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VIEWS

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Differential Effects of Various Carbon Sources, Nitrogen Sources, pH and Temperature on Growth Rate of Baker's Yeast

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ABSTRACT

Relative effects of Various carbon sources, Nitrogen Sources, various P^H values and wide Temperature Range on growth and Biomass yield of Baker's Yeast in Shake flask cultures was determined in vitro. In YPD fermentation media Dextrose is replaced with 4 other Carbon sources like Lactose, Maltose, Fructose and Sucrose. Similarly, Peptone is replaced with Urea, Ammonium Sulphate and Ammonium Orthosulphate. Meanwhile, the effects of these said Nutritional factors are studied in combination with environmental factors like P^H and Temperature to analyze their relative effect on Yeast Biomass Yield. Wide range of P^H and Temperature were kept as constant variables in YPD fermentation media. The effect of PH at 5.5, 6.0, 6.5, 7.0 and 7.5 was analyzed. Similarly effect of Temperature at 25^0 C, 30^0 C, 35^0 C and 40^0 C. The findings showed that out of these five carbon sources Maltose has a great influence on Baker's Yeast growth, a dry weight of 1.01g/100ml broth was obtained at P^H 5.5 and Temperature 30^0 C. Similarly Dextrose is found to be the second most favored carbon source in YPD media. With Dextrose a dry weight of 0.97g/100ml broth was recorded in P^H 5.5 at 30^0C. Mean While, Sucrose has supported for a moderate yeast growth which has given a dry weight of 0.81g/100ml broth at 35^0C in 5.5 P^H. But this growth rate is less compared to the growth rate given by Fructose and Lactose. With Lactose and Fructose, growth rate is too less and it is negligible. Similarly, among 4 Nitrogen sources used namely Peptone, Urea, Ammonium Sulphate, and Ammonium Orthosulphate, Peptone in combination with Maltose can be considered as the best Nitrogen source since a highest dry weight of 1.10g/100ml broth is obtained in P^H 5.5 at 30^0C.

Key Words: Baker's yeast, Maltose, Lactose, Sucrose, Fructose, Dextrose, Urea, Peptone, ammonium sulphate, ammonium ortho sulphate, pH and Temperature, Biomass Productivity.

Introduction

Baker's Yeast perhaps the most useful Yeast having been Instrumental to Baking and Brewing since ancient times. Baker's Yeast is the common name for the strains commonly used as the leavening agent in baking bread and bakery products. Baker's Yeast is the species of *Sacchoromyces cerevisiae* which is same species commonly used in alcoholic fermentations and is also called brewer's Yeast. Among several Yeasts *Sacchoromyces cerevisiae* is the most important species present during fermentation process and baking process. Many nutritional and environmental factors affect the course of fermentation [1,2,3]. Since growth behavior varies with variable factors. Normally yeast can grow either aerobically or anaerobically. Under aerobic conditions they can grow by oxidizing simple carbon sources such as Ethanol, Acetate or Glycerol [4, 7]. If they have adequate oxygen they will completely oxidize carbon sources usually sugars to carbon dioxide and water[4, 5]. However under Anaerobic conditions, deprived of oxygen Yeast can convert sugars only to carbon dioxide and ethanol, recovering less of energy[4,5]. In either case growth will be limited by essential nutrients or accumulation of toxins. All Yeast strains can use Ammonia and Urea as the sole nitrogen source but cannot use Nitrate, since they lack the ablity to reduce them to Ammonium ions [7]. They can also use most amino acids, Small peptides, Nitrogenous bases as Nitrogen sources [6, 8, 9]. Histidine, Glycine, Cystine and Lysine are however not readily used [6,8,9] . Yeast also have requirement for phosphorous which is assimilated as a dihydrogen phosphate ion and Sulphur which can be assimilated as a sulphate ion or as organic sulphur compound such as the Amino Acids Methionine and Cystine [2, 10,11]. Some metals like

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magnesium, iron, calcium, Zinc are also required for good growth of yeast [12]. Other significant variables or environmental parameters are P^H and Temperature. Both temperature and P^H are interdependent environmental factors, which together affect the growth and biomass yield of Yeasts [13, 14]. Several studies have shown that yeasts thrive well when the P^H is slightly acidic [7,8.12.15]. However it grows over a wide range even when P^H is neutral or even slightly alkaline. If the P^H is too acidic or too basic the yeast just won't grow [15, 16]. A neutral to slightly acidic P^H will give fastest rate of fermentation [7,8,9]. Foods such as bakery products have P^H values that range from 3 to 5, therefore seemed important to focus on the PH effects. Usually, Yeasts are mesophilic organisms which favor a moderate temperature between 30^0C to 39^0C.

Materials and Methods

Yeast cultures

Yeast used for investigation was a Prosper brand of commercial Baker's yeast supplied by Genie. Yeasts used were immobilized bakers Yeast Granules. Through out the work same brand of Yeast granules were used. Baker's Yeast is the common name for the strain Sacchoromyces cerevisiae commonly used for baking bread and bakery products.

Inoculum Preparation

100 grams Yeast granules were suspended in 600ml in Luke warm water and the slurry was shaken for 90mins at 34^0 C on a shekar or magnetic shaker the suspension was subjected to centrifugation at 5000 rpm for 20 minutes. The supernatant which has yeast cells was transferred to conical flasks aseptically. 10 ml of this inoculum is inoculated into 100 ml YPD Broth and incubated for 12 hrs at 30^0 C in an orbital shaker incubator at 180 rpm. This is performed to make cells activated.

Chemicals and Media

All chemicals were obtained from Sigma Aldrich unless otherwise stated. Micro Biological products were purchased from Genie. Complex YPD medium contained "Glucose, 20 g/ L; Yeast extract 10g/L; Bacto peptone, 20 g/L; Autoclaved for 30 minutes at 110^0c. Fructose Maltose Sucrose, Lactose, Urea, Ammonium sulphate, Ammonium ortho sulphate, Phosphate buffer chemicals - Monosodium phosphate, disodium phosphate, phosphoric acid, sodium hydroxide, were of good quality and supplied by Sigma Aldrich. Five sets YPD broth was prepared in which YPD with dextrose as the carbon source was standard media remaining four sets contained fructose , lactose, maltose, Sucrose in each set. In the second stage four sets of YPD was prepared where one set with peptone as nitrogen source remaining three sets were made with urea ammonium sulphate, ammonium OrthoSulphate respectively. With all these Nitrogen sources Maltose and Sucrose is the Carbon Sources. PH Was maintained using Phosphate buffer.

Shake Flask Cultivation

Batch fermentations were carried out in 250 ml Erlenmeyer's flasks with 50 ml sterile medium. Flasks were inoculated with 10% activated cultures of Baker's Yeast, to all the 5 sets with altered sugar sources and four sets of altered nitrogen sources. Broth cultures were incubated at 25^0C, 23^0C, 35^0C, 40^0C respectively and P^H was adjusted at 5.5, 6.0, 6.5, 7.0, 7.5 in an orbital shaking incubator at 180 rpm. Optical density was read at 550 nm for every 24 hrs to analyze growth response.

Biomass Determination

Wet Mass of the yeast were determined Spectrophotometrically taking the absorbance at 550nm at every 24 hrs time interval and dry weight is recorded after 96 hrs of incubation by allowing biomass on Watsmann filter paper at 30 C for 36hrs. Dry Weight of each experimental set was recorded and compared with each other to analyze relative effect of P^H, Temperature , Carbon source and Nitrogen source on growth of Baker's Yeast.

RESULTS

The *Saccharomyces Cerevisiae* is normally grown in YPD media where Dextrose and peptone are the carbon and Nitrogen sources respectively. These sources are replaced further with various other carbon and Nitrogen sources for obtaining better or higher yield of yeast Biomass. The sugars that replace Dextrose are Sucrose, Maltose, Lactose and Fructose. Similarly, Nitrogen source peptone is replaced by Urea, Ammonium sulphate and Ammonium orthosulphate. Meanwhile, the effect of altered pH and Temperature effects is also analysed. P^H range opted was 5.5, 6.0, 6.5, 7.0 and 7.5. Similarly wide range of temperature employed was 25^oC, 30^oC, 35^oC and 40^oC. The relative effects of these factors on the growth and Biomass productivity was assessed.

Effect of Carbon Source

5 sets of YPD Fermentation media was prepared, in duplicates. For each of the set Dextrose, Fructose, Sucrose, Maltose, and Lactose was carbon sources. These altered Carbon Source altered YPD broths were incubated at 25^oC, 30^oC, 35^oC and 40^oC in P^H 5.5, 6.0, 6.5, 7.0, 7.5. The Differential effecs of Maltose, Sucrose, Dextrose, Lactose, Fructose with pH and Temperature are compared in the Table 1.1 to Table 1.4.

Table-1.1
Combined effect of Carbon Sources and Various P^H values at 25^0C

Sl. No.	Carbon Source	pH5.5	pH6.0	pH7.5	pH7.0	pH7.5
1	Maltose	0.8	0.72	0.71	0.68	0.65
2	Sucrose	0.72	0.7	0.66	0.63	0.6
3	Doctrose	0.65	0.64	0.6	0.57	0.55
4	Fructose	0.58	0.55	0.5	0.41	0.4
5	Lactose	0.43	0.41	0.4	0.36	0.33

Table-1.2
Combined effect of Carbon Sources and Various P^H values at 30^0C

Sl. No.	Carbon Source	pH5.5	pH6.0	pH7.5	pH7.0	pH7.5
1	Maltose	1.01	0.9	0.79	0.7	0.62
2	Sucrose	0.58	0.51	0.43	0.41	0.32
3	Doctrose	0.97	0.46	0.41	0.37	0.31
4	Fructose	0.4	0.35	0.33	0.31	0.28
5	Lactose	0.32	0.31	0.31	0.27	0.22

Table-1.3
Combined effect of Carbon Sources and Various P^H values at 35^0C

Sl. No.	Carbon Source	pH5.5	pH6.0	pH7.5	pH7.0	pH7.5
1	Maltose	0.91	0.86	0.84	0.79	0.75
2	Sucrose	0.89	0.89	0.91	0.88	0.8
3	Doctrose	0.81	0.78	0.76	0.75	0.71
4	Fructose	0.51	0.47	0.44	0.4	0.38
5	Lactose	0.39	0.33	0.31	0.28	0.27

Table 1.4
Combined effect of Carbon Sources and Various P^H values at 40 ^{0}C

Sl. No.	Carbon Source	pH5.5	pH6.0	pH7.5	pH7.0	pH7.5
1	Maltose	0.61	0.53	0.53	0.51	0.49
2	Sucrose	0.68	0.61	0.6	0.58	0.55
3	Doctrose	0.65	0.63	0.61	0.6	0.6
4	Fructose	0.55	0.5	0.51	0.48	0.47
5	Lactose	0.4	0.41	0.4	0.38	0.33

The combined effect of these sugars as below :-

Maltose:- The usual YPD media contain Dextose as the carbon source. Here Dextrose is eplaced with Maltose. A maximu dry weight of 1.01 g/100ml broth was observed at 30 ^{o}C with P^H 5.5.

Dextrose:- With Dextose in YPD media, it was observed that Dextrose is found to be the 2nd most favoured carbon source. A dry weight of 0.97 g/100ml broth was obtained at 30 ^{o}C at pH 5.5

Sucrose:- Here Sucrose is subsitituted for Dextrose in YPD media is used . A dry weight of 0.89 g/100 ml Broth was obtained at 35^{o}C in P^H 5.5.

Fructose:- Fructose is substituted for Dextrose in YPD at all said P^H and temperature experiments were carried out. A dry weight of 0.58 g/100 ml broth was observed at 25 °C in P^H 5.5 at other temperature and P^H values, dry weight is still less.

Lactose:- Dextrose is replaced with Lactose. A dry weight of 0.41 g/ 100ml broth was recorded at 40 °C in pH 6.0. Previous investigations have shown that Lactose is not Fermented by Baker's yeast.

Based on the experimental results, it is found that Maltose and sucrose are able to support for higher growth rate and Biomass productivity of yeasts.

In the next step of our experiments, Maltose and sucrose were choosen as best carbon sources, P^H 5.5 and 6.0 as optimum P^H and 30 °C and 35 °C as the optimum temperature values. These 3 parameteres, were later experimented with 3 various altered Nitrogen sources, such as Urea, Ammonium sulphate and Ammonium orthosulphate. The Repeated experimental rials with altered Nitrogen sources, revealed following facts.

Effect of Nitogen Sources

Urea :- Urea is substituted for peptone in YPD. In which one set has Maltose and other set has Dextrose. A dry weight of 1.10 g/100 ml broth was recorded wih urea and sucrose combination at 30 °C in P^H 5.5. YPD with Urea and Maltose combination a dry weight 1.02 g/100ml broth was observed at 30 °C in P^H 5.5. This is the highest growth value obtained.

Peptone :- Peptone is the most common Nitrogen source used in most of the microbial growth media. Standard YPD too has peptone. This standard YPD in combination with Maltose has given a Maximum growth rate of Baker's Yeasts, a dry weight of 0.72 g/100 ml broth at 30 °C in P^H 5.5. YPD in combination with sucrose has given a dry weight of 0.80g/100 ml broth at 30 °C at P^H 6.0

Ammonium Sulphate :- Ammonium Sulphate is substituted to peptone in YPD. In which Dextose is also replaced with one set of Maltose and sucrose each. A dry weight of 0.76 g/100 ml broth was recorded at 35°C in P^H 5.5 with ammonium sulphate and sucrose combination. Similarly, a dry weight of 0.66 g/100 ml broth was obtained for Ammonium sulphate and Maltose combination t 35 °C in P^H 5.5.

Ammonium OrthoSulphate :- A Dry weight of 0.71 g/ 100 ml broth at 35°C in P^H 5.5 was observed with Ammonium ortho Sulphate and sucrose combination. Maltose and Ammonium orthosulphate combination has given a dry weight of 0.65 g/100 ml broth at 30 °C at P^H 5.5.

The Differential effecs of Nitrogen sources such as Peptone, Urea, Ammonium sulphate, and Ammonium orthosulphate with Maltose, Sucrose, and P^H , Temperature effects are compared in Table 2.1 and Table 2.2

Table-2.1

Effect of Various Nitrogen Sources in combination with Maltose in P^H 5.5 at 30^{0}C on Biomass productivity of Baker's Yeast

Sl. No.	Nitrogen Source	Dry Wt g/100ml
1	Peptone	0.72
2	Urea	1.1
3	Ammonim Sulphate	0.66
4	Amm Orthosul	0.65

Table-2.2

Effect of Various Nitrogen Sources in combination with Sucrose in P^H 5.5 at 30^{0}C on Biomass productivity of Baker's Yeast

Sl. No.	Nitrogen Source	Dry Wt g/100ml
1	Peptone	0.8
2	Urea	1.02
3	ammonium sulphate	0.76
4	amm ortho	0.71

Discussion

This study clearly demonstrated that carbon source, Nitogen source, temperature and pH has a great influence on yeast growth and Biomass productivity.

Relative effect of Carbon Source

Dextrose is a Monosaccharide sugar. Maltose, Fructose, Sucrose and Lactose are Disaccharides. According to the study conducted, Maltose, which is a Disaccharide formed from 2 units of glucose joined with an α (1 – 4) bond, is the most favoured carbon source for yeast fermentation activity. Yeast cells fermented Maltose at a higher rate and grow profusely with Maltose as carbon source. Yeasts have zymogen or Maltase which is able to hydrolyse Maltose. Yeasts favour Maltose for carbon source requirement and utilized Maltose at a higher rate. A dry weight of 1.01 g/100 ml broth was recorded at 30°C in P^H 5.5. This is the highest value of Dry weight recorded when compared to Sucrose, Dextorose, Lactose and Fructose. The failure of Yest cells to Ferment Maltose above P^H 6.0 is a valid evidence that, Maltose is not actively Functioning at P^H 6.0, 6.5, 7.0 and 7.5. Maltose is vigorously Fermented by living Baker' yeast cells at acid P^H Values but was not Fermentd at all by same cells at P^H near Neutrality and at neutral P^H.

Dextrose which is alo a Monosaccharide is found to be the second most favoured carbon source for yeasts. Yeasts have Invertase which hydrolyse and make utlize of sucrose in the growth media. A Biomass dry weight of 0.97

g/100 ml broth at 30°C in P^H 5.5 was recorded. This value is the 2nd highest growth value in the experiments conducted.

In terms of Dry weight value Dextrose is later followed by Sucrose, Fructose and lastly by Lactose. With Lactose growth rate is very poor because Baker's yeast lack the the enzyme that can hydrolyse lactose to Glactose and Glucose [14,15,17]. Hence Baker's yeast cannot utlize Lactose as carbon source. And can utilize Maltose and Dextrose.

Relative Effect of Nitrogen Source

Peptone is the most common Nitrogn source in most of the Microbial growth media. To study the effect of ther Nitrogen sources on Yeast growth, peptone was replaced by Urea, Ammonium sulphate, Ammonium orthosulphate. The experimental studies revealed that Urea is the most favoured and best suited Nitrogen source when compared to Peptone, Ammonium sulphate, and Ammonium ortho sulphate. The Relative importance of Maltose and Dextrose with peptone is obviously dependent on P^H and Temperature. A maximum dry weight of 1.02 g/ 100 ml broth was recorded in Urea and Dextrose combination at 30°C din P^H 5.5 .With Maltose Urea combination, a dry weight of 1.10 g/100 ml broth was obtained.

With Peptone growth Rate is moderate. Ammonium sulphate, and Ammonium ortho sulphate in combination with Maltose and Sucrose growth rate is too Poor.

P^H and Temperature

At various P^H values 5.5, 6.0, 6.5, 7.0 and 7.5, growth rate was observed. p^H 5.5 which is slightly aidic is found to be the optimum P^H values for yeasts growth and Biomass productivity. At P^H valus 6.5, 7.0, 7.5 the growth was declined with all the carbon and Nitrogen source combinations.

Mean while, resuts reveeled that, Yeasts Favour Mesophilic Temperature. Maximum growth values were obtained at 30 ^{0} C. At 25^{0}C, 35^0 C and 40^{0}C a reduction in growth value was observed. All the combinations of Experimental trials supported this mesophillic concept.

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Acute Toxicity Study of Poly Herbal Formulation in Albino Mice

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ABSTRACT

Herbal formulations plays an important role in the treatment of various disorders. All most all herbs are considered to be safe, because of which toxicity studies were not performed routinely, but standardization and evaluation of toxicity profile of herbal formulation is very much important in order to determine the safety of respective formulation. Hence the present study focuses in assessing acute toxicity studies of a polyherbal formulation comprises of *Gymnema sylvestris*, *Curcuma longa*, *Trigonella foenum graceum, Emblica officinalis* and *Cuminum cyminum*. The results of the study weren't exhibit any abnormalities in biochemical profile of albino mice.

Key Words: Albinomice, Polyherbal formulation, Acute toxicity

Introduction

Herbal plants play an important role in the treatment of various disorders. Medicinal plants have occupied a vital place in the socio cultural, development of rural people of India[1]. Recently researchers are focusing much in formulation and standardization of polyherbal formulations along with evaluation of their pharmacological actions. Hence the present study focuses in assessing acute toxicity studies of a polyherbal formulation claimed to be useful in treatment of metabolic syndrome. The poly herbal formulation comprises of *Gymnema sylvestris*, *Curcuma longa*, *Trigonella foenum graceum* , *Emblica officinalis* and *Cuminum cyminum*.

Material and Methods

Polyherbal formulation

- Cuminum cyminum 150mg.
- Curcuma longa 200mg.
- Emblica officinalis 300mg..
- Gymnema sylvestrae 300m.
- Trigonella foenum-graceum 50mg.

The above plant materials were procured from Sri Srinivasa Ayurveda Pharmacy, T.T.D. Srinarasingapuram, shade dried, coarsely powdered thoroughly mixed to obtain PHF.

Experimental animals

The albino mice weighing 30 to 60 g of either sex were procured from Sri Raghavendra enterprises, Bangalore and they were fed with standard palatable laboratory diet and ordinary tap water. Prior to start the study, albino mice of either sex were housed at four animals per cage. They were acclimatized for one week and were provided with food and water *ad-libitum*. After a week time, mice were housed one per cage for 5 days for food training (i.e. they were provided food only for 6hrs / day) and average food intake for three days was measured in all animals, before starting the experiment. All experimental protocols were approved by I.A.E.C of Sri Padmavathi school of pharmacy, Tiruchanoor, Tirupati.

Toxicity Studies

Mice were then randomly divided into 4 groups comprising of six mice in each group. First group received plain water only and was used as control. Group 2, 3and 4 were treated orally with 1gm, 3gm and 10gm per kg of the body weight (single dose) of aqueous extract of polyherbal formulation, thus making the exposed doses of the extract in the animals were 10, 30 and 100 times the therapeutic dose of the drug respectively. The following parameters were carried out immediately after administrating the drug that is at 0hr, followed by at 1hr, 2hrs, 4hrs, 6hrs, 8hrs, 24hrs and 72hrs[2].

Parameters under Observation

Mortality of animals, motor activity, tremors, convulsions, posture, spasticity, opisthotonicity, ataxia, righting reflex, sensations, pilo-erection, ptosis, lacrimation, exopthalmos, salivation, diarrhea, writhing, change in skin color and respiratory rate were monitoreda as mentioned above.

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Daily food intake and weight of the animals were also recorded. Blood was drawn from each of the animal by cardiac puncture, three days (72hrs) after oral administration of the drug to determine hemoglobin, R.B.C. count, W.B.C. count, blood urea, blood glucose, serum creatinin, serum cholesterol and S.G.P.T. blood chemistry was carried out in the Pharmacology Laboratory using semi auto analyser. Then the animals were sacrificed immediately after cardiac puncture. Liver and the Kidney dissected out and their gross and histo-pathological examinations were done[3,4].

Statistical analysis

Data was collected and was analyzed by ANOVA using computer based fitting programme (prism graph pad) and statistical significance set at $p<0.05$.

Results and Discussion

Experimental screening method is imperative in order to establish the safety and efficacy of traditional and herbal products and also to set up the active components of the herbal products[6].

Results obtained during various experiments are summarized in table-1. In the present study, mortality of mice was not observed upto 10g/ kg (100 times the normal therapeutic dose) dose of polyherbal formulation.

No significant clinical findings were noted in any of the group. Body weight did not change significantly in any treated group IV, day 2nd as compared to control group. Food intake was significantly lowered on day 2 and 3 in group –IV.

Table-1

Effect of aqueous extract of Polyherbal formulation on biochemistry of albino mice.

Sr. No.	Animal groups	Group-I Control (n=6)	Group-II Treated(10x) (1gm/kg) (n=6)	Group-III Treated(30x) (3gm/kg) (n=6)	Group-IV Treated(100x) (10gm/kg) (n=6)
	Tests	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
1	**Clinical observations**	**Nil**	**Nil**	**Nil**	**Nil**
2	**Body weight(g)**				
a)	Pre-treatment	40.16±4.2	31.16±1.75	44.5±1.92	31.33±1.52
b)	After 24 hrs.	40±4.13	31.33±1.68	44.33±1.87	30.5±1.66
c)	After 48 hrs.	40.33±4.2	30.33±1.66	44.16±1.97	29.66±1.49*
d)	After 72 hrs.	40.16±4.15	30.33±1.42	43.66±1.94	30±1.52
3	**Food intake(g)**				
a)	Pre-treatment (avg.)	7.66±0.86	6.77±0.43	6.02±0.39	7.44±0.42
b)	Day-1	6.66±1.02	3.33±0.88*	5.83±0.83	3.16±1.16*
c)	Day-2	7.66±0.84	5.50±0.76	6.66±0.98	4.66±0.88*
d)	Day-3	7.8±0.83	5.50±0.76	5.83±1.22	5.55±0.56*
4	**Hematology**				
a)	Hb.(g/dl)	13.66±0.2	12.83±0.26*	13.23±0.14	13.66±0.15
b)	Total W.B.C.(cells per cc)	8166.5±300	7600±850	7150±584	6266±436***
5	**Blood chemistry**				
a)	S. Cholesterol (mg/dl) Normal (150-220)	155.6±5.47	142.8±8.3	153±2.86	156±2.75
b)	S. Creatinine (mg/dl) Normal (0.4-1.2)	0.7±0.06	0.93±0.09	0.68±0.07	0.6±0.08
c)	Blood urea (mg/dl) Normal (20-40)	28.8±3.14	36.66±3.12	28.83±2.63	27.66±1.56
d)	B.G (mg/dl) Normal (upto 140)	77.5±2.71	60.4±3.04****	59.33±3.28	52.66±5.8
6	**Liver Functions**				
a)	S.G.P.T. (U/L) Normal (40)	25.83±2.79	62±10.20***	41.83±4.77**	31.5±3.47

*(*p < 0.05 ; **p < 0.02 ; ***p < 0.01 ; ****p < 0.001) as compared to control.*

Although Hb concentration turned to be significantly different in group II (10x), no other group showed any changes, and even in group II, the difference dose not has any clinical significance. Total W.B.C. (per cmm) showed statistically significant difference in group IV as compared to control group.

Serum cholesterol remained unchanged, while Blood glucose (B.G) was significantly lower in group II. There was no effect on B.G in group treated with higher doses(30x and 100x)[6].

Serum creatinine and blood urea remained unchanged during acute toxicity study. However, on histo-pathological examination, seven out of total eighteen animals had developed hyaline degenerative changes as compare to none in the control group.

Liver function was not significantly altered and S.G.P.T. showed significant rise as compared to normal (control). Histo-pathological examination further established possible acute hepato- toxicity. 12 out of 18 animals developed fatty changes to a greater or lesser extent.

Present study thus, led us to conclude that aqueous extract of polyherbal formulation did not have any lethal effect up to 100 times of the therapeutic dose in albino mice. Although not dose dependent, this poly herbal formulation did not produced any significant abnormality in liver and kidneys during acute toxicity studies.This study has attested the safety of the present poly herbal formulation and further studies to establish pharmacological activities might be fruitful.

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Synthesis and Evaluation of Antibacterial Activity of Substituted Fluoro Cinnolino Pyrimidines

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ABSTRACT

In view of various biological activities of cinnoline derivatives such as antibacterial, antioxidant, antineoplastic etc. it was our interest to prepare substituted fluoro cinnolino pyrimidines. Diazonium salt of 4-fluoro-3-chloroaniline was treated with ethylcyanoacetamide and the resulting aryl-hydrazine(cyano)acetamide formed was then reacted with chlorobenzene in the presence of anhydrous aluminiumchloride to give 4-amino-3-cinnolinocarboxamide which was further refluxed to give fluoro cinnolino pyrimidine. Continued thereafter the title compounds were prepared using different phenols.

Keywords: diazonium salt, chlorobenzene, fluorocinnolinopyrimidine

Introduction

Pyrimidines have a unique place and have contributed significantly to biological and medicinal fields [1]. Pyrimidine ring system containing substituted 6-membered ring exhibited anti cancer and herbicidal activities [2, 3]. In recent years pyrimidine derivatives have received significant attention owing to their diverse range of biological properties particularly as antitubercular [4] and calcium channel blockers [5]. The close association of pyrimidine nucleus with biologically important compounds like nucleic acids, vitamins, co-enzymes and a wide variety of drugs has drawn the attention of a number of workers. Condensed pyrimidine systems have been extensively investigated [6-13]. But there are only few reports related to condensed system such as cinnolino-pyrimidine in which cinnoline heterocycle is fused to pyrimiding ring.

In view of the above observations that pharmacological activity is invariably associated with a large variety of heterocyclic compounds, the present investigation of some heterocycles belonging to cinnolinopyrimidine class of compounds was undertaken and evaluated for their antibacterial activity.

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Experimental

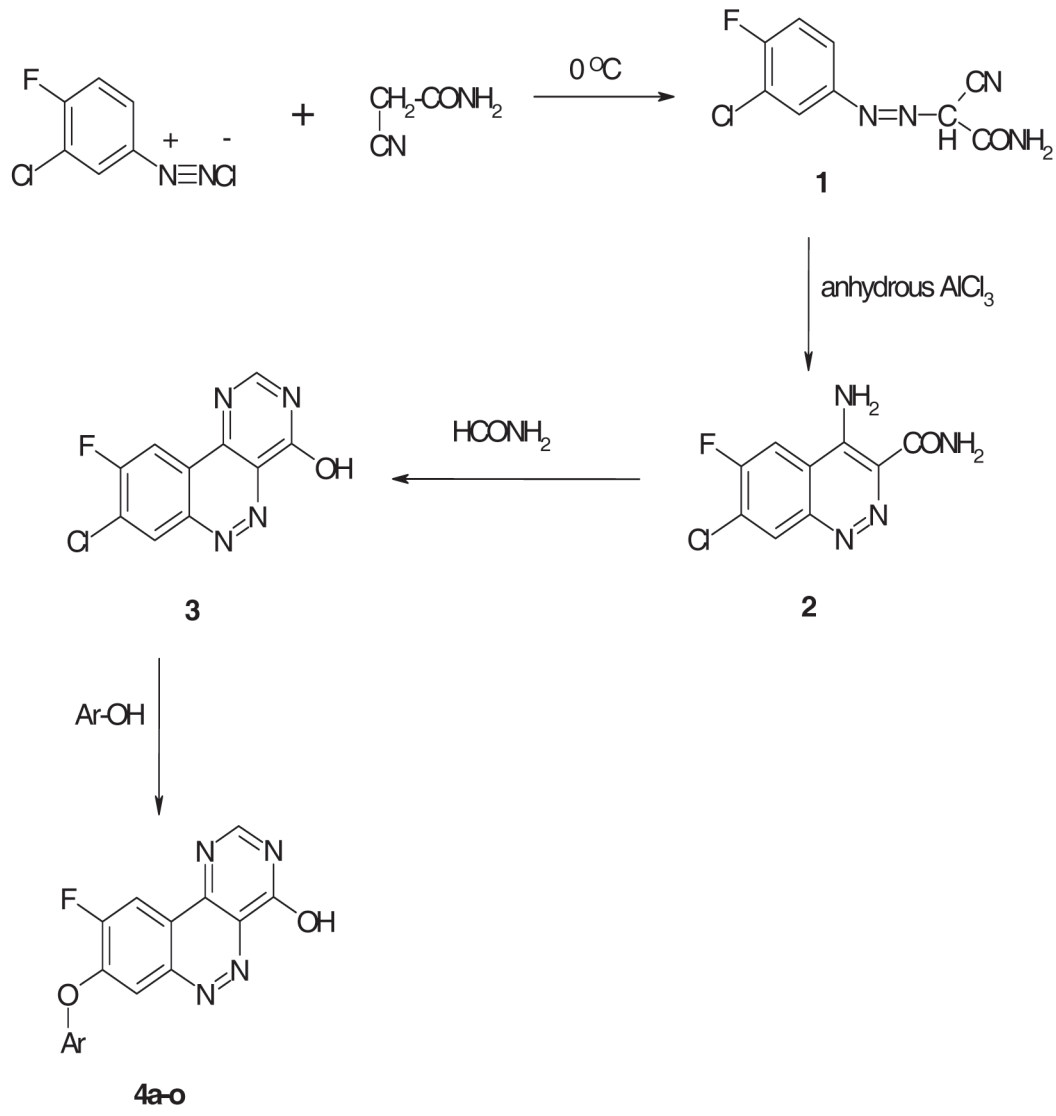
All the melting points have been determined in an open capillary and are uncorrected. IR Spectra (in KBr) were recorded on a Shimadzu FT-IR 8400S instrument and ^{1}H NMR spectra on Bruker Spectrospin- 200 spectrometer in DMSO-d_6 using TMS as internal standard. Elemental analysis (C, H, N) of the newly synthesized compounds were carried out on Perkin-Elmer 240 analyzer and satisfactory.

Synthesis of substituted aryl-hydrazino(cyano)acetamide (1)

The diazonium salt solution was prepared by adding drop wise a solution of 1.4 gm (0.02 mol) of $NaNO_2$ in 20 ml of water to a suspension of 2.9 gm (0.02 mol) of chloro-fluoro-aniline in 100 ml of 1N hydrochloric acid. The reaction mixture was stirred for 1 hour at 0-5^{o}C and filtered.

The filtered diazonium salt solution was added at 0^{o}C to a well stirred mixture of 0.02 mol of cyanoacetamide, ethanol (30 ml) and water (400 ml). Sodium acetate was added in small portions to keep the mixture alkaline. After 3 hours of stirring at 0^{o}C, the crude precipitate of azo compound was collected washed thoroughly with water, air dried and recrystalized from ethanol. IR: 3500 cm^{-1} and 3315 cm^{-1} N-H str; 3035 cm^{-1} C-H str. Aromatic; 2250 cm^{-1} CN str; 1650 cm^{-1} C=O str. Amide; 1585 cm^{-1} N=N

Scheme of Synthesis:



vib; 1200 cm^{-1} C-F str.; 750 cm^{-1} C-Cl str. ^{1}H NMR: d 9.1-9.3 (2H, s, $CONH_2$), 8.1-8.2 (3H, m, Ar-H); 3.2 (1H ,s,-CH).

Synthesis of substituted 4-amino3-cinnolinocarboxamide (2)

A suspension of substituted arylhydrazino (cyano) acetamide (45 m mol) and anhydrous aluminium chloride (180 m mol) in dry chlorobenzene (100 ml) was heated at 100^{0}C under vigorous stirring for 2 hours. After cooling 2 M hydrochloric acid (100 ml) was added slowly to the homogenous reaction mixture which was then heated at 90^{0}C for further 1.5 hours. The precipitate was filtered off, washed with ethanol and recrystallized from dimethylformamide. IR: 3395 cm^{-1} and 3280 cm^{-1} N-H str; 3040 cm^{-1} C-H str aromatic; 1645 cm^{-1} C=O str amide; 1210 cm^{-1} C-F str; 750 cm^{-1} C-Cl str; ^{1}H NMR d 9.0-9.2 (2H, s, $CONH_2$); 8.2-8.3 (2H, m, Ar-H); 4.2-4.3 (2H, s, -NH_2).

Synthesis of substituted pyrimido(5,4-c)cinnoline (3)

A mixture of substituted-4-aminocinnoline-3-carboxamide (5 mmol) was refluxed for 2 hours in formamide (15 ml). The separated solid was filtered after cooling. The crude product was recrystallized from dimethylformamide. IR: 3512 cm^{-1} O-H str (phenolic); 3042 cm^{-1} C-H str aromatic; 1215 cm^{-1} C-F str; 776 cm^{-1} C-Cl str. ^{1}H NMR d: 8.25-8.35 (3H, m, Ar H); 5.4 (1H, s, Ar-OH)

Table -1

Physical and analytical data of the synthesized compounds

Comp Code	Ar	M.P (^{0}C)	Yield (%)	Mol. Formula	Mol. Wt.	Elemental analysis					
						C %		H %		N%	
						Calc	found	Calc	found	Calc	found
4a	-C_6H_5	222	56	$C_{16}H_9FN_4O_2$	308	62.24	62.21	2.94	2.95	18.17	18.15
4b	3- $C_6H_4NO_2$	218	54	$C_{16}H_8FN_5O_4$	353	54.4	54.39	2.28	2.29	19.82	19.8
4c	4- $C_6H_4NO_2$	224	57	$C_{16}H_8FN_5O_4$	353	54.4	54.41	2.28	2.27	19.82	19.81
4d	3,5- $C_6H_3(NO_2)_2$	204	62	$C_{16}H_7FN_6O_6$	398	48.25	48.26	1.77	1.75	21.1	21.12
4e	2,4,6- $C_6H_2(NO_2)_3$	219	65	$C_{16}H_6FN_7O_8$	443	43.36	43.35	1.36	1.37	22.12	22.11
4f	3- C_6H_4Cl	195	63	$C_{16}H_8ClFN_4O_2$	342	56.07	56.1	2.35	2.36	16.35	16.34
4g	4- C_6H_4Cl	189	58	$C_{16}H_8ClFN_4O_2$	342	56.07	56.09	2.35	2.34	16.35	16.36
4h	3,5- $C_6H_3(Cl)_2$	215	53	$C_{16}H_7Cl_2FN_4O_2$	377	50.95	50.93	1.87	1.89	14.85	14.86
4i	3- $C_6H_4CH_3$	204	50	$C_{17}H_{11}FN_4O_2$	322	63.35	63.34	3.44	3.43	17.38	17.36
4j	4- $C_6H_4CH_3$	192	49	$C_{17}H_{11}FN_4O_2$	322	63.35	63.36	3.44	3.42	17.38	17.37
4k	4- C_6H_4OH	179	51	$C_{16}H_9FN_4O_3$	324	59.26	59.28	2.8	2.82	17.28	17.28
4l	3- C_6H_4OH	201	54	$C_{16}H_9FN_4O_3$	324	59.26	59.24	2.8	2.83	17.28	17.29
4m	3,5- $C_6H_3(OH)_2$	209	48	$C_{16}H_9FN_4O_4$	340	56.48	56.44	2.67	2.65	16.47	1648
4n	3- C_6H_4Br	217	53	$C_{16}H_8BrFN_4O_2$	387	49.64	49.63	2.08	2.05	14.47	14.48
4o	4- C_6H_4Br	188	55	$C_{16}H_8BrFN_4O_2$	387	49.64	49.67	2.08	2.06	14.47	14.46

Synthesis of aryl ethers of substituted pyrimido(5,4-c)cinnoline (4)

To the substituted pyrimido(5,4-c)cinnoline (0.01 mol) were added various phenols (0.01 mol), 1, 4-dioxan (10 ml) and triethylamine (2-3 drops) and the mixture was then refluxed for 16 hours. The contents of the flask were transferred to ice cold water and the precipitate obtained was filtered, dried and recrystallized from dimethyl-formamide. IR (4c): 3495 cm^{-1} O-H str (phenolic); 3039 cm^{-1} C-H str aromatic; 1550 cm^{-1} C-NO_2 str (aromatic), 1315 cm^{-1} Ar-O-Ar/; 1216 cm^{-1} C-F str. ^{1}H NMR (4c) d: 8.1-8.2 (3H, m, Ar H); 7.9-8.0 (4H, m, Ar H); 5.45 (1H, s, Ar-OH)

Results and Discussion

Compound **1** had been prepared by addition of cyanoacetamide to the diazonium salt. Compound **2** has been prepared from arylhydrazino(cyano)acetamide, chlorobenzene and anhydrous aluminium chloride by heating for 2 hours. Cyclisation of **2** was carried out by refluxing with formamide for 2 hours. Various derivatives (4a-o) were synthesized from **3** and different phenols. The structures of the synthesized compounds have been confirmed by their analytical and spectral data. Disappearance of the absorption band of nitrile group at 2250 cm^{-1} confirms the cyclisation to give compound **2**. Furthermore the appearance of peak in PMR spectrum showing 2 protons of aromatic amino group at d 4.2-4.3 confirmed the structure of **2**. Appearance of phenolic OH proton in the region d 5.4 in PMR spectrum reveals the pyrimidine cyclisation to give compound **3**. Disappearance of C-Cl stretching band in IR spectrum of **4c** reveals the replacement of chlorine by p-nitrophenol. Further appearance of C-NO_2 absorption band in IR spectrum of **4c** confirms the substitution reaction.

The synthesized compounds were screened for their antibacterial activity (table-2) against S. aureus and B. subtilis taking Ampicillin as standard. Zone of inhibition was measured in mm after an incubation period of 24 hours. Compounds 4d, 4e, 4h and 4m showed good antibacterial activity, compound 4j was least active and remaining compounds showed moderate activity against S. aureus and B.subtilis.

Table-2

Antibacterial activity of synthesized compounds

Comp. Code	Zone of inhibition (in mm) after 24 hours			
	S. aureus		B. subtilis	
	50 µg/ml	100 µg/ml	50 µg/ml	100 µg/ml
4a	4.5	4.8	4.7	5.1
4b	3.9	4.8	4.4	4.8
4c	3.8	4.7	4.5	6.1
4d	6.7	7.6	7.1	7.3
4e	6.7	7.4	6.9	7.5
4f	4.7	5.1	5.1	5.3
4g	4.6	4.9	4.7	5.2
4h	6.8	7.8	7.2	8.7
4i	4.2	5.2	4.8	5.7
4j	3.7	4.2	3.9	4.8
4k	4.1	5.1	4.2	6.7
4l	4.7	5.3	6.2	6.9
4m	6.6	7.5	7	7.8
4n	4.3	5.1	4.8	6.5
4o	4.1	5.2	4.8	6.3
Standard	8.5	9.5	8.9	10.1

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Synthesis and Antibacterial Activity of Chalcones of 2- Acetyl-6-chloro-5-fluro-3-phenylindole

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ABSTRACT

Phenyl hydrazone 1. of 3-chloro-4-fluro aniline was prepared by coupling diazonium salt of chloro-fluor aniline with ethyl-a-benzylacetoacete. This hydrazone was then subjected to cyclisation in the presence of dry HCl to give 2-acetyl-6-chloro-5-fluoro-3-phenylindole 2. Various chalcones 3a-f were synthesized using various aromatic aldehydes. These chalcones were further brominated to give dibromo chalcones 4a-f. These compounds were characterized by elemental analysis & UV, IR and ^{1}HNMR spectral data. Finally the chalcones & dibromochalcones were screened for antibacterial activity.

Key words: 2-acetyl-6-chloro-5-fluoro-3-phenylindole, chalcones, dibromo chalcones, antibacterial activity.

Introduction

Indoles and their derivatives find a prominent place in synthetic organic chemistry [1], as they are found to be potent pharmacophores next only to purines and pyrimidines. It has been found that, very often, the 2- and 3- substituted indoles are found to be biologically active [2]. A perusal of litrature revealed that indole ring system is associated with a large number of biological activities [3-7].

Phenylhydrazone of 3-chloro-4-fluoroaniline **1** was obtained by coupling the diazonium chloride of chloro fluro aniline with ethyl a-benzyl acetoacetate in the presence of sodium acetate under the condition of modified Japp-Klingemann reaction [8]. On passing HCl for 1hr the hydrazone underwent Fischer indole cyclisation to give 2-acetyl-6-chloro-5-fluoro-3-phenylindole **2**. It was then condensed with various aromatic aldehydes in the presence of sodium hydroxide in ethanol to get substituted chalcones 3a-f. Bromination in acetic acid gave dibromo chalcones 4a-f.Chalcones and dibromo chalcones were prepared according to the general procedures [9].

It is suggested that the compounds having antibacterial activity may act either by killing the bacteria or blocking their active sites [10-12]. In view to improve the activity, chalcones and dibromo chalcones of fluoro-indoles were prepared and screened for antibacterial activity.

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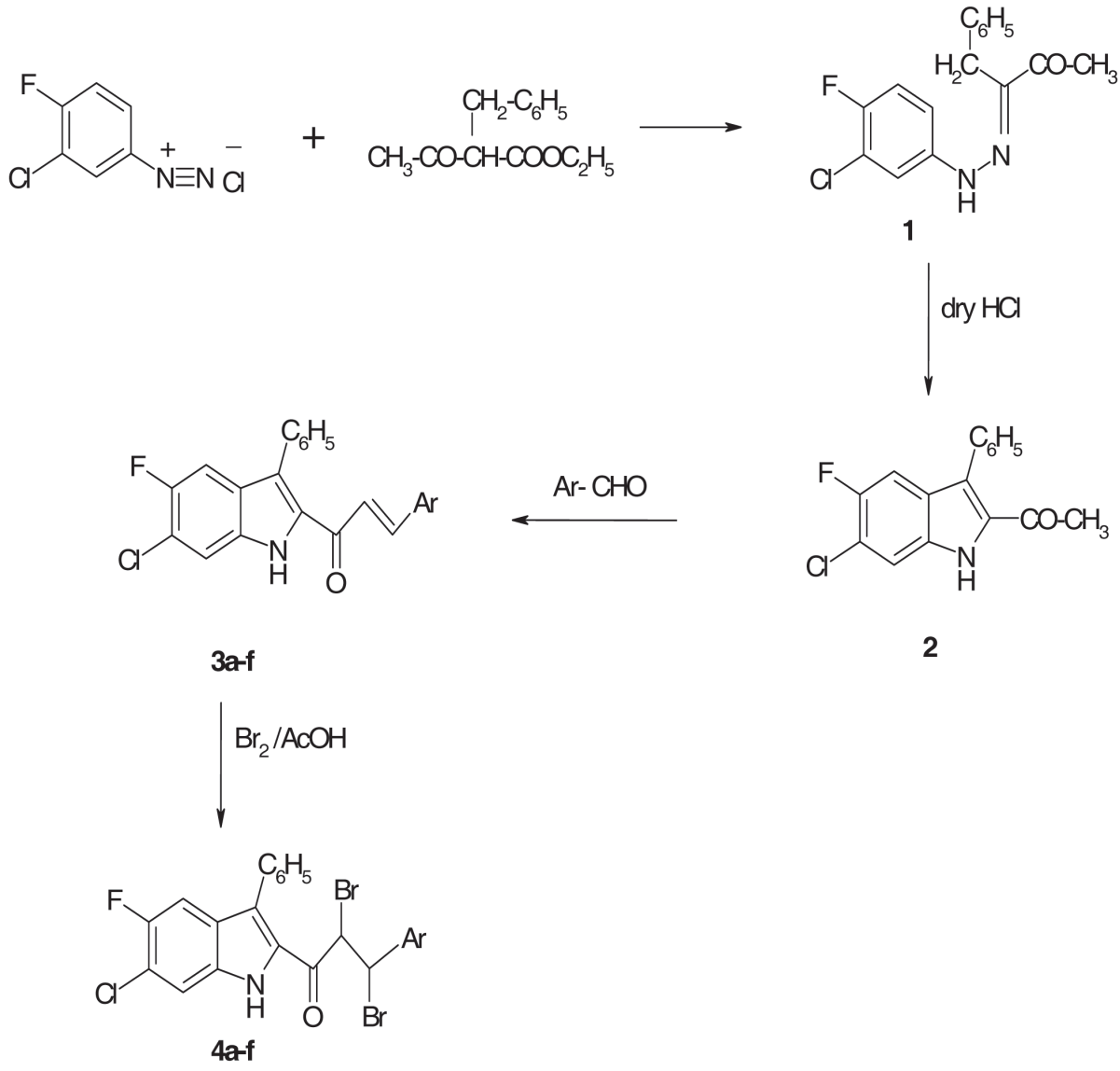
Experimental Section

All the melting points were determined in open glass capillary tubes and are uncorrected. UV spectra were recorded on Schimadzu 1700 UV-VIS spectrophotometer and spectral grade ethanol used. IR spectra were recorded on Schimadzu FTIR 8400S. ^{1}H NMR spectral study was done using $CDCl_3$ as solvent on BRUKER spectrospin 200. Elemental analysis (C,H,N) determined by means of a Perkin-Elmer 240 CHN elemental analyzer, and results were found within the range of theoretical values.

Synthesis of Phenylhydrazone of 3-chloro-4-fluoroaniline (1)

To a vigorously stirred solution of ethyl a–benzyl acetoacetate(4.4g) in absolute ethanol (5ml) was added a solution of sodium hydroxide(0.9g) in water(2.5ml). Immediately after precipitation of a gelatinous mass, water (50ml) was added and stirring was continued for 4 hours. To the above aqueous layer was added aryl diazonium chloride solution prepard from 3-chloro-4-fluoroaniline(0.02mol).After adding crystalline sodium acetate (10g) and further stirring for 1hr.,the phenyl

Scheme of Synthesis



hydrazone was precipitated.The solid product was filtered, washed with water, aqueous sodium carbonate and then again with water and dried. Crude product was recrystallised from ethanol. M.P. 118 ^{0}C. IR (KBr, cm^{-1}); 3273 (N-H str), 3075 (Ar-H str), 1654 (C=O str), 1570 (C=N str), 1203 (C-F str), 700 (C-Cl str)

Synthesis of 2-Acetyl-6-chloro-5-fluoro-3-phenylindole (2)

Phenyl hydrazone **1** (0.01mol) was dissolved in absolute ethanol (30ml) and dry HCl gas was passed for about 2 hrs.Solution was kept aside for crystallization.Product was filtered & dried.Recrystallised from ethanol. M.P. 205^{0}C. IR (KBr, cm^{-1}); 3312 (N-H str), 3056(C-H str aromatic), 1641(C=0 str ketone), 1259 (C-F str), 710(C-Cl str). ^{1}HNMR ($CDCl_3$, in d ppm): 9.4 (s, 1H), 7.15-7.57 (m, 7H), 2.26 (s, 3H)

Synthesis of Indolyl Chanlcones (3 a-f)

2-Acetyl-6-chloro-5-fluoro-3-phenylindole **2** (0.01 mol) in ethanol (15 ml) was stirred with sodium hydroxide (0.5g in 5ml water and 5ml ethanol) for 10 minutes at room temperature. Then appropriate aromatic aldehyde (0.01 mol) was added to it and stirring was continued for 2 hrs till yellow solid was separated. The product was collected by filtration, washed thoroughly with water and finally washed with little ethanol. It was recrystalized from ethanol. 3a. IR (KBr, cm^{-1}); 3292 (N-H str), 3058 (C-H str), 1660 (C=0 str ketone), 1244 (C-F str), 704 (C-Cl str). ^{1}HNMR ($CDCl_3$, in d ppm); 9.5 (s, 1H); 7.0-7.75 (m, 12H), 6.8 (d, 1H), 7.8 (d, 1H).

Table -1

Physical and analytical data of the synthesized compounds

Comp Code	Ar	M.P (^{0}C)	Yield (%)	Mol. Formula	Mol. Wt.	Elemental analysis					
						C %		H %		N%	
						Calc	found	Calc	found	Calc	found
3a	-C_6H_5	232	73	$C_{23}H_{15}ONClF$	375	73.6	73.58	4	3.99	3.73	3.72
3b	2-C_4H_3O	208	78	$C_{21}H_{13}O_2NClF$	365	69.04	69.03	3.56	3.54	3.84	3.83
3c	2-C_6H_4-NO_2	220	75	$C_{23}H_{14}O_3N_2ClF$	420	65.71	65.69	3.33	3.31	6.66	6.64
3d	4-C_6H_4-$N(CH_3)_2$	210	68	$C_{25}H_{20}ON_2ClF$	418	71.77	71.73	4.78	4.77	6.7	6.68
3e	4-C_6H_4Cl	218	72	$C_{23}H_{14}ONCl_2F$	410	67.32	67.3	3.41	3.39	3.41	3.4
3f	4-$C_6H_4(OCH)_3$	227	74	$C_{24}H_{17}O_2NClF$	405	71.11	71.1	4.2	4.18	3.46	3.43
4a	-C_6H_5	220	70	$C_{23}H_{15}ONBr_2ClF$	535	51.59	51.58	2.8	2.78	2.62	2.61
4b	2-C_4H_3O	228	72	$C_{21}H_{13}O_2NBr_2ClF$	525	48	47.98	2.48	2.47	2.66	2.64
4c	3-C_6H_4-NO_2	230	69	$C_{23}H_{14}O_3N_2Br_2ClF$	580	47.58	47.55	2.41	2.4	4.83	4.82
4d	4-C_6H_4-$N(CH_3)_2$	215	65	$C_{25}H_{20}ON_2Br_2ClF$	578	51.9	51.88	3.46	3.44	4.84	4.83
4e	4-C_6H_4Cl	240	71	$C_{23}H_{14}ONBr_2Cl_2F$	570	67.32	67.31	2.46	2.45	2.46	2.44
4f	4-$C_6H_4(OCH_3)$	230	68	$C_{24}H_{17}O_2NBr_2ClF$	565	50.1	50.08	3.01	3	2.48	2.46

Synthesis of dibromo chalcones (4 a-f)

To a solution of chalcone (0.01 mol) in acetic acid (15ml) was added a solution of bromine in acetic acid (25% w/v, 6.4 ml) under cooling and stirring. Yellow crystalline solid which separated after stirring for further 30 minutes was collected by filtration, washed with acetic acid & finally with petroleum ether. These were Recrystalized from benzene and petroleum ether mixture. 4a. IR (KBr, cm^{-1}); 3320 (N-H str), 3050 (C-H str), 1635 (C=0 str ketone), 1500 (C-F str), 750 (C-Cl str), 550 (C-Br str). ^{1}HNMR ($CDCl_3$, in d ppm); 9.57 (s, 1H), 6.9-7.7 (m, 12H), 5.0 (d, 1H), 5.3(d, 1H).

Results and Discussion

Phenyl hydrazone of 3-chloro-4-fluoroaniline (**1**), synthesized by modified Japp-Klingemann reaction. The IR spectrum has revealed the presence of C=N group exhibiting a strong absorption at 1570 cm^{-1} and absorption at 3273 cm^{-1} due to N-H stretching and a strong absorption at 1654 cm^{-1} due to C=O stretching. On passing dry HCl gas for 1 hour the hydrazone underwent Fischer-Indole cyclisation to give 2-acetyl-6-chloro-5-fluoro-3-phenylindole (**2**). The disappearance of C=N peak at 1570 cm^{-1} in IR Spectrum and the peaks observed in ^{1}H NMR spectrum (N-H proton of indole at d 9.4, 7 aromatic protons at d 7.15-7.57 and 3 aliphatic protons at d 2.26) confirm the structure of **2.** The structure of chalcone of 2-acetyl-6-chloro-5-fluoro-3-phenylindole (**3a**) has been confirmed by ^{1}H NMR spectrum, in which appearance of two doublets at d 6.8 and d 7.8 characterize olefinic protons of chalcone. Disappearance of these olefinic doublets and appearance of two doublets at d 5.0 and d 5.3 confirmed the structure of dibromochalcone (**4a**).

Synthesized compounds were screened for antibacterial activity against S.aureus, B.subtilis, P.vulgaris and E.coli. Results are reported in table 2. Activity was determined by using agar diffusion method. Zone of inhibition was measured after incubation for 24 hours at 37^0C. Compounds 3c, 3e, 3f, 4c, 4e & 4f were highly active against E.coli, 3a, 3b & 4a were significantly active against B.subtilis, 3b & 4c were effective against P.vulgaris and 3d against S.aureus.

Table: 2

Antibacterial activity of synthesized compounds

Comp. code	Zone of inhibition (in mm) at concentration 500µg/ml after 24 hours			
	S. aureus	B. subtilis	P. vulgaris	E. coli
1	+	+	+	+
2	+	+	+	+
3a	+	++	+	+
3b	+	++	++	+
3c	+	+	+	++
3d	++	+	+	+
3e	+	+	+	++
3f	+	+	+	++
4a	+	++	+	+
4b	+	+	+	+
4c	+	+	++	++
4d	+	+	+	+
4e	+	+	+	++
4f	+	+	+	++
std	++	++	++	++

Std. Norfloxacin. (+)= 12-15 mm; (++)= 16-24 mm

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Formulation and Evaluation of Aceclofenac Buccal Patches

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ABSTRACT

The present investigation was to formulate and evaluate mucoadhesive drug delivery system for aceclofenac using bioadhesive polymers like Hydroxy propyl methyl cellulose 15cps and Carbopol 934 P along with ethyl cellulose as impermeable layer. The patches were evaluated for weight variation, thickness, drug content, swelling index, mucoadhesive strength and rate of drug release from the patches. The patches were also tested for their surface pH and even for SEM analysis to understand the effect of pH of patch on surrounding environment and to understand morphological characters of the patches. The surface pH of the patches was satisfactory and the patches were with sufficient strength and even with good swelling characters which shows that the patches release drug in slow and sustained manner.

Key Words: buccal patches, mucoadhesive drug delivery, aceclofenac, mucoadhesive polymers.

Introduction

Oral route has been most preferred route of administration for systemic delivery of many active drugs but it has been reported that many therapeutics agents has been subjected to extensive presystemic elimination by gastrointestinal degradation and / or hepatic metabolism resulting in formation of inactive or toxic substances, short duration of therapeutic activity and low bioavailability. These draw backs has encouraged investigation for administration of drugs through absorptive mucosa in various easily accessible body cavities like ocular, nasal, buccal, rectal and vaginal cavities, as these routes have an advantage of bypassing hepato-gastro intestinal first pass elimination [1] and avoidance of presystemic elimination [2]. Administration of drugs through oral cavity is highly acceptable by patients because of presence of mucosa which is highly permeable and with rich blood supply. The total surface of oral cavity is about 100cm^2 to 170 cm^2 [4] and can be further divided into five regions such as floor of mouth (sublingual), the buccal mucosa (cheeks), the gums (gingival), palatal mucosa and the lining of lips. These regions are different from each other not only in their anatomy but also in permeability of drugs as well as in capability of retaining a dosage form for required length of time. Sublingual mucosa being more permeable to drugs than buccal mucosa is associated with drawback that it is relatively unsuitable for holding a retentive system undisturbed for longer time [3]. Success of buccal drug delivery system depends on factors like physicochemical properties of drug, buccal environment and formulation factor etc. Thus present study was about developing buccal mucoadhesive patches of an analgesic drug, aceclofenac, using HPMC 15cps, and Carbopol 934 as polymers to provide mucoadhesive property to the patch and to modify the rate of drug release.

Materials and Methods

Materials

Aceclofenac was purchased from SL Drugs, Hyderabad, Carbopol 934 P and HPMC 15cps were from Himedia, Mumbai, ethanol from Hayman's LTD, England, Monobasic phosphate and Potassium di- hydrogen phosphate from Ranbaxy, Fine chemicals SAJ Nagar and Hydrochloric acid was purchased from Merck , Mumbai, India. All the other reagents and chemicals used were of analytical grade.

Methods

Formulation of Aceclofenac buccal patches

The patches were prepared by using solvent casting method. The required quantity of polymers were weighed and mixed together with 10- 15ml of solvent and mixed for about 20 min and kept aside for 15min to hydrate the lumps formed due to polymers. 5% plasticizer was added and remaining 5- 10 ml of solvent was added to the polymer mixture and stirred by using mechanical stirrer for about 30minutes. The system was removed from the stirrer and kept aside undisturbed to eliminate air bubbles entrapped if any. The solution was then casted into clean

and dry petridish and covered with an inverted funnel and allowed to dry till a flexible film was formed. The dried films were carefully cut into 2x2cm patch and stored carefully in aluminum foil till further studies. Patches containing drug was prepared by adding drug dissolved solvent to the polymer solution before subjecting to mechanical stirring. The prepared patches were carefully removed from the petridish cut into desired size 2X2cm and stored in aluminum foil till further studies. Patches of different formulations were prepared with varying the ratio of polymers. The composition of different patches was given in the table 1 below

Table1

Composition of different formulations of aceclofenac patches

Sl. No.	Name of the ingredient	Formulations		
		F1	F2	F3
1	Carbopol 934P (Polymer)	300mg	500mg	700mg
2	Hydroxy Propyl Methyl Cellulose (Polymer)	700mg	500mg	300mg
3	Glycerol (Plasticizer)	1ml	1ml	1ml
4	Aceclofenac (Drug)	150mg	150mg	150mg
5	Ethanol (Solvent)	15-20ml	15-20ml	15-20ml

Preparation of backing layer

The backing layer was fabricated by preparing a solution of ethyl cellulose (5%) by using ethyl alcohol as solvent. The well mixed ethyl cellulose was poured in petri dish. The petri dish was placed on level surface undisturbed and the solvent was allowed to evaporate at a controlled rate by placing an inverted glass funnel covering the petridish.

Evaluation of the patches

1. Weight variation:

Three patches were selected randomly from each formulation and each patch was individually weighed and average has been calculated.

2. Thickness variation:

Thickness of the prepared patch was determined by measuring thickness at different places in the same patch using a screw gauge. Three readings have been and average was reported.

3. Swelling Studies:

a. Area of swelling:

A patch with dimensions 2X2 cm was placed in a petri dish. A graph paper was placed under the petridish to facilitate easy measuring of the change in dimensions of the patch after swelling. 50ml of phosphate buffer pH 6.2 was placed in the petridish and area of swelling was measured at 5 min interval for 20 minutes.

b. Weight of swelling:

A patch with dimensions 2X2cm was placed on a pre weighed cover slip and weighed before placing in the a petridish containing 50ml of phosphate buffer pH 6.2 and the weight was measured at an interval of 5 min for 20 minutes and percentage hydration was calculated.

$$\% \text{ hydration} = \text{weight (swollen)} - \text{weight (dry)} / \text{weight (dry)}$$

4. Content uniformity:

Five different patches cut from different corners of the film were selected and placed in five different petri dishes containing 50ml of 0.01M HCl solution for 30 minutes. The patches were then crushed to release the drug from them suitably diluted and each solution was measured for absorbance by using UV spectrophotometer at 276nm. The procedure was repeated with a drug free patch to get blank solution. Uniformity of drug of each patch was determined.

5. Drug content in the patch:

Amount of drug present in patch was determined by selecting a patch of 2 x2 cm randomly. The selected patch was placed in 10ml phosphate buffer pH 6.2 for 30min and crushed with glass rod to release the entire drug, filtered, suitably diluted and measured for its absorbance by using UV Spectrophotometer at 276nm to determine drug content from the patch.

6. Folding endurance

To understand the strength the patches were subjected for folding endurance test. Three patches of each formulation were selected and repeatedly folded and stretched at same place till break. The number of times patch could be folded at same place without breaking gave the value of folding endurance. A mean of the three readings was recorded.

7. Determination of surface pH:

To study the effect of patch on surrounding pH, the pH of patch surface was determined. To determine surface pH three patches from each formulation were dissolved separately in 1 ml of distilled water for 30min. The electrode of pH meter was touched to the surface of patch and sufficient time was given for attaining equilibrium and pH was measured. A mean of three readings were reported.

8. In vitro drug release studies:

Invitro studies were carried out by attaching sigma dialysis membrane to one end of the open cylinder which acts as donar compartment. The prepared buccal patch containing drug was placed inside donar compartment which was agitated continuously using magnetic stirrer and then temperature was maintained at 37 ± 1^0 C. Receptor compartment consists of 50ml of phosphate buffer pH 6.4, a sample of 2 ml was withdrawn at regular interval from receptor compartment and replaced with fresh 2 ml of solution immediately and drug released was analyzed at 276nm.

9. Measurement of mucoadhesive strength:

A study of bond between patch and mucous membrane was excised from sheep buccal mucosa. The sheep buccal tissue was collected from local slaughter house and was used within two hours after collection. The buccal mucosa was prepared by removing under lying tissue and washed thoroughly with isotonic phosphate buffer pH 6.8. The fresh buccal mucosa was cut and tied to open mouth of a glass vial which was completely filled with phosphate buffer pH 6.8. The vial was securely fitted at the centre of a glass beaker. The glass beaker was filled with phosphate buffer pH 6.8 till it touches the surface of buccal mucosa. The patch was stuck to a rubber stopper with cyanoacrylate glue. Two pans of physical balance were balanced with 5g weight. A weight of 5g was removed right hand side pan which lowers pan attached with rubber stopper which in turn attached to patch over mucosa. The balance was kept in the same position for 5 minutes. On the left hand side water was slowly added with infusion set (100drops/ min) into a beaker until the patch gets detached from mucosal surface. The weight in grams to detach the patch from the mucosal surface provides the measure of mucoadhesive strength.

10. Morphological studies

Surface morphology and cross section of the patch were studied by Scanning Electronic Microscopy. The patches were prepared for SEM analysis by sprinkling the cut patches on to one side of double adhesive stub. The stub were then coated with gold using polaran SC 500 sutter coater and observed under scanning electron microscope.

Result and Discussion

The buccoadhesive patches of analgesic drug aceclofenac were prepared and characterized for parameters like weight, thickness, surface pH, folding endurance, swelling characteristics, content uniformity, drug content, mucoadhesive strength, Invitro release studies and morphology characters. Results were given in table 2.

From the results obtained it was evident that the formulation F2, that one containing both the polymers in same quantity, was exhibiting uniform thickness and less variation in weight. Whereas the formulation i.e. F1 was having good swelling index than other formulations which may be because of more Carbopol in the formulation.

The weight of patches was more or less same in all formulations. The percentage of swelling was in the order F3> F2>F1. A little variation was found in thickness of the patches which may be because of variation in density because of change in combinations of polymers in formulations. The folding endurance values for all patches were in the range 146 – 176. Therefore patches were with good physical and mechanical properties. It was found that folding endurance was decreased with raise in concentration of carbopol. Surface pH of the patches being in the range of 6.23 to 6.84 implies that it is well in the range of salivary pH and hence should not cause any irritation and ultimately improve patient compliance. The mucoadhesive strength of all patches was found to be good. The formulation F2 showed maximum mucoadhesive strength while F1 exhibited minimum mucoadhesion. The drug release rate from different formulations was given in Figure 1. The formulation F1 delivered aceclofenac in faster rate than other formulations and this was may be because of more HPMC present in the formulation. In other words the formulation with more amount of carbopol exhibited slower rate of drug release from which it is evident that raise in carbopol concentration will bring change in viscosity[7], and raise in viscosity will decrease the drug release rate. Scanning Electron Microscopy (figure 2 and figure 3) of the aceclofenac patches revealed that the surface of the patch was uniform, smooth and without the evidence of pores. Cross section of the patch shows porous and layered like structure which indicates mucus secretion may enter into patch, swell and facilitate drug release[8].

Table 2

Physical parameters of Aceclofenac Buccal Patches

Sl. No.	Formu-lations	Weight	Thickness (mm) ± SD	Drug Content(mg)	Surface pH	Mucoadhesive strength	Folding Endurance (num)
1	F1	0.37	0.152 ± 0.017	12.89 ± 0.007	6.78 ± 0.07	8 ± 0.32	< 146
2	F2	0.34	0.174 ± 0.010	12.57 ± 0.03	6.84 ± 0.14	6 ± 0.42	< 158
3	F3	0.42	0.166 ± 0.013	12.63 ± 0.02	6.23 ± 0.14	10 ± 0.47	< 176

(Note: All the test was performed on triplicate i.e. n=3)

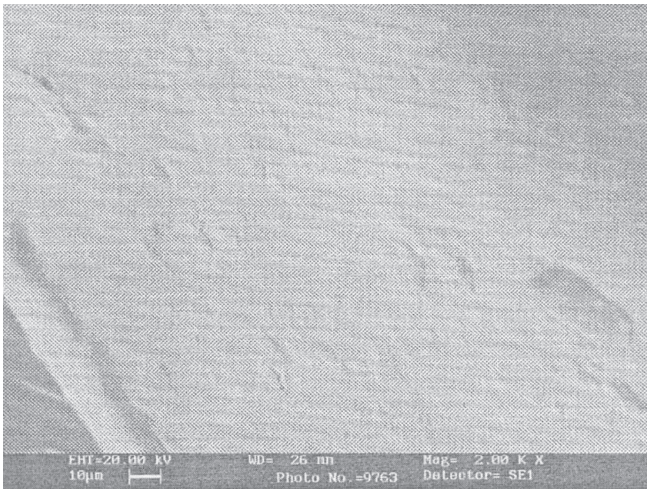

Fig. 2: Surface view of mucoadhesive patch of aceclofenac

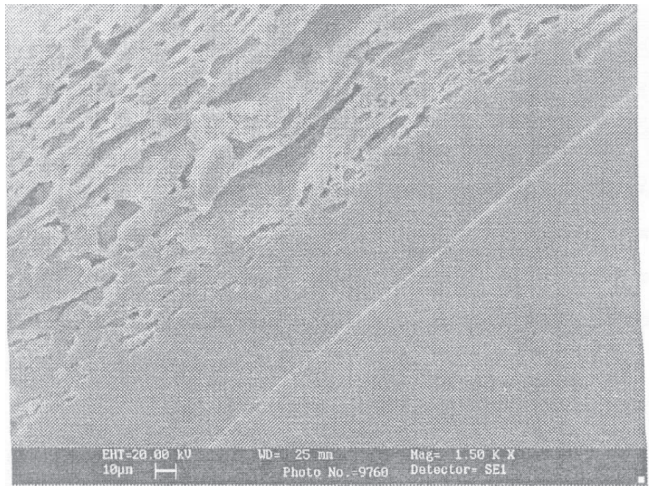

Fig. 3: Cross section view mucoadhesive patch of aceclofenac

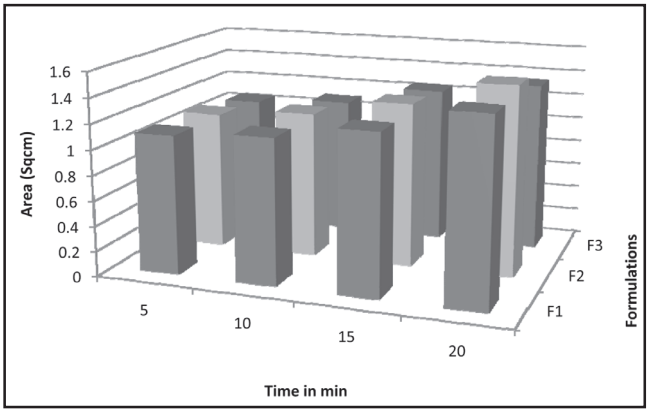

Fig. 4: Swelling profile of patches in terms of Area

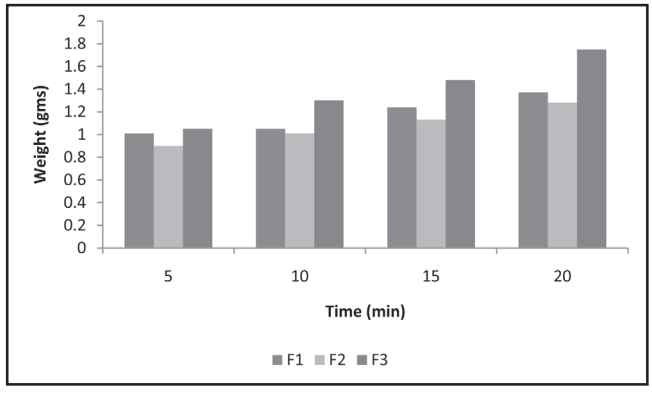

Fig. 5: In Vitro Bioadhesive profiles of Buccal Patches

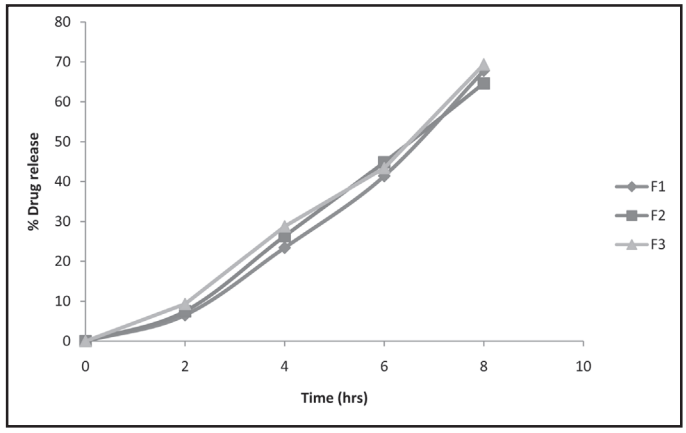

Fig. 6: Drug release profile form different buccal patches of aceclofenac

Conclusion

The mucoadhesive patches were prepared by using HPMC and Carbopol as polymers and by using solvent casting method. The polymers were used in different ratios. From the present study it can be concluded that buccoadhesive drug delivery system for analgesic drug like aceclofenac can help in delivering drug at slower rate. The patches of formulation F2 can be a better formulation than other formulations. The ratio of carbopol and HPMC had significantly effected the characteristics like swelling index, Invitro mucoadhesive strength and in vitro drug release. So it can be understood that such mucoadhesive patches with combination of HPMC and Carbopol can be a promising combination for buccal delivery of aceclofenac

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Synthesis and Spectral Characterization of Metal Complexes of Quinazolyl Schiff Bases

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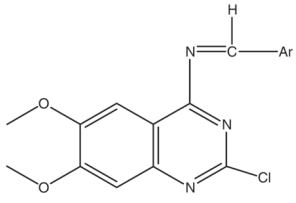
ABSTRACT

The VO(IV), Pd(II), Zn(II), Cd(II), Hg(II), Ru(III), Au(III) and UO_2(VI) complexes of Schiff bases derived from 2-chloro-6,7-dimethoxy-4-quinazolinamine and 2-hydroxybenzaldehyde, 2-hydroxynaphthaldehyde and 2-hydroxy-3-methoxy-benzaldehyde have been synthesized and characterized by physico-chemical data. All the three ligands behave towards the metal ions as mononegative, bidentate ones co-ordinating through phenolic oxygen and azomethine nitrogen. The geometry and the bonding characteristics associated with the complexes have been deduced from the relevant spectral data.

Keywords: Quinazolyl Schiff base complexes, Synthesis, Characterization.

Introduction

Quinazoline derivatives are biologically active and find application in medicinal use[1]. These compounds have potent donor groups and despite this, the studies directed towards exploring the ligational behaviour of these compounds are limited. For this reason, we report, herein, the synthesis and chracterization of VO(IV), Pd(II), Zn(II), Cd(II), Hg(II), Ru(III), Au(III) and UO_2(VI) complexes of some quinazolyl Schiff bases namely 4-(2-hydroxybenzylamino)-2-chloro-6,7-dimethoxyquinazoline (HBCMQ), 4-(2-hydroxy naphthylamino)-2-chloro-6,7-dimethoxyquinazoline (HNCMQ) and 4-(2-hydroxy-3-methoxybenzylamino)-2-chloro- 6,7-dimethoxyquinazoline (HMCMQ).



Ar = C_6H_5O, $C_{10}H_7O$, $C_7H_7O_2$

Experimental

2-Chloro-6,7-dimethoxy-4-quinazolinamine was procured from Aldrich and all other chemicals used were of A.R. or B.D.H. grade. The ligands HBCMQ HNCMQ and HMCMQ were synthesized by refluxing equimolar methanolic solutions of 2-chloro-6,7-dimethoxy-4-quinazolinamine and the respective aldehydes in presence of a few drops of piperidine, for 6 hrs. The solids that separated during reflux were filtered, washed with methanol and recrystallised from hot dry methanol. The colour, yield%, mp (^oC) and elemental analysis (%) of HBCMQ, HNCMQ and HMCMQ are dirty white, 75, 278 (Found C 59.37, H 4.07, N 12.18; $C_{17}H_{14}ClN_3O_3$ requires C 59.40, H 4.10, N 12.22); light yellow, 80, 290 (Found C 63.96, H 4.03, N 10.52 $C_{21}H_{16}ClN_3O_3$ requires C 64.05, H, 4.09, N 10.67) and dirty white, 75, 280 (Found C 57.54, H 4.29, N 11.18, $C_{18}H_{16}ClN_3O_4$ requires C 57.84, H 4.31, N 11.24).

The Zn(II), Cd(II) and UO_2(VI) complexes were prepared taking respective metal acetates, Pd (II), Ru(III) and Au(III) complexes using metal chlorides and VO(IV) complexes using vanadyl sulphate. In the preparation of VO(IV), Pd(II), Zn(II), Cd(II), Hg(II), Au(III) and UO_2(VI) complexes, the metal and the ligand were combined in 1:2 mole ratio while in the case of Ru(III) complexes they were mixed in 1:3 ratio using aqueous methanol for the metal salts and methanol-DMF (20:1) mixture for ligands. The contents were refluxed on a water bath for about 3h, the solid that separated was filtered, washed with water, hot methanol and ether and dried in air.

The elemental analyses of the ligands and the complexes were carried out by CDRI, Lucknow. Conductance measurements were made in DMF at 10^{-3} M

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concentration on a Digisun digital conductivity meter DI 909 model. Gouy balance calibrated with $Hg[Co(SCN)_4]$ was used to measure the magnetic susceptibility of the metal complexes at room temperature. The IR spectra of the ligands and the metal complexes in KBr were recorded in the range 4000-400 cm^{-1} using JASCO FT/IR 5300 spectrophotometer. The electronic spectra of the metal complexes in DMF were recorded on Systronics UV-VIS spectrophotometer. The Varian E4 X-band spectrophotometer operating in the frequency range 8.8-9.6 GHz available with RSIC, IIT, Chennai was employed in recording the ESR spectra of VO(IV) complexes in DMF solution at LNT.

Results and Discussion

All the metal complexes (Table 1) are stable at room temperature and are non-hygrogcopic. The metal complexes decompose upon heating without melting. The ligands and their metal complexes are insoluble in water, slightly or very slightly soluble in methanol and acetone and fairly soluble in dimethyl formamide. With the exception of Au(III) complexes which are 1:1 electrolytes with molar conductance in the range (78-85), all others are non-electrolytes showing only residual conductance (10-14). The magnetic moment data indicate that VO(IV) and Ru(III) complexes are paramagnetic to the extent of one unpaired electron while all others are diamagnetic.

Table - 1
Analytical & Physical Data Of Metal Complexes

Metal Complex	Per cent			Molar cond.	μ_{eff}
	C	H	N	Ω^{-1} cm^2 mol^{-1}	B.M.
$[VO(C_{17}H_{13}ClN_3O_3)_2]$	54.11(54.27)	3.44(3.48)	11.14(11.17)	10	1.78
$[Pd(C_{17}H_{13}ClN_3O_3)_2]$	51.48(51.57)	3.28(3.31)	10.58(10.61)	12	—
$[Zn(C_{17}H_{13}ClN_3O_3)_2]$	54.29(54.38)	3.44(3.49)	11.14(11.19)	14	—
$[Cd(C_{17}H_{13}ClN_3O_3)_2]$	51.11(51.18)	3.25(3.28)	10.49(10.53)	11	—
$[Hg(C_{17}H_{13}ClN_3O_3)_2]$	46.01(46.09)	2.91(2.96)	9.42(9.48)	10	—
$[Ru(C_{17}H_{13}ClN_3O_3)_3]$	54.19(54.24)	3.42(3.48)	11.12(11.16)	10	1.79
$[Au(C_{17}H_{13}ClN_3O_3)_2]$ Cl	46.19(46.27)	2.94(2.97)	9.48(9.52)	80	—
$[UO_2(C_{17}H_{13}ClN_3O_3)_2$	42.68(42.74)	2.71(2.74)	8.76(8.80)	11	—
$[VO(C_{21}H_{15}ClN_3O_3)_2]$	59.06(59.17)	3.49(3.55)	9.78(9.86)	11	1.80
$[Pd(C_{21}H_{15}ClN_3O_3)_2]$	56.49(56.55)	3.32(3.39)	9.38(9.42)	10	—
$[Zn(C_{21}H_{15}ClN_3O_3)_2]$	59.16(59.28)	3.51(3.55)	9.76(9.88)	13	—
$[Cd(C_{21}H_{15}ClN_3O_3)_2]$	56.01(56.17)	3.32(3.37)	9.31(9.36)	10	—
$[Hg(C_{21}H_{15}ClN_3O_3)_2]$	51.02(51.15)	3.01(3.07)	8.49(8.52)	12	—
$[Ru(C_{21}H_{15}ClN_3O_3)_3]$	59.08(59.14)	3.51(3.54)	9.81(9.85)	11	1.80
$[Au(C_{21}H_{15}ClN_3O_3)_2]Cl$	51.29(51.34)	3.02(3.08)	8.49(8.55)	78	—
$[UO_2(C_{21}H_{15}ClN_3O_3)_2]$	47.71(47.79)	2.82(2.86)	7.89(7.96)	14	—
$[VO(C_{18}H_{15}ClN_3O_4)_2]$	53.17(53.22)	3.69(3.72)	10.29(10.34)	10	1.79
$[Pd(C_{18}H_{15}ClN_3O_4)_2]$	50.68(50.75)	3.49(3.55)	9.81(9.86)	11	—
$[Zn(C_{18}H_{15}ClN_3O_4)_2]$	53.28(53.32)	3.68(3.73)	10.29(10.36)	10	—
$[Cd(C_{18}H_{15}ClN_3O_4)_2]$	50.31(50.40)	3.47(3.52)	9.76(9.80)	12	—
`$[Hg(C_{18}H_{15}ClN_3O_4)_2]$	45.66(45.70)	3.18(3.20)	8.75(8.88)	10	—
$[Ru(C_{18}H_{15}ClN_3O_4)_3]$	53.12(53.19)	3.66(3.72)	10.28(10.34)	12	1.79
$[Au(C_{18}H_{15}ClN_3O_4)_2]Cl$	45.76(45.87)	3.18(3.21)	8.88(8.92)	85	—
$[UO_2(C_{18}H_{15}ClN_3O_4)_2]$	42.49(42.57)	2.94(2.98)	8.19(8.28)	13	—

Values in parentheses are the calculated ones

All the ligands show, in their spectra, a medium intensity band in the region 3190-3200cm^{-1} that has been assigned to νO-H. This band disappears in the spectra of their complexes indicating that deprotonation of the group has taken place. A small or medium intensity band around 1230cm^{-1} in the ligands assignable to νC-O is seen to have undergone a positive shift by 30-50cm^{-1} in the complexes suggesting coordination through phenolic oxygen[2]. The positive shift observed may be attributed to the drift of electron density from oxygen to the metal ion resulting in greater ionic character of the νC-O bond and a consequent increase in its vibration frequency[3]. Further, the ligands reveal bands around 1660cm^{-1} due to free νC=N and around 1580cm^{-1} due to ring νC=N. While the band due to free νC=N has got lower shifted by 10-20cm^{-1} in all the complexes, the band due to ring νC=N remains unshifted in the complexes. These observations suggest that the ligands act as mononegative, bidentate ones bonding through phenolic oxygen and nitrogen of free νC=N group [4-6] .

An intense band that appears around 750cm^{-1} in all the ligands and their metal complexes has been assigned to νC-Cl [7] and a fairly intense band that figures around 950cm^{-1} in all the VO(IV) complexes has been attributed to νV=O[8].

The coordination through phenolic oxygen and azomethine nitrogen is further substantiated by the appearance, in all the complexes, of non-ligand bands in the far infrared region around 590 and 450cm^{-1} assignable repectively to νM-O and νM-N vibrations [9-10].

The electronic spectral data for the VO(IV), Ru(III) and Pd(II) complexes are presented in Table 2.

The VO(IV) complexes each show three peaks in the region 11100 – 28570 cm^{-1} which may be assigned in the increasing order of frequency to the transitions $^2B_2 \rightarrow {}^2E$, $^2B_2 \rightarrow {}^2B_1$ and $^2B_2 \rightarrow {}^2A_1$ of square pyramidal geometry [11].

The Pd(II) complexes each show three peaks in the region 12195-25000 cm^{-1} that are assignable, in the increasing order of frequency to the transitions, $^1A_{1g} \rightarrow {}^1A_{2g}$, $^1A_{1g} \rightarrow {}^1B_{1g}$ and $^1A_{1g} \rightarrow {}^1E_g$ of square planar geometry [12]. Based on these data, the complexes have been assigned square planar geometry.

The Ru(III) complexes reveal three peaks in the region 11490 – 25000 cm^{-1} which may be assigned in the increasing order of frequency to the transitions $^2T_{2g} \rightarrow {}^4T_{1g}$, $^2T_{2g} \rightarrow {}^4T_{2g}$ and $^2T_{2g} \rightarrow {}^2A_{2g}$, $^2T_{1g}$ of octahedral geometry[13].

The Zn(II), Cd(II) and Hg(II) complexes of HBCMQ, HNCMQ and HMCMQ show, as expected, no d-d transitions in their electronic spectra and on the basis of analytical, conductance and infrared spectral data, they have been assigned tetrahedral geometry. The Au(III) and

Table - 2
Electronic Spectral Data Of Metal Complexes

Metal complex	Frequency (cm^{-1})		
VO-HBCMQ	11710	17240	28570
Pd-HBCMQ	12195	20000	25000
Ru-HBCMQ	11490	18870	25000
VO-HNCMQ	11200	17820	28200
Pd-HNCMQ	12620	19860	24600
Ru-HNCMQ	12120	18800	24490
VO-HMCMQ	11100	17280	28120
Pd-HMCMQ	12800	19980	24620
Ru-HMCMQ	11860	18760	24480

Table - 3
Esr Parameters Of Vo(iv) Complexes

Metal complex	$g_{\parallel}$	$g_{\perp}$	g_{av}
VO-HBCMQ	1.94	1.99	1.97
VO-HNCMQ	1.94	2.00	1.98
VO-HMCMQ	1.95	1.99	1.98

UO_2(VI) complexes show only charge transfer (above 35000cm^{-1}) but no d-d bands and based on this observation and the other data obtained for the complexes, Au(III) complexes have been assigned square planar geometry and the UO_2(VI) complexes, octahedral geometry.

The ESR spectral parameters calculated for the VO(IV) complexes using appropriate methods and equations [14] are presented in Table-3. The spectra of all the three complexes are well resolved with eight parallel and eight perpendicular components due to hyperfine coupling with vanadium nucleus I = 7/2. The g values observed for the present complexes are in agreement with those generally observed for a vanadyl complex with a square pyramidal geometry[15]. For all the complexes, $g_{\parallel} < g_{\perp} < g_e$ (where g_e is free electron value) which indicates that the unpaired electron is in the d_{xy} orbital with ^2B as the ground state[16].

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Hepatoprotective activity of the Methanolic extract of *Commelina Clavata. Clarke* against Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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ABSTRACT

The methanolic extract of whole plant *Commelina Clavata*. C*larke* was screened for hepatoprotective activity in carbon tetrachloride induced hepatotoxicity in rats**.** Alteration in the whole plant of biochemical markers of hepatic damage like SGPT, SGOT, ALP, bilirubin were tested in both treated and untreated groups. Carbon tetrachloride has enhanced the SGPT, SGOT, ALP and bilirubin levels. Treatment with methanolic extract of whole plant of Commelia Clavata (100, 200 and 400 mg/kg) has brought back the altered levels of biochemical markers to the near normal levels in the dose dependent manner. This was evident from significant reduction in serum enzyme, SGOT, SGPT, ALP and Total bilirubin (TB).Various pathological changes like centribular necrosis and vacuolization were observed in CCl_4 treated rats, which were significant protective activity in groups treated with SP and silymarin. It was concluded from the study that methanolic extracts of *Commelina Clavata* possess hepatoprotective activity against CCl_4 induced hepatotoxicity in rats.

Keywords: Hepatoprotective, *Commelina Clavata,* Carbontetrachloride, Silymarin.

Introduction

Liver damage is always associated with cellular necrosis, increase in lipid peroxidation and depletion in the tissue GSH levels. In addition serum levels of many biochemical markers like SGOT, SGPT, ALP and bilirubin levels are elevated [1, 2]. However there are several herbs/ herbal formulations claimed to possess beneficial activity in treating hepatic disorders. *Commelina clavata* is a perennial herb with thick, non tuberous roots. Shoots are rising to climbing, rooting on contact with ground. They grow up to 1 meter long. Leaves are narrowly lanceolate shaped to elliptic and 2.5 to10 cm long and 0.4 to1.5 cm wide with pointed or long pointed tip. Flowers are borne in 2-flowered clusters. Flowers are sky-blue, about 1.5 cm across, with sepals 3 mm long. Paired petals are 7 mm wide, sky-blue. The third petal is 5 mm wide. The family is important for its ornaments as day flower (commelina). In South Africa the young shoots and leaves of Commelina clavata are edible[3]. *Commelina* is a genus of approximately 170 species, commonly called dayflowers due to the short lives of their flowers. They are less often known as widow's tears. It is the largest genus of its family Commelinaceae. The Asiatic dayflower (*Commelina communis*) is probably the best known species in the West. It is a common weed in parts of Europe and throughout eastern North America. Several species, such as *Commelina benghalensis*, are eaten as a leaf vegetable in Southeast Asia and Africa. Previously isolated compounds are Ursolic acid and oleanolic acid. However, there are no significant basis or reports in the modern literature regarding its usefulness as hepatoprotective agent. Thus the present study was conducted to evaluate the hepatoprotective activity of the methanol extract of the whole plant of *Commelina clavata* by using CCl_4 induced hepatic injury in rats.

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Materials and Methods

Plant Material Collection

Whole plant of *Commelina Clavata* (Commelineaceae) were collected from the dry lands, Anantapur district area, India in the month of December 2009 and authenticated by the taxonomist, Department of Botany, Sri Krishna Devaraya University and the specimen voucher was preserved in the Department.

Acute toxicity studies

Acute toxicity studies were performed for extract of

selected plant according to the toxic classic method as per guidelines. None of these extract showed mortality even at a dose of 1000mg/kg and therefore considered safe. Toxicological studies were conducted in mice (N=6) for all the extracts as per the Irvin's method [4] at the doses of 100, 300 and 1000 mg/kg, no mortality were observed.

Materials

All the materials used for this experiment are of Pharmacopoeial grade. Carbon tetrachloride (E. Merck), silymarin (Sigma Chemical Co.,) and olive oil were purchased from the local supplier. Diagnostic kits for the estimation of SGOT, SGPT, SALKP and serum bilirubin were purchased from local supplier (Sai chemicals) manufactured by Ranbaxy Diagnostics Ltd., New Delhi, India. Water represents the double distilled water, standard orogastric cannula was used for oral drug administration.

Animals

Albino wistar rats weighing 200-250g and Albino mice 20-30 g was procured from Biogen, Bangalore. They were maintained in the animal house of Gautham College of Pharmacy. Animals were maintained under controlled condition of temperature at $27^{o} \pm 2^{o}$ C and 12-h light-dark cycles. They were housed in polypropylene cages and had a free access to standard pellets (Amruth) and water *ad libitum*.

All the studies conducted were approved by the Institutional Animal Ethical Committee (IAEC) of Gautham College of Pharmacy, Bangalore (REF-IAEC/023/5/2011) according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (Reg No: 491/01/c/CPCSEA), Govt. of India.

Carbontetrachloride-induced Hepatotoxicity

The animals were divided into six groups of six animals each. Group-I served as normal control received 5% acacia mucilage (1 ml/kg.p.o) daily once for 7 days. Group-II served as toxic control and received CCl4 (1 ml/kg i.p) daily once for 7 days [5]. Group-III was treated with the standard drug Silymarin (50 mg/kg .p.o) and followed by CCl_4 (1 ml/kg i.p) daily once for 7 days[6]. Groups IV-VI were treated with methanol extract of whole plant of *Commelina Clavata* at doses of 100, 200 & 400mg/kg p.o. in acacia mucilage respectively followed by CCl4 (1 ml/kg i.p) daily once for 7 days. After completion of treatment blood was collected, serum was separated and used for determination of biochemical parameters.

Collection Of Blood Samples

All the animals were sacrificed on 7 th day under light ether anesthesia.The blood samples were collected separately in sterilized dry centrifuge tubes by puncture retro-orbital plexes and allowed to coagulate for 30 min at 37°C. The clear serum was separated at 2500 rpm (Microcentrifuge) for 10min and subjected to biochemical investigation viz.., serumglutamic oxaloacetate transe aminase (SGOT), serum glutamic Pyruvate transe aminase (SGPT), Alkaline phosphatase (ALP) and Total Bilirubin (TB).

Assessment Of Liver Function

The Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were estimated by UV kinetic method in which both SGOT and SGPT were assayed based on enzyme coupled system; where keto acid formed by the aminotransaminase reacts in a system using NADH.The coenzyme is oxidized to NAD and the decrease in absorbance at 340 nm for SGOT malate dehydrogenase (MDH) reduces to malate with simultaneous oxidation of NADH to NAD.The rate of oxidation of NADH is measured, where as SGPT [7] the pyruvate formed in the reaction is converted to lactate by lactate dehydrogenase.Estimation of Alkaline phosphate (ALKP)[8] involves hydrolysis of P-nitrophenyl phosphate by alkaline phosphatase to give Pnitrophenol, which gives yellow color in alkaline solution. The increase in absorbance due to its formation is directly proportional to alkaline phosphate (ALKP) activity. Estimation of total bilirubin (TB) [9] involved the reaction of bilirubin with diazotized sulphanic acid to form an azocompound, the color of which is measured at 546 nm .All the estimations were carried out using standard kits in semi auto analyzer Screen Master 3000.

Statistical Analysis

Results of biochemical estimation were reported as mean ±SEM for determination of significant inter group difference was analyzed separately and one-way analysis of variance (ANOVA) was carried out[10].Dunnet's test was used for individual comparisons [11].

Results and Discussions

Serum levels of SGOT, SGPT, SALKP and total bilirubin were significantly increased ($p<0.01$) in carbon tetrachloride treated Group-2 rats. Group-3 rats treated with Silymarin produced significant reduction ($p<0.01$) in SGOT, SGPT, SALKP and total bilirubin levels.

In Groups: 4-6 treated with methanol extract of ***Commelina Clavata*** at doses of 100, 200 and 400mg/kg; p.o respectively, there is significant decrease in SGOT, SGPT, SALKP and total bilirubin levels when compared to Group-2 rats. The activity of the extracts is found to be dose dependant. The results were given in Table-(1 & 1.1).

Carbontetrachloride (1ml/kg.i.p) intoxication in normal rats produced elevated levels of serum biochemical parameters significantly SGOT(160.5 ± 0.62, 295.5 ± 0.39), SGPT(96.95 ± 1.34, 269.5 ± 1.8), ALKP(179.5 ± 0.99, 296.5 ± 1.45), T.B(0.82 ± 0.06, 2.02 ± 0.03) indicating acute hepatocellular damage and biliary obstruction. The percentage reduction of various serum biochemical

TABLE- 1

Effect of methanol extract of *Commelina Clavata* on biochemical estimation of SGOT, SGPT, SALKP and total bilirubin of CCI4 induced toxicity in rats

Groups	SGOT(IU/ml)	SGPT(IU/ml)	SALKP(IU/ml)	TotalBilirubin(mg/dl)
Control1ml/kg	160.5 ±0.62	96.95 ± 1.34	179.5 ± 0.99	0.82 ± 0.06
$CCl_4$1ml/kg	295.5 ±0.39+	269.5 ± 1.8+	296.5 ±1.45+	2.02 ± 0.03+
Silymarin50mg/kg	174.8 ±1.88***	107.5 ±1.45***	187.7 ±2025***	0.89 ±0.04***
CCM100mg/kg	253.1 ±1.09*	228.3 ±2.13**	246.5 ±3.40*	1.48 ± 3.26*
CCM200mg/kg	231.3 ±4.73**	214.0 ±3.26**	237.3 ±2.24**	1.37 ±2.31**

Values are mean ± SEM for six observations
P: +<0.001 Compared to respective control group-1
P: *<0.05, **<0.01, ***<0.001 Compared to respective control CCl_4 group-2
CCM- Commelina Clavata methanol extract

Table - 1.1

Percentage Reduction Of Various Biochemical parameters Due To Treatment With Methanol Extracts Of *Commelina Clavata* Against CCl_4 Induced Hepatotoxicity In Rats

TREATMENT	SGOT(IU/ml)	SGPT(IU/ml)	SALKP(IU/ml)	T. B(mg/dl)
Silymarin50mg/kg	89.63	93.8	92.99	94.16
CCM100mg/kg	33.77	39.5	60.68	53.33
CCM200mg/kg	67.62	57.64	79.48	69.16
CCM400mg/kg	77.92	81.46	83.76	75.83

parameters in case of standard drug Silymarin(50mg/kg.p.o) in CCl_4 intoxicated rats revealed a significant reduction ($p<0.01$) in the levels of SGOT(89.63%), SGPT(93.8%), ALKP(92.99%) and T.B(94.16%).

In Groups: 4-6 treated with methanol extract of ***Commelina Clavata*** at doses of 100, 200 and 400mg/kg; p.o respectively, there is significant decrease in SGOT, SGPT, SALKP and total bilirubin levels when compared to Group-2 rats. The activity of the extracts is found to be dose dependant.

The comparative efficacy of the extracts tested for their hepatoprotective activity, the relationship between dose and percentage reduction in each case were depicted in the form of a bar diagram as shown.

Carbon tetrachloride (1ml/kg.i.p) intoxication in normal rats produced elevated levels of serum biochemical parameters significantly SGOT (160.5 ± 0.62, 295.5 ± 0.39), SGPT(96.95 ± 1.34, 269.5 ± 1.8), ALKP(179.5 ± 0.99, 296.5 ± 1.45), T.B(0.82 ± 0.06, 2.02 ± 0.03) indicating acute hepatocellular damage and biliary obstruction. The percentage reduction of various serum biochemical parameters in case of standard drug Silymarin(50mg/kg.p.o) in CCl_4 intoxicated rats revealed a significant reduction ($p<0.01$) in the levels of SGOT(89.63%), SGPT(93.8%), ALKP(92.99%) and T.B(94.16%).

When compared to the CCl_4 toxic control group, the group treated with the methanol extracts of ***Commelina Clavata*** at doses of 100, 200 and 400mg/kg; p.o in CCl4 intoxicated rats exhibited a significant reduction ($p<0.01$) of SGOT(31.40%, 47.55%, 61.55%), SGPT(23.87%, 32.15%, 45.88%), ALKP(42.73%, 50.59%, 55.72%) and T.B(44.99%, 54.16%, 64.99%) levels respectively.

Though the extract were recorded with significant hepatoprotective activity with same "p" valu ($p<0.01$). The methanol extract was found to be more potent because of effect on percentage reduction in elevated levels of biochemical parameters and effect was dose dependant. The effect of methanol extract of whole plant of *Commelina clavata* on CCl_4 induced liver damage in rats with reference to biochemical changes in serum was shown. Percentage decrease or increase was calculated by Histopathology of liver tissues. Group I (vehicle control)—section shows central vein surrounded by hepatic cord of cells (normal architecture). Group II (toxic control)—section shows

patches of liver cell necrosis with inflammatory collections, around central vein. Group III (standard silymarin)—almost near normal. Group IV (CCM 100mg/kg)— inflammatory collections around central vein and focal necrosis. Group V (CCM 200mg/kg)— inflammation decreasing around central vein. Group VI (CCM 400mg/kg)— less inflammatory cells around central vein, absence of necrosis (Fig-1).

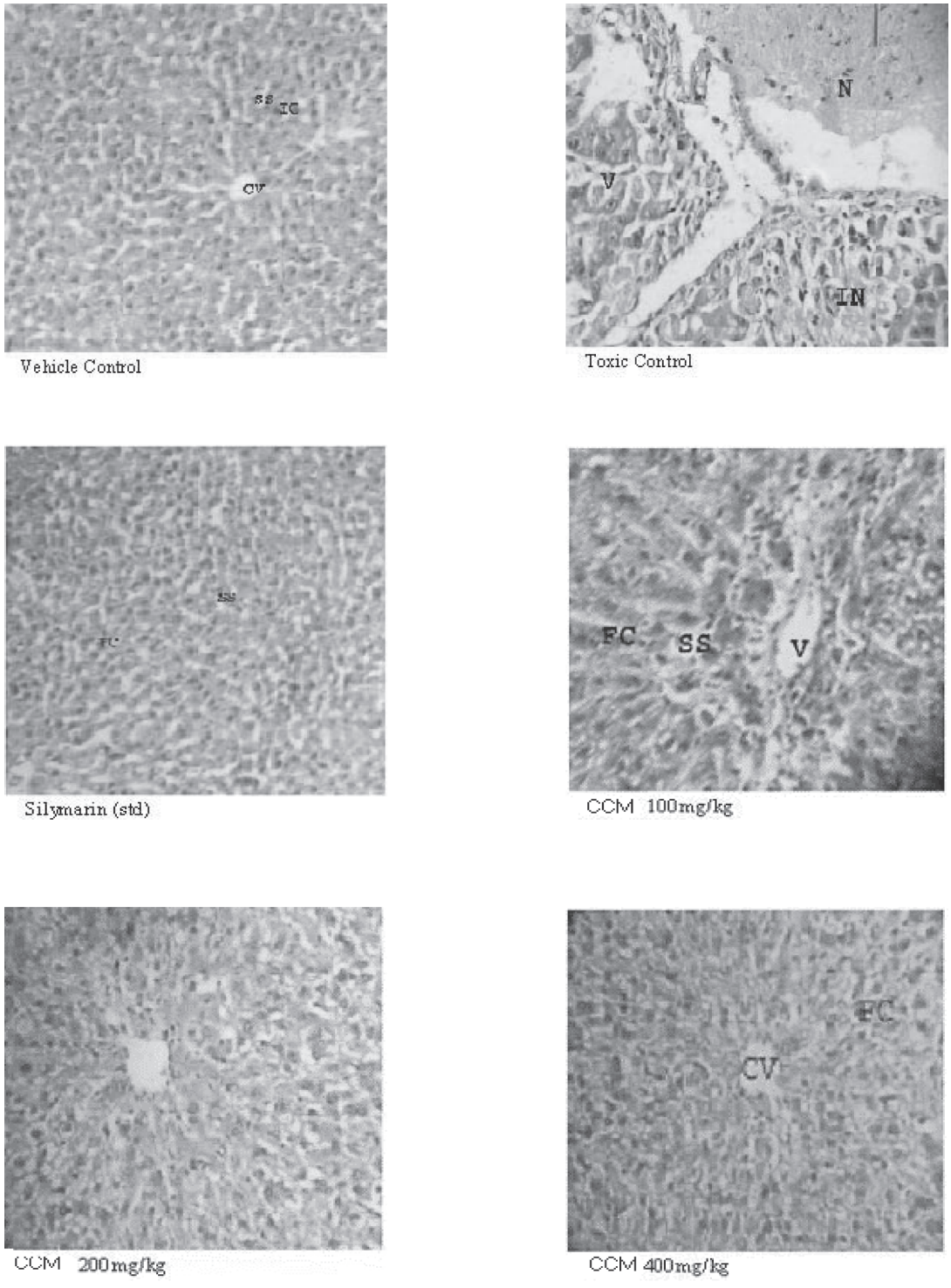

Figure-1: Representative photographs of histopathological changes showing effect of the test material on the rats intoxicated with carbon tetrachloride. CCM- *Commelina Clavata* methanol extract.

Conclusion

The efficacy of any hepatoprotective drug is essentially dependent on its capability of either reducing the harmful effects or in maintaining the normal hepatic physiological mechanism, which have been imbalanced by a hepatotoxin.

Orally administered doses of 100, 200 and 400mg/kg of methanol of whole plant of *Commelina Clavata* produced significant decrease in SGOT, SGPT, SALKP and total bilirubin levels. The activity of the extract is found to be dose dependant. In CCl_4 induced toxic hepatitis, toxicity begins with the changes in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures [12]. Administration of methanolic extracts of *Commelina Clavata* showed recovery against the toxic effects of CCl_4. The hepatoprotective effect of the drugs was further concluded by the histopathological examinations of the liver sections which reveal that the normal liver shape was disturbed by hepatotoxin intoxication. In the liver sections of the rats treated with methanolic extract and intoxicated with CCl_4 the normal cellular shape was retained as compared to silymarin, thereby confirming the protective effect of the extract of *Commelina Clavata.*

The hepatoprotective activity of *Commelina Clavata* could be due to the presence of triterpenoids which have hepatoprotective properties [13-15]. The result of this investigation indicated that the methanolic extract of whole plant of *Commelina Clavata* possess hepatoprotective activity against CCl_4 induced liver damage in rats. Attempts are being made to isolate and characterize the active principle to which the hepatoprotective activity can attribute.

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Isolation and Structure Confirmation of Piperine from *Piper Trioicum Roxb*

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ABSTRACT:

Many plant derived molecules have shown a promising effect in therapeutics. Among the plants investigated to date, one showing enormous potential is the Piperaceae. Piperine is an alkaloid found naturally in plants belonging to the pyridine group of Piperaceae family. It is widely used in various herbal cough syrups and it is also used in anti-inflammatory, anti malarial, anti leukemia treatment. The present study was sought to isolate piperine from *Piper trioicum* ROXB, its structure confirmation by spectral analysis and its hydrolysis to piperic acid.

Key Words: piperine, anti-inflammatory, anti-analgesic, anti malarial, anti leukemia, *piper trioicum Roxb*

Introduction

Plants have been the source of medicines since thousands of years. Species of the genus *Piper* are among the important medicinal plants used in various systems of medicine[1, 2] The plant family Piperaceae is a source of many biologically active phytochemicals[3,4] with great potential for medicinal[5] and agricultural use[6]. The genus *Piper* is from the Piperaceae family which has over 700 species distributed in both hemispheres. Species of *Piper* contain a wide array of secondary metabolite compounds, principally alkaloids and amides[3]. '**Piperine'** is one of the major alkaloid present in most of the piper species. Its benefits are substantial as it enhances the assimilation of other key nutrients including beta-carotene, selenium, vitaminB_6, curcumin, amino acids, and even blood sugar (glucose). Scientific research has yielded preliminary evidence which suggest that piperine may aid in the digestion of food. Studies have proven that piperine does increase thermogenic activity as it increases the body's metabolic rate, which does help weight loss. The substance may also have some anticonvulsant, anti carcinogenic and anti-inflammatory properties[7].

***Piper Trioicum* ROXB** belongs to Piperaceae family, distributed in South Asian countries. The whole plant is used as rubefacient, diuretic, hepatoprotective and used for diabetes, muscular pains, headache, toothache and cholera in folk medicine; the root is used as diuretic[8]. The present work has been carried out in continuation of our previous work on *Piper Trioicum* ROXB. This paper reports the isolation of piperine **(Fig- 1)** from the seeds of *Piper Triocum* ROXB, its hydrolysis to piperic acid (**Fig-2**) and piperidine hydrochloride (Fig-**3**).

Materials And Methods

Plant material

Dried fruit of *Piper Trioicum* ROXB was collected from Andhra Pradesh, India, and authentified by Asst. Prof. Dr. K. Madhava Chetty, Dept. of Botany S.V.University, Tirupathi. A. P.

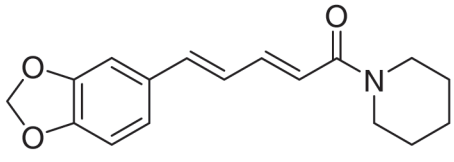

Fig-1 piperine

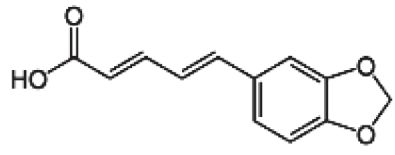

Fig-2 piperic acid

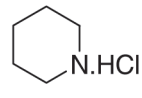

Fig-3 piperidine Hydrochloride

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Preparation of the extract (EEPT)

About 100g of powdered fruits material of P*iper trioicum Rpxb* was taken into a clean, round bottomed flask, 400 ml of 95% ethanol was added to it and refluxed for 85-90mins. Solid was filtered by using Buchner funnel, and then it was taken into the same R.B flask. The above procedure was repeated two times with another 800ml of fresh Ethanol. The whole filtrate (ethanol extract) obtained was evaporated using rotary evaporator. It rendered a gummy concentrate of reddish brown color. The gummy concentrate was designated as crude extract of ethanol (EEPT). The extract was transferred into a closed container for further use.

Identification test

The piperine was subjected on to the precoated silica gel TLC plates. The mobile phase is Hexane: Ethyl acetate in 4:1 ratio. After the TLC run the yellow spot (**Fig- 4**) of piperine were identified visually as well as in UV-visible light. Rf value was calculated[9].

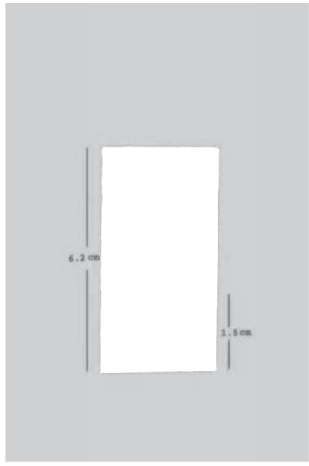

Fig-4 Rf value of isolated piperine 0.24

Results and Discussion

Piperine is 1-2E, 4E-piperinoyl piperidine (**Fig- 1**) with two trans double bonds in the chain containing the methylenedioxyphenyl and piperidine groups. In the present study it was isolated as yellow needles, with melting point 126-128^{0}C (literature 130^{0}C). After isolation it is identified by TLC. The standard Rf- value of piperine from the literature was 0.25. The Rf- value of purified piperine from TLC was found to be 0.24. So it was confirmed that the product obtained from the *Piper Triocum* ROXB contains piperine (**Fig- 1**). The structure was further confirmed on the basis of the spectral data (FTIR, ^{1}H-NMR and MS) which are in agreement with the published data [10, 11].

FTIR spectral analysis

FTIR spectrum (**Fig- 5**) was run by using conventional KBr pellet method from 4,000-600cm^{-1}. It's characteristic peaks are at 3,066-3,008cm^{-1} (aromatic C-H stretching);

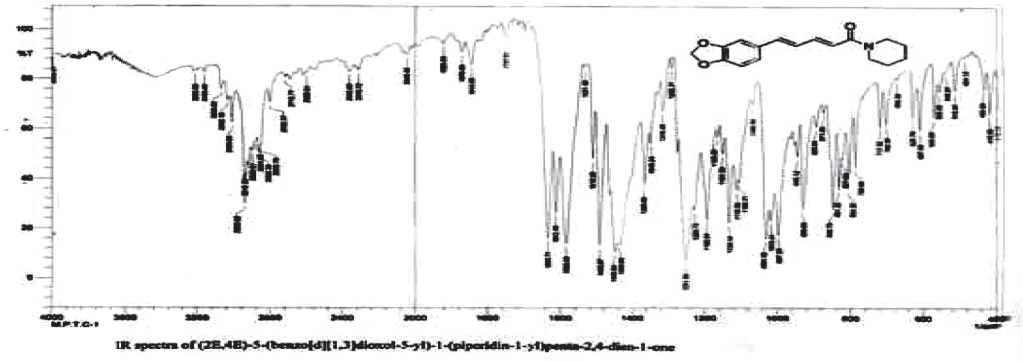

Fig- 5 IR spectrum of piperine

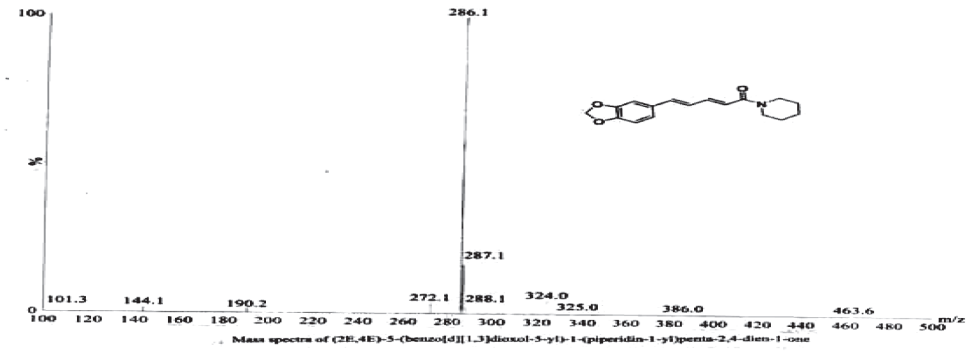

Fig- 6 MS spectrum of piperine

symmetric and asymmetric stretching of C=C diene at 1633, 1612; aromatic stretching of C=C(benzene ring) 1612, 1583, 1490; stretching of –CO-N at 1633, for ethylene dioxy group asymmetric, symmetric stretching of CH_2 and aliphatic stretching comes at 2939, 2850$cm^{-1}$and the most characteristic C-O stretching comes at 930cm^{-1}.

Mass Spectra

ESI-MS *m/z* in positive-ion mode for the piperine peak gave a quasimolecular ion at *m/z* 286 (100%) $[M + H]^+$ **(Fig- 6)**.

^{1}H-NMR analysis

^{1}H-NMR spectrum (**Fig- 7**) of piperine exhibited a methylene type signal at d 6.75 (1H, s). The geometry of the double bond on C2-3 and C4-5 was also presumed as E or Z relation based on the characteristic IH-1H spin-coupling constants. The coupling constants of the olefinic protons (on C2 and C5) indicated a 2E, 4E configuration for 4 (J= 14.6; 15.5). Peaks obtained were listed below in **Table-1.**

Experimental

Melting points were determined in open capillaries and are uncorrected. The purity of all the compounds was routinely checked by TLC on silica gel coated plates. IR spectra was recorded in KBr pellets. ^{1}H-NMR spectra on a Varian 400 MHz instrument with TMS as internal standard, chemical shifts are expressed in δ ppm and Mass spectra on a Hewlett Packard Mass spectrometer operating at 70eV.

Piperine isolation and purification:

100g of *Piper Triocum* ROXB seeds powder and 400ml ethanol was taken into a 1000ml R.B flask at room temperature. It was heated to reflux for 90 minutes, and the solid was filtered by using Buchner funnel. The clear brown colour filtrate was kept aside, the above same procedure was repeated two times with the left over solid by using 800 ml (400 x 2) of ethanol and the solid was filtered out. Combined the ethanol extracts and concentrated under reduced pressure at below 50^{0}C. Reddish Brown

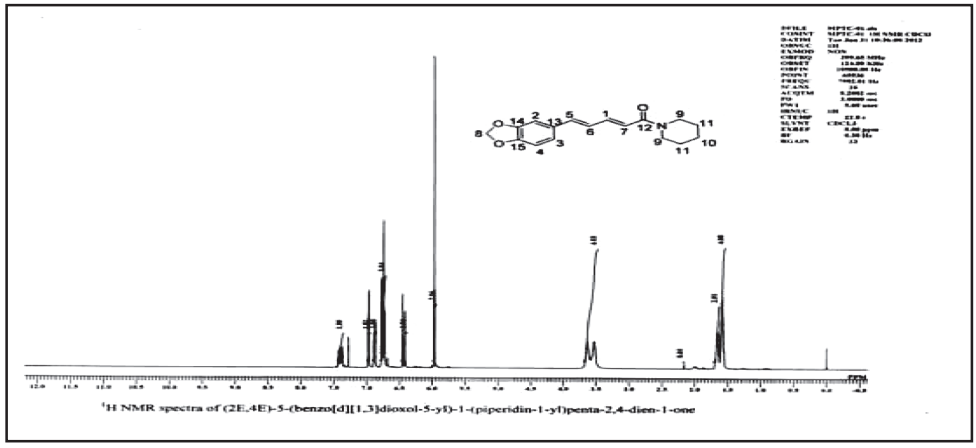

Fig- 7 ^{1}H-NMR spectra of piperine

Table- 1

^{1}H-NMR values of piperine

d ppm	multiplicity	Number of H	assignment
1.49-1.54	m	4	11-H
1.56-1.59	m	2	10-H
3.45-3.56	m	4	9-H
5.90	s	2	8-H
6.36	d	1	7-H
6.66	d	1	6-H
6.67	d	1	5-H
6.70	d	1	4-H
6.80-6.82	dd	1	3-H
6.90	m	1	2-H
7.30-7.36	dd	1	1H

colour oily residue (EEPT) was obtained (15g), this crude material was fractionated on a silica gel column, eluting with hexane + diethyl ether (1+2 by volume).the fractions obtained were combined according to their similarities as analysed by thin layer chromatography (TLC) and this led to the isolation of 4g piperine.

M.P: -126-128^{0}C.

IR: - 3,066-3,008; 2939, 2850; 1633, 1612; 1583, 1490; 930cm^{-1}

^{1}**H-NMR**:- d 1.49-1.54 (m, 4H), 1.56-1.59 (m, 2H), 3.45-3.56(m, 4H), 5.90(s, 2H), 6.36(d, 1H, J=14.6Hz), 6.66(d, 1H, J=15.5), 6.67(d, 1H, J=10.2),6.70(d, 1H, J=1.7),6.80-6.82(dd, 1H, J=8.0, 1.7), 6.90(m, 1H),7.30-7.36 (dd, 1H, J=14.5, 10.2Hz).

MS: - 286[M+H$^+$]

Hydrolysis of Piperine to Piperic acid and Piperidine hydrochloride

1g piperine and 10mL 10% alcoholic potassium hydroxide are placed in a 100ml RB flask and refluxed for 90min. The ethanolic solution was evaporated to dryness under reduced pressure at below 50^{0}C, and then it was cooled in an ice-salt bath. The solid potassium piperinate was suspended in hot water and acidified with 2N HCl, it was cooled for 30mins then the yellow precipitate was collected on a Buchner funnel, washed with minimum amount of ice-cold water, and recrystallized from ethanol to yield piperic acid (0.3g) as yellow needles with Mp 216-217°C (lit:218^{0}C). The strongly basic ethanolic distillate in the receiver is saturated with hydrochloric acid and evaporated to dryness to give piperidine hydrochloride, which melts at 244°C after recrystallization from ethanol.

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