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ORIGINAL ARTICLE

Molecular complexes of aspirin with humic acid extracted from shilajit and their characterization

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Abstract Aspirin possesses antipyretic, anti-inflammatory, analgesic and anti-aggregatory activity. The acetylsalicylic acid molecule has a carboxyl group and an ester group. The ester group can be easily hydrolyzed, which reduces the medicinal value and causes side effects on humans. The aim of the present study was to prepare solid complexes between aspirin and humic by lyophilization and solvent evaporation technique in the molar ratio 1:1 and 1:2. Molecular interaction between aspirin and humic acid were studied by DSC, XRD, FT-IR and scanning electron microscopy. This technique clearly demonstrated the existence of solid inclusion complex formation. The lyophilized complex in the molar ratio 1:2 showed enhanced stability and dissolution rates of aspirin significantly. A highly significant ($p < 0.05$) anti-inflammatory action of the treatment of optimized freeze dried (1:2) aspirin complex with humic acid was evidenced by inhibition of rat paw edema and anti-ulcerogenic action was measured by lowest score (0.63 ± 0.10) with significant reduction in ulceration as compared to aspirin alone.

Keywords Aspirin · Shilajit · Humic acid · Characterization · Stability · Bioavailability

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Introduction

Shilajit, a wonder medicine of ayurveda, neither a plant nor animal origin, it is a mineral pitch that comes out from the rocks of the Himalayas, as they become warm during summer months [1–5]. Shilajit contains a variety of organic compounds that can be broadly classified into humic and non-humic substances [6–9]. Humic substances are further classified into humic and fulvic acid. Humic acid (HA) and fulvic acid (FAs) have relatively open, flexible structure punctured by voids (micropores) of different diameters (200–1,000 Å) as reported in literature [4, 10, 11]. The interior of these humic and fulvic acid hydrophobic and thus are capable of forming inclusion complexes with non-polar solutes and unstable drug molecules [10, 11]. These drug molecules can be entrapped in the hydrophobic interior so as to increase their solubility, rate of dissolution and stability, thereby enhancing their bioavailability [12, 13]. We recently reported the influence of humic and fulvic acid on enhancement of bioavailability through complexation with poorly bioavailable drugs [4, 11].

Aspirin (acetylsalicylic acid) is very old drug but still having a very high market value. It possesses antipyretic, anti-inflammatory, analgesic and anti-aggregatory activity due to decreased production of prostaglandins and thromboxanes [14]. The acetylsalicylic acid molecule has a carboxyl group and an ester group. The ester group can be easily hydrolyzed, which reduces the medicinal value and causes side effects on humans [15, 16]. A strategy designed how to inhibit the hydrolytic decomposition and enhancement of dissolution of aspirin inside the void of humic acid of shilajit. We propose to investigate the effects of humic acid as carrier on aspirin (acetylsalicylic acid) in enhancing the dissolution rate and bioavailability, increasing the

stability and decreasing the toxicity of aspirin through complexation.

Materials and methods

Materials

Shilajit was kindly supplied by Dabur Research Foundation, India and aspirin was purchased from sigma–Aldrich, Germany. All other chemical were of analytical reagent grade.

Extraction of humic acid form shilajit

Humic acid is extracted from shilajit by increasing the polarity of solvent as reported in literature [17]. The method consisted of successive extraction of raw shilajit with hot organic solvents of increasing polarity to remove the bioactive components. The residue (marc) was dissolved in 0.1 N NaOH with intermittent shaking in the presence of nitrogen. The suspension was filtered and the filtrate was acidified to a pH of less than 3 to precipitate the humic acids. The resulting humic acid is dried, pulverized in glass mortar pestle and stored in desiccator.

Preparation of inclusion complexes

The preparation of solid complexes aspirin-humic acid were performed by different techniques in the molar ration 1:1 and 1:2.

Solvent evaporation (SE)

Complex of Aspirin with humic acid were prepared by dissolving the required quantity of aspirin and humic acid in 50 mL of chloroform [18]. The mixture was sonicated and solvent was then removed in rotary evaporator (Hahn shin science Co., Hs- 2001N) under reduced pressure at 60 °C. The complex was dried in oven and collected. The complex was sieved through sieve no. 60 and was stored in a vacuum desiccator.

Freeze drying (FD)

Aspirin-humic acid complex was prepared by dissolving the required quantity of both in double distilled water. The mixture was sonicated and sonicated for 2 hrs to get a clear solution. The solution was frozen in ultra freezer by keeping for 24 h and freeze dried over 12 h in Lyph-lock apparatus (Drywinner, DW-8-85 Heto Holten, Denmark). The resulting amorphous powder is powdered in glass mortar and pestle and passed through 100-mesh sieve to obtain a uniform size fine powder.

Characterization of the solid complexes

Differential scanning calorimetry (DSC)

Thermal behaviour of aspirin, and humic acid and their inclusion complexes were examined by using a Perkin Elmer Pyris 6 DSC. Inert nitrogen gas was used as carrier gas and the DSC analysis was carried out at a heating rate of 10 °C/min and an nitrogen gas flow rate of 20 mL/min. The sample size was 1 mg and examinations were made in the temperature intervals between 50 and 400 °C.

Fourier transforms infra-red spectroscopy (FT-IR)

The FT-IR spectra of aspirin, humic acid and inclusion complexes of aspirin were recorded on the Win-IRrez (Bio-Rad) using the potassium bromide (KBr) disc technique. Sample equivalent to 2 mg of aspirin was mixed with potassium bromide (about 100 mg) using a clean glass pestle and mortar and compressed to get a pellet. Base line was corrected and scanning was done from 4,000 to 400 cm^{-1}.

X-ray diffraction of solid complexes (X-RD)

X-ray diffraction of samples were obtained by using X-ray diffractometer (PW 1830, Phillips, Japan). The scanning rate was 4°/min. The voltage/current used was 30 kV/25 mA and the target/filter (monochromator) was copper.

Scanning electron microscopy (SEM)

SEM of samples were performed using *Jeol scanning Microscope JSM-840* with a 10 kV accelerating voltage. The surface of the samples for SEM were previously made electrically conductive in a sputtering apparatus (Fine coat ion sputter JFC-1100) by evaporation of gold. A magnification of 1500 was used.

Release study of aspirin from their complexes

The dissolution rate studies were performed according to the USP XXVI rotating paddle type method. The sample corresponding to 100 mg of aspirin were placed in hard gelatin capsules. Dissolution medium was acetate buffer (pH 4.5). The stirring speed was 50 rpm and temperature 37 ± 0.5 °C. 5 mL samples were withdrawn at a settled time interval using a syringe and analyzed by HPLC method.

Stability studies

All the complexes and ASA alone were packaged in well labeled sealed polythene lined aluminium pouches and stored in stability chamber at ±40 °C and 75 ± 5% RH for 120 days. Accurately weighed equivalent to 18 mg of the ASA present in complex and dissolved in 100 mL of ethyl alcohol (180 μg/mL). The solution was vigorously shaken putting the conical flask in sonicator bath for 15 min. Samples were analyzed by HPLC for salicylic acid content at 0, 30, 60, 90 and 120 days.

Pharmacodynamic studies

Anti-inflammatory studies: the rat paw edema method

Anti-inflammatory activity was performed using carrageenan induced rat hind paw edema model. Male wistar albino rats, each weighing 150–200 g were divided into three different groups each containing four rats ($n = 4$). The animals were starved overnight and deprived of water only during the experiment. At first, paw volume of each animal of different groups was determined before giving any treatment to them. Oedema was induced by sub-planter injection of 0.1 mL of 1% carrageenan suspension in 1% sodium carboxy methyl cellulose (CMC) into the right hind paw of each rat. Animals of group I served as control and received vehicle only (10 mL/kg body weight of 1% CMC).

Group II received pure drug aspirin suspended in 1% sodium carboxy methyl cellulose at dose of 100 mg/kg and Group III received optimized freeze dried complex of aspirin with humic acid (1:2) suspended in 1% sodium carboxy methyl cellulose at dose equivalent to 100 mg/kg aspirin 1 h prior to the carrageenan injection. The paw volume was measured at 0, 1, 2, 3, and 4 h after the injection of carrageenan by using digital plethysmometer. The paw is inserted onto water in a clear acrylic cell, up to the mark.

Gastric ulceration studies

Male albino rats weighing between 140 and 170 g were selected for pyloric ligation ulcer model [19]. Rats were divided into three groups, each group consisting of five animals. Animals were fasted for 24 h. Group I received 1% sodium carboxy methyl cellulose (1% Na-CMC), Group II received pure drug aspirin suspended in 1% Na-CMC at dose of 100 mg/kg and Group III received optimized freeze dried complex of aspirin with humic acid (1:2) suspended in 1% Na-CMC at dose equivalent to 100 mg/kg aspirin. Animals were sacrificed 4 h later using ether and the stomach was opened to collect the gastric contents. The stomach is removed and slightly inflated by injection of 1% formalin solution through esophageal junction. Then the stomach is maintained in 1% formalin solution for 10 min for fixation of the inner and outer layer of the gastric wall. The stomach is opened along the greater curvature and the length of lesions (dark blue areas against pale blue background) in the glandular portion is measured under a dissecting microscope (×40) provided with square grid [20].

0.0—Normal (no injury, bleeding and latent injury)
0.5—Latent injury or widespread bleeding.
1.0—Slight injury (2–3 dotted lines)
2.0—Severe injury (continuous lined injury or 5–6 dotted injuries)
3.0—Very severe injury (several continuous lined injury)
4.0—Widespread lined injury or widened injury.

Results and discussion

Characterization of the solid complexes

Differential scanning calorimetry (DSC)

The DSC thermogram of pure aspirin drug powder showed a sharp endotherm near 135 °C, which is indicative of its melting temperature. Humic acid exhibits a thermogram devoid of any endothermic peak which indicates that it does not have any sharp melting point. An exotherm is observed above 330 °C indicating decomposition. The DSC pattern of aspirin-humic acid inclusion complexes (1:1 and 1:2) prepared by solvent evaporation showed complete absence of peaks of pure aspirin Thermogram of aspirin-humic acid complexes prepared by freeze drying method (1:1 and 1:2) showed complete disappearance of the endothermic peaks characteristic of aspirin, thus suggesting maximal/complete complex formation (Fig. 1).

Fourier transforms infra- red spectroscopy

FT-IR spectra of aspirin showed a characteristic peak at 1,754 cm^{-1} (acetoxy C=O group stretching), 1,693 cm^{-1} (carboxyl C=O group stretching) and 1,606 cm^{-1} (C=C aromatic stretching). Humic acid exhibited its characteristics broad band at about 3,400 cm^{-1} (hydrogen bonded OH group) and a band in the region of 1,640 cm^{-1} (conjugated C=C double bond), 1,400 cm^{-1} (OH bending of carboxylic acid) and 1,140 cm^{-1} (C–O stretching).

Complex of aspirin-humic acid prepared by solvent evaporation in the molar ratio of 1:1 exhibited spectra where characteristic peaks of aspirin 1754, 1693 and

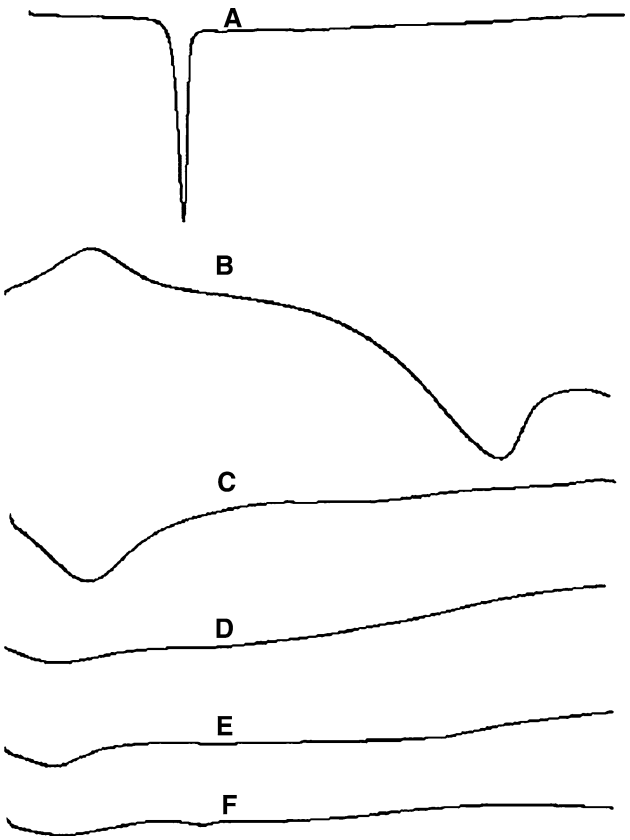

Fig. 1 DSC spectra of humic acid complexes (*A*) aspirin, (*B*) humic acid, (*C*) 1:1 SE, (*D*) 1:2 SE, (*E*) 1:1 FD, (*F*) 1:2 FD

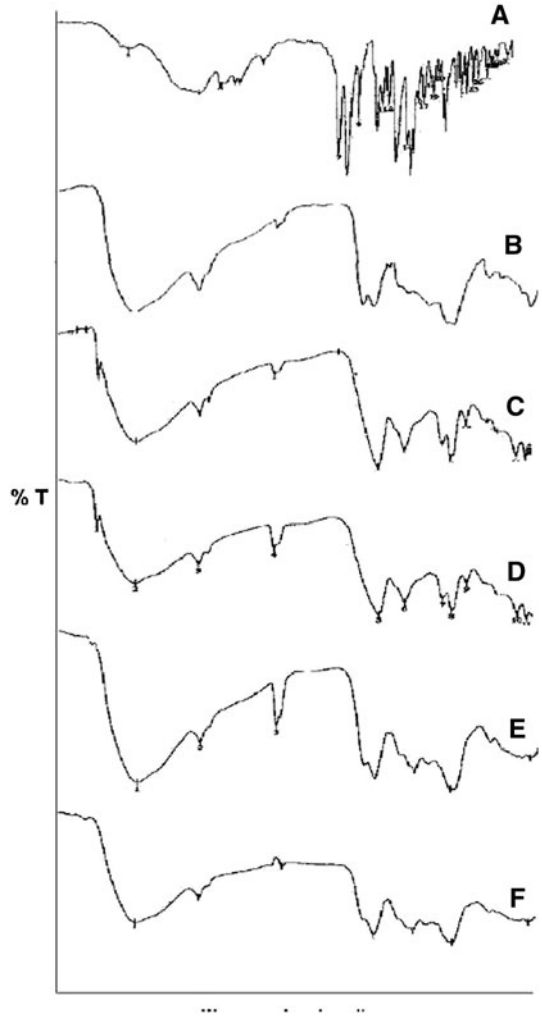

Fig. 2 FT-IR spectra of humic acid complexes (*A*) aspirin, (*B*) humic acid, (*C*) 1:1 SE, (*D*) 1:2 SE, (*E*) 1:1 FD, (*F*) 1:2 FD

1606 cm^{-1} were missing. A shifting of drug peak was observed from 1,754 to 1,578 cm^{-1}, revealing interaction between drug and humic acid. Whereas FTIR spectra of solvent evaporated complex in the molar ratio 1:2 showed complete disappearance of drug indicating complete entrapment of drug within humic acid.

All the characteristic peaks of drug in the finger print region were absent in the complex of freeze dried 1:1 molar ratio but at 1,609 cm^{-1} due to C=C aromatic stretching present indicating strong interaction of drug with complexing agent. However in the freeze dried 1:2 molar ratio all the representative peaks of drug were disappeared, indicating complete complexation (Fig. 2).

X-ray diffraction of solid complexes

X-ray diffraction pattern of aspirin showed a most intense peak at an angle 15.5° (100%) followed by 15.33° (44.4%), 7.67° (40.5%), 7.79° (35.5%), 26.8° (15.9%), 27.01° (13.1%), 32.47° (5.5%). These intense peaks reveal the crystalline nature of aspirin. However X-ray diffraction pattern of humic acid showed some intense peak at an angle of 31.8° (100%), 45.53° (32.7%), 56.55° (18.4%), 26.72° (16.9%) revealing its amorphous nature. Inclusion complexes of aspirin with humic acid showed undefined, broad, diffused peaks with low intensities (Fig. 3). Though this signifies amorphous nature but a few sharp peaks having less intensities of was observed.

Scanning electron microscopy

Scanning electron microscopy is useful tool to examine the microscopic aspects of the drug, the complexing agent and the complexes formed. Although this method is not a very authentic technique to confirm complex formation nevertheless it helps to asses the existence of a microparticulate present in the preparations obtained. Aspirin is characterized by the presence of crystalline particle of different sizes. Pure humic acid appears as amorphous particles with irregular shape. The optimized freeze dried complex (1:2) of aspirin-humic acid showed typical morphology of preparations, that is aggregated particles, suggesting the

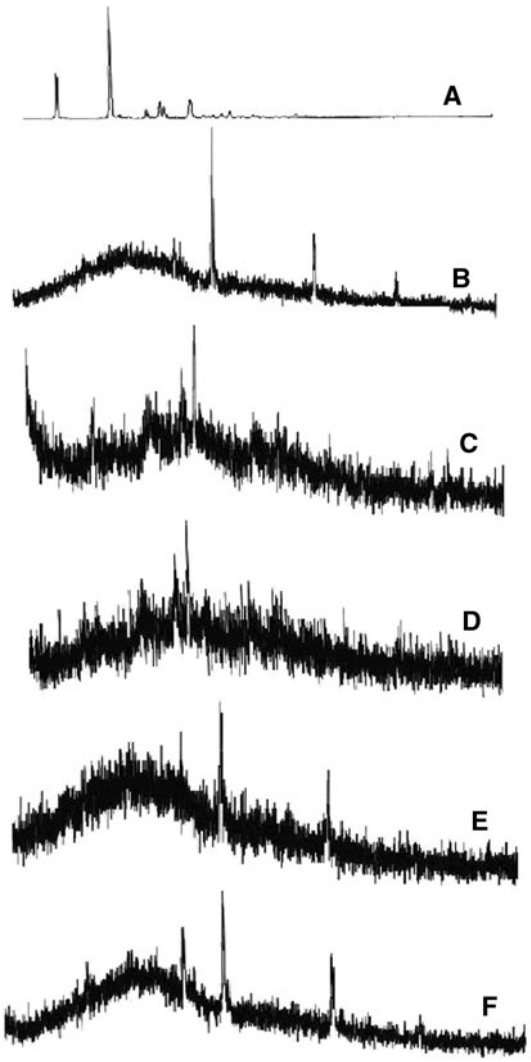

Fig. 3 XRD spectra of humic acid complexes (*A*) aspirin, (*B*) humic acid, (*C*) 1:1 HAC, SE, (*D*) 1:2 SE, (*E*) 1:1 FD, (*F*) 1:2 FD

existence of an amorphous product with the presence of a single component in the complex, thus suggesting maximum or complete complex formation (Fig. 4).

In vitro dissolution rate studies

The release profile of aspirin-humic acid systems prepared by solvent evaporation and freeze drying method are shown in Fig. 5. The dissolution profiles of the complexes were studied in acetate buffer (pH 4.5) to gain information about the dissolution of the drug in the stomach. The release rate profiles were drawn as the percentage of drug dissolved versus time. According to these results, Dissolution of aspirin alone was found to be 30% in 30 min. However, release of freeze dried complex (1:2) was increased to 65% (2 fold) in 30 min, which may be attributed due to amorphous nature of complex, as confirmed by XRD studies. The study clearly demonstrates that when aspirin is complexed with humic acid there is a significant increase in its dissolution rate. It is also seen that the freeze dried and solvent evaporated complexes in different molar ratios (1:1, 1:2) exhibit higher dissolution rates than the pure drug alone. The enhancement of the release of aspirin from complexes were found to be dependent on the preparation method and molar ratios both, since the freeze dried (1:2) exhibited the highest dissolution rates.

Stability studies

The results of stability studies are shown in Figs. 6 and 7 of aspirin -humic acid complexes in different molar ratio prepared by different techniques were found to be stable. The overall profile of ASA degradation in the aspirin-humic acid complex were studied at ±40 °C and 75 ± 5% RH for 120 days indicated by the rate of appearance of salicylic acid. Degradation of aspirin was rapid in solvent evaporated complex of humic acid (1:2) among others. However, very less content of salicylic acid (6.21%) was determined in 1:2 freeze dried complex among other humic acid complex, when compared to aspirin alone (14.20%). It was concluded that a significant improvement in aspirin stability were observed with 1:2 Freeze dried aspirin-humic acid complex. Complex formation may be regarded as an encapsulation of the aspirin molecule inside the void of humic acid which protect hydrolabile ester moiety of the molecule from hydrolysis.

Pharmacodynamic studies

Anti-inflammatory studies: the rat paw edema method

The optimized humic acid complex prepared by the freeze drying method showed a faster onset of anti-inflammatory activity as compare to the control (1% CMC), indicating maximum inhibition of edema. A 56, 43, 60 and 67% inflammation inhibition in the 1:2 freeze dried humic acid-aspirin complex was obtained after 1, 2, 3 and 4 h respectively, where as for aspirin, a 31, 37, 31, 23% inflammation inhibition was observed after 1, 2, 3 and 4 h respectively. A highly significant ($p < 0.05$) anti-inflammatory action of the treatment of optimized freeze dried (1:2) aspirin complex with humic acid was evidenced by inhibition of rat paw edema as compared to aspirin alone (Table 1).

Gastric ulceration studies

Accumulation of gastric juice and blood are responsible for the induction of ulceration or injury to the stomach during

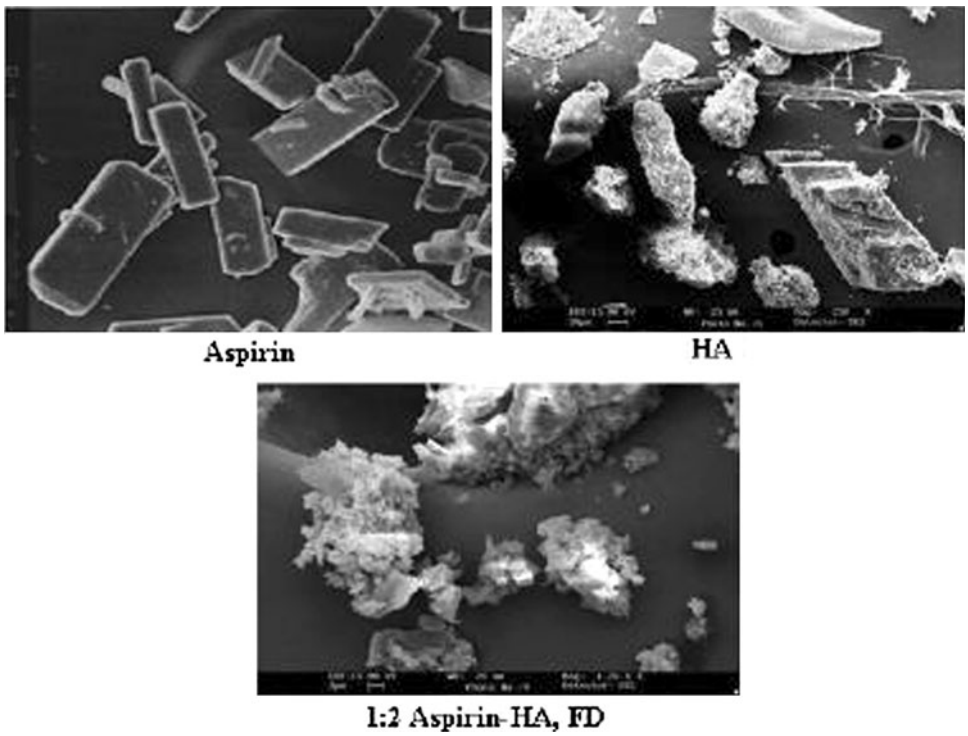

Fig. 4 SEM of aspirin, humic acid and optimized humic acid complex

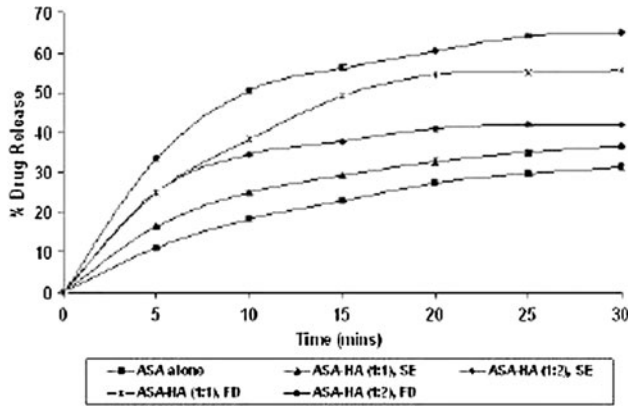

Fig. 5 Release profile of humic acid complexes in acetate buffer (pH 4.5)

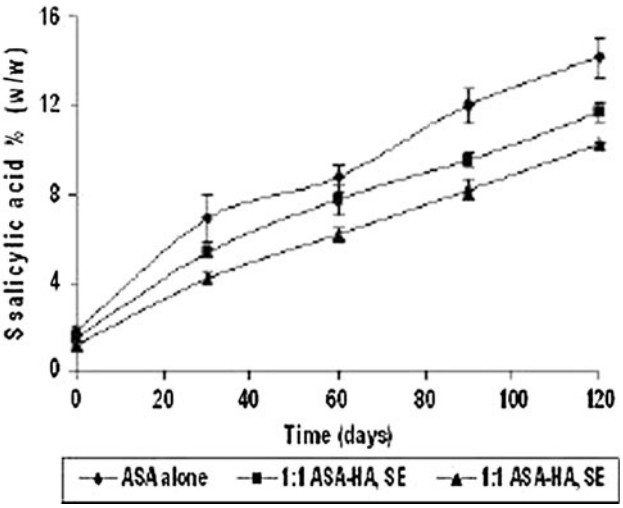

Fig. 6 Appearance of salicylic acid (w/w) content in freeze dried humic acid complex

pyloric ligation. Table 2 shows the degree of ulceration after treatment at a dose of 100 mg/kg equivalent to aspirin. Freeze dried complex of aspirin with humic acid in the molar ratio 1:2 showed lowest score (0.63 ± 0.10) with significant reduction in ulceration as compared to aspirin alone. Crystals of aspirin being partially soluble in gastric acid remain in contact with the stomach wall for a longer period of time, resulting in a dangerously high local concentration. This leads to mucosal damage by inhibiting the prostaglandin synthesis, increasing acid secretion and back diffusion of H^+ ions. It is expected that in the complexed form, the drug will dissolve fast and show an accelerated absorption. Moreover, it will not come in direct contact with the stomach wall in crystalline state since until it is dissolved it remains encapsulated within the humic acid matrix.

Conclusions

Desirable enhancement in dissolution and stability of aspirin can be achieved through humic acid complexation. However, such a novel approach appears to be beneficial to overcome the problem of poor bioavailability. The aspirin

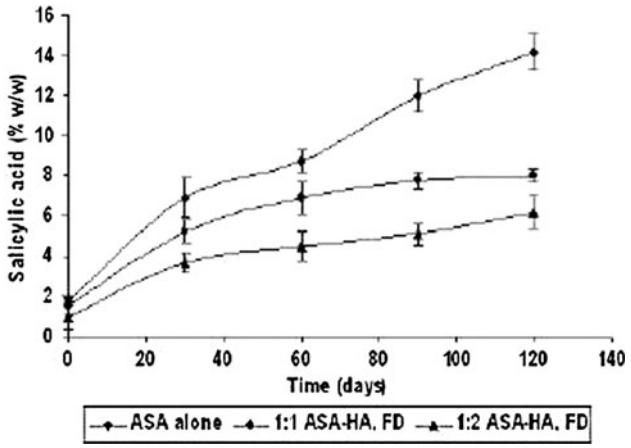

Fig. 7 Appearance of salicylic acid (w/w) content in solvent evaporated humic acid complex

Table 1 Inhibition of rat paw edema by aspirin and their optimized complexes

Treatments	% Inhibition/time (h)			
	1	2	3	4
Aspirin	31.25	36.96	31.11	22.92
1:2 ASA-HA (FD)	56.25	43.48	66.67	60.42

Table 2 Degree of injury to the stomach of rats

Treatments	Ulcer score
Control (1%) CMC	0.68 ± 0.17
ASA as such	1.12 ± 0.08
HA-ASA 1:1 FD	0.63 ± 0.10

molecule has a carboxyl group and an ester group. The ester group can be easily hydrolyzed, which reduces the medicinal usefulness and has gastrointestinal side effects on humans. To reduce its toxicity, complexation was tried with humic acid extracted from shilajit. Complexes were made in the molar ratio 1:1, 1:2 by solvent evaporation and freeze drying techniques. The complexes were identified by FT-IR, DSC, XRD and SEM spectral studies. Release and stability of aspirin from 1:2 freeze dried complex showed significant improvement as compared to other complex. Thus significant enhancement of bioavailability parameters such as drug dissolution and stability were observed when aspirin was complexed with humic acid. A highly significant anti-inflammatory and anti-ulcerogenic action were evidenced by the treatment of optimized complex. This has potential for industrial application in developing and improved dosage form of aspirin.

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