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Preparation and antihepatotoxicity activity of *Fagonia indica* extract and its solid dispersion formulation

Naglaa Gamil Shehab[1,2*], Aliasgar Shahiwala[3], Ihcene Benouared[3] and Rawoof Khan[4]
[1]Pharmaceutical Chemistry and Natural Products Department, Dubai Pharmacy College, Dubai, UAE
[2]Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Egypt
[3]Pharmaceutics Department, Dubai Pharmacy College, Dubai, UAE
[4]Pharmacology and Toxicology Department, Dubai Pharmacy College, Dubai, UAE

Abstract: This study aimed to evaluate and compare the antihepatotoxicity effect of *Fagonia indica* extract and its solid dispersion formulation (SD) against paracetamol-induced hepatotoxicity in rats. Dried Ethanolic plant extract was prepared by cold maceration in ethanol followed by solvent evaporation under reduced pressure. Quality control of crude extract was performed and the total phenolic and flavonoid contents were determined. Solid dispersion (SD) formulations were prepared by solvent evaporation technique and optimized with respect to drug solubility. Antihepatotoxicity activities of *Fagonia indica* extract and optimized solid dispersion were performed against paracetamol-induced hepatotoxicity in rats. Quality control parameters like total ash, acid insoluble ash, water soluble ash, crude fiber content and moisture content were within the acceptable limits. Total flavonoid and phenolic contents were found to be 31.289mg quercetin equivalents/g and 40.28mg gallic acid equivalent/g respectively. TLC Investigation of the plant extract revealed the presence of gallic acid, kaempferol and quarcetin. Optimized SD formulation with 200 mg of the dried extract, 350mg of PEG 4000 and 50mg of Tween 20 showed almost four-fold increasing in the solubility of the extract in water. The average hydrodynamic diameter of extract particles was reduced from 1972 nm to 437.6nm when prepared as SD. SD formulation showed highest antihepatotoxicity activity compared with plain plant extract at the same concentration. Optimized SD formulation at 500mg dose showed complete recovery from hepatotoxicity induced by paracetamol in rats. Therefore, SD is found to be one of the promising strategy to enhance the antihepatoxicity activity of *Fagonia indica* plant.

Keywords: Antihepatoxicity activity, *Fagonia indica,* solid dispersion.

INTRODUCTION

The liver diseases prevalence is increasing globally. A recent study across England showed that there is premature mortality rates of 18.3 per 100000 populations with an average of approximately 8500 deaths per year due to liver disease (Liver Disease Profiles, 2017) . Nevertheless, the mortality rate from the liver disease is increasing instead of decreasing. Nonalcoholic Fatty Liver Disease (NAFLD) is the most common liver disorder which was reported to have prevalence of 6-35% with the median of 20%. While in Western industrialized countries, the prevalence is found to be even more (Bellentani, 2017).

As the liver is the primary organ for drug metabolism, electrophilic chemicals or free radicals promotes different types of chemical reactions in the body such as depletion of reduced glutathione; covalently binding to proteins, lipids, or nucleic acids; or inducing lipid per oxidation. All these lead to either apoptosis or necrosis followed by cell death. This is called a Drug-Induced Liver Injury (DILI). The risk factor to develop the DILI is the concomitant drug usage and diseases like HIV, chemical properties of the drug itself, age, sex, environmental factors and genetic factors (Kaplowitz, 2004).

The Drug-Induced Liver Injury can be of either predictable like acetaminophen or unpredictable like phenytoin and isoniazid (Kaplowitz, 2004). It is estimated that approximately 2000 people experience Acute Liver Failure (ALF) annually in the United States and nearly 60% of these are caused by acetaminophen or idiosyncratic drug reactions (Lee, 2013). DILI has become one of the most important concerns in modern drug development as it is a leading cause of drugs failing clinical trials and being withdrawn from the market (Li *et al*., 2018).

There is no specific treatment for the drug-induced liver injury. The treatment is usually supportive and dependent on the symptoms. Hence, preventive measures can be an optimal choice to halt the liver injury. The preventative measures may include increasing the awareness, vigilance and identification of the risk factors in addition to monitoring of ALT enzyme with certain drugs (Verma and Kaplowitz, 2009). On the other hand, other measures can be used to help in the management of hepatotoxicity, which is the medicinal plants.

**Corresponding author:* e-mail: naglaa@dpc.edu

Nowadays, the use of natural products for various diseases becomes of a very great interest, which is considered as a traditional or alternative medicine. The reason behind the utilization of alternative medicine over the conventional medicine is that the patients are seeking ways to improve their well-being or to help in relieve of symptoms associated with the illness or to avoid the side effects of the conventional therapies. It was found in one study that traditional medicine is most commonly used in the following illnesses; back pain, joint pain, neck pain, and anxiety (Barnes *et al*., 2008). On the other hand, there are many plants that are used in the traditional medicine were candidate for drug discovery and development. There are 122 compounds obtained from 94 plant species that are globally used as drugs (Fabricant and Farnsworth, 2001).

Fagonia indica (Mushikka or white spine) belongs to Zygophyllaceae family, is available mainly in Asian and African deserts. This plant is safe (high LD_{50}) and contain a wide range of triterpenoids, sterols and bioactive flavonoids, for example, quercetin and isorhamnetin aglycones and their glycosides. *Fagonia's* alcoholic extract showed an analgesic and antitumor activities (Shehab *et al*, 2015).

In a previous investigation of the plant that was done by the authors, it was proved that the alcoholic extract of the *Fagonia indica* plant possess high antioxidant and hepatoprotective activities in carbon tetrachlrode induced hepatotoxicity (Shehab *et al*, 2015). As per the findings of one of the researches it reveales that the crude extract of *F. indica* has antimicrobial activity against different microorganism (Farheen *et al*., 2017).

Solid dispersion (SD) is one of the promising approach to improve oral bioavailability and increases drug wettability (Vasconcelos *et al*., 2007). The main aim of this investigation was to assess and compare the antihepatotoxicity activity of *Fagonia indica* extract and its solid dispersion formulation (SD) against paracetamol-induced hepatotoxicity in rats.

MATERIALS AND METHODS

Plant material

Whole plant of *Fagonia indica* Burm. F was collected (September 2016) from the Dubai desert, UAE. The plant was identified and was authenticated by Prof. Naglaa Shehab, Pharmaceutical Chemistry and Natural Products Department, Dubai Pharmacy College, Dubai. The Herbarium specimens were saved at of the Department (# 5-9-16). The plant, air-dried in shade, was powdered and was kept for the other studies.

Chemicals

Methanol, ethanol and chloroform were purchased from Fisher Scientific, USA. Folin-Ciocalteu reagent, gallic acid, sodium nitrate, quercetin and aluminium chloride were purchased from Merck, Darmstadt, Germany. Carboxymethyl cellulose, Polyethylene glycol 4000, hydrochloric acid, sodium hydroxide, Tween 80, Polyvinyl pyrrolidone and Monobasic Potassium Phosphate were purchased from S.D. Fine Chem Ltd., Mumbai, India. Reagent kits for Alanine amino transferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), Total Bilirubin and Total Protein were purchased from Adaltis S.r.l., Guidonia Montecelio, Italy.

Preparation of the ethanolic extract

Cold maceration method, in ethanol, (5 L X 2) was used for the extraction of the powdered plant (852gram). The solvent was evaporated under reduced pressure at 50°C. The dried extract was saved and was used for further investigation.

Standardization of the plant

Total ash, acid –insoluble ash, water soluble ash, moisture content and crude fiber were determined for the plant according to the British Pharmacopeia (British Pharmacopoeia, 1980). Results were calculated as the average of three determinations**.**

Total phenolic and flavonoid contents of the crude ethanolic extract

Folin- Ciocalteu reagent was used for the determination of the total phenolic content as described by Oktay *et al*. (2003). Results were calculated as mg/g dry weight gallic acid. The absorbance was measured at 750 nm.

Total flavonoid content was determined by the aluminum chloride method using quercetin as a standard and following the procedure described by Dewanto *et al*. (2002). The absorbance was measured at 510 nm. The experiments were carried out in triplicate.

Composition of Plant Extract and Formulation

The composition of both plant extract and formulation were investigated by Thin Layer Chromatography by using solvent system; Chloroform: Methanol in a ratio of 9:1.

Pharmaceutical Formulation

Solid dispersion formulation was prepared by solvent evaporation technique. Different amounts of dried plant extract, polymer and surfactant were dissolved in 25 ml ethanol. The resultant ethanolic solutions were then slowly evaporated to dryness on the hot plate at temperature 60-70^{o}C till the smell of the ethanol did not detect. The compositions of different solid dispersion formulations prepared are shown in table 1.

Assay

To determine the solubility of the extract and SDs, the standard solutions of plant extract in ethanol were prepared (80µg, 160µg, 240µg, 320µg and 400µg). The

calibration curve was generated by taking absorbance at λ_{max} 269 nm using UV spectrophotometer.

Particle size distribution

The particle size distribution (average hydrodynamic diameter and polydispersity index) of the plant extract and the prepared formulae were determined by utilizing the Malvern Zetasizer.

Animal studies

Acute Toxicity

The acute toxicity studies of *Fagonia indica* had been previously reported (Naglaa and Amina, 2009).

Evaluation of Antihepatotoxicity activity for the extract and the formulation

Treatment Protocol

Thirty healthy male albino rats, 5 rats in each group, weight 200-250g were used. Each group of rats were housed in a separate cages receiving standard hygienic (fed with well-balanced normal diet and water supplied ad libitum) and environmental (temperature 25.0±2.0°C, relative humidity 50-60%, with 12h day/night lighting cycle) conditions. Animal experiments were conducted as per ethical standards for the proper care and use of laboratory animals (Animals National Research Council, 2011). The animal study protocol was approved by the Research Ethical Committee of the Dubai Pharmacy College, Dubai, United Arab Emirates. One group was kept as control received 1% CMC. The other groups received 1g/kg paracetamol as injection to induce hepatotoxicity. The treatment was started after 2 days of the induction of the hepatotoxicity. The groups were divided as per table 2.

Assay of liver enzymes, Total Bilirubin and Total Protein

Blood samples were collected separately, from the eye of the rats with the use of capillary tubes and were transferred into non-heparinized tubes immediately. The level of the enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), Total Bilirubin and Total Protein were measured by kinetic photometric test using Diasys Diagnostic systems GmbH, Holzheim, Germany. Each result is reported as mean ± SD from five repeated determinations.

STATISTICAL ANALYSIS

The results of liver enzymes, total bilirubin and total protein are compared by one-way analysis of variance (ANOVA) followed by post hoc analysis using Tukey using IBM SPSS Statistics for Windows, Version 20.0 and results are considered to be significant at p-value of <0.05.

RESULTS

Quality Control Tests for the plant

The percentage yields of the proximate analysis tests of *Fagonia indica* showed that the plant has 4.2% of total ash, 1.4 % of acid insoluble ash, 1.0 % of water soluble ash and 26.92% from the crude fiber content. The plant showed 12.82% of moisture content.

Total Flavonoid and phenolic contents

Total flavonoid and phenolic contents were calculated by utilizing the calibration curves and the results were found to be 31.289mg quercetin equivalents/g and 40.28mg gallic acid equivalent/g respectively.

Composition of plant extract

TLC Investigation of the plant extract revealed the presence of gallic acid (R_f =0.166), kaempferol (R_f =0.54) and quarcetin (R_f =0.3) using solvent system chloroform: methanol in a ratio of 9:1.

Analytical method development

The calibration plot of concentration over the absorbance is shown in fig. 1.

The optical characteristics such as regression equation and correlation coefficient, mean absorbance value, and statistical data of the calibration curve were calculated and results are presented in table 3.

From the UV spectrum obtained, the wavelength of maximum absorbance (λ_{max}) was found to be at 269 nm. The absorbance values which were obtained corresponding to different dilutions were shown in table 3. Using absorbance-concentration data; calibration curve was plotted as shown in fig. 1 with regressed equation & r^2 value. As shown from the graph, the linear correlation exists between extract concentrations of 80-400 µg/ml and absorbance with high correlation coefficient values of 0.9995. This confirms that the developed calibration plot obeys Beer-Lambert's law in the same concentration range.

Preparation and characterization of SDs

Initial trials of second generation SDs were taken using extract with either PEG 4000 or PVPK30 polymer at ratio of 1:2. Compared to extract, both the SD showed increase in solubility (table 4, fig. 2). However, solubility with PEG was higher compared to SD prepared with PVP. Hence further SDs were prepared using PEG. When ratio of extract to PEG was increased to 1:4, no further increase in the solubility was observed (table 4, fig. 3). To further enhance the solubility, third generation SDs were prepared by combining PEG (375mg) with surfactants Brij 35 and Tween 20 (25mg) respectively. The trial with Tween 20 shown better results. When Tween 20 was increased to 50 mg, solubility was increased to 788.24

µg/ml (table 4, fig. 4). Further increase in Tween 20 beyond 50 mg resulted into liquid like consistency of SD. Optimized SD formulation with 200mg of the dried extract with 350 mg of PEG 4000 and 50 mg of Tween 20 shown almost four-fold increase in the solubility of the extract and was selected for further animal studies.

Particle size distribution

The particle size distribution of the plant extract and the prepared formula were determined by utilizing the Malvern Zetasizer (fig. 5 and 6). The average hydrodynamic diameter and polydispersity index were found to be 1972nm and 0.425 for the plant extract aqueous dispersion while 437.6 nm and 0.492 for the optimized SD formulation.

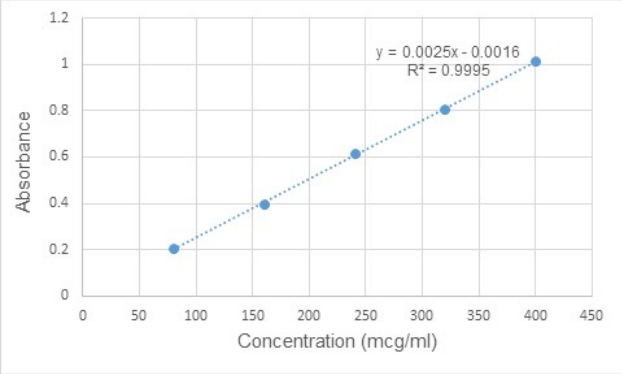

Fig. 1: Calibration curve of plant extract in ethanol

Evaluation of Antihepatotoxicity activity for the extract and the formulation

From the results shown in table 5, it showed that Paracetamol induced sharp hepatotoxic effect which was indicated by the elevation in the liver enzymes ALT, ALP, AST and Total Bilirubin (146.00±37.59, 525.60± 44.76, 148.00±9.54 and 0.99±0.03 respectively) and decrease in the total protein (4.17±.31). The plant extract given in a high dose of 500 mg/kg, showed a more significant reduction in the liver enzyme, total bilirubin than the plant extract given in a lower dose 250mg/kg. The 500mg plant extract of Fagonia indica (Group IV) significantly reduced the plasma enzymes of ALT, ALP, AST and Total Bilirubin (88.67±1.53, 369.53±5.97, 119.67±1.53, 0.72±0.04, respectively). On the other hand, significant elevation in total protein was observed by the two doses of the extract of the plant (6.96±.28 for 250 mg/kg and 7.40±.39 for 500mg/kg). SD formulations of plant extract were shown to have better hepato-protective activities compared to plain extract treated animals at same dose levels. According to these finding, the antihepatotoxicity activity of Fagonia indica can be arranged in an ascending order as follow: Extract in dose of 250mg/kg, 500mg/kg and SD formulation with 250mg/kg and 500mg/kg.

DISCUSSION

Even though plants are valuable source of active ingredients with high antioxidant and hepatoprotective activities, their pharmacological effect is often limited due to the poor water solubility of plant extracts. The results of this study demonstrated that pharmacological activities of plant extract could be enhanced by using suitable formulation approach.

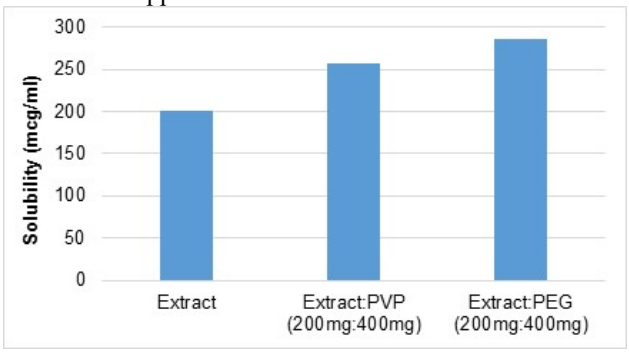

Fig. 2: Solubility of Extract, Extract PVP and Extract PEG formulation

Table 1: Composition of solid dosage form with the plant extract

No. of trials	Material Name	Amounts
1.1	Plant Extract	250 mg
	PVP	500 mg
1.2	Plant Extract	250 mg
	PEG	500 mg
2.1	Plant Extract	200 mg
	PEG	800 mg
2.2	Plant Extract	200 mg
	PEG	750 mg
	Tween 20	50 mg
2.3	Plant Extract	200 mg
	PEG	750 mg
	Brij35	50 mg
3.	Plant Extract	200 mg
	PEG 350	350 mg
	Tween 20	50 mg

Table 2: Animal Studies Groups

Group Name	Oral Treatment
Group I	1% CMC
Group II (negative Control)	Paracetamol 1g/kg
Group III	250 mg/kg of Plant Extract
Group IV	500 mg/kg of Plant Extract
Group V	250 mg/kg of the optimized SD (eq.to 250 mg extract)
Group VI	500 mg/kg of the optimized SD (eq.to 500 mg extract)

Table 3: Optical characteristics and precision of the analytical method

Parameter	Results (PBS)
Wavelength	269 nm
Regression equation (Y= mx+b)	y = 0.0025 x – 0.0016
Slope (m)	0.0025
Intercept (C)	-.0016
Correlation Coefficient (R^2)	0.9995

Table 4: Absorbance results of different SD formulations

No. of Trials	Material Name	Amounts	Solubility (µg/ml)
1	Plant Extract	--	201.04
1.1	Plant Extract	200 mg	257.44
	PVP K30	400 mg	
1.2	Plant Extract	200 mg	285.44
	PEG 4000	400 mg	
2.1	Plant Extract	200 mg	281.84
	PEG 4000	800 mg	
2.2	Plant Extract	200 mg	476.24
	PEG 4000	375 mg	
	Tween 20	25 mg	
2.3	Plant Extract	200 mg	388.27
	PEG 4000	375 mg	
	Brij35	25 mg	
3	Plant Extract	200 mg	788.24
	PEG 4000	350 mg	
	Tween 20	50 mg	

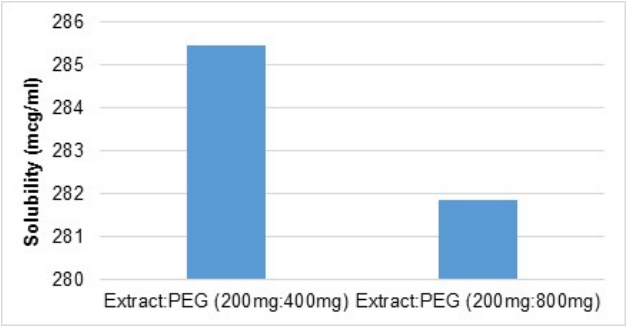

Fig. 3: Solubility of SD prepared at different PEG Ratios

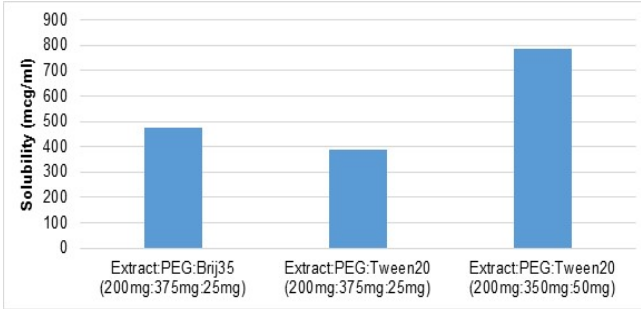

Fig. 4: Solubility of Third generation solid dispersions

Total ash, acid insoluble ash and water soluble ash are used to indicate the purity as well as the quality of the herbal medicine. Total ash includes both active components which are derived from the plant itself and non-active components from the environmental contamination (Moreira *et al*., 2014). The dry form of the plant material was used, so the moisture content should be kept low to protect the plant from any growth of microorganisms.

The selection of analytical method largely depends on the type of study to be conducted or samples to be assayed such as solubility study, partition coefficient determination, drug release study and drug permeation

Table 5: Antihepatotoxicity activity for extract and SD formulation

Group Name	ALT	ALP	AST	Total Bilirubin	Total Protein
Group I (Normal group (1%CMC))	46.67±4.51*	116.00±7.00*	107.33±5.86*	0.66±038*	6.20±0.7*
Group II (Paracetamol treated group (negative control))	146.00 ± 37.59	525.60 ± 44.76	148.00±9.54	0.99±0.03	4.17±0.31
Group III (250mg/kg extract)	93.67±9.81*	418.17±7.75*	141.67±13.31	0.84 ± 0.04*	6.96±0.28*
Group IV (500 mg/kg extract)	88.67 ± 1.53*	369.53±5.97*	119.67±1.53*	0.72±0.04*	7.40±0.38*
Group V (SD eq. to 250mg extract)	76.33 ± 6.03*	266.33±11.24*	133.00±2.65*	0.61±0.01*	6.93±0.09*
Group VI (SD eq.to 500mg extract)	61.33 ± 2.52*	163.33±13.43*	114.10±8.62*#	0.64±0.02*#	6.60±0.01*#

*Significantly different ($p<0.05$) from paracetamol treated group. # No significant difference ($p>0.05$) from normal group.

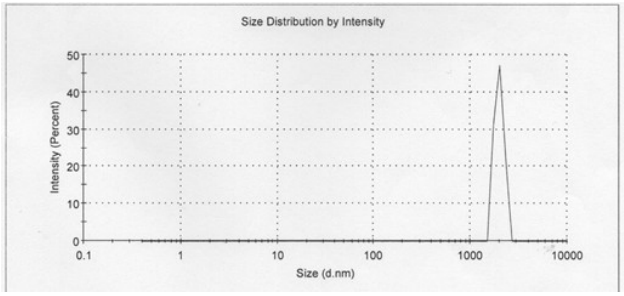

Fig. 5: Size Distribution of Plant Extract

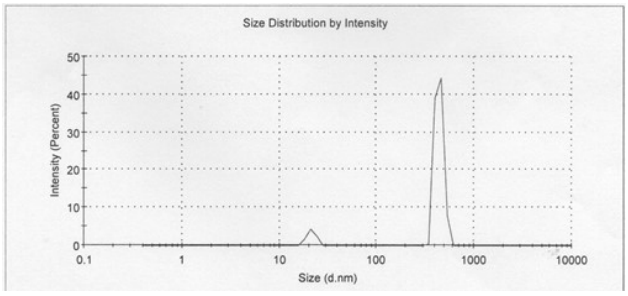

Fig. 6: Size distribution of the optimized SD formulation

study. RP-HPLC profiling of phenolics of the plant extract was done previously (Shehab, 2015). Extract was completely soluble in ethanol, hence calibration curve for the extract was prepared in ethanol.

SD is simple, effective and economic strategy for improving dissolution and bioavailability of poorly water-soluble drugs (Kaur *et al*., 1980). SD is basically a dispersion of one or more active ingredients with the inert carrier/s and based on the carrier/s used, SD is classified into first generation (crystalline carriers such as urea and sugar), second generation (amorphous carriers usually polymers) and third generation (carriers with surface activity or self-emulsifying properties, usually contains surfactant or a mixture of amorphous polymer and surfactant). In this investigation, second generation SD were developed since first generation SD are thermodynamically more stable and do not release the drug easily. PEG and PVP were selected as polymers since they are highly water soluble and listed as generally regarded as safe (GRAS) excipients. Solvent evaporation method was preferred when both active ingredients and carriers are soluble in common volatile solvent (Weerapol *et al*., 2017). Ethanol was used as a solvent since both the ingredients were soluble in it and is safe compare to other organic solvents.

Paracetamol is known to induce liver toxicity. The reactive metabolite of paracetamol, N-acetyl-p-benzoquinoneimine causes depletion of reduced glutathione (GSH) leading to liver cell nacrosis, with increased formation of reactive oxygen and nitrogen species in hepatocytes undergoing necrotic changes and increased oxidative stress (Hinson *et al*., 2010). Liver diseases are one of the major causes of morbidity and mortality and affects people of all ages throughout the world especially in the Arab countries. The drugs that are currently available to treat this condition pose serious drawbacks (Shehab *et al.,* 2015), which justifies the search for new antihepatotoxicity agents. In this context, the use of plants extracts and isolates therefrom with antihepatotoxicity properties can provide beneficial means for prevention and treatment of liver conditions. It was found that phenolic and polyphenolic compounds such as flavonoids are very efficient scavengers of free radicals (Halliwell, 1994) because of their molecular structures, which include an aromatic ring with hydroxyl groups containing mobile hydrogen. The flavonoid and the phenolic contents of *Fagonia indica* were found to be 31.289 mg quercetin equivalents/g and 40.28 mg gallic acid equivalent/g, respectively which consider as a high content from the antioxidant.

The reduction in liver enzymes in plasma that were elevated due to liver toxicity induced by paracetamol may be attributed to bioactive antioxidant principles detected in the extract. New compounds from *Fagonia indica* were isolated including a flavonoid glycoside; 3-O-[α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl]-kaempferol and triterpenoid saponin; 28-O-[β-Dglucopyranosylester-(1→3)-β-D-glucopyranosyl] oleanolic acid along with known quinovic acid-3-O-(α-L-rahmnopyranosyl-)-28-O-β-Dglucopyranoslyester (Kamel *et al*., 2013). The mechanism of lowering free radical levels by *Fagonia indica* increased expression of Cu-Zn Superoxide dismutase, decreased expression of *inducible nitric oxide synthase* (*iNOS*) with simultaneous scavenging of the fee radicals (Ali *et al*., 2008). In

another study, methanolic extracts of *Fagonia indica* Burm showed dose dependent increase in the free radical scavenging activity (Bagban and Roy, 2012). The scavenging of free radicals and thus boosting of antioxidant capacity may be responsible for this hepatoprotective effect of plant extract.

The prepared SD formulation of both doses showed a significant decrease in the liver enzymes and total bilirubin especially for SD at 500 mg dose compared to the negative control. Most of the liver parameters are not significantly different ($p>0.05$) from the normal group when animals were treated with the SD formulation at 500mg dose. This indicates, SD formulation at 500 mg completely eliminated liver toxicity induced by the paracetamol.

Highest antihepatotoxicity activities were demonstrated by the SD formulations prepared under this investigation compared with plain plant extract at the same concentration. These results may be attributed to the fact that SD improves the oral bioavailability of poorly-water soluble drugs by reducing the particle size of the drug and improving the drug wettability (Vasconcelos *et al*., 2007). In SD, extract particles were molecularly dispersed in the water soluble polymer/surfactant matrix, greatly reduces the particle size, increases the surface area and improves the wettability (Wairkar and Gaud, 2013). Similarly, improved dissolution of *Kaempferia parviflora* plant extract was demonstrated by solid dispersion approach. In another study, enhanced oral absorption and antihypothermic action of ginger extract was demonstrated when formulated as SD (Sato *et al*., 2017).

CONCLUSION

Poor water solubility of alcoholic plant extracts demands suitable formulation strategy to improve its solubility and bioavailability in order to enhance its efficacy and ease of administration. In this investigation, third generation SD with plant extract, PEG 4000 and Tween 20 at ratio of 200 mg: 350 mg: 50 mg was optimized with respect to water solubility and particle size. The developed SD under this investigation significantly enhances antihepatotoxicity activity of crude ethanolic extracts of *Fagonia indica*. Optimized SD formulation at 500mg dose showed complete recovery from hepatotoxicity induced by paracetamol in rats. The results of this investigation confirms that SD of *Fagonia indica* could be considered as a potential treatment option for hepatotoxicity. However, further trials are needed to confirm the findings of this investigation with higher animal species and in clinical settings.

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