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CONFIRMATORY EVIDENCE OTHER THAN ^{1}HNMR SPECTROSCOPIC TECHNIQUE FOR COMPLEX FORMATION OF DICLOFENAC SODIUM WITH GO-GHRITA

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Abstract: Present investigation showing confirmatory evidence for complex formation of Diclofenac sodium (Dic) with Go-Ghrita (GG) by various sophisticated techniques like FT-IR, *In-vitro* release study, DSC, X-ray diffraction and SEM other than ^{1}NMR (which is reported earlier). Diclofenac sodium binary mixture in (1:0.5), (1:1), (1:2), (1:3) w/w proportions were prepared by adding to molten Go-Ghrita kept over a water bath at 65-70°C with continuous stirring. Organoleptic and Physico-chemical properties of GG (procured from Magan Sangrahalaya, Wardha) were as per the specifications given in Ayurvedic Pharmacopoeia (AP) and Indian Pharmacopoeia (IP). All proportions were subjected for FT-IR and *in-vitro* release behaviour (SGF, P^H 1.2 for 2 Hrs and SIF, P^H 6.8 for 7 Hrs) and on basis of it, optimized (1:1) w/w proportions were further analyzed and characterized for Differential Scanning Calorimetry, X-ray diffraction and Scanning Electron Microscopy. Carbonyl stretching of benzene acetic acid group to slight higher frequency 9.23 % cm^{-1} (increase) and (–C=O) linked (-OH) band stretching to slight lower frequency 0.57 cm^{-1} (decrease) shown in FT-IR spectra, supported by *in-vitro* release of Diclofenac sodium 99.13±0.31% of (1:1) w/w in sustenance form than remaining. Retention of peaks with lesser intensity in binary mixture of drug with GG, revealed crystalline as well as non-crystalline (amorphous) nature in X-RD, Slight lowering of 17.6°C Tm and enthalpy change of 8.51 % (loss) in DSC, and entrapment of Diclofenac sodium crystals in Dic-GG binary mixture shown in SEM photographs, reflecting possible evidence for the formation of inclusion type complex between Diclofenac sodium and GG.

Key Words: Diclofenac sodium, Go-Ghrita, Ayurvedic Pharmacopoeia, Physico-chemical, Inclusion complex, FT-IR., *In-vitro* release study, DSC, X-ray diffraction, SEM.

INTRODUCTION

A sanskrit Indian word Go-Ghrita (GG), is common name for cow ghee. GG, along with other substances, composed of numerous saturated fatty acids like myristic, stearic, lauric, butyric, capric, caprylic and unsaturated fatty acids like linoleic, linolenic, vaccenic and arachidonic acids [1], leads to difficulty in proposing any single chemical structure of it. Among these fatty acids, palmitic acid, a 16:0 saturated fatty acid, constitutes 29.95%, while oleic acid, which is 18: 1 monounsaturated acid with a double bond between 9-10 carbon atoms, is present to the extent of 27.42% [2]. GG has been shown to exhibit excellent wound healing property [3] as well as substantial anticonvulsant action [4]. A formulation containing some herbs and GG has been shown to exert remarkable memory enhancing activity [5] and patented in U.S. as an ointment base [6]. Literature repleting with reports on use of GG in designing the sustenance release formulation [7], as well as few of its interaction study with NSAIDs like Acetaminophen [8,9,10] and Diclofenac sodium by ^{1}H NMR spectroscopy [11].

Keeping in mind all such few and rare interactions study of NSAIDs with GG, attempt has been made to examine the nature, type of interaction and complex formation of Diclofenac sodium NSAID with GG (composing saturated and unsaturated fatty acids) by the techniques other than ^{1}H NMR. To investigate such phenomenon one or more sophisticated analytical techniques like FT-IR, cumulative % Diclofenac sodium release from binary mixtures (1:0.5), (1:1), (1:2), (1:3) w/w proportions, DSC, X-rd, and SEM were studied. In addition to this preliminary analysis of GG were carried out prior its used to confirm its purity in binary mixture.

Diclofenac sodium [12] – BCS class II NSAIDs, having strongest anti-inflammatory and pain killing effects. It has also relaxant effect on smooth muscle and reduces the concentration of uric acid in the blood. Chemically, Diclofenac sodium (Fig.1) is 2-[(2, 6-dichlorophenyl) amino] benzene acetic acid.

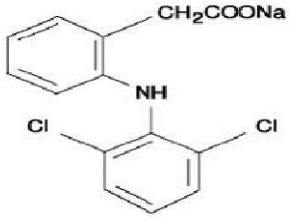

Figure 1: Diclofenac sodium

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MATERIALS AND METHODS

Diclofenac sodium as a gift sample was kindly supplied by Zim Laboratories Ltd., Kalmeshwar, Nagpur. Go-Ghrita was purchased from Magan Sangrahalay, Wardha, India. All other chemicals and reagents used were of analytical grade and were procured.

Preliminary analysis of Go-Ghrita

Organoleptic analysis: Colour, Odour, Taste and Texture of GG sample was evaluated as described in AP [13].

Physical characterization: Moisture content and Refractive index (reading at 40°C) of GG sample was determined by the method described in AP [13].

Chemical analysis:

Acid and Saponification values: Acid and Saponification values of GG were determined as per the method described in AP [13].

Iodine and Peroxide values: Iodine and Peroxide values of GG were determined by pyridine bromide method and titration method as described in AP [13].

Ester value of GG: Ester value, difference between Saponification value and Acid value was determined as described in AP [13].

Baudouin test for GG: Sample response for this test is checked to verify purity and adulterant present in it, as described in AP [13].

Free fatty acids (% oleic acid) and Unsaponifiable matter in GG

Free fatty acids levels of GG sample was determined by the method as described in AP [13] and IP [14].

Preparation of sample:

To the molten GG kept over a water bath at 65-70°C an amount of Diclofenac sodium was added and uniformly dispersed by continuous stirring to prepare (1:0.5), (1:1), (1:2), (1:3) w/w proportions. The 1:0.5 to 1:3 w/w proportions were selected for observing minimize to maximize the interaction (if any) [15] involved in it. The fused mixtures were homogenized and allowed to cool slowly to room temperature with stirring.

Fourier Transform Infrared Spectroscopy (FTIR):

Fourier Transform Infrared Spectroscopy (FTIR) is a rapid analytical technique that measures vibrations of bonds within functional groups. FTIR spectral studies were carried out using an FTIR spectrometer (Perkin Elmer Spectrum 2000, Norwalk, CT). Diclofenac sodium, GG and their binary mixtures (1:0.5), (1:1), (1:2), (1:3) w/w proportions were smeared onto KBr windows and the spectra were recorded from 500 to 3500/cm.

In-Vitro Diclofenac sodium Release Study

The % cumulative Diclofenac sodium release from the binary mixtures (1:0.5), (1:1), (1:2), (1:3) w/w proportions were studied in 900 ml Simulated Gastric fluid (SGF), P^H 1.2 without pepsin for first 2 Hrs and subsequent 7 Hrs in Simulated Intestinal fluid (SIF), P^H 6.8 Phosphate buffer, stirred at 50 rpm, 37°C ± 0.5°C by USP - I (Rotating Basket type) method, VIII stations Dissolution Test Apparatus, Electrolab, Mumbai. Scanning of Diclofenac sodium was carried out between 200-400 nm in both SGF and SIF and λ_{max} was reported to be at 368 nm and 278 nm respectively. Absorbance of standard calibration curve of Diclofenac sodium in SGF and SIF were analyzed, after adequate dilutions, at λ_{max} 368 nm and 278 nm on UV Spectrophotometer (UV-1700; Pharmaspec, Shimadzu, Japan) equipped with UV probe software (2.01 version). Data was depicted in Microsoft excel and had correlation coefficient (R^2) 0.998, 0.997 and equation of regression lines Y = 0.006X - 0.002 and Y = 0.048X + 0.001 respectively.

Drug content

The percent drug content of each binary mixture was determined. Weighed accurately about 50 mg of Diclofenac sodium binary mixtures (1:0.5), (1:1), (1:2), (1:3) w/w proportions and dissolved in 20 ml of alcohol using the magnetic stirrer for 20 min. To the solution obtained, simulated gastric fluid or simulated intestinal fluid was added and volume was made upto 100 ml. It was then filtered through Whatman filter paper no. 42 and required dilutions were made and absorbance was taken at 264 nm.

Differential Scanning Calorimetry (DSC)

5-10 mg of Diclofenac sodium sample, GG and their 1:1 w/w binary mixture was weighed into pin holed platinum pans (TG/DTA instruments) and heated under dry nitrogen in 0 - 340°C scanning range at a rate of 10°C/min. An empty pan was used as reference. Experiments were carried out in duplicate.

X-Ray Diffraction

X-ray diffraction of Diclofenac sodium, GG and their 1:1 w/w binary mixture was carried out on a Rigaku Rotating Anode Diffractometer RUH3R (Tokyo, Japan). Measurement conditions were 40 kV voltage, 30 mA current, at a scanning speed of 2°/min, step size 0.02 and scanning range from 10–80° 2Theta.

Scanning Electron Microscopy

Scanning electron microscopy of Diclofenac sodium, GG and their 1:1 w/w binary mixture mounted

on scanning electron microscope stubs with double-sided carbon tape and observed under 370701-14, S-3700, Scanning Electron Microscope.

Statistical analysis

The t-test was performed on all collected mean data obtained from physiological evaluation as well as dissolution studies. Significance was accepted at p≤0.05 [16].

RESULT AND DISCUSSION

Physico-chemical analysis of GG

Physico-chemical properties of GG given in Table 1 revealed the purity and adulterant free GG. All the tested parameter of GG passes the standards and limit given in Ayurvedic Pharmacopoeia [13] and Indian Pharmacopoeia [14] respectively.

Table 1: Physico-chemical analysis of GG

Sr. No	Physiological parameters of GG	Observations (Mean ± S.D.)	A.P. standards
1.	Moisture content	0.087% ± 0.0290	NMT 0.5%
2.	Refractive index	42 ± 0.0090	40 – 45
3.	Acid value	0.22 ± 0.0190	NMT 0.15 - 0.25 %
4.	Saponification value	190.74 ± 0.0210	NMT 225
5.	Ester value	189.79 ±0.0030	NMT 225
6.	Iodine value	25.88 ± 0.0199	NMT 35
7.	Free fatty acids (% oleic acid)	2.73 ± 0.0171 %	NMT 3 %
8.	Unsaponifiable matter (%)	0.4 ± 0.0025 %	NMT 1.5 %
9.	Baudouin test	No pink color formation	No pink colour
10.	Peroxide value	0.00	Less than 0.5

All the determinations are carried out five times with significance (p≤0.05)

Fourier Transform Infrared Spectroscopy

Fig. 2 showing FT-IR spectra of Diclofenac sodium (A), GG (B) and binary mixture of Dic with GG in different w/w proportions (C – F), reveling retention of characteristics bands at 1571.36 cm^{-1} (C=O), 714.43 cm^{-1} (-Cl stretch), 1466.80 cm^{-1} (CH bend), 1555.12 cm^{-1} (NH bend) as reported in literature [17, 19]. The ability of the GG to form a complex with Diclofenac sodium depends on the nature of the core-surface groups of GG (composed of saturated and unsaturated fatty acids), electrostatic interactions between the GG and Dic, and the ability of the Dic to form a conjugate with the GG through chemical bonding. One might expect that the Dic with the carboxylic group may form a complex with surface unsaturated -C=C- fatty acids group (may exists as dimer involving hydrogen bonding) [19] of GG and may physically encapsulate the Diclofenac sodium structure as bands shown in Dic at 3386.64 cm^{-1} (NH stretch), 3256.04 cm^{-1} (Aromatic CH), is disappear in all binary mixture.

Pure Diclofenac sodium (Fig. 2A) shows a strong carbonyl band absorbance at 1571.36 cm^{-1}, which corresponds to the carboxyl acid group (COOH) where as other smaller peaks in the region 1500–500 cm^{-1} are contributions from the benzene ring [16]. FTIR spectrum of all binary mixture (Fig. 2C - F) 1:0.5, 1:1, 1:2, 1:3 w/w shows disappearance of strong carbonyl band at 1571.36 cm^{-1} of Diclofenac sodium and shifted to slight higher frequency 1741.52 cm^{-1} (i.e. 9.23 % cm^{-1} increase), as well as (–C=O) linked (-OH) band stretching to slight lower frequency 1458.39 cm^{-1} (i.e. 0.57 cm^{-1} decrease) band due to observed band respective at 1571.36 cm^{-1} and 1466.94 cm^{-1} (Fig. 2B) of carboxylic and OH group of saturated or unsaturated fatty acid [8, 9] present in GG.

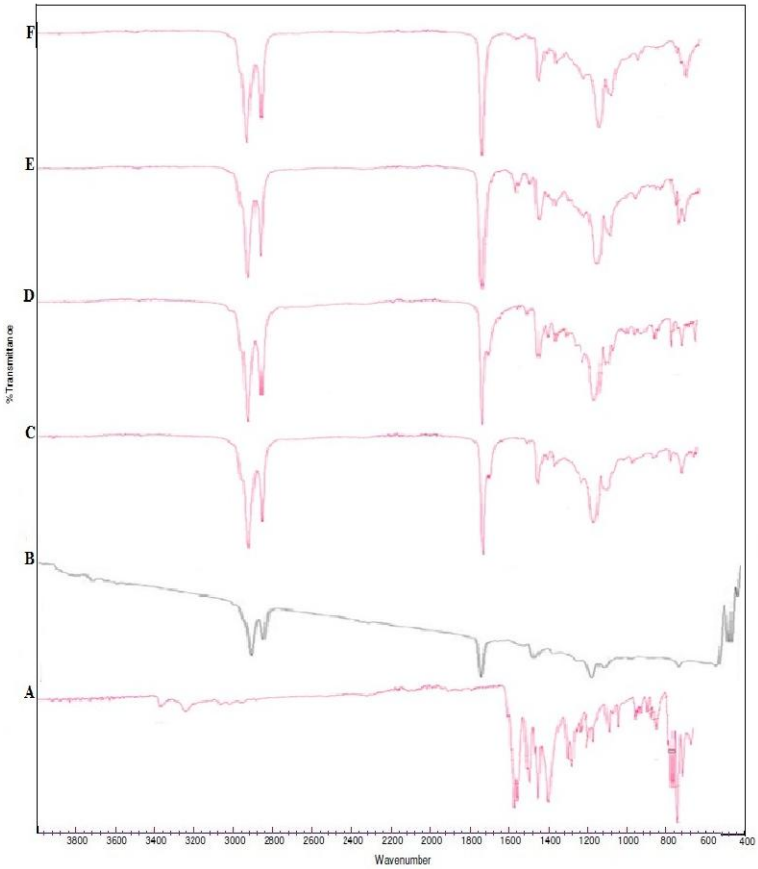

Figure 2: FT-IR Spectra of A) Diclofenac sodium, B) GG, C) Binary mixture of Dic-GG 1:0.5 w/w, D) 1:1 w/w, E) 1:2 w/w, F) 1:3 w/w proportion.

Table 2: FTIR absorption bands Interpretation

Characteristic Peaks	Dic sod	1:0.5	1:1	1:2	1:3	% cm^{-1} Stretching
C=O (cm^{1})	1571.36	1741.43	1741.52	1740.82	1741.34	9.23 Increase
Cl stretch (cm^{1})	714.43	720.77	720.70	716.32	720.80	0.88 Increase
CH bend (cm^{1})	1466.80	1458.24	1458.39	1457.19	1457.78	0.57 Decrease
NH bend (cm^{1})	1552.12	1707.31	1706.75	1706.28	1705.29	8.96 Increase

Drug content and % Diclofenac sodium Release

Binary mixtures of various w/w proportions were subjected for Diclofenac sodium determination and 1:1 w/w content was found to be highest 98.95±

0.32 in SGF, 98.79± 0.24 in SIF observed slightly less (Table 3) in 1:0.5, 1:2, 1:3. The strength and stability of the Dic-GG complex was examined using in vitro release study. Fig. 3 represents more uniform and sustenance zero order release 99.13±0.31 (Table 4) of Diclofenac sodium with R^2 value 0.9896 (Table 5) from 1:1 binary complex in both SGF and SIF, represents the efficacy, integrity and entrapment of Dic in Dic-GG binary mixture. The order of % cumulative Diclofenac sodium release is 1:1 > 1:0.5 > 1:2 > 1:3.

Table 3: Percent drug content of Diclofenac sodium from binary mixtures

Sr. No.	Binary mixtures w/w proportion	Drug content* (%) SGF	SIF
1.	1:0.5	95.51± 0.42	96.25± 0.11
2.	1:1	98.95± 0.32	98.79± 0.24
3.	1:2	96.75± 0.43	97.10± 0.29
4.	1:3	97.45± 0.21	96.08± 0.18

*Represents mean ± S. D. (n=3)

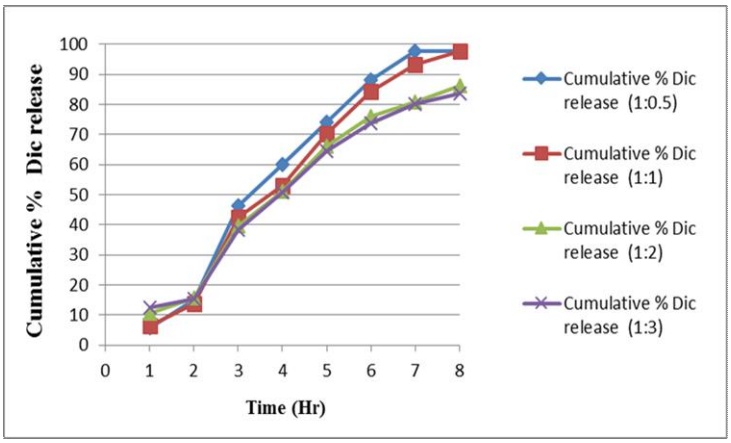

Figure 3: Cumulative % Diclofenac sodium release from (1:0.5), (1:1), (1:2), (1:3) w/w proportions.

Table 4: Cumulative % Diclofenac sodium release of various binary mixtures

Sr. No.	Medium	Time (Hr)	Cumulative % Diclofenac sodium release* 1:0.5	1:1	1:2	1:3
1.	0.1N HCl, P^H 1.2	1	5.7±0.23	6.45±0.11	10.5±0.20	12.6±0.19
2.		2	15.16±0.31	13.81±0.23	15.76±0.32	15.31±0.17
3.		3	46.24±0.26	42.49±0.20	39.61±0.11	38.41±0.16
4.	Phosphate Buffer, P^H 6.8	4	60.06±0.41	53.11±0.22	51.20±0.05	50.83±0.15
5.		5	74.15±0.13	70.38±0.31	66.09±0.16	64.57±0.13
6.		6	88.24±0.24	84.47±0.17	76.37±0.26	73.83±0.09
7.		7	98.05±0.41	93.45±0.21	81.21±0.27	80.31±0.25
8.		8	98.14±0.13	99.13±0.31	86.53±0.19	84.12±0.11

(*Represents mean ± S.D.) (n=3)

Table 5: Release kinetics of various binary mixtures

Release Model	1:0.5 R^2	1:1 R^2	1:2 R^2	1:3 R^2
Zero Order	0.984165	0.989610	0.988003	0.987788
First Order	0.865161	0.873075	0.8540151	0.8511787
Higuchi Release	0.952255	0.950297	0.960853	0.962079
Corse Mayer Release	0.138432	0.116643	0.067218	0.049848
Hixson Crowell Model	0.939045	0.945876	0.932448	0.932683

Differential Scanning Calorimetry

The DSC thermograms of Diclofenac sodium, GG and Dic-GG 1:1 w/w proportions are presented in Fig. 4. The following general results can be derived from Table 6, 7; the melting temperature of binary systems (Fig.4C) is lower than those of single Diclofenac sodium (Fig.4A). The thermogram of Diclofenac sodium-GG 1:1 w/w records two sharp endothermic peaks corresponding to their melting point with onset at 53.9°C and 269.9°C respectively along with approximately 8.51 % loss in enthalpy ($\Delta H_{observed}$) in comparison to ($\Delta H_{calculated}$) values of system, suggesting the possibility of interaction [20,21].

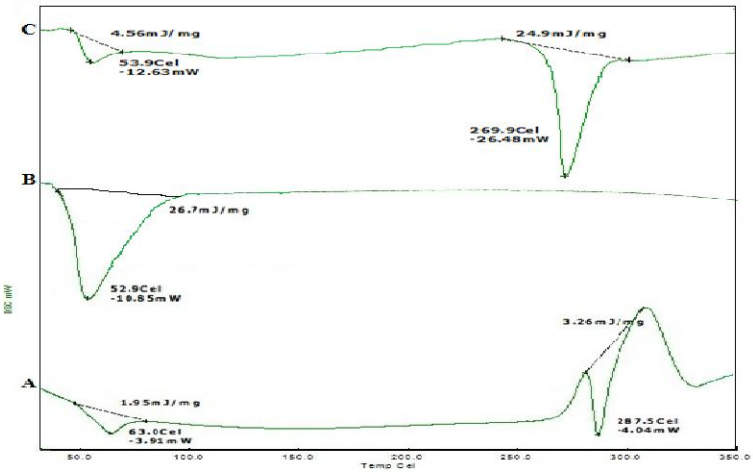

Figure 4: DSC of A) Diclofenac sodium, B) GG, C) Binary mixture of Dic-GG 1:1 w/w proportion.

Table 6: Thermal parameters of Diclofenac sodium, GG and binary mixture 1:1 w/w proportions

Sr. No.	Type	T_{peak} (°C)	ΔH (J/g)
1	Dic	287.5	3.26
2	GG	52.9	26.7
3	Dic-GG 1:1	269.9	24.9

Table 7: Thermal parameters of Dic-GG 1:1 w/w proportions

Binary Mixture	1ST Transition		2ND Transition		ΔH_{cal} (J/g)	ΔH_{obs} (J/g)	ΔH % /Result
	T_{peak} (°C)	ΔH (J/g)	T_{peak} (°C)	ΔH (J/g)			
Dic-GG 1:1w/w	53.9	4.56	269.9	24.9	27.15	29.46	8.51(loss)

X-Ray Diffraction

The diffraction pattern of crystalline nature of Diclofenac sodium, GG and binary mixture are shown in Fig. 5. The characteristic diffraction peaks of Diclofenac (Fig. 5A) were also found in binary mixture of drug with GG (Fig. 5C). It can be also noticed that, the intensity of peak in the binary mixture of drug with GG is lesser than that in X-ray diffraction pattern of the drug alone. That is may be due to the lipidic polymorphism of GG, indicating there is a complete change in the crystallinity of the drug and suggesting that Diclofenac is in either amorphous form [22] or entrapped in GG matrix. Accordingly it can be concluded that, the drug in binary mixture are in two forms, one crystalline form and the other in non-crystalline one.

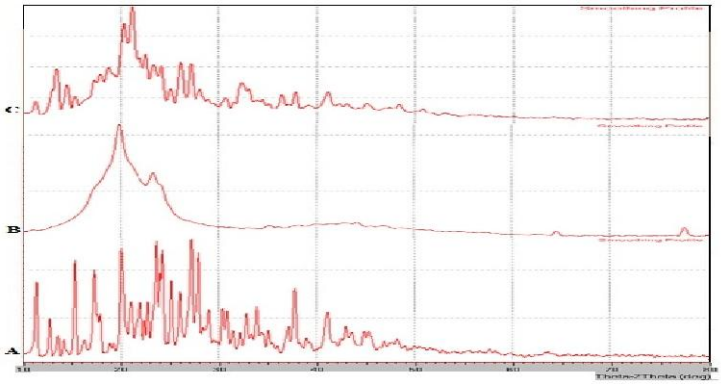

Figure 5: X- RD of A) Diclofenac sodium, B) GG, C) Binary mixture of Dic-GG 1:1 w/w proportion.

Scanning Electron Microscopy

Scanning electron micrographs of crystals of Diclofenac sodium appear more discrete and irregular in shaped a as illustrates in Fig. 6 (A) at 100X magnification. In the admixture of Diclofenac sodium with GG, the open lattice matrix or cage like crystal structures of GG Fig. 6 (B) entrapped discrete and irregular shaped Diclofenac sodium crystals with them (inclusion type complex) [23]; some of them lost their crystal habit while the habit of remaining was modified in the presence of GG, highlighted by arrow in Fig. 6 (C). The degree of entrapped discrete and irregular shaped Diclofenac sodium crystals in open lattice matrix or cage like crystal structures of GG may be the determining factor in the diffusion or dissolution of Diclofenac sodium.

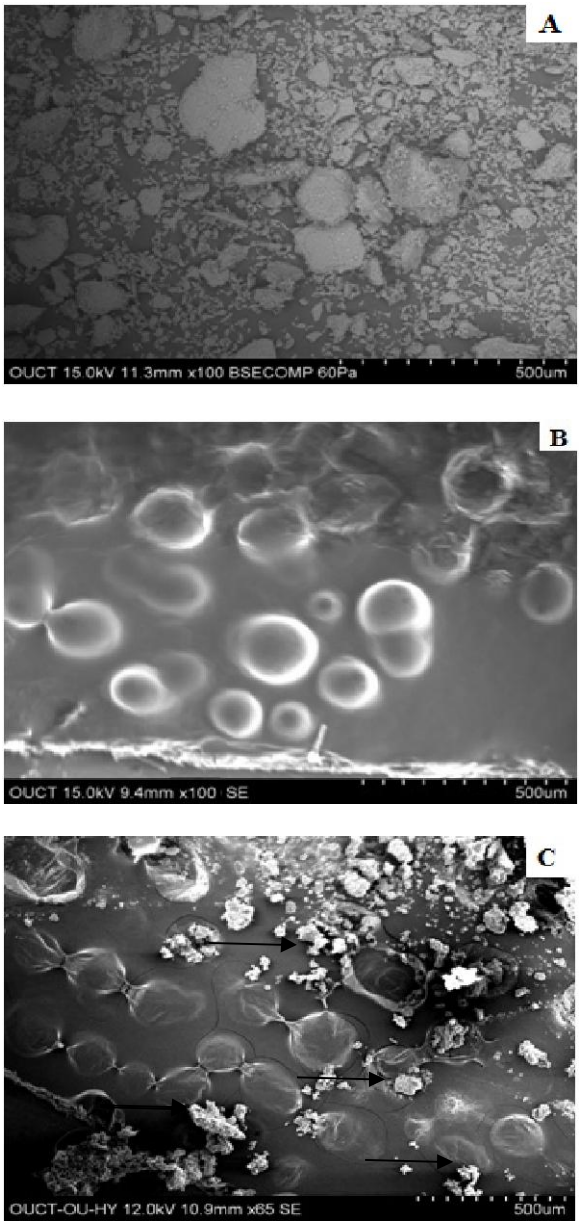

Figure 6: Scanning electron microscopy at a magnification of 100X A) Diclofenac sodium, B) GG, C) Binary mixture of Diclofenac sodium with GG (marked arrow showing Diclofenac sodium crystals get trapped in cage like structures of GG)

CONCLUSION

In present investigation, we report the evidence of complex formation of Diclofenac sodium with Go-Ghrita other than ^{1}NMR technique. The FT-IR spectrum of binary mixtures detected characteristics increase in strong carbonyl band and decrease in (–C=O) linked –OH band. More uniform and sustenance zero order release of 1:1 w/w from *in-vitro* dissolution

study (render its selection for optimization), % loss in enthalpy ($\Delta H_{observed}$) in comparison to ($\Delta H_{calculated}$) values of DSC system, suggesting the possibility of interaction. Moreover such complexation phenomenon can be verified by X-RD, and SEM. Retention of respective peaks at their positions with lesser intensity of peak in the 1:1 w/w binary mixture of drug with GG, revealed crystalline as well as non-crystalline (amorphous) nature of Diclofenac sodium in X-RD, confirming possible interaction between Diclofenac sodium and fatty acids present in GG, substantiated by SEM photographs. SEM further elucidated entrapment of Diclofenac sodium crystal structure in open lattice matrix or cage like crystal structures of GG suggesting inclusion type complex. However, an interesting part is, such sustenance release inclusion complex of Diclofenac sodium with GG (or other NSAIDs which may form a complex with GG) may be useful in increasing the oral bioavailability or comparing such complex concentration in various biological fluids in experimental animals is under study.

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