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Organochlorine Residues in Bees and Hive Products in Karnataka

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Abstract: ***In the present study, Aldrin, BHC, DDT and Endosulfan were simultaneously determined in honey, pollen, wax and bees by using liquid-liquid extraction followed by clean-up and determination by GC/ECD and GC/MS techniques. In the present investigation 67 samples were collected throughout the state of Karnataka, India and analysed of which only 18 samples were contaminated with OCPs such as Aldrin, BHC and Endosulfan. Aldrin was detected only in the honey samples whereas BHC and endosulfan were found in all the matrices with highest recorded value of 3.8mg/kg in wax and 2.78mg/kg in brood samples respectively. Thus results clearly indicated that, there is no significant pesticide residues in honey samples from Karnataka.***

Keywords: Honey, pollen, wax, organochlorine, pesticide contamination

1. Introduction

Organochlorine pesticides (OCPs) are organic compounds hazardous to human health, animal life and non-target insects like the honeybees. In the past they have brought great benefits by increasing crop yields and controlling harmful diseases [1]. However, on account of their extreme stability, long persistence and probable indiscriminate use in the past, have found an entry into the food chain and ultimately into the human system [2]. Most organochlorine pesticides including DDT, Aldrin and BHC are considered as persistent organic pollutants (POPs) [3] and may affect endocrine, reproductive and immune systems [4]. Thus, once considered wonder chemicals for agriculture the OCPs are restricted /banned in several countries worldwide including North America and Europe [5]. Previously the annual consumption of DDT and BHC in India was about 17 and 42 million kilograms respectively and together both formed more than 70% of total insecticide usage [6], [7]. However, DDT and BHC have been restricted or banned in India for use in agriculture since 1987-88 [8].

The environmental contamination by organochlorine pesticides has been widely documented in several countries such as USA [9], United Kingdom [10], Spain [11], France [12], Italy [13], Belgium [14], Greece [15], Egypt [16], Poland [17], Australia [18] and India [8], [19]-[22]. Literature reveals the existence of OCPs in foodstuffs [23] including honey [24] and other hive products such as pollen [25]-[27] and wax [28]. Bee products are natural food products rich in minerals, vitamins, antioxidants and simple sugars contributing to health protection of people for thousands of years. Organochlorine pesticides can be present in honey because of the crop treatment or migration from pollen/ or nectar to bee hives. However, the persistence of OCPs residues in flowers, plants, soil and water previously treated with OCPs constitutes a form of environmental contamination, thus bees might encounter these chemicals and carry them back to their hives [29], [30]. Since honeybees travel long distances and come close to many flowering plants, honey and other hive products may be an easily accessible environmental pollution indicator [31]-[33]. Pesticide determination in bee products is necessary to monitor contamination and guarantee consumer health. Honey is a natural product that must be free of any chemical contaminants and safe for human consumption, hence its quality must be proved [15], [34].

Apicultural matrix analysis such as honey, wax, pollen and bees themselves can provide useful indications of the diffusion of pesticides within the environment. Many organocholorines such as DDT, BHC and Aldrin are no longer used in agriculture, but are still present in the environment. Since honey is a product for human consumption, its contamination by OCPs has been studied in many countries such as Poland [35], Turkey [36], Jordan [37], Italy [38], France [26], [27], USA [39], Romania [40], Columbia [41] and India [42], [43].

The determination of low concentration of OCPs in apicultural matrices requires the application of effective extractions and sample purification techniques, followed by a final chromatographic determination. A multiresidue method for the determination of organochlorine residues in honey was developed and performed by capillary gas chromatography (GC) with electron capture detector (ECD) [15], the simple and traditional liquid-liquid partitioning followed by clean-up and analysis by GC [8] and the recent methods used to detect pesticide residue in honey[44]. Gas chromatography in combination with ECD has become a routine technique in the analysis of organochlorine pesticides. However, the high sensitivity of this detector contrasts with its lack of identification power. Hence an additional confirmation technique is necessary and detection by mass spectrometry (MS) is frequently used because of its identification capacity. This ensures reliable results, in order to confirm the presence/lack of compounds in the analysis of biological samples. It was also concluded that both GLC and HPLC methods were effective in assay, however, GC methods with MS were the most promising.

In the present study, aldrin, BHC, DDT and endosulfan were simultaneously determined in honey, pollen, wax and bees by using liquid-liquid extraction followed by clean-up and determination by GC/ECD and GC/MS techniques. Total of 67 samples throughout the state of Karnataka, India were collected and analysed for the selected pesticides.

2. Materials and Methods

2.1 Study Area

The State of Karnataka lies towards the south of peninsular India, 18° 30´ North latitude, 74° East and 78° 30´ East longitude, extending 750 km from north to south and about 400 km from east to west having an area of 191,791 km^2. Agri-horticultural profile of the state includes oil seeds, sugarcane, jowar, maize, cotton, coconut, mango cashewnut, ragi, red chilli, potato, paddy, vegetables including cabbage, cauliflower, carrot, beet root, beans, brinjal, lady's finger and others. Extensive agriculture, indiscriminate spraying of various pesticides and ignorance of the farmers about importance of pollinators have caused pesticide contamination of the honeybees and hive products (honey, pollen and wax) obtained directly from nature and known to be pristine in origin. The location map of the study area is shown in Fig 1.

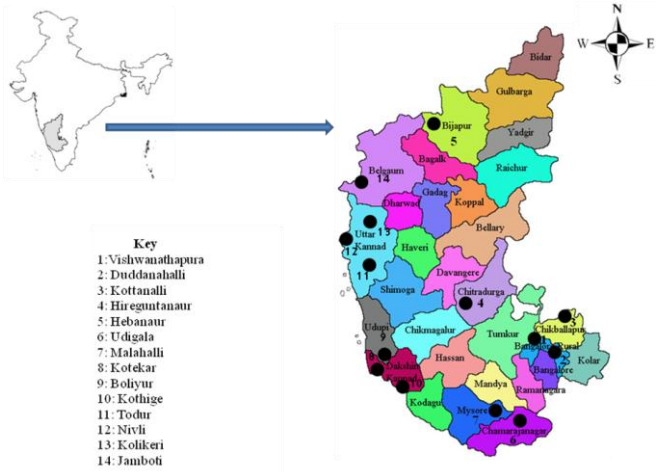

Figure 1: Map showing the study area including the 14 locations where pesticides were detected

2.2. Collection of Samples

A total of 67 samples of honeybees and hive products like honey, pollen, wax and brood samples were collected directly from the bee hives of Indian honeybee, *Apis cerana* and wild bee, *Apis dorsata* from Karnataka, India (Fig.1). The collected honey samples were stored in food grade plastic bottles (Duropet 1) under room temperature whereas the wax, pollen and brood samples were stored in vials at -4°C.

2.3. Research Method

Standards of the pesticides were obtained from RANKEM, with purity of 99% (technical grade). Stock standard solutions (100µg/ml) was prepared by exact weighing and dissolving them in n-hexane. Working standards were prepared by appropriate dilutions and stored in a refrigerator at 4°C. HPLC (analytical) grade solvents: n-hexane, methylene chloride, acetone , diethyl ether were supplied by Spectrochem (Bangalore) and other chemicals and reagents like sodium chloride, sodium sulphate, ammonium chloride, Orthophosphoric acid was obtained from SD Fine chemicals (Bangalore, India).

Five grams of honey samples were blended with 50 ml of 35% acetone for 2 minutes and filtered with whatmann filter paper (No.1). The sample was extracted thrice with 30 ml methylene chloride and using liquid-liquid partitioning. The organic phase was pooled together and finally concentrated using rotary evaporator at 40°C. Column for clean up was prepared using a glass column 15mm diameter and 30 cm height. The tip of the column was plugged with a piece of cotton. About 1cm of anhydrous sodium sulphate was then placed in the column followed by the mixture of silica gel and activated carbon.10g of silica gel and 500mg activated charcoal was grounded well in a mortar and pestle and homogenised. The adsorbent material was then packed properly to a height of 10 cm followed by 1 cm sodium sulphate.

The column was pre-wetted with 20ml of the eluting solvent mixture [methylene chloride and n-hexane (1:1)]. The concentrated sample extract was then loaded to the column followed by elution of the column with 100 ml of the eluant (methylene chloride + n-hexane, 1:1). The elutes were then evaporated to dryness in rotary evaporator at 40 °C and redissolved in 1ml n-hexane. This is the final extract ready for injection in GC/GCMS.

Three grams of honeybees/brood samples were homogenized with 20 ml acetone using mortar and pestle. The mixture was filtered and washed with 20 ml acetone. 100 ml of 1% ammonium chloride and 2% orthophosphoric acid were added and allowed to stand for 30 minutes. After filtration the sample was diluted to 200 ml with 2% sodium chloride and extracted twice with 30 ml methylene chloride. The combined organic phase was passed through a filter containing anhydrous sodium sulphate and evaporated to dryness in a rotary evaporator at 40 °C. The residue was redissolved in 2 ml of the solvent mixture of diethyl ether and petroleum ether (1:1) [45]. Florisil column was prepared using a 15mm diameter glass column packed with 10 cm of activated florisil between 1cm of anhydrous sodium sulphate. The column was pre-wetted with 20 ml of the eluant diethyl ether and petroleum ether (1:1) followed by loading of the extracts of honey, honeybees and brood respectively and their elution with 50 ml each of 6%, 15% and 50% diethyl ether in petroleum ether. The eluates were collected and evaporated to dryness in rotary evaporator at 40°C, further the residue was re-dissolved in 1 ml n-hexane for injection in GC/GCMS.

Two grams of beewax was dissolved completely in 40 ml of hexane and immediately centrifuged for 15 minutes at 4500 rpm. The supernatant fraction was collected and concentrated followed by addition of 6 ml of hexane and 20 mL of hexane and acetonitrile (1: 9). The solution was extracted twice using liquid-liquid partitioning and the acetonitrile phase was collected, pooled and concentrated using rotary evaporator [28] followed by column clean-up. The column was prepared as mentioned above and pre-conditioned by 20 ml of successive elutions of 30% and 50% methylene chloride in n-hexane. The extract was then loaded to the column and eluted with 50 ml each of 30% and 50% methylene chloride in n-hexane. The solution was dried in a rotary evaporator and the residue was dissolved in 1 ml n-hexane and analysed.

One gram of pollen sample was centrifuged for 4 minutes with 50 ml acetone, petroleum ether and dichloromethane in a ratio of 1:2:2. The liquid phase was decanted, evaporated to approximately 2 ml. and dried. The residue was dissolved in 5 ml of n-hexane and methylene chloride mixture in a ratio of 1:1 and cleaned on a silica gel (60-120 mesh) and activated carbon column as mentioned above. After evaporation, 1 ml of the mixture of n-hexane and analysed [25].

The analysis was carried out on Varian auto sampler CP-8410 with GC – 3800 and Varian MS/MS 4000. The oven temperature was as follows: initial temperature of 80°C, held for 1 minute, increased to 180°C at 30°C/minute, held for 5 minutes, then increased to 305°C at 5°C/minute and held for 1 minute. The injector and detector temperatures were 180°C and 305°C respectively. The total run time was 35:20 minutes with a flow rate of 5 µl/s and injection volume was 2 µl injected in splitless mode.

3. Result and Discussion

In the present investigation 67 samples were collected from the study area where 18 samples were found contaminated with organochlorine pesticides. Samples from 14 locations exhibited contamination (Table 1). The pesticides detected were aldrin (Fig 2.), BHC (Fig 3.) and endosulfan (Fig 4.).

The analysis revealed that, Aldrin was found only in two honey samples from Uttar Kannad and Belgaum districts of Karnataka with concentration 0.027mg/kg and 0.025mg/kg respectively. The values were comparatively lower than the ones reported by [20] in the honey samples from Haryana, India with maximum concentration up to 0.10 mg/g. Also in a study carried out on 101 Spanish honeys by [46] in which Aldrin was detected in 36 samples amounts up to 150 µg/kg. and in 6 honey and 2 pollen samples from Turkey with maximum concentration up to 43.05 ppb and 18.68 ppb respectively [47]. Aldrin was also detected in the honey samples of Aragon, Spain with mean concentration of 0.57µg/kg [11]. Aldrin was also reported in 38 Polish honey samples with concentrations up to 14.27µg/kg [35]. Similar study was carried out by [48], where 109 honey samples were collected from Konya, Turkey and analysed for OCPs and Aldrin was reported to contaminate all the 109 honey samples with concentration up to 0.0401µg/g with only one sample exceeding the limit of Turkish alimentarius Codex for honey rescript (2005/49). Aldrin is an insecticide, a nerve poison and seems to attack ganglia region. It is highly dangerous chemical and a persistent OCP totally undesirable in any food stuff. Aldrin is banned for manufacture, import and use in India [49].

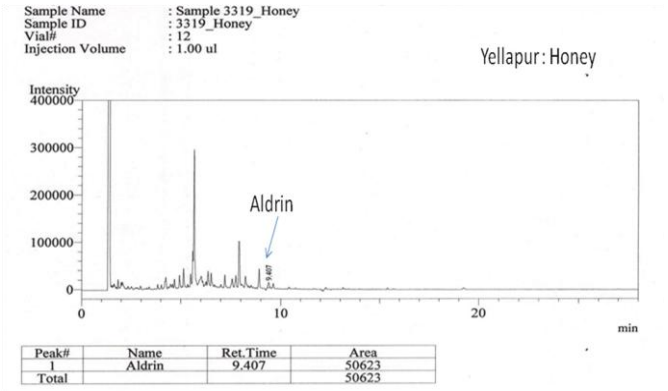

Figure 2: Chromatogram showing the detection of aldrin in honey sample from Yellapur.

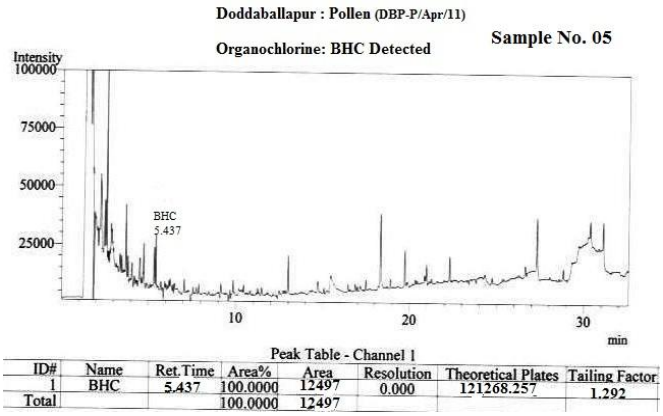

Figure 3: Chromatogram showing the detection of BHC in a pollen sample from Doddaballapur.

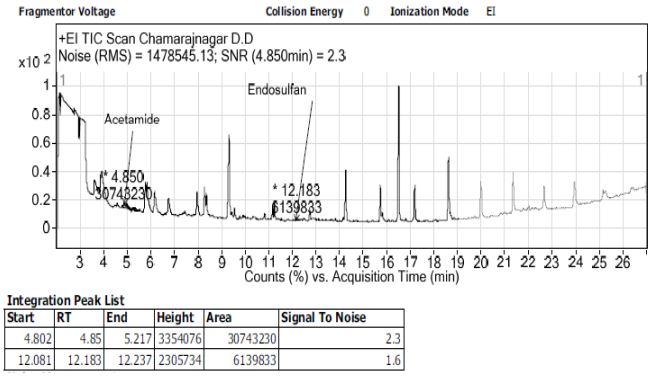

Figure 4: Chromatogram showing the detection of Endosulfan in honey sample from Chamrajnagara.

In the present investigation BHC was detected in nine samples which includes one honey, three wax, four pollen and one brood sample. Concentration of BHC ranged from 0.51mg/kg in honey from Puttur to 3.8mg/kg in wax from Bangalore. In India [42], reported BHC in the honey samples from Punjab, with a maximum concentration of 101 ppb in one of the commercial samples of honey. BHC in 14 Valencian honey samples with an average c oncentration of 0.017mg/kg and that of the Portugal ranged up to 0.39 mg/kg [24] was reported. BHC in the honey samples of Aragon, Spain with maximum concentration of

Table 1: Showing the details of 18 samples contaminated with organochlorine pesticide residues from Karnataka, India

Sl. No.	Place (Village name)	Taluk	District	Date of Collection	Sample Type	Species Sampled	Source of Sample	Pesticides Detected (mg/kg)
1	Vishwanathpur	Doddaballapur	Bangalore	10-09-2010	Brood	*A. cerana*	Bee box	BHC (1.89)

2	Duddanahalli	Doddaballapur	Bangalore	10-09-2010	Pollen	*A. cerana*	Bee box	BHC (0.7)
3	Duddanahalli	Doddaballapur	Bangalore	10-09-2010	Wax	*A. cerana*	Bee box	BHC (3.7)
4	Hireguntanaur	Chitradurga	Chitradurga	17-10-2010	Pollen	*A. dorsata*	Tree Hive	BHC (1.7)
5	Hireguntanaur	Chitradurga	Chitradurga	17-10-2010	Wax	*A. dorsata*	Tree Hive	BHC (3.8)
6	Hebanur	Bijapur	Bijapur	23-08-2010	Wax	*A. cerana*	Bee box	BHC (2.9)
7	Nivli	Karvar	Uttar Kannad	11-10-2010	Honey	*A. cerana*	Tree Hive	Endosulfan (0.52)
8	Nivli	Karvar	Uttar Kannad	11-10-2010	Brood	*A. cerana*	Tree Hive	Endosulfan (2.78)
9	Todur	Karvar	Uttar Kannad	11-10-2010	Pollen	*A. cerana*	Tree Hive	Endosulfan (1.67)
10	Todur	Karvar	Uttar Kannad	11-10-2010	Wax	*A. cerana*	Tree Hive	Endosulfan (0.91)
11	Kotekar	Mangalore	Dakshin Kannad	03-04-2011	Honey	*A. cerana*	Bee box	Endosulfan (1.67)
12	Boliyur	Mangalore	Dakshin Kannad	03-04-2011	Wax	*A. cerana*	Bee box	Endosulfan (1.13)
13	Malahalli	Mysore	Mysore	23-05-2012	Pollen	*A. cerana*	Bee box	BHC (0.966)
14	Kottapalli	Chintamani	Kolar	16-08-2012	Pollen	*A. cerana*	Bee box	BHC (2.601)
15	Kothige	Puttur	Dakshin Kannad	19-05-2013	Honey	*A. cerana*	Bee box	BHC (0.51)
16	Kolikeri	Yellapur	Uttar Kannad	07-01-2013	Honey	*A. dorsata*	Bee box	Aldrin (0.027)
17	Jamboti	Khanapur	Belgaum	29-03-2013	Honey	*A. cerana*	Bee box	Aldrin (0.025)
18	Udigala	Chamrajnagar	Chamrajnagar	24-03-2014	Honey	*A. dorsata*	Tree Hive	Endosulfan (0.045)

5.97µg/kg [11] and from Konya, Turkey [48] where 92 honey samples were found to be contaminated with BHC with concentration upto 0.0443µg/g with 29 samples exceeding the limit of Turkish alimentarius Codex for honey rescript (2005/49). BHC is cheap, reliable and broad spectrum insecticide and was the first one to be introduced in the country for use in agriculture [19]. It is stable, highly fat-soluble and having low water solubility, it tends to stay in the environment hence, is an ideal environmental pollutant. Accumulation of toxic BHC ranging from 0.006 to 0.130 ppm in human blood serum samples was reported by [21]. Presently BHC is banned for manufacture, import or formulate in India. However, it was allowed for use up to March 2013 for termite control in buildings and for wood, also for termite control in agriculture as per approved label claims by the Registration Committee [49]. Thus contamination of the samples in the present study may be explained, as BHC is quite stable and persistent might have entered the hive due to termite treatment of the wood (bee box) which accumulated in the wax and gradually got transferred to stored pollen contaminating the emerging brood and the pristine hive product 'the honey'. The contamination level of the hive products with BHC are as follows: wax > brood > pollen > honey.

Endosulfan was the third OCP detected in seven of the samples of which were three honey, two wax, one pollen and one brood with concentration ranging from 0.045mg/kg in honey to 2.78mg/kg in brood sample from Karwar, Karnataka. In India endosulfan was reported in honey samples from Haryana with a maximum concentration upto 0.20mg/kg [20]. The residues of endosulfan with a maximum concentration upto 340.0 µg/kg in 5 pollen load samples from France [26]. Endosulfan was reported in 11 beewax samples from France with an average value of 88.8 µg/kg and a maximum concentration upto 243.1 µg/kg [28]. Also another study revealed that, endosulfan with average concentrations of 45.8 µg/kg in pollen, 51.0 µg/kg in beewax, 8.3 µg/kg in honeybees whereas, the values were < LOD for honey [27]. Endosulfan was also detected in wax, pollen and bee samples with maximum concentration upto 132 ppb, 157 ppb and 9ppb respectively in North American apiaries [39].

Endosulfan was reported in 108 honey samples from Konya, Turkey [48] with mean concentration of 0.0016 µg/g. In a study conducted by [50], two of the 31 collected honey samples in Uruguay, endosulfan was detected but the values were below the limit of detection (< LOD). Endosulfan (35% EC) is an insecticide used for control of ahpids and insect control against vegetable crops. Currently endosufan is banned for production, use and sale all over India since May 2011 [49]. Endosulfan is subjected to long range atmospheric transport from where it is used hence contamination is frequently found in the environment at considerable distances from the point of its original applications [22]. Endosulfan has been shown to have a genotoxic effect on human liver carcinoma cells, hepatocytederived transformants and abnormalities in germ cells [51].

The total area of cultivation in the State is 105.45 lakh hectares (ha) up to 2000, Out of which, cereals and millets occupy 53.94 lakh ha, oil seeds 25.30 lakh ha (24.25 %), sugar cane 3.08 lakh ha, Cotton 6.06 lakh ha, tobacco 0.67 lakh ha. The area under different horticultural crops includes spices and condiments (2,51,135 ha), fruit crops (1,32,724 ha), vegetables (179,984 ha), plantation crops (653,675 ha) and flower crops (5,412 ha). Numerous pesticides, combination of pesticides and formulations are used throughout the State for control of various diseases and pests like insects, thrips and aphids. As per the manufacturers association of Karnataka, the average sales of

Table 2: Showing the Maximun, minimum and average concentration of the OCPs detected

Pesticides	Pesticide Group	Purpose of Use	LOD (µg/kg)	LOQ (µg/kg)	Min. Conc. (mg/kg)	Max. Conc. (mg/kg)	Average Conc. (mg/kg)
Endosulfan	Organochlorine	I, A	0.07	0.5-1.0	0.045	2.78	1.24
BHC	Organochlorine	I	0.003	0.5-1.0	0.7	3.8	2.17
DDT	Organochlorine	I	0.003	0.5-1.0	-	-	
Aldrin	Organochlorine	I	0.07	0.5-1.0	0.025	0.027	0.026

Insecticide [11321 metric tonnes (MT)] is highest followed by Fungicide (2424 MT) and then Herbicide (683 MT) up to 2002. The use of insecticides is more as India is a tropical country and attack by pests are common and as a consequence it is the non-target honeybees that are affected the most. The study area is marked by intensive agri-horticultural practices, with the major crops being grapes, mango, banana, sapota, guava, coconut, cashew, tamarind, jackfruit, jamun, tomato, potato, brinjal, lady's finger, cabbage, cauliflower, carrot, beans, beet root, groundnut, ragi, maize, paddy, sunflower and cotton. Some of the common pesticides recommended and used in these regions are dimethoate (Roger), methomyl (Lannate), metalaxyl (Ridomil), spinosad (Success), copper oxychloride, cypermethrin (Cymbush), and lamda cyhalothrin (Karate), monocrotophos, fenvalerate, chlorpuriphos, ecalaux, dicofal, hostalin and quinolphos.

The present study was conducted to assess the persistant OCP contamination in different ecosystems by using bees and bee products as indicators. The analysis revealed that, Aldrin was detected only in the honey samples whereas BHC was recorded value of 3.8mg/kg in wax and 2.78mg/kg in brood samples respectively (Table 2.). This reflects the degree of endosulfan found in all the matrices with highest accumulation in the hive products and depicts hive condition. The pollen samples were also contaminated with BHC (2.601mg/kg) and endosulfan (1.67mg/kg). Inspite of its natural origin, pollen was contaminated with pesticides due to the extensive and intensive application of pesticides in agriculture. The analysis of OCP residues in pollen is necessary in order to protect consumer health and guarantee the quality of bee pollen. High residue values of BHC were exhibited in a wax sample from Chitradurga (3.8 mg/kg) and Doddaballapur (3.7 mg/kg), Bangalore. Contamination of beewax is undesirable as it is an important and valuable by-product of the beekeeping industry with an extremely wide spectrum of useful applications and occupies a very special position among waxes [52].

Brood sample from Karwar, Karnataka exhibited maximum endosulfan (2.78 mg/kg). Pesticide exposure in broods might result in increased brood mortality and may contribute to reduced honeybee population in the colony. The contamination of the hive products are as follows: wax > brood > pollen > honey (Fig 5.). whereas honeybees were not found contaminated with any of the OCPs.

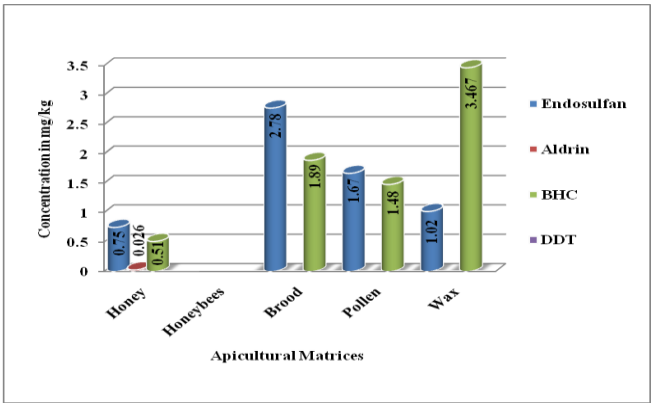

Figure 5: Concentration of OCPs in bees and bee products in Karnataka.

It was also observed that samples collected from both honeybee species exhibited pesticide residues. Samples from *A. cerana* colonies (bee box) were more frequently contaminated than samples from the *A. dorsata* colonies (tree hives) and the frequency of contamination was 20.89% and 5.97% respectively.

4. Conclusion

The pesticide analysis in honey and other hive products revealed that residues of organochlorine insecticides that are banned long back still existed in the honey samples of Karnataka state. In honey samples the organochlorine residues were Aldrin, BHC and endosulfan. The differential content of these pesticides revealed the possibility that bees and bee products are the bio-monitors of pesticide contamination in the environment. Thus, honeybees are biological indicators, picking up chemicals and other pollutants from their environment both external and internal to their lives [54]. Although it is hard to conclude with the limited data, the results from the present investigation clearly indicated contamination honey and other hive products with the persistant OCPs from Karnataka state. Although honey samples were not significantly contaminated when compared to other hive products. Hence honey from Karnataka is safe for consumption. Wax being lipophilic by nature tends to accumulate these OCPs for a longer duration thereby contaminating the colony. Beekeeping products in general have low residual contamination that affects less the consumer's health but the ones do suffer from pesticide poisoning are the honeybees.

5. Acknowledgements

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