CHAPTER-1

INTRODUCTION
1.1 INTRODUCTION

Shilajit, also known as salajit, shilajatu, mumie or hummiyo is a pale-brown to blackish-brown exudation, of variable consistency, coming out from layer of rocks in many mountain ranges of the world, especially the Himalayan ranges of the Indian subcontinent (Chopra et al., 1958; Ghosal, 1992a; Agarwal et al., 2007a). It is also found in many other mountain ranges of the world, e.g. Afghanistan (Hindukush), Australia (Northern Pollock Ranges), and in the former USSR (Tien-Shan, Pamir, Caucasus, Ural), where it is collected in small quantities from steep rock faces at altitudes between 1000 and 5000m (Ghosal, 2002a). We have recently reported the physico-chemical, spectral and thermal properties of Shilajit which further confirm its humic nature (Agarwal et al., 2007b).

Shilajit has been reported to contain a number of components including resins, fatty acids, sterols, triterpenes, aromatic carboxylic acids, 3,4-benzocoumarins and \( \alpha \)-aminoacids (Ghosal et al., 1976). The biological effects of shilajit have been ascribed to two distinct classes of compounds (Ghosal et al., 1991).

❖ The low molecular weight bioactive organic compounds such as oxygenated dibenzo-a-pyrones, and

❖ The medium molecular weight Fulvic acids (FA) and Humic acids (HA).

While the benzopyrones act as the active principles, fulvic and humic acids acts act as carrier molecules for \textit{in-vivo} transportation of these bioactive substances (Anwer et al., 2007). The interior of this complexing agents are thus capable of forming inclusion complexes with non-polar solutes and drug molecules with low bioavailability. These drug molecules can be entrapped in the hydrophobic interior so as to increase their solubility, dissolution and stability, thereby enhancing their bioavailability (Khanna, 2006).

Such entrapment is also capable of enhancing the stability of the drug molecules. In fact, it has been reported that the bioactive principles of shilajit owe their stability in the natural habitat due to their entrapment in the voids (micropores) of the fulvic acids of shilajit humus (Ghosal, 1992b, Agarwal et al., 2008a, c). A purified fulvic acid carrier...
having a sponge like structure punctured by voids of about 200-1000 Å in diameter and an average molecular weight of about 700-2500 to which a water insoluble and unstable active ingredient added to fill the voids (Ghosal, 1992c).

So far, except Jamia Hamdard (Saluja, 2001; Khanna, 2006; Karmarkar, 2007; Anwer, 2005, Ahmad, 2006; Vashisht, 2006; Mirza, 2007 and Tyagi, 2007) there is no report in the literature on the use of fulvic and humic acids in enhancing the bioavailability of any drug. There are some scattered reports of their use as bioenhancers of trace elements and vitamins.

Aspirin (ASA) is an old drug but still possesses high medical value, and its health protection function such as antipyretic, anti-inflammatory, analgesic and anti-aggregatory activity has received more and more attention. The acetylsalicylic acid molecule has a carboxyl group and an ester group. The ester group can be easily hydrolyzed, which reduces the medical value and has side effects on humans. A need exists to learn how to inhibit the hydrolysis of acetylsalicylic acid and to reduce its toxicity.

In the present project we use humic and fulvic acids extracted from shilajit as complexing agents for such moisture sensitive drug can be a potential approach to inhibit the degradation, such an interaction or association between the drug molecule either with humic or fulvic acid can lead to an increase in the drug bioavailability, decreasing toxicity and a better pharmacodynamic profile. A comparative study has been done between complexes of humic acid/fulvic acid and hydroxy propyl-β-cyclodextrin (HP-β-CD) - aspirin complex.

1.2 LITERATURE REVIEW

1.2.1 Shilajit

Shilajit is a pale-brown to blackish-brown exudation, of variable consistency, oozes from the rocks of the Himalayas, as they become warm in the summer months. It is said to carry the healing power of these great mountains (Chopra et al., 1958; Ghosal, 1993; Ghosal et al., 2000; Frawley, 2001). It is also found in Russia, Tibet, Norway and other countries, where it is collected in small quantities from steep rock faces at altitudes...
between 1000 and 5000m. Shilajit samples from different regions of the world however vary in their physiological properties (Chopra et al., 1958; Ghosal, 1992c).

Shilajit is an important drug of the ancient Hindu materia medica and is to this day used extensively by the Hindu physicians for a variety of diseases. Early ayurvedic writings from the Charaka Samhita and Susruta Samhita describe shilajit as a cure for all diseases as well as a rasayana (rejuvenative) able to increasing longevity from 100 to 1000 years of age. Shilajit is one such remedy, which has been in use as a folk medicine for over 3,000 years as a rejuvenator and adaptogen (Sharma et al., 2000). It has been used by Vaidyas and Hakims for ages and has a unique place in the ancient texts. It has been said that there is hardly any curable disease, which cannot be controlled or cured with the aid of shilajit. Although this is a tall order, scientific studies over the last 20-25 years have shown that it is indeed a panacea of traditional medicine, effective in a number of ailments. We present here a brief review of the ancient claims for this panacea and the modern scientific findings, which have validated these claims.

1.2.1.1 Synonyms of shilajit

<table>
<thead>
<tr>
<th>Languages</th>
<th>Name</th>
<th>References</th>
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<tr>
<td>Sanskrit</td>
<td>Shilajit, Silajit, Silaras</td>
<td>(Chopra et al., 1958)</td>
</tr>
<tr>
<td>Hindi, Gujarati and Marathi</td>
<td>Silajita</td>
<td>(Chopra et al., 1958)</td>
</tr>
<tr>
<td>Hindi</td>
<td>Ral-yahudi</td>
<td>(Nadkarni, 1954)</td>
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<td>Bengali</td>
<td>Silajatu</td>
<td>(Chopra et al., 1958)</td>
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<tr>
<td>Arabic</td>
<td>Hajar-ul-musa</td>
<td>(Chopra et al., 1958)</td>
</tr>
<tr>
<td>English</td>
<td>Vegetable Asphalt</td>
<td>(Tirtha, 1998)</td>
</tr>
<tr>
<td>Botanical description</td>
<td>Bitumen mineral</td>
<td>(Puri, 2003)</td>
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<tr>
<td>Russian</td>
<td>Mummio, Mumie</td>
<td>(Bucci, 2000)</td>
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<td>Persian</td>
<td>Momiai Faqrual Yahud</td>
<td>(Nadkarni, 1954)</td>
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<td>Tamil</td>
<td>Perungyum, Uerangyum</td>
<td>(Nadkarni 1954)</td>
</tr>
<tr>
<td>Latin</td>
<td>Asphaltum</td>
<td>(Tirtha, 1998)</td>
</tr>
</tbody>
</table>
1.2.1.2 Shilajit in ancient texts

Tribal villagers of Himalaya, who were observing white monkeys moving to the higher mountains in summer months, made the discovery of shilajit. The monkeys were observed to lick the semi-solid substance exuding out the rock crevices. Since observing the animal behaviors was an important part of healthcare research in ancient times, those villagers attributed the great strength, longevity and wisdom of those monkeys to this substance. Curious by the thought, they themselves started taking the substance and reported a broad spectrum of improvement in their health and stamina. It gave them more energy, relieve digestive problems, increase sex drive, improve to memory etc., with the passage of the time traditional health practitioners established the methods to purify the substance (Dabur, 2003; Tewari et al., 1973).

1.2.1.3 Source of Shilajit

The statement of Charaka Samhita

“Stones of metal like gold, silver, black iron etc in the mountains get heated up by the sun and form exudates that comes out of them and results in the formation of smooth and clean gum called cilajatu”. Sharma adds that metals like gold do not produces exudate and what was actually intended was that stones containing gold would produce shilajit (Sharma et al., 2000).
The statement of Sushruta Samhita

“A gelatinous substance that is secreted from the side of the mountains when they have become heated by the rays of the sun in the months of Jyaishta and Ashadha. This substance is what is known as Çilájatu and it cures all distempers of the body.” (Bhishagratna, 1998). It is found in abundance in the lower Himalayan hills near Haridwar, Simla and also in Nepal. (Chopra et. al., 1958).

1.2.1.4 Varieties of Shilajit

According to Charaka Samhita four types of shilajit were found based upon four types of metals on stone from which it exudes: gold, silver, copper and black iron. The shilajit obtained from the stone containing gold is the best. If administered according to proper procedure, it produces rejuvenating and aphrodisiac effects and cures diseases (Sharma et. al., 2000).

The Sushruta Samhita states that there are six types based on their origins. In addition to the four types of metal associated with shilajit listed above he explained presence of tin and lead. Each type has the same taste (rasa) and potency (virya) as the metal to whose essence it owes its origin. He goes on to note that tin, lead, iron, copper, silver and gold are progressively more efficacious, so the different types of shilajit that derive from these metals are also progressively more efficacious in their application (Bhishagratna, 1998).

The Astanga Hardayam also noted the six types of shilajit but they mentioned that the shilajit coming out of iron is the best (Murthy, 2001).

The description of six types in Sushruta relates to both the rejuvenation therapy and treatment of diseases. Charaka describes only the rejuvenating effects of shilajit, and this effect is available in all four types of shilajit that he lists. (Sharma et. al., 2000).

Chopra (1958) states there are four types each with its own unique color; gold (red), silver (white), copper (blue), iron (blackish brown).

There are several varieties of the substance, of which the black color has the main therapeutic properties (Frawley, 2001).
CHAPTER -1

INTRODUCTION

The black form of shilajit is the most commonly used medicinal form (Halpern, 2003).

1.2.1.5 Chemical Constituents of Shilajit

Extensive research has been carried out to know the exact chemical nature of shilajit. Earlier work on shilajit showed that its major organic constituents included benzoic acid, hippuric acid, fatty acids, resin and waxy materials, gums, albuminoids and vegetable matter with benzoic acid being the active ingredient (Kong et al., 1987; Ghosal et al., 1976). Extensive research in the eighties showed that the major organic mass of shilajit comprised of humus (60-80%) along with other components such as benzoic acid, hippuric acid, fatty acid, ichthyol, ellagic acid, resin, triterpenes, sterol, aromatic carboxylic acid, 3,4-benzocoumarins, amino acids and phenolic lipids (Ghosal et al., 1988a & b). The major physiological action of shilajit was found to be due to the presence of the bioactive dibenzo-alpha-pyrones along with humic and fulvic acids which acted as a carrier molecules for the active ingredients (Ghosal, 1990; Ghosal, 1980).

The composition of shilajit is influenced by factors such as the plant-species involved, the geological nature of the rock, local temperature profiles, humidity and altitude, etc. For example, it was found that shilajit obtained from India in the region of Kumoan contains higher percentage of fulvic acids (21.4%) as compared to shilajit obtained from Nepal (15.4%), Pakistan (15.5%) and Russia (19.0%). On the other hand the bioactive low molecular weight compound found to be high in shilajit obtained from Nepal. Similarly pH of the 1% aqueous solution of shilajit is different obtained from different countries, viz., 6.2 for India (Kumoan), 7.5 for Nepal (Dolpa), 6.8 for Pakistan (Peshawar) and 8.2 for Russia (Tien-Shan). Similarly, humic constituents in shilajit samples obtained from these countries also varied (Ghosal et al., 1991).

1.2.1.6 Purification and Formulation of Shilajit

Modern research has shown that shilajit in its natural form is often contaminated with varied amount of impurities such as mycotoxins, heavy metal ions, polymeric quinones, reactive free radical etc. Mycotoxins are produced by mold or fungi and can cause illness or death in man. Free radicals can be harmful to cells and are believed to be a causative factor in aging. Polymeric quinones are an oxidation product of quinic acid which is...
found in some plants. Hence, it is necessary to purify the shilajit before it is consumed. The findings are consistent with the ancient texts which recommend purification of shilajit before consumption (Ghosal et al., 1996).

1.2.1.7 Biological effects of shilajit

The biological effects of shilajit were evaluated by pharmacological and immunological screening of pure shilajit and its major components. The biological effects of shilajit are attributed to a combination of two broad groups of compounds:

(1) DCPs (DBP-chromoproteins), comprising several low and medium molecular weight compounds, as prosthetic group, and intercalated entities, and low and medium molecular weight conjugated proteins (e.g protamines and histones), and

(2) Low and medium molecular weight fulvic acids (FAs) and fusoms obtained from shilajit humus. FAs and fusoms act as systemic carrier of the bioactive molecules.

Appropriate combination of these two groups of compounds has exhibited the biological effects enumerated and detailed below;

(a) Anti-ulcerogenic activity

Shilajit possesses both anti-inflammatory and anti-ulcerogenic activity and can be safely utilized in clinical practice (Goel et al., 1990). Shilajit increases the thickness of protective layer of mucous secreted by the mucus secreting cells in the lining of the stomach. This protects the wall of the stomach from the acid preventing and allowing ulcers to heal, and allows proper digestion and assimilation of food (Fortan, 1978).

FAs containing DBPs and 4'-methoxy-6-carbomethoxy-biphenyl (MCB), isolated from shilajit significantly reduced the resistant-stress-induced ulcer index in pylorus ligated albino rats, compared to the control and the aspirin treated group (Ghosal et al., 1989; Ghosal et al., 1988b).
(b) Anti-Diabetic activity

Subcutaneous administration of shilajit alone and in combination with insulin on plasma glucose level were determined in streptozotocin-induced diabetic rats. Shilajit alone did not alter the glucose level. But same dose of shilajit with insulin significantly potentiated and prolonged the hypoglycemic action of insulin (Kanikkannan et al., 1995).

Purified shilajit was found to attenuate streptozotocin induced Diabetes mellitus and decrease in pancreatic islet superoxide dismutase activity in albino rats (Bhattacharya et al., 1995a). Shilajit produced a significant reduction in blood glucose level and also produced beneficial effects on lipid profile. (Trivedi et al., 2004).

(c) Immunomodulatory activity

Purified shilajit was found to augment the lytic potential of activated lymphocyte. When treated according to methods reported in literature (Zarling and Bach, 1976), shilajit produced T-cell mediated cytotoxicity. This was evident from the ability of the shilajit treated lymphocyte to lyse 51Cr labeled tumor cells. Shilajit produced significant morphometric and functional changes in macrophages (Bhaumik et al., 1993; Ghosal et al., 1995a). Effect of shilajit was determined on the level of brain monoamine in rats. It was observed that shilajit administered at a dose of 25 and 50 mg/kg i.p., for five days, has significantly reduced the level of 5-hydroxy tryptamine and 5-hydroxy indole acetic acid and enhanced the level of dopamine, noradrenaline and their metabolites in rat brain (Ghosal, 1992b). These changes in neurotransmitter levels showed an increase in humoral activity (immune activity).

(d) Antioxidant activity

The antioxidant property of processed shilajit was compared to unprocessed shilajit and vitamin C (ascorbic acid). Processed shilajit exhibited significant antioxidant activity of itself and also had the ability to regenerate (recycle) ascorbic acid after it had neutralized free radicals. The dihydroxybenzo-alpha-pyrones in shilajit caused recycling (regeneration) of ascorbic acid. Unprocessed shilajit did not consistently exhibit the antioxidant activity (Salil et al., 1995).
In another experiment, processed shilajit was tested for its ability to neutralize sulphite anion, hydroxy and nitric oxide free radicals. Chemical polymerization by free radicals was measured with and without processed shilajit. Processed shilajit provided almost complete protection of methyl methacrylate against hydroxyl radical-induced polymerization and significantly inhibited the polymerization of methylmethacrylate by the sulphite free radical. Processed shilajit efficiently trapped nitric oxide free radicals. The study showed concentration dependent antioxidant effects. Higher concentrations of processed shilajit provided greater free radical protection (Ghosal et al., 1995b; Bhattacharya et al., 1995a & b).

In a separate experiment, the effect of shilajit on lipid peroxidation and glutathione content in rat liver homogenates was also investigated. It was found that shilajit inhibited lipid peroxidation induced by cumene hydroperoxide and ADP/Fe^{3+} complex in a dose dependent manner (Ghosal, 2000). Shilajit also decreased the rate of oxidation of reduced glutathione content and inhibited the ongoing lipid peroxidation which was induced by these agents immediately after its addition to the incubation system (Tripathi et al., 1996).

(e) Learning augmentation

The study was carried out to test the validity of use of shilajit as an Ayurvedic medha rasayana (enhancer of memory and learning) in albino rats. Processed shilajit, native shilajit and a preparation consisting of a mixture of ethyl acetate extractive and fulvic acids obtained from processed shilajit were evaluated in an active avoidance, elevated plus-maze and open field behavior paradigms. It was found that processed shilajit and its active constituents (total ethyl acetate fraction and fulvic acids) significantly increased the learning acquisition and memory retention in old albino rats (Ghosal et al., 1993). However native shilajit produced erratic response (both augmentive and retendative) in the above parameters.

(f) Anxiolytic activity

The effect of shilajit was investigated for putative nanotropic and anxiolytic activity in charles foster strain albino rats. The nanotropic activity was assessed by passive
avoidance learning acquisition and retention while the anxiolytic activity was studied and evaluated by the elevated plus-maze technique. The results of these studies indicated that shilajit had significant nanotropic and anxiolytic activity. The biochemical studies carried out for level of monoamines indicated that acute treatment with shilajit had insignificant effect on rat brain monoamines and monoamine metabolite levels. However, it was observed that subacute (5 days) dose treatment caused a decrease in 5-hydroxy indole acetic acid concentration and an increase in the level of dopamine, homovallanic acid and 3,4-dihydroxyphenyl acetic acid concentration with insignificant effect on noradrenaline and 3-methoxy-4- hydrophenylethylene glycol levels. The observed neurochemical studies on shilajit indicate a decrease in rat brain 5-hydroxytryptamine turnover, associated with an increase in dopaminergic activity leading to an increase in memory and anxiolytic activity in albino rats (Jaiswal et al., 1992).

(g) Antistress activity

Shilajit collected from India, Nepal, Pakistan and Russia and organic constituents isolated from them were studied for their antistress effect in albino mice. It was found that shilajit from Kumoan (India), Dolpa (Nepal) and a combination of the total ethyl acetate extract and fulvic acids extracted from Kumoan shilajit produced statistically significant improvement in forced swimming induced immobility in albino mice (Ghosal et al., 1991).

(h) Antiallergic activity

The effect of shilajit and its main active constituents fulvic acids, 4'-methoxy-6-carbomethoxybiphenyl and 3,8-dihydroxy-dibenzo-alpha-pyrone were studied in relation to the degranulation and disruption of mast cell against noxious stimuli. Shilajit and its active constituents provided satisfactory significant protection to antigen-induced degranulation of sensitized mast cells, markedly inhibited the antigen induced spasm of sensitized guinea-pig ileum and prevented mast cell disruption (Ghosal et al., 1989). These findings are consistent with the therapeutic use of shilajit in the treatment of allergic disorders.
(i) Anti AIDS activity

Shilajit is endowed with both immunopotentiating (Ghosal, 1990; 1992 a &b; Ghosal, 1998; Ghosal et al 1995c & d; Bhaumik et al., 1993) and viral load reducing properties (Ghosal, 2000; Ghosal, 2002a). Clinical studies were conducted in AIDS patients with a multi-component natural product-formulation, comprising three essential and three supportive ingredients, in which shilajit was one of the essential constituent. Out of 36 patients enrolled, 22 who received the treatment with the formulation containing shilajit, for 6 months showed positive sign of improvement. Their CD4 and CD8 cell counts were increased from 259 ± 119 (CD4) and 733 ± 483 (CD8) to 356 ± 203 and 984 ± 356, respectively. Ten patients who received the treatment for one year, showed distinct improvement in the symptoms and augmentation in the CD4, 526 ± 272; CD8 1157 ± 428 cell counts.

(j) Spermatogenic and Ovogenic activity

In the shilajit treated male rats, the number of sperms in the testes and epididymides was significantly higher than in the control. A histological examination revealed an apparent increase in the number of seminiferous tubular cell layers in the testes of the treated rats. However, there were no significant differences in the weights of heart, spleen, liver, kidney, brain, testes and epididymides. In the female rats, the effect of Shilajit was estimated by the ovulation inducing activity. Over a 5-day, ovulation was induced in seven out of nine rats in the Shilajit administration group and in three out of nine rats in the control. It was estimated that Shilajit had both a spermiogenic and ovogenic effect in mature rats (Park et al., 2006).

1.2.1.8 Patents on Shilajit

There are several patents filed with United States Patents and Indian patents & Intellectual Property Rights office on Shilajit. Extensive proof has been submitted on the healing, anti-aging and restorative properties of shilajit. This has been verified, approved.
CHAPTER - 1

INTRODUCTION

1.2.1.3.1 United States Patents on Shilajit

(i) Patent Number 6,440,436 (Ghosal, 2002b)

“Process for preparing purified shilajit composition form native shilajit”

This invention relates to shilajit compositions, and particularly to purified shilajit compositions obtained from native shilajit, which compositions have an abundance of defined bio-active constituents and are devoid of toxic components, and their application to personal care, pharmaceutical and nutritional use formulations thereof.

Aging and its associated problems are degenerative diseases. The aging process involves the action of highly reactive free radicals, produced systemically, which interact with other cellular compounds and produce oxidative damages and eventually kills cells and tissues and impairs the immune function of the organisms. Such free radical damage accumulates and increases with age, creating degenerative diseases, such as Alzheimer's, cardiovascular, arthritis, cancer and over a hundred other diseases.

(ii) Patent Number 6,558,712 (Ghosal, 2003)

“Delivery system for pharmaceutical, nutritional and cosmetic ingredient”

This invention relates to delivery systems for active ingredients, and more particularly, to a water soluble delivery system for pharmaceutical, nutritional and cosmetic active ingredients, which includes a purified shilajit composition obtained by extraction from native shilajit containing a carrier which is a purified fulvic acid and wherein the active ingredient is added to and present in voids of the carrier.

A feature of the invention is the provision of a stable delivery system including a purified fulvic acid carrier having a predetermined molecular weight and void sizes which can accept different active ingredients advantageously to deliver and release them smoothly at cell-receptor sites.
The invention will be described hereinafter with reference to the following examples:

(1) Purified fulvic acid - glibenclamide drug delivery system

(2) Pentazocin (Ptz) - Purified fulvic acid carrier compositions

(3) Potentiation of Anti-diabetic effect of insulin (p.o) by purified fulvic acid-Insulin compositions

(iii) Patent Number 5, 405, 613 (Rowland, 1995)

"Vitamin/mineral composition"

The present inventor has found that shilajit over and above its nutritional and herbal content has novel energetic properties. Measurement of subtle energy changes indicate that shilajit has a vibratory field that is substantially stronger than any vitamin, mineral, food substance or herb.

The present inventor has also surprisingly found that when a small amount of shilajit is added to a vitamin or mineral preparation, the energetic properties of the vitamin or mineral preparation are enhanced. In particular, the present inventor has found that the addition of a small amount of shilajit to a vitamin or mineral preparation increases the energy field of the entire preparation to at or near the vibratory level of pure shilajit.

(iv) Patent Number 10/128, 832

"Herbo-Mineral compositions"

This invention relates to herbo-mineral compositions for treating mineral-deficient conditions, and more particularly, to compositions which include a bioactive metal-complexing agent which is purified Shilajit containing purified dimeric and oligomeric α-pyrones (DBPs), obtained from native shilajit, and, optionally, in synergistic combination with gallo/ellagitannoids (GET) extracted from the Emblica officinalis plant, and added mineral supplement, such as iron, copper,
chromium and the like, which compositions can be readily absorbed in the body without gastric upset or side effects (Ghosal, 2002a)

1.2.1.8.2  Indian Patents on Shilajit

(i) Patent application Number 531/Del/2005

(Khanna, R, Agarwal, SP and Khar RK, 2005a)

"A novel complexing agent"

The invention relates to isolation and characterization of humic and fulvic acids from shilajit. The physiochemical properties of HA and FA obtained have been determined. These complexing agents could increase the solubility, wettability, dissolution characteristics, permeability and hence bioavailability of poorly water soluble drugs.

(ii) Patent application Number 532/Del/2005

(Khanna, R, Agarwal, SP and Khar RK, 2005b)

"A novel complexes"

Complexes of itraconazole, ketoconazole, artesunate and acyclovir were prepared with fulvic and humic acids from shilajit. Complexation resulted in a significant increase in the solubility, dissolution rate, permeability and bioavailability of drugs. Complexes were prepared in molar ratio 1:1 by solvent evaporation or freeze drying methods. The complexes were characterized by differential scanning calorimetry and powder X-ray diffractometry and fourier transform infra red spectroscopy.

(iii) Patent application Number 814/Del/2001 (Saluja, A and Agarwal SP, 2001)

"A new non-steroidal anti-inflammatory and analgesic drug composition of piroxicam and humic acid extracted from shilajit."
Piroxicam, a non-steroidal anti-inflammatory drug, was complexed with humic acid extracted from shilajit. The complex prepared by freeze drying technique was characterized by differential scanning calorimetry and powder X-ray Diffractometry. Improved solubility, bioavailability, and reduced gastrointestinal side effects were obtained. The anti-inflammatory activity of the complex was improved when evaluated by carrageenan induced edema test.

1.3 PROPERTIES OF HUMIC SUBSTANCES

1.3.1 Components of Humic substances

Humic substances along with other colloidal organic materials are fascinating substances that can have profound environmental consequences. Researchers have recognized their ability to complex metals and radio nuclides for sometime. The micellar properties of humic and fulvic acids also give them the ability to play important role in the solubilization and transport of hydrophobic chemical entities by acting as surfactant like agents (Stevenson, 1982).

These materials are usually divided into the following three main fractions.

1) **Humic acids**: The fraction of humic substances that is not soluble in water under acidic conditions (pH < 2) but is soluble at higher pH values. They are dark brown to black in color.

2) **Fulvic acids**: The fraction of humic substances that is soluble in water under all pH conditions. They remain in solution after removal of humic acid by acidification. They are light yellow to yellow-brown in color.

3) **Humin**: The fraction of humic substances that is not soluble in water at any pH value. Humin is black in color.
Fig. 1.2: Chemical properties of humic substances (Stevenson, 1982)

(i) A profile of fulvic acid (Schnitzer and Khan, 1978)

<table>
<thead>
<tr>
<th>Description</th>
<th>Light yellow to yellowish brown powder</th>
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<tr>
<td>Solubility</td>
<td>Soluble in water at all pH values</td>
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<tr>
<td>Elemental composition</td>
<td>C=28-39%, H=4-6%, N=3-8%, O=46-62%</td>
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<tr>
<td>Molecular weight</td>
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(ii) A profile of humic acid (Schnitzer and Khan, 1978)

<table>
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<th>Synonyms</th>
<th>Ulmic acids</th>
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<tr>
<td>Description</td>
<td>Dark brown to black amorphous powder</td>
</tr>
<tr>
<td>Solubility</td>
<td>Soluble in water above pH 3.0</td>
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<tr>
<td>Elemental composition</td>
<td>C=41-56%, H=4-6%, N=13-20%, O=20-38%</td>
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<td>Melting point</td>
<td>&gt; 300 °C</td>
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1.3.2 Colloidal characteristics

The colloidal state represent a phase intermediate between true solution, where species are of ionic or molecular dimensions, and suspended particulates, where species are sufficiently large to settle under the force of gravity. Chemical and physical reactions are generally enhanced in colloidal systems due to large surface area of colloidal particles. At the same time, mobility through water or groundwater is also enhanced, approaching that for true solutions. The range of molecular size for humic acids places them in the colloidal range when in solution i.e. from 0.001 to 1 μm (Gaffney et al., 1996).

Humic colloidal material is thought to consist of coiled, long chained or three-dimensional cross-linked macromolecules with electrical charges variously distributed on the particle. The presence of charged sites arising from ionized groups, results in mutual repulsion and causes maximum expansion of the macromolecule. Trapping of biologicals (peptides, carbohydrates) and anthropogenic substances such as pesticides and plasticizers in the voids of these macromolecular substances has been investigated (Gaffney et al., 1996).

1.3.3 Micellar nature

It was shown that humic acids have surfactant properties. Humic acids are predominantly hydrophilic (except at lower pH) but they also contain a substantial concentration of aromatic rings, fatty acid esters, aliphatic hydrocarbon and other hydrophobic substances, which together with the hydrophilic groups account for the surface activity of these materials. The hydrophilic oxygen containing functional groups (COOH, C=O, OH) are thought to play a significant role in lowering the surface tension of water and so increasing aqueous wettability of hydrophobic materials.

It has been recognized that the presence of even a small amount of humic acid in aqueous solution can significantly enhance the water solubility of hydrophobic organic compounds (Schnitzer and Khan, 1978). This solubilization in solution is often attributed to the presence of micelle (Guetzloff et al., 1996).
Huminic acids being highly aromatic as compared to fulvic acids become insoluble at low pH values when the carboxylate groups become protonated that may also lead to formation of intramolecular pseudomicelles.

The structure of humic acids is such that it allows them to function as surfactants with the ability to bind both hydrophilic and hydrophobic materials. This function in combination with their colloidal properties makes humic acids effective agents in transporting both organic and inorganic materials in the environment.

1.4 METHODS FOR BIOAVAILABILITY ENHANCEMENT

A review of monographs in the European Pharmacopoeia has revealed that more than 40 per cent of the drug substances have aqueous solubilities below 1 mg/ml, and that 32 per cent have an aqueous solubility below 0.1 mg/ml (Philip et al.; 1986) The implementation of dissolution and absorption enhancing methods is, therefore, a major field in the formulation of drug dosage forms, in particular for the oral route of administration. A wide range of principles and methods for enhancing dissolution rate of low-solubility substances, are available which include:

❖ Selection of salt form for weak acids and bases,
❖ Reduction of particle size,
❖ Preparation of solid dispersions,
❖ Change of crystal form by precipitation with hydrophilic polymers,
❖ Lipophilic formulations, i.e. emulsions, microemulsions etc.,
❖ Use of surfactants for increased wettability,
❖ Complexation with cyclodextrins
(i) Salt formation

Selection of an appropriate salt form or in case of liquid preparations, adjustment of pH is first choice for weakly acidic and basic drug substances. Substances having aqueous solubilities above 10 mg/ml are formulated as hydrochlorides, sulphates, maleates, citrates etc. of basic drugs or for acidic drugs potassium, sodium, calcium or other salts. But it is limited to drug with ionizable groups only. Moreover poor crystallinity and hygroscopicity are major problems.

(ii) Micronization

In principle, the lower the aqueous solubility, the lower particle size is required to achieve a satisfactory dissolution rate. For the practically insoluble substances (< 0.1 mg/ml), the required particle size is so low that it may be technically difficult to prepare the desired size range. On the other hand, physical instability of the drug as well as the size distribution is introduced when the size range is reduced to micrometer and sub-micrometer scale (Dalmora et al., 2001).

(iii) Solid dispersions

Several solid dispersions and coprecipitates with hydrophilic polymers have been prepared where the drug substance typically is present in an amorphous state but these are highly energetic systems (Chiou et al; 1971). Therefore physical instability is a major problem and that is why only a few products based on solid dispersions have been marketed (Arias et al., 1994).

(iv) Microemulsion

The newest trend in the formulation of low-solubility drugs is accordingly the use of so-called lipidic formulations, in particular microemulsions and other self-emulgating systems which can dissolve a sufficient amount of the drug substance. But physical and chemical instability are again major problems.
1.5 CYCLODEXTRIN COMPLEXATION

Physical and chemically stable compounds can be made by the preparation of inclusion complexes of drugs with cyclodextrins. The potentials of the various types of cyclodextrins for solubility and absorption enhancement are well documented in the literature (Baboota et al., 2000, 2001, 2002a & b, 2003a & b, 2004a & b) Complexation with cyclodextrins has been used as a novel approach for designing drug delivery system because of numerous advantages provided by these carrier systems like:

❖ Cyclodextrins being natural carriers have low toxicity and are almost inert.
❖ They are usually safe and can be used almost through every possible route of administration like oral, ocular, nasal, buccal, parenteral and rectal.
❖ They are insignificantly absorbed through intestinal mucosa.
❖ A large number of cyclodextrins with different cavity sizes are available
❖ They have a well defined chemical structure which provides a number of potential sites for chemical modification or conjugation
❖ They are versatile complexing agent and can accommodate almost every type of organic molecule
❖ They are equally applicable for both ionizable and non-ionizable drugs
❖ They are stable enough to withstand high temperature (upto 280 °C) during manufacturing processes like preparation and sterilization
❖ They protect the included drugs from biodegradation.
1.5.1 Complexation phenomenon

A "complex" is a species formed by interaction of two or more molecules or ions with a definite substrate to ligand stoichiometry (Eccleston et al.; 1994).

\[ mS + nL \leftrightarrow SmLu \]

Substrate Ligand Complex

A "substrate" \( S \) is the interactant whose physical or chemical properties are observed experimentally.

A "ligand" \( L \) is the second interactant whose concentration may be varied independently in an experimental study.

1.5.1.1 Types of complexes

The definition of a complex leads to a classification into two groups (Higuchi and Connors, 1995).

A. Chemical Bonding

1) Co-ordinate complexes These are formed by co-ordinate bonds in which transfer of a pair of electrons takes place e.g metal and ammonium ion co-ordination complexes between metal ions and bases (Amiji et al., 2003).

   - Inorganic complexes e.g. \([\text{Ag} (\text{NH}_3)_2]^+, [\text{Co} (\text{NH}_3)_6]^{3+} \text{Cl}^\) etc.

   - Chelates: Ligand have more than one donor groups e.g. EDTA

2) Molecular complexes These are mainly formed by non covalent interaction between the substrate and the ligand such as electrostatic induction and dispersion interactions with the exception of charge transfer or electron donor acceptor complexes which may have some covalent character hence appearance of new UV absorption bands (Amiji et al., 2003).
B. Type of Bonding or Interaction

1) Charge transfer e.g. nitrobenzene complex

2) Hydrogen bonding e.g. Caffeine complexes

3) Hydrophobic interaction

4) Stacking interaction

Type or structure of interactants

1) Small molecule - Small molecule interaction

2) Small molecule - macromolecule binding

3) Drug-protein binding

4) Drug-receptor binding

5) Enzyme-substrate complex

1.5.1.2 Type or structure of complex

1) Self association: It is a complexation of molecule with others of its own species for example benzene forms dimer.

2) Micelle: A special form of self aggregated complex in which interactant is a surfactant.

3) The inclusion complex: One interactant (guest) is entrapped within the cavity formed by other macrocyclic interactant called as host (Szejtli, 1998 and Eastburn et al., 1994).

1.5.2 Methods of preparing inclusion complexes

Several methods have been described in literature for preparing complexes of drugs. Since cyclodextrins has been studied in great details as a complexing agents and the following methods have been used to prepare cyclodextrin...
complexes, these are given here as these methods can be modified to form complexes with humic and fulvic acids as well. By only trial and error one can find a method which will give the best result for a given drug. Complexes can be formed by a variety of techniques that depend on the properties of drug, the equilibrium kinetics, the formulation ingredients and processes and final dosage form desired. However, each of these process depends on a small amount of water to help drive the thermodynamics. Among the methods used are simple dry mixing, mixing in solutions and suspensions followed by a suitable separation, the preparation of pastes and several thermo-mechanical techniques.

1) Grinding

Inclusion complexes can be prepared by simply grinding the guest with a complexing agent such as cyclodextrin. This is a very slow process for making inclusion complex and degree of complexation achieved is very low (Szejtli, 1988).

2) Solid dispersion/Co-evaporated dispersion

The drug is dissolved in ethanol and cyclodextrin is either dissolved in a alcoholic solution or dissolved separately in water or other suitable medium. The cyclodextrin solution is then added to the drug solution or vice versa and stirred to attain equilibrium. The resulting solution is evaporated to dryness preferably under vacuum.

3) Neutralization method

Martin and Udupa 1995, reported this method for various fluoroquinolones. In this method equimolar concentration of drug and cyclodextrin are separately dissolved in 0.1 N NaOH, mixed and stirred for about half an hour, pH is recorded and 0.1 N HCl is added dropwise with stirring until pH reaches 7.5, upon when the complex precipitates. The residue is filtered and washed until free from Cl⁻. It is dried at 25⁰C for 24 hours and stored in a dessicator.
4) Kneading

In this method cyclodextrin is not dissolved but kneaded like a paste, either with small amount of water to which the guest component has been added. Guest component can be added without a solvent or in small amount of ethanol in which guest has been suspended. Several hours of grinding of paste in mortar results in evaporation of solvent and formation of powder like complex (Otero-Espiner et al., 1992).

5) Precipitation

The guest which shows Bs type phase solubility curve are suitable for this method of complex formation. In this method the drug (guest) and cyclodextrin are dispersed in water and the solution is heated to obtain concentrated, viscous and translucent liquid. The solution is left to give a precipitate of inclusion complex. Precipitate obtained is separated and dried to get solid inclusion complex.

6) Spray drying

In this method first a monophasic solution of drug and cyclodextrin is prepared using a suitable solvent (generally hydroalcoholic solution is used). The solution is then stirred to attain equilibrium following which the solvent is removed by spray drying (Bietti et al., 1992).

7) Freeze-drying

Freeze drying method is similar to spray drying method except that in this method, the solvent is removed by freeze-drying after attaining the equilibrium (Becirevic-Lacen et al., 1996).

8) Preparation of suspension

Cyclodextrin need not be dissolved. Simply stirring the guest in an aqueous suspension of CD can achieve complexation within 2-24 hrs at ambient temperature. This is recommended method for industrial application (Szelti, 1988).
9) Melting

Complexes can be prepared by simply melting the guest, mixed with finely powdered cyclodextrin. In this procedure there has to be a large excess of guest, and after cooling this excess is removed by careful washing with weak complex forming solvent or by vacuum sublimation. This latter is preferred method and is used to sublimate guests such as menthol (Szetli, 1988).

1.5.3 Characterization of inclusion complexes

Several methods have been proposed for the analytical characterization of drug/cyclodextrin complexes, according to the physical state considered, i.e. solution or solid. The formation of inclusion complex can be studied and characterized in two ways (Szetli, 1988).

1) In solid state (by DSC, XRD, FTIR, SEM)

2) In solution (by solubility studies, dissolution tests; UV spectral studies, \(^1\)H-NMR studies, TLC)

1) Characterization in solid state

(i) Differential scanning calorimetry (DSC)

DSC is the measurement of rate of heat evolved or absorbed by the sample, during a temperature program. It is extensively employed to check any variation of crystalline properties due to the interaction with the CD. The reduction in the degree of crystallinity of the drug is often taken as an indication of complexation. The DSC curve of cyclodextrin generally show an endotherm near 100°C which signifies removal of water. The DSC curve of the guest molecule shows a sharp intense peak (endotherm) at its melting temperature (m.p.) and then it starts decomposing. In DSC curve of cyclodextrin-guest inclusion complex, these peaks are either diminished or absent. Partial complex formation may be shown by varying patterns e.g. small exotherm adjacent to the melting endotherm of guest molecule.
The DSC curve of simple mixture would resemble the combination of curves of pure substance i.e. guest and cyclodextrin.

(ii) Powder X-ray diffraction (XRD)

This is an important technique for the determination of three-dimensional structure of molecule and distinguishes between amorphous and crystalline forms. The diffraction pattern is characteristic of a substance. The crystalline substance has sharp intense peaks in its powder diffraction pattern whereas amorphous substance shows only undefined, broad, diffused peaks of low intensity. Generally the complex has an amorphous nature i.e. broad, undefined peaks with low intensities.

(iii) Fourier transforms infra-red spectroscopy (FT-IR)

It is another useful technique to verify formation of inclusion complexes. The guest molecule within the cavity show shift in its peaks or shows peaks of less intensity. Basically, peaks which lie in the fingerprint region and peaks due to C=O or O-H stretching are affected (shifted or intensity is changed). FTIR technique is known to have superior sensitivity and resolution, absolute wavelength accuracy and higher precision of measurement than conventional IR technique.

(iv) Scanning electron microscopy (SEM)

SEM is done to observe the crystalline structure of the sample. SEM studies help us to observe the changes that occur in the crystal structure during or after the preparation procedure. Generally a change from crystalline to amorphous nature (of the drug) can be seen upon complexation with fulvic and humic acids.

2) Characterization of inclusion complexes in solution

(i) Phase solubility studies and dissolution tests

The most common and widely used method to evaluate the ability of CD to complex a drug is by phase solubility studies. Phase solubility analysis allows the determination of both the stability constant and the stoichiometry of the complex formed in solution. Higuchi and Connors, 1965 have classified the various
solubility behavior seen during complex formation as A-type (a soluble inclusion complex is formed) or B-type (an inclusion compound of finite solubility is formed).

(ii) TLC (Thin layer chromatography)

TLC may also be useful for verification of complex formation, since the Rf values are altered considerably. Rf values are usually diminished provided the complex is sufficiently stable in the solvent mixture used.

(iii) Proton nuclear magnetic resonance ($^1$H-NMR)

It is useful not only for verification of complex formation but also to guess how the guest is geometrically aligned in the cyclodextrin cavity. The inclusion of guest molecule into the cyclodextrin cavity clearly induces some changes in the chemical shift values. The chemical shift values are also indicative of the interactions, if any between protons of cyclodextrin and guest (Djedani et.al., 1991).

1.5.4 Studies Carried on Complexation of drugs with Humic Substances

Karmarkar, R.R (2007) developed ketoconazole complexes with fulvic and humic acid isolated from shilajit. Solubility, dissolution and antimicrobial activity were improved as compared to ketoconazole alone. A successful bioequivalence study was performed on healthy human volunteers with significant increase in bioavailability of ketoconazole complex as compared to uncomplexed dosage form of ketoconazole (Agarwal et.al., 2008b).

Tyagi, B (2007) prepared complexes of paclitaxel, an anticancer drug with humic and fulvic acid of shilajit. Complexes were evaluated for solubility, dissolution and characterized by FT-IR, DSC, XRD and SEM.

Mirza, A (2007) investigated the effect of fulvic acid on the solubility, dissolution and bioavailability of Carbamazepine. The complexes were prepared using different techniques like freeze drying, solvent evaporation, kneading and physical mixture and evaluated for solubility, dissolution, differential scanning calorimetry (DSC), fourier
Ahmad, D (2006) investigated the influence of shilajit extracted fulvic acids on complexation with melatonin and to develop an oral dosage form of melatonin in order to increase the solubility, dissolution rate. The complexes were characterized by using differential Scanning calorimetry (DSC), X-Ray diffraction (XRD), Fourier transform Infra-red spectroscopy (FT-IR) and Scanning Electron Microscopy (SEM) and it was concluded that maximum complexation was achieved by lyophilized complex.

Anwer, M.K (2005) prepared complexes of furosemide with humic and fulvic acid using different techniques, Freeze drying, solvent evaporation and grinding and characterized them by FT-IR, DSC, XRD and SEM. A significant enhancement in solubility, dissolution profile and diuretic activity were observed in comparison to the uncomplexed furosemide dosage form (Agarwal et.al, 2008a).

Khanna, R (2005) developed and validated a new method to extract humic and fulvic acid using ion exchange resins. Method gives better yield of humic and fulvic acids and even the solubility of fulvic acids obtained by this method is better than obtained by established Ghosal method. Complexes of itraconazole and acyclovir were prepared with humic and fulvic acid and these complexes were found to have improved solubility, dissolution and therapeutic efficacy than the uncomplexed drugs. Permeability of the drugs was also found to be improved in comparison to the uncomplexed form. Bioequivalence study was conducted on healthy human volunteer, it was found that bioavailability of itraconozole increased significantly.

Sahuja, A (2001) proved that humic acid-piroxicam complex has better solubility than pure piroxicam powder, it also, found that dissolution profile of the tablets of these complexes are better than the marketed preparation containing piroxicam in uncomplexed form. Gastric ulceration was significantly reduced as compared to uncomplexed piroxicam.
1.6 STRATEGIES TO IMPROVE ASPRIN STABILITY

Mroso et al. (2006) identified salicylsalicylic acid and acetylsalicylsalicylic acid as decomposition products of aspirin when mixtures of the drug with magnesium stearate were stored in the solid state at 60° and 75% relative humidity. The effect of increasing the concentration of magnesium stearate and the addition of other alkali stearates on the rate of decomposition of aspirin were studied. The validity of the theory that pH changes induced by the alkali stearates account for the catalytic effect of the lubricants on the decomposition was tested. The changes observed were modeled and the mechanism involved elucidated. The potential use of the melting points of aspirin mixtures in predicting the stability of the drug in such drug-excipient mixtures is demonstrated.

Williams et al. (1999) investigated the effect of formulation technique for 2-hydroxypropyl-β-cyclodextrin (HPβCD) on the stability of aspirin in a suspension based pressurized metered dose Inhaler (pMDI) formulation containing a hydrofluoroalkane (HFA) propellant. The chemical stability of aspirin in pMDI formulation was determined over 6 month storage at 5, 25 and 40 °C. Aspirin in the lyophilized inclusion complex exhibited the most significant degree of degradation during 6 month storage, while aspirin alone in the pMDI demonstrated a moderate degree of degradation.

Mario et al. (1965) studied the effect of ultrasound on the hydrolysis of aspirin solutions at various temperature and pH values. The reaction kinetics followed a pseudo first-order rate, both with and without the influence of ultrasound. The rate of hydrolysis was increased in all cases by applying sound energy.

Gore et al. (1968) investigated the significance of salicylic acid sublimation in stability testing of aspirin-containing solids. Under conditions of accelerated stability testing, the loss of salicylic acid from the system by sublimation can incur appreciable errors in the detection of overestimating aspirin stability. Since aspirin was not detected to sublime under these same conditions, its residual content is an improved indication of its stability. A method for its simultaneous determination with salicylic acid is presented.

Chang et al. (1984) conducted study on 0.2% w/v aspirin liquid formulation in a wide range of water-propylene glycol mixture and water-triethylene glycol diacetate mixture at...
four temperatures. The effect of surfactant, polyoxyethylene (20) sorbitan monolaurate, on aspirin stability was investigated. There was a linear relationship between water content and degradation rate constants. Formulation containing the higher concentration of the surfactant showed the greater aspirin degradation.

Mihranyan et.al (2005) studied the effect of cellulose powder structure on moisture induced degradation of acetylsalicylic acid. Different cellulose powders were manufactured and characterized by X-ray diffraction and N2 BET gas adsorption. Cellulose with lower crystallinity index exhibited lower degradation rate than the sample with the higher crystallinity index. It should be noted that higher ASA degradation rate were observed in the sample with comparably higher moisture content.

Mizobuchi et.al (2001) prepared the external preparation containing aspirin which were stored for a long term and was superior in dermal absorbability. Formulation were prepared by mixing aspirin together with at least one substance selected from an ester of an organic acid ester having 2 to 20 carbon atoms, a glycerol fatty acid ester, silicon oil, hydrocarbon oil and crotamiton.

Snavely et.al (1993) conducted the study on the stability of a direct compression tablet formulation containing aspirin as a model hydrolabile drug with Emdex (a mixed sugar diluent containing approximately 8 percent moisture) and stearic acid. Compressed tablet and uncompressed powder blend were packaged in storage container and placed on stability at different temperatures. Analysis of the aspirin data showed that the rate of aspirin decomposition increased with temperature. The formulation showed good stability with less than one percent decomposition occurring after 1.75 year of storage at room temperature.

Choi, H.S (1989) investigated the molecular nature of aspirin hydrolysis as biometric model for esterase. The structural specificity and the chemical dynamics of these inclusion complexes in the solid state and in the solution state were determined by FT-IR, UV, FAB-MS, ¹H NMR and ¹³C NMR spectroscopy. Dissociation constants were obtained by the kinetic method under alkaline condition (Choi, 1992).
1.7 OBJECTIVE OF THE STUDY

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used of all therapeutic agents. They are drugs of choice for the management of a variety of acute and chronic inflammation. They are frequently prescribed for long-term treatment of rheumatic musculo-skeletal complaints. The major drawback to anti-inflammatory drug use is the occurrence of gastrointestinal side effects with majority of agents. Aspirin is very old drug but still having a very high market value. It possesses antipyretic, anti-inflammatory, analgesic and anti-aggregatory activity (Chang et.al., 1984). The acetylsalicylic acid molecule has a carboxyl group and an ester group. The ester group can be easily hydrolyzed, which reduces the medical value and causes side effects on humans (Gore et.al, 1968 and Mario et.al, 1965). A strategy was designed how to inhibit the hydrolytic decomposition and enhance solubility and dissolution of aspirin inside the void of humic and fulvic acid of shilajit. We propose to investigate the effects of humic and fulvic acid as carrier on aspirin in enhancing the solubility, dissolution rate, bioavailability, stability, decreasing the toxicity and obtaining a better pharmacodynamic profile of aspirin through complexation and compare the prepared complex with hydroxy-propyl-β-cyclodextrin.

The main objectives of study were,

1. To isolate pure fulvic acid and humic acid from shilajit and to characterize them.

2. To prepare complexes of drugs with fulvic, humic acids and HP-β-CD and study their stoichiometry and nature of complexation through techniques like FT-IR, DSC, SEM and X-ray diffraction.

3. To carry out saturation solubility studies of prepared complexes and compared with HP-β-CD complexes

4. To carry out in-vitro dissolution behaviour of the solid complexes of humic/fulvic acid and compare with HP-β-CD complex and drug alone

5. Selection of preparative technique and optimization of complexation method
6. Effect of fulvic/humic acid on solubility/dissolution of aspirin and comparison with HP-β-CD

7. Effect of humic/fulvic acid on stability of aspirin and comparison with HP-β-CD

8. Effect of fulvic acid on permeability of aspirin and comparison with HP-β-CD

9. Effect of fulvic/humic acid on anti-inflammatory activity and comparison with HP-β-CD

10. Effect of fulvic/humic acid on gastric ulceration and comparison with HP-β-CD

11. Preparation of dosage forms and in-vitro release of the drug from the prepared dosage form

12. Stability studies of the prepared dosage forms of fulvic acid complex and compare with dosage form of HP-β-CD complex and innovator product

13. To determine the stability of complexes by using computational method

1.8 HUMIC SUBSTANCES AS CARRIER

From the literature the following properties of the humic substances were observed. This encouraged us to test their bioavailability and stability enhancing potential

1. They have a sponge like structure punctured by voids of about 200-1000Å in diameter. A water insoluble and unstable active ingredient can be added to fill the voids.

2. They are naturally occurring and are toxicologically safe.

3. They have surfactant properties which gives them an advantage over cyclodextrin as a bioenhancer.

4. Their ubiquitous occurrence in nature can provide the pharmaceutical industry a large amount of ready to use bioenhancers.
5. Established pathways of the formation of humic substances provides a scientific basis for exploring the possibilities of in house production of humic substances.

6. As the traditional literature boasts of various pharmacological activities of humic substances, establishment of their pharmacological and safety profile could give us magic molecules which will not only have their own pharmacological activity but will also help in enhancing the bioavailability of various poorly bioavailable and unstable drugs, thus reducing the amount required to produce their pharmacological effect.

1.9 SELECTION OF MODEL DRUG CANDIDATE

Aspirin is a very old drug but still having excellent medicinal value, and its health protection function such as analgesic, anti-inflammatory, antithrombotic, and antipyretic, has received more and more attention (Choi, 1992). The aspirin 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 (Connors et.al. 1986). A need exists to learn how to inhibit the hydrolysis of aspirin. A number of papers are available describing decomposition of aspirin. Aspirin is degraded into salicylic acid and acetic acid by influence of moisture. (Connors et.al., 1986). The decomposition of aspirin complexes with cyclodextrin has been studied and found significant degradation during 6 month of storage as compared to aspirin (Williams et.al., 1999). In another study, Influence of cellulose powder with lower crystallinity index exhibited lower degradation rate of aspirin than the sample with the higher crystallinity index (Mihranyan et al., 2005). The degradation of aspirin increased by increasing the specific surface area of excipient (dicalcium phosphate dihydrate powders).

Fulvic acids are the major constituent of shilajit, having relatively open, flexible structure punctured by voids (micropores) of different diameters (Agarwal et.al., 2007a & b). These compounds were, presumably, loosely held in the core structure of shilajit (Agarwal et.al., 2008a). The plant secondary metabolites which are trapped in the internal voids of fulvic acids are spared from and become resistant to common chemical and biological decomposition (Agarwal et al., 2007c). Taking a clue
on this point we started to investigate the potential of fulvic and humic acid as a novel complexing agent in order to increase the stability of aspirin. Theses fulvic and humic acid provide the protective layer around aspirin in which water is excluded as much as possible and reduce the decomposition.

1.10 ASPIRIN- A DRUG PROFILE

Aspirin is employed as an analgesic-antipyretic and as an effective non-steroidal anti-inflammatory agent.

\[
\text{CH}_3 - \text{C} = \text{O} - \text{COOH}
\]

1.10.1 Physicochemical properties

**Synonyms**
Acetylsalicylic acid; Salicylic acid acetate.

**Proprietary names**
Adprin-B; Angettes; Ascriptin; Aspergum; Asprimox; Aspro etc

**Molecular weight**
180.16

**Physical state**
White crystals or white crystalline powder or granules

**Dissociation Constant**
\( pK_a 3.5 \) (25 °C).

**Partition Coefficient**
Log P (octanol-buffer pH 7.4), -1.1

**Melting point**
135°C

**Solubility**
Soluble 1 in 300 of water, 1 in 5 of ethanol, 1 in 17 of chloroform, and 1 in 10 to 15 of ether; soluble in solutions of acetates and citrates and, with decomposition, in solutions of alkali hydroxides and carbonates.
1.10.2 Pharmacology

Aspirin is analgesic, anti-inflammatory, antipyretic and an inhibitor of platelet aggregation. It inhibits prostaglandin G/H synthase (Roth et al; 1975). This enzyme catalyses the first step in the synthesis of prostaglandins and thromboxanes from arachidonate. Aspirin is relatively specific for type-I isoenzyme which is constitutively expressed in platelets and other tissues and are involved in platelet/endothelial cell interaction (Smith et.al., 1992). Aspirin acetylates the hydroxyl group of a serine residue at a position 529 of the polypeptide chain, thereby preventing access of substrate to the active site by steric hindrance and causing irreversible loss of cyclooxygenase activity (DeWitt et.al., 1988 and Funk et al., 1991). Aspirin also inhibits type-II prostaglandin G/H synthase which is not expressed constitutively but is induced by cytokines during the inflammatory response (Xie et. al., 1991 and Kujubu et.al., 1991). Most of the pharmacological effects of aspirin are caused by inhibition of formation of prostaglandins and thromboxanes.

Aspirin has an active metabolite (salicylate) which in addition to possessing some anti-inflammatory properties in its own right also has important effects on respiration acid-base balance, and the stomach. Salicylates stimulate respiration by a direct effect on medulla, and at high concentration, uncouple oxidative phosphorylation in muscle, increasing oxygen consumption and carbon dioxide production. Salicylate have a direct irritant effect on the gastric mucosa and further predispose to ulceration by inhibiting synthesis of vasodilator and cytoprotective prostaglandins. Large doses of salicylates (greater than 5 g per day) are uricosuric, but such doses are poorly tolerated and salicylates are no longer used to treat gout.

1.10.3 Toxicology

An in-vitro study (Joshko et.al., 1993) looked at rat embryos cultured for 48 h (days 9.5-11.5 of gestation) in 100-300 μg/ml salicylic acid, a metabolite of aspirin. When compared with growth in control embryo, a significant dose-dependant decrease in crown rump lengths, somite members, and yolk sac diameters was observed in the rat embryos cultured with salicylic acid. There was also a significant increase in overall
dysmorphology, including eye, bronchial arch and anomalies, and an absence of forelimb buds. The neural tube was especially vulnerable and had frequently failed to close. Clinical and experimental evidence indicates that exposure to relatively large doses of aspirin prolongs parturition. A study of the dose-response relationship for salicylic acid on labor and gestation times in rat used, as a positive control pregnant rat exposed to 260 mg/kg per day of aspirin from day 15 to day 21. The aspirin-treated rats had both prolonged labor and gestation times, as well as increased maternal peripartum death.

1.10.4 Clinical Pharmacology

Aspirin is generally well tolerated at doses up to 2 g daily. Higher doses are associated with numerous side effects, including tinnitus, abdominal discomfort, nausea, vomiting and gastrointestinal bleeding. Increasingly toxic concentrations cause deafness, vertigo, headache, hyperpnea, acid-base disturbance, fever, sweating, tachycardia, hallucination, delirium, loss of consciousness, circulatory collapse, respiratory failure and death.

The analgesic effect of aspirin is a peripheral effect owing to its inhibition of the cyclooxygenase enzyme. In areas of inflammation, increased amounts of PGE\(_2\) and PGF\(_{2\alpha}\) are produced. These lower the threshold for triggering pain fibers. This can be demonstrated by injecting prostanoids into the skin, which causes an area hyperesthesia. PGE\(_2\) also act as a vasodilator in areas of inflammation and this combined with other substances which increase vascular permeability, contributes to the vascularity and swelling in areas of inflammation. The anti-inflammatory effects of aspirin and salicylate related to the decreased vascularity which results from inhibition of PGE\(_2\) synthesis.

The inhibition of platelet prostaglandin G/H synthase has been investigated through measurement of serum thromboxane B\(_2\) (Patrignani et al., 1982; Patrono et al., 1985) and urinary thromboxane metabolite (FitzGerald et al., 1983 and Ritter et al., 1989). Single oral dose of 5-100 mg aspirin cause dose-dependent inhibition of serum thromboxane B\(_2\) generation, with 100 mg causing near maximal inhibition.

Aspirin irreversibly acetylates prostaglandin G/H synthase, its duration of action on platelets substantially outlives its presence in the body. Its effect on platelet thromboxanae biosynthesis and on bleeding time persist for many days after dosing is

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discontinued, recovery being determined by the entry of new platelets into the circulation (Patrano et al., 1980). When aspirin is used as an analgesic in postoperative dental pain, large doses are required (around 1200 mg), analgesia only lasts for few hours and there is a significant correlation between analgesia and plasma salicylate concentration (Seymour et al., 1982). However, the correlation between analgesia and plasma salicylate concentration is fortuitous, and sodium salicylate does not cause significant analgesia (Seymour et al., 1984). It therefore seems likely that the analgesic action of aspirin depend on acetylation of cyclooxygenase and transience of the analgesia is probably explained by biosynthesis of new prostaglandin G/H synthase.

1.10.5 Pharmaceutics

Aspirin is available froms many manufacturers. Preparations are available for oral and rectal administrations. In some countries lysine aspirin is available for intramuscular or intravenous administration.

Oral dosage form are available as capsule, plain uncoated tablets, dispersible tablets, effervescent tablets, soluble tablets, enteric-coated tablets, enteric-coated capsules, buffered tablets and modified-release tablets. The usual strengths available include 300 mg, 75 mg and 500 mg. Plain tablets or capsules and dispersible or soluble tablets should be taken with or after food. Dispersible or soluble tablets should be dispersed or dissolved in water. Enteric-coated tablets or enteric-coated capsules should be swallowed whole, not chewed and should not be taken at the same time as indigestion remedies. Modified-release tablets should be swallowed whole, not chewed.

Suppositories are available containing 60 mg to 1.2 g aspirin in USA and 150 or 300 mg in the UK. Generally aspirin preparations should be stored at room temperature, protected from moisture, in air-tight container.

Aspirin is available in combination with many other drugs - for example, analgesic such as aloxiprin and acetaminophen; opioid analgesic such as codeine-dihydrocodeine, propoxyphene napsylate and ethohepazine citrate; muscle relaxants such as meprobamate and methocarbamol; histamine H1-receptor antagonist such as chlorpheniramine and
cyclizine. Other drugs include aluminium or magnesium hydroxide, ascorbic acid and phenylephrine.

1.1.6.6 Mechanism of pH dependent hydrolysis of aspirin

Esters group of aspirin are susceptible to catalytic hydrolysis by both aqueous acids and bases. The possible mechanisms are given below:

1.1.6.1 Acidic hydrolysis

![Chemical reaction diagram]

**Fig. 1.3: Scheme for acidic hydrolysis of aspirin**

If the proton is hydronium ion (H$_3$O$^+$) the catalysis is known as specific acid catalysis. Source of proton is from dissociated acid and the ester of aspirin is protonated in the transition state of the reaction. For specific acid catalysis, the observed rate constant, $k_{obs}$ is described by equation 1.

$$k_{obs} = k_0 + k_1[H_3O^+]$$

Where $k_0$ is the rate constant for the uncatalyzed process and $k_1$ is the rate constant for the acid catalyzed process. Note that there is no term in the equation for any undisassociated acid present in the reaction mixture.

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1.10.6.2 Basic hydrolysis

When the base is hydroxide (HO\(^-\)), the catalysis is termed specific base catalysis. The ultimate source of the base is hydroxide in the reaction mixture, and the ester group of aspirin is attacked by the hydroxide in the transition state of the reaction. The observed rate constant, \( k_{obs} \) for the reaction is described by equation 2, where \( k_0 \) is defined as above, and \( k_{OH} \) is the rate constant for the hydroxide catalyzed process.

\[
k_{obs} = k_0 + k_{OH}[\cdot\text{OH}]
\]

Aspirin, acetylsalicylic acid, is an ester. The equation for its hydrolysis to salicylic and acetic acids may be written very simply.

\[
\begin{align*}
\text{Aspirin} & \quad \text{H}_2\text{O} \quad \text{Salicylic acid} \quad \text{Acetic acid} \\
& \quad \text{H}_2\text{C} - \text{CO}_2\text{H} \quad \text{H}_3\text{C} - \text{CO}_2\text{H}
\end{align*}
\]

Fig. 1.4: Scheme for basic hydrolysis of aspirin

Fig. 1.5: Reaction for basic hydrolysis of aspirin
The exact mechanism of hydrolysis is a bit more difficult to describe, since the hydrolysis of aspirin may occur by one or more of the mechanisms described above.

1.10.7 Method of Analysis

The preferred analytical method is high performance liquid chromatography (Lo et al., 1980). The sensitivity is 0.5 mg/L for both aspirin and salicylate. Blood samples are collected in the presence of potassium fluoride to inhibit plasma esterases and must be analyzed immediately if reliable information on aspirin itself is needed. For routine toxicological purposes, determination of salicylate suffices, and efforts to prevent hydrolysis of acetylsalicylic acid are unnecessary. Absorption after oral administration of a solution of aspirin is usually complete, while enteric-coated tablet are less reliably absorbed (Levy et al., 1960).

However, GC-MS is also used to detect aspirin in plasma (Tsikas et al., 1998). Limit of detection of aspirin, salicylic acid, and salicyluric acid is 50 μg/L in plasma (Buskin et al., 1982). Limits of detection in plasma are 100 μg/L for aspirin and salicyluric acid, 500 μg/L for salicylic acid, and 200 μg/L for gentisic acid (Rumble et al., 1981). In plasma, LOD for aspirin and salicylic acid is 100 μg/L (Kees et al., 1996). In plasma or skin, LOD for aspirin and salicylic acid is 0.1 μg/mL and 0.1 μg/cm² respectively (Pirola et al., 1998).
### Table 1.1: Reported HPLC methods of aspirin

<table>
<thead>
<tr>
<th>S. No</th>
<th>Sample</th>
<th>S.P (column)</th>
<th>M.P</th>
<th>F.R</th>
<th>Detection</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Formulation/Human serum</td>
<td>C&lt;sub&gt;H&lt;/sub&gt;</td>
<td>acetonitrile:0.1% aqueous orthophosphoric acid</td>
<td>2.0 ml/min</td>
<td>fluorescence detection</td>
<td>(Ibrahim et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150 mm x 4.6 mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Tablet</td>
<td>3.0 μm non-porous silica ODS</td>
<td>98:2 v/v 50 mM phosphate buffer pH 3.0-acetonitrile</td>
<td>1.5 ml/min</td>
<td>UV-220 nm</td>
<td>(Xu X et al., 2000)</td>
</tr>
<tr>
<td>3</td>
<td>Tablet</td>
<td>C&lt;sub&gt;18&lt;/sub&gt; column (150×4.60 mm, 5 μ)</td>
<td>0.1% v/v triethylamine (pH 4.0):acetonitrile in the ratio 25:75% (v/v)</td>
<td>1.0 ml/min</td>
<td>UV 225 nm</td>
<td>(Gandhimathi et al., 2007)</td>
</tr>
<tr>
<td>4</td>
<td>Dosage form</td>
<td>Bondapak C18 reverse phase column</td>
<td>methanol: water (35:65; v/v)</td>
<td>1.8 ml/min</td>
<td>UV-235 nm</td>
<td>(Akay et al., 2008)</td>
</tr>
<tr>
<td>5</td>
<td>Capsule of combination dosage form</td>
<td>C-18, 5 mm column having 250 x 4.6 mm</td>
<td>0.02 M potassium dihydrogen phosphate: methanol (20:80) adjusted to pH 4</td>
<td>1.0 ml/min</td>
<td>UV-240 nm</td>
<td>(Shah et al., 2007)</td>
</tr>
</tbody>
</table>

S.P- Stationary phase; M.P- Mobile phase; F.R - Flow rate
**Table 1.2: Reported HPTLC methods of aspirin**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Formulations</th>
<th>Mobile Phase</th>
<th>Detection</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tablets of Aspirin, Paracetamol, Caffeine</td>
<td>n-hexane-ethylacetate-glacial acetic acid (6 : 4 : 0.1 v/v)</td>
<td>UV-254 nm or 273 nm</td>
<td>Sethi., 1996</td>
</tr>
<tr>
<td>2</td>
<td>Tablets of aspirin, Paracetamol, Caffeine</td>
<td>Ethyl acetate-glacial acetic acid (95 : 5, v/v)</td>
<td>UV-254 nm</td>
<td>Sethi., 1996</td>
</tr>
<tr>
<td>3</td>
<td>Tablets of Aspirin, Paracetamol, Caffeine and Ascorbic acid</td>
<td>Toluene-n-propanol-formic acid (7.5 : 2.5 : 0.1, v/v)</td>
<td>UV-254 nm or 273 nm</td>
<td>Sethi., 1996</td>
</tr>
<tr>
<td>4</td>
<td>Tablets of Aspirin and Dipyridamol</td>
<td>Chloroform-methanol-glacial acetic acid (9.5 : 0.5 : 0.04, v/v)</td>
<td>UV-230 nm or 245 nm</td>
<td>Sethi., 1996</td>
</tr>
<tr>
<td>5</td>
<td>Tablets, Drages of Aspirin, Salicylamide and Salicylic acid</td>
<td>Cyclohexane-chloroform-glacial acetic acid (60 : 5 : 5, v/v)</td>
<td>UV-225 nm or 200 nm</td>
<td>Sethi., 1996</td>
</tr>
</tbody>
</table>
CHAPTER -I

INTRODUCTION

1.11 PLAN OF WORK
1.11.1 Literature search
1.11.2 Procurement of shilajit and model drug candidate, aspirin
1.11.3 Method development and standardization for aspirin analysis
1.11.3.1 Analytical methods for routine analysis (HPLC and HPTLC)
1.11.4 Characterization and identification of aspirin
1.11.4.1 Physico-chemical characterization
1.11.4.2 Spectral characterization.
1.11.5 Characterization and authentication of shilajit of different sources
1.11.6 Extraction of humic and fulvic acid from shilajit
1.11.6.1 Standardization of reported method
1.11.6.2 Development of an improved method.
1.11.7 Characterization of humic and fulvic acid
1.11.7.1 Spectral characterization
1.11.7.2 Elemental composition analysis by FT-ICR mass spectrometry
1.11.8 Phase solubility studies
1.11.9 Preparation of complexes using following methods
1.11.9.1 Solvent evaporation
1.11.9.2 Freeze drying
1.11.9.3 Spray drying
1.11.10 Characterization of complexes
1.11.10.1 Differential scanning calorimetry
1.11.10.2 FT-IR spectroscopy
1.11.10.3 X-ray diffraction
1.11.10.4 Scanning electron microscopy.
1.11.10.5 Nuclear magnetic resonance spectrometry
1.11.10.6 Saturation solubility of prepared complexes
   1.11.10.6.1 in 0.1N HCl
   1.11.10.6.2 in acetate buffer pH 4.5
   1.11.10.6.3 in phosphate buffer of pH 6.8
1.11.10.7 Dissolution studies of solid complexes
1.11.10.8 Stability studies of aspirin and their complexes as per ICH Guideline
1.11.10.9 Accelerated stability studies according to WHO for shelf life determination
1.11.10.10 Drug permeation study across rat everted gut sac
1.11.10.11 pH stability profile of aspirin and their optimized complexes
1.11.10.12 Forced degradation of aspirin and their complexes
1.11.11 Pharmacodynamic studies of the optimized complexes using established animal model
   1.11.11.1 Anti-inflammatory studies: The rat paw edema method
   1.11.11.2 Pylorus ligated gastric ulceration
   1.11.11.3 Histopathological study of stomach
1.11.12 Formulation of tablets using fulvic acid and HP-β-CD complexes
   1.11.12.1 Release and in-vitro equivalence study
   1.11.12.2 Stability studies of the optimized formulation
1.11.13 Molecular method: Computational methods