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Cannabis as homeopathic medicine in extreme dilutions: Thermal analysis for their differentiation and action on a protein

Priya Mondal[1], Nirmal Chandra Sukul[1, 2]*, Atheni Konar[1], Tandra Sarkar[1], Md Amir Sohel[3], Asmita Sengupta[3], Indrani Chakraborty[4] & Anirban Sukul[1]

[1]Sukul Institute of Homeopathic Research, Santiniketan, West Bengal- 731 235, India

[2]Department of Zoology; [3]Department of Physics, Visva-Bharati University, Santiniketan, West Bengal- 731 235, India

[4]Department of Zoology, Jogamaya Debi College, Kolkata, West Bengal- 700 026, India

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Cannabis indica and *C. sativa* have been used in homeopathy in extreme dilutions, called potencies, for therapeutic purposes since 1841. The purpose of the present study is to see whether *Cannabis* dilutions have specific levels of free water molecules which characterize other homeopathic potencies. The second objective is to see whether *Cannabis* mother tincture (MT) and potencies act on the binding sites of a protein. The three potencies 8, 14 and 32 cH were prepared from *Cannabis* mother tincture (MT) by successive dilution followed by succussion in 8, 14 and 32 steps, respectively. The 3 potencies of diluent medium 90% EtOH were similarly prepared. Each potency was analysed by differential scanning calorimetry (DSC) to determine the free water level in it. The drug potencies and unpotentised EtOH were tested for their binding reaction with a protein human serum albumin (HSA) by isothermal calorimetry (ITC). MTs and the potencies differ from each other and also from water control and EtOH with respect to free water content as revealed by DSC. MTs, their potencies and EtOH bind to HSA showing difference in thermodynamic parameters in terms of stoichiometry, binding constant, change in enthalpy, entropy and Gibbs free energy. Potencies may initiate their individual effect through binding with a protein thereby leading to subsequent biochemical events inside the cell.

Keywords: Albumin, Binding, *Cannabis*, Differential scanning calorimetry (DSC), Extreme dilutions, Free water, Isothermal calorimetry (ITC)

Cannabis (Cannabinaceae) is a tall herb, native to central Asia and is cultivated in India and other parts of the world. Two different parts of the plant have been used in Indian subcontinent for recreational purposes. Dried leaves and flowering shoots of both male and female plants have been used as a drink mixed with milk, sugar, and other spices like cardamom, black pepper *etc* on festive occasions. This is called "Bhang" or "Siddhi". Dried, unfertilized inflorescences have been used for smoking. This form called "Ganja" is used as a strong narcotic. The plant yields oil from seeds. Crude resin is also smoked as a narcotic and is known as "Charos"[1,2]. However, *Cannabis* has also been used for treatment of neurological disorders. It is reported to improve multiple sclerosis, epilepsy, spasticity, schizophrenia, insomnia and glaucoma[3]. *Cannabis* contains many compounds of medicinal value including Δ^9-tetrahydrocannabinol (Δ^{9-}THC) and cannabidol (CBD). These have antioxidant and anticancer properties[4]. Two strains of this plant, namely *C. indica* and *C. sativa* show some differences in shape of leaves, height of plant, internodal length, bud size and density, flowering time and odour (Wikipedia retrived on 17.04.19). The plant products are also called "Marijuana". *Cannabis* has been mentioned in the oldest Sanskrit literature like the Rigveda (1700-1100 BCE), the Atharva veda (1500-1000 BCE) and Sushrata Sanhita (600 BCE)[5,2] (Wikipedia retrived 17.04.19). Sushruta, considered as father of Indian medicine and plastic surgery, used *Cannabis* as anesthesia before surgery[6].

Cannabis was introduced into homeopathy by Dr. Trinks in 1841[7]. In high doses it produces euphoria and hallucination. Both the varieties of this plant have been used as good medicine for different ailments in homeopathy[8]. Homeopathy uses extreme dilutions of drugs whereby the toxic effects of the crude drugs are eliminated. The dilutions, though devoid of original drug molecules, assume a new set of therapeutic properties[7,9]. Vibrational and Raman

*Correspondence:
E-mail: ncsukul@gmail.com

spectroscopy and thermogravimetry showed that homeopathic potencies differ from each other with respect to the amount of free water[10-13]. One objective of the present study is to determine free water content in *Cannabis* potencies. Ultra high dilutions (UHDs) of both the drugs, their mother tinctures (MT) and ethanol were analysed by differential scanning calorimetry (DSC) with a view to finding their difference from each other in terms of free water content. There is evidence that UHDs initiate their action on proteins[14-16]. HSA is a circulating protein. We, therefore, tested MTs, their UHDs and the unpotentized medium ethanol on HSA in order to find out their action on this protein by isothermal calorimetry (ITC). Since alcohol is itself a homeopathic drug[17], its potencies were not tested for their action on HSA.

Materials and Methods

Preparation of UHDs

Dried leaves with flowering tops of both *C. indica* and *C. sativa* were purchased from an authorized retail outlet under the control of the Excise Department, Government of India at Ghatal, West Midnapore, West Bengal. They were powdered and extracted separately with 90% ethanol in which the materials were kept for 7 days. The powder was mixed with 90% ethanol in the proportion of 1 g/mL EtOH. Optical density (OD) of each extract was measured in a UV-VIS spectrophotometer at a wave length of 550nm, and found to be 80 for *C. indica* and 75 for *C. sativa*. Each extract was designated as MT. Three UHDs like 8 cH, 14 cH and 32 cH were prepared from each MT by the standard process of successive dilution with the solvent 1:100 followed by mechanical agitation or succussion in 8, 14 and 32 steps, respectively. This UHD is called a potency[9,7]. Each MT was diluted with the solvent, 90% ethanol 1:100 (*v/v*) and poured into a hard glass vial in such a way that ¼ th of its space remained empty. The vial was corked and succussed by hand 10 times over a pad to prepare the first centesimal potency called 1 cH. This potency was further mixed with the solvent 1:100 (*v/v*) and succussed 10 times in a similar way to prepare the 2nd potency called 2cH. Subsequent potencies were prepared through successive dilution followed by succussion by the same process to prepare the 3 test potencies like 8cH, 14cH and 32cH. The three potencies of EtOH were prepared in a similar way.

Differential scanning calorimetry (DSC) of Drugs and medium used

The three potencies of EtOH, and also of *C. indica* and *C. sativa* were diluted with DD water 1:1000 just before DSC experiments. For ITC experiments only drug potencies and unpotentized EtOH were tested. The purpose was to reduce ethanol content in test potencies to a negligible amount of 0.09%. The MT was undiluted. Each diluted potency and undiluted MT were mixed with lactose in the proportion of 100 µL drug per 1 g lactose. Lactose $C_{12}H_{22}O_{11}$ is a disaccharide, formed of D-galactose and D-glucose. It occurs in α and β-forms. Lactose used here contained both the forms[18]. DSC measurements were conducted by using 200 F3 Maia model instrument with intra cooler 70 version (NETZSCH), Germany. DSC of each sample was measured from a starting temperature of 28°C down to −35°C at a scanning rate of 5 K $min^{-1}$and kept at −35°C in an isothermal condition for 5 min. Then the temperature was raised at 3 K min^{-1} upto 50°C. In this way melting point of each sample was recorded, and endothermic (melting) enthalpies were calculated. All DSC experiments were conducted in dry nitrogen atmosphere with constant pressure of 0.3 bar in order to prevent any oxidation of the samples. A vacant pan was measured first as a reference. Pure lactose, commonly used by homeopathic pharmacists, was obtained from SRL, Mumbai. Each sample was put into an aluminium sample pan of 5 mm diameter which was sealed by a sealing machine.

Determination of free water in lactose samples mixed with a fixed amount of undiluted MT and diluted *Cannabis* potencies and EtOH potencies by DSC

Endothermic enthalpies of each sample were calculated from the peak area of melting points. The mass of freezable water was calculated by the formula $W_C = Q/\Delta H$ (g) where, ΔH is the melting enthalpy of this type of water which is presumed to be same as bulk water (ΔH = 333.5 Jg^{-1}), and Q is the heat absorbed during melting process. Q is calculated from the endothermic peak[19]. It may be mentioned here that freezable water occurs in two states in hydrophilic polymer, here lactose. These are freezable bound water and free water. Besides that water also exists in non-freezable bound water which would not freeze even at −100°C. While free water melts at 0°C, freezable bound water does so below 0°C (Hitachi High Tech Sc Corporation, TA 14, Dec, 1983).

Binding reaction of *Cannabis* MTs, their potencies and EtOH on HSA

The instrument used for calorimetric measurements is ITC 200 GE Health Care Bioscience Ltd, Sweden. ITC experiment provides accurate data on binding interaction between HSA and ligands, here *Cannabis* MT and potencies, under optimal conditions in a single experiment[20]. The binding reaction causes release of heat (exothermic) or its absorption (endothermic). Drug solution (ligand) was injected at 2 µL/ injection every 2 min into a measurement cell containing 300 µL of 16 µM HSA solution in water (pH 6). The *Cannabis* MTs and potencies (8, 14, 32 cH) were in 90% ethanol which were diluted with DD water 1:1000 (pH 6) just before the experiments EtOH was also similarly diluted. Both the sample and the reference cell containing water only were maintained at a constant temperature of 25°C. Once the thermal equilibrium was established injections of drug solutions were started[18]. All injections and cell cleaning after each experiment were fully automated, controlled and operated through a software. Thermodynamic interactions involving HSA and drug solutions calculated according to standard equations provided values for linear refression, change in enthalpy (ΔH), entropy (ΔS) and Gibb's free energy and binding constant[21]. The built-in software in the ITC instrument analysed the data and gave the desired values.

Results

DSC: *Cannabis* and EtOH potencies

Results are presented both in figures and a table. Figure 1 shows melting points and melting enthalpies of *C. indica* MT, and its potencies like 8, 14 and 32. There are 2 peaks one around 0°C and another around −7°C. Melting enthalpies of *Cannabis* MT and potencies show distinct variation from each other. The 14th potency occupied the highest rank followed by 32, MT and 8. These enthalpies are related to free water which melt around 0°C. The smaller peak close to −7°C represents enthalpies of bound water molecules of the same samples. These enthalpies also show distinct difference from each other. Figure 2 shows melting points and enthalpies of *C. sativa* MT and potencies around 0°C. Here the MT occupied the highest point followed by the potencies 32, 8 and 14. The enthalpies show distinct variation from each other and can be ranked from the highest to the lowest as 32>MT>8>14. There is no second peak here. The enthalpies here represent only free water molecules. The results are also presented in (Table 1) which shows differences in free water content in the test samples and water control. In case of EtOH enthalpies 14th potency occupied the highest rank, followed by 32 and 8 (Fig. 3 & Table 1).

ITC experiments

Results are presented in (Fig. 4 & Table 2). Each figure has two panels A and B. Panel A shows heat

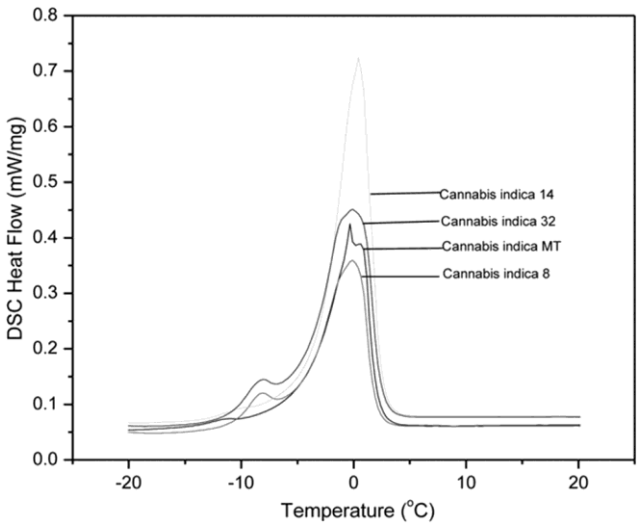

Fig. 1 — DSC curves showing two melting points of *Cannabis indica* mother tincture (MT) in 90% EtOH and three potencies 8, 14 and 32 cH in 90% EtOH diluted with water 1:1000 and mixed with lactose at 100 µL/g lactose. The samples were cooled from 28°C down to −35°C at 5 k min^{-1}, kept for 5 min and then heated to 50°C at 3 k min^{-1} in nitrogen

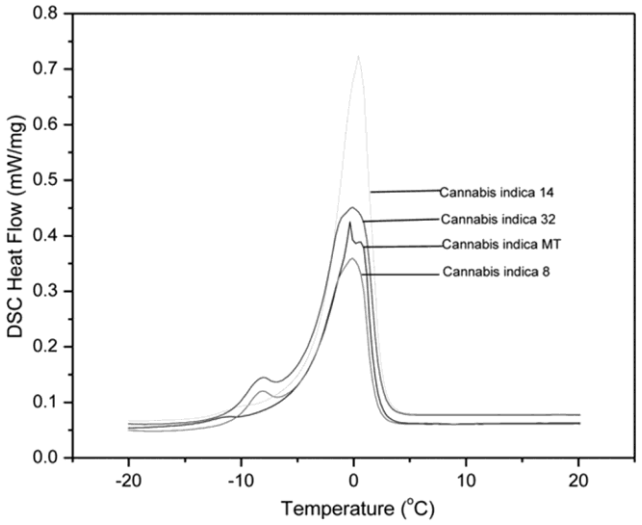

Fig. 2 — DSC curves showing single melting points of *Cannabis sativa* mother tincture (MT) in 90% EtOH and three potencies 8, 14 and 32 cH in 90% EtOH. The potencies were diluted with water 1:1000 and mixed with lactose at 100 µL/ g lactose. The samples were cooled from 28°C down to −35°C at 5 k min^{-1}, kept for 5 min and then heated to 50°C at 3 k min^{-1} in nitrogen

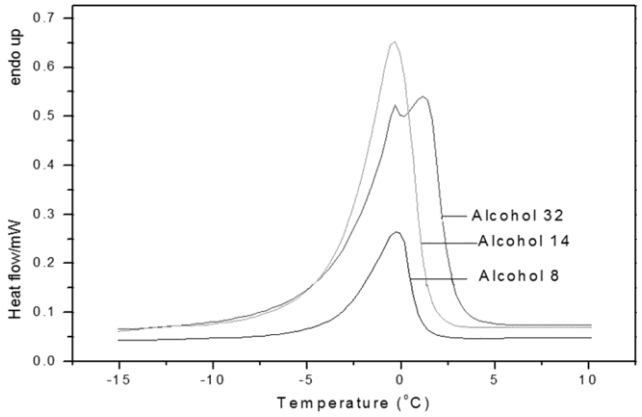

Fig. 3 — DSC curve showing melting points of three potencies 8cH, 14cH and 32cH of alcohol/ethanol and water mixed with lactose at 100 µL/g^{-1} lactose. The potencies were in aqueous ethanol having 0.03 molar ethanol. The samples were cooled from 28°C down to −35°C at the rate of 5 K min^{-1}

Table 1 — Melting temperature, enthalpy of melting and free water content in the mother tincture (MT) and 3 potencies of *Cannabis indica* and *Cannabis sativa*. The potencies in 90% EtOH were diluted with water 1:1000, mixed with lactose 100 µL/g lactose and analysed by differential scanning calorimetry (DSC). The samples were cooled from 28°C down to −35°C, kept for 5min and heated to 50°C

Sample	Temp. of melting	Enthalpy of melting	Free water (%)
Cannabis indica MT	−0.3	27.51	8.09
Cannabis sativa MT	0.0	33.3	9.79
Cannabis indica 8	−0.2	23.17	6.81
Cannabis sativa 8	−0.2	31.26	9.19
Cannabis indica 14	−0.4	47.92	14.09
Cannabis sativa 14	−1.3	15.68	4.61
Cannabis indica 32	0.0	44.55	13.10
Cannabis sativa 32	−1.2	37.27	10.96
Lactose +water	0.2	45.58	13.40
Ethanol 8	−0.3	12.54	3.67
Ethanol 14	−0.4	44.72	12.89
Ethanol 32	−0.4	41.07	11.88

rate (µcal/sec) versus time in min. Each peak represents an injection of ligand (drug) into a sample cell containing HSA. Panel B represents heat released per mole of ligand during interaction with HSA in relation to molar ratio (ligand/HSA) in the form of a nonlinear regression. Best fit parameters like reaction stoichiometry (N), binding constant (K), change in enthalpy (ΔH), change in entropy (ΔS) and number of binding sites for complex formation were recorded. Gibbs free energy change ΔG was calculated from ΔH, ΔS and T (absolute temperature in Kelvin). ΔG= ΔH- TΔS. All these parameters for each sample are given in (Table 2). The ITC data were analysed by a soft ware Origin 7.

Binding between HSA and *C. indica* MT shows endothermic reaction with a maximum heat change of 1 µcal/sec, 3 sequential binding sites and a tendency to gradual saturation (Fig. 4A & Table 2). Binding between HSA and *C. indica* potencies (8, 14, 32 cH) shows exothermic reaction, maximum heat change 0.2-4 µcal/sec, 3-6 sequential binding sites and gradual saturation (Fig. 4B-D & Table 2).

Binding of HSA and *C. sativa* MT and potencies (8, 14, 32 cH) involves exothermic reaction, maximum heat change 0.6-2 µcal/sec, 2-6 sequential binding sites and gradual saturation (Fig. 5A-D, Table 2). In case of sequential binding one site induces opening up of the next site after saturation. Results with water control ethanol are given in (Table 2). Binding between water and HSA is shown in (Fig. 5E). Water shows 3 binding sites, exothermic reaction and gradual saturation (Fig. 5E & Table 2). Ethanol shows single binding site, small heat change, endothermic reaction and increasing trend of heat change without any saturation (Fig. 5F & Table 2).

Discussion

The dilution of *Cannabis* potencies tested in the present study are 10^{16} for the 8th potency, 10^{28} for the 14th one and 10^{64} for the 32nd one. The latter two potencies have crossed the Avogadro number and are, therefore, devoid of any original drug molecule. The 8th potency is too dilute to produce any tangible effect of the original drug like the mother tincture. So, the *Cannabis* potencies cannot produce any intoxication like their mother tinctures. The acute effect of *Cannabis* involves deep sleep, some sort of tranquility, excessive hunger, dry mouth *etc*. The effects depend on age. The drug also produces some addiction[22]. Depending on the dose *Cannabis* can produce exaggerated sensations and emotions like uncontrollable laughter, hallucination, confusion regarding time and space *etc*[8].

However, *Cannabis* potencies, although free from drug substance and identical with respect to EtOH content (0.09%), differ from each other and also water control and also ethanol with respect to free water molecules (Table 1). This is evident from the DSC results which are in agreement with other homeopathic potencies so far tested. Earlier studies using FTIR, Raman spectroscopy, DSC and thermogravimetry have demonstrated that the free

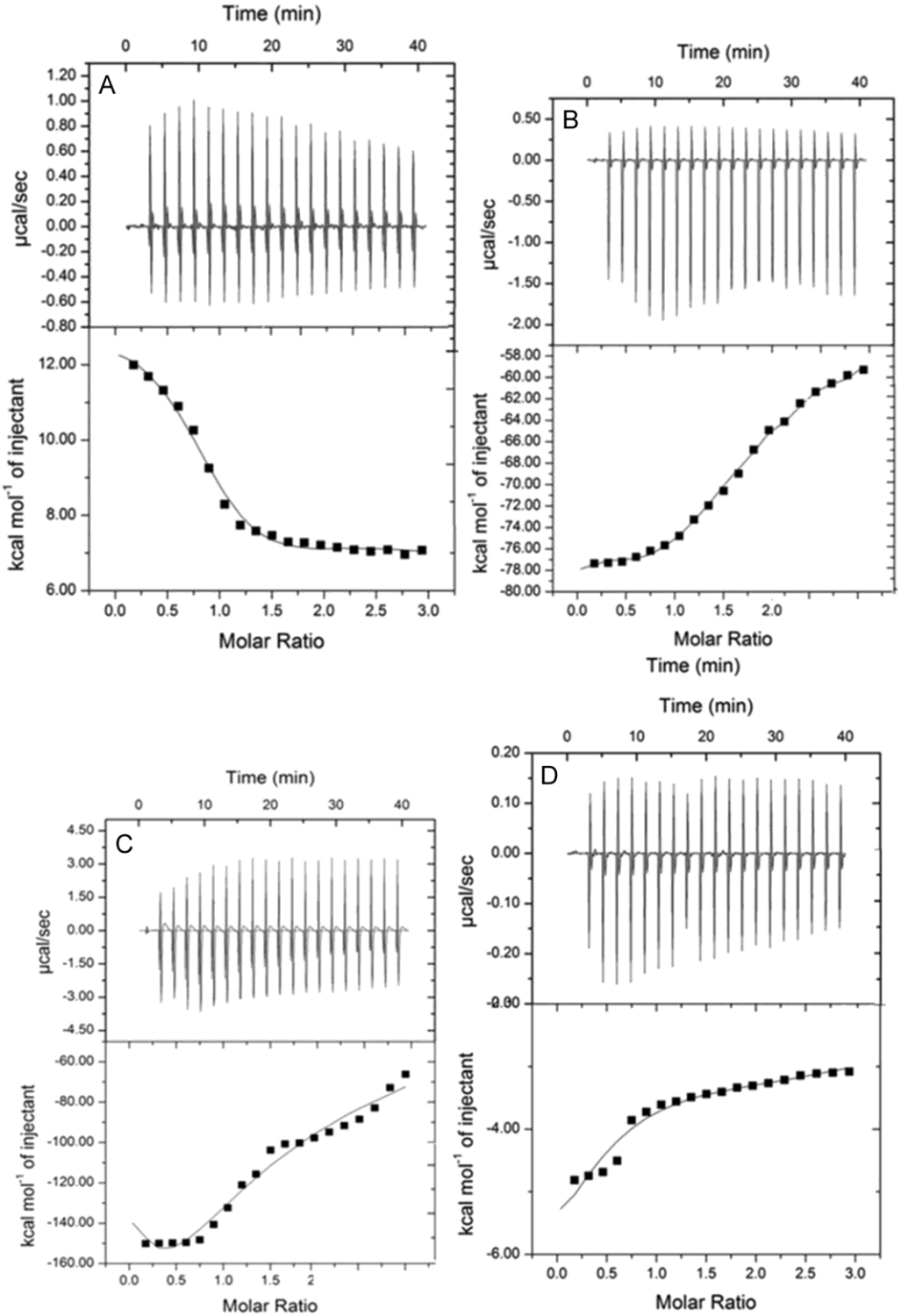

Fig. 4 — Raw data showing heat change (µcal/sec) due to injection of ligand (A) (mother tincture of *Cannabis indica*); (B) (*Cannabis indica* 8 cH); (C) (*Cannabis indica* 14 cH); and (D) (*Cannabis indica* 32 cH) at 2 µL/ injection every 2 min into 300 µL of 16 µM HSA in a sample cell. Non-linear regression derived from raw data showing heat released per mole of ligand during interaction with HSA in relation to molar ratio ligand/HAS

Table 2 — Thermodynamic parameters of interactions between human serum albumin (HSA) and ligands like *Cannabis indica* and *Cannabis sativa* MT, 8 cH, 14 cH, and 32 cH potencies. All potencies were in 0.09% ethanol. Each ligand was injected 20 times every 2 min at 2 µL/injection into 16 µM HSA at 25°C in an isothermal titration calorimetry (ITC) instrument. Control was water

Ligands injected 2 µL/2 min into 16 µM HAS	KM^{-1} (Binding constant) $\times 10^4$	ΔH cal/mol$\times 10^4$	ΔS Cal/mol/deg	ΔG Cal/mol$\times 10^3$	Binding sites, maximum heat change, Stoichiometry (N)
Cannabis indica MT (0.09% EtOH)	K_1:1.25×10^2	ΔH_1:1.37	ΔS_1:73.8	ΔG_1:−1.84	Sequential 3, 1 µcal/s, endothermic, N= 3
	K_2:1.69	ΔH_2:4.45	ΔS_2:169	ΔG_2:−4.22	
	K_3:4.06	ΔH_3:7.07	ΔS_3:258	ΔG_3:−6.44	
C. indica 8cH (0.09% EtOH)	K_1:1.02×10	ΔH_1:−1.87×10	ΔS_1:−604	ΔG_1:1.50×10	Sequential 6, 1.5-2 µcal/s, exothermic, N=6
	K_2:9.85	ΔH_2:−8.63	ΔS_2:−267	ΔG_2:6.67	
	K_3:9.80	ΔH_3:−5.43	ΔS_3:−159	ΔG_3:3.97	
	K_4:1.02×10	ΔH_4:−7.31×10	ΔS_4:−2.43×10^3	ΔG_4:6.07×10	
	K_5:1.03×10	ΔH_5:2.20×10^2	ΔS_5:7.40×10^3	ΔG_5:−1.85×10^2	
	K_6: 1.01×10	ΔH_6:−2.71×10^2	ΔS_6:−9.06×10^3	ΔG_6: 2.26×10	
C. indica 14cH (0.09%EtOH)	K_1:1.06×10	ΔH_1:−3.24×10	ΔS_1:−1.06×10^3	ΔG_1:2.65×10	Sequential 4, 4 µcal/s, exothermic, N=4
	K_2:1.20×10	ΔH_2:−4.50×10	ΔS_2:−1.48×10^3	ΔG_2:−3.69×10^3	
	K_3:8.39	ΔH_3:8.78×10	ΔS_3:2.96×10^3	ΔG_3:7.39×10	
	K_4:1.06×10	ΔH_4:−9.49×10	ΔS_4:−3.16×10^3	ΔG_4:7.89×10	
C. indica 32cH (0.09% EtOH)	K_1:2.34×10	ΔH_1:−0.8566	ΔS_1:−4.15	ΔG_1:1.02×10^{-1}	Sequential 3, 0.2 µcal/s, exothermic N=3
	K_2:4.00	ΔH_2:−0.3228	ΔS_2:10.2	ΔG_2:−2.55×10^{-1}	
	K_3:6.57	ΔH_3:−2.40×10^{-03}	ΔS_3:−58.5	ΔG_3:−1.46	
Cannabis sativa MT (0.09% EtOH)	K_1:1.07×10	ΔH_1:−5.54	ΔS_1:−163	ΔG_1:4.06	Sequential 3, 2 µcal/s, exothermic, N=3
	K_2:7.22	ΔH_2:−7.04	ΔS_2:−214	ΔG_2:5.34	
	K_3:1.25×10	ΔH_3:4.56×10	ΔS_3:1.55×10^3	ΔG_3:1.50	
C. sativa 8cH (0.09% EtOH)	K_1:1.07×10	ΔH_1:−3.34	ΔS_1:−89	ΔG_1:2.25	Sequential 6, 1 µcal/s, exothermic, N=6
	K_2:9.29	ΔH_2:−2.37×10	ΔS_2:−772	ΔG_2:1.92×10	
	K_3:1.03×10	ΔH_3:1.08×10^2	ΔS_3:3.36×10^3	ΔG_3:9.11×10	
	K_4:1.07×10	ΔH_4:−3.72×10^2	ΔS_4:−1.24×10^4	ΔG_4:−1.20×10	
	K_5:9.99	ΔH_5:6.79×10^2	ΔS_5:2.28×10^4	ΔG_5:−5.69×10^2	
	K_6: 9.75	ΔH_6:−6.26×10^2	ΔS_6:−2.09×10^4	ΔG_6:5.21×10^2	
C. sativa 14cH (0.09%EtOH)	K_1:6.42	ΔH_1:−4.20	ΔS_1:−119	ΔG_1:2.97	Sequential 6, 0.6 µcal/s, exothermic, N=6
	K_2:9.61	ΔH_2:4.27×10	ΔS_2:1.45×10^3	ΔG_2:3.62×10	
	K_3:1.32×10	ΔH_3:−2.75×10^2	ΔS_3:−9.20×10^3	ΔG_3:2.29×10^2	
	K_4:1.10×10	ΔH_4:5.02×10^2	ΔS_4:−1.89×10^4	ΔG_4:4.71×10^2	
	K_5:1.88×10	ΔH_5:−2.55×10^2	ΔS_5:−8.52×10^3	ΔG_5:2.12×10^2	
	K_6: 1.20×10	ΔH_6:−1.16×10^2	ΔS_6:−3.86×10^3	ΔG_6:9.63×10	
C. sativa 32cH (0.09% EtOH)	K_1:1.29×10	ΔH_1:−3.36	ΔS_1:−89.4	ΔG_1:2.23	Sequential 2, 1.5 µcal/s, exothermic, N=2
	K_2:1.54×10	ΔH_2:−2.96×10	ΔS_2:−967	ΔG_2:2.41×10	
Water (control)	K_1:5.48	ΔH_1:−1.69×10^2	ΔS_1:−5.65×10^3	ΔG_1:1.41×10^2	Sequential 3, 2.3 µcal/s, exothermic, N=3
	K_2:4.80	ΔH_2:3.85×10^2	ΔS_2:1.29×10^4	ΔG_2:3.22×10^2	
	K_3:5.70	ΔH_3:−5.92×10^2	ΔS_3:−1.99×10^4	ΔG_3:4.96×10^2	
Ethanol (0.09%)	K:3.12×10	ΔH:1.26	ΔS:67.2	ΔG:−1.67	1, 0.45 µcal/s, endothermic, N=1

water molecules and hydrogen bond strength contribute to the individual identity of homeopathic potencies tested[10,11,23]. It is, therefore, presumed that the possible therapeutic effect of *Cannabis* potencies, like other homeopathic potencies, may result from free water molecules.

ITC experiments have demonstrated that the MT and potencies (8, 14, 32 cH) of both the strains of *Cannabis* bind to HSA, and the heat change resulting from the binding reaction is substantially high. This binding between ligand and protein molecules is reversible and non-covalent in nature. While *Cannabis* MTs contain active compounds, their potencies are virtually water with very negligible amount of ethanol (0.09%). Water molecules are known to bind to many parts of a protein nonspecifically[24]. In this study and in our earlier experiments we have demonstrated that water

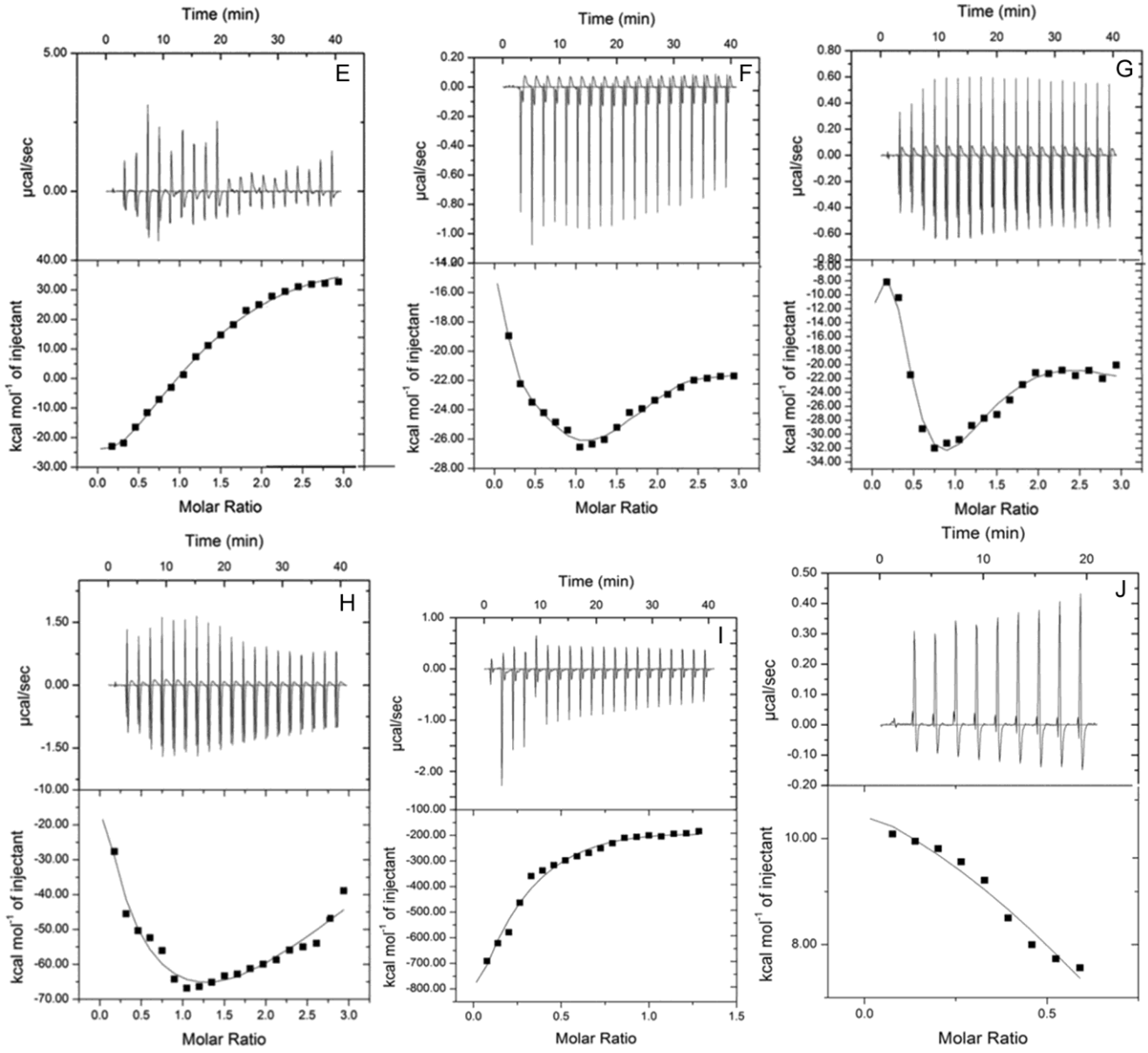

Fig. 5 — Raw data showing heat change (µcal/sec) due to injection of ligand (A) (Mother tincture of *Cannabis sativa*); (B) (*Cannabis sativa* 8 cH); (C) (*Cannabis sativa* 14 cH); (D) (*Cannabis sativa* 32 cH); (E) (Water); and (F) (Ethanol) at 2 µL/ injection every 2 min into 300 µL of 16 µM HSA in a sample cell. Non-linear regression derived from raw data showing heat released per mole of ligand during interaction with HSA in relation to molar ratio ligand/HAS

molecules bind to proteins[14-16]. It is interesting to note that binding parameters of ethanol are totally different from those of water control and *Cannabis* potencies. Major differences shown by ethanol, are number of binding site (1), stoichiometry (1) and increasing heat change (Fig. 5F & Table 2). Proteins have flexible binding pockets lined with amino acid residues. The internal motion of proteins determines and designs binding properties[25]. It is possible that MTs and potencies of *Cannabis* may initiate their action by binding at specific sites of HSA or other proteins thereby inducing subsequent biochemical events inside the cell.

Conclusion

Cannabis indica and *C. sativa* have been used in homeopathy in the form of extreme dilutions since 1841 for their specific therapeutic effects. These extreme dilutions, called potencies, differ from each other with respect to the level of free water molecules.

They also differ from ethanol potencies. The drug potencies bind to HSA showing exothermic reaction. MTs and ethanol show endothermic reaction. Potentized drugs and their solvent, though identical in chemical composition (0.09% EtOH and 99.91% water) show major difference in binding properties. It is possible that the therapeutic effect of *Cannabis* potencies might have been mediated through binding with a protein.

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