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Drug loaded essential oil microemulsions enhance photostability and evaluation of *in vitro* efficacy

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ABSTRACT

Loss of stability of pharmaceutical APIs (Active Pharmaceutical Ingredient) due to photolytic activity has been a major concern in the pharmaceutical industry as it leads to loss of activity of API and excipients, the formation of toxic by-products, change in color and flavor. Itraconazole (ITZ) bulk drug was exposed to UV−C (254 nm) irradiation in an environmental chamber (37 °C, 75 %RH) and its photoprotection by cinnamon, clove, eugenol, and oregano based microemulsion was analyzed. No significant change in the spectra was observed at various time points, confirming the photo-protective activity of microemulsions, unlike the bulk drug. FTIR spectra illustrate the fundamental peaks of the functional groups of ITZ and ITZ loaded MEs. The overlaid spectra showed that there was a minor change in peaks of UV exposed ITZ bulk drug but the ITZ loaded microemulsions were able to protect all the major functional groups. The *in vitro* anti-microbial assay against *C. albicans* demonstrated no significant change in the activity of ITZ loaded microemulsion between untreated, 7th day and 15th day while the activity of bulk drug was reduced drastically in the UV−C exposed sample. It was concluded that microemulsions can be used as an effective photo-protective drug delivery vehicle for light-sensitive compounds.

1. Introduction

Fungal infections affect approximately two-thirds of the population around the world [1]. Recently, there has been a drastic increase in the number of fungal infections caused by genera Candida, Aspergillus, and Cryptococcus. The clinical manifestations of the disease caused by the fungal agent can be highly variable and depend on the physiological and immunological status of the host [2]. Itraconazole (ITZ), belonging to the triazole family of antifungal agents, is widely used against serious fungal infections in normal and immunocompromised hosts compared to other triazoles like fluconazole, posaconazole and amphotericin B [3]. ITZ is a class II drug according to Biopharmaceutical Classification System (BCS). Its action against fungal cell membrane is by inhibiting lanosterol 14α demethylase in ergosterol biosynthesis. ITZ is a hydrophobic weak base possessing high inter and intraindividual variability in its oral bioavailability [4].

Pharmaceutical stability is an important criterion to assess the quality of the pharmaceutical formulation under storage stress conditions. In recent years, investigations into the impact of light on drugs have gained significance. Strict storage conditions are mandatory to maintain integrity and product efficiency [5]. Many pharmaceutical products undergo photo-degradation, which is one of the major factors leading to the degradation of drugs [6]. Photodegradation eventually leads to undesirable circumstances such as loss or alteration of the active pharmaceutical ingredient (API) or the excipients, reduced efficacy, the formation of toxic by-products, changes in flavor and discoloration. Solvent loss, crystal growth, reduced shelf life, and formation of toxic particles are a result of photolysis in biphasic preparations such as emulsions and suspensions [7,8].

Stability is defined as the capacity of an API or product to remain within the established specifications and to maintain its identity, strength, quality, and purity until the respective expiration period. Stability testing of an API or drug product can provide evidence regarding the drug quality and the impact of the various environmental factors such as temperature, humidity, and light [9]. Photosensitized reactions may also lead to the degradation of drug substances [10,11]. The photoproducts of a drug may be harmful and cause phototoxic, photoallergic, or photosensitization reactions upon administration [12–14]. Pharmaceuticals must be tested for photo-degradation to ensure the safety, efficacy, and quality of the final product [15].

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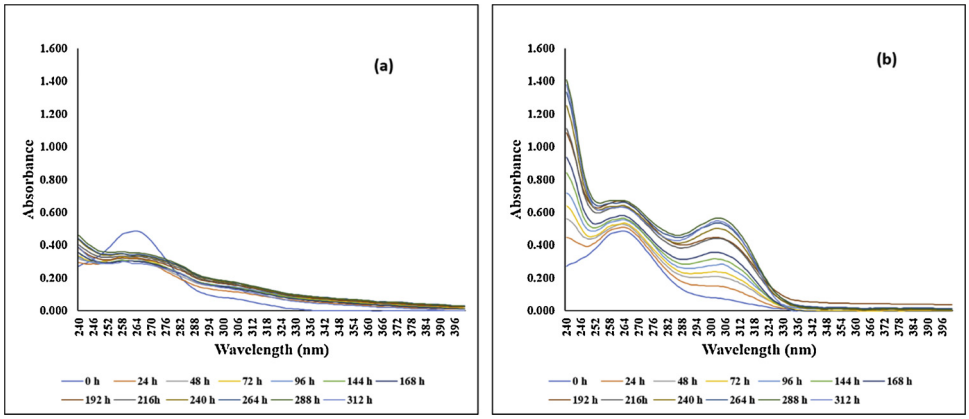

Fig. 1. UV absorption spectrum at different time exposure (a) UV-C exposed bulk ITZ (b) dark/light exposed bulk ITZ.

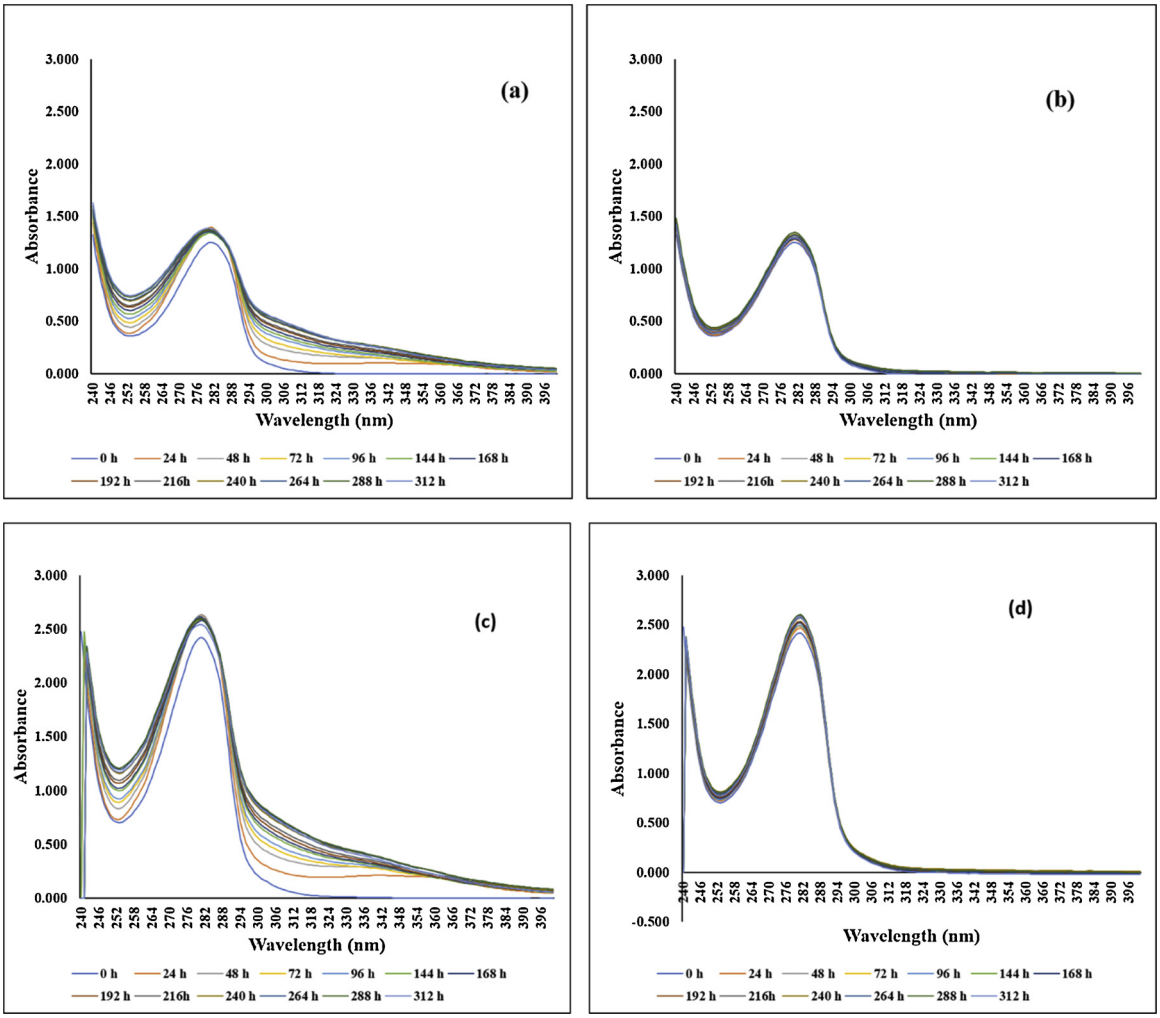

Fig. 2. UV absorption spectrum at different time exposure (a) UV-C exposed cinnamon ME system (b) dark/light exposed cinnamon ME system (c) UV-C exposed ITZ loaded cinnamon ME (d) dark/light exposed ITZ loaded cinnamon ME.

Physically and chemically stable microemulsions confer protection from external factors to the API [16]. Microemulsions (MEs) have an ability to incorporate high amounts of lipophilic and hydrophilic active compounds. The use of microemulsion formulation could help to overcome the poor solubility of ITZ [17]. In comparison with other colloidal systems such as nanoparticles, liposomes or noisomes, the microemulsions have a greater advantage in ease of preparation and better thermodynamic stability [18]. Subsequently, it is helpful to apply ME for incorporation of photolabile compounds (*e.g.* ITZ). The suitability of the ME carrier has to be confirmed by stability testing and biopharmaceutical characterization of the formulation.

The aim of the present study was to investigate the photo-lability of ITZ exposed to UV−C radiation as well as in dark/light conditions. The ability of different formulations to overcome this photochemical degradation was compared. ITZ and ITZ loaded MEs were monitored by UV–vis double beam spectrophotometer and the functional groups were

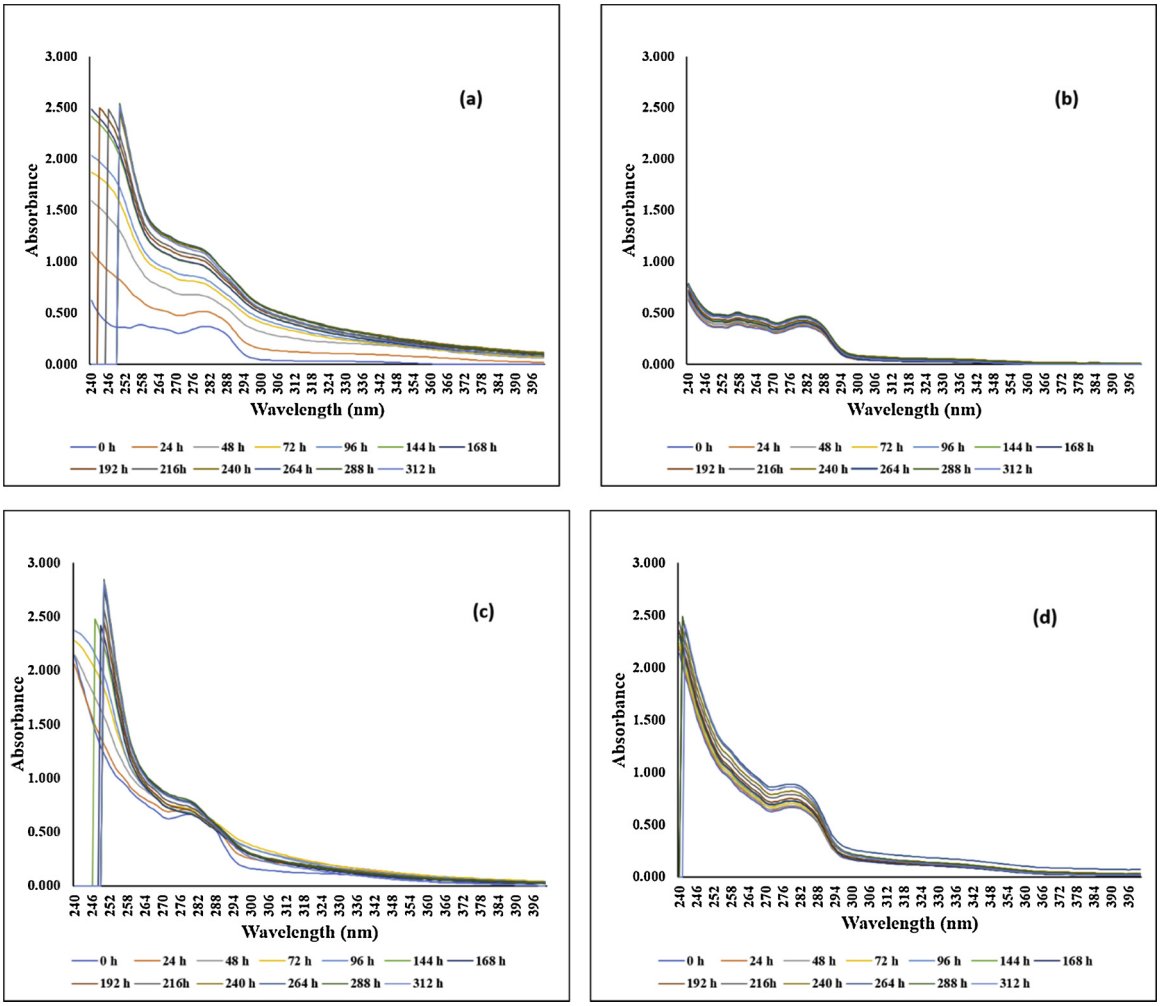

Fig. 3. UV absorption spectrum at different time exposure (a) UV-C exposed clove ME system, (b) dark/light exposed clove ME system, (c) UV-C exposed ITZ loaded clove ME, (d) dark/light exposed ITZ loaded clove ME.

analyzed for any changes using FTIR. The antifungal activity was checked using *in vitro* well diffusion assay.

2. Materials and methods

2.1. Chemicals

Itraconazole was a kind gift from Microlabs limited, India. Pure essential oil of Cinnamon, clove, eugenol, and oregano were purchased from Falcon, India. Tween 20 (Polyethylene glycol sorbitan monolaurate), and Tween 80 (Polyethylene glycol sorbitan monooleate) were obtained from Sigma–Aldrich, India. Mueller Hinton agar, glucose, methylene blue and *Candida albicans* (ATCC 90028) were purchased from HiMedia, India. Acetonitrile was purchased from Merck, India. For all experiments, reagent grade chemicals and ultrapure water with a resistivity of 18.2 MΩ/cm (Cascada™ Biowater System, Pall Corporation, USA) were used.

2.2. Preparation of drug loaded microemulsion

1 % w/v of ITZ was dissolved in the oil core by initial vortexing followed by mixing in an orbital shaker (Orbitek, Scigenics Biotech, India) for 72 h. Based on our previous experiments, optimized drug loaded and drug free oil in water MEs for cinnamon (6:24:70 %), clove (5:25:70 %), eugenol (5:25:70 %) and oregano oils (5:25:70 %) were formulated using Tween 20 or Cremophor EL as surfactants [19,20].

2.3. Photostability testing

2.3.1. UV-C irradiation

Each drug-free and drug-loaded ME formulations, and pure drug ITZ were stored in 10 ml PP/HDPE vials (Tarsons, India). Similar samples photo-protected using an aluminium foil were used as controls. All the samples were exposed to UV-C (Philips UV lamp, 254 nm, 8 W) radiation, at 75 % RH in an environmental chamber (CHM-12S, Remi, India) at 37 ± 1 °C. UV–vis spectra (240 nm–400 nm) was determined for all the samples using a UV–vis spectrophotometer (Eppendorf Biospectrophotometer, Germany) at every 24 h for 15 days.

2.3.2. Dark and light (D/L) stability

Another set of similar samples were exposed to dark and light conditions for 15 days, at room temperature and was periodically analyzed using UV–vis spectrophotometer. For maintaining the dark conditions, the samples in 10 ml PP/HDPE vials were wrapped with aluminium foil and stored inside dark cupboard. For the light conditions the samples were exposed to tubelights with an average of 550 lx.

2.4. Fourier transform infrared spectroscopy (FTIR)

The Fourier transform infrared (FTIR) spectra of ITZ API and ITZ loaded emulsions (UV-C and D/L treated) were recorded using FTIR spectrophotometer (FTIR-8400S, Shimadzu, Kyoto, Japan). Samples were mixed with potassium bromide (FT-IR grade) and compressed into

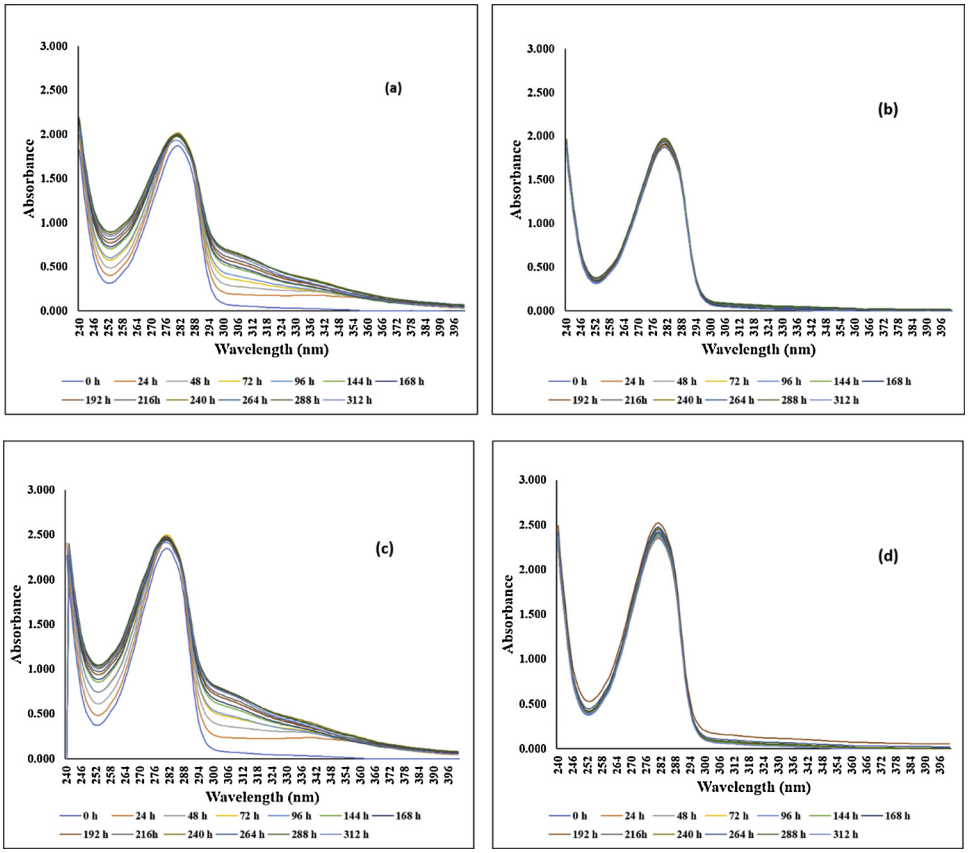

Fig. 4. UV absorption spectrum at different time exposure (a) UV-C exposed eugenol ME system, (b) dark/light exposed eugenol ME system, (c) UV-C exposed ITZ loaded eugenol ME, (d) dark/light exposed ITZ loaded eugenol ME.

disks using hydraulic press before scanning from 4000 to 500 cm^{-1} [21].

2.5. Determination of anti-microbial activity

Well diffusion method was carried out to analyze the anti-microbial activity of ITZ-API and ITZ loaded microemulsions exposed to UV-C and ambient conditions, at 0th, 7th and 15th. The fungal culture, *Candida albicans* (ATCC 90,028) was cultivated in Sabouraud dextrose agar (SDA).

Antifungal susceptibility testing by well diffusion method was carried out according to CLSI guidelines (CLSI document M44-A2) [22]. The standard medium used for disk diffusion test was Mueller-Hinton agar supplemented with 2 % glucose and 0.5 μg/ml methylene blue. Inclusion of methylene blue in the medium has been found to improve the yeast growth and provide sharp zones of inhibition for the azole group of drugs [22]. The yeast colonies from SDA plate was suspended in 3 ml of sterile 0.85 % saline, and the turbidity was adjusted to yield 1 × 10^5 - 1 × 10^6 cells/ml (0.5 McFarland standard). Subsequently, wells of 6 mm diameter were punched into agar medium. The test samples equivalent to 25 μg (100 μl) was added to the wells. The antifungal disk, fluconazole (25 μg) was tested along with the test samples. The plates were then incubated in the upright position at 37 ± 1 °C, for 48 h. The diameter of the growth inhibition zones were measured in triplicates [23].

2.6. Statistical analysis

All the tests were performed in triplicates. Statistical difference in significance was determined ($p < 0.05$), using two-way ANOVA followed by Dunnett's multiple comparison tests with 95 % CI. The experimental data where analyzed using GraphPad Prism 6 (version 6.01, GraphPad Software, Inc., California)

3. Results and discussion

3.1. Photostability testing

Photostability testing was done using accelerated degradation testing in order to evaluate overall photosensitivity and photochemical stability of microemulsions to protect the drug from UV-C radiation [24]. The API and drug-loaded microemulsions along with drug-free microemulsions were exposed to UV-C radiation and the comparison on the obtained absorption spectra explains the photostability of the exposed test samples. The photostability studies were carried out for ITZ and ITZ loaded ME formulations before exposure, at regular time intervals by diluting the samples appropriately with acetonitrile.

UV exposed ITZ degradation over 15 days was determined using UV–vis double beam spectrophotometer and compared with the degradation of ITZ in drug-loaded emulsions to analyze the protective behavior of the microemulsions against UV and Dark/light conditions. In order to determine the UV degradation of the vehicle, the microemulsion systems were exposed to the same condition.

The spectral data was compared for its significance using Dunnett's multiple comparison tests between bulk ITZ and ITZ loaded MEs (Fig. 1). Cinnamon ITZ ME (Fig. 2) showed significant protective activity from 277 – 280 nm (****), 272 – 276 nm and 281 – 285 nm (***), 267 – 271 nm and 286 – 287 nm (**), 263 – 266 nm and 288 – 289 nm (*). Clove ITZ ME (Fig. 3) could not prevent degradation action completely. Eugenol ITZ ME (Fig. 4) demonstrated significant protective activity from 274 – 284 nm (***), 269 – 273 nm and 285 – 287 nm (**), 265 – 268 nm (*). Oregano ITZ ME (Fig. 5) showed minimal protective activity at 265 – 279 nm (*).

Figs. 1–4 shows the spectral graph of ITZ loaded ME and ME system

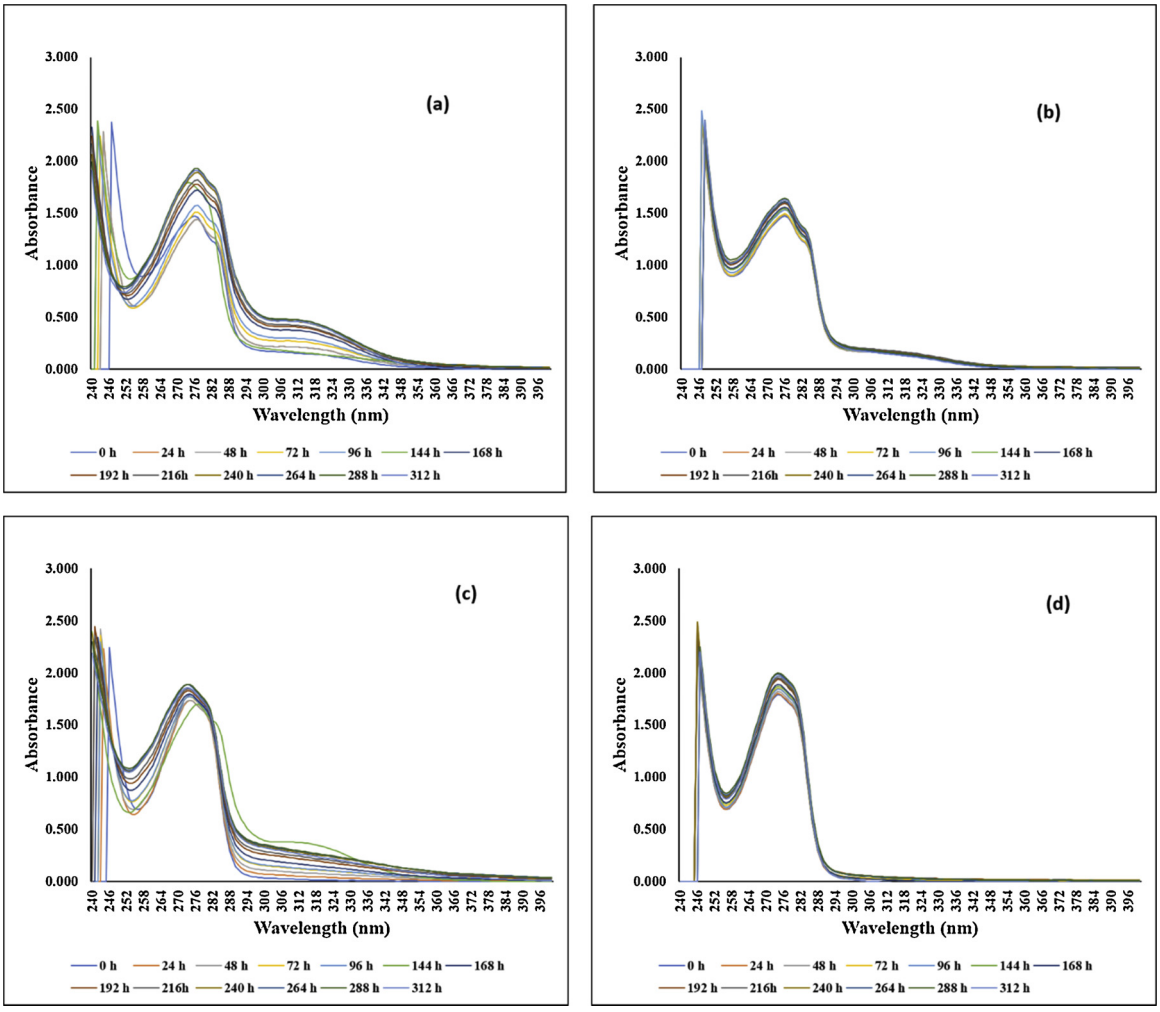

Fig. 5. UV absorption spectrum at different time exposure (a) UV-C exposed oregano ME system, (b) dark/light exposed oregano ME system, (c) UV-C exposed ITZ loaded oregano ME, (d) dark/light exposed ITZ loaded oregano ME.

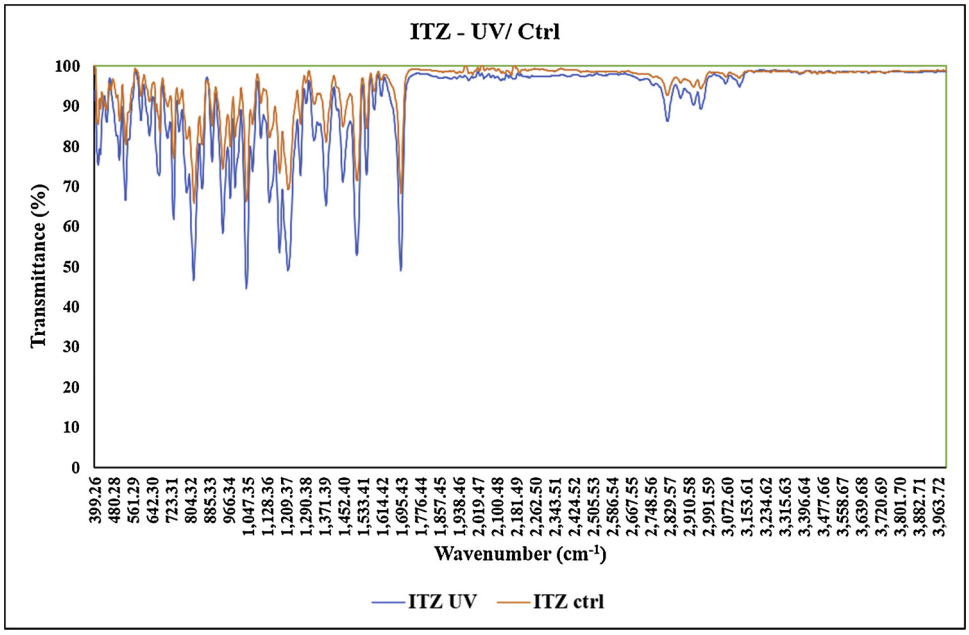

Fig. 6. FTIR spectrum for pure drug ITZ exposed to UV radiation along with control.

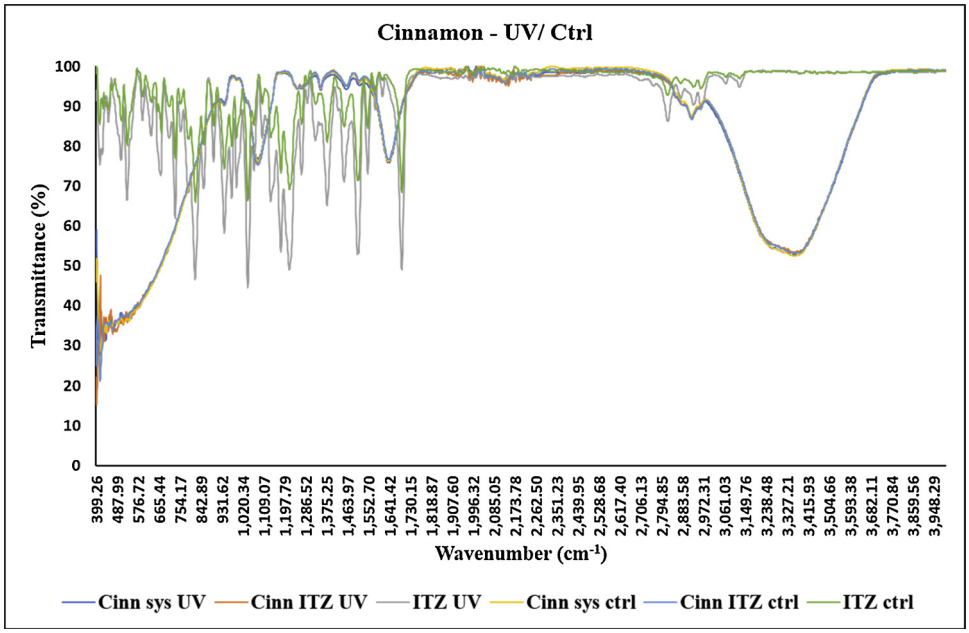

Fig. 7. FTIR spectrum for cinnamon system, Cinnamon ITZ, ITZ exposed to UV-C radiation along with respective controls.

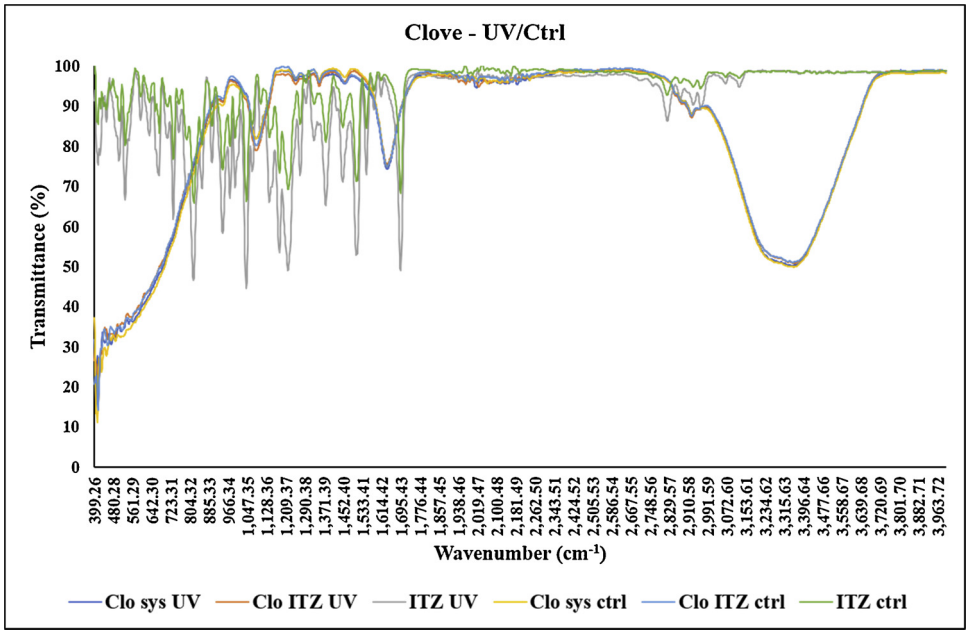

Fig. 8. FTIR spectrum for clove system, Clove ITZ, ITZ exposed to UV-C radiation along with respective controls.

at sequential exposure times with UV-C and Dark/light conditions. In order to evaluate the photostability of ME formulation, system control was also exposed to similar experimental conditions. On comparison with the ITZ pure drug, the ME carriers strongly reduced the photo-degradation process except clove ME, which have lesser photo-protective activity compared to other ME carriers. No significant change was observed in the absorption spectra recorded before and after visible light exposure, which shows that any photo-degradation occurring due to visible light was negligible. UV and visible light were considered to stimulate the autoxidation by prompting the hydrogen abstraction that leads to the formation of alkyl radicals [25]. ME provides efficient protection to ITZ under dark/light conditions and shows significantly reduced UV-C photodegradation when compared to ITZ alone. Recent studies have demonstrated the protective role of the novel formulation matrix as carrier systems for a number of photosensitive drugs [26].

The photostability studies were carried out for ITZ and ITZ loaded ME formulation before and after exposure to UV−C radiation at regular time intervals. ITZ shows 2 major absorption band at 265 nm and 306 nm. The peak at 265 nm and 306 nm may be due to π- > π* transition and n- > π* transition respectively. UV exposed ITZ shows complete degradation within 24 h due to the presence of highly strained chiral carbon unit in drug moiety. Whereas, dark/ light exposed ITZ was not degraded completely. It shows certain photochemical changes or dissociation occurring. The π- > π* transition was not changing much, while there was a gradual increase in n- > π* transition over temporal evolution of the absorption spectra [27].

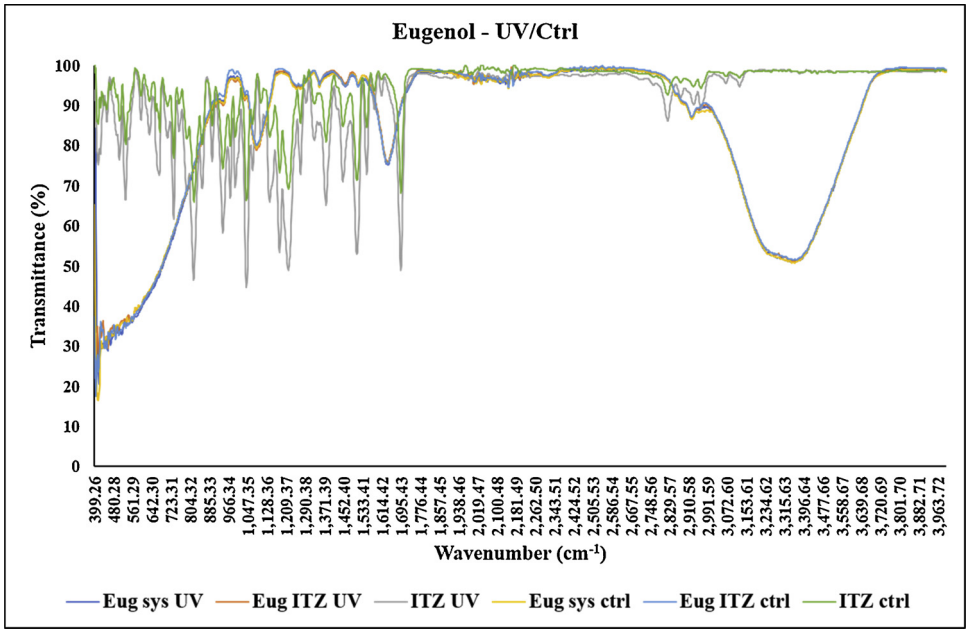

Fig. 9. FTIR spectrum for Eugenol system, Eugenol ITZ, ITZ exposed to UV-C radiation along with respective controls.

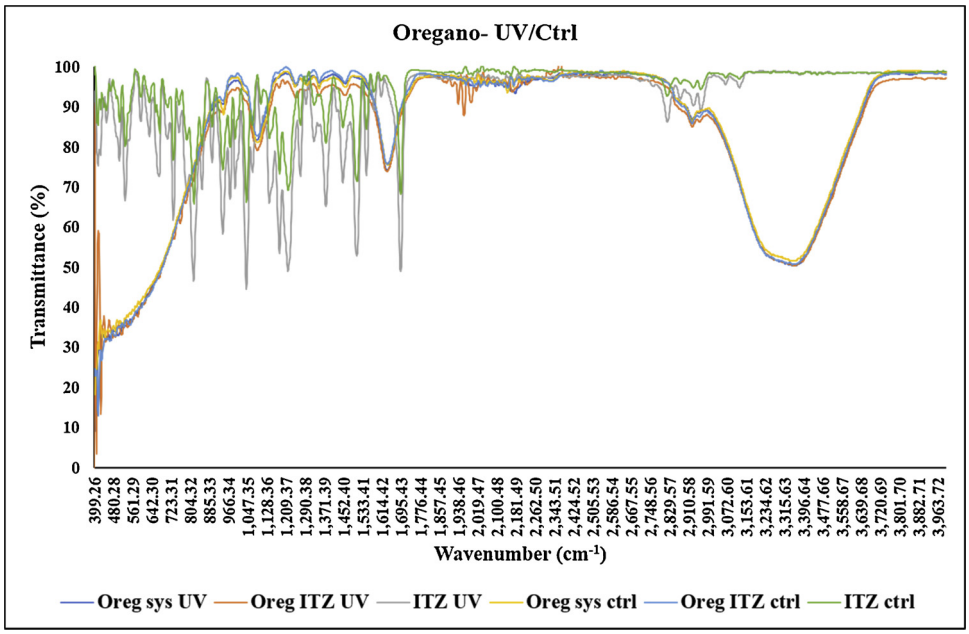

Fig. 10. FTIR spectrum for Oregano system, Oregano ITZ, ITZ exposed to UV-C radiation along with respective controls.

3.2. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum shows the fundamental peaks corresponding to the chemical nature of the drug. The FTIR spectrum of pure drug ITZ (Fig. 6) shows the characteristic peaks at 2821.86 cm^{-1} due to -C–H- vibrations, at 3138.18 cm^{-1} due to NH– stretching of amide groups, at 2962.66 due to the presence of $-CH_3$ group. Peaks at 1550.77 cm^{-1}, 1510.26 cm^{-1}, 1271.09 cm^{-1}, 1184.29 cm^{-1} corresponds to -NH, C=C, -CN, -COC respectively. Peaks at 734.88 to 898.83 cm^{-1} arises due to the groups occupying different substitution positions on the benzene ring. The spectrum from 532.35-671.01 cm^{-1} represents C-Cl stretching. UV irradiation of sample caused systemic changes in the FTIR spectra of ITZ, the N–H stretching peak was shifted to 3126.61 cm^{-1} and new peak splitting at 1219.01 cm^{-1} occurred. The integral band of absorbance has increased with exposure to UV–C.

The FTIR spectrum of Cinnamon ME, Cinnamon ITZ ME (Fig. 7) exposed to Dark/Light and UV-irradiation conditions showed a broader peak at 3346.50 to 3356.14 cm^{-1} was due to slight variation in vibrational frequency as well as due to superimposability of ME components to the drug. The major functional group of Cinnamon ITZ ME at 1641.42 cm^{-1} representing N-H shows a shift to 1637.56 cm^{-1}. Comparing pure drug and ME, the delocalization of Pi electrons might be the probable reason for a negligible peak shift.

Clove ME and Clove ITZ ME (Fig. 8) spectra exposed to Dark/light and UV irradiation demonstrated minor shift from 3352.28 cm^{-1} to 3329.14 cm^{-1} for N–H group and 1251.80 cm^{-1} to 1253.73 cm^{-1} for CN– group. Peaks at 2926.01 cm^{-1}, 1637.58 cm^{-1}, 1460.11 cm^{-1},1085.92 cm^{-1},943.19 cm^{-1} represents C–H, -C-H, $-CH_3$,C-N and O-H groups respectively.

Eugenol ME and Eugenol ITZ ME (Fig. 9) spectra exposed to dark/

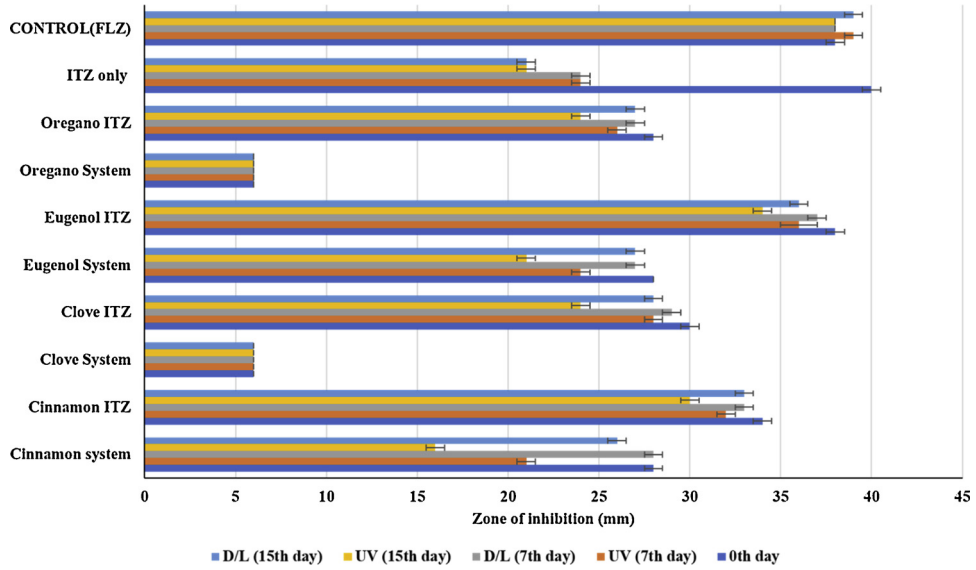

Fig. 11. Comparison of the zone of inhibition.

light and UV irradiation shows broad spectrum peak from 3332.99–3354.21 cm^{-1} denoting NH– stretching. Peaks at 2924.09 cm^{-1}, 1641.42 cm^{-1},1514.12 cm^{-1},1350.17 cm^{-1}, 1271.09 cm^{-1},947.05 cm^{-1} represents C-H, =C-H, C=C, C-N and O-H groups respectively.

Oregano ME and Oregano ITZ ME (Fig. 10) represents similar N—H stretching for dark/light and UV exposed samples along with a peak at 3275.13 cm^{-1} for OH– (carboxylic acid). Peaks at 2927.94 cm^{-1}, 1462.04 cm^{-1}, 1253.73 cm^{-1}, 939.33 cm^{-1}, 763.81 cm^{-1}, 540.07 cm^{-1} represents CH, $-CH_3$, C–N, -OH, -CH and C-Cl respectively.

Drug photosensitivity was mainly caused due to alteration in the functional groups like carbonyls, alkenes, aryl chlorides, polyenes, weak C—H and O-H bonds [28]. The functional groups in pure bulk drug show few modifications in the fingerprint region. Whereas, the excipients were found to be compatible with the drug and able to protect major functional groups present in bulk form.

3.3. Determination of anti-microbial activity

The inhibitory activity of the ITZ may be determined against the fungal strain, *C. albicans* as the test organism. Therefore, ITZ and all four ITZ loaded MEs exposed to UV and Dark/light conditions were checked for its antifungal activity against *C. albicans* at 0th, 7th and 15th day respectively. Fluconazole disc (25 μg) were used as an inhibition control.

The comparison of the results depicts a drastic change in the zone size of bulk ITZ in both UV exposed and dark/light condition, which shows the loss in activity of the drug against the test organism. All drug loaded ME showed comparable activity against *C. albicans* even after the 15th day of exposure in both UV and dark/light condition. Although a mild reduction in activity of UV-C exposed ME samples were observed on the 15^{th} day compared to the 0th and 7th day, this was not statistically significant. There is no demonstrable change in the activity of test samples exposed to dark/light condition even after a 15^{th} day. The drug-free system showed similar results as of the drug-loaded MEs. Eugenol ITZ ME showed the maximum potency against *C. albicans* and was found to be sensitive even after the 15th day of UV exposure by holding its activity. Bulk ITZ showed a significant difference between the zone size of untreated ITZ to the 15th day of UV exposed ITZ. All the ITZ loaded MEs did not show any significant difference in the zone size between the untreated, 7th and 15th day of UV exposure (Fig. 11).

Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparison tests with 95 % CI. There was a significant difference ($p < 0.0001$) in the zone size between UV-C exposed bulk ITZ and all the UV-C exposed drug loaded MEs, when compared between 0th day and 7th day or 0th day and 15th day. A similar observation was seen in the zone sizes compared between 7th day and 15th day except for clove ME ITZ and oregano ME ITZ. Clove and Oregano based ITZ MEs showed decreased zone sizes demonstrating minimal photoprotective activity when compared to Cinnamon ITZ ME and Eugenol ITZ ME. This phenomenon has been earlier seen in the changes in the UV spectra over time (Figs. 3 and 5).

In case of dark/light conditions the bulk ITZ *vs* ITZ loaded MEs showed significant difference with ($p < 0.0001$), as the drug was progressively degraded even with the alternating exposure of dark and visible light.

4. Conclusion

ITZ was found to be photolabile when exposed to UV radiation and dark/visible light conditions. Inclusion of ITZ in ME shows the photoprotective activity of ME on the drug. Microemulsions (MEs) were formulated to provide a protective eff ;ect on ITZ and minimize possible isomerization. Further, FTIR studies elucidated the functional group compatibility between the drug and excipients of the various MEs. *In vitro* microbiological assay of ITZ MEs showed negligible decreased activity in dark/light condition and was found to be active against *C. albicans* in case of UV exposed samples too. The study confirms that o/w MEs can be recommended for photoprotection of photolabile drugs, providing protection during storage, handling, and packaging.

Consent for publication

Not applicable

Ethics approval

Not applicable

Authors' contribution

Dr. N. Chandrasekaran, Dr. A. Sivakumar and Dr. Amitava Mukherjee planned and supervised the work. Nisha Tiwari, Andrew Ebenazer and Jonathan Sampath Franklyne processed the experimental data, performed the analysis, designed the figures, performed statistical analysis and drafted the manuscript.

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Declaration of Competing Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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