ISSN 0973 – 9874 J.Pharm.Chem CODEN: JPCOCM

Journal of Pharmacy and Chemistry

(An International Research Journal of Pharmaceutical and Chemical Sciences)
Indexed in Chemical Abstract
www.stfindia.com

ISSN 0973 – 9874 J.Pharm.Chem CODEN: JPCOCM

Journal of Pharmacy and Chemistry

(An International Research Journal of Pharmaceutical and Chemical Sciences)

Volume 4 • Issue 1 • January – March 2010

Contents

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VIEWS

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Antiurolithiatic and Antioxidant Effects of Cassia *occidentalis* L. on Experimentally Induced Calcium Oxalate Urolithiasis in Rats

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ABSTRACT

In the indigenous system of medicine in India, the seeds of *Cassia occidentalis* L. are claimed to be useful in the treatment of urinary stones. Present study evaluates the effect of ethanolic extract of *C. occidentalis* seeds on experimentally induced calcium oxalate stones in male albino rats, employing 0.75% v/v ethylene glycol and 2% w/v ammonium chloride in drinking water for 15 days. Calculogenic rats exhibited enhanced levels of calcium and oxalate deposition in the kidney, urinary excretion of calcium, oxalate, creatinine and an increase in the kidney weights on formation of the renal calculi. Rats treated with the ethanolic extract of *C. occidentalis* (0.5g and 1g/kg, oral), showed a marked reduction in the elevated urolithic parameters, lipid peroxidation and enhanced levels of reduced glutathione and catalase. Results of the present study revealed that *C. occidentalis* seeds are effective in treating calcium oxalate stones.

Key words: *Cassia occidentalis*, Calcium oxalate renal stones, Ethylene glycol/Ammonium chloride, Antiurolithiatic activity, Oxidative stress, Antioxidant activity.

Introduction

Urolithiasis or formation of stones in the urinary system is a problem even in today's developed world, due to a number of extrinsic and intrinsic factors that influence kidney stone formation. Though it's not a life threatening disease, most of the times, the discomfort is intolerable. In humans, calcium oxalate (CaOx) is the most common component (70-80%) of kidney stones [1]. Studies show that oxalate (Ox) induced peroxidative injury is also involved in the process of CaOx crystal deposition [2, 3].

Surgical removal and Extracorporeal Shock Wave Lithotripsy are the most common ways to treat urinary stones. However, stone recurrence is very common with these methods. Urolithiaisis can also be treated with drugs, but many of these drugs produce a number of metabolic adverse effects that limit their long term use. Hence, a safe and effective alternative treatment employing natural resources may be beneficial. Many plants have been used to treat urinary stones in the traditional systems of medicine throughout the world for centuries. *Cassia occidentalis* L. (family: Caesalpinaceae), a common weed of waste lands, popularly known as stinking weed is one among them. The seeds are widely used in the Indian indigenous system of medicine to treat urinary stones [4]. Earlier researchers reported the antimicrobial activity of the plant [5]. Literature survey on *C. occidentalis* revealed that systematic pharmacological studies have not been carried out to verify its use in treating urinary stones. Therefore, the objectives of the present study were to evaluate the antiurolithiatic and antioxidant activities of the ethanolic extract of the seeds of *C. occidentalis* against ethylene glycol (EG) and ammonium chloride (AC) induced CaOx urolithiasis in rats.

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Materials and Methods

Plant material

C. occidentalis plant was authenticated by Dr. K. Madhavachetty, Department of Botany, Sri Venkateswara University, Tirupati. Dried pods were collected from Nalanda nagar, Tirupati during January-February of 2008. A voucher specimen was preserved at Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati.

Extraction

Coarsely powdered seeds of *C. occidentalis* (500 g) were extracted with 2L of ethanol (95%) by refluxing over a water bath for 4 h at 70°C. The extract was filtered and the procedure was repeated three times. The filtrates were pooled and converted to a semisolid consistency in vacuum (yield 10.5% w/w). The semisolid extract thus obtained

was stored in a refrigerator (8^0 C) until further use. From the extract a 10 %w/v aqueous suspension was constituted before dosing of the rats. The extract was subjected to phytochemical testing.

Animals

Adult male albino rats of Wistar strain, weighing 150-200g were used in the present study. The rats were housed in polypropylene cages under hygienic conditions and maintained on standard pellet diet (Gold Mohur, Bangalore) and water *ad libitum.* The animals were acclimatized and maintained at 25±2°C in a well ventilated room under 12h light /12h dark cycle throughout the study. All experimental procedures were performed in accordance to animal ethical norms and all experimental protocols were approved by Institutional Animal Ethical Committee.

Acute toxicity and gross behavioral changes study

Five groups of rats consisting of six per group were fasted overnight with free access to drinking water. Group 1 acted as normal and received distilled water (10 ml/kg, oral). Group 2 to 5 animals received 0.5, 1.0, 2.0 and 4.0 g/kg of ethanolic extract of *C. occidentalis* (ECO) respectively, orally. After administering the extract, the animals were observed continuously for 2 h and then intermittently at one hour interval up to 4 h and at the end of 48 h, the number of deaths was recorded to calculate $LD_{50.}$ During acute toxicity studies, the animals were also observed for gross behavioral changes [6, 7].

Assessment of antiurolithiatic activity

Induction of urolithiasis

Current study employed drinking water containing 0.75% (v/v) ethylene glycol (EG) and 2% (w/v) ammonium chloride (AC) ad *libitum* for 15 days to induce CaOx urolithiasis in rats [8].

Experimental design

Rats were divided into seven groups comprising of six per group and were subjected to different treatments, as shown in Table-1.

Determination of urinary parameters

After hydrating with 5 ml distilled water orally, the rats were placed in separate metabolic cages. From normal, preventive control and preventive treated groups, 24 h urine samples were collected from overnight fasted rats on day 15, whereas the samples from curative control and curative treated groups were collected on day 30. The supernatant obtained upon centrifugation of the urine samples at 2,500 rpm at 30 ±2°C for 5 min was used to determine pH and quantitative estimation of calcium, oxalate [9, 10] and creatinine [11].

Kidney homogenate analysis

At the end of the experimental periods, the rats were sacrificed by decapitation. Kidneys were perfused with ice-cold normal saline. One kidney from each animal was carefully separated, washed in ice-cold 0.15 M KCl and weighed. Then the kidney was homogenized in 10N HCl to get 10% w/v of homogenate. The homogenate was centrifuged at 2,500 rpm at 30 ± 2°C for 3 min and the supernatant was used to estimate calcium [9] and oxalate [10].

Determination of oxidative stress (OS)

Other kidney from each animal was carefully incised and washed with chilled normal saline and weighed. Then the kidney was homogenized with ice cold phosphate

Table 1

Treatment Schedule	Group Treatment	Purpose
1. Normal	——	Control for group 2 & 5
2. Preventive control	EG/AC in drinking water *ad libitum* from day 1-15	Control for group 3 & 4
3. Preventive treated	EG/AC in drinking water *ad libitum* and ECO 0.5 g/kg (p.o.) from day 1-15	To assess the ability of ECO in preventing the renal stone formation
4. Preventive treated	EG/AC in drinking water *ad libitum* and ECO 1.0 g/kg (p.o.) from day 1-15	To assess the ability of ECO in preventing the renal stone formation
5. Curative control	EG/AC in drinking water *ad libitum* from day 1-15 and distilled water 10ml/kg (p.o) from day 16-30	Control for group 5 & 6
6. Curative treated	EG/AC in drinking water *ad libitum* from day 1-15 and ECO 0.5 g/kg (p.o.) from day 16-30	To assess the ability of ECO in dissolving the pre-formed renal stones
7. Curative treated	EG/AC in drinking water *ad libitum* from day 1-15 and ECO 1.0 g/kg (p.o.) from day 16-30	To assess the ability of ECO in dissolving the pre-formed renal stones

buffer (pH 7.4) to get 10% w/v homogenate. Nuclear debris was separated by centrifuging the homogenate at 800 rpm at 4°C for 5 min. The resultant supernatant was further centrifuged at 10,000rpm at 4°C for 20min to get post mitochondrial supernatant, which was used to estimate lipid peroxidation as malondialdehyde (MDA) [12] and antioxidants, reduced glutathione (GSH) [13] and catalase [14].

In vitro antioxidant studies

DPPH free radical scavenging

The antioxidant activity of the extract was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical *in vitro* scavenging [15]. The extract in ethanol (3 ml) at different concentrations (100, 200, 400, 800 and 1000 µg/ml) was incubated with 1ml of the ethanolic solution of DPPH (100µM) at 30±2°C for 20 min and the absorbance was read at 517 nm. For each concentration, the assay was carried out in triplicate. Percentage scavenging of the DPPH by the extract was expressed by taking the difference in absorbance between the control and the test. Ascorbic acid was used as the standard. For ascorbic acid and ECO, IC_{50} values (concentration required to scavenge 50% of the free radicals) were also determined. The results were expressed as means of triplicates.

Nitric oxide free radical scavenging

Nitric oxide free radical scavenging was determined by studying the inhibition of the generation of nitric oxide from sodium nitroprusside. 3ml of each 100, 200, 400, 800 and 1000 µg/ml of ECO was dissolved in ethanol and incubated with 1ml of sodium nitroprusside (10mM) in phosphate buffer (pH 7.7) at 25ºC for 120 min. After incubation, 0.5 ml of the reaction mixture was diluted with 0.5 ml of Griess reagent (2% *o*-phosphoric acid, 1% sulphanilamide and 0.1% N-napthylethylenediamine). The absorbance of the pink chromophore, formed during diazotization of nitrite with sulphanilamide and subsequent coupling with N-napthylethylenediamine was measured at 546 nm against the corresponding blank solution. For each concentration, the assay

was carried out in triplicate. The degree of free radical scavenging in the presence and absence of different concentrations of the extract was measured. The difference in absorbance between the control and the test was taken and expressed as percentage free radical scavenging of the NO by the extract [16]. Ascorbic acid was used as standard. IC_{50} values (concentration required to scavenge 50% of the free radicals) for ascorbic acid and ECO were also determined. The results were expressed as means of triplicates.

Statistical analysis

The results were presented as Mean ± SEM. One way analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons were used for the measurement of inter-group variation. Statistical significance was considered at $P<0.05$.

Results

Preliminary phytochemical evaluation

The preliminary phytochemical screening of ECO showed the presence of flavonoids, tannins, saponins, steroids, anthraquinones and carbohydrates.

Acute toxicity and gross behavioral changes studies

Ethanolic extract of ECO was found to be safe, as no animal died on administration of the extract up to 4 g/kg, orally and there were no significant gross behavioral changes except diuretic and laxative effects.

Antiurolithiatic activity

Kidney weight

In both the preventive and curative control groups, on administration of EG/AC in drinking water, a significant increase in kidney weight was observed when compared to the normal group. On treatment with ECO at 0.5 and 1.0 g/kg orally, a significant reduction in kidney weight was observed in both the preventive and curative treated groups, when compared to their respective controls (Table-2).

Urinary pH

In normal rats, the pH was 7.0 to 7.5. EG/AC in drinking water a*d libitum* reduced urinary pH to 5.0 – 6.0 in the preventive and curative control groups. In these groups, treatment with ECO restored the pH to normal (6.5 – 7.0).

Deposition of calcium and oxalate in the kidney

In the control group 2 and 5 animals, administration of EG/AC in drinking water resulted in a significant enhancement in kidney calcium and oxalate deposition. On ECO treatment, a dose dependent reduction in kidney calcium and oxalate deposition was observed in the preventive and curative treated groups (group 3,4,6 & 7) (Table- 2).

Urinary excretion of calcium, oxalate and creatinine

A significant enhancement in the urinary excretion of calcium, oxalate and creatinine was observed in the preventive and curative control groups on CaOx renal stone formation. On treatment with ECO, a dose dependent significant reduction in urinary calcium, oxalate and creatinine excretion was observed in the preventive and curative treated groups (Table-3).

Oxidative stress

In vivo lipid peroxidation

On administration of EG/AC in drinking water, the MDA content of the kidneys was increased in the preventive and curative control groups, when compared

Table 2

Effect of ECO on kidney weight and deposition of Calcium and oxalate in the kidney

Group	Treatment	Kidney weight (g/100g body weight)	Deposition (mg/g kidney tissue)	
			Calcium	Oxalate
1	Normal	0.33 ± 0.02	0.32 ± 0.02	0.95 ± 0.09
2	Preventive control	0.48 ± 0.02[a]	0.95 ± 0.02[a]	3.74 ± 0.18[a]
3	ECO 0.5g/kg	0.37 ± 0.02[b]	0.44 ± 0.02[b1]	1.54 ± 0.05[b1]
4	ECO 1.0g/kg	0.34 ± 0.02[b1]	0.29 ± 0.02[b1]	0.93 ± 0.03[b1]
5	Curative control	0.44 ± 0.02[a1]	0.93 ± 0.02[a]	3.65 ± 0.17[a]
6	ECO 0.5g/kg	0.34 ± 0.02[c]	0.60 ± 0.02[c1]	2.43 ± 0.04[c1]
7	ECO 1.0g/kg	0.33 ± 0.02[c]	0.53 ± 0.02[c1]	1.91 ± 0.10[c1]

Values are expressed as Mean±SEM

a-p<0.001, a1-p<0.01 compared to normal group I
b-p<0.01, b1-p<0.001 compared to preventive- control group II
c-p<0.01, c1-p<0.001 compared to curative -control group V
ECO-Ethanolic extract of *C. occidentalis*

Table 3

Effect of ECO on urinary excretion of creatinine, calcium and oxalate

Group	Treatment	(mg/dl)		
		Creatinine	Calcium	Oxalate
1	Normal	7.83 ± 0.48	8.56 ± 0.19	1.70 ± 0.14
2	Preventive control	28.83 ± 0.40[a]	21.75 ± 0.66[a]	9.12 ± 0.23[a]
3	ECO 0.5g/kg	11.67 ± 0.56[b]	11.34 ± 0.19[b]	2.14 ± 0.14[b]
4	ECO 1.0g/kg	7.83 ± 0.48[b]	7.61 ± 0.14[b]	1.31 ± 0.10[b]
5	Curative control	26.17 ± 0.54[a]	20.66 ± 0.17[a]	8.47 ± 0.29[a]
6	ECO 0.5g/kg	19.83 ± 0.48[c]	15.04 ± 0.20[c]	4.90 ± 0.17[c]
7	ECO 1.0g/kg	17.50 ± 0.62[c]	13.18 ± 0.17[c]	3.49 ± 0.10[c]

Values are expressed as Mean±SEM

a-p<0.001 when compared to normal group I
b-p<0.001 when compared to preventive- control group II
c-p<0.001 when compared to curative-control group V
ECO-Ethanolic extract of C. *occidentalis*

to the normal rats. ECO administration produced a dose dependent sharp reduction in the kidney MDA levels of the preventive and the curative treated groups when compared to their respective controls (Table -4).

Antioxidant activity

***In vivo* antioxidant enzymes**

On EG/AC administration, a sharp decline in GSH and catalase levels of kidneys was observed in the preventive and curative control groups. A dose dependent significant enhancement in GSH and catalase levels was observed in the preventive and curative treated groups on treatment with ECO (Table-4).

Table 4

Effect of ECO on in *vivo* lipid peroxidation and antioxidant parameters

Group	Treatment	MDA (nM/mg tissue)	GSH (nM/mg tissue)	Catalase (µM /mg tissue)
1	Normal	67.00 ± 4.30	74.99 ± 4.14	365.70 ± 14.15
2	Preventive control	119.30 ± 3.02[a]	24.50 ± 1.64[a]	81.80 ± 4.34[a]
3	ECO 0.5g/kg	31.87 ± 0.55[b]	93.62 ± 8.81[b]	276.80 ± 9.52[b]
4	ECO 1.0g/kg	25.93 ± 1.07[b]	171.56 ± 12.67[b]	324.20 ± 10.44[b]
5	Curative control	93.20 ± 4.03[a]	26.95 ± 2.57[a]	109.80 ± 2.66[a]
6	ECO 0.5g/kg	49.52 ± 0.70[c]	63.23 ± 6.0[c1]	181.9 ± 8.92[c1]
7	ECO 1.0g/kg	40.51 ± 2.42[c]	99.02 ± 10.24[c]	259.9 ± 20.09[c]

Values are expressed as Mean±SEM

a-$p<0.001$ when compared to normal group I

b-$p<0.001$ when compared to preventive- control group II

c-$p<0.001$, c1-$p<0.01$ when compared to curative -control V

ECO-Ethanolic extract of *C. occidentalis*

***In vitro* antioxidant studies**

DPPH scavenging

NO induced free radical scavenging ability of ECO was concentration dependent and IC_{50} value of ascorbic acid was 58.45µg/ml whereas IC_{50} for ECO was 566.50 µg /ml.

Nitric Oxide radical scavenging activity

ECO exhibited concentration dependent DPPH free radical scavenging and IC_{50} of ascorbic acid was 77.04µg/ml while it was 776.09µg/ml for ECO.

Discussion

The process of renal stone formation in rats closely mimics the etiology of human kidney stone formation. So rats are commonly used to study the pathogenesis of renal stone disease in humans [17]. When EG/AC in drinking water is administered orally, EG is converted to endogenous oxalic acid by the liver [18] and AC lowers urinary pH [19], thus promoting adhesion and retention of CaOx particles within the renal tubules [20]. Association of oxalate crystals with renal tubular cells is considered as a potential factor in the renal stone formation [21]. Hence in the present study, rats were rendered lithogenic by administering EG and AC in drinking water for 15 days.

Enhanced calcium and oxalate deposition in the kidneys and their urinary excretion in the preventive and curative control groups, manifest that the EG/AC administration promoted hyperoxaluria. The lithogenic effects of EG/AC were also evident through enhanced kidney weights. In addition, increased urinary excretion of creatinine indicates that the hyperoxaluria, promoted renal impairment [22]. These results were in consistent with the reports of Atmani *et al* (2003) [23]. In humans, the type of stones formed can be predicted by determining the urinary pH of fasting subjects. Urinary pH of 5.0-6.5, favors mostly CaOx type stone formation [24]. As crystalluria is pH dependent, dissolution of calculi can be attained by changing urinary pH [25]. In the present study, the decrease in the urine pH from 7.0 - 7.5 to 5.5 - 6.0 in both the control groups, supports the formation of CaOx type of stones.

In both the preventive and curative treated groups, the reduction in calcium and oxalate deposition in the kidneys and also their urinary excretion indicate the dose dependent efficacy of ECO in preventing the formation and also in dissolving the pre-formed CaOx stones. In these groups, the reduction in elevated urinary creatinine levels indicates improved renal function by ECO against hyperoxaluria induced renal damage. The increase in urinary pH to 6.5-7.5, also supports the antilithiatic effect of ECO.

Supersaturation of urine with CaOx is the main factor in crystallization, followed by crystal nucleation, growth and aggregation [26]. Thus, it is important to minimize supersaturation or the subsequent steps in crystallization to prevent urolithiasis to a great extent. Agglomeration of particles is the limiting factor in urinary stone formation, as larger crystals are more likely to remain in urinary tract [27]. Studies indicate that saponin rich fraction from Herniaria *hirsuta* pre-coats CaOx crystals and decreases their adhesion to renal epithelia [28]. Similarly, in the present study after treating with ECO, the decrease in the levels of urinary calcium and oxalate implies reduced urinary supersaturation, and deposition of CaOx. Saponins

may be responsible for these effects, as the preliminary phytochemical tests indicated the presence of saponins, anthraquinones, steroids and flavonoids in *C. occidentalis*.

In addition, herbal remedies with antimicrobial activity protect the anti-adherent layer of renal mucosa, a protective barrier against urinary stone deposition [29]. Anti-microbial activity of anthraquinones from *C. occidentalis* has been reported [5] which also might be responsible for the antiurolithic activity of the extract in the present study, as anthraquinones, flavonoids and steroids were isolated by earlier investigators from the plant [30,31].

The lithogenic effects of EG are mainly attributed to its oxidative damage [20]. Polyunsaturated fatty acids of renal membrane are prone to ROS mediated LPO on exposure to Ox/CaOx [32, 33]. The ROS uses up antioxidants considerably thus constitutes OS. In addition, low levels of renal cellular glutathione favor LPO and subsequent retention of calcium and oxalate in the kidneys [34]. Enhanced LPO and reduced levels of GSH and catalase in the preventive and curative control groups indicate that hyperoxaluria, promoted extensive generation of ROS. These ROS may have been responsible for cellular damage and subsequent enhancement in LPO and accumulation and retention of Ox and deposition of CaOx crystals. These results were in agreement with earlier reports of Toblli *et al* (2002) [34].

Studies in rats indicate that treatment with herbal extracts and antioxidants reduce EG induced CaOx crystallization in the kidney and subsequent OS [35-37]. In addition, Curhan *et al* (1998) reported that daily consumption of tea reduces kidney stone formation in humans as its flavonoids act by directly quenching ROS and by chelating metal ions like iron and copper [38-40]. For this reason, the present study was also focused on evaluation of the antioxidant effect of ECO in urolithic rats.

In the preventive and curative treated groups, the dose dependent decline in kidney MDA content reflects, suppression of LPO and alleviation of OS, providing convincing evidence for the effect of the extract in decreasing hyperoxaluria induced LPO. The dose dependent increase in the levels of antioxidant enzymes GSH and catalase reflects the potential of the extract in resisting hyperoxaluria induced OS. *In vitro* antioxidant studies also revealed that the extract is a rich source of antioxidants. Hence, flavonoids present in ECO might have prevented OS through scavenging ROS and by chelating metal ions.

Thus, the extract may have exhibited antiurolithiatic activity through inhibiting crystal aggregation by pre-coating of CaOx crystals with saponins, protecting the antiadherent layer of renal mucosa by anthraquinones and scavenging of ROS by flavonoids. From the present study results, *C. occidentalis* seems to be a promising plant in treating CaOx urinary stones. However, further chemical and pharmacological studies on the isolated components of *C. occidentalis* seeds may be helpful in ascertaining the antiurolithiatic and antioxidant effects of the plant.

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Spectral, electrochemical and molecular modeling studies of Schiff bases of some hydrazinylthiazocoumarins and 2-acetyl pyridine

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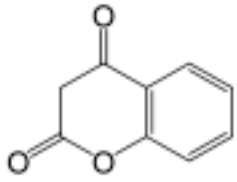
ABSTRACT

The spectral and voltammetric behavior of a series of 3-{2-[N-(1-pyridin-2-yl-ethylidene) hydrazino]thaizol-4-yl}coumarins **(1a-1d)** have been studied. Plots of absorbance vs pH were used for evaluation of their pK_b values. The electrochemical reductions of **(1a-1d)** have been studied over a wide *p*H range at mercury electrode. All the compounds exhibit a characteristic two-electron irreversible reduction wave corresponding to the electrochemical reduction of the azomethine group. Relevant electrochemical data such as diffusion coefficient, charge transfer co-efficient ($\pm_{na}$) and forward rate constant (k^0_h), etc have also been evaluated. Photometric and voltammetric methods of assaying **1a-1d** have been developed. Molecular modeling was done on the compounds to correlate the stability and structural aspects of them to the observed data.

Key words: Thiazolylcoumarins, Cyclic voltammetry, Molecular modeling, Pyridine-2-hydrazones, Acid-base equilibria

Introduction

Hydrazinylthiazocoumarins are extended derivatives of chromone-2-one (**2**) nucleus and find numerous therapeutic applications [1-6]. A variety of Schiff bases have been generated by adducting the hydrazine of this series with suitable carbonyl compounds. These Schiff bases have evinced considerable research interest among electrochemists for their versatile electrochemical behavior with mechanism that varies with the solvent, *p*H, nature of electrode material and other electrochemical experimental conditions [7-9]. Review of the literature, reveals that reports on electrochemical and spectral investigations of compounds possessing all the three, viz, thiazocoumarine, hydrazone and pyridyl moieties are scanty. Hence, we considered it important to study the spectral and electrochemical characteristics of a few representative such molecules **(1a-1d)** which are the Schiff bases of hydrazinylthiazocoumarins and 2-acetyl pyridine.



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Experimental

All the chemicals used were of AnalaR grade. Stock solutions of **(1a-1d)** ($1x10^{-3}$ M) were prepared in aqueous alcohol media. Buffers of different *p*H (ionic strength = 0.05 M) were prepared according to literature procedure [10]. An ATI Orion Model 902 Ion Meter was used for pH-metry. The UV-visible spectra of the solutions were recorded on an AnalyticJena Specord Ratio Recording Spectrophotometer. The electrochemical measurements such as cyclic voltammetry, differntial pulse polorography etc., were recorded on a Metrohm 663 VA Stand whereas coulometry was on a BAS Model CV-27 Voltammograph. Molecular modeling was carried out on ChemOffice Pro 10.0 platform.

The electrochemical recordings were run on a 25 mL sample solution, containing 5 mL of stock solution of the sample and 20 mL of the desired buffer, transferred into the electrochemical cell. Before each run, nitrogen or argon gas was purged for about 4 minutes to dispel the dissolved oxygen. Buffer (20 mL) and 5 mL of water were mixed for blank run.

Results and Discussions

Spectral Studies and Acid-Base Equilibria: The electronic spectra of **1a** in various aqueous buffers are shown

in **Figure 1**. The spectra exhibit a small amount of bathochromic shift with increased pH. The spectra of the other compounds are similar to this one.

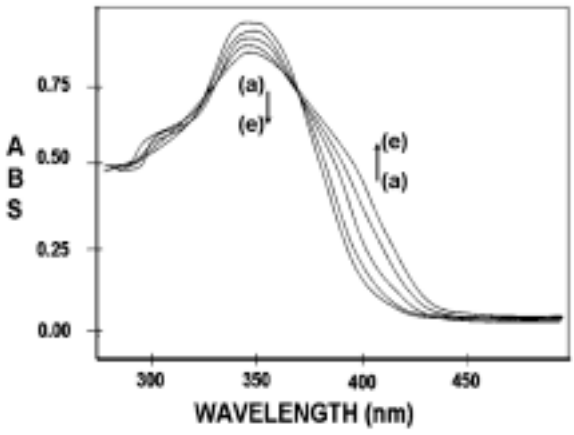
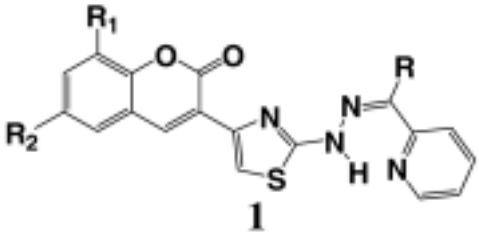


Fig. 1: Uv-vis spectra of 1a at pH (a) 2.17, (b) 5.35, (c) 7.85, (d) 9.64, and (e) 10.28



1

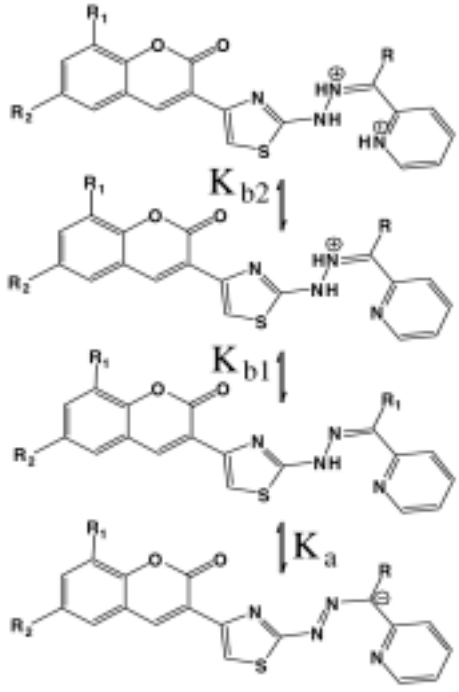
$R_1 = R_2 = H; R = CH_3$ **a**

$R_1 = Br; R_2 = H; R = CH_3$ **b**

$R_1 = Br; R_2 = Br; R = CH_3$ **c**

$R_1 = R_2 = H; R = H$ **d**

in **1a-1d**, viz, the pyridinic, thiazolic and the hydrazonic Schiff base nitrogens. The hydrazenic hydrogen has been reported to be usually acidic and that it undergoes Wolf-Kischner kind of transformation [11]. It may be tentatively suggested that the compounds exhibit acid-base equilibria as in **Scheme 1**.



Scheme 1

Even though it is likely that **1** is triply protonated to render $\mathbf{1H_3^{3+}}$, the pK_a of the latter ($\mathbf{1H_3^{3+}}$) might be very low due coulombic repulsions. Instead, the species, $\mathbf{1H_2^{2+}}$, must be very stabilized by intramolecular hydrogen bonds and tautomerism, as indicated in **Scheme 1**. Whether the nitrogens are protonated or not, the À'‡À* and n'‡À* transitions may hardly be influenced because the electron pairs of all sp^2 nitrogens are orthogonal to the ring systems. Thus one expects a poor effect of pH on the low energy uv-visible spectral profile of **1**. **Figure 1** is indicative of this fact. Molecular modeling done on all these species also gave varied values for the HOMO and LUMO energies but the energy gap between the HOMO and LUMO of **1** and hence, λ_{max} were found to be marginally variable with acid-base conjugate species. By plotting absorbance at ~ 350 nm with pH, we evaluated the pK_a (for $\mathbf{1H^+}$) and pK_b (for **1**). The spectral and thermodynamic data of 1 are presented in **Table 1**.

Table 1

Spectral and thermodynamic data# of 1

Compound	$\lambda_{low}(\varepsilon)$	$\lambda_{high}(\varepsilon)$	$\lambda_{iso}(\varepsilon)$	pK_a	pK_b	$K_a \times 10^5$	ΔG^o
1a	351.6(5405)	399.1(2276)	367.3(3756)	4.4	9.6	3.981	25.12
1b	349.0(5303)	401.4(2232)	365.3(3639)	4.3	9.7	5.011	24.55
1c	354.5(5292)	402.2(2198)	369.6(3617)	4.2	9.8	6.310	23.98
1d	348.2(5523)	403.4(2363)	377.3(3796)	4.6	9.4	2.512	26.26

λ in nm; ε (in lt mol^{-1} cm^{-1}); K_a and ΔG^o (in kJ mol^{-1}) are for the process, $\mathbf{1H^+ \rightarrow 1}$; λ_{low} and λ_{high} are wave lengths below and above the isosbestic wave length, λ_{iso}, respectively.

Electrochemical studies: It has been shown above that the compounds do not exhibit appreciable pH dependence in their electronic spectral profiles due to poor effect of proton transfer on the energy gaps of the HOMO and LUMO orbitals. However, the electron transfer characteristics are expected to very much species-dependent.

The cyclic voltammograms of **1a**, as a representative of **1**, in several buffers, are presented in **Figure 2.** All the compounds offer irreversible reduction peaks in aqueous solution in the *p*H range 2-11. It is also obvious that the peak potentials drift cathodically with increased pH indicating the involvement of H^+ ions in the electrochemical reduction mechanism. Further, at very low *p*H, the reduction peak is slightly split whereas at elevated *p*H one observes a single peak.

Even though, **1a-1d** appear to have several electrochemically active sites, many of them are hard to be reduced due to aromatic stability. However, the azomethine site sandwiched between an aromatic pyridine and amino thiazole rings must be readily reducible. The electrochemical reduction behaviour of Schiff bases is well documented [12]. Curve fitting of Randles-Sevscik equation besides coulometric analyses establishes the number of electrons involved as 2.

Since the compounds undergo neither hydrolysis like many Schiff bases are famous for [13] nor any other chemical reaction in these buffers, the 2-electron transfer is attributed to the reduction of the -C=N- moiety of **1** or protonated **1** i.e., $\mathbf{1H}^{2+}$ or $\mathbf{1H}^{+}$ as

$$-C{=}N- + 2e^- + 2H^+ \xrightarrow{k} -CH-NH- \qquad (1)$$

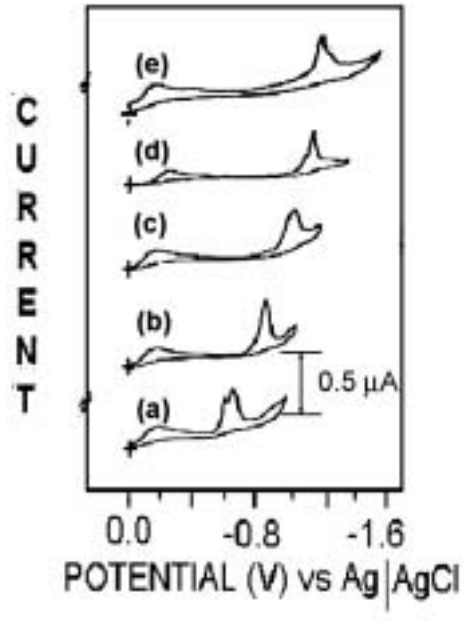


Fig. 2: Cyclic voltammograms of 1a (2.06 x 10^{-4} M) in buffers (ionic strength = 0.05M) of pH (a) 2.56, (b) 5.93, (c) 7.80, (d) 9.62 and (e) 10.48

In many cases, the 2-electron reduction of the azomethine group of the Schiff bases intrinsically happens in a consecutive pair of single electron transfer processes as

$$-C{=}N- + e^- + H^+ \xrightarrow{k_1} -\dot{C}H-NH- \qquad (2a)$$

$$-\dot{C}H-NH- + e^- + H^+ \xrightarrow{k_2} -CH_2-NH- \qquad (2b)$$

giving two successive reduction current peaks [14]. If $k_2 >>> k_1$, one obtains a single but two electron reduction peak with k. The second consecutive reaction has a parallel dimerization process [15] as

$$2\,(-\dot{C}H-NH-) \xrightarrow{k_d} \begin{pmatrix} -CH-NH- \\ | \\ -CH-NH- \end{pmatrix} \qquad (3)$$

Usually, the k_d value is far slower (due to steric restrictions) than k_2 and the chemical dimerization step, (3), would influence the cyclic voltammetric profile when only the k_2 is slow. If the on-setting of the potential responsible for the second consecutive reduction, (2b), is delayed, there is a finite probability of the formation of the dimer which would undergo an easier electrochemical reduction as

$$\begin{pmatrix} -CH-NH- \\ | \\ -CH-NH- \end{pmatrix} + 2e^- + 2H^+ \longrightarrow 2\,(-CH_2-NH-) \qquad (4)$$

The presence of only a single but two electron-two proton reduction indicates that the k_2 is very fast and k_d is very slow. The effect of scan rate on the cyclic voltammetric response of **1** in low pH buffers was studied. A shoulder was observed at lower cathodic potential due to (4) at slow scan rates because of the finite life for the product of (2a) to engage in (3). At faster scan rates the potential needed for (2b) is too quickly reached to allow any scope for (3). That the E_p values of **1a-1c** are considerably more cathodic than those of **1d** and others with more open –C=N– moiety supports the fact that the methyl substitution on the azomethine group hinders a facile electron transfer at the -C=N- site.

The linearity of the plots of peak current, i_p, vs $\nu^{1/2}$, the square root of the scan rate, suggests that the electrochemical reduction in the entire pH range, 2 - 12, is diffusion-controlled. The cathodic shift of peak potential, E_p, with increased scan rate also supports the irreversible nature of the electrode reaction. From plots of E_p vs log ν, at different pH values, the transfer coefficients, α_{na}, were evaluated. The plots of E_p vs *p*H of **1a-1d** are linear with the slopes compatible to a 2-proton participation. With the knowledge of *n* and α_{na}, we could evaluate the diffusion coefficient, D,

for the electroactive species, of **1a-1d**, from the Randles-Sevcik equation,

$$i_p = 2.69 \times 10^{-5} n(\alpha n_a)^{½} A D^{½} ½^{½} c \quad (5)$$

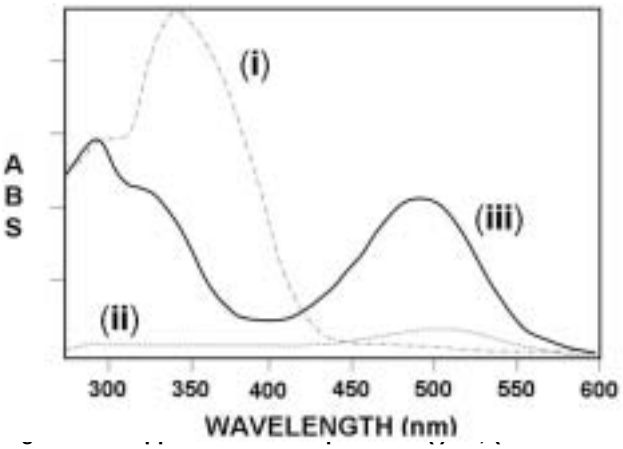
applicable for an irreversible but diffusion controlled electron transfer cyclic voltammetirc peaks, where i_p is the peak current A is the area of the electrode, ½ is the scan rate and c is the concentration of the electro active species.

Compounds, **1b** and **1c** have additional reducible steps in their C–Br bonds. The electrochemistry of organo halogen compounds is well documented [16]. The –C–X– bond (where X is a halogen substituent) is known to offer an irreversible electron transfer due to the process,

$$-C-X \xrightarrow{e^-, H^+} -CH + X^- \quad (6)$$

in aqueous media. However, in the present case of **1**, wherein the C-X is on a stable aromatic ring, such reduction has been found to be very near to the hydrogen evolution and hence could not be investigated further. The electrochemical data of 1 are presented in **Table 2**.

Metal coordination: Compounds, **1a-1d**, are expected to be a set of facile chelating agents given the presence of several nitrogen atoms at appropriate locations of the molecular skeleton. In **Figure 3** are shown the ethanolic electronic spectra of (i) **1a**, (ii) $CoCl_2.6H_2O$, and (iii) the mixture of $CoCl_2.6H_2O$ and **1a** in a 1:2 molar ratio. Neither (i) nor (ii) nor the additive of spectrum of (i) and (ii) matches with (iii), indicating a metal-ligand interaction between Co^{2+} and **1**. A Job's plot also gave the M:L ratio as 1:2.



(ii) $CoCl_2.6H_2O$ (1.87 x 10^{-5} M) and (iii) solution containing 1a (3.74 x 10^{-5} M) and $CoCl_2.6H_2O$ (1.87 x 10^{-5} M)

Assaying of 1: Based on the electrochemical response of **1**, an excellent electrochemical assaying of **1**, in differential pulse polarography (DPP), has been developed. The DPP curves of **1** at *p*H=8.00 along with the calibration curve (inset) are shown in **Figure 4**.

Table 2

Cyclic voltammetric data of 1

Compound	pH	$-E_p$ (V)# vs Ag|AgCl	α_{na}	k^o_h x10^9 (cm^2 /s)	D_o x 10^6 (cm^2 /s)
	2.77	0.645	0.43	5.12	8.88
	4.85	0.774	0.44	4.31	8.23
1a	6.57	0.813	0.45	3.44	8.15
	8.66	0.936	0.47	2.2	8.82
	10.23	1.143	0.58	2.01	9.13
	2.95	0.734	0.39	5.47	5.46
	4.75	0.863	0.45	4.41	5.03
1b	6.52	0.904	0.47	4.07	5.71
	8.79	1.025	0.49	3.34	6.02
	10.93	1.257	0.53	2.74	5.53
	2.55	0.721	0.42	6.91	2.45
	4.65	0.833	0.44	6.09	2.16
1c	6.37	0.879	0.45	5.41	2.33
	8.89	1.038	0.48	4.97	1.38
	11.13	1.271	0.56	4.3	1.27
	2.38	0.614	0.43	7.42	8.99
	4.21	0.746	0.44	6.57	9.05
1d	6.19	0.807	0.45	5.49	9.54
	8.76	0.918	0.51	4.63	9.34
	11.01	1.122	0.55	3.8	9.21

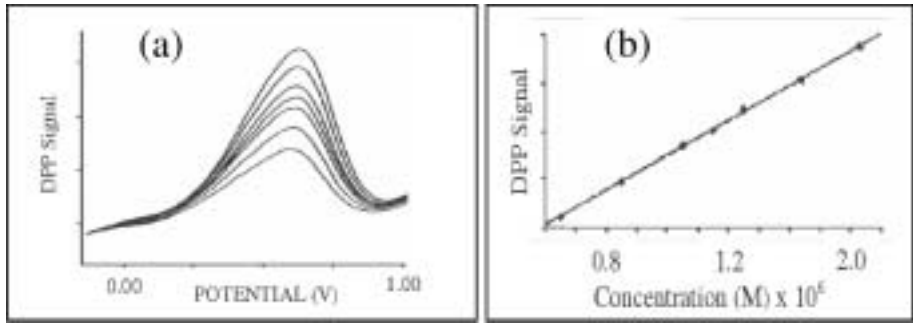


Fig. 4: Differential pulse polarograms (a) and the voltammetirc calibration plot (b) of 1a (scan rate = 5 mV s-1and pulse height = 25 mV) in buffer of pH = 6

Table 3

Molecular modeled thermodynamic, dipole moment, dihedral angle (ϕ) and spectral data of 1

Property	System#	$1H_3^{3+}$	$1H_2^{2+}$			$1H^+$			1	1^-
			AP	AT	PT	A	P	T		
ΔH^o_f (kJ mol^{-1})	a	150.9	62.7	49	33.4	6.6	7.2	3.1	16.6	19.1
	b	159.5	65.6	55.6	37.4	11	10.1	6.6	18.3	19.7
	c	162.5	67.8	58.3	40	13	11.8	8.9	19.7	21.1
	d	142.2	60.4	51.2	32	5.4	5.6	2.5	15	17.5
Dipole Moment (DB)	a	4.1	6.0	3.4	3.4	3.4	5.4	2.6	2.8	6.8
	b	6.0	7.9	5.5	5.2	5.4	7.1	5.1	5	7.7
	c	7.0	9	6.6	6.3	6.5	8.2	6.2	6	8.8
	d	4.2	6.8	4.1	4.1	3.2	6.1	4	3	6.4
Dihedral Angles	a	2.6	11.1	12.0	5.1	25.1	10.9	7.7	21.5	6.8
		13.3	45	18.2	14.1	26.7	2.4	10.7	12.3	11.4
		8.9	0.3	4.1	6	25.6	8.3	31.3	36.1	89
$\phi_{7\text{-}8\text{-}12\text{-}13;}$ $\phi_{14\text{-}15\text{-}17\text{-}18;}$ $\phi_{18\text{-}19\text{-}21\text{-}22\ (deg)}$	b	29.7	7.9	8.4	6.4	25.7	10.1	11.3	1.9	7.1
		14.8	46	18	4.5	27.1	1.9	9.2;	1.7	9.9
		7.9	37.7	8.4	10.1	27.3	7.7	33.1	22.9	95.6
	c	30.1	8.8	8.3	6.5	27.2	11.2	8.7	0.5	5.8
		15.8	46.3	19	3	28.1	2	9.7	1.7	9.7
		6.9	38	9.8	9.6	29.3	7.4	32.6	23	97.2
	d	13	1.8	13.2	7	10	10.5	12.3	15.8	9.4
		19	19.7	13.8	9	18.5	0.1	12.8	5.8	3.3
		3.5	25.5	7.7	4.3	8.9	0.5	11.7	17	64.3
$\Delta^\$$ (eV) λ_{max} (nm)	a	2.5	1.7	1.1	2.4	1.8	2.2	2.9	3.2	0.9
		482	692	1070	502	676	541	411	370	1332
	b	2.6	1.5	1	2.1	1.6	2.2	2.6	3.3	0.8
		456	771	1175	582	747	551	456	365	1577
	c	2.6	1.6	1	2	1.7	2.6	2.6	3.1	0.8
		451	765	1158	585	707	449	455	380	1546
	d	2.9	1.7	1.3	2.1	1.9	2.3	2.4	3	0.9
		419	685	893	558	622	530	489	392	1293

A, P and **T** mean hydrazone, pyridine and thiazole nitrogen sites respectively; $ energy gap between LUMO and HOMO orbitals of the modeled global minimum energy structure

*Molecular Modeling of **1***: Molecular modeling studies were carried out on **1a-1d** using CambridgeSoft ChemOffice Ultra software. The calculated thermodynamic values of various acid-base conjugate species in several of their tautomeric forms are collected in **Table 3** while their standard heats of formation are shown pictorially in **Figure 5**. The global energy-minimized structures of the most thermodynamically stable species of **1a** are shown in **Figure 6** along with the numbering scheme for the atoms. It is observed that coumarin and thiazole are slightly away from coplanarity with less than 20° dihedral angle between them whereas the hydrazone moiety makes as much as 35° dihedral angle with the pyridine ring system in **1**. However, the dihedral angle between the coumarin and thiazole rings falls considerably in **1H**$^+$ (especially in **1H(III)** with little change between the hydrazone and pyridine π-systems. This preference to establish an acute angle between adjacent ring-planes far less than electrostatically preferred 90° is attributed to the possibility of Huckel's aromaticity for the whole molecular π-system that has 26 π electrons including the lone pairs of electrons on the sp^3 nitrogen of the hydrazine moiety, oxygen and sulfur (with n=6 in 4n+2 rule) in **1**. Electron pair orbitals on the hydrazonium and pyridine nitrogens are orthogonal to the molecular π-system and hence do not contribute to the aromaticity. The fact that **1H** is more stable than any other acid-base conjugates of **1** supports this conclusion because the additional hydrogen bonds possible in **1H** favour greater planarity between the successive rings. Conformational energy trends of **1a** over these planes for its most stable acid-base conjugates, viz., **1** and **1H**$^+$, can be visualized from their double-dihedral plots over the congruent atoms, C(15)-N(17)-N(18), shown in **Figure 7**.

The quantum mechanical HOMO-LUMO energy calculations have been used for computing the expected gas-phase electronic transitions for various conjugates and tautomers of **1**. These values are collected in **Table 3** for **1** and **1H**$^+$ along with the experimental spectral data. The trends of the theoretical and experimental absorption maxima are in good agreement.

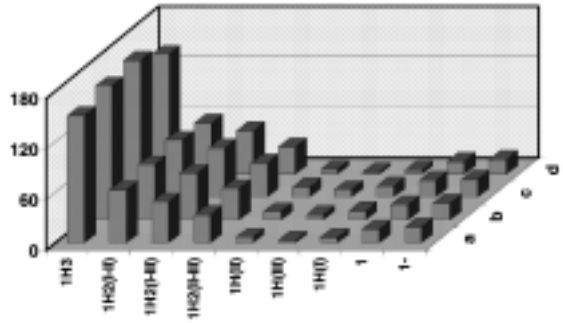


Fig. 5: Histographic representation of the standard heats of formation of various acid-base conjugates of 1 (1Hn is n-many protonated 1 and 1- is deprotonated 1; I, II, and III are azo, pyridine and thiazole nitrogen sites respectively

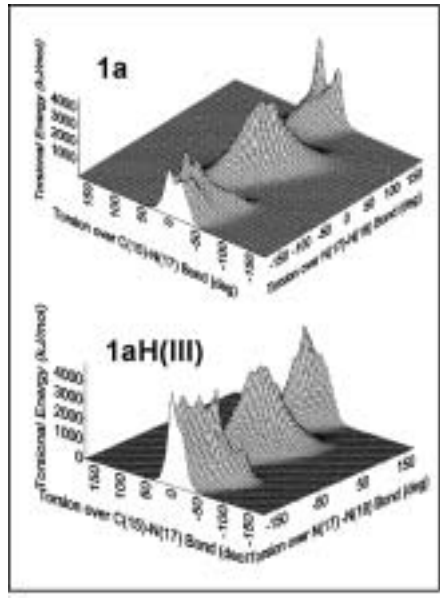


Fig. 7: Double dihedral drive torsional energy diagrams over C(15)-N(17)-N(18) link of 1a and 1aH(III) (1a protonated on thiazole nitrogen

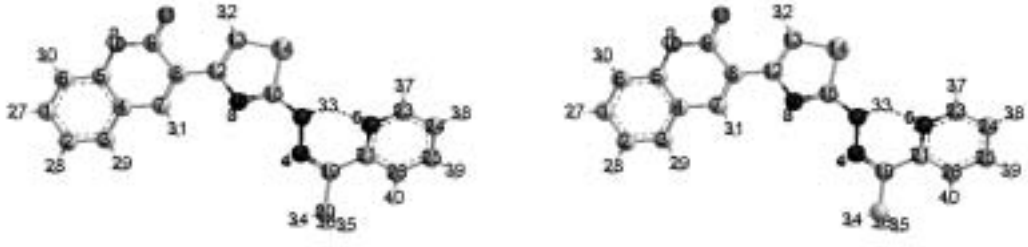


Fig. 6: Stereographic ball and stick model of 1a with the numbering scheme of atoms

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Formulation, Characterization And Evaluation Of Granisetron Buccal Patches

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ABSTRACT

The aim of this investigation was to develop and evaluate mucoadhesive buccal patches of Granisetron(GRN). Permeation of GRN was determined *in vitro* using porcine buccal membrane. Buccal patches were developed by solvent-casting technique using Hydroxypropyl-methylcellulose(HPMC) as mucoadhesive polymer. The patches were evaluated for *in vitro* release, moisture absorption,mechanical properties and *ex vivo* permeation studies. The optimized formulation, based on *in vitro* release, *ex vivo* permeation studies and moisture absorption studies, was subjected for *in vitro* residence time using porcine buccal membrane. *In vitro* flux of GRN was calculated to be 3.19±0.27µg.hr^{-1}.cm^{-2}. *In vitro* drug release and moisture absorbed was governed by HPMC content. Increasing concentration of HPMC delayed the drug release. All formulations followed Zero order release kinetics where as the release pattern was non-Fickian. The mechanical properties, tensile strength (9.58±3.45kgmm^{-2} for formulation F3) and elongation at break reveal that the formulations were found to be strong but not brittle. Formulations showed a significant permeation through porcine buccal membrane and convenient residence time. The results indicate that suitable bioadhesive buccal patches of GRN with desired permeability and suitable mechanical properties could be prepared using HPMC.

Keywords: Buccal,Granisetron,Mucoadhesive, Mechanical properties.

Introduction

Absorption of therapeutic agents from the oral mucosa overcomes premature drug degradation within the gastro intestinal tract, as well as active drug loss due to first pass metabolism that may be associated with other routes of administration. Buccal delivery of drugs became an attractive alternate to the oral route of drug administration by providing rich blood supply that drains drug directly into the jugular vein and chance to administer drugs to patients who cannot be dosed orally[1-3]. Various mucoadhesive formulations were suggested for buccal delivery that includes buccal patches[4], adhesive tablets[5] and adhesive gels[6]. Buccal patches overcome some of the drawbacks of other dosage forms. They have unique characteristics including flexibility, relatively rapid onset of drug delivery, sustained drug release and rapid decline in the serum drug concentration when the patch is removed. The patch is confined to the buccal area over which it is attached and therefore the absorption profile may have less inter and intra-individual variability [7, 8].

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Granisetron is a selective 5-HT_3 receptor antagonist used in treatment of chemotherapy-induced, radiation-induced and post-operative nausea and vomiting. The oral route of administration of GRN is also impractical for patients who are vomiting or who have impaired gastric emptying [9, 10]. Both parenteral and oral formulations have also been used, but buccal route may obviate the need for repeated injections, repeated oral dosing and also be useful in patients who cannot tolerate oral dosage forms. It is less invasive than IV or subcutaneous administration. Physicochemical (low molecular weight 312.4g/mol, low dose 1-2mg , Log *P* 2.6) and pharmacokinetic ($t_{1/2}$ 4-6hr, absolute bioavailability about 60%) parameters made GRN to be suitable for buccal delivery[11-13].

In this investigation we developed GRN buccal patches with a dissolvable matrix using HPMC E 15, with an insoluble backing membrane. The developed patches were evaluated for *in vitro* release, *ex vivo* permeation through porcine buccal membrane and mechanical properties.

Materials and Methods

Granisetron was a gift from by Natco pharma, Hyderabad, A.P, India. Hydroxypropyl methylcellulose E

15(HPMC E 15) was procured from Loba Chemie Pvt. Ltd., India. Phenol red was obtained from Hi Media Laboratories Pvt. Ltd. Mumbai, India. All reagents used were of analytical grade.

Tissue Preparation (Isolation)

Porcine buccal tissue was taken from local slaughter-house. It was collected within 10 minutes after slaughter of the pig and tissue was kept in Kreb's buffer solution. It was bought immediately to the laboratory and was mounted within 2 hours of isolation of buccal tissue. The tissue was rinsed thoroughly using phosphate buffer saline to remove any adherent material. The epithelium was separated from the underlying connective tissue using surgical procedure. Sufficient care was taken to prevent any damage to the buccal epithelium [14].

***In vitro* Drug Permeation Studies**

The buccal epithelium was carefully mounted in between the two compartments of a Franz diffusion cell with internal diameter of 1.1cm (4.15cm^2 area) with a receptor compartment volume of 24ml. Phosphate buffer solution (PBS) pH (7.4) was placed in receptor compartment. The donor compartment contained a solution of 5ml of phosphate buffer pH 6.6 in which 5 mg of GRN was dissolved. The donor compartment also contained phenol red at a concentration of 20µg/ml. This is because phenol red acts as a marker compound and is not supposed to permeate through the porcine buccal membrane. The entire set-up-was placed over magnetic stirrer and temperature was maintained at 37° C by placing the diffusion cell. The samples were collected at predetermined time intervals and stored under refrigerated conditions till the analysis was carried out using UV-Visible spectrophotometer (Elico, India) at 302nm. All the experiments were performed in triplicates.

Fabrication of GRN patches

`Buccal patches were prepared using solvent casting technique with HPMC E 15 as polymer and propylene glycol as plasticizer[15].Polymer was added to 20ml of dichloromethane and methanol (1:1) solvent system and allowed to stand for 6hrs to swell. GRN and propylene glycol were dissolved in 5 ml of solvent system and added to the polymeric solution. This was set aside for 2hrs to remove entrapped air, transferred to a petriplate and dried at room temperature for overnight and then in vacuum oven for 8-12 hrs. The formed patches were removed carefully, cut to size and stored in a desiccator. The composition of the patches is shown in Table 1. Patches with any imperfections, entrapped air, differing in weight or GRN content were excluded from further studies.

***In vitro* Release Studies**

The drug release rate from buccal patches was studied using Franz diffusion cells. Patches were supposed to release the drug from one side only; therefore an impermeable backing membrane (aluminium foil) was placed on the other side of the patch. The patch was sandwiched in dialysis membrane (Hi Media molecular weight 5000) and, was further placed between receptor and donor compartments. The entire set up was placed over magnetic stirrer and temperature was maintained at 37°C by placing the diffusion cell. The contents of receptor compartment were stirred with teflon bead at a speed of 500 rpm. One ml sample was collected at predetermined time intervals from receptor compartment and replaced with an equal volume of the buffer. The samples were analyzed using UV-Visible spectrophotometer (Elico, India) at 302 nm. The experiment was performed in triplicates [15].

Table 1

Formulation ingredients of mucoadhesive buccal patches of Granisetron

Formulation code	Ingredients		
	GRN (mg)	HPMC E 15 (mg)	Propylene glycol(µl)
F1	140	2000	300.0
F2	140	2250	337.5
F3	140	2500	375.0
F4	140	2750	412.0
F5	140	3000	450.0

Note: 25ml of solvent system, 1:1 ratio of dichloro methane and methanol was used.

***Ex vivo* Permeation Studies**

Ex vivo permeation [16,17] of GRN from buccal patches through porcine buccal membrane was studied. Porcine buccal mucosa was obtained and buccal membrane was isolated. The membrane was mounted over a Franz diffusion cell and a buccal patch was placed over the membrane. A dialysis membrane was placed over the membrane so as to secure the patch tightly from getting dislodged from the membrane (the buccal patch was sandwiched between the buccal mucosa and the dialysis membrane). The donar compartment of diffusion cell was filled with PBS pH 7.4. The setup was placed over a magnetic stirrer with temperature maintained at 37^{0}C. Samples were withdrawn and replenished immediately from the receiver compartment at at predetermined time intervals. They were stored under refrigerated conditions till the analysis was carried out. The content of GRN in the samples was analyzed by UV-Visible Spectrophotometer at the wavelength of 302nm. All the experiments were performed in triplicates.

Moisture Uptake Studies

The moisture uptake studies give an indication about

the relative moisture absorption capacities of polymers and an idea whether the formulations maintain their integrity after absorption of moisture. The study was carried out as per procedure reported earlier [18] . Briefly, agar (5% w/v) was dissolved in hot water, transferred into petriplates and allowed to solidify. Six patches from each formulation series were placed in vaccum oven overnight prior to the study to remove moisture if any and laminated on one side with water impermeable backing membrane. They were then incubated at 37°C for one hour over the agar surface. The initial and final weights were recorded and percentage moisture absorption was calculated by using the formula.

$$\%\text{Moisture absorption} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

Measurement of Mechanical Properties

Mechanical properties of the patches were evaluated using a microprocessor based advanced force gauze equipped with a motorized test stand (Ultra Test, Mecmesin, West Sussex, UK), equipped with a 25 kg load cell. Film strip with the dimensions 60 x 10 mm and without any visual defects were cut and positioned between two clamps separated by a distance of 3 cm. Clamps were designed to secure the patch without crushing it during the test, the lower clamp was held stationary and the strips were pulled apart by the upper clamp moving at a rate of 2 mm/sec until the strip broke. The force and elongation of the film at the point when the strip broke was recorded. The tensile strength and elongation at break values were calculated using the formula[19].

$$\text{Tensile strength (kg. mm}^{-2}) = \frac{\text{Force at break (kg)}}{\text{Initial cross sectional area of the sample (mm}^2)}$$

$$\text{Elongation at break (\%mm}^{-2}) = \frac{\text{Increase in length (mm)}}{\text{Original length(mm)}} \times \frac{100}{\text{Cross sectional area (mm}^2)}$$

Measurement of *in vitro* Residence Time

The *in vitro* residence time was determined using USP disintegration apparatus. The disintegration medium was 800 ml of PBS (pH 6.6) maintained at 37±2^{0}C. The segments of porcine buccal mucosa, each of 3 cm length, were glued to the surface of a glass slab, which was then vertically attached to the apparatus. Three mucoadhesive films of each formulation were hydrated on one surface using PBS (pH 6.6) and the hydrated surface was brought into contact with the mucosal membrane. The glass slab was vertically fixed to the apparatus and allowed to move up and down. The film was completely immersed in the buffer solution at the lowest point and was out at the highest point. The time required for complete erosion or detachment of the film from the mucosal surface was recorded[20].

Drug-Polymer Interaction Study

To study the possible interaction between Granisetron and Polymeric materials of the patches, infrared (IR) spectroscopy was carried out on pure substances and their physical mixture. The IR spectra were recorded using IR-Spectrophotometer (Perkin Elmer FT-IR, Perkin Elmer Inst.USA) by KBr pellet method.

Results and Discussions

Drug Permeation Studies through the Porcine Buccal Membrane

Porcine buccal mucosa has been the most frequently chosen model for *in vitro* permeation studies because of its similarity to human tissue and is available in large quantities from slaughter houses. Cumulative amount of GRN permeated through the porcine buccal epithelium is shown in Figure.1. The isolated membrane was intact as no detectable level of phenol red, which was used as a non-absorbable marker compound, was found in the receiver compartment. The thickness of the isolated membrane, measured with a digital micrometer (Mitutoyo, Japan), ranged from 1040 to 1880 microns. Cumulative amount of GRN permeated in 6 hr was about 71.52 ± 4.12 % and flux was calculated to be 3.19±0.27µg. $hr^{-1}.cm^{-2}$.

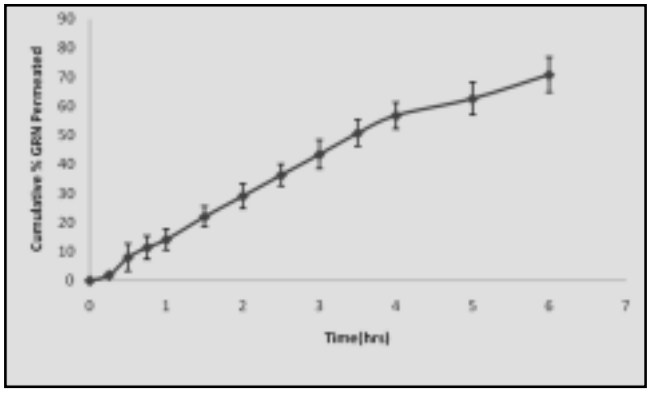

Fig. 1: *Ex vivo* permeation of GRN (5.0 mg) through porcine buccal mucosa, values represented as Mean ±S.D (n = 3)

***In vitro* Drug Release Studies**

The drug release profiles of GRN patches were shown in Figure. 2. The drug release was governed by the amount of matrix forming polymer. An increase in polymer concentration causes an increase in the viscosity of the gel as well as formation of a gel layer with a longer diffusional path. This could cause a decrease in the effective diffusion coefficient of the drug and therefore a reduction in the drug release rate. Formulation F1 showed maximum drug release (83.08± 3.36%) where as formulation F5 showed lowest release of (58.43±2.67%), among the series. Data of the *in vitro* release was fit into different equations and kinetic models to explain the release kinetics of GRN from

buccal patches. Zero order model seemed to be the most appropriate model describing release kinetics from all patches (0.986, 0.987, 0.988,0.995, 0.976 for formulation F1 to formulation F5).On other hand '·' values indicted that amount of released drug was by non Fickian diffusion[21,22].Increasing the concentration of the polymer in the formulations showed a sustained effect on GRN release. This is because, as the proportion of these polymers in the matrix increased, there was an increase in the amount of water uptake and proportionally greater swelling leading to a thicker gel layer. Zero-order release from swellable hydrophilic matrices occurs as a result of constant diffusional pathlengths. When the thickness of the gelled layer and thus the diffusional pathlengths remain constant, zero-order release can be expected. In this investigation similar behavior was predicted and obtained.

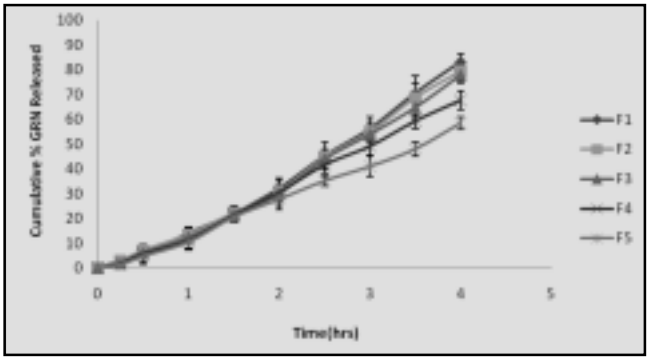

Fig. 2: Release profiles of GRN from mucoadhesive buccal patches, values represented as Mean ±S.D (n=3)

Ex vivo Permeation Studies

Figure 3 shows the graphical representation of cumulative percentage drug permeated from the buccal patches.The results of drug permeation from buccal patches of Granisetron through the porcine buccal mucosa reveal that drug was released from the formulation and permeated through the porcine buccal membrane,hence could possibly permeate through the human buccal membrane. The results indicated that the drug permeation was more in F3 among the last three formulations and about 72.25% of Granisetron could permeate through the buccal membrane in 4 hrs.

Moisture Uptake Studies

Moisture absorption studies evaluate the integrity of

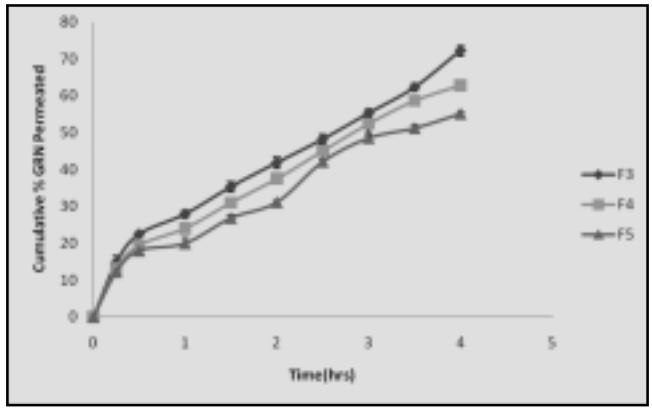

Fig. 3. *Ex vivo* permeation studies of selected mucoadhesive buccal patches of GRN, values represented as Mean±S.D (n=3)

the formulation upon exposure to moisture and the results were shown in Table 2. The percentage moisture observed ranged from about 59.6±11.99% to 156.34±9.27 % w/w for different formulations. Formulations F1 and F2 were deformed during the study. The results reveal that, percentage of moisture absorption was increased with increase in polymer content of formulations(Table 2). When the patches were placed without backing membrane complete swelling followed by erosion was observed indicating that the drug release mechanism involves swelling of the polymer initially, followed by drug release from the swollen matrix by diffusion.

Mechanical Properties of Patches

Ideal buccal film, apart from good bio adhesive strength, should be flexible, elastic and strong enough to withstand breakage due to stress caused during its residence in the mouth. The tensile strength (TS) and elongation at break (E/B) shows the strength and elasticity of the film. A soft and weak polymer is characterized by a low TS and E/B; a hard and brittle polymer is defined by a moderate TS, and low E/B; a soft and tough polymer is characterized by a moderate TS and a high E/B; whereas a hard and tough polymer is characterized by high TS and E/B. It is suggested that an ideal buccal film should have a relatively high TS and E/B. The results of the mechanical properties i.e. TS and E/B were presented in Table 2. TS increased with the increase in polymeric content but E/B values

Table 2

Moisture absorption, Mechanical properties of GRN buccal patches, values represented as mean±S.D (n=3)

Formulations	Moisture absorbed (%w/w)	TensileStrength (Kg.mm^2)	Elongation at break (mm^{-2})
F1	Deformed	3.87±1.28	117±8.24
F2	Deformed	8.74±1.74	94±7.58
F3	59.67±11.99	9.58±3.45	75±6.42
F4	63.72±1.94	11.58±2.42	70±5.24
F5	156.34±9.27	13.24±2.74	53.6±3.72

decreased with the increase in polymer content. Maximum TS was exhibited by F5 patch (13.24±2.74kg.mm^{-2}) and minimum was exhibited by F1(3.87±1.28 kg.mm^{-2}). Maximum E/B was seen with F1(117±8.24% mm^{-2}) and the least was observed with F5 (53.6±3.72% mm^{-2}).

***In vitro* residence time**

In vitro residence time was determined for the formulations F3, F4, F5.The formulations F1, F2 were eroded in moisture absorption study. So they were not suitable as buccal patches. The *in vitro* residence time of the formulations was in order of F4> F3> F5.

Drug - Polymer Interaction Study

The IR spectral analysis of Granisetron alone showed that the principal peaks were observed at wave numbers of 1648.15, 1559.88, 1228.95 and 3235.58. In the IR spectra of the physical mixture of Granisetron and HPMC E15 1647.90, 1560.15, 1242.12 and 3448.42 were observed for the Granisetron. However, some additional peaks were observed with physical mixtures, which could be due to the presence of polymer. These results suggest that there is no interaction between the drug and polymer used in the present study. It is already well known that the common polymers such as HPMC popular in controlled/sustained release matrix type formulations because of their compatibility with a number of drugs.

Selection of optimized formulation

Based on *in vitro* release and moisture absorption studies formulation F3 was selected as the best formulation. Formulation F1 showed maximum drug release 83.08 ± 3.36 %, where as formulation F2 showed 79.37±4.46% drug release. Formulations F1 and F2 were deformed during Moisture absorption studies, these formulations could not be expected to maintain the integrity after administration. The results indicated that the drug permeation was more in F3 among the last three formulations therefore, formulation F3 was selected as best formulation and subjected for further investigation.

Acknowledgements

The authors would like to thank Natco Pharma, Hyderabad, A.P, India for providing gift sample of Granisetron.the authors also like to thank principal and management of Vaagdevi College of Pharmacy for providing necessary facility useful in conduction of this work.

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Gastroretentive Delivery Systems: A Short Review

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ABSTRACT

Recent scientific and patent literature indicates increased interest among academics and industrial research groups regarding the novel dosage forms that can be retained in the stomach for a prolonged and predictable period of time. One of the most feasible approaches for achieving a prolonged and predictable dug delivery profiles in the gastrointestinal tract is to control the gastric residence time, using gastroretentive dosage forms that will provide us with new and important therapeutic options. From the formulation and technological point of view, the preparation of a floating drug delivery system is a considerably easy and logical approach. In this paper, the gastric physiology and the reported intragastric delivery systems have been presented briefly.

Key words: Gastro retentive delivery systems, effervescent floating systems, non effervescent floating systems, single unit and multiple units.

Introduction

Under certain circumstances prolonging the gastric retention of a delivery system for achieving greater therapeutic benefit of the drug substance is desirable. For example, drugs that are absorbed in the proximal part of the gastrointestinal tract [1], and drugs that are less soluble in or are degraded by the alkaline pH may benefit from prolonged gastric retention [2, 3]. In addition, for local and sustained drug delivery to the stomach and proximal small intestine to treat certain conditions, prolonged gastric retention of the therapeutic moiety may offer numerous advantages including improved bioavailability and therapeutic efficacy, and possible reduction of dose size [4-6]. It has been suggested that prolonged local availability of antibacterial agents may augment their effectiveness in treating Helicobacter pylori (H. Pylori) related peptic ulcers [7]. H. pylori live deep within the gastric mucus layer and prolonged local application of drug product is needed for sufficient drug to diffuse to the bacteria. Menon et al. [8] have compared the absolute bioavailability of furosemide in dogs from commercial conventional products and a floating dosage form. Higher bioavailability from floating dosage form when compared to the non-floating commercial products of furosemide has been attributed to the fact that the upper gastrointestinal tract is the primary site of absorption for the drug.

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It was suggested that compounding narrow absorption window drugs in a unique pharmaceutical dosage form with gastroretentive properties would enable an extended absorption phase of these drugs. After oral administration, such a dosage form would be retained in the stomach and would release the drug there in a controlled and prolonged manner, so that the drug would be supplied continuously to its absorption sites in the upper gastrointestinal tract. This mode of administration would best achieve the known pharmacokinetic and pharmacodynamic advantages of controlled release dosage forms for these drugs [9].

Gastro retentive delivery systems (GRDS), however, are not suitable for drugs that may cause gastric lesions, e.g., non-steroidal anti-inflammatory agents. Also, the drug substances that are unstable in the strong acidic environment of the stomach are not suitable candidates to be incorporated into such systems. In addition, these systems do not offer significant advantages over the conventional dosage forms for drugs, which are absorbed throughout the gastrointestinal tract [10].

The need for gastroretentive dosage forms (GRDFs) has led to extensive efforts in both academia and industry towards the development of such drug delivery systems. These efforts resulted in GRDFs that were designed in large part based on the following approaches: (a) low density form of the dosage form that causes buoyancy above gastric fluid; (b) high density dosage form that is retained in the bottom of the stomach; (c) bioadhesion to

the stomach mucosa; (d) slowed motility of the gastrointestinal tract by concomitant administration of drugs or pharmaceutical excipients; (e) expansion by swelling or unfolding to a large size which limits emptying of the dosage form through the pyloric sphincter. However, it is recognized that there are many physiological constraints which may limit development of such delivery systems. Following is a brief description of the physiological considerations pertaining to designing GRDS.

Basic Gastrointestinal Tract Physiology

It is well recognized that the stomach is used as a depot for controlled release dosage forms. The stomach is situated in the left upper part of the abdominal cavity immediately under the diaphragm [11]. The stomach is composed of the following parts: *fundus*, above the opening of the esophagus into the stomach; *body*, the central part; and *antrum*. The proximal portion of the stomach comprising fundus and body regions, serves as a reservoir for ingested materials, secretes digestive juices and propels chyme, a milky mixture of food with gastric juices, to the distal part of the stomach i.e., antrum. Antrum serves as the major site for mixing and for pumping which leads to gastric emptying [12] Its size varies from 25 to 50 ml according to the amount of distention. Following a meal it is 1500ml, after the food emptied, a 'collapsed' state is obtained with a resting volume of only 25–50 ml. The pylorus is an anatomical sphincter situated between the most terminal antrum and the duodenum [13] In the fasting state pH of the stomach is 2. But after ingestion of food pH gradually rises to a level of 6.5 and then gradually declines to the pH of fasting state over a period of a few hours.

The intrinsic properties of the drug molecule and the target environment for delivery are the major determining factors in the bioavailability of the drug. Factors such as pH, enzymes, nature and volume of secretions, residence time, and effective absorbing surface area of the site of delivery play an important role in drug liberation and absorption. A brief survey of the relevant physiological features that pose challenges to the development of an effective gastroretentive delivery system is presented below.

Gastric pH

The gastric pH is not constant; it is rather influenced by various factors like diet, disease, presence of gases, fatty acids, and other fermentation products. In addition, the gastric pH exhibits intra-as well as inter-subject variation. This variation in pH may significantly influence the performance of orally administered drugs. It has been reported that the mean value of gastric pH in fasted healthy subjects is 1.1±0.15 [14 – 16]. On the contrary, the mean gastric pH in fed state in healthy males has been reported to be 3.6±0.4, [17] and the pH returns to basal level in about 2 to 4 hours. However, in the fasted state, basal gastric secretion in women is slightly lower than in men.

Gastric pH may be influenced by age, pathological conditions and drugs. About 20% of the elderly people exhibit either diminished (hypochlorohydria) or no gastric acid secretion (achlorohydia) leading to basal pH value over 5.0[18]. Pathological conditions such as pernicious anemia and AIDS may significantly reduce gastric acid secretion leading to elevated gastric pH. [19, 20] In addition, drugs like H_2 receptor antagonists and proton pump inhibitors significantly reduce gastric acid secretion.

Gastric pH is an important consideration in selecting a drug substance, excipients, and drug carrier(s) for designing intragastric delivery systems.

Gastric Emptying

The process of gastric emptying occurs both during fasting and fed states: however, the pattern of motility differs markedly in the two states. In the fasted state, it is characterized by an interdigestive series of electrical events that cycle both through the stomach and small intestine every 2-3 hrs [21]. This activity is called the interdigestive myoelectric cycle or migrating myoelectric complex (MMC), which is often divided into four consecutive phases as described by Wilson and Washington [22].

Phase I is a quiescent period lasting from 30 to 60min with rare contractions.

Phase II is a period of similar duration consisting of intermittent action potentials and contractions that gradually increase in intensity and frequency as the phase progresses and it lasts for about 20 to 40 min.

Phase III is a short period of intense, large regular contractions lasting from 10 to 20 min. It is this phase, which gives the cycle the term, "housekeeper wave", since it serves to sweep undigested materials out of the stomach and down the small intestine. As phase III of one cycle reaches the end of the small intestine, phase III of the next cycle begins in the duodenum.

Phase IV is a brief transitional phase that occurs between phase III and phase I of two consecutive cycles. The motor activity in the fed state is induced 5-10 min after ingestion of a meal and persists as long as food remains in the stomach. It consists of regular and frequent contractions. These contractions are not as severe as those in the third phase of fasted motility pattern.

Some of the important aspects of the physiology of the gastric emptying process are as follows [23]

- The rate of movement of the dosage form from stomach to intestine is affected by the multiple chemical factors and the physical size of the medication.
- The chemical components of the gastric fluid will interact with the intestinal receptors. These receptors will control the rate of gastric emptying by neuronal or hormonal means.

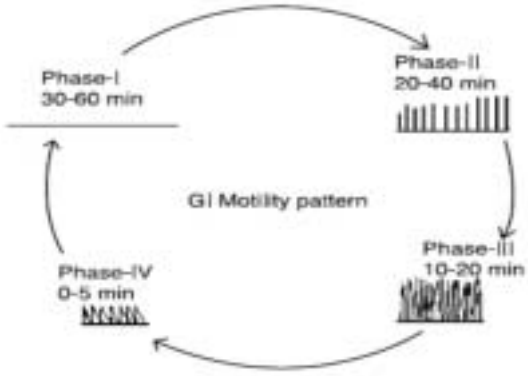

Fig.1: Schematic representation of interdigestive motility pattern. (Adapted from Ref. [25].)

- Emptying of the dosage form is also influenced by whether it is taken on an empty stomach, in an interdigestive state (with or soon after a meal), or in a digestive state.

- Small particles, regardless of size, density, or texture that are ingested during the interdigestive state become coated by mucus and these coated dosage forms are emptied uniformly from the stomach.

- During the digestive state, the larger particles are retained in the stomach until the meal is essentially emptied.

- Emptying times of the solid dosage forms range from 5 min to 5 h depending on the size of the medication and whether the individual is in the interdigestive or digestive state when the medication is administered.

Thus, an understanding of the physiology of gastric emptying is important in developing intragastric drug delivery systems especially in the case of the floating drug-delivery systems.

Approaches to Prolong Gastric Residence Time (GRT) of Drug Delivery System:

Various devices such as mucoadhesive, floating, swelling and high-density systems have been developed to increase GRT of a dosage form. Physiological features of the upper gastrointestinal tract pose a considerable challenge to develop such systems. Nevertheless, in vivo studies of certain systems have shown promising results.

Mucoadhesive Systems

The idea of bioadhesive began with the clear need to localize a drug at a certain site in the GI tract. Therefore a primary objective of using a bioadhesive system orally would be achieved by obtaining a substantial increase in the residence time of the drug for local drug effect and to permit once daily dosing [24].

The mucoadhesive systems extend their gastroretentive time by adhering themselves to the gastric mucous membrane. Bioadhesion on soft tissues of certain natural or synthetic polymers has been exploited to control as well as to prolong the gastric retention of the delivery systems. The adhesion of the polymers with the mucous membrane may be mediated by hydration, by bonding, or by receptor mediation. In hydration mediated adhesion, the hydrophilic polymers become sticky and mucoadhesive upon hydration. Bonding mediated adhesion may involve mechanical or chemical bonding. Chemical bonds may involve covalent or ionic bonds or Van der Waals forces between the polymer molecules and the mucous membrane. Receptor mediated adhesion takes place between certain polymers and specific receptors expressed on gastric cells. The polymers could be anionic or cationic or nonionic. A brief classification of these polymers is mentioned in Table 1.

Table 1

A list of bioadhesive polymers

Anionic	Cationic	Nonionic
Carboxymethyl cellulose	Polylysine	Polyethylene glycol
Chondroitin sulfate	Polybrene	Polyvinyl pyrrolidone
Poly acrylic acid		Dextran
Pectin		
Carageenan		
Chitosan		
Alginic acid		

Robinson et al [26] studied the bioadhesive properties of a broad spectrum of polymers; his group also reported that albumin beads containing chlorthiazide mixed with polycarbophil offered sustained release for eight hours after being administered orally in the form of capsules to rats. Although these materials have shown good bioadhesion in vitro and in vivo in rats, results in the humans were disappointing because of two problems. One is that the bioadhesive formulation must attach to the surface of the mucus layer, which is a continuously eroding surface and the other is that the delivery system must withstand the GI tract motility.

Smart and Kellaway [27] reported prolonged gastric retention of dosage forms coated with Carbomer in mice. In vivo data of granules containing microcrystalline chitosan and furosemide showed higher AUC than that of the conventional dosage form. [28] Also, the granules exhibited slow release characteristic with a marked lag time. It appeared that due to its mucoadhesive properties, chitosan retained the drug in the gastric mucosa for longer period of time. Although the in vivo data exhibited promising results for intragastric delivery, further characterization and evaluation of the system is necessary. Freeze-dried non-covalent polyionic complexes with porous structure based on polyacrylic acid and chitosan intended for delivering

antibiotics to stomach have been developed by de laTorre. [29] The systems released about 70% of their contents in two hours.

Preda and Leucuta [30] developed a sustained release delivery system based on bioadhesive polymers: polyacrylic acid in gelatin microsphere. In vivo experiments with rat have shown significant retardation of gastric emptying of the beads due to adhesive characteristic of the gelatin and polyacrylic acid.

Illum and Ping [31] developed microspheres that released the active agent in the stomach environment over a prolonged period of time. The active agent was encased in the inner core of microspheres along with the rate-controlling membrane of a water-insoluble polymer. The outer layer was composed of bioadhesive chitosan. The microspheres were prepared by spray drying an oil/water or water/oil emulsion of the active agent, the water-insoluble polymer, and the cationic polymer.

Floating Systems

The floating system is intended to float in and over the gastric contents resulting in prolonged GRT. Floating systems can be of effervescent or non effervescent in nature.

Effervescent systems are prepared with the help of swellable polymers such as methylcellulose and chitosan and various effervescent compounds, eg, sodium bicarbonate, tartaric acid, and citric acid. They are formulated in such a way that when in contact with the acidic gastric contents, CO_2 is liberated and gets entrapped in swollen hydrocolloids, which provides buoyancy to the dosage forms.

Systems with floatation chambers filled with gaseous materials, [32] or with inflatable chambers containing liquids that generate gas at body temperature [33] have been reported to remain afloat over gastric contents. Stockwell et al. [34] devised a system of alginate matrix capsule containing sodium bicarbonate that released carbon dioxide, which remained entrapped in the gel network. Ichikawa et al. [35] prepared a multiple-unit floating pill, which consisted of a core seed surrounded by two different layers. The primary layer contained sodium bicarbonate and tartaric acid, which generated carbon dioxide in aqueous media. The outer layer was composed of a swellable membrane that trapped the gas resulting in floatation of the system. The system started floating within 10 minutes of immersion into the test media and remained floating over a period of 5 hours. Matrix tablets containing hydroxypropyl methylcellulose, drugs, and gas generating agents have shown duration of floating over 8 hours with a floating lag time of 30 minutes [36]. Radiological studies suggested no adherence of the tablet to the gastric mucosa and a mean gastric residence time of over 4 hours.

Yang et al [37] developed a swellable asymmetric triple-layer tablet with floating ability to prolong the gastric residence time of triple drug regimen (tetracycline, metronidazole, and clarithromycin) in H. pylori–associated peptic ulcers using hydroxy propyl methyl cellulose (HPMC) and poly (ethylene oxide) (PEO) as the rate-controlling polymeric membrane excipients. The design of the delivery system was based on the swellable asymmetric triple-layer tablet approach. Hydroxypropylmethylcellulose and poly (ethylene oxide) were the major rate-controlling polymeric excipients. Tetracycline and metronidazole were incorporated into the core layer of the triple-layer matrix for controlled delivery, while bismuth salt was included in one of the outer layers for instant release. The floatation was accomplished by incorporating a gas-generating layer consisting of sodium bicarbonate: calcium carbonate (1:2 ratios) along with the polymers. The in vitro results revealed that the sustained delivery of tetracycline and metronidazole over 6 to 8 hours could be achieved while the tablet remained afloat. The floating feature aided in prolonging the gastric residence time of this system to maintain high localized concentration of tetracycline and metronidazole (Figure 2).

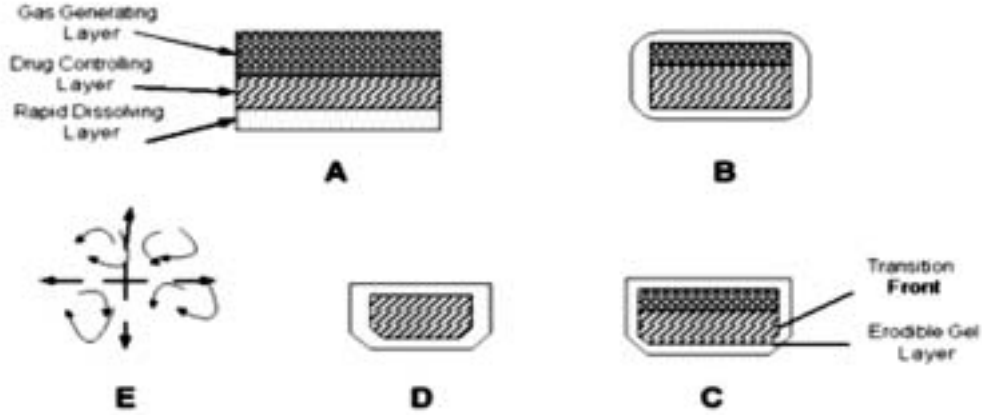

Fig. 2: Schematic presentation of working of a triple-layer system. (A) Initial configuration of triple- layer tablet. (B) On contact with the dissolution medium the bismuth layer rapidly dissolves and matrix starts swelling. (C) Tablet swells and erodes. (D) and (E) Tablet erodes completely [Adapted from reference no.37].

Ichikawa et al [38] developed a new multiple type of floating dosage system composed of effervescent layers and swellable membrane layers coated on sustained release pills. The inner layer of effervescent agents containing sodium bicarbonate and tartaric acid was divided into 2 sublayers to avoid direct contact between the two agents. These sublayers were surrounded by a swellable polymer membrane. When this system was immersed in the buffer at 37ºC, it settled down and the solution permeated into the effervescent layer through the outer swellable membrane. CO_2 was generated by the neutralization reaction between the two effervescent agents, producing swollen pills (like balloons) with a density less than 1.0 g/mL. It was found that the system had good floating ability independent of pH and viscosity of the solution in the G.I.T and the drug (para-amino benzoic acid) was released in a sustained manner. This finding is shown in Fig.No.3.

Fassihi and Yang [39] developed a zero-order controlled release multilayer tablet composed of at least two barrier layers and one drug layer. All the layers were made of swellable, erodible polymers and the tablet was found to swell on contact with aqueous medium. As the tablet dissolved, the barrier layers eroded away to expose more of the drug. Gas evolving agent was incorporated in either of the two barrier layers, which caused the tablet to float and increased the retention of the tablet in the patient's stomach.

Non-effervescent floating dosage forms use a gel forming or swellable cellulose type of hydrocolloids, polysaccharides, and matrix-forming polymers like polycarbonate, polyacrylate, polymethacrylate, and polystyrene. The formulation method includes a simple approach of thoroughly mixing the drug and the gel-forming hydrocolloid. After oral administration this dosage form swells in contact with gastric fluids and attains a bulk density of G I fluids. The air entrapped within the swollen matrix imparts buoyancy to the dosage form. The swollen gel-like structure which is formed thus acts as a reservoir and allows sustained release of drug through the gelatinous mass.

Kawashima et al [40] prepared multiple-unit hollow microspheres by emulsion solvent diffusion technique. Drug and acrylic polymer were dissolved in an ethanol-dichloromethane mixture, and poured into an aqueous solution of PVA with stirring to form emulsion droplets. The rate of drug release in micro balloons was controlled by changing the polymer to drug ratio. Micro balloons were floatable in vitro for 12 hours when immersed in aqueous media. Radiographical studies proved that micro balloons orally administered to humans were dispersed in the upper part of stomach and retained there for three hours against peristaltic movements.

El-Gibaly [41] developed floating hollow microcapsules of chitosan by ionic interaction with a negatively charged surfactant. The system remained buoyant for over 12 hours in simulated gastric fluids and exhibited sustained drug release. Streubel et al [42] prepared single-unit floating tablets based on polypropylene foam powder and matrix-forming polymer. Incorporation of highly porous foam powder in matrix tablets provided density much lower than the density of the release medium. A 17% w/w foam powder (based on mass of tablet) was achieved in vitro for at least 8 hours. It was concluded that varying the ratios of matrix-forming polymers and the foam powder could alter the drug release patterns effectively.

Thanoo et al [43] developed polycarbonate microspheres by solvent evaporation technique. Polycarbonate in dichloromethane was found to give hollow microspheres that floated on water and simulated biofluids as evidenced by scanning electron microscopy (SEM). High drug loading was achieved and drug-loaded microspheres were able to float on gastric and intestinal fluids. It was found that increasing the drug to polymer ratio increased both their mean particle size and the release rate of the drug.

Fell et al [44] prepared floating alginate beads incorporating amoxycillin. The beads were produced by dropwise addition of alginate into calcium chloride solution, followed by removal of gel beads and freeze-drying.

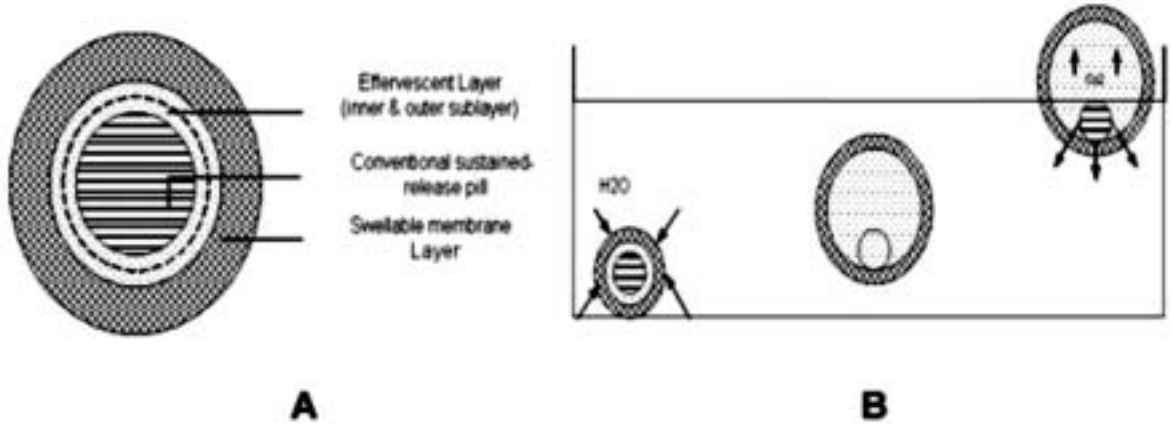

Fig. 3: (A) Multiple-unit oral floating drug delivery system. (B) Working principle of effervescent floating drug delivery system [Adapted from reference no.38].

The beads containing the dissolved drug remained buoyant for 20 hours and high drug-loading levels were achieved.

El-Kamel et al [45] prepared floating microparticles of ketoprofen by emulsion solvent diffusion technique. Four different ratios of Eudragit S 100 with Eudragit RL were used. The formulation containing 1:1 ratio of the two above mentioned polymers exhibited high percentage of floating particles in all the examined media as evidenced by the percentage of particles floated at different time intervals. This can be attributed to the low bulk density and high packing velocity.

Swelling and Expanding Systems

A dosage form in the stomach will withstand gastric transit if it is bigger than the pyloric sphincter. However, the dosage form must be small enough to be swallowed, and must not cause gastric obstruction either singly or by accumulation. Thus, three conditions are required: a small configuration for oral intake, an expanded gastroretentive form and a final small form enabling evacuation following drug release [46]. Unfoldable and swellable systems have been investigated for this purpose.

Unfoldable systems are made of biodegradable polymers. The concept is to make a carrier, such as a capsule, incorporating a compressed system which extends in the stomach. These systems may be referred to as “plug type systems” since they exhibit a tendency to remain lodged at the pyloric sphincter.

Swellable systems are retained because of their mechanical properties. The presence of polymers in the systems promotes their swelling to a size that prevents their passage through pyloric sphincter resulting in prolonged GRT. The swelling usually results from osmotic absorption of water (Fig. 4). The dosage form is small enough to be swallowed, and swells in gastric liquids. The bulk enables gastric retention and maintains the stomach in a “fed” state, suppressing housekeeper waves. However, a balance between the rate and extent of swelling and the rate of erosion of the polymer is crucial to achieve optimum benefits and to avoid unwanted side effects.

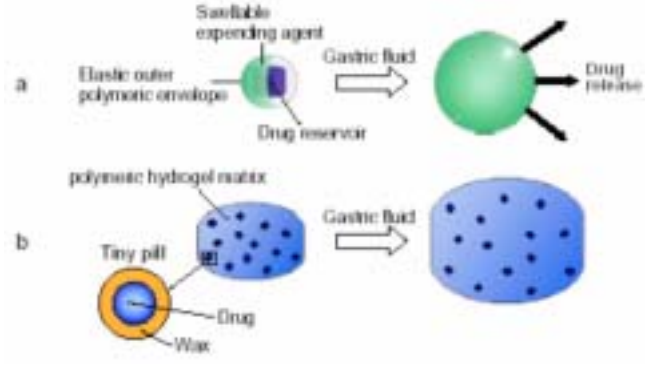

Fig. 4: Swellable type gastric retentive systems

High-Density Systems

High-density systems are intended to lodge in the rugae of the stomach withstanding the peristaltic movements. Systems with a density of 1.3 g/ml or higher are expected to be retained in the lower part of the stomach. A density close to 2.5 g cm^3 seems necessary for significant prolongation of gastric residence time and barium sulphate, zinc oxide, iron powder, titanium dioxide are used as excipients. The formulation of heavy pellets is based on the assumption that the pellets might be positioned in the lower part of the antrum because of their higher density **(Fig. 5).** Devreux et al [47] reported that the pellets with density of at least 1.5 g/ml have significantly higher gastric residence time both in fasted and fed state.

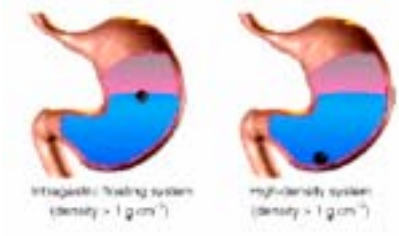

Fig. 5: Schematic localization of an intragastric floating system

Conclusion

Drug absorption in the gastrointestinal tract is a highly variable procedure and prolonging gastric retention of the dosage form extends the time for drug absorption. Mucoadhesive, swelling, high-density, and floating systems promise to be potential approaches for gastric retention. Although there are a number of difficulties to be worked out to achieve prolonged gastric retention, a large number of companies are focusing towards commercializing these techniques.

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Preparation and characterization of gliclazide microcapsules

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ABSTRACT

In recent years, polymer microcapsules have attracted more and more attention because of their specific properties and applications in encapsulation and drug delivery. Polymer microcapsules can be employed to deliver medication in a rate – controlled and sometimes targeted manner. In this single emulsion solvent evaporation method, ethyl cellulose microcapsules containing Gliclazide were prepared. The surface and morphology of microcapsules were characterized by Scanning Electron Microscopy (SEM) and the result showed that the mean diameter of microcapsules was approximately 3µm and possess smooth surface .The compatibility of Gliclazide with ethyl cellulose polymer was studied using Fourier Transform Infra red spectroscopy (FT-IR) and Differential Scanning calorimeter (DSC).The particle size, encapsulation efficiency and drug loading behaviors of ethyl cellulose microcapsules was investigated. *In-vitro* drug release of these microcapsules was performed in phosphate buffer solution pH 7.4. The release profiles of Gliclazide from Ethyl cellulose microcapsules were found to be monophasic with gradual drug release. A prolonged *in - vitro* drug release profile was observed and they are in appreciable extent.

Key words: Ethyl cellulose, Microcapsules, Gliclazide, Drug delivery.

Introduction

In recent years, polymer microcapsules have attracted much attention because of their specific properties, biocompatibility, biodegradability and applications in the pharmaceutical industry [1,2]. Various drug delivery systems, such as liposomes, micelles, emulsions and polymer micro / nanoparticles, facilitate application in controlled and targeted delivery [3-7]. Such delivery systems offer numerous advantages compared to conventional dosage forms including improved efficacy, reduced toxicity and improved patient compliance and convenience. A number of microencapsulation techniques have been studied in the various fields concerning the protection and controlled release of active materials from microcapsules or microspheres. A typical controlled release device, i.e microcapsules is one of the important tools for controlled release of chemical reactants and can consist of active liquid or solid core materials and protective wall.

Many biodegradable polymers have been investigated. The major advantage of biodegradable polymers is that they do not require removal after application. A large number of natural and synthetic polymers are potentially suitable for production of the sphere-forming polymer. Among different polymers, ethyl cellulose have been extensively used as microparticle carriers in controlled release delivery systems for many bioactive molecules due to their non-toxic, good bioavailability and biocompatibility, and approval by the food and drug administration for human use [8].

Ethyl cellulose (EC) is frequently used as hydrophobic polymeric coating materials for extended drug release applications [9]. Microcapsules were prepared from EC using various methods such as phase separation, coacervation, solvent evaporation, either by addition of a non-solvent, or of an incompatible polymer [10-13].

Gliclazide is an oral hypoglycemic agent (anti-diabetic drug) and is classified as a first generation sulfonyl urea derivative [14]. The structure of gliclazide is shown in fig.1.

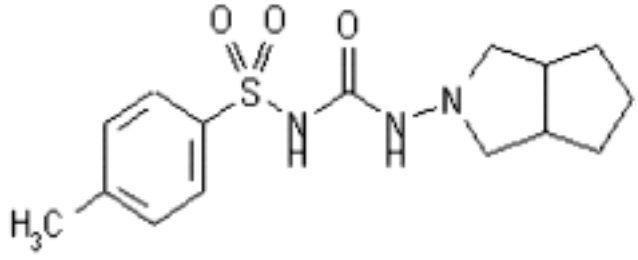

Fig.1 Chemical structure of gliclazide.

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In general, rapid (GI)absorption is required for oral hypoglycemic drugs,in order to prevent a sudden increase in blood glucose level after food intake in patients with diabetes mellitus.Hence there is always a need for the development of sustained release patient compliant formulation of gliclazide,as it is short acting sulfonyl urea[15].The use of extended release products offers potential advantages like sustained blood levels, attenuation of adverse effects and improved patient compliance. Hence to develop gliclazide formulations in controlled release form is important and useful.

In this research, the drug-loaded microspheres were prepared by new technique offered a short time process and consuming less quantity of solvent for their preparation and easy to proceed. *In-vitro* drug release profile was performed in a phosphate buffer solution of pH 7.4. This formulation of controlled delivery of gliclazide has not been reported previously.

Materials and methods

Ethyl cellulose (EC) polymer was purchased from Aldrich (Germany). Acetone, used as solvent was purchased from SISCO research laboratories pvt.ltd (India). Liquid Paraffin a lubricant was obtained from *sd* fine-chem ltd (Mumbai). Gliclazide was purchased from Sigma (Germany).The other reagents and solvents were analytical grade and were used as received

Preparation of microcapsules

The single emulsion solvent evaporation technique was applied to the fabrication of hydrophobic drug loaded microcapsules. Ethyl cellulose was dissolved in 10 ml acetone and specified amount of gliclazide was added and this mixture was sonicated for 5 minutes using Ultra sonicator .The above solution was added into 100ml of liquid paraffin and allowed to homogenize at 1000 rpm for 30 minutes using Ultra turrax homogenizer. Then the obtained microcapsules were separated by vacuum filtration. The microcapsules were washed with petroleum ether (60-80) and dried at room temperature for 4 hrs.

Characterization of Microcapsules

Morphology of microcapsules

The morphology of the microcapsules were observed by a Scanning Electron Microscope (Philips XL 30 ESEM).An appropriate sample of microcapsules was mounted on metal stubs ,using double – sided adhesive tape. Samples were platinum coated and observed for morphology, at acceleration of 20 KV.

Particle size analysis of microcapsules

The particle size and distribution of the microcapsules were measured with a Photon correlation spectroscopy (PCS: size mode V161/NBIO2 SZ2).Microcapsules were first suspended in distilled water and subjected to sonication and vortexing before analysis.

Encapsulation efficiency and drug loading

Microcapsules(100 mg) were crushed in a glass mortar and pestle , and the powdered microcapsules were suspended in 20 ml of phosphate buffer (pH 7.4).After 24 hours ,the solution was filtered and the filtrate was analyzed for drug content using UV visible spectrophotometer(Perkin Elmer) at 227nm .The drug loading and encapsulation efficiency was calculated using the equation Eqs.(1) and (2).

$$\text{Drug Loading} = \frac{\text{Amount of drug in microcapsules}}{\text{Microcapsules sample weight}} \times 100 \quad \text{--- (1)}$$

$$\text{Drug encapsulation efficiency} = \frac{\text{Amount of drug in microcapsule}}{\text{Microcapsules sample weight}} \times 100 \quad \text{--- (2)}$$

FT-IR analysis

Infrared spectra for the drug, ethyl cellulose and prepared microcapsules were obtained after prepartion of KBr pellet, using a Perkin Elmer FTIR spectrophotometer combined to PC with Spectrum 2000 analysis software.

Differential scanning Calorimetry

The thermo gram of gliclazide, ethyl cellulose and gliclazide loaded ethyl cellulose microcapsules were obtained using **Differential Scanning Calorimetry (DSC)** using DSC Q 200 V 23 .10 Build 79 apparatus .The operating temperature used was between 30 °C and 300°C.The temperature gradient was 10 °C per minute.

In- vitro release studies

The *In-vitro* release studies were performed by taking gliclazide loaded ethyl cellulose microcapsules (equivalent to 10mg of gliclazide) were suspended in 50ml of phosphate buffer pH 7.4 in a mechanical shaking bath (100 cycles / min),with temperature adjusted to37^{o} C. At selected time intervals, 2ml of the sample was removed and replaced with same amount of fresh buffer. The sample was then filtered through 0.2 µ membrane and analysed by UV-spectrophotometry at λ_{max} of 227nm.

Results and Discussion

Size and morphological characterization of microcapsules

Fig 2. Shows the morphology of gliclazide(1 part) loaded ethyl cellulose (4 parts)microcapsules. The microcapsules obtained under these conditions were spherical, smooth, free flowing and individually homogeneously distributed without evidence of collapsed spheres and diameter was about 2µm. Smooth surface reveals complete removal of acetone and liquid paraffin from microcapsules. The particle size and distribution of

the microcapsules were measured with a laser diffraction particle size analyzer and found that all microcapsule formulations have particle size smaller than 24µm diameter with narrow size distribution, and the mean particle size of microcapsules ranged from 1 to 3 µm.

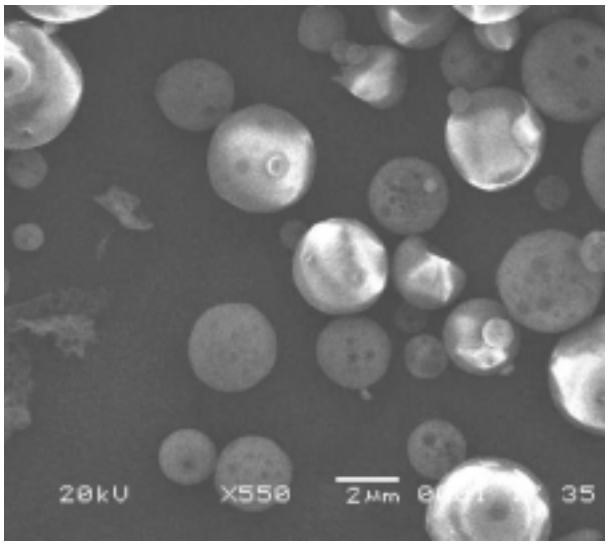

Fig. 2: Scanning electron micrographs of Gliclazide loaded microcapsules

Fourier Transform Infra Red spectroscopy analysis

Fourier transform infra red spectroscopy was used to analyze the interaction between gliclazide and ethyl cellulose (Fig No.3) .The spectra of gliclazide showed peaks of – NH stretching (3272 **cm^{-1}**),= CH stretching (2945 **cm^{-1}**),O=C (1708 **cm^{-1}**),C=C aromatic (1595 **cm^{-1}**),C-H deformation (1435 **cm^{-1}**),SO_2 NH (1348 **cm^{-1}**).Similar peaks were seen in gliclazide – loaded ethyl cellulose microcapsules .This observation further confirmed by DSC studies.

Differential scanning Calorimetry

DSC observation confirmed that there was no interaction between gliclazide and ethyl cellulose polymer .Gliclazide showed endotherm at 100 °C (Fig No.4) ethyl cellulose showed exotherm at 225°C (Fig No.5) and gliclazide loaded ethyl cellulose microcapsules showed both exotherm and endotherm which was identical to the plain drug and ethyl cellulose polymer thermogram (Fig No.6).Hence the compatability of gliclazide in ethyl cellulose microcapsules was evaluated. The DSC study apparently revealed that there was no drug decomposition nor drug- polymer interactions occurred in the freshly prepared microcapsules.

Effect of processing parameters on ethyl cellulose microcapsules preparation

The effect of polymer: drug ratio on encapsulation efficiency and drug loading is shown in Table.1.Gliclazide and ethyl cellulose microcapsules in three different ratios were prepared. From these 4:1 ratio was selected as best ratio because of high drug loading and encapsulation

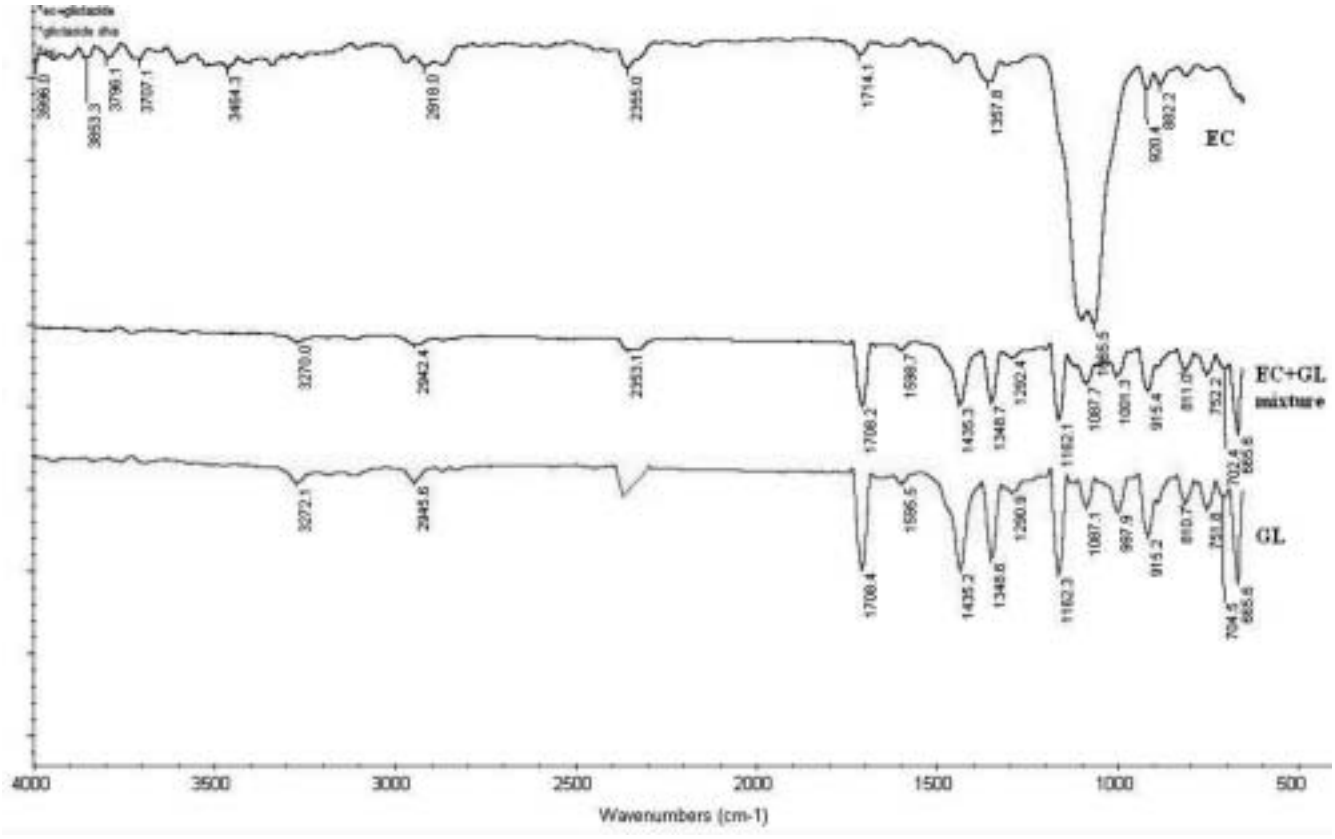

Fig. 3: FT IR Spectra of pure drug localized (GL), polymer Ethyl cellulose (EC) and microcapsule formulation (EC+GL mixture)

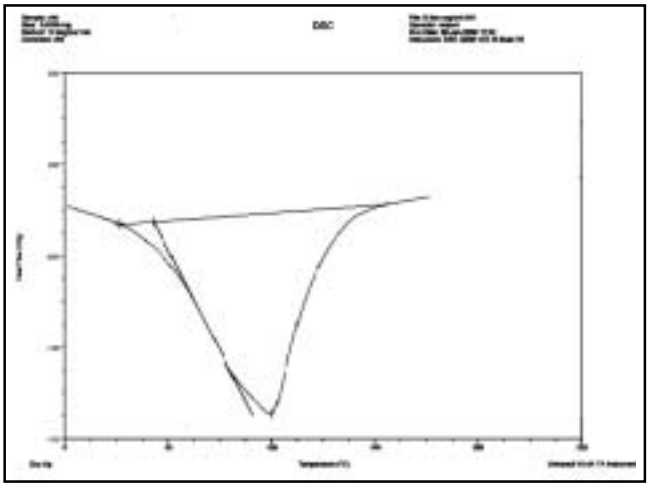

Fig. 4: Thermogram of Gliclazide core material

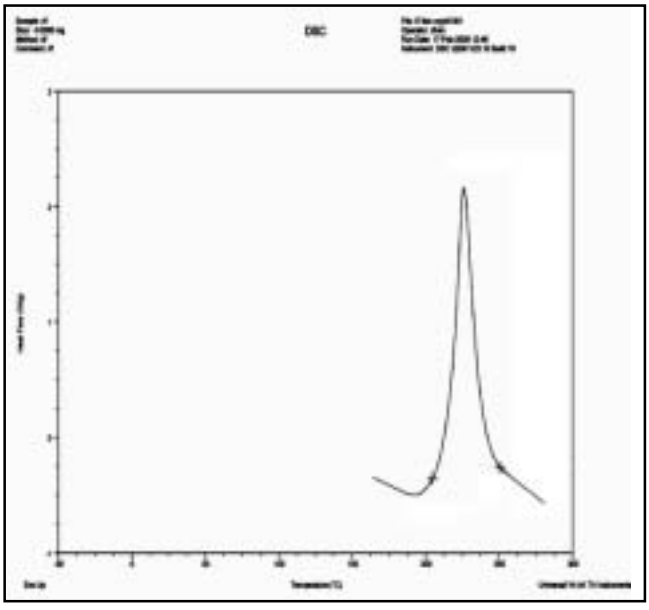

Fig. 5: Thermogram of Ethyl Cellulose polymer

efficiency when compared to other formulation. The drug loading and encapsulation efficiency were mainly affected by the polymer drug ratios. Encapsulation efficiency increases with increase polymer drug ratio. This improved encapsulation efficiency may be due to greater proportion

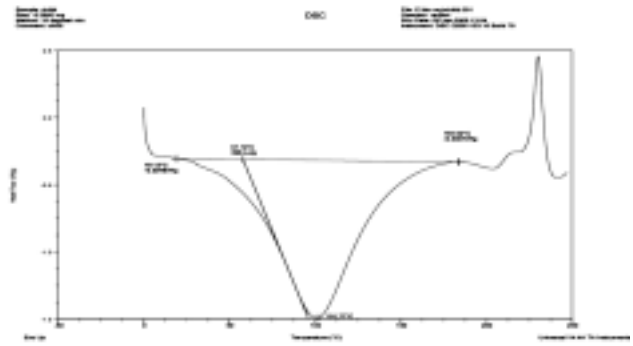

Fig. 6: Thermogram of gliclazide loaded ethyl cellulose microcapsules

of polymer with respect to the amount of drug [16]. Based on above concept 4:1 ratio show high entrapment efficiency and drug loading. The mean particle diameter of 4:1 ratio was approximately 2.23µm.

***In -vitro* release of gliclazide**

In vitro release behavior of gliclazide from the EC microcapsules were studied in Phosphate Buffer Saline pH 7.4. The polymer: drug ratios on release profiles from three batches were investigated and shown in Figure 7. The gliclazide release profiles from the microcapsules were found to occur in a monophasic manner, with gradual release of gliclazide from microcapsules. Gliclazide microcapsules in the ratio of 4:1 released 88 % of the loaded gliclazide in 7 days. The increase in drug content influences the absolute release profiles such that both the cumulative amount of drug released at any time and the induction period increases. This suggests that the level of drug loading was a main factor that controlled the rate of drug release, the microcapsules with a higher loading released gliclazide faster [17-,19]**.**The increase in drug content increased the amount of drug close to the surface which is responsible for an increased initial burst [19-21].This initial burst helps to attain initial therapeutic plasma concentration of drug. Here we found that in the three different formulations (F1, F2, and F3) the release rates vary depending upon the amount of the drug loaded in gliclazide microcapsules (Fig 7), hence these results suggested that drug loading amount should be taken into

Table.1

Properties of the ethyl cellulose-loaded microcapsule prepared by Single emulsion solvent diffusion method (±S.D.,n =3)

Formulation Codes	Polymer Ratio and Drug	Drug Loading (%)	Encapsulation Efficienc (%)	Mean Diameter, (µm)
F1	2:01	13.78(±0.68)	59.87(±1.47)	2.39(±0.12)
F2	3:01	11.98(±0.60)	64.92(±1.69)	2.59(±0.11)
F3	4:01	18.55(±0.54)	71.93(±2.55)	2.23(±0.08)

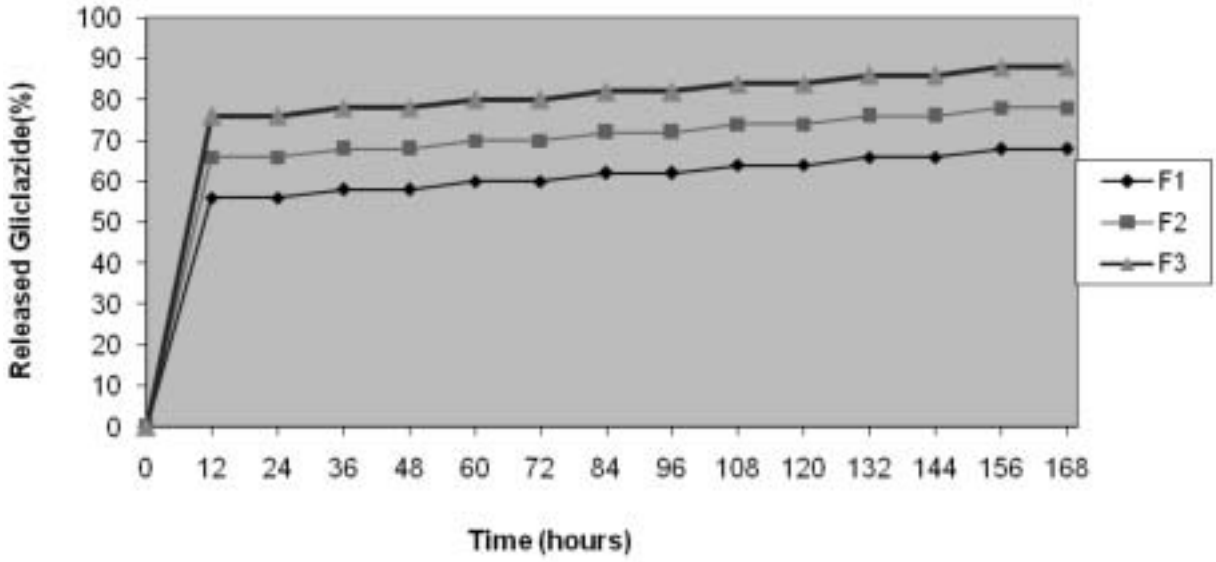

Fig. 7: Release of gliclazide from microcapsules prepared using different ratios of EC: gliclazide in pH 7.4 PBS, 2:1 for F1,3:1 for F2 and 4:1 for F3 (±S.D ., n=3).

consideration when trying to achieve a desired release profile [22]

Conclusion

The gliclazide loaded EC microcapsules were prepared and evaluated. The microcapsules had a spherical, smooth morphology and a diameter of approximately 2μm. More than 70% encapsulation efficiency and a controlled release rate were obtained by varying the process parameters. The gliclazide loaded EC microcapsules had a long release period of about 7 days. The present results suggest that the obtained microcapsules could be useful as a prolonged drug delivery system for diabetic treatment. Accordingly, the next step of this work will be to study the therapeutic effect of these microcapsules in vivo.

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Anti-inflammatory and antipyretic activities of *Smilax perfoliata.* Lour.

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ABSTRACT

The ethanolic extracts of *Smilax perfoliata.* Lour (EESP). (200, 400 and 600 mg/kg orally) were tested in carrageenan-induced rat paw oedema and brewer's yeast-induced pyrexia in rats to assess their anti-inflammatory and antipyretic activities respectively. The paw volumes and pyrexia in rats were reduced significantly ($P < 0.05$) compared to that of control. These results indicate that the extract possess anti-inflammatory and antipyretic properties.

Key words: *Smilax perfoliata,* Anti-inflammatory and Antipyretic activity, EESP.

Introduction

Smilax perfoliata (Smilacaceae) is used in folklore medicine for treating various disorders like venereal diseases, urinary diseases, leprosy, hemiplegia, psychosis, parkinsonism, muscle wasting and rheumatism [1]. The literature survey showed that there is no specific evaluation of this plant for its role in the above mentioned disorders. In the current research, an effort was made to study the antipyretic and anti-inflammatory potential of ethanolic extract of *Smilax perfoliata* by following standard pharmacological screening methods.

Materials and Methods

Plant material

The shrubs of *Smilax perfoliata* Lour (Smilacaceae) were collected from the hill regions of Tirupathi, Chittor District of Andhra Pradesh, India. The plant was authenticated and certified by taxonomist Dr.K.Madhava Chetty, Asst.Prof., Department of Botany, S.V. University and Tirupathi. A.P. The entire plant was collected in the month of August, washed with water several times, shade dried, pulverized by a mechanical grinder and subjected to extraction process.

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Preparation of extract

5000 grams of the powdered drug was accurately weighed and extracted using analytical grade solvents starting with Highly non polar petroleum ether (60 – 80°C) to successively increasing the polarity viz. chloroform, acetone, ethyl acetate and ethanol (95%) following soxhlation method. All the extracts were concentrated by using vacuum dryer until a semisolid extract is obtained, dried at less than 50°C and kept in desiccators for further studies.

Preliminary phytochemical investigation

A preliminary phytochemical investigation [2] was carried out for all the extracts obtained from the *Smilax perfoliata* using analytical grade chemicals, solvents and reagents. The respective yields and the preliminary phytochemical investigation results were given in Table 1.

Animals

Albino rats (Wistar) weighing 150-200g and albino mice weighing 20-25g of either sex were used in this study. They were procured from Sri Venkateshwara Enterprises, Bangalore. The animals were acclimatized for one week under laboratory conditions. They were housed in polypropylene cages and maintained at 27°C ± 2°C under 12 hrs dark / light cycle. They were fed with standard rat feed (Gold Mohr Lipton India Ltd.) and water *ad libitum* was provided. The litter in the cages was renewed thrice a week

to ensure hygeinicity and maximum comfort for animals. Ethical clearance for handling the animals was obtained from the Institutional Animals Ethical Committee prior to the beginning of the project work.

Acute oral toxicity studies

The acute oral toxicity was performed according to OPPTS following up and down procedure. Colony bred female albino rats Wistar strain (150-200gm) were maintained under controlled animal house condition with access to food and water *ad libitum.* The limit test carried out first at 5000mg/kg. b.w. All animals were observed for toxic symptoms and mortality for 72 h.

Anti-inflammatory activity

Acute inflammation was produced by injecting 0.1 ml of (1%) carrageenan into plantar surface of rat hind paw. The test extract (200, 400 and 600 mg/kg orally), and phenylbutazone (100 mg/kg orally) as reference agent were administered 60 min before carrageenan injection. The paw volume was measured at 0, 1, 2, 3 and 4 h, using a thread to determine the diameter of oedema formation size. The difference in diameter between the left and right hind paws was taken as a measure of oedema [3].

Antipyretic activity

Rats were given 20 ml/kg (20%) suspension of brewer's yeast subcutaneously. Initial rectal temperature was recorded. After 18 h animals that showed an increase of 0.3–0.5^{0}C in rectal temperature were selected. The test extract (200, 400 and 600 mg/kg orally) was administered to three groups. Control group received 0.3 ml normal saline. Paracetamol (100 mg/kg orally) was used as reference drug. Rectal temperature was determined by thermal probe Ellab themistor thermometer 1, 2, 3 and 4 h, after test extract/reference drug administration [4].

Statistical analysis

The results and data obtained in this study were evaluated using the one-way analysis of variance (ANOVA) test between two mean groups; control and test groups, followed by Student's *t*-test. Significant levels were at $P < 0.05$

Results and Discussion

Preliminary phytochemical studies and percentage of yield shown in Table No 1. The EESP found to be non toxic up to 5000 mg/kg.

Anti-inflammatory activity

The results of the anti-inflammatory effect of the EESP on carrageenan-induced oedema in rat's right hind paws are presented in Table 2. There was a gradual increase in oedema paw volume of rats in the control (carrageenan treated). However, in the test groups, the extract showed a significant reduction in the oedema paw volume. As indicated in Table 1, a dose-related inhibition of hind paws oedema between 2 and 4 h was observed. Phenylbutazone as reference drug (100 mg/kg orally) produced a significant inhibitory effect comparable to tested extract.

Antipyretic activity

Effect of EESP extract on normal body temperature in rats is presented in Table 2. It was found that the extract at a dose of 200 mg/kg caused significant lowering of body temperature at 4 h following its administration. This effect was maximal at doses of 400 and 600 mg/kg in dose dependent manner and it caused significant lowering of body temperature up to 4 h after its administration. The subcutaneous injection of yeast suspension markedly elevated the rectal temperature after 18 h of administration. Treatment with EESP extract at a dose of 200, 400 and 600 mg/kg

Table 1

Percent yield of extracts and preliminary phytochemical studies

	Inference				
	Pet. ether extract	**Chloroform extract**	**Acetone extract**	**Ethyl acetate extract**	**Ethanolic extract**
% Yield (w/w)	2.7	3.5	4.1	6.3	8.4
Alkaloids	Absent	Absent	Absent	Absent	Absent
Amino acids	Absent	Absent	Absent	Absent	Absent
Flavonoids	Present	Present	Present	Present	Present
Glycosides	Absent	Absent	Present	Absent	Present
Triterpenoids	Absent	Absent	Absent	Absent	Present
Steroids	Absent	Absent	Absent	Absent	Absent
Carbohydrates	Absent	Absent	Present	Absent	Present
Gums	Absent	Absent	Absent	Absent	Present
Tannins	Absent	Absent	Present	Present	Present
Saponins	Absent	Absent	Present	Absent	Present

decreased the rectal temperature of the rats in dose dependent manner (Table 3).

The antipyretic effect started as early as 1 h and the effect was maintained for 4 h, after its administration. Both the standard drug paracetamol 100 mg/kg and tested EESP *extract* significantly reduced the yeast-elevated rectal temperature compared to control group.

Conclusion

Based on the results of the present study it can be concluded that the ethanolic extract of *Smilax perfoliat* has potential dose-dependent anti-inflammatory and antipyretic activity, it may due to active principles present in this extract. The activity is in dose dependent manner.

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Table 1

Anti-inflammatory activity of EESP and phenylbutazone (100 mg/kg) on carrageenan-induced rat paw oedema in the right hind-limb paw of rats

Treatment	Dose (mg/kg)	Time (h)					Average oedema formation
		0	1	2	3	4	
Control (CT)		-	0.47±0.09	0.71±0.01	0.88±0.31	0.72±0.14	0.69
EESP	200	-	0.38±0.22	0.34±0.11*	0.28±0.11*	0.22±0.01*	0.30*
	400	-	0.32±0.16	0.31±0.09*	0.24±0.14*	0.20±0.09*	0.26*
	600	-	0.25±0.09	0.22±0.12*	0.20±0.13*	0.18±0.08*	0.21*
phenylbutazone	100	-	0.20±0.03	0.18±0.08*	0.18±0.12*	0.17±0.01*	0.18*

Values are mean ± S.E.M. (n = 6), *P < 0.05 of the difference between the left and the right hind paws
EESP : Ethanolic extract of *Smilax perfoliata.*

Table 2

Effect of EESP on normal body temperature

Treatment	**Dose(mg/kg)**	**Rectal temperature (C°) before and after treatment**				
		0	**1**	**2**	**3**	**4**
Control (saline)	5ml/kg	37.2±0.2	37.3±0.1	37.2±0.1	37.3±0.1	37.1±0.1
EESP	200	37.1±0.1	36.6±0.1*	36.6±0.1*	36.6±0.2*	36.4±0.1*
	400	37.2±0.3	36.4±0.1*	36.4±0.1*	36.2±0.1*	36.2±0.1*
	600	37.4±0.1	36.9±0.1*	36.2±0.1*	36.2±0.1*	36.0±0.1*

Values are Mean ± S.E.M. (n = 6), *P < 0.05 compared with control values
EESP : Ethanolic extract of *Smilax perfoliata.*

Table 3

Antipyretic activity of EESP and paracetamol (100 mg/kg) on Brewer's yeast-induced pyrexia in rats

		Rectal temperature (C°) before and after treatment					
Treatment	**(Dose**	**Before treatment**			**After treatment**		
	mg/kg)	**0h**	**18h**	**1h**	**2h**	**3h**	**4h**
Control (saline)	5 ml/kg	37.6±0.2	39.2±0.1	39.2±0.1	39.2±0.1	39.2±0.1	39.2±0.1
EESP	200	37.5±0.1	39.9±0.1	38.6±0.1*	38.6±0.2*	38.2±0.1*	38.0±0.1*
400	37.4±0.3	39.8±0.1	38.5±0.1*	38.4±0.1*	38.0±0.2*	37.6±0.1*	
600	37.4±0.1	39.9±0.1	38.6±0.2*	38.2±0.1*	37.9±0.1*	37.4±0.1*	
Paracetamol	100	37.8±0.2	39.5±0.1	38.3±0.1*	38.3±0.1*	37.9 ±0.1*	37.3±0.1*

Values are mean ± S.E.M. (n = 6), *P < 0.05
EESP : Ethanolic extract of *Smilax perfoliata.*

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