



Exploration of fulvic acid as a functional excipient in line with the regulatory requirement

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ABSTRACT

Fulvic acid, a humic substance often considered as a geopolymer, extracted from different natural resources like Shilajit, Peat, dissolved organic matters, etc. There are several reports of its pharmacological properties and its potential as pharmaceutical excipients. So, we have devised a project to strengthen its claim as a functional excipient. For the given project, lyophilized sample of a dietary supplement product (an aqueous solution of peat derived Fulvic acid) was used. The selected studies were typical for an excipient development like physico-chemical properties, flow properties, compatibility with other excipient and stability studies, non-clinical safety studies (acute toxicity in mice whereas sub-acute toxicity in rats) and some functionality tests. We also suggest its ability to form co-crystal with natural phytochemicals. Our group has already reported its ability to enhance solubility and or bioavailability of different BCS class II drugs. Henceforth, we can propose that Fulvic acid appears a good candidate to be further explored as a functional excipient and should be evaluated as per the remaining recommendations of IPEC, USFDA, and USP.

1. Introduction

Humic substances are heterogeneous organic compounds which are ubiquitous in nature obtained from both terrestrial and marine sources, produced naturally by microbial degradation (Senesi, 2010). Nowadays, these are also synthetically produced through radical polymerizations, abiotic oxidation, and other enzymatic techniques (Kiprop et al., 2013). Although, humic substances like Fulvic acid (FA) and humic acid (HA) were employed in various fields from agricultural application to biomedicine (Peñ a-M é ndez et al., 2005) but unfortunately its usage as a pharmaceutical excipient is still under consideration only when it has a potential to resolve the hurdles encountered during drug development process. Surprisingly, FA has an ability to enhance solubility (Mirza et al., 2011a, 2011b) thus

creating an opportunity for developing novel drug delivery systems and improve manufacturing process and above all there are enough evidences of different pharmacological potentials of FA like, anti-oxidant activity (Rodríguez et al., 2011; Visser, 1987), anti-inflammatory activity (Chien et al., 2015), and improving gut health (Winkler and Ghosh, 2018), etc. Depending upon the sources, the uses of FA varies from dietary supplements to drug product. In fact, FA can be considered as an "Atypical Actives" since it is of natural origin and used either as a drug or nutritional supplement globally over a period of time with a proven history of clinical safety. Furthermore, the need and scope in development of Fulvic acid (FA) as a pharmaceutical excipient can be understood from Fig. 1.

As aforementioned it is already being consumed as a dietary supplement in different dosage forms (> 150 products) for diverse potentials in different parts of the world (USA, EU, UK, Canada, Australia,

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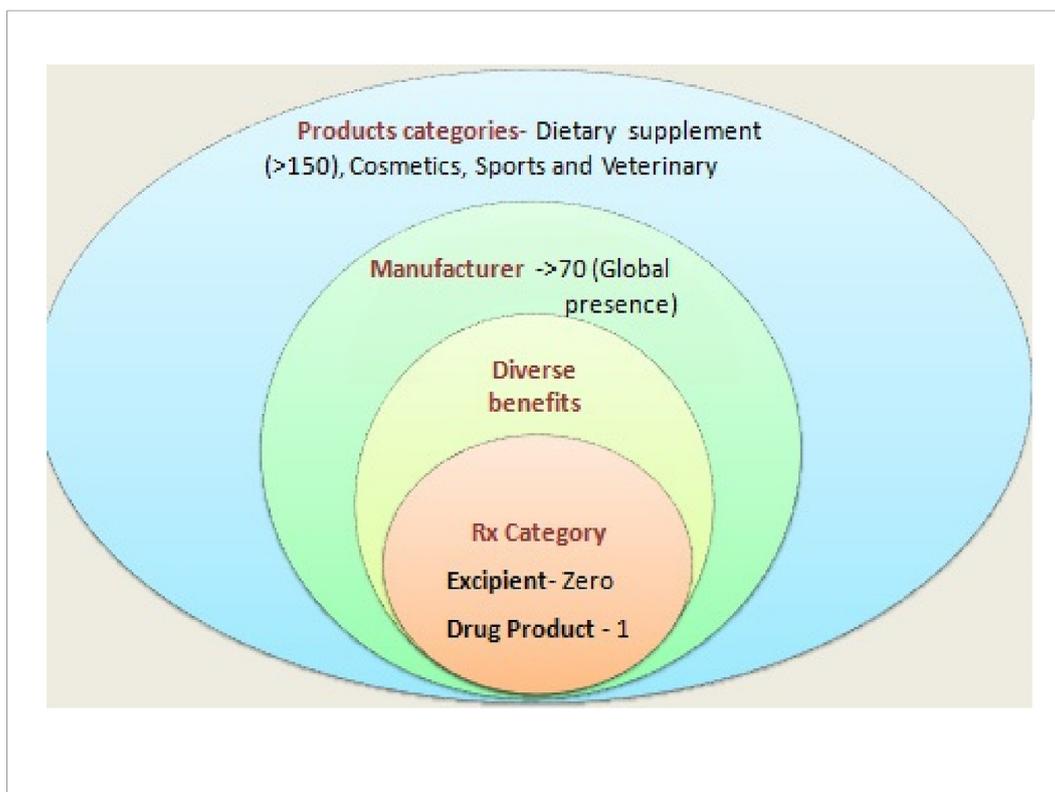


Fig. 1. Scope of humic substances based products in pharmaceutical industry. There are more than 150 products available in the categories of dietary supplements, cosmetics, sports and veterinary medicine, by more than 70 manufacturers. It is also being explored as a drug product which supports its safety profile. There should not be much data required if thought of developing it as an excipient.

South Africa and Hong Kong). Few clinical trials have also been done for the FA based products (Gandy et al., 2011, 2012; Botes et al., 2018). On the other hand, the bulk of research so far in the area of exploration of pharmaceutical excipient potential is based on the premise of solubility/bioavailability enhancement potential of FA (obtained from Shilajit) for BCS class II and IV drugs (Mirza et al., 2011a, 2011b; Javed et al., 2018), because it is supposed to form inclusion complexes (like Cyclodextrin) with hydrophobic drugs. There is also a report of acid buffering, mucoadhesion and modified drug-releasing potential of FA (Mirza et al., 2016). So, there is enough PoC (Proof of Concept) to explore it as a functional excipient in commercial drug products. The products listed in Table 1 claim several beneficial properties.

But there may be several reasons, why it has not been done so far:

1. In the majority of the pharmaceutical excipient based researches, Shilajit has been used as a source for obtaining FA. The major bottleneck in the exploration of this type of FA in commercial drug product is the high cost of Shilajit and less availability.
2. The Humic substances (HS) or Fulvic acid is not a simple substance to deal with. A sample would typically contain a range of different size of molecules. With the change of source, chances of variation in chemical composition and structures are always there. The molecules may vary with respect to both number and type of functional groups and also with respect to size. A special characteristic of HS is its capacity to show spontaneous changes in their conformation and aggregation state as a function of solution conditions like pH and ionic strength (Mirza, 2018). It is assumed that any change in the chemical composition of an excipient produces a new excipient, no matter how minor the modification to the chemical composition is. So this may trigger an entirely different course of the developmental pathway of a drug product. Furthermore, an excipient is new or novel (which is also the case with FA), if it is not listed in
 - i. The FDA Inactive Ingredient database (<https://www.accessdata.fda.gov/scripts/cder/iig/index.Cfm>).
 - ii. Any of the three major compendia, U.S. Pharmacopeia (USPNF), European Pharmacopeia (Ph. Eur.), or Japanese Pharmacopeia (JP).
 - iii. Other widely known compendia such as the “Handbook of Pharmaceutical Excipients” or “Fiedler: Lexikon der Hilfsstoffefür Pharmazie, Kosmetik und angrenzende Gebiete” (Encyclopedia of excipients for pharmaceutical, cosmetic and related use).

3. The finished product manufacturer is highly dependent on the excipient manufacturer to provide materials that are uniform in chemical and physical properties. This is particularly important in the context of the pharmaceutical product approval process where bioequivalence (BE) comparisons are made between pivotal, clinical trial batch (“bio-batch”) and commercial scale-up lots. The excipient used to manufacture commercial lots should not differ significantly from those used in bio-batches to provide adequate assurance of finished product performance. Therefore, it is important to minimize variation between the different batches of excipient. However, if significant differences do occur between excipient lots used in clinical and commercial drug product lots, additional testing by the finished product manufacturer may be required to establish the BE of the drug product.

It is actually a cross-functional job including at least humic-chemistry, regulatory and product development scientist to deal with the nuances mentioned above. With the same intention a team was constituted with different expertise and an attempt has been made to address some of the conundrums and to generate some amount of data to establish FA as a functional excipient.

To deal with regulatory and product development challenges, we have taken following steps in designing this study:

Table 1

A collection of few humic substances (mainly FA) based dietary supplement available in global market. Thus as per the regulatory recommendations, the safety studies can be abridged as it is already being consumed by different races. The dose of FA is much higher in several cases as compared to what we are expecting in the case of pharmaceutical excipient.

Oral dosage forms			
S. No	Product	Manufacturer	Max Adult Dose (per day)
1.	Miessencefulvic acids	Miessence	5–10 ml (3% FA), dilute 150–200 times
2.	Trace minerals	Mineralife	10 ml (15 mg)
3.	Best Fulvic	Mother Earth	1–3 ounce (FA-887-2661 mg) diluted with water
4.	Nanonized Fulvic acid	Nano Health Solutions	30 ml
5.	Fulvic force (Coal driven)	Fulvic force	16–32 ml (up to 15%FA)
6.	FulHum	Earth water	20 floz (400 mg HA FA complex)
7.	Liquid Concentrate Mix		0.75 fl. Oz (1252 mg HA-FA complex)
8.	POPTOPS Mineral concentrate		8 fl. oz (1252 mg HA-FA complex)
9.	FulHum One Litre Glass bottle		Fulvic/Humic Liquid concentrate- 1%
10.	Fulvic acid liquid mineral supplement	Fulvic X cell Products Ltd	5 ml (2.7–5.7% FA)
11.	Ionic fulvic acid	Trace Minerals Research	1 ml (FA-250 mg)
12.	Fulvonic minerals	Elixia	0.5–1 ml diluted with water (10–15% FA)
13.	Pure fulvic minerals	Pure fulvic minerals	Adult-7–10 drops (10–18% FA)
14.	MLG-A50™	Mineral logic	0.5 ml–1.0 ml/day (1%–5% FA)
15.	Liquid mineral support	Alpha Health Support	¼ fl. oz (6.3% FA)
16.	Fulvic mineral complex	Vital Earth Minerals	1 floz (18.75 mg Fulvic Bio mass)
17.	Mineral Blend Fulvic-Humic		1 ounce daily (FA- 14.04 mg)
18.	Fulvic acid minerals	Ancient Purity	7 drops with water, 21% FA
19.	intraMIN®	Drucker Labs	1 fl.oz (FA-2.7 g)
20.	intraKID®		1 fl.oz (FA-1.2 g)
21.	intraMAX®		1 fl.oz (FA- 2.7 g)
22.	Wujinsan Fulvic Acid	Mana life laboratory (a division of BioAg Corp)	0.5 ounce (0.663% FA)
23.	Fulvic Acid Concentrate	Humalife	3-10 drops (3% FA) 2-3 times daily
24.	Pure Concentrated Fulvic Acid	I.S.Natura	15 drops (60% FA), 4–5 times/day
25.	Purified Fulvic Acid Extra Set		2.5 ml (60% FA), 2 times a day
26.	HUMINIQUUM syrup	Hymato	10 ml contains (FA- 480 mg)
27.	Metal-X-Synergy™ capsule	Designs for Health	6 capsule (FA-900 mg)
28.	Liquid Multiple Vitamin And Mineral	New Sun®	30 ml (FA-100 mg)
29.	Liquid Multi	NOW®	15 ml (FA-50 mg)
30.	OrthoLiquid Multi	Protocol For Life Balance	15 ml (FA-50 mg)
31.	Fulvic Acid powder	Biosil	1/4 tsp daily
32.	Fulvic acid Caps		1 capsule (FA- 500 mg)
33.	Very Cranberry	Irwin Naturals	2 capsule (FA-40 mg)
34.	Test HD™	MuscleTech™	2 caplets (FA-100 mg) in Shilajit
35.	Joint DX	Natural Dynamix	2 tablets (FA-460 mg)
36.	Rejuvenate One™	Patient One MediNutritionals™	2 capsule (FA-70 mg)

1. Peat is available abundantly across the globe and is relatively cheaper source of FA than Shilajit. So, peat derived FA was selected as a suitable alternative.
2. In the product list mentioned in Table 1, there are several dietary supplement products, sourced from peat. Furthermore, the amount of FA being consumed in different products is variable and too high in many cases. So it is not required to conduct a full battery of safety studies as FA is already being consumed (even in different races).
3. Our group has recently finished another project in which the bio-availability enhancement potential of FA, obtained from peat (exactly same source as used in this project) has been evaluated with the same drugs reported earlier with Shilajit derived FA. Thus authenticating the potential of peat derived FA after changing the source (Shilajit to Peat) and bridging the data set of two differently sourced FA.

Thus it was felt worthwhile to study its physicochemical, microbial count, functional properties, and compatibility with other excipients as well as the toxicological profile of FA. In this project we have also studied the cytotoxicity by using MTT assay to further explore its functionality.

2. Materials and methodology

2.1. Materials

Peat sourced FA was obtained from NZFulvic Ltd (Mount Maunganui, New Zealand) as ex-gratis. The sample was obtained after lyophilizing the dietary supplement (a commercial product) available in global market, which is claimed to be an aqueous solution of FA. Authors were not revealed about the process of extraction of FA from peat, broadly it was based on acid-base method of extraction. All other chemicals and reagents used in the study were of AR grade.

2.2. Physico-chemical evaluation

Solubility, pH, powder flow properties of FA were evaluated as per the pharmacopeia recommendation (Indian pharmacopeia 2007).

a. Solubility

Solubility study was done in water and different solvents like methanol, ethanol, acetone and ether.

b. Determination of pH

The pH of 1% w/v aqueous solution of FA was determined by calibrated pH meter (M/s. Systronics, Model No: 361) at room

temperature 25-27 °C.

c. Powder flow properties

Flow properties like Bulk density, Tapped density, Carr's index (I) Hausner ratio (H) and angle of repose were evaluated (Shishir et al., 2014).

2.3. Characterization of FA using different analytical techniques

2.3.1. Atomic force microscopic studies (AFM)

In AFM studies; FA was dispersed in acetone, followed by sonication at 50 °C for 30 min. Then a small drop of the colloidal solution developed on glass and allowed to dry, later on examined under park systems XE7 atomic force microscopy.

2.3.2. Fourier transform infrared spectroscopy studies (FTIR)

The IR spectral analysis of FA powder and FA compatibility with other excipients was done using press pellet technique using potassium bromide. The IR spectrum was recorded by using ALPHA Bruker, IR spectrometer (Eco-ATR) in the region between 4000 and 400 cm^{-1} .

2.3.3. Differential scanning calorimetry (DSC)

Thermal properties of pure FA and FA compatibility with other excipients were characterized by using differential scanning calorimeter (Model: Q20, TA Instruments, Inc., New Castle, DE, USA). Accurately weighed samples (5 mg) in sealed aluminum pans were subjected to a single heating cycle from 30 °C to 400 °C at a heating rate of 10 °C/min. The instrument was calibrated using indium. The data obtained from the thermal analysis of the samples were analyzed using the software (Universal Analysis, V.4.5A, build 4.5.0.5, TA Instruments, Inc., New Castle, DE, USA).

2.3.4. Powder X-ray diffractometry (XRD)

Powder X-ray diffraction (XRD) studies of the FA and FA compatibility with other excipients was performed with the Rigaku X-ray diffractometer (D/MAX-B) using Ni-filtered Cu-K(α) radiation, a voltage of 40 kV and current of 30 mA. The width of receiving slit is 0.3 mm. The sample was analyzed over the 2θ range of 10–90°, scan speed of 4.0°/min with scan step and scan time of 0.020° and 0.3 s respectively. The XRD technique is non-destructive nature and therefore the sample is available for further analytical confirmations. Nevertheless, X-ray diffraction provides valuable information on polymorphism, degree of crystallinity and amorphous character of solid formulations. In other words it is an ideal fingerprinting technique to evaluate compatibility between any physical mixtures.

2.3.5. Scanning electron microscopic studies (SEM)

The morphological properties of FA powder were characterized by scanning electron microscopy (JEOL JSM-6360, Japan). A dried sample of FA was placed on an electron microscope brass stub and coated with gold in an ion sputter. Digital images of FA were taken by random scanning of the stub at 100, 300, and 500 X magnifications.

2.3.6. Elemental analysis by SEM-EDX

Elemental analysis of FA sample was performed by using scanning electron microscopy SEM-EDX (JEOL JSM-6360, Japan). The sample was subjected to an electron beam with primary energies of 10–20 keV which produces an x-rays that are characteristic of the elements found in the sample. SEM-EDX is equipped with auto-identification software that enables to ascertain the peaks and calculate the atomic percentage of each element detected.

2.4. Compatibility study with other excipients

FA along with different excipient were sealed in clear glass vials (1:1

w/w), were punctured to facilitate exposures to humidity conditions. These were then charged into stability chambers (40 ± 2 °C/ $75 \pm 5\%$ RH) for 1 month. The vials were inspected periodically to observe any change in appearance. Finally after 1 month, FTIR, DSC and XRD analysis were carried out as afore mentioned to observe any chemical and physical changes (Balestrieri et al., 1996; Stulzer et al., 2008).

2.5. Stability studies

Stability studies were carried out according to ICH guidelines' (ICH Q1A R2) Zone IVb recommendations (long term - 25 ± 2 °C/ $60 \pm 5\%$ RH and accelerated- 40 ± 2 °C/ $75 \pm 5\%$ RH). The samples were securely packed in high density polyethylene (HDPE) bottles and kept in stability chambers (M/s. Remi Instruments Ltd, Mumbai, India). As per ICH stability guidelines, FA was subjected to accelerated studies and analyzed at regular intervals (0, 1, 2, 3 and 6 months) for appearance, pH, moisture content, FTIR and DSC.

2.6. Microbial count studies

Microbial count was performed as outlined in Indian Pharmacopeia 2000 edition for total aerobic count, fungal count and pathogenic microorganisms. The plate count method used to estimate the number of viable bacteria and fungi cells in the sample. The media used were sterilized liquefied agar and potato dextrose agar medium for bacteria and fungi respectively. Solution of 1 g/mL of FA was placed into each liquefied sterilized agar medium and the medium was poured into Petri plate and allowed to solidify. The plates were kept at 5–10 °C for 1hr and were incubated at 37 °C for 18 h. The number of bacterial colonies formed in each plate after the incubation period was counted. Fungal count was determined in similar procedure using potato dextrose agar medium and the plates were incubated at room temperature (20–25 °C) for a period of 5 days. The presence of pathogenic microorganisms in the FA was estimated using specific individual media like mannitol salt agar medium (*Staphylococcus aureus*), cetrimide agar (*Pseudomonas aeruginosa*), MacConkey agar medium (*Escherichia coli*) and deoxycholate citrate agar medium (*Salmonella* sp.).

2.7. Toxicity studies

2.7.1. Acute toxicity studies

Acute oral toxicity study of FA was carried out according to OECD guidelines 423. The acute toxicity is a step wise procedure, where three animals of single sex (female mice) were used per step. All three animals were fasted prior to dosing. After the period of fasting, the animals were weighed and FA was administered in a single dose i.e. 300 mg/kg and followed by 2000 mg/kg by oral gavages and observed for any sign of toxicity for first 30 min, periodically during the first 24 h, special attention was given during the first 4 h, and daily thereafter, for a total of 14 days. The work was approved by institutional animal ethical committee IAEC Ref NO 12/IAEC/SVCP/2018–19.

2.7.2. 28 days sub-acute toxic studies

Sub-acute oral toxicity studies on FA were carried out as per OECD guidelines 407, repeated dose 28-day oral toxicity study in rodents (Okazaki et al., 2001). A single dose of 1000 mg/kg was selected, 2 groups each of 10 animals (five male and five female rats) were taken. Throughout the study the animals were observed closely, each day for any sort of toxicity signs. The animals were given FA daily for a period of 28 days. Quite similar to that of acute toxicity studies, toxic effects were monitored through the study period like any sort of clinical signs and symptoms, changes in body weight, food consumption, haematological parameters, and organ weights. Finally, animals were sacrificed and histopathological examinations were carried out. All the animals from vehicle control group and treatment group were euthanized by carbon dioxide asphyxiation followed by exsanguinations. Necropsy

was carried out on all euthanized animals and subjected for gross and histopathological examination. Furthermore Livers, kidneys and Lungs were collected from all the animals and preserved in 10% neutral buffered formalin. The tissues were trimmed, processed and embedded in paraffin blocks, sectioned at 3–5 μm and stained with haematoxylin and eosin. The histopathology slides belonging to these animals were evaluated microscopically.

2.7.3. MTT assay

In MTT assay, HEK 293 were selected and seeded on to 96-well plates at 5.0×10^4 cells/well in 100 ml of a medium. The cells were incubated (37 °C, 5% CO₂ overnight to allow them to attach to the wells. After this incubation, the cultured cells were washed twice with PBS, FA dissolved in the medium was added to obtain final concentrations 5–300 $\mu\text{g/ml}$ (5, 50, 100, 150, 200, 250, 300 $\mu\text{g/mL}$) in triplicate. The cells were incubated for 24 h, and then 15 μL of 0.5 mg/mL of the MTT solution was added to each well. The cells were incubated for further 3 h at 37 °C and absorbance was measured at OD 570 nm for each well on an absorbance plate reader. Blanks DMSO were prepared at the same time to correct for the absorbance caused by sample colour and by the inherent ability of a sample to reduce MTT in the absence of the cells. The optical density of the formazan produced by the untreated control cells is considered as representing 100% viability (Riss and Moravec, 2004).

2.8. Formation of co-crystals

Co crystal were prepared by using solution methods viz. solution crystallization technique where 1:1 wt ratio of FA (co-former) and desired phytochemical were co-dissolved in water: methanol: acetone (1:0.5:0.5) and the solution was concentrated by continued boiling until the volume was reduced to half and finally the solution was allowed to cool instinctively to room temperature to crystallize in an open beaker. Several days were necessary for the solvent evaporation to obtain microcrystalline material (Namjesnik-Dejanovic and Maurice, 1997).

3. Results and discussion

3.1. Physico-chemical evaluation

In solubility studies, FA was found to be freely soluble in water at all pH. The pH, solubility in different solvents and flow properties data has shown in Table 2. It is evident that flow ability is critical parameter which has its impact not constrain to production level but also has its deleterious consequences throughout formulation process. For any successful formulation one should have awareness of particle size distribution and colloidal stability. The mobility of any particle is intensely affected by its size. While, in our SEM observations, although we noticed that the size of the given FA sample has particles size range 2.3–6.23 micrometres however they are in the form of agglomerates

Table 2

Results of Physico-chemical evaluation of FA sample. The results are sync with literature reports. The data supports its claim as pharmaceutical excipient.

S.No	Parameters	Results
1.	Solubility	Water- freely soluble at all pH Acetone- Slightly soluble Methanol- Very slightly soluble Ethanol- Very slightly soluble Ether- Insoluble
2.	pH	5.8
3.	Flow properties	Bulk density- 0.85g/cc Tapped density-1g/cc Compressibility index (I)-15% Hausner's ratio- 1.7 Angle of repose-36°

with angular to sub angular shapes may exhibit medium to high sphericity. This might be the reason for its good Carr's index, Hausner's ratio and passable angle of repose, suggesting decent flow ability.

3.2. Characterization of FA using different analytical techniques

3.2.1. Atomic force microscopic studies

Normally, AFM can show valuable information about particle morphology with higher resolution. Additionally it can provide three-dimensional surface plots. In our AFM observation, FA appeared as the platy particles with irregular shapes such as sand heaps. The height of FA was in the range from 34 nm to 59 nm; while width was in the range of 214–385 nm (Fig. 2 A). It has displayed dark spots on particle surfaces indicating the uneven nature of surface and composed of diverse components. Similar kind of observations was noticed by another group (Namjesnik-Dejanovic and Maurice, 1997).

3.2.2. Fourier transforms infrared spectroscopy studies

The FTIR spectrum of FA has been shown in Fig. 2B. It shows the presence of functional groups like alcoholic hydroxyls, aromatic and aliphatic carboxyls and carbonyls, aliphatic C–H and amides, which are in accordance with literature (Francioso et al., 1998). In our observation FA has shown a broad band at 3427.63 cm^{-1} which corresponds to O–H stretching of hydroxyl groups from phenol and alcohol and/or to those from the carboxylic groups and also to amide and amine N–H stretching. Furthermore, a peak can be seen in 2920.43 cm^{-1} , attributed to the asymmetrical C–H stretching of methyl (–CH₃) or aromatic CH-stretching (Silverstein et al., 1991). A broad band at 1645.31 cm^{-1} attributes to the carbonyl stretching of carboxylic acid or NH bending. A band was noticed at 1094.92 cm^{-1} which corresponds to C–O stretching.

3.2.3. Differential scanning calorimetry

The DSC thermogram of FA (Fig. 2 C) exhibited two endothermic peaks initially a minor curve at 60 °C (not interpreted in the graph) it corresponds to the evaporation of incorporated water or adsorbed onto FA (Kucerik et al., 2012). Subsequently, a broad endotherm at 229 °C may be attributed to the decomposition of simple and labile organic structures. An earlier report suggests that FA doesn't have a fixed and sharp melting point (Mirza et al., 2016). Nevertheless, based on the origin of humic substances the thermal behaviour can differ (Provenzano et al., 2000).

3.2.4. Powder X-ray diffractometry

In general, the diffraction pattern of FA of natural origin exhibited amorphous nature (Mirza et al., 2011a,b, 2016). In our current observation the diffractogram of FA has exhibited fine and well defined seven peaks at 2θ position 24.50, 31.36, 34.67, 39.03, 46.29, 59.36, and 70.21 respectively (Fig. 2 D). According to the reason behind crystalline peaks might be due the residues of inorganic portion derived from humin (as discussed in 3.6 and 3.7). Moreover, the sand is basically composed of SiO₂, the fine peaks may be due to the referred oxide.

3.2.5. Scanning electron microscopic studies

SEM can be of use to prove homogeneity of solid phase. FA can exhibit different possible structures by SEM analysis, influenced by factors like based on soil, method of extraction and pH (Tan, 1985). Furthermore, especially the freeze dried sample can exhibit six types of possible structures e.g., fibrous, spheroids connected by fibers, sheets interwoven by fibers, perforated sheets, bladed, and shredded sheets (Chen and Schnitzer, 1976). have explored the effect of varying the pH on the shape, size, and degree of aggregation of humic and FA particles in SEM analysis. In our SEM observations it revealed the presence of fibrous structure to platy shape (angular/sub angular shape) (Fig. 2 E).

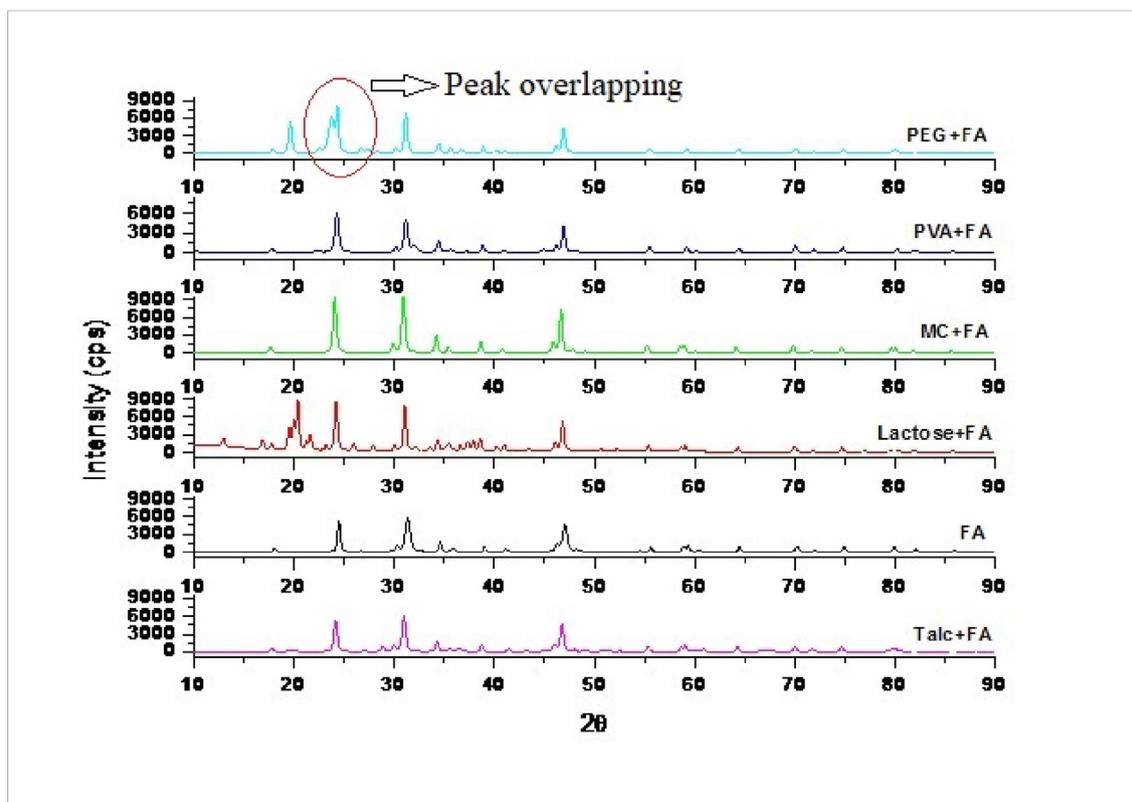


Fig. 3. XRD overlay image of results of compatibility studies with other excipients (with respect to FA) where a peak overlapping is noticed with PEG requires further investigation to prove its compatibility. The samples were kept for 30 days as per the protocol and analyzed thereafter.

observed that could lead to change in FTIR spectra, even there was no change in the peak positions or in their relative intensities (Table 3). But whereas in case of excipients of carbohydrate source like lactose, methyl cellulose have shown deviation in FTIR values of carbonyl group by $80\text{--}100\text{ cm}^{-1}$. It may be due to H-bonding or tautomerism which is a reversible in nature, quite common phenomenon in excipients with functional groups. It is a physical interaction and should not be considered as physical incompatibility. However, the presence of overlapping peaks in the spectra may hinder the overall analysis.

In Powder X-ray diffraction (XRD) analysis assists in case of incompatibilities which may occur in processes like compression, wet granulation etc. (Stulzer et al., 2008). In our study, XRD pattern of FA along with other excipients were analyzed and plotted (Fig. 3). The XRD diffraction pattern of FA had exhibited all its peaks predominantly among all the excipients mixture studied even there were no major differences in intensities and sharpness in peaks of FA in corresponding physical mixture and almost similar pattern of peaks were retained except in case of PEG where there was a superimposition effect of PEG or peak attenuation was observed on FA pattern at 24.50° , indicating its interaction with FA.

In compatibility screening studies, thermal analysis also plays a crucial role and frequently employed for the rapid assessment of physicochemical incompatibility. Over the past few decades, DSC is often considered as the principal thermal analytical technique to screen incompatibility studies. Nevertheless, it requires a lesser amount of sample and shorter time for the analysis. Generally, in this technique, the DSC thermogram of pure components is compared to that of the thermogram obtained from 1:1 physical mixtures. It is presumed that if the thermal properties (melting point, change in enthalpy, etc.) are retained the components are supposed to be compatible. Non-appearance of a curve, a substantial shift in the melting points of the components or presence of a new exo/endothemic peak and/or deviation in the corresponding enthalpies of reaction in the physical mixture

specifies incompatibility (Balestrieri et al., 1996). However, slight variations in peak shape, height and width are anticipated due to possible differences in the mixture geometry. In the present study DSC thermograms of FA along with other excipients have been shown in Fig. 4. The characteristic FA endothermic peaks in the excipients were present except with PEG where it disappears and leading to an unexpected exothermic peak. This might be due to presence of peroxide content which may accumulate on storage in PEG and affect the stability (Visser, 1987).

In summary, by the experimental studies like FTIR, DSC, and XRD data all the excipients studied were almost compatible with FA except in case of PEG 6000. We recommend further detailed investigation to validate its incompatibility with FA.

3.4. Stability studies

The stability (chemical or physical) of any drug molecule is an intrinsic property directed by its chemical structure. Perhaps the drug's stability in a finished product may depend on various other factors like manufacturing process, package, and storage conditions. The presence of other excipients also plays an important role. Generally, the excipients are originated from biological or natural sources while they may be inorganic or organic, synthetic or semi-synthetic. Most of them have functional groups that can interact with other materials. Additionally, there might be probability of presence of trace amounts of impurities that may react with the drug or other excipients and further it may manifest undesirable effects which may be toxic (formation of degradation products) or end up with compromised clinical efficacy (loss of potency).

In the six months study maximum changes were observed in accelerated study like 3.4% decrease in pH might be due to high temperature, 2.1% increase in LOD is due to loss of moisture which may exist either in the unbound state or as part of crystal structure. No

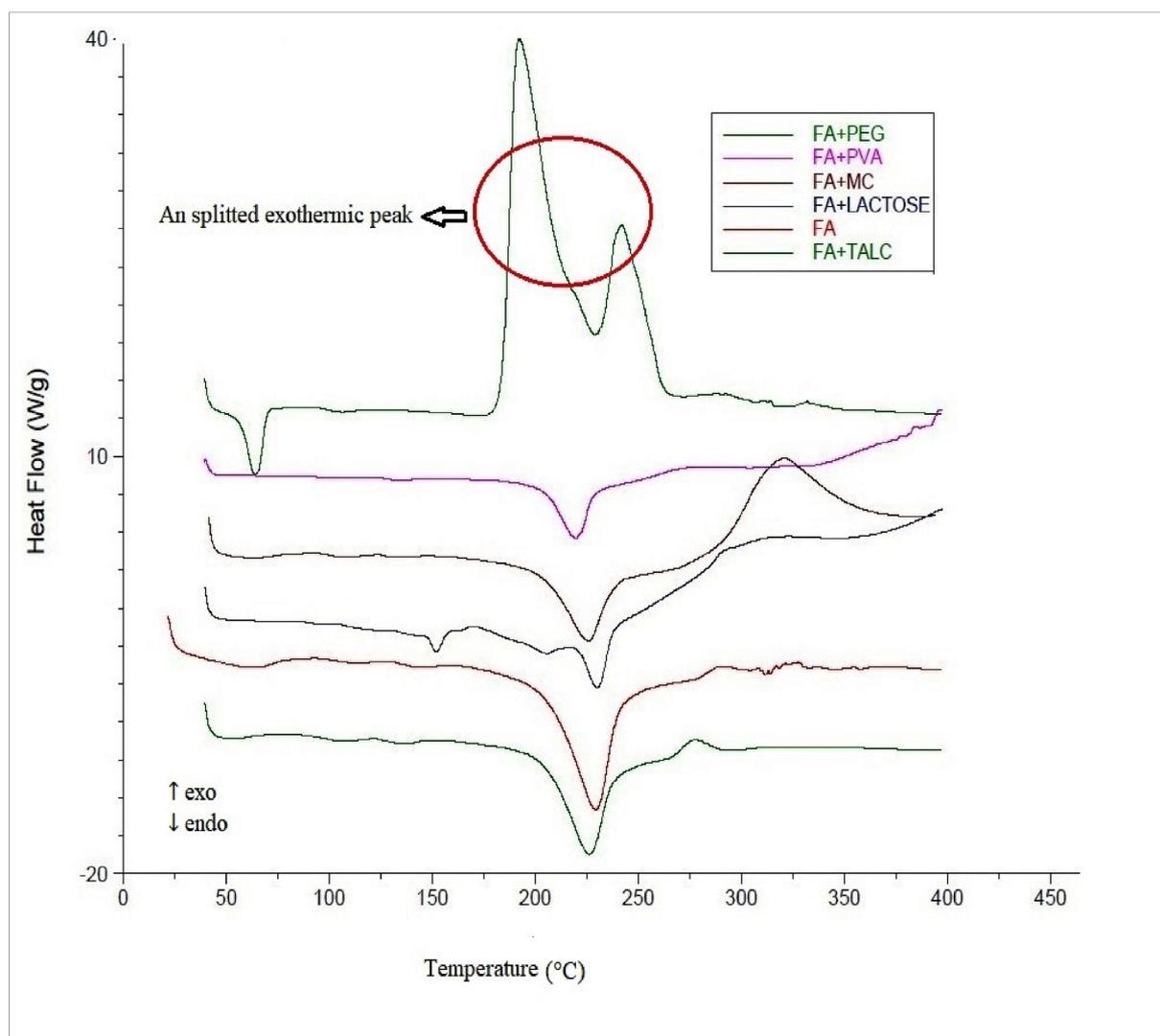


Fig. 4. DSC overlay images of results of compatibility studies with other excipients (with respect to FA). Like the results of other analysis, the FA + PEG is not in sync with others. It doesn't indicate any incompatibility but augurs further evaluation.

difference was observed in appearance, FTIR and DSC studies.

3.5. Microbial count studies

In compliance to the previous works FA exhibited absence of microbes (supplementary data Table S2). FA didn't show any pathogenic microorganisms like *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella* sp. Results clearly indicates FA doesn't support the growth of neither bacteria nor fungus. Thus microbial quality of FA was satisfactory and met the Indian Pharmacopeia requirements required for the pharmaceutical preparations.

3.6. Toxicity studies

3.6.1. Oral toxicity studies

Any new excipient has to undergo nonclinical studies (<http://academy.gmp-compliance.org/guidemgr/files/NONCLINICAL%20STUDIES.PDF>), with the key note that excipients may be potential toxicants and therefore it is essential to evaluate accordingly. It is compulsory to establish the toxicology and pharmacokinetics profile of a novel excipient and the testing approaches recommended by IPEC and FDA. Keeping this in view we have examined whether FA has the potential to be claimed as a safe pharmaceutical excipient or not. Peat

derived FA is being consumed across the world in different healthcare products, so it may be supposed safe. While from the oral toxicity studies, all the three tested mice were found to be safe even at dose of 2000 mg/kg.

3.6.2. Sub-acute toxicity studies

Sub-acute toxicity was done for 28 days, to evaluate its safety after prolonged usage. FA showed neither any toxic effect, nor any lethal effect. Additionally, no change in skin and fur, body weight, eyes and mucous membrane, respiratory, circulatory, autonomic and central nervous system, and behaviour pattern were noticed. Even there were no signs of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma throughout the experiment period. In gross pathology, no treatment related external and internal gross pathological findings were observed in any animals. In histopathology, there was no treatment related histopathological findings observed in liver, kidneys and lungs (shown in supplementary data Fig S1a, S1b and S1c).

3.6.3. MTT assay

In MTT assay generally cytotoxic potential is ascertained by characteristic features like cell shrinkage, cytoplasmic condensation, rounding and detachment from the surface and changes in filopodial length etc. whereas in case of FA against HEK 293 cell lines no such

changes were noticed moreover cells remained to be viable even after 72 h up to the concentration of 500 µg/ml. Results clearly indicated that FA doesn't have any cytotoxicity suggesting its compatibility to mammalian cells.

3.7. Formation of co-crystals

Both binary and ternary Co-crystals were formed successfully with few phytochemicals examined (data not presented). We are suggesting co-crystal synthesis of FA with phytochemicals having poor water solubility. Hence in future FA can be utilized as co-former in supramolecular synthesis.

4. Conclusion

Under current drug approval processes, novel excipients are not independently evaluated by the regulatory agency; they are only reviewed in the context of the first drug application containing the excipient. There are DMFs of excipients in USFDA. There is no regulatory approval process specifically for a new excipient as a unique molecule. Globally, the International Conference on Harmonization (ICH) does not have specific excipient safety evaluation guidelines, but FDA guidance on excipient safety evaluation and guidelines by the IPEC cite several ICH safety-testing guidelines (e.g., ICH S1A, S2B, S3A, S5A, S7A and M3) as reference materials for the conduct of safety tests. Being consumed in different dietary supplement products across the globe assures its safety. Hence we can abbreviate the safety studies. So based on the shared data we can advocate further exploration of FA as a pharmaceutical excipient.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2020.109642>.

References

- Balestrieri, F., Magri, A.D., Magri, A.L., Marini, D., Sacchini, A., 1996. Application of differential scanning calorimetry to the study of drug-excipient compatibility. *Thermochim. Acta* 285 (2), 337–345. [https://doi.org/10.1016/0040-6031\(96\)02904-8](https://doi.org/10.1016/0040-6031(96)02904-8).
- Botes, M.E., Gilada, I.S., Snyman, J.R., Labuschagne, J.P., 2018. Carbohydrate-derived fulvic acid wellness drink: its tolerability, safety and effect on disease markers in pre-ART HIV-1 positive subjects. *S. Afr. Fam. Pract.* 60 (3), 91–96. <http://hdl.handle.net/2263/68622>.
- Chen, Y., Schnitzer, M., 1976. Scanning electron microscopy of a humic acid and of a fulvic acid and its metal and clay complexes 1. *Soil Sci. Soc. Am. J.* 40 (5), 682–686. <https://doi.org/10.2136/sssaj1976.03615995004000050024x>.
- Chien, S.J., Chen, T.C., Kuo, H.C., Chen, C.N., Chang, S.F., 2015. Fulvic acid attenuates homocysteine-induced cyclooxygenase-2 expression in human monocytes. *BMC Compl. Alternative Med.* 15 (1), 61. <https://doi.org/10.1186/s12906-015-0583-x>.
- Francioso, O., Sanchez-Cortes, S., Tugnoli, V., Ciavatta, C., Gessa, C., 1998. Characterization of peat fulvic acid fractions by means of FT-IR, SERS, and ¹H, ¹³C NMR spectroscopy. *Appl. Spectrosc.* 52 (2), 270–277. <https://doi.org/10.1366/0003702981943347>.
- Gandy, J.J., Snyman, J.R., Van Rensburg, C.E., 2011. Randomized, parallel-group, double-blind, controlled study to evaluate the efficacy and safety of carbohydrate-derived fulvic acid in topical treatment of eczema. *Clin. Cosmet. Invest. Dermatol.* 4, 145. <https://doi.org/10.2147/CCID.S23110>.
- Gandy, J.J., Meeding, J.P., Snyman, J.R., van Rensburg, C.E., 2012. Phase 1 clinical study of the acute and subacute safety and proof-of-concept efficacy of carbohydrate-derived fulvic acid. *Clin. Pharmacol.: Adv. Appl.* 4, 7. <https://doi.org/10.2147/CCID.S23110>.
- Hartauer, K.J., Guillory, J.K., 1991. A comparison of diffuse reflectance FT-IR spectroscopy and DSC in the characterization of a drug-excipient interaction. *Drug Dev. Ind. Pharm.* 17 (4), 617–630. <https://doi.org/10.3109/03639049109044268>.
- Kiprop, A.K., Marie-Camille, J., Pourtier, E., Kimutai, S., Kirui, S., 2013. Synthesis of humic and fulvic acids and their characterization using optical spectroscopy (ATR-FTIR and UV-Visible). *Int. J. Appl. Sci. Technol.* 3 (8), 28–35.
- Kucerik, J., Bursakova, P., Prusova, A., Grebikova, L., Schaumann, G.E., 2012. Hydration of humic and fulvic acids studied by DSC. *J. Therm. Anal. Calorim.* 110 (1), 451–459. <https://doi.org/10.1007/s10973-011-2178-1>.
- Mirza, M.A., 2018. Future of humic substances as pharmaceutical excipient. *Pharma. Sci. Anal. Res J* 1 (1), 180004.
- Mirza, M.A., Agarwal, S.P., Iqbal, Z., 2011a. Effect of fulvic acid on oral delivery of Carbamazepine. *Sci. Adv. Mater.* 3 (2), 223–232. <https://doi.org/10.1166/sam.2011.1149>.
- Mirza, M.A., Ahmad, N., Agarwal, S.P., Mahmood, D., Anwer, M.K., Iqbal, Z., 2011b. Comparative evaluation of humic substances in oral drug delivery. *Results Pharm. Sci.* 1 (1), 16–26. <https://doi.org/10.1016/j.rinphs.2011.06.001>.
- Mirza, M.A., Talegaonkar, S., Ahmad, F.J., Iqbal, Z., 2016. A novel and multifunctional excipient for vaginal drug delivery. *J. Excipients Food Chem.* 2 (4), 1119.
- Namjesnik-Dejanovic, K., Maurice, P.A., 1997. Atomic force microscopy of soil and stream fulvic acids. *Colloids Surf., A* 120 (1–3), 77–86. [https://doi.org/10.1016/S0927-7757\(96\)03678-3](https://doi.org/10.1016/S0927-7757(96)03678-3).
- Okazaki, K., Okazaki, S., Nishimura, S., Nakamura, H., Kitamura, Y., Hatayama, K., Nakamura, A., Tsuda, T., Katsumata, T., Nishikawa, A., Hirose, M.A., 2001. Repeated 28-day oral dose toxicity study of methoxychlor in rats, based on the 'Enhanced OECD Test Guideline 407' for screening endocrine-disrupting chemicals. *Arch. Toxicol.* 75 (9), 513–521. <https://doi.org/10.1007/s002040100273>.
- Peña-Méndez, E.M., Havel, J., Patočka, J., 2005. Humic substances—compounds of still unknown structure: applications in agriculture, industry, environment, and biomedicine. *J. Appl. Biomed.* 3 (1), 13–24. <https://doi.org/10.32725/jab.2005.002>.
- Pharmacopoeia, J., 2002. *European Pharmacopoeia*. Council of Europe, Strasbourg.
- Provenzano, M., Ouattmane, A., Hafidi, M., Senesi, N., 2000. Differential scanning calorimetric analysis of composted materials from different sources. *J. Therm. Anal. Calorim.* 61 (2), 607–614. <https://doi.org/10.1023/A:1010146207459>.
- Riss, T.L., Moravec, R.A., 2004. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev. Technol.* 2 (1), 51–62. <https://doi.org/10.1089/154065804322966315>.
- Rodríguez, N.C., Urrutia, E.C., Gertrudis, B.H., Chaverri, J.P., Mejía, G.B., 2011. Antioxidant activity of fulvic acid: a living matter-derived bioactive compound. *J. Food Agric. Environ.* 9 (3&4), 123–127. <https://doi.org/10.1234/4.2011.2236>.
- Senesi, N., 2010. Humic substances as natural nanoparticles ubiquitous in the environment. In: *Molecular Environmental Soil Science at the Interfaces in the Earth's Critical Zone*. Springer, Berlin, Heidelberg, pp. 249–250. https://doi.org/10.1007/978-3-642-05297-2_72.
- Shishir, M.R.I., Taip, F.S., Aziz, N.A., Talib, R.A., 2014. Physical properties of spray-dried pink guava (Psidiumguajava) powder. *Agric. Agric. Sci. Procedia* 2 (7), 74–81.
- Silverstein, R.M., Bassler, G.C., Morrill, T.C., 1991. *Infrared spectroscopy*. In: *Spectrometric Identification of Organic Compounds*. John Wiley & Sons Inc, New York, pp. 91–164.
- Stulzer, H., Rodrigues, P., Cardoso, T., Matos, J., Silva, M., 2008. Compatibility studies between captopril and pharmaceutical excipients used in tablets formulations. *J. Therm. Anal. Calorim.* 91 (1), 323–328. <https://doi.org/10.1007/s10973-006-7935-1>.
- Tan, K.H., 1985. Scanning electron microscopy of humic matter as influenced by methods of preparation. *Soil Sci. Soc. Am. J.* 49 (5), 1185–1191. <https://doi.org/10.2136/sssaj1985.03615995004900050023x>.
- Visser, S.A., 1987. Effect of humic substances on mitochondrial respiration and oxidative phosphorylation. *Sci. Total Environ.* 62, 347–354. [https://doi.org/10.1016/0048-9697\(87\)90521-3](https://doi.org/10.1016/0048-9697(87)90521-3).
- Winkler, J., Ghosh, S., 2018. Therapeutic potential of fulvic acid in chronic inflammatory diseases and diabetes. *J. Diabetes Res.* 2018. <https://doi.org/10.1155/2018/5391014>.