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## **Bioactive aqueous extract of *Nigella sativa* L. seed waste: Formulation and evaluation**

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### **ABSTRACT**

The bioactive aqueous extract of *Nigella sativa* L. seed waste was formulated into soft gelatin capsules (Su1-Su6) in a dose of 200 mg/kg b. w. using different pharmaceutical excipients: soya lecithin/ span80/ sodium taurocholate, PEG 400, and Meglyol 810. The formulations were evaluated for their immunostimulant and hepatoprotective activities using biochemical methods and for their release by dissolution studies according to USP XXII. Formula Su3 showed a potent effect by significantly reducing tumor necrosis factor-alpha (TNF-alpha), interferon-gamma (INF- $\gamma$ ) and interleukin-beta (IL-1 $\beta$ ) levels compared to CCl<sub>4</sub>-treated animals. A significant reduction in the glutamic pyruvic transaminase (GPT) and bilirubin levels and a decrease in both lipid peroxidases (LP) and glutathione content (GSH) have been noticed when compared to CCl<sub>4</sub>-treated mice. Formula Su3 revealing the highest activity and optimum release, was selected and coated with two films – coating solutions (Su3: Eu. L-100, Su3: Eu. S-100) for site-specific delivery and sustained release. Eudragit L-100 and Eudragit S-100 were selected as coating materials and PEG 4000 as plastifying agent. Dissolution and disintegration tests were carried out for coated Su3 in order to verify the gastric-resistant properties of the coated product in simulated gastric medium (SGM) at pH 1.2 for 2 h and in simulated intestinal medium (SIM) at pH 7.4 for additional 2 h using a normal basket-rack. Quality control of the formulated extract, either uncoated or coated was performed using protein as active marker by adopting Bradford protein assay for comparison with plain extract in both SGM and SIM.

**Key words:** *Nigella sativa*, bioactive seed waste, formulation, evaluation, coated and uncoated soft gelatin capsules, Eudragit L-100, Eudragit S-100.

### **INTRODUCTION**

*Nigella sativa* L. known as seed of blessing is one of the most exciting of all plants in terms of healing properties. It was discovered in King Tutankhamen's tomb implying that it played an

important role in ancient Egyptian practices. Best seeds come from Egypt where they grow under almost perfect conditions in Oases with plenty of water supply until the seed pods form [1]. Many effects have been described for the seeds of *Nigella sativa* and their constituents, specially the volatile oil including its antioxidant role [2, 3] especially against Hepatotoxicity [3]. It has been demonstrated that *Nigella sativa* oil (NSO) can significantly prevent Hepatotoxicity [4] and might have protective effects against nephrotoxicity induced by either disease or chemicals [5]. Moreover, the immunomodulatory effect of NSO was proved recently [6]. One of the mechanisms responsible for antioxidant potentials of NSO could be the inhibition of 5-lipoxygenase [7, 8]. Carbon tetrachloride (CCl<sub>4</sub>) is one of the oldest and most widely used toxins for experimental induction of liver fibrosis in laboratory animals [9]. CCl<sub>4</sub>-induced reactive free radicals initiate cell damage through two different mechanisms of covalent binding to the membrane proteins and causing lipid peroxidation [10]. *Nigella sativa* L. seed waste aqueous extract had been previously evaluated [11] for its immunostimulant and hepatoprotective activities. The aqueous extract attenuated the CCl<sub>4</sub>-induced liver damage which was noticed by a significant decrease in both serum and tissue cytokines; tumor necrosis factor-alpha (TNF- $\alpha$ ), interferon-gamma (INF- $\gamma$ ) and interleukin-beta (IL-1 $\beta$ ), in the markers of liver functions; bilirubin and glutamic pyruvic transaminase (GPT) and in the oxidative stress markers; malondialdehyde (MDA) and glutathione (GSH) content. The aqueous extract was fractionated into its components: protein, saponin and polyphenol fractions to identify and analyze the component(s) responsible for activity. Proteins (36.85%) showed the most significant activity and were chosen as active marker [11].

Soft gelatin capsules have been available since the nineteenth century and were used to dispense a variety of liquids and solids [12,13] with multitude of advantages over the conventional dosage forms [14]. They are pharmaceutically elegant dosage forms offering an improved drug stability due to the tight enclosure of the content by the capsule shell protecting the drug from oxygen, moisture and light and from physiological fluids until it is released [15-17]. Soft gelatin capsules are used to enclose powders or water-insoluble liquids dissolved in a non-polar solvent for several reasons, such as masking flavors or unpleasant smells, reducing contamination of the product and protecting the active drug against oxidation. Nowadays many particulate products are enteric-coated to give the product specific functionalities. Capsules with gastric resistant properties are employed to avoid degradation of the active substances by the gastric juice, to reduce gastric irritation caused by the medicine, to avoid caking during storage, to facilitate dosage and mixing of the products, for shelf-life enhancement and prolongation and to allow the release of the medicine only at enteric pH 7 [18,19]. Many pharmaceutical polymers exhibit brittle properties and require the addition of a plasticizing agent to obtain an effective coating that is free of cracks, edging or splitting [20-22]. In recent years, colonic drug delivery has gained increased importance not just for the delivery of drugs for the treatment of local diseases of colon but also for its potential for the delivery of proteins and peptides [23]. Most of the previous literature reports on the development of a colonic delivery system based on time-dependent delivery, pH dependent systems and systems that utilize bacteria that colonize the colon or enzymes produced by these bacteria to affect drug release [24,25]. Among these approaches, the pH dependent systems have found practical application. The pH of the gastrointestinal tract gradually increases as one move down the GI tract from stomach (pH 1.5–3) to terminal ileum (pH 7–8). On entry into the colon, the pH drops to 5.6–7.0 [26]. The dosage forms which disintegrate at suitably high pH levels have the potential for site-specific delivery into this region. Most commonly used pH-dependent coating polymers are methacrylic acid copolymers – Eudragit L-100 and Eudragit S-100 which dissolve at pH 6.0 and 7.4, respectively. In our previous publication [11], the protein fraction was found responsible of the hepatoprotective and immunopotentiating activities of the aqueous extract of *Nigella sativa* seed

waste. This stimulated the authors to perform the present study in view to provide an economic and efficient formulation which could help in management of liver disorders. In this respect, several formulae were prepared and tested; this aiming to ensure highest activity and optimum release for the enteric coating allowing site-specific delivery of proteins and sustained release. Moreover proteins were, as previously described used as active markers, were used to evaluate the selected formula (uncoated and coated) in both SGM and SIM in comparison to plain extract.

## EXPERIMENTAL SECTION

**Plant material:** *Nigella sativa* L. seeds waste were collected from local mills in Cairo, Egypt (2008).

### **Material for formulation and coating soft gelatin capsules:**

Soya lecithin/ span80/ sodium taurocholate, PEG 400 and Meglyol 810 (Hüls AG, Witten, Ruhr, Germany). Soft gelatin capsules were gift samples supplied from Glaxo Co., Egypt. Eudragit L-100 and Eudragit S-100 (Rohm Pharma, Germany) are selected as coating materials. Hydroxypropyl methyl cellulose (HMPC), PEG 4000 and PEG 6000 were purchased from Sigma and Fluke respectively and used as plastifying agent. All chemicals used were of the analytical grade.

### **Material for dissolution media:**

Hydrochloric acid (Riedel de-Haën, Germany), monobasic potassium phosphate (Sigma–Aldrich Laborchemikalien GmbH, Germany), sodium hydroxide (Tamro, Finland), potassium chloride (Riedel-de Haën, Germany). Phosphate buffer (pH 7.4): monobasic potassium phosphate (6.805 g), sodium hydroxide (1.56 g) in 1000 ml purified water (USP 24, 2004).

### **Material for protein assay:**

Coomassie Brilliant Blue R-250 and G-250 Dyes, to prepare protein assay reagent, orthophosphoric acid and Bovine albumin (used as reference standard of protein) were purchased from Sigma – Aldrich Com., Saint Louis, USA.

### **Animals:**

Male Albino mice were obtained from the National Scientific Research Centre (Giza, Egypt), fed a standard pellet chow (El-Nasr Chemical Company, Cairo, Egypt) and had free access to water. All animals were maintained on a 12-h light, 12-h dark cycle and housed for 1 week before experimentation. Mice weighing between 26-30 g on the day of the experiment were used. This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Pharmacy Cairo University, Cairo, Egypt.

### **Apparatus:**

ELISA was used for estimation of TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$ . USP release tester (Hanson SR6, Hanson Research Corporation, California, U.S.A); UV-Spectrophotometer (Shimadzu, Kyoto, Japan); Electrical blender, Philips, Holland; Hot plate with Magnetic stirrer (Thermolyne Corporation, Dubuque, Iowa, U.S.A); Heating mantle 5 liters capacity; Electrical balance (Sartorius GMBH, Göttingen, Germany); Thermostatically controlled electric water bath (Julabo Laborer Hank GMBH, Sellback, Germany); Sieves of mesh size 250  $\mu$ m and 100  $\mu$ m (VEB, Metal-Weberei, DDR, Italy) were used. Mini coating machine (Taizhou LiMing Medical Machinery Co.LTD-China). Disintegrator tester Dr.Schleuniger® Pharmaton Solothurn, Switzerland.

**Immunostimulant Kits:**

Tumor necrosis factor-alpha (TNF-  $\alpha$ ), interleukin-beta (IL-1 $\beta$ ) and interferon-gamma (IFN-  $\gamma$ ) (R&D Systems, Minnesota, USA).

**Formulation of the aqueous extract:**

Hot maceration was performed using one kilogram of seed waste with distilled water (4L x 4, 4h, with continuous stirring), filtered and concentrated to dryness under reduced pressure at 40°C to afford the aqueous extract (160 g). The dried extract was kept in desiccators for 4 weeks, pulverized into free powder and kept in tightly closed glass containers for further investigation. One hundred grams were extracted by ultrasonication with distilled water at room temperature for 30 minutes. The extract was filtered, clarified by centrifugation, transferred into 500 ml volumetric flask and the volume adjusted with distilled water to give 200 mg extract/ml (stock solution).

**Bradford Protein assay:**

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins [27]. The dye exists in three forms: cationic (red), neutral (green), and anionic (blue) [28]. Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form ( $\lambda_{\max} = 470$  nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form ( $\lambda_{\max} = 595$  nm) [29-31]. It is this blue protein-dye form that is detected at 595-620 nm in the assay using a spectrophotometer. The assay is accomplished by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol; add 100 ml 85% (w/v) orthophosphoric acid. Dilute to one liter when the dye has completely dissolved and filtered through Whatman paper No. 42 just before use [32]. One ml tested solution was transferred to 5 ml volumetric flask and the volume adjusted with the prepared dye reagent, incubated for 5 min and then vortex. The produced blue colour was measured spectrophotometrically at 620 nm.

**Preparation of standard curves (at pH 1.2 and pH 7.4):**

Two standard calibration curves were conducted by plotting absorbance (y) against concentration (x,  $\mu\text{g/ml}$ ) using standard protein Bovine albumin. A stock solution of 100 mg% (S1) was prepared in 100 ml volumetric flask and the volume was adjusted by buffer solution (pH 1.2). An aliquot (1 ml, S1) was transferred to 10 ml volumetric flask and the volume was adjusted with buffer solution to contain 100  $\mu\text{g/ml}$  (S2). The first calibration curve was established using serial dilutions of S2 (1, 2, 4, 6 and 8 ml completed to 10 ml with buffer solution pH 1.2), mixed with prepared dye as previously described and the obtained blue colour measured spectrophotometrically. Triplicate measurements were performed to obtain absorption plots ranging from 20-80  $\mu\text{g}$  standard protein. The second calibration curve was established by repeating the same steps using buffer solution pH 7.4 over a concentration range of 25-200  $\mu\text{g/ml}$  standard protein. Regression equations and coefficient were computed.

**Formulations of powdered seed waste extract of *Nigella sativa* (SWEN) uncoated soft gelatin capsules as a test (Su1-Su4):**

Formulae (Su1-Su4) were prepared by encapsulating 200 mg powdered (SWEN) separately with the different excipients in soft gelatin capsules to give total weight of 400 mg, which were then sealed with gelatin solution (Table 1). The control formulation was prepared similarly by encapsulating 200 mg powdered SWEN in soft gelatin capsules.

### Formulations of aqueous seed waste extract (200/0.25 w/v- stock solution) in uncoated soft gelatin capsules as a test (Su5 and Su6):

Formulae (Su5-Su6) were prepared using the previously prepared stock solution (1ml equivalent to 200 mg extract: effective dose) [11] as shown in Table (1).

**Table 1: Different formulations of seed waste extract of *Nigella sativa* (SWEN).**

Symbols	Name	Contents
(SWEN)	Plain extract as a control	Seed waste extract of <i>Nigella sativa</i>
SU1	Formulated powdered seed waste extract (SWEN-200 mg/kg) in soft uncoated gelatin capsules as a test	Powdered SWEN/ soya lecithin/ span 80/ sodium taurocholate and PEG 400 in soft gelatin capsules
SU2		Powdered SWEN/ span 80/ sodium taurocholate/ PEG 400 in soft gelatin capsules
SU3		Powdered SWEN/ span 80/ tween 80/ soya lecithin/ PEG 400 and Meglyol 813 in soft gelatin capsules
SU4		Powdered (SWEN)/ span 80/ tween 80 /sodium taurocholate and PEG 400 in soft gelatin capsules
SU5	Formulated aqueous seed waste extract (SWEN) (200/0.25 w/v) in	Aqueous extract (200/0.25 w/v)/span 80/tween 80 /sodium taurocholate/PEG 400 in soft gelatin capsules
SU6	soft uncoated gelatin capsules as a test	Aqueous extract (200/0.25 w/v)/span 80/tween 80 /soya lecithin/PEG 400 in soft gelatin capsules

### Dissolution studies for uncoated soft gelatin capsules (Su1-Su6):

These studies were conducted adopting the USP basket method (apparatus I). The release studies of the individual plain extract (SWEN), as well as, its incorporation in the various formulations (Su1-Su6) were performed by accurately weighing amount of soft gelatin capsules equivalent to 200 mg of extract and placing in USP dissolution basket. The basket was rotated at 100 rpm in 1000 ml 0.1N HCL at  $37 \pm 0.5^\circ\text{C}$ . At each time interval, 3 ml samples were withdrawn and replaced with 3 ml dissolution media (SGM). Each release experiment was allowed to last for 2 h. Protein in the different formulae was determined spectrophotometrically at each specified time intervals (5,10,15,30,45,60,90 and 120 min) at  $\lambda$  620 nm by using Bradford protein assay to select the formula explicating the highest release and comparing it with the plain extract.

### Biochemical experiments

#### Experimental design:

LD<sub>50</sub> of the aqueous extract was previously determined to be 5g/kg b.w. [11].

#### Preparation of the blood samples:

The animals were divided into eight groups of 6-8 mice each: Group I: normal control 1% tween 80 treated; Group II: CCl<sub>4</sub> positive control treated; Group III-VIII: hepatotoxic treated with 10 mg/kg soft gelatin capsules (Su1-Su6). The different formulations were dissolved in 10% tween 80 and administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl<sub>4</sub> (10 mg/kg b wt of 0.25% (v/v) solution in corn oil. One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation and used for estimation of TNF-  $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  levels in the serum of mice together with estimation of GPT activity and bilirubin level.

#### Preparation of the liver samples:

Different formulations (Su1-Su6) were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl<sub>4</sub> (10 mg/kg b wt of 0.25% (v/v)). One day thereafter, mice were sacrificed by cervical dislocation and livers were

rapidly excised and homogenized in chilled 1.15 KCl (PH 7.4) to yield 10% homogenates then used for estimation of TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$  levels; LP level and GSH content in liver homogenates of mice.

**Determination of tumor necrosis factor-  $\alpha$ , interleukin-1  $\beta$  and interferon- $\gamma$  assays:**

TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  levels were measured in serum and tissue homogenates by using a Quantikine rat TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  ELISA kits (R&D Systems).

**Determination of MDA and GSH levels:**

Lipid peroxidation products were estimated by the determination of the content of the thiobarbituric acid-reactive substances (TBARS) that was measured as malondialdehyde [33] and expressed as nmol/g wet tissue. Estimation of GSH content was performed spectrophotometrically at 412 nm, using Elman's reagent [34, 35] and expressed as  $\mu$ mol /g wet tissue.

**Determination of GPT and Bilirubin:**

Serum activities of ALT enzyme were measured using test reagent kits based on the method of Reitman and Frankel, 1957 [36]. Serum bilirubin levels were estimated according to Jendrassik and Grof, 1938 [37].

**Statistical Analysis:**

Comparisons between different groups were carried out by one way analysis of variance (ANOVA) followed by "*Turkey-Kramer multiple comparisons test*". The level of significance was set at  $p < 0.05$ . "*Graph Pad Software Instate (version 2)*" was used to carry out these statistical tests.

**Manufacturing process of the enteric-coated capsules:**

Based on the aforementioned biochemical investigation and release studies, Su3 was selected to be coated with two film – coating solutions (I and II) of Eudragit L-100 and Eudragit S-100. Su3 was prewarmed for 30-60 min before coating was initiated by the coating machine. Outlet bed temperature was held between 30 and 35 °C.

I-

Eudragit L-100.....	5.00 g
Ethanol (96%).....	86.25 g
PEG 4000.....	1.25 g
Distilled water .....	7.5 g

II-

Eudragit S-100 .....	5.00 g
Ethanol (96%).....	86.25 g
PEG 4000.....	1.25 g
Distilled water .....	7.5 g

Patches were subcoated with 2% hydroxypropyl methylcellulose (HPMC) and PEG 4000 which was used as plastifying agent. Capsules were divided into two groups and were separately coated with the above two film–coating solutions by the spray technique. After coating the capsules, their specifications were investigated and the results were recorded in Tables (2, 3).

**Table (2): Coating parameters for the soft gelatin capsules.**

Process parameter	Capsules coating
Inlet temperature (°C)	35-40
Outlet temperature (°C)	30-35
Nozzle diameter (mm)	1.0
Atomization pressure (bar)	2.0
Spray rate (g /min)	1

**Table (3): Coated capsules specifications' (n=6)**

Method	Eudragit L-100 coated capsules	Eudragit S-100 coated capsules
Average weight	401mg SD ± 1.086	400 mg SD ± 1.934
<b>Disintegration time</b>		
In SGM	Not disintegrated within 2 h	Not disintegrated within 2 h
In SIM	10.5 min	9.5 min

**Disintegration tests of the enteric-coated capsules:**

The disintegration time of the enteric-coated capsule (Su3: Eu.L-100, Su3: Eu.S-100) was defined as the time needed for the enteric film coating to rupture and to release its contents. The disintegration test was carried out in simulated gastric medium (SGM) at pH 1.2 followed by simulated intestinal medium (SIM) at pH 7.4 using the normal basket-rack.

**In-Vitro dissolution studies of the film coating capsules:**

Four hundred ml of SGM and SIM (pH 7.4) were used separately as dissolution media using USP XXII, apparatus I according to the basket method. SGM at pH 1.2 was the dissolution medium for the initial 2 h period and then SIM at pH 7.4 for capsules coated with Eu.L-100 and Eu.S-100 for the following 2 h at  $37 \pm 0.5^\circ\text{C}$  100 rpm. The amount of protein dissolved was measured spectrophotometrically at 620 nm as previously mentioned.

**Quality control of uncoated (Su3) and coated (Su3: Eu. S-100) soft gelatin capsules in comparison to plain extract:**

Formulae Su3 and Su3:Eu.S-100 (coated with Eudragit S-100) were selected for quality control in comparison to plain extract. Su3 was selected based on its significant hepatoprotective activity and best release while Su3:Eu.S-100 for the significant rise of lag time and lowest released amount of extract during the 4 h of dissolution test. One uncoated capsule containing 200 mg extract was dissolved in 100 ml SGM (0.1 N HCl, pH 1.2) for 2 h. An aliquot (1 ml) of the dissolved capsule was completed to 5 ml prepared dye in a 5 ml volumetric flask and measured as previously mentioned. One coated capsule equivalent to 200 mg extract was dissolved separately in 100 ml SGM (0.1 N HCl, pH 1.2) and SIM (phosphate buffer, pH 7.4) for initial of 2 h and for additional 2 h in case of SIM respectively. For each time, one ml of each sample was mixed separately with prepared dye reagent and measured spectrophotometrically at 620 nm as previously discussed. From the plotted calibration curves, the protein concentration was calculated in triplicates. The same procedure was repeated using 100 mg plain aqueous extract of *Nigella sativa* seed waste in 50 ml SGM and SIM respectively and the protein concentration of the plain extract was calculated in triplicates to be used as standard.

## RESULTS

### Biochemical studies

The bioactive aqueous extract of *Nigella sativa* seed waste was formulated into soft uncoated gelatin capsules (Su1-Su6, Table 1) in a dose of 10 mg/kg b.w. using different pharmaceutical excipients and was tested for their hepatoprotective and immunostimulant activity. They were administered orally for five consecutive days to mice before treating them intraperitoneally with CCl<sub>4</sub> at a dose of 10 mg/kg b. w. of 0.25 (v/v) solutions in corn oil. Liver injury produced was evaluated by biochemical parameters. Notably, administration of CCl<sub>4</sub> caused liver injury manifested by a one-fold increase of TNF- $\alpha$ , 3- fold rise of IL-1 $\beta$  and 2-fold increase of INF- $\gamma$  in the serum and by 4-fold increase of TNF- $\alpha$ , 3- fold rise of IL-1 $\beta$  and 2-fold increase of INF- $\gamma$  in liver homogenate, as compared to the normal control mice (Table 4, 5). Later, the acute liver injury was evidenced in the plasma by about 6- fold rise in glutamic pyruvic transaminase (GPT) activity and 2-fold increase in bilirubin level as compared to the normal control group (Table 6). Hepatotoxicity was further manifested by 2-fold increase in the hepatic level of MDA and by 54% increase of the GSH level as compared to the normal control group (Table 7). Administration of the different formulations of the soft gelatin capsules showed that Su3 possessed a potent effect by a significant reduction of TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  levels by 56%, 60% and 49% respectively in serum as compared to CCl<sub>4</sub>-treated mice. Similarly, Su4 produced a significant reduction of TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  levels by 52%, 54% and 54.5% respectively in the liver homogenates compared to CCl<sub>4</sub>-treated mice (Table 4, 5). Su3 caused a significant reduction in the GPT and bilirubin levels by 84% and 62% respectively and Su4 produced a significant reduction in GPT and bilirubin by 83% and 66% respectively as compared to CCl<sub>4</sub>-treated animals (Table 6). Concerning the antioxidant parameters, Su3 showed significant decrease of GSH by one fold as compared to CCl<sub>4</sub>-treated mice (Table7).

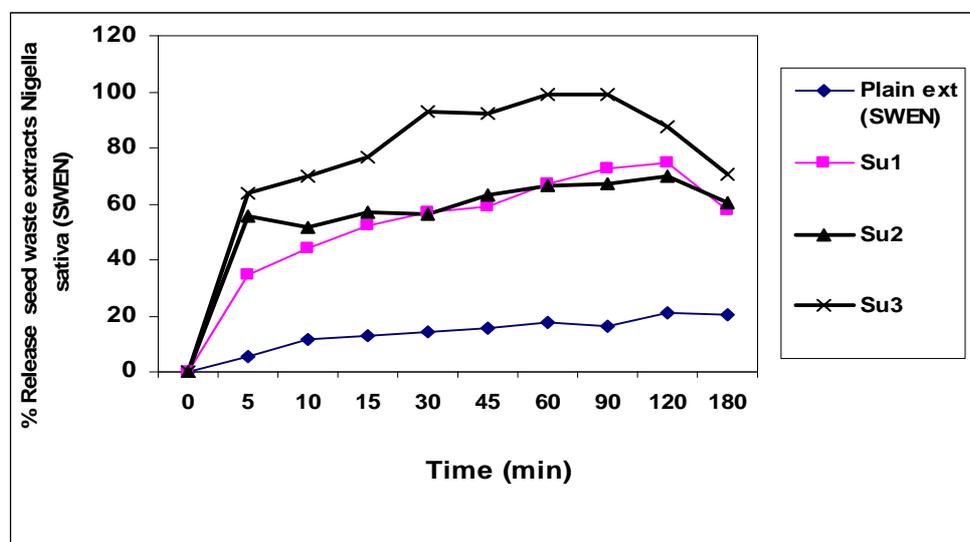


Figure 1: Dissolution profile of seed waste extract of *Nigella sativa* (SWEN) released from soft uncoated gelatin capsules (Su1-Su3) in comparison to plain extract

### Dissolution Studies of uncoated soft gelatin capsules (Su1-Su6)

The percentage of protein release from plain and formulated extracts packed in uncoated soft gelatin capsules were screened spectrophotometrically at 620 nm using Bradford assay for proteins [27-32]. It decreased in the following order: Su3 (98.90%) > Su5 (81.51%) > Su4 (67.21%) > Su1 (66.55%) > Su2 (57.20%) > Su6 (50.21%) indicating that the highest protein release was obtained from Su3 formula as illustrated in Figures (1, 2). Su3 protein release rate

was found to be  $98.90\% \pm 0.99$  after 60 min. in comparison to plain extract (SWEN) which gave  $20.52\% \pm 1.06$  after 120 min. The kinetic analysis of the extract release data from different formulae was calculated by linear regression according to zero, first order kinetics and simplified Higuchi model and followed diffusion model kinetics. Based on highest immunostimulant activity and optimum release studies. Su3 was selected for enteric coating using Eudragit L-100 and Eudragit S-100 by spray technique.

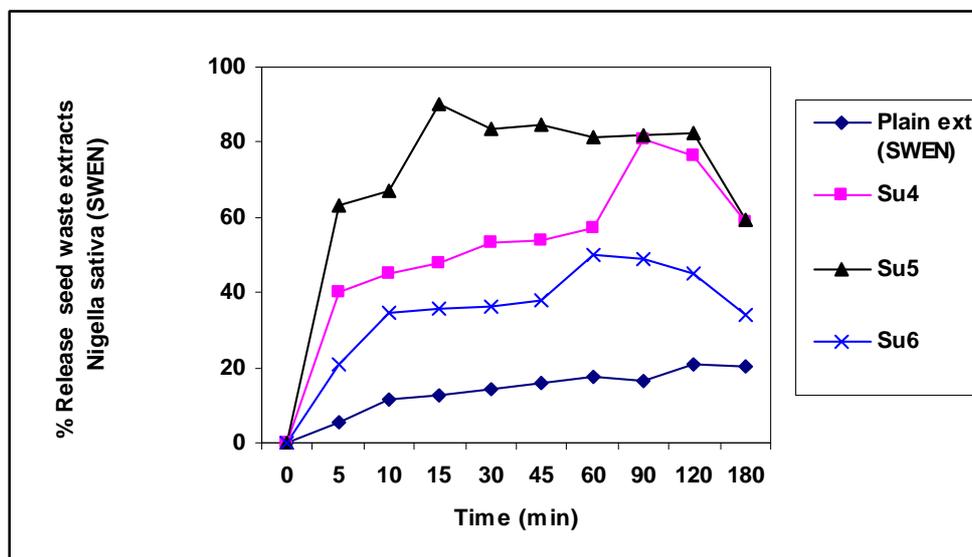


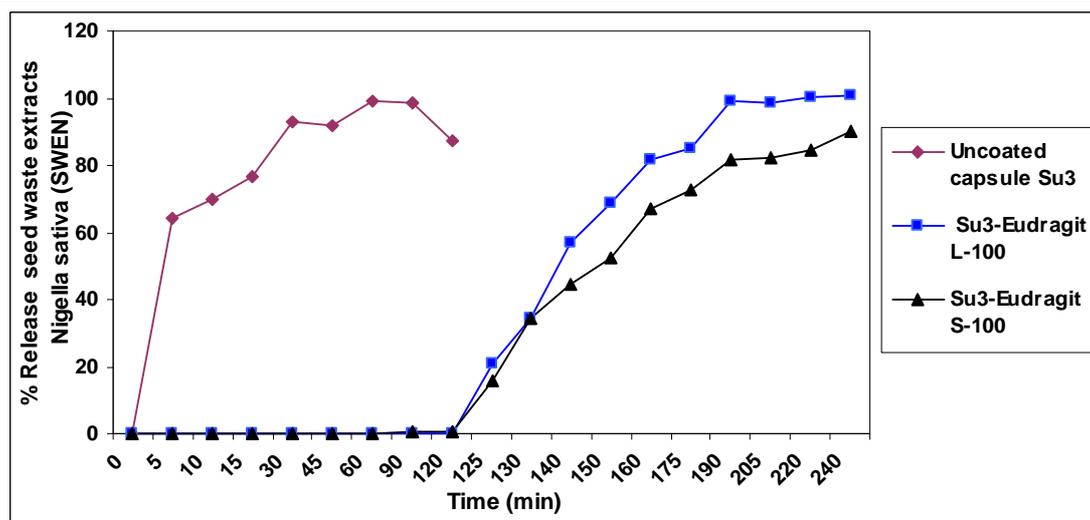
Figure 2: Dissolution profile of seed waste extract of *Nigella sativa* (SWEN) released from soft uncoated gelatin capsules (Su4-Su6) in comparison to plain extract

### Disintegration tests of the enteric-coated capsules (Su3:Eu.L-100, Su3:Eu.S-100)

By comparing coated to uncoated capsules, uncoated capsules (Su3) were disintegrated in SGM after 5 min. while coated capsules (Su3:Eu.L-100, Su3:Eu.S-100) did not disintegrate in SGM within 2 h, but they disintegrated in SIM in about 10 min as shown in Table (3).

### In-Vitro dissolution studies of film coating capsules

The calibration curves have correlation coefficients close to 1. The resulting data and the mean regression equations were computed and found to be: First curve:  $y = 0.00536x + 0.00399$ , where  $y$  is the absorbance,  $x$  is the concentration in  $\mu\text{g/ml}$  and correlation coefficient  $R^2 = 0.9998$  over the concentration range of (20 – 80  $\mu\text{g/ml}$ ) in SGM (pH 1.2). The second curve:  $y = 0.00516 + 0.00633x$  and  $R^2 = 0.9997$  over the range (25 – 200  $\mu\text{g/ml}$ ) in GIM (pH 7.4). Dissolution studies were carried out for both coated and uncoated capsules as shown in Figure (3). The percentage of protein release from uncoated capsules (Su3) was  $98.90\% \pm 0.99$  after 60 min when dissolved in SGM, whereas coated capsules with Eudragit L-100 and S-100 showed 0.32% and 0% release after 2 h respectively when dissolved in SGM. On the other hand a high percentage of protein release was obtained from coated capsules with Eudragit L-100 (101.12%) and Eudragit S-100 (90.04%) dissolved in SIM at the end of the additional 2 h. Figure (3) shows the effect of two formulation factors on lag time in GIM. Eudragit S-100 rises lag time significantly than Eudragit L-100, at the same time and less amount of extract was released [90.04%] during the 4 h of dissolution test. Thus it is important for a drug formulated at the terminal ileum at pH 7.4 to be released in GIM after a suitable lag time.



**Figure 3: Dissolution profile of seed waste extract of *Nigella sativa* (SWEN) released from soft uncoated (Su3) and coated (Su3-Eudragit L-100, Su3-Eudragit S-100) gelatin capsules.**

### Quality control of Su3 and Su3:Eu.S-100

The percentage of protein in Su3 was found to be  $99.5 \pm 0.77$  when dissolved in SGM (pH 1.2) for 2 h in comparison to the plain extract while it showed zero % for Su3:Eu.S-100 under the same condition. Protein percentage for Su3:Eu.S-100 was found to be  $97.7 \pm 2.22$  when dissolved in SIM (pH 7.4) for additional 2 h when compared to plain extract. Concentration of proteins in Su3 as calculated using regression equation was found to be 10.6 mg proteins/capsule after 2 h in SGM, while concentration of proteins from Su3: Eu. S-100 was 5.59 mg proteins/capsule after additional 2 h in SIM.

## DISCUSSION

Based on economical criteria and previous bioactivity-guided investigation of the aqueous extract of *Nigella sativa* seed waste [11], soft gelatin capsules were prepared incorporating the extract into a series of pharmaceutical excipients. Formula Su3 containing 200 mg powdered SWEN blended in a mixture of soya lecithin (50 mg), span 80 (25 mg), tween 80 (25 mg), PEG 400 (50 mg), Meglyol 813 (50 mg) [total weight of each capsule, 400 mg] was further subjected to enteric coating (Eudragit L-100 and Eudragit S-100). Selection of this formula was based on its potential hepatoprotective effect and optimum release of the bioactive protein.

In the current study,  $\text{CCl}_4$  produced an acute liver inflammation with significant increase in  $\text{TNF-}\alpha$ ,  $\text{IL-1}\beta$  and  $\text{IFN-}\gamma$  levels in both the serum and liver homogenates of mice, in bilirubin and GPT, as well as, GSH and LP compared to the normal control groups (Tables 4-7). Treatments with Su3 and to some extent Su4 significantly prevented  $\text{CCl}_4$ -induced increase in the cytokines production (Tables 4 and 5); in bilirubin and GPT (Table 6) and in GSH for Su3 only as compared to  $\text{CCl}_4$ -treated groups (table 7). The reduction of the elevated levels of  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$  in serum suggested that inhibition of pro-inflammatory mediators and cytokines is partly the mechanism of action of the aqueous extract and especially the protein fraction's protective effect. Results showed also that the level of  $\text{IFN-}\gamma$  was positively correlated with serologic markers of hepatic injury.

**Table 4: Effect of uncoated soft gelatin capsules Su1-Su6 (10mg/kg) of *Nigella sativa* seed waste on the TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  levels in the serum of mice**

Groups	Normal Control	CCl <sub>4</sub> -treated	Su1 (10mg/kg)	Su2 (10mg/kg)
TNF- $\alpha$ (pg/ml)	95.25 $\pm$ 3.56	216.75 $\pm$ 6.65*	200.15 $\pm$ 7.65*	198.23 $\pm$ 12.33*
IL-1 $\beta$ (pg/ml)	41.44 $\pm$ 9.42	170.34 $\pm$ 14.66*	150.68 $\pm$ 8.54	166.12 $\pm$ 6.08
IFN- $\gamma$ (pg/ml)	457.65 $\pm$ 51.78	1247 $\pm$ 67.21*	1089 $\pm$ 66.98*	1119.68 $\pm$ 41.99*

Groups	Su3 (10mg/kg)	Su4 (10mg/kg)	Su5 (10mg/kg)	Su6 (10mg/kg)
TNF- $\alpha$ (pg/ml)	94.01 $\pm$ 10.11* <sup>@</sup>	103.67 $\pm$ 11.45* <sup>@</sup>	180.17 $\pm$ 8.45* <sup>@</sup>	204.67 $\pm$ 6.55* <sup>@</sup>
IL-1 $\beta$ (pg/ml)	68.56 $\pm$ 4.82 <sup>@</sup>	78.56 $\pm$ 9.22 <sup>@</sup>	138.16 $\pm$ 10.43	129.56 $\pm$ 7.51
IFN- $\gamma$ (pg/ml)	632.45 $\pm$ 37.57 <sup>@</sup>	567.11 $\pm$ 14.98* <sup>@</sup>	1186 $\pm$ 66.98*	1290.09 $\pm$ 32.22*

SG1-SG6 were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl<sub>4</sub> (10 mg/kg b wt of 0.25 (v/v) solution in corn oil). One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation. Data are expressed as mean values  $\pm$  SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Turkey-Kramer multiple comparisons test. \* Significant difference from the control group at p<0.05. @ Significant difference from the CCl<sub>4</sub> group at p<0.05

**Table 5: Effect of uncoated soft gelatin capsules Su1-Su6 (10mg/kg) of *Nigella sativa* seed waste on the TNF- $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  levels in liver homogenate of mice.**

Groups	Normal Control	CCl <sub>4</sub> -treated	Su1 (10mg/kg)	Su2 (10mg/kg)
TNF- $\alpha$ (pg/ml)	65.25 $\pm$ 4.56	350.75 $\pm$ 12.65*	330.67 $\pm$ 10.45* <sup>@</sup>	300.76 $\pm$ 18.45* <sup>@</sup>
IL-1 $\beta$ (pg/ml)	39.66 $\pm$ 6.12	170.34 $\pm$ 24.66*	128.56 $\pm$ 10.22 <sup>@</sup>	143.45 $\pm$ 16.54*
IFN- $\gamma$ (pg/ml)	442.02 $\pm$ 42.17	1347 $\pm$ 101.23*	1236 $\pm$ 51.98*	1111.35 $\pm$ 39.66

Groups	Su3 (10mg/kg)	Su4 (10mg/kg)	Su5 (10mg/kg)	Su6 (10mg/kg)
TNF- $\alpha$ (pg/ml)	74.54 $\pm$ 10.67 <sup>@</sup>	298.67 $\pm$ 16.03* <sup>@</sup>	299.67 $\pm$ 17.45* <sup>@</sup>	289.11 $\pm$ 20.34* <sup>@</sup>
IL-1 $\beta$ (pg/ml)	57.92 $\pm$ 9.22 <sup>@</sup>	128.56 $\pm$ 12.22*	178.56 $\pm$ 15.29	150.18 $\pm$ 20.88
IFN- $\gamma$ (pg/ml)	641.23 $\pm$ 27.67 <sup>@</sup>	1244 $\pm$ 32.24*	943.26 $\pm$ 19.87	1145 $\pm$ 41.90*

Su1-Su6 were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl<sub>4</sub> (10 mg/kg b.wt of 0.25 (v/v) solution in corn oil). One day thereafter, rats were sacrificed by cervical dislocation and livers were rapidly excised and homogenized in chilled 1.15 KCL (PH 7.4) to yield 10% homogenates. Data are expressed as mean values  $\pm$  SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Turkey-Kramer multiple comparisons test. \* Significant difference from the control group at p<0.05. @ Significant difference from the CCl<sub>4</sub> group at p<0.05.

**Table 6: Effect of uncoated soft gelatin capsules: Su1- Su6 (10mg/kg) of *Nigella sativa* seed waste on CCl<sub>4</sub>-induced biochemical changes (glutamic pyruvic transaminase (GPT) activity and bilirubin level in plasma.**

Groups	Normal Control	CCl <sub>4</sub> -treated	Su1 (10mg/kg)	Su2 (10mg/kg)
GPT (units/ml)	193 $\pm$ 18.56	1366 $\pm$ 34.76*	1257.90 $\pm$ 68.17*	12926.52 $\pm$ 45.12*
Bilirubin ( $\mu$ mol/l)	2.4 $\pm$ 0.09	8.55 $\pm$ 0.32*	7.25 $\pm$ 0.55*	8.90 $\pm$ 0.39*

Groups	Su3 (10mg/kg)	Su4 (10mg/kg)	Su5 (10mg/kg)	Su6 (10mg/kg)
GPT (units/ml)	208.56 $\pm$ 12.12 <sup>@</sup>	227.56 $\pm$ 9.22 <sup>@</sup>	1190.16 $\pm$ 20.43*	1209.56 $\pm$ 19.51*
Bilirubin ( $\mu$ mol/l)	3.2 $\pm$ 0.07 <sup>@</sup>	2.9 $\pm$ 0.18 <sup>@</sup>	8.6 $\pm$ 1.98*	8.09 $\pm$ 0.42*

SSu1-Su6 were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl<sub>4</sub> (10 mg/kg b wt of 0.25 (v/v) solution in corn oil). One day thereafter, blood samples were collected from 18 h food-deprived animals and plasma was separated by centrifugation. Data are expressed as mean values  $\pm$  SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Turkey-Kramer multiple comparisons test. \* Significant difference from the control group at p<0.05. @ Significant difference from the CCl<sub>4</sub> group at p<0.05.

**Table 7: Effect of uncoated soft gelatin capsules: Su1-Su6 (10mg/kg) of *Nigella sativa* seed waste on CCl<sub>4</sub>-induced biochemical changes [lipid peroxides (LP) level and reduced glutathione (GSH)] content in liver homogenates of mice.**

Groups Parameters	Normal Control	CCl <sub>4</sub> -treated	SG1 (10mg/kg)	SG2 (10mg/kg)
LP(nmol MDA/g)	325.11±20.23	768.48±32.44*	677.14± 26.76*	626.52±29.10*
GSH (µmol/g)	9.87±0.58	19.12±1.54*	16.77±1.64*	18.71±0.45*

Groups Parameters	SG3 (10mg/kg)	SG4 (10mg/kg)	SG5 (10mg/kg)	SG6 (10mg/kg)
LP (nmol MDA/g)	348.66±12.12*	730.14±30.44*	690.25±29.87*	709.08±33.43*
GSH (µmol/g)	10.92±1.67 <sup>@</sup>	17.96±1.18*	18.16±2.04*	17.66±0.83*

S Su1-Su6 were administered orally, once daily, for 5 consecutive days. On day 5, liver injury was induced in animals by a single i.p. injection of CCl<sub>4</sub> (10 mg/kg b wt of 0.25 (v/v) solution in corn oil). One day thereafter, rats were sacrificed by cervical dislocation and livers were rapidly excised and homogenized in chilled 1.15 KCL (PH 7.4) to yield 10% homogenates. Data are expressed as mean values ± SEM (n=8). Statistical analysis was carried out by one-way ANOVA followed by Turkey-Kramer multiple comparisons test. \* Significant difference from the control group at p<0.05. @ Significant difference from the CCl<sub>4</sub> group at p<0.05

The significant increase in lipid peroxidation observed after CCl<sub>4</sub>- induced hepatotoxicity was not attenuated after treatment with all formulations, while the increased levels of GSH as compared to the respective normal values were significantly decreased by Su3.

In the present study, the GPT and bilirubin activities were dramatically increased in the CCl<sub>4</sub>-treated group compared with the normal control group, indicating severe hepatocellular damage. In contrast, treatments with Su3 and to a less extent Su4 significantly reduced CCl<sub>4</sub>-induced increase in bilirubin and GPT as compared to the control CCl<sub>4</sub> group. These results suggest that the Su3 may be clinically applied to treat liver diseases.

Enteric coating of Su3 with Eudragit S-100 sustained the release of proteins manifested by a low concentration of proteins (5.59 mg/capsule) at pH 7.4 (SIM) after additional 2h compared to that of Su3 (10.6 mg/capsule) at pH 1.2 (SGM) after 2 h. Absorption of nutrients occur mainly (95%) through the small intestine. The rates of absorption of various amino acids were influenced by the proportions and composition of proteins, peptides and free amino acids in the hydrolysates and by the interaction between amino acids involving inhibition or stimulation of transport. Proteases and peptidases in the intestinal mucosa play an important role in the process of amino acid absorption. The rate of absorption of arginine, leucine, phenyl alanine and methionine were found to be higher than those of threonine, histidine and lysine irrespective to the hydrolysate source [38]. Site-specific release of proteins at the terminal ileum at pH 7.4 offered maximum absorption of proteins leading to maximum activity.

In summary, this study demonstrates that Su3 formulation can protect against CCl<sub>4</sub>-induced acute Hepatotoxicity through restoration of the anti-oxidative defense system and down-regulation of the pro-inflammatory pathway, providing evidence that *Nigella sativa* seed waste aqueous extract may be an alternative treatment for liver diseases caused by xenobiotics. Enteric coating of Su3 with Eudragit S-100 sustained the release of proteins and increased their absorption at the terminal ileum.

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