practical applications in therapy. The present study opens up a new perspective for future research in cancer science through in- vivo evaluation of Indian Propolis.

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