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Research Article

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Synthesis, Physicochemical Studies and Evaluation of Antifungal Activity of Clotrimazole Ionic Liquid.

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Abstract

The combination of cationic Clotrimazole, an antifungal drug and anionic ascorbic acid were used to synthesize active pharmaceutical ingredients based ionic liquid. The low water soluble Clotrimazole was converted into its liquid salt form to improve the physicochemical characteristics and evaluate its biological activity. The solvent evaporation method was applied to synthesize the ionic liquid using ethanol as a solvent. The analytical evidences generated by differential scanning calorimetry, Fourier transformation infrared spectroscopy and X-ray powder diffractometry confirmed the formation of ionic liquid. The increase in solubility as compared to Clotrimazole and decreased log *P* value indicated reliable improvement in physicochemical properties of ionic liquid. The synthesized ionic liquid showed the antifungal activity against the *candida albicans.*

Keywords: Clotrimazole, ionic liquid, antifungal, ascorbic acid, physicochemical.

Introduction

Clotrimazole (CTZ) is a broad spectrum antifungal agent used in the treatment of vaginal and skin infections [Sawyer et al.,1975].It is a synthetic imidazole derivative chemically known as 1-[(2-chlorophenyl) diphenyl) methyl]-1H imidazole (Fig.1) formulated in tablet and topical form [Crowley et al., 2014]. It is an FDA approved drug used in the treatment of dermatophytosis, oral candidiasis and vaginal candidiasis [Kaur et al., 2020, Amrouni et al., 2000, Tonglairoum et al., 2014]. It primarily acts by damaging the permeability barrier in the fungal cytoplasmic membrane [Haller I (1985)]. It inhibits the cytochrome P 450- lanosterol 14 α demethylase, an enzyme required in the fungal cell membrane synthesis and thus impair ergosterol synthesis leading to the cascade of membrame abnormalities in fungus [Tripathi

(2013)]. CTZ has a remarkable *in vitro* activity against *candida* spp., *Cryptococcus* spp. and other fungal genera for systemic mycoses [Paradkar et al., 2015].

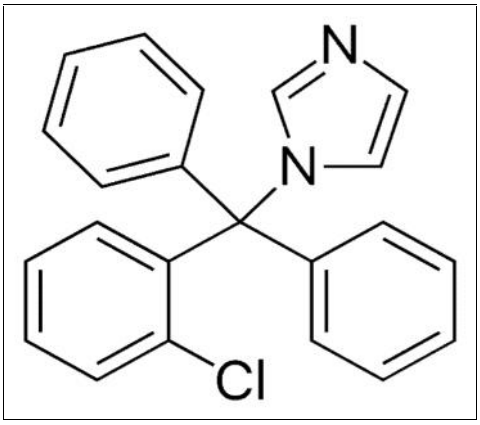

Fig. 1: Structure of Clotrimazole

CTZ is a highly lipophilic drug (log K o/w = 4.1) with low aqueous solubility (0.49 mg/ml) [Pradines et al., 2015, Balat et al., 2011]. Various techniques like solid dispersions [Balat et al., 2011], cyclodextrin complexes [Pedersena et al., 1998, Mohammed et al., 2016], soluble salts and cocrystals [Mittapalli et al., 2015] were adopted in order to enhance the solubility of CTZ. One of the techniques used for the enhancement of the physicochemical characteristics is ionic liquids (IL) [Mangrule et al., 2017]. The liquid salts synthesized by the combination of prudent choice of cations and anions are in liquid form at room temperature, depending on their chemical composition are called as ionic liquids [Romeli et al., 2014, Singh et al., 2008, Wilkes (2002), Khupse et al.,2010]. These new compounds are third generation ionic liquids named as active pharmaceutical ingredient (API) based-IL [Ferraz et al., 2011]. They have the prominent use in pharmaceutical field in order to improve physicochemical properties and biological activity [Miwa et al., 2016].

The low water solubility of API- fluconazole was enhanced by converting it into ionic liquid by using API as cation and ascorbic acid as anion [Mangrule et al., 2017]. The anionic etodolac and cationic lidocaine was combined to synthesize IL to prepare the etodolac patches to improve its skin permeation [Miwa et al., 2016]. Diclofenac, as anion and tetra butyl ammonium bromide, as cation were combined to form the diclofenac IL [Pore et al., 2017]. IL was also prepared by combining cholinium as cation and anionic APIs like nalidaxic acid, 4 amino salicylic acid, pyrazinoic acid and picolonic acid that improved physical, chemical and biopharmaceutical properties of those APIs [Araujo et al., 2014]. The literature survey exhibited that, ILs are classified into three types, first type via ionic bonding in which API used either as cation or anion, second type via covalent linkage and third type by combining similar or different APIs that produce dual activities [Egorova et al.,2017]. Hence, CTZ-IL was synthesized based on the first type of IL as no literature review reported its formation.

Therefore, the objective of present work was intended to synthesize the IL by combination of cationic CTZ and anionic ascorbic acid to improve the physicochemical properties. The solvent evaporation technique was used to synthesize the CTZ-IL. The ionic hydrogen bond formation between the imidazole ring of CTZ and carboxylic acid group of ascorbic acid lead to the formation of API based-IL. The synthesized CTZ-IL was characterized using differential scanning calorimetry (DSC), X-ray diffractometry (XRD), Fourier transformation- infrared spectroscopy (FTIR). The saturation solubility of pure CTZ and corresponding IL was determined in distilled water, conclusively confirmed by the determination of octanol/water coefficient (log *P*). The pure CTZ and its IL was further investigated for antifungal activity against *Candida albicans*.

Materials and Methods

CTZ was kindly gifted by J. Duncan Health Care Pvt.Ltd, Atgaon, Thane. Ascorbic acid was purchased from Loba chemie Pvt, Ltd. Mumbai, India. *Candida albicans* was collected from Department of Microbiology, Shivaji University, Kolhapur, Maharshtra, India. Analytical grade agents and glass distilled water was used for all experimental procedures. The substances were used without any further purification.

Synthesis of CTZ-IL:

CTZ-IL was synthesized using solvent evaporation technique. The molar ratio of 1:1 of CTZ and ascorbic acid were added to 70 ml of ethanol separately and was dissolved by sonication for 20 min. The obtained clear solution was left for 4 days for solvent evaporation. The product was collected and stored in desiccator for further analysis [Keramatnia et al., 2016]

Differential scanning calorimetry (DSC):

The thermal analysis of pure CTZ, ascorbic acid and corresponding IL was done using DSC analyzer (TA Instruments SDT Q600 USA). A 5 mg of sample was heated under a nitrogen atmosphere at a heating rate of 10^{o} C/min over the temperature range of 20-500^{o}C. The DSC thermograms were further investigated.

X-ray powder diffractometry (XRPD):

X-ray diffractometer (Bruker- D2 PHA-SER, Germany) with tube anode Cu was used to record XRPD patterns of all systems over the interval 10-90^{o}/2θ. The operational data was as follows: Generator tension (voltage) 30 kV, Generator current 10 mA.

Fourier transformation- infrared spectroscopy (FTIR):

FTIR is a sampling technique used in the conjunction with the infrared spectroscopy which enables the sample to be investigated is placed directly in the infrared beam by preparing its pellet of film. FTIR (BRUKER-ECO-ALPHA 100508) was used for the IR analysis. The samples were directly placed in infrared beam in the form of pellet and analyzed in the range of 400-4000 cm^{-1}.

Saturation Solubility Studies:

An excess amount of CTZ and its IL was added to 10 ml distilled water in the solubility tubes. These samples were shaken on a orbital shaker (BTI-05) at room temperature (25± 2^{o}C) for 24 hours until they reach equilibrium. The solution was then withdrawn, filtered through Whatman filter paper no. 41. The solution was further diluted sufficiently with distilled water. The amount of soluble drug was determined at 262 nm and that of IL at 265.20 nm by UV Spectrophotometer (Shimadzu 1780 Japan).

Determination of partition coefficient (log *P*):

The partition coefficient was estimated by adding 10 ml each of n-octanol and water in glass tubes. They were allowed to stand for 24h at room temperature. An accurately weighed 50 mg of drug and IL were added to the tubes and shaken on an incubator shaker (REMI-CIS24plus Incubator shaker, Mumbai India) for 24h at room temperature. These mixtures were then transferred to the separating funnel and allowed to stand for 4h until equilibration. The separation of organic and aqueous phase took place. The concentrations of pure drug and its IL were analyzed spectrophotometrically (Shimadzu 1780, Japan) at 262 nm and 265.20 nm respectively. The formula used to calculate the partition coefficient was,

Partition Coefficient (log *P*) = Log ($C_{Octanol}$/ C_{Water}),

Where C is the concentration of drug in octanol and / or water phase.[Jadhav et al., 2016]

Antifungal Activity:

The antifungal activity of CTZ and its IL was estimated by performing zone of inhibition using *Candida albicans* culture. The potato dextrose agar plate was used as growth media and was prepared according to the given formula. The assay was performed using the *Candida albicans* strain. The colonies of *Candida albicans* were transferred (with a sterile loop) to test tube containing 5 ml of saline solution and resulting suspension was stirred for 15 seconds. The concentration of 100 µg/ml was prepared by dissolving CTZ in methanol and its IL in distilled water. Agar well diffusion method was adopted to evaluate the zone of inhibition. The surface was inoculated by spreading a volume of microbial

inoculum over the entire agar surface. Then, a hole was punched aseptically with a sterile cork borer. The volume of 100 µL of CTZ and its IL at desired concentrations were introduced into the well with 0.7 cm diameter. These agar plates were kept for diffusion in refrigerator for 5 min. The plates were incubated at 37^{o}C for 24 h [Khan et al., 2010].

Results and Discussion

Synthesis of Ionic Liquid:

The white crystalline solid compound CTZ with melting point 148^{o}C and ascorbic acid with melting point 194^{o}C after dissolving in ethanol and complete evaporation for 4 days appeared to be sticky red colored substance in the investigated molar ratio of CTZ and ascorbic acid. This indicated the formation of API based IL as the conversion of clear solution into red colored substance is the common phenomenon of IL to be observed. Photograph of prepared IL of CTZ-ascorbic acid is shown in Fig 2.

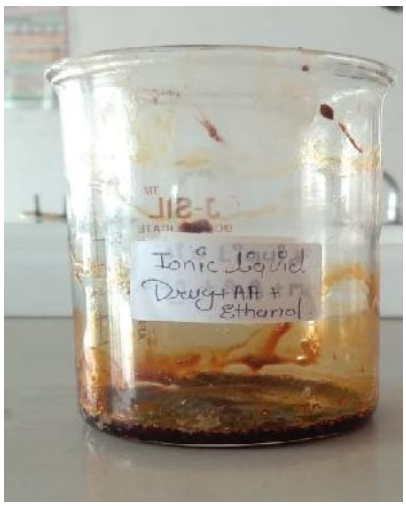

Fig.2: photograph of prepared CTZ-IL

Differential Scanning Calorimetry (DSC):

DSC plays a crucial role in the study of interaction between the API and conformers. As white crystalline CTZ was converted into IL, its melting point shifted to different temperatures or disappeared. DSC patterns of all systems are shown in fig.3.

DSC thermograms of CTZ and ascorbic acid exhibited sharp melting endothermic peaks at 145^{o}C and 194.31^{o}C, respectively. The DSC thermogram of IL did not show any peaks. This was because of conversion of solid form of drug into liquid salt form. The absence of melting endotherm stated its amorphous character.

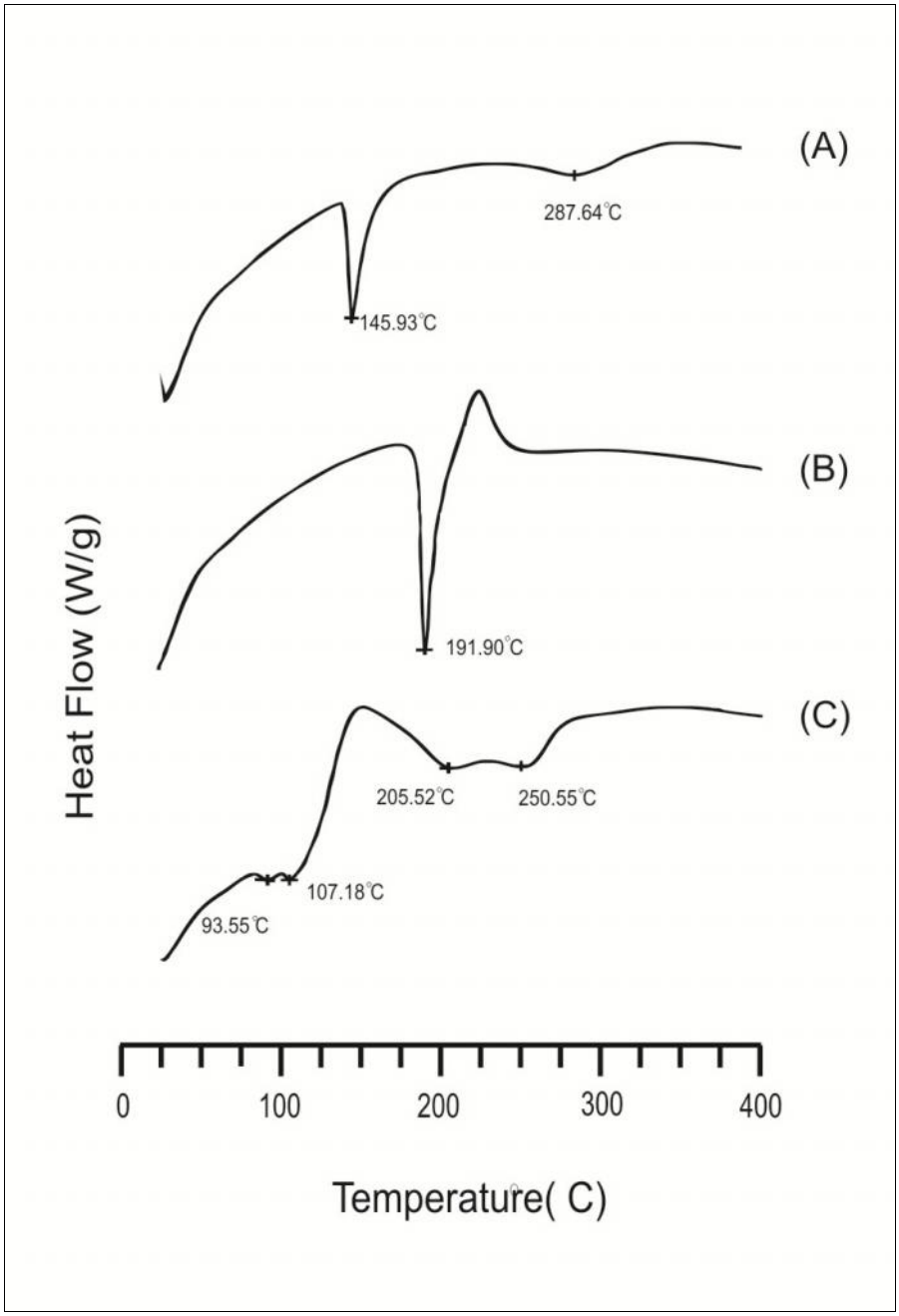

Fig. 3: DSC thermograms of CTZ (A), ascorbic acid (B), IL(C).

Fourier transformation-infrared spectroscopy (FTIR):

The possible interaction between drug and carrier was studied by FTIR spectroscopy. The IR patterns of all systems are shown in Fig 4. The principle absorption peaks of CTZ were observed at 3012.47 cm^{-1} (C-H aromatic stretch), 2891.37 cm^{-1} (C-H aliphatic stretch), 1575, 1473.92 cm^{-1}(C=C aromatic stretch), 1434.35 (C=N stretch), 1204.12 (C-N stretch), 691.58 (C-Cl). The IR spectra of ascorbic acid showed four absorption peaks of O-H groups. The major absorption peaks were observed at 3623.75, 3521.72, 3405.01, 3309.50 (OH stretch), 2907.52 (CH_2 stretch aliphatic), 1749.75 (lactone).

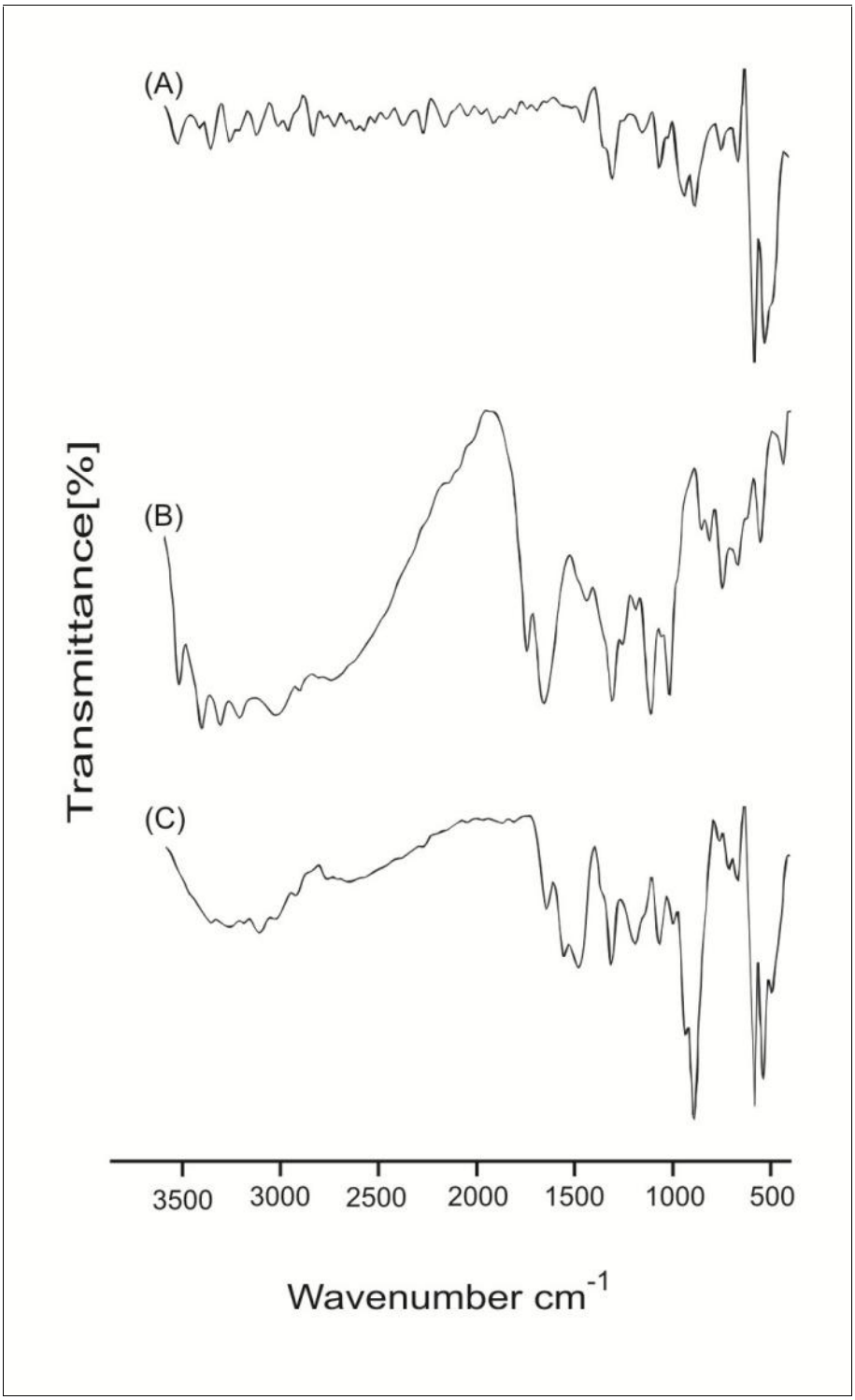

Fig.4: FTIR spectra of CTZ (A), ascorbic acid (B), IL (C)

The functional groups of CTZ (aromatic C=C, C=N, C-N, C-F) and lactone group of ascorbic acid were observed in IL and appeared to be shifted. Therefore, the absorption peaks of IL appeared at 3388.07 (O-H stretch broad), 1753.25 (lactone), 1596.96 (C=C aromatic stretch), 1439.24 (C=N stretch), 1205.42 (C-N stretch), 699.09 (C-Cl). The broad peak in IR spectra of IL indicated that heterocyclic imidazole ring of CTZ might be involved in the interaction with hydroxyl group of ascorbic acid with the transfer of proton from dissociated ascorbic acid.

X-ray powder diffractometry (XRPD):

The XRPD patterns of all samples are presented in Fig 5. The diffractogram of CTZ showed 2θ at diffraction angle of 9.33769 (250), 10.3899 (155), 16.8105 (163) 18.8935 (141), 19.5592 (312), 20.8691 (403) while that of ascorbic acid showed the angles at 10.7979 (1633), 22.8232 (503), 24.7129 (108), 30.0813 (12532), 33.8606(135). The XRPD pattern of IL exhibited different 2θ angles as compared to CTZ and ascorbic acid. The peak intensity of the IL decreased as compared to CTZ and ascorbic acid. This stated the transformation of solid form of pure drug into its liquid form which was in full agreement of thermal analysis of sample.

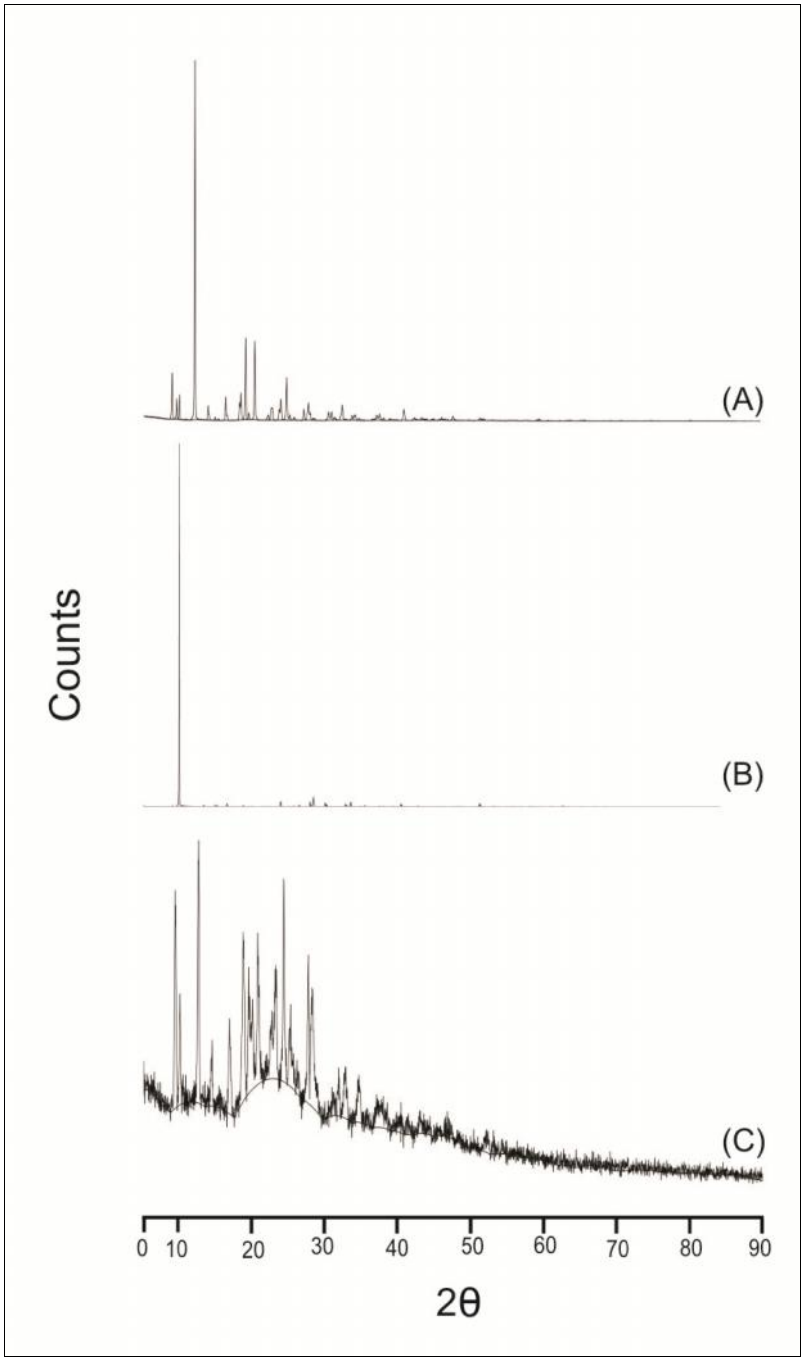

Fig.5: XRPD patters of CTZ (A), ascorbic acid (B), IL(C)

Saturation solubility studies:

The saturation solubility of pure CTZ and IL was found to be 0.52 ± 0.14 mg/ml and 4.20 ± 0.182mg/ml respectively. The synthesized IL exhibited 8.09-fold increase in solubility as compared to pure CTZ drug. The notable improvement in solubility of CTZ was imputed to its conversion into IL with decreased crystallinity, hydrophilicity of ascorbic acid and wetting property.

Partition coefficient studies:

The log *P* values of pure drug CTZ and its IL were observed as 5.4 ± 0.1 and 2.32 ± 12 respectively. The decreased log *P* value of IL as compared to CTZ clearly stated the increase in hydrophilicity of IL.

Antifungal activity:

The antifungal activity of CTZ and its IL against *candida albicans*was evaluated by measuring the diameter of zone of inhibition (Fig 6). The assay was performed in triplicate and results were expressed as Mean ± SD given in Table no. 1. The results of antifungal activity of pure drug CTZ and its IL were promising against *Candida albicans*. There was no significant difference between the antifungal activity of CTZ and its IL. The results indicated that activity of IL retained.

Fig.6: photographs of antifungal activity of CTZ and its IL

Table 1: Antifungal activity (zone of inhibition, mm) of CTZ and its IL

Organisms	Samples (Zone of inhibition in cm)	
	Ionic liquid	Clotrimazole sample
Candida albicans	3.0	3.1

*Values are expressed in mean ± SD where n=3(CTZ vs IL– No significant difference)

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