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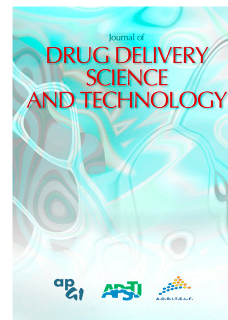

Pharmacokinetic evaluation of fulvic acid-ketoconazole complexes: A validation and line extension study

Rahmuddin khan, Pooja Jain, Mohd. Aqil, Suraj P. Agarwal, Mohd Aamir Mirza, Zeenat Iqbal

PII: S1773-2247(19)31623-5

DOI: https://doi.org/10.1016/j.jddst.2019.101469

Reference: JDDST 101469

To appear in: *Journal of Drug Delivery Science and Technology*

Received Date: 23 October 2019

Revised Date: 9 December 2019

Accepted Date: 14 December 2019

Please cite this article as: R. khan, P. Jain, M. Aqil, S.P. Agarwal, M.A. Mirza, Z. Iqbal, Pharmacokinetic evaluation of fulvic acid-ketoconazole complexes: A validation and line extension study, *Journal of Drug Delivery Science and Technology* (2020), doi: https://doi.org/10.1016/j.jddst.2019.101469.

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CRediT author statement

Contribution of each and every author in this research is listed below-

Rahmuddin Khan- Conceptualization, methodology, Investigation & Data Curation

Pooja Jain- Software, validation, Writing - Original Draft, Writing- Review and editing & Formal analysis.

Mohd. Aqil- Resources & Supervision

Suraj P. Agarwal - Supervision

Mohd Aamir Mirza[*]- Project administration & Funding acquisition

Zeenat Iqbal- Supervision, Resources & Project administration

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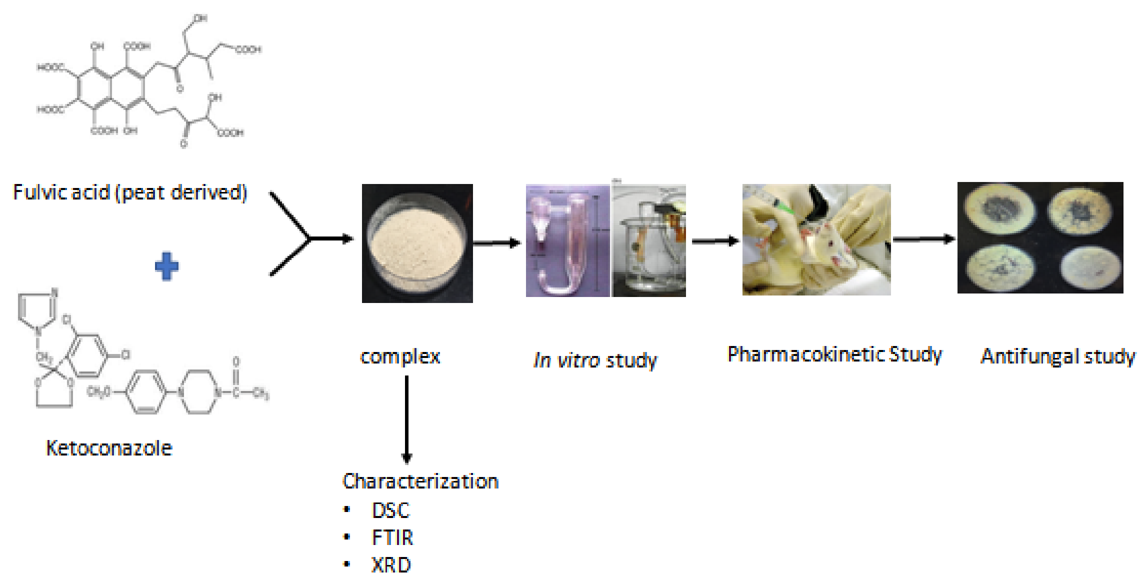

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Pharmacokinetic evaluation of Fulvic acid-Ketoconazole complexes: a validation and line extension study

Rahmuddin khan, Pooja Jain, Mohd. Aqil, Suraj P. Agarwal, MohdAamir Mirza*, Zeenat Iqbal*

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Abstract

Pharmaceutical product development is a challenging field and it requires regular introduction of new technology and excipients, in a cost effective manner. One such technology, complexation of drugs using humic substances is being tested across the globe. Complexation has already proven its suitability as evident in different available commercial pharmaceutical products but the humic substances (Fulvic acid and humic acid) have yet to address the major impediments of commercial success like its potential across a wide range of challenging drugs, availability of consistent quality material at cheaper rate and safety issues. This manuscript is a part of a series of projects where on one hand our research group is testing different classes of drugs and on the other hand exploring more commercially viable sources of humic substances, thus accruing the

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data and validating the previous results. Complex of humic acid (obtained from Indian Shilajit) and ketoconazole has already been published; now we share the data of complex of Fulvic acid (obtained from New Zealand peat) with ketoconazole, which appears a better alternative in solubility/bioavailability enhancement.

Keywords- Solubility; Permeability; Bioavailability; Complexation; Humic substances; Antifungal; Pharmacokinetics,

Introduction

Humic substances like humic acid (HA) and fulvic acid (FA) have been used in drug delivery for various potentials [1]. Formation of complexes and thereby addressing the solubility/bioavailability issues of BCS class II and IV drugs is a major area where FA and HA are being tested [2]. These macromolecules are supposed to form complexes (mainly inclusion type) with low soluble drugs. Poor aqueous solubility of the active pharmaceutical ingredient (API) is a persistent problem, only to be intensified by the advances in drug design techniques [3]. Interestingly, the paradigm of this long existing problem is now shifting to a point where some of the new chemical entities (NCE) have both poor 'aqueous and organic' solubility [4]. In this case inclusion complexes appear to be a good alternative.Advocacy for humic substances (mainly FA) to explore as pharmaceutical excipient is based on the premise like- abundant presence of raw material across the globe at a cheaper rate, obtained from different sources and no safety concern as it is already being consumed as dietary supplement.

This manuscript deals with several challenges of drug delivery. It addresses the poor aqueous solubility of Ketoconazole (KTZ) by the development of inclusion complexes, tests the alternative source of FA (New Zealand peat, other than shilajit as used before) and generates a data set in comparison to HA for the same experimental design. Our group has been collating data on different facets of a larger concept i.e., bolstering the claim to explore humic substances in commercial drug products. We have already tested the complexes of KTZ and HA (obtained from Shilajit) [5].From commercial point of view, Shilajit can't be an acceptable source of raw material, hence switching over to peat (obtained from the mines of New Zealand). Furthermore, in our series of experiments we have observed that FA has pharmaco-technical superiority over HA. So in the given project we have amalgamated the goodness of humic substances (i.e., FA) with commercial viability (source change).

Materials &Methods

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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)which is claimed to be an aqueous solution of FA. Ketoconazole was provided by Jubilant life sciences (Noida, India) as gift sample. All other chemicals and reagents used in the study were AR grade.

Phase solubility study- Phase solubility study was carried out at room temperature (25 ±2°C) in triplicate manner as per the reported method [6]. Excess amount of KTZ was added into distilled water containing various concentrations (2–12 % w/v) of FA in a series of stopper glass vials and shaken for 48 h in a biological shaker. The suspensions were then passed through membrane filter (0.45μm) and analyzed for KTZ using UV spectroscopy at 226 nm against blanks prepared using same concentration of FA in distilled water.

Preparation of complex- From the phase solubility studies optimum ratio of KTZ and FA solution was chosen to develop the drug–FA complex. 4% w/v FA solution was prepared under magnetic stirring at 200 rpm, calculated amount of drug was added into the solution and left for48 hrs.The resultant solution was frozen for 24 hrs and then lyophilized. Sucrose solution (2% w/v) was added as a cryoprotectant.The resulting mass was then powdered in glass mortar-pestle and passed through 100-mesh sieve to obtain a uniform size fine powder[7][8].

In general, during freeze-drying there is a risk of distortion because of an increase in specific volume during the conversion of water to ice. Moreover, the freezing of a solution may create a concentration gradient, which can lead to non- desirable mass movements within the specimen. To overcome these disadvantages rapid freezing of thesamples has been suggested, prior to freeze-drying[9]. Simple freeze drying of FA and HA solutions normally results in material with a loose spongy structure [10] and also sheets with a laminated structure are sometimes observed [11].

Characterization of the solid complex- Following methods were followed to characterize the solid complex.

Differential Scanning Calorimetry (DSC) -The samples of KTZ, FA and complex (about 5 mg) were loaded and sealed into DSC pan. The samples were scanned between 20-350°C at a heating rate of 10°C/ min, under nitrogen atmosphere, using a differential scanning calorimeter (Perkin Elmer Pyris 6 DSC, Massachusetts, U.S.A). Low scan rates are preferable in terms of peak resolution and investigation of the sample having close peaks while high scan rates increase the sensitivity of the measurement as they lead to the exchange of heat within a comparatively short time period. Although DSC is able to monitor and quantify even minute thermal events in the sample (depending on the sensitivity of the instrument) and to identify the

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temperatures at which these events occur. But it does not directly reveal the cause of a thermal event. The exact nature of the thermal transitions is determined with complementary methods such as microscopic observations, X-ray diffraction or spectroscopic techniques to distinguish, for example, between melting, polymorphic transitions, loss of water from hydrates or decomposition of the substance.

Fourier Transforms Infra-Red Spectroscopy (FT-IR)-FTIR spectroscopic analysis of the drug, FA and complex were carried out using Potassium Bromide (KBr) pellet technique. An accurately weighed quantity of sample (5 mg) was mixed with KBr (1:1) and later converted into a pellet using hydraulic press. The pellet was scanned between4500 to 500 cm^{-1} to get the characteristic spectra.

X-Ray Diffraction (XRD) - X-ray diffraction of KTZ, FA and complex were studied by using X-Ray diffractometer (PW 1830, Phillips, Japan). The samples (1000 mg) were rotated during data collection to reduce orientation effects. XRD pattern was recorded between 2Θ = 10 to 70 at 35 kV and 30 mA.

HPLC analysis of KTZ- For the determination of KTZ in both aqueous media and plasma, RP-HPLC with C-18 silica column (Waters, 5-micron, 250 x 4.0mm) of a variable wavelength programmable PDA detector with automatic injector was used. An already reported method of analysis was used after validation studies [12]. *For aqueous media*, mobile phase comprises of Acetonitrile: Water (80:20) and wavelength of 226 nm was set. The injection volume was 20 µL and the mobile phase was pumped through the column at a flow rate of 1ml/min. The run time was set for 10 minutes, retention time was observed at 4.58 minutes and linearity was found in the range of 0.39-50 µg/mL (R^2=0.998). *Plasma analysis*- Drug was extracted from the plasma using liquid-liquid extraction method [13]. In Brief, pooled plasma sample was spiked with different concentration of drug (0.5-15µg) and vortexed for 15 minutes. Ethyl acetate was then added to the plasma samples in a ratio of 1:4 and vortexed for 10 minutes. It was then centrifuged to separate the plasma and the organic layer. The organic layer was then separated in Eppendorf tube and evaporated to dryness using nitrogen gas. The dry residue was dissolved in acetonitrile and injected into the HPLC column for analyses. For RP-HPLC analysis in plasma, mobile phase comprises of Methanol: Water (68:32) with a flow rate of 1ml/min. Wavelength of 226 nm with the run time of 10 min was set. Retention time was observed at 4.54 minutes and linearity was found in the range of 0.1-15µg/mL (R^2=0.996) [12].

Aqueous solubility determination of solid complex

Excess amount of complex was kept in amber colored bottles containing 10 ml of distilled water and stirred on thermostated mechanical shaker (Grower enterprises, New Delhi, India) at 25°C

for 5 days. Suspensions were filtered through 0.22μm "*Millipore*" filter, adequately diluted with distilled water and analyzed by UV-Visible spectroscopy.

Release of ketoconazole from the complex- Drug release study of pure drug (5 mg drug solution) and drug complex (equivalent to 5mg drug) was performed using USP II dissolution apparatus (Hanson Research SRS, USA) in 900 mL of SGF pH1.2 at 37.5±0.5 °C (75 rpm, 60 min). The study was carried out by putting the constituted suspension (5 ml) in dialysis bag (Spectra-Por dialysis bag, Sigma Aldrich, St. Louis, MO with cutoff 12000–14000 Da). The concentration of the drug in solution at various time intervals was analyzed by UV-Visible spectroscopy [8]. All dissolution studies were carried out in triplicate.

***In-vitro* everted intestinal sac permeation study**- Rats were anesthetized in CO_2 chamber. After making a midline incision in the abdomen, the small intestine was cut at two positions, at about 18 cm distal to the stomach and at about 30 cm (being the medial jejunum). This segment was then removed and ligated with silk thread to one end of a glass rod and carefully everted on the rod, rinsed with saline solution and then cut and secured to the tip of a 1 ml disposable syringe barrel. The gut sac was filled with the modified Tyrode solution and was then placed inside the bath containing 100 ml of modified Tyrode solution (isotonic to intestinal fluid) continuously bubbled (95% O_2 and 5% CO_2*)*. After stabilization 2 ml of complex solution (equivalent to about 1mg drug), and pure drug were added separately into the sac. The tubes were maintained at 37 °± 2C and shaken continuously on magnetic stirrer at 60 rpm with bubbling oxygen supply.1ml Samples were withdrawn at an interval of 0, 15, 30, 45, 60, 75 and 90 minutes from the dissolution medium (modified Tyrode solution). After filtering through Millipore filter (0.45 μm) these were analyzed by HPLC method [14].

Pharmacokinetics study Healthy male Wistar rats (180–200 g) were obtained from the Central Animal House Facility of Jamia Hamdard, and were divided into three groups (n = 6) and placed in plastic animal cages with a 12hr light/ dark cycle (25±2°C) in accordance with the animal facilities guidelines (Approval No. 1426). The rats were provided water ad libitum and fed standard rat chow diet. The animals were acclimatized to laboratory conditions for a week prior to experiments and fasted for 12 h before the experiment, while water was allowed ad libitum. All the groups Group were treated with normal saline orally for 6 days and then Group 1 received pure KTZ (40 mg/kg p.o.) whereas Group 2 received KTZ complex (equivalent to KTZ 40 mg/kg p.o.) on day 7. Group 3 rats were treated with normal saline for 7 days. Blood samples were collected from jugular vein in tubes containing di-sodium EDTA at 0, 1, 2, 4 and 8h after KTZ administration. Plasma was separated by centrifugation at 2500 rpm for 10 min and

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transferred to pre-labeled Eppendorf tubes for subsequent simultaneous analysis of KTZ by RP-HPLC [15].

***In-vitro* antifungal efficacy study**

Agar cup-plate method was used to determine *in vitro* antifungal activity against *Candida albicans* and *Tricophytonrubrum*. Nutrient agar plates were prepared and sterilized by autoclaving at 120 ºC, 15 pounds pressure for 15 min.30 mL nutrient agar media was then inoculated with fungal strain i.e. *Candida albicans and Tricophytonrubrum* (2 mL of inoculum to 100 mL of nutrient agar media). The mixture was then poured in two sterilized petriplates and a well of 5 mm diameters was prepared via sterile borer in each petriplate. Stock solutions of 25 µg/mL of pure ketoconazole, complexed ketoconazole and fulvic acid were prepared in Methanol.0.1 ml of stock solution was then pipetted onto the strains (T. rubrum and C. albicans).The prepared petri plates were incubated at 28°C and zone diameters were measured after 24,48 and 120 hr to estimate the marked reduction in colony sizes.The diameter of zone of inhibition surrounding each of the well was recorded using Antibiotic zone reader, HICON, New Delhi[16].

Results

Phase Solubility Behavior- Phase solubility was performed in triplicate manner and the Figure.1 show the pattern of drug solubility with increasing concentration of FA. At 4%w/v concentration of FA, amount of KTZ solubilized was the highest, after which there has not been increase in solubility. So, it was selected for further studies. In the FA obtained from Shilajit, the maximum solubility of KTZ was observed at 1.5% of FA andafter that a plateau was obtained. Thus, solubility enhancement property of FA was found at a higher concentration (4%) in peat derived FA, which may be attributed to the purity of FA. In commercial scale extraction of FA, heavy metals and earth material related impurities are minimized. On the other hand, the certificate of analysis (CoA) of peat derived FA indicates the presence of some elements (Ca, P, Mn, Mg, K, Na etc). These were organic in nature and were not removed owing to its health benefits in dietary supplement. Presence of elements along with FA is expected to adversely affect the complexation capacity of FA hence after removal of these elements we may expect a further enhancement of solubility profile of FA.

Formation of micelle may be another mechanism for the solubility enhancement (other than complexation). FA concentration is a critical factor for the aggregation. When the concentration is increased, the system gives rise to the formation of aggregates. No aggregation takes place when the molecule is ionized. The ionized FA has a higher electric negative charge, which increases the energetic barriers and inhibits the approximation of FA caused by the Brownian

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movement [17]. The non-ionized aggregated species are more stable than single ones because of the increment in their interaction due to H-bonding and non-bonding forces. So, all these factors play a cumulative role in demonstration of solubility enhancement property of FA.

Characterization of the complex- The complex was characterized by different techniques and the results are as follows.

Differential Scanning Colorimetry (DSC)- DSC spectra of KTZ and FA displayed peaks at 153.968°C and223.257°C, respectively. This indicates the crystallinity of the samples. The peak of KTZ disappeared in the complex as shown in Figure2.This suggests the interaction of two entities and loss of crystalline pattern. It appears that the KTZ-FA complex has some physical interaction and a different pattern of lattice arrangement has developed. Melting enthalpy (ΔH value) of developed complex (2309.011 j/g) was found to be between FA (3567.080 j/g) and KTZ (824.456 j/g). ΔH value is a characteristic of crystal order if the impurities influence could be ignored. Furthermore, an increased melting range could be correlated with impurities or less ordered structure. A substantial expansion in melting range in KTZ-FA complex can also be observed. So, from both the values (ΔH and melting range) it can be inferred that KTZ-FA is less ordered. The CoA of the FA also shows the presence of some elements.
Generally, there is a shift in melting temperature when a new physically interacted state forms due to the formation of different particle size and its distribution. It is explained by Gibbs–Thomson equation which in itself is derived form of the Kelvin equation. It reflects the decrease in melting temperature for a particle of given size compared with the bulk material and becomes particularly more pronounced in the nanometre size range:

$$\ln \frac{T}{T_0} = -\frac{2\gamma_{sl} V_S}{r \Delta H_{fus}}$$

Where, T is the melting temperature of a particle with radius r, T_0 is the melting temperature of the bulk material at the same external pressure, γ_{sl} is the interfacial tension at the solid–liquid interface, Vs is the specific volume of the solid, ΔH_{fus} is the specific heat of fusion. In our case there was a very little change in melting point of KTZ-FA complex as compared to FA, 220.55°C and 223.25°C respectively. So, it appears that the size of both are not significantly different, may be a case of entrapment of KTZ inside FA. The electron microscopic data reported by Schnitzer and Kodama [18] also indicates the same i.e., a relatively "open" structure, perforated by voids of varying dimensions which can trap or fix organic and inorganic compounds that fit into the voids, provided that the charges are complementary.

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Fourier Transforms Infra-Red Spectroscopy **(FTIR)** **-**Pure ketoconazole as shown in figure3 displayed characteristic peaks of C=O stretching vibration of carbonyl group, C-O stretching of aliphatic ether group and C-O stretching of cyclic ether at 1645.28 cm^{-1}, 1041.56 cm^{-1} and 1247.94 cm^{-1} respectively.Pure FA displayed major absorption bands in the regions of 3650–3600 cm^{-1} (H-bonded OH groups), 2940–2900 cm^{-1} (aliphatic C–H stretching), 1720–1700 cm^{-1} (C O stretching of COOH), 1380–1330 cm^{-1} (C–O stretching and OH deformation of COOH) and 1099 cm^{-1} (C–O stretching of polysaccharide or Si–O of silicate impurities).Whereas, in the FTIR spectra of complex, amide stretching is slightly shifted upwards and appeared at 1680- 3473cm^{-1}. The aromatic stretching is observed at 1676 cm^{-1} and –NH stretching is observed at 3461 cm^{-1}.

X-Ray Diffraction- XRD spectra of the pure KTZ shows the crystalline nature. Two sharp peaks were observed which were present in the complex also. The XRD pattern of earlier reports (Shilajit derived FA) doesn't show any crystalline pattern. The reason for the peaks may be attributed to the presence of minerals as evident in the CoA. Presence of the peaks in the complex also, strengthens our claim.

In case of KTZ, peaks were observed at 17°, 19°, 20, 27 ° and 28° 2Ө which almost disappeared in complex as shown in figure4. So, it may be inferred that crystallinity of the KTZ has lost in the complex after the interaction of FA. Use of these two techniques (DSC and XRD) often lead to complementary information on the systems of interest and data evaluation from these methods is usually straightforward.

Aqueous solubility determination of solid complex- Aqueous saturation solubility of KTZ was found to be 13.5µg/ml. After complexation the solubility was found to be 97.335µg/ml. Hence the maximum percentage increase in solubility of the KTZ was found to be 621± 2.3 %.The percentage increase in solubility in case of Shilajit derived FAwas 645.2% to 2265.76%.With pure FA (after removal of minerals and ions present in given sample) we can expect a better solubility profile.

Release of KTZ from complex-Release study of KTZ showed that for pure drug 45% release obtained in 60 minutes, whereas for the complexed drug 82% release observed in 60 minutes as shown in the figure5. Hence the drug release was enhanced with the complexation. Thus, a better release profile was obtained. Similarly, with Shilajit derived FA ~81% drug released in 60 min.

***In-vitro* everted intestinal gut sac permeation study for KTZ-FA complex**- The permeability of optimized complex (4% w/v complex) across gut sac was significantly increased as compared

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to KTZ suspension in water in 90 minutes. For pure drug it was found to be 68% whereas for complexed drug is was 95% as shown in the Figure.6 In case of Shilajit derived FA-KTZ complex the enhancement of permeation was ~3.7 times.

Better permeation of KTZ has been observed than solubility because during determination of solubility we pass the solution through membrane filter. It should be considered that, in ultrafiltration, very large concentrations of organic matter may occur at a membrane surface, such is not the case in the bulk of the solution. Because membranes are themselves generally hydrophobic, they may even assist the dehydration process. This can result in a decrease in the size of organic matter particles which can then attain a size permitting penetration of the membrane. Two phenomena associated with this latter process are expected: some particles can remain adsorbed on hydrophobic sites lining the pores inside the membrane; when all these hydrophobic sites are saturated, the rest of the particles will pass the membrane [18]. These hindrances are not expected during intestinal permeation.

Pharmacokinetics of Ketoconazole- KTZ plasma concentrations were measured using a pre developedvalidated high-performance liquid chromatographic analytical method. The calculated parameters was area under plasma concentration-time curve (AUC) using the linear trapezoid method. The maximum plasma concentration (Cmax) and time to maximum concentration (Tmax) were determined empirically directly from the time-concentration curve. Table 1 shows the values of non-compartmental pharmacokinetic parameters of pure KTZ and complexed KTZ and figure7 shows the plasma concentration vs time curve of KTZ.

***In-vitro* antifungal efficacy study**-The prepared petri plates were incubated at 28°C and zone diameters were measured after 24,48 and 120 hr to estimate marked reduction in colony sizes. The diameter of zone of inhibition surrounding each of the well was recorded using (Antibiotic zone reader, HICON, New Delhi). It was observed that the zone of inhibition with complexed-KTZ was larger as compared to the pure KTZ as shown in the figure 8. Also, the zone inhibition with fulvic acid alone was very less. Where figure 8.a indicates zone inhibition with complexed KTZ, figure 8.b represents alone fulvic acid and figure 8.c represents alone KTZ. This indicates that the fulvic acid alone does not have promising anti-fungal activity but when complexed with KTZ it enhances the antifungal activity of the pure drug.

Discussion

Poor aqueous solubility of Ketoconazole (API) is a persistent problem since many years. This issue is prominently relevant to Biopharmaceutics Classification System (BCS) Class II and Class IV drugs. The current research work particularly aimed at resolving this issue by using the

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complexation approach using a novel peat source Fulvic acid. Although FA has been used for solubility enhancement, but peat sourced fulvic acid which is purer, cheaper and low on contaminants is yet to be exploited as a pharmaceutical excipient with improved functionality. So, the novelty of this studies relies in the fact that extra-virgin peat sourced fulvic acid is explored for its pharmaceutical potential.

Phase solubility study is primarily a basic tool to select an optimum amount of fulvic acid solution in which maximum drug was soluble and was found to be 4% (w/v) solution of FA. From the phase solubility study, a certain fixed amount of drug was dissolved in FA solution for the complex development by freeze drying method. Further the developed complex is characterized physically on the basis of DSC, FTIR and XRD. Next to this maximum aqueous solubility, *in-vitro* drug release and *ex-vivo* drug permeation studies were carried out to compare the pure drug with the complexed drug. Other than this, pharmacokinetic and pharmacodynamic studies with the pure drug and complexed drug were carried out on wistar rats. The results suggest that the complexed drug performs better than the pure drug hence the fulvic acid was successful in enhancing the solubility related properties of the pure drug, re-authenticating our previous results.

But due to the presence of high mineral impurity in the FA sample, excess amount of FA was required to solubilize the small amount of drug, this leads to development of a complex with high ratio of FA to drug. If such complex will be further converted for drug delivery purpose then it may lead to the large dosage form.

Hence there is a need to further purify the fulvic acid sample to that a better yield can be obtained.

CONCLUSION

On the basis of the above studies The results suggest that the complexed drug perform better than the pure drug hence the fulvic acid was successful in enhancing the solubility of the pure drug, re-authenticating our previous results. So, complexation of CBZ with FA could be opted as a promising tool for oral drug delivery and needs to be evaluated clinically.

DECLARATION OF INTEREST: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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ACKNOWLEDGMENTS

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors wish to thank the New Zealand Fulvic Ltd, company (New Zealand) for the generous gift of Fulvic acid (Peat derived).

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Table 1. Non-compartmental pharmacokinetic parameters of pure KTZ and complexed KTZ

Parameters	**Group 1(Pure drug) Mean±SD (N=3)**	**Group 2 (Complexed drug) Mean±SD (N=3)**
Cmax (µg/ml)	2.64±0.20	5.1 ±0.13
Tmax (h)	0.5 ±0.14	1 ±0.32
$AUC_{0\text{-}t}$ (µg/ml*h)	7.39 ±2.8	15.51±0.43

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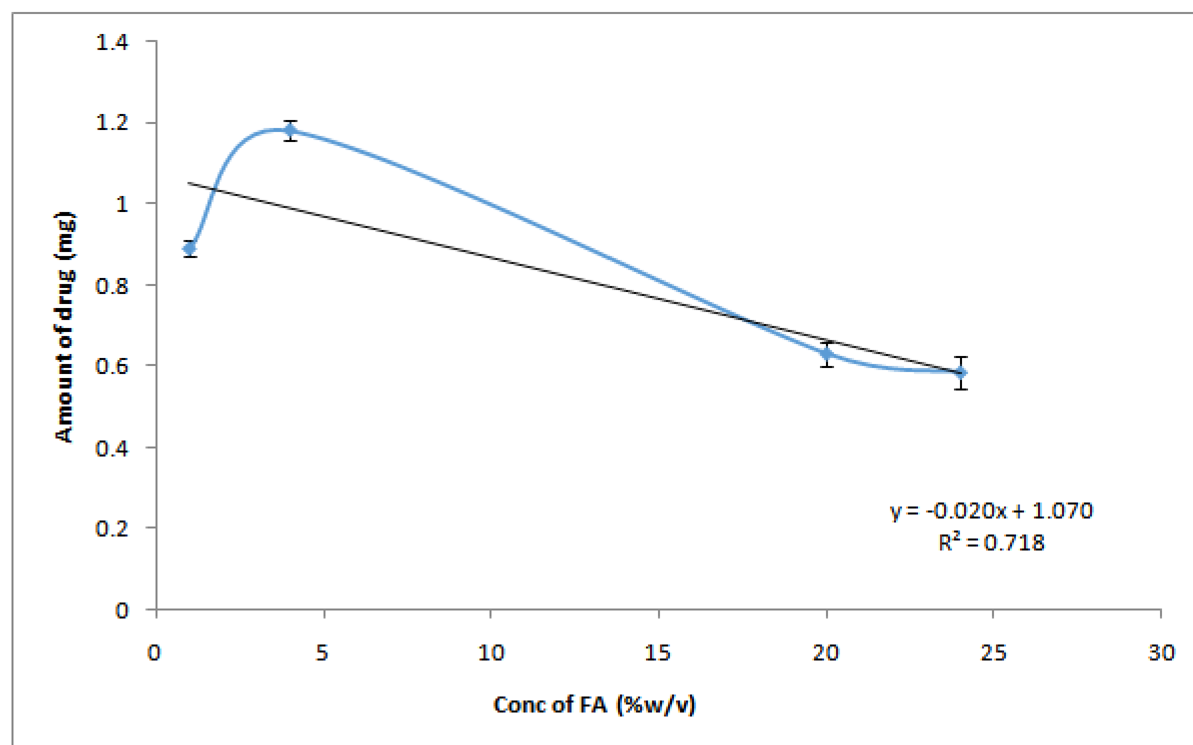

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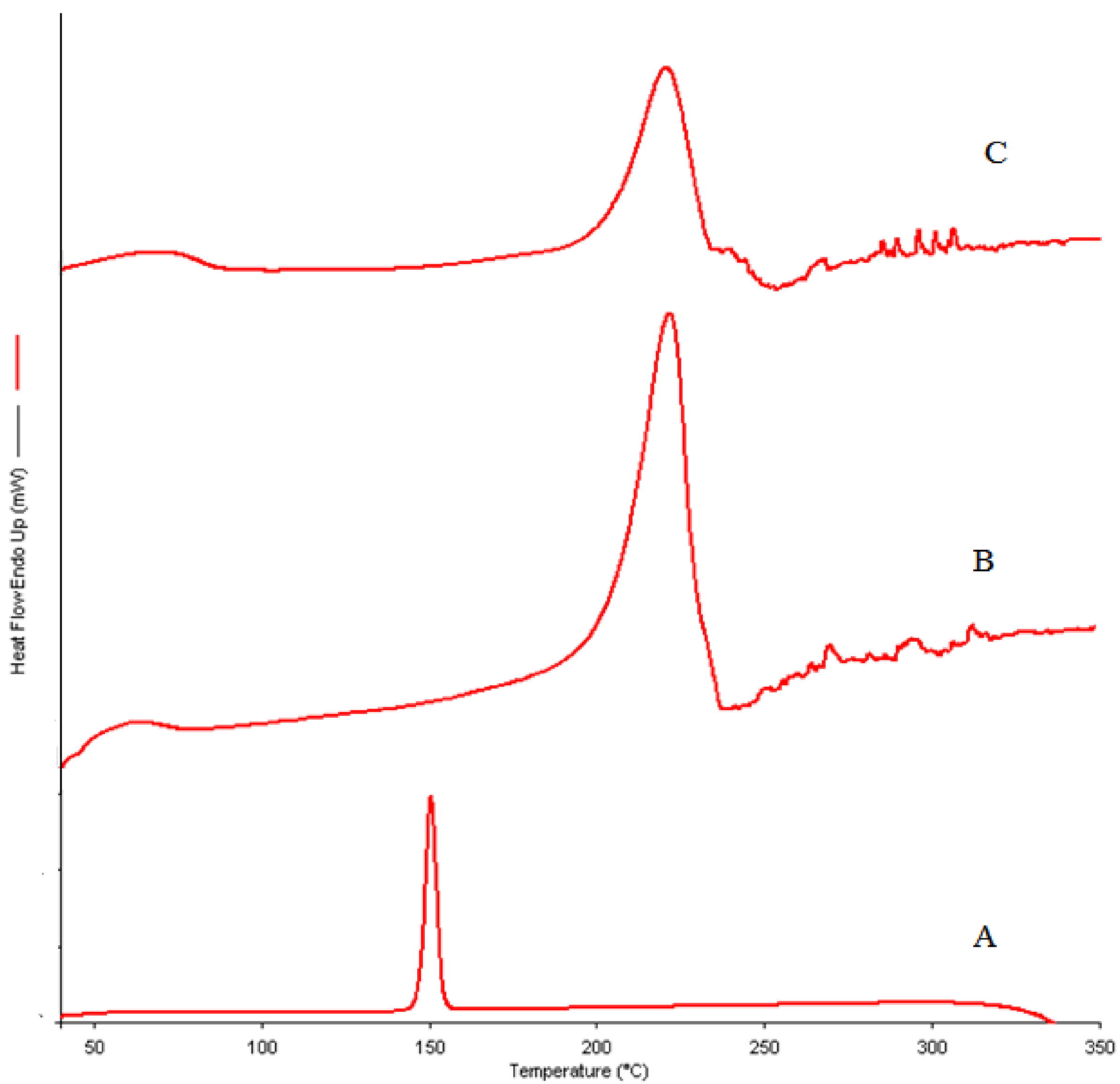
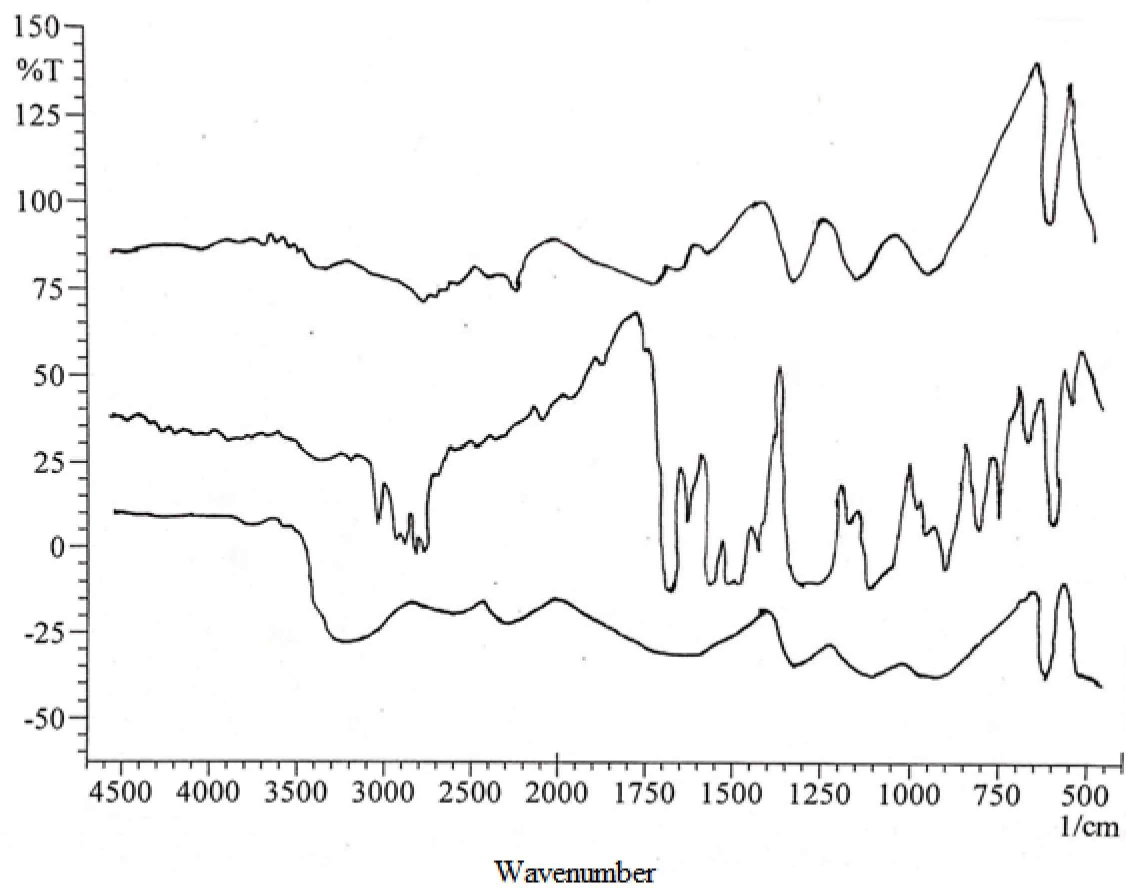

C
B
A
Heat FlowEndo Up (mW)
50
100
150
200
250
300
350
Temperature (°C)



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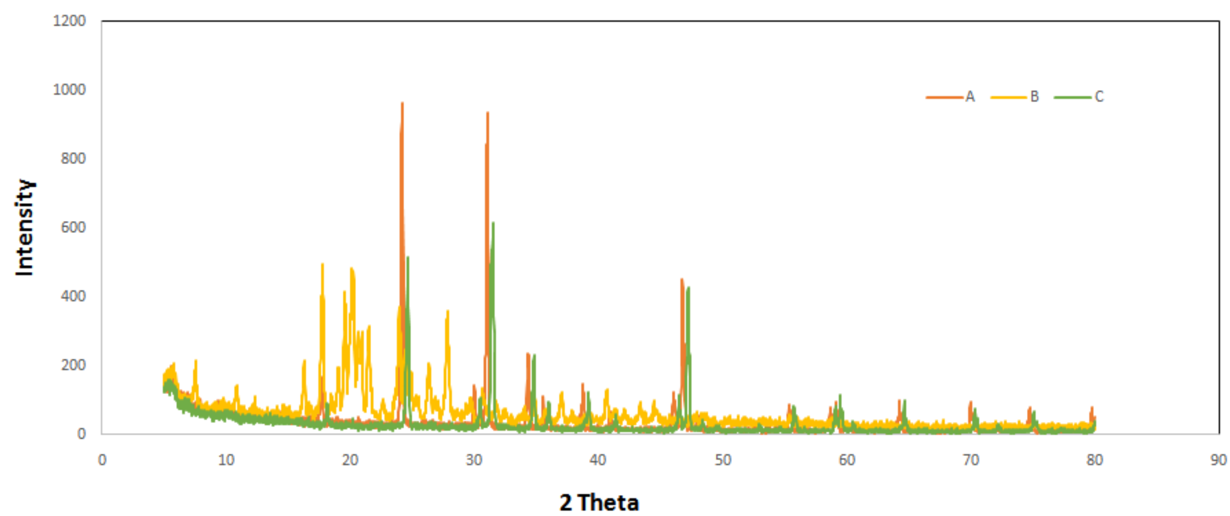

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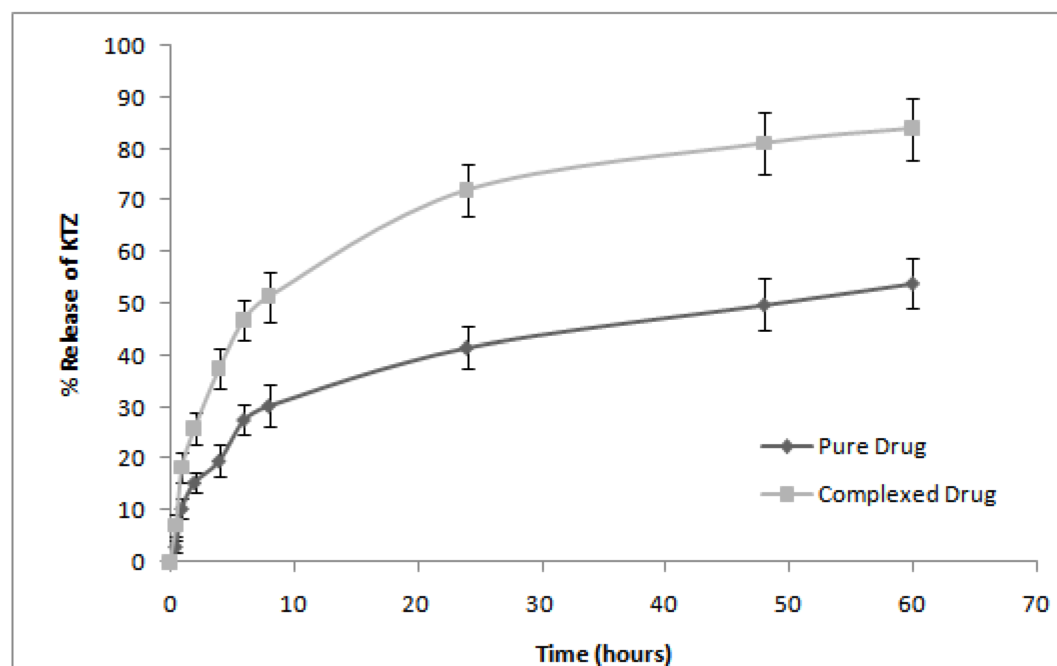

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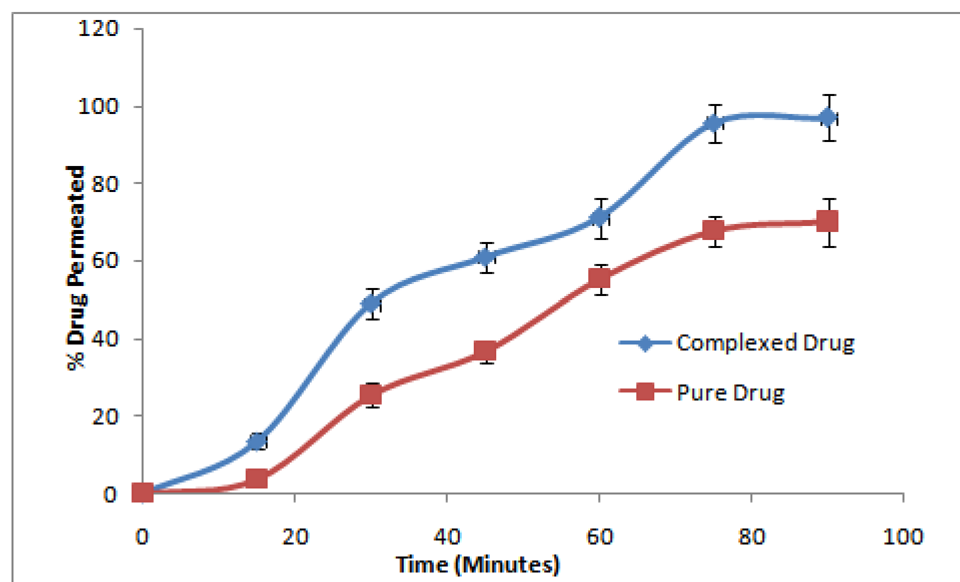

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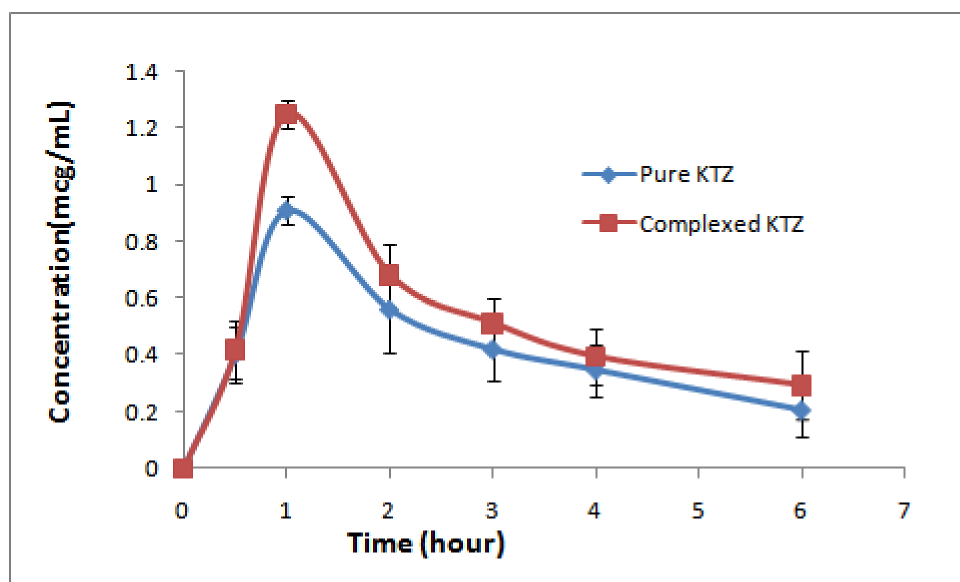

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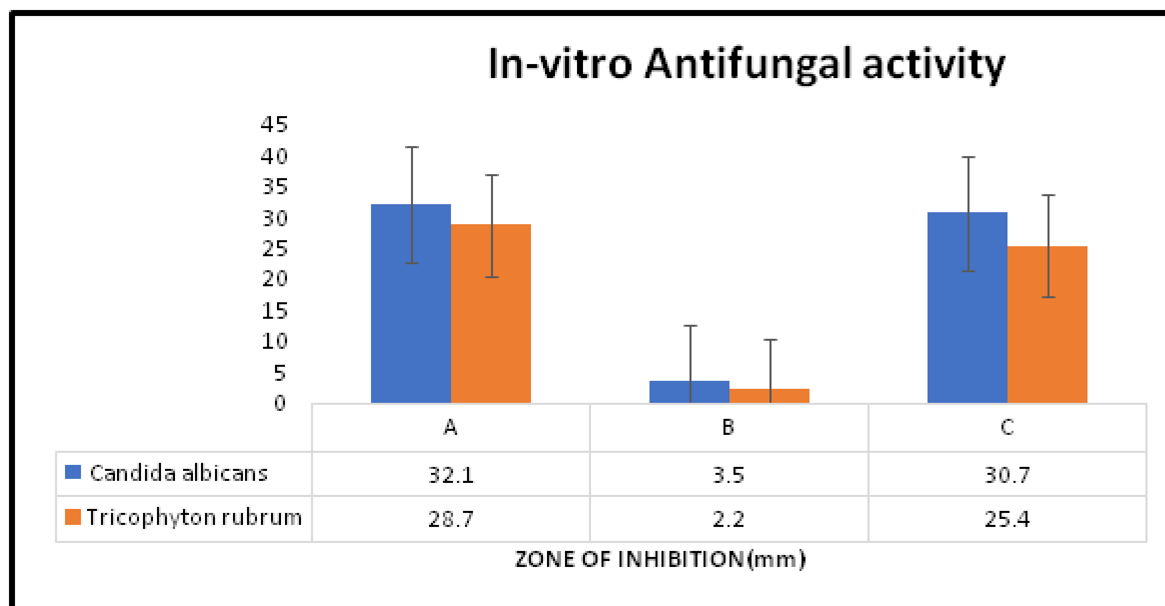

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DECLARATION OF INTEREST: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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