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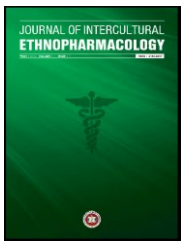

Original Research

Drosera indica L: Potential effect on liver enzyme, lipid profile and hormone change in Dalton's lymphoma ascites (DLA) bearing mice

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Keywords:
D. indica L, hormone, liver enzyme, lipid profile changes, Dalton's lymphoma ascites (DLA) cells

Abstract
Aim: In this study, the ethanol and aqueous extracts of *Drosera indica* L were prepared and cancer induced liver enzyme, lipid profile and hormone changes were studied in mice using the Dalton's lymphoma ascites (DLA) cells.
Method: Animals were divided into seven groups as the normal control, DLA control, standard (5FU) and the ethanol and aqueous extracts (250 and 500mg/kg each) of *D. indica* L + DLA (four groups) were given the respective treatments 24 h after tumor cell inoculation, for 14 days.
Result: Both ethanol and aqueous extracts of *D. indica* L at doses of 250 and 500mg/kg extracts showed significant ($p<0.001$) effects on the elevated liver enzyme, lipid profile and hormonal changes to normal.
Conclusion: The results of the present study demonstrated that both extracts were able to normalize the cancer induced liver enzyme, lipid profile and hormone changes in DLA bearing mice.

INTRODUCTION

A number of natural products have been studied for anticancer activity on various experimental models. This has resulted in the availability of nearly 30 effective anticancer drugs [1]. The aim of naturopathic cancer treatment is to support normal metabolism, decrease side effects of treatment and boost the body's immune system. These treatments also provide strategies for long term health maintenance and improve energy, well-being and overall quality of life [2]. In certain cancer therapies there is an increased risk of development of characteristics metabolic syndrome. It will be important to evaluate cancer therapy itself to overcome a risk factor for the development of metabolic syndrome. Metabolic syndrome often associated with elevated triglycerides, reduced high density lipoprotein (HDL), low testosterone levels and overall connection with sex hormones [3]. These problems were overcome by plant drug treatment. Ayurvedic, a system of Indian medicine in which the treatment involves the use of whole plant extracts either alone or a combination of several plant extract for better efficacy and reduction in toxicity. Each herbal formulation contains multiple active principles that may operate synergistically, producing therapeutic benefits and lowering the risks on adverse effects [4].

Drosera is a cosmopolitan genus of insectivorous plants and consists of approximately 170 species. In India, *D. indica* L., *D. burmannii* and *D. peltata* J.E.Sm. ex Wild have been reported from many different locations. These species are used as vital components in an Ayurvedic preparation called 'Swarnabhasma' (Golden

ash). Macerated *D. indica* is a vital components in an Ayurvedic preparation called 'Swarnabhasma' [5, 6]. In Thai traditional herbal remedy, *D. indica* L., has been commonly used for treatment of stomache, eczema, and hepatitis [7]. The major naphthoquinone found in *D. indica* is plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) [8]. This present study was carried out to evaluate the effect of *D. indica* on development of metabolic syndrome by treatment with ethanol and aqueous extract of *Drosera indica* L., in Dalton lymphoma ascites (DLA) bearing mice mice.

MATERIAL AND METHODS

Plant material

The whole plant of *D. indica* L was collected from the forests of Savanadurga, Karnataka, India during December 2010. The plant material was identified and authenticated by Dr. S.N.Yoganarasimhan, Taxonomist and Research Coordinator at M. S. Ramaiah College of Pharmacy, Bangalore, Karnataka, India. The material was washed with tap water, shade dried, powdered, passed through sieve no. 60 and stored in air tight containers for further experiments.

Preparation of the extracts

Alcoholic extract: A weighed quantity of the air-dried powdered drug was extracted with ethanol (90 %v/v) in a Soxhlet apparatus. The extract was concentrated in a rotary flash evaporator at a temperature not exceeding 50ºC. The ethanol extract was suspended in distilled water for experimental purposes.

Aqueous extract: The marc from the ethanol extract was macerated with chloroform- water for 24h to obtain the aqueous extract. Aqueous extract was concentrated under vacuum and dissolved in distilled water for experimental studies.

The ethanol (EEDI) and aqueous (AEDI) extracts of *D. indica* L were stored in air tight containers.

Acute toxicity studies

Acute toxicity study was carried out on EEDI and AEDI following OECD guidelines (OECD 423) [9].

Induction of cancer using DLA cells

Dalton lymphoma ascites (DLA) cells were supplied by Amala Cancer Research Center, Trissur, Kerala, India. The cells maintained (*in vivo)* in Swiss albino mice by intraperitoneal transplantation ($2X10^6$ cells/ mouse) for 7 days.

Treatment protocol [10, 11]

Mature male and female (virgin) Swiss Albino mice weighing 20-25g (n=10) were kept in identical laboratory condition and were fed with standard pellet diet and water *ad libitum.* Study protocol was approved by the Institution Animal Ethical Committee (Protocol.No.A.Raju 0903PH2254/ JNTUH 2009). They were divided into seven groups as Normal group (G1), DLA control group (G2), DLA + 20mg/kg of 5-Fluorouracil treated group (G3), 250,500mg/kg of EEDB (G4 and G5) and 250 and 500mg/kg AEDB (G6 and G7) of ten each and used for the study. The DLA cells were injected intraperitoneally ($2X10^6$ cells/ mouse) to all groups of animals except G1. On the second day the animals of G3 with 5- fluorouracil (20 mg/kg, i.p), G4 and G5 were treated with 250 and 500 mg/kg of EEDB and G6 and G7 with 250 and 500mg/kg of AEDB orally. The treatment was continued for 14 days. G1 was treated with vehicle.

On day 15, the mice were sacrificed before that blood was withdrawn by retro - orbital plexus method and the following parameters were measured.

Assay of Hormones [12, 13]

Hormones such as LH, FSH, E2and progesterone levels in virgin female mice blood were measured by RIA method using commercially available standard kits according to the manufacturer's instruction (Sigma, US)

Lipid profile [14]

Cholesterol, triglyceride, HDL cholesterol was estimated using kits from Agappe Diagnostics, Kerala, India. The estimation was carried out on fully automated analyzer Hitachi 717(Italy)

Liver marker enzymes [15]

Serum enzymes such as Aspartate amino Transferase (AST), Alanine amino Transferase (ALT), Alkaline Phosphatase (ALP) and Lactate dehydrogenase (LDH) were analyzed using Agappe Diagnostics, Kerala, India.

Statistical analysis

The results are expressed as mean ± S.E.M. The evaluation of the data was performed using one way ANOVA followed by Newman-Keul's multiple comparison test; $p< 0.05$ implied significance.

RESULT

Acute toxicity studies

Ethanol and aqueous extract of *D. indica* L were administered separately up to 3000 mg/kg body weight and since these extracts did not produce any toxic manifestation like increased motor activity, salivation, acute convulsion, coma and death. Hence they were considered safe for further pharmacological screening.

Table 1. Effect of EEDI and AEDI on Liver enzyme of DLA bearing mice

Parameters	AST (U/l)	ALT (U/l)	ALP (U/l)	LDH (U/l)
Normal	70.55±0.93	25.1±0.17	0.98±0.05	293.4±12.63
DLA control	134.9±2.76	16.33±1.48	5.7±0.1	563.3±19.19
DAL+5FU (20mg/kg)	67.18±1.9	24.1±0.27	1±0.09	297.97±6.32
DLA+EEDI (250mg/kg)	107.38±4.6[a]	23.5±1.01[a]	3.13±0.05[a]	373.2±28.4[a]
DLA+EEDI (500mg/kg)	68.7±1.1[a]	23.7±0.45[a]	0.93±0.06[a]	310.9±3.45[a]
DLA+AEDI (250mg/kg)	117.2±1.76[a]	21.15±0.26[a]	4.05±0.06[a]	462.5±6.87[a]
DLA+AEDI (500mg/kg)	87.62±1.94[a]	22.4±0.36[a]	2.3±0.17[a]	386.4±6.32[a]

The data were expressed as mean ±S.E.M. n = 10. The data analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keul's multiple comparison test
a- ***p<0.001, compared to the DAL control group

Table 2. Effect of EEDI and AEDI on Hormone level of DLA bearing mice

Parameters	LH (ng/ml)X10^{-2}	FSH (ng/ml)	E2 (β-estradiol) pg/ml	Progesterone (ng/ml)
Normal	20±1.74	6.7±0.23	24.1±1.32	15.5±0.14
DLA control	35.6±2.2	1.63±0.2	5.33±0.15	5.05±0.34
DAL+5FU (20mg/kg)	16.42±0.51	6.27±0.32	20.53±1.11	14.82±0.23
DLA+EEDI (250mg/kg)	24.07±0.9[a]	4.1±0.09[a]	12.83±1.37[a]	10.57±0.19[a]
DLA+EEDI (500mg/kg)	23.95±0.96[a]	5.57±0.17[a]	21.72±0.58[a]	15.27±0.49[a]
DLA+AEDI (250mg/kg)	23.4±1.85[a]	2.95±0.23[a]	10.75±1.01[b]	8.67±0.21[a]
DLA+AEDI (500mg/kg)	22.57±1.1[a]	3.95±0.06[a]	12.9±1.38[a]	12.82±0.39[a]

The data were expressed as mean ±S.E.M. n = 10. The data analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keul's multiple comparison test
a- ***p<0.001, compared to the DAL control group
b- **p<0.01, compared to the DAL control group

Table 3. Effect of EEDI and AEDI on Lipid profile of DLA bearing mice

Parameters	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL(mg/dl)	LDL(mg/dl)
Normal	152.7±5.3	95.58±1.3	34.6±0.4	117.7±1.2
DLA control	118.3±3.08	127.8±0.7	21.5±0.8	79.5±2.6
DAL+5FU (20mg/kg)	142.9±0.9	89.05±1.5	32.2±1.1	115.1±0.9
DLA+E EDI (250mg/kg)	135.3±1.4[b]	98.35±0.6[a]	30.5±0.9[a]	116.7±0.7[a]
DLA+EEDI (500mg/kg)	148.05±2.0[a]	94.75±0.8[a]	34.9±0.8[a]	119.5±0.4[a]
DLA+AEDI (250mg/kg)	128.7±3.8[c]	114.1±0.7[a]	28.6±0.5[a]	104.7±1.1[a]
DLA+AEDI (500mg/kg)	144.4±0.9[a]	103.5±1.3[a]	30.9±0.9[a]	112.05±1.5[a]

The data were expressed as mean ±S.E.M. n = 10. The data analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keul's multiple comparison test
a- ***p<0.001, compared to the DAL control group

Administration of daily doses of 250, 500 mg/kg for 14 days altered the serum liver enzyme, as shown in Table 1. There is an increased AST, ALP and LDH and there is a decreased ALT in DLA bearing mice. Treatment with both the doses of EEDI and AEDI significantly ($p<0.001$) reduced the elevated levels of the altered parameters to normal level.

The serum hormone levels in virgin female mice were greatly altered in DLA bearing mice (Table 2). Continuous 14 days oral administration of 250 and 500mg/kg of EEDB and AEDB restored the hormone level near to normal in female virgin mice.

The serum Cholesterol, HDL and LDL cholesterol significantly ($p<0.001$) decreased in DLA control mice and were restored by the doses of EEDI and AEDI treatment. Serum triglycerides values were significantly elevated in DLA control mice and were brought back to normal value by the extract treatment (Table 3).

DISCUSSION

The present study was carried out to evaluate the extracts of *D. indica* L on liver enzyme, lipid profile and hormone changes in DLA bearing mice, doses of 250 and 500 mg/kg of EEDB and AEDB were given orally for 14days to DLA bearing mice.

Endogenous level of sex steroids and gonadotrophic hormones and increased receptor population are associated with caner development [12]. The steroids which are likely to influence tumorigenesis, are namely estrogen, progesterone. The steroids are synthesized and secreted mainly from ovaries and adrenal glands on the stimulation of follicle stimulating hormone (FSH) and lutenizing hormone(LH), are involved in regulation of steroidogenesis. In this study DLA-bearing virgin female mouse showed altered hormone level which was brought back to normal.

Since liver is considered to be the main organ of drug detoxifying organ, some liver marker enzyme levels were measured from serum. AST, ALP and LDH levels were increased in DAL controlled mice, whereas ALT level was decreased. Yet there is no explanation has been given such changes but Abu Sienna et al., 2003 suggested that , the consumption of free amino acid for building the proteins of rapidly dividing tumor cells might result in the disturbance of the enzyme activity in the liver. On treatment with 250, 500mg/kg of EEDI and AEDI, altered liver enzyme level was restored as that of the normal group. It was compared with DAL control group, this result indicating that the plant extracts play a protective role on the liver.

Several studies have reported clear relationship between low cholesterol levels and cancer [14,16,17].Alterations of cholesterol metabolism, including increased cholesterol synthesis and accumulation of cholesterol esters in tumor tissues associated with a decrease of high density lipoprotein cholesterol in serum, were previously observed in different models of neoplastic cell proliferation including haematological malignancies [18].In our study showed that the serum cholesterol, HDL cholesterol and LDL cholesterol showed significantly decreased in DLA control mice which was restored by the doses of 250,500 mg/kg of EEDI and AEDB treatment, whereas the serum triglycerides values were significantly elevated in DLA control mice and are brought back to normal value by the extract treatment.

CONCLUSION

Results concluded that the ethanol and aqueous extract of *D. indica* L was effective in inhibiting the tumor growth (reported) in DLA-bearing mice. The results of the present study demonstrated that both extracts normalized the cancer induced metabolic change and lipid profile. The higher dose of ethanol extract showed a significant good activity when compared with the lower dose, similarly higher dose of aqueous extract showed better activity than lower dose but ethanol extract was comparatively better than aqueous extract.

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Original Research

Nephroprotective effect of Corn Silk extract on oxalic acid-induced nephrocalcinosis in rabbit model

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Keywords: Nephrocalcinosis, Oxalic acid, Renal tubular acidosis, Saponins, Zea Mays

Abstract

Background: Nephrocalcinosis is a state of deposition of calcium phosphate or oxalate in the renal parenchyma. It may occur in patients with renal tubular acidosis, vitamin D intoxication, and hyperparathyroidism. Corn silk was used in traditional Chinese medicine to relieve renal pains. Aim: To evaluate the effect of Corn silk aqueous extract in reducing calcium deposits from renal parenchyma in oxalic acid-induced nephrocalcinosis model.

Methods: Fourteen healthy rabbits were allocated to two groups. Two hours before induction of nephrocalcinosis, one group received water and the other received aqueous extract of corn silk and continued feeding for ten days. Blood samples were collected for biochemical analysis before induction and in the fifth and tenth post-induction day. Urine samples were taken to estimate urinary ca+2 levels and crystals. The histopathological examination was carried to check for crystal deposits in renal tissues.

Results: Corn silk aqueous extract produced a significant reduction of blood urea nitrogen(5.2±0.08 vs 7.3±0.2) mmol/l, serum creatinine (85.9±0.2 vs 97.3±0.5) mmol/l and serum Na+ levels (137±0.2 vs 142.16±0.7) mmol/l with non-significant reduction in serum K+ (4.0±0.02 vs 4.2±0.05). There is a significant reduction in calcium deposition in renal parenchyma in comparison to the control group after ten days of treatment.

Conclusion: Corn silk had a significant diuretic effect that accelerates the excretion of urinary calcium.

INTRODUCTION

Nephrocalcinosis (NC) is a state of deposition of calcium in the form of phosphate or oxalate in the renal parenchyma that can impair kidney function [1]. It is liable to occur in patients with renal tubular acidosis, hyperparathyroidism, vitamin D intoxication and healing of renal tuberculosis [2] The fragments of calcium salts may break freely from the kidney to provide nuclei for the formation of different types and sizes of stones [3].

In traditional Chinese medicine, some medicinal plants like corn silk, barley and celery were used to relieve renal pains [4]. Corn silk resembles soft threads of either light green or yellow-brown in color. It contains [5]: proteins, carbohydrates, volatile oils, steroids such as sitosterol, saponins, and flavonoids. Extract of corn silk showed an anti-oxidative [6] and anti-TNF activity [7].

Few studies were made determining the diuretic effect of Barley [8] and Celery [9], but withoutavailable data to evaluate the possible nephroprotective effect of corn silk.Therefore, this study was made to evaluate this potential effect in an experimental model of Nephrocalcinosis.

MATERIALS AND METHODS

Fourteen local domestic healthy rabbits weighing 900 to 1200 grams were used in this study, which was approved by Animal Ethics Committee of the college of pharmacy, Al-Yarmouk University (Approval No.AEC/31/10/CPAYU). The rabbits were supplied by the animals' house of college of medicine. They were housed in separated cages, which were provided with a wide wire mesh floor at a controlled temperature of 27±2°C with a 12-hour light/dark cycle. They were fed standard oxoid pellets and water *ad libitum*.

The animals were allocated to two groups(seven animals in each) and were given the following as a single daily dose for 10 days (at 9 a.m.) ;

G1 (control group) -received 3 ml/kg of distilled water

G2 -received 1gm/kg of aqueous extract of Corn silk [10]

At 9 a.m. of 10^{th} day, the last doses were given and the rabbits were fasted for 24 hours.

At 11a.m. of next day, all animals were given 333 mg/kg of oxalic acid (H&W England) as a single dose per gastric tube for induction of NC [11]

Blood samples were collected from a marginal ear vein for biochemical analysis and renal functions, before induction of NC to determine the normal values of blood urea nitrogen (BUN), serum creatinine, Na^+ and K^+ using spectrophotometer and in the 1^{st} , 5^{th} and 10^{th} day after induction. Urine samples were taken from the animals by catheterization after anesthetizing them in the last day of the study to determine the urinary Ca^{+2} levels and present crystals. The histopathological examination was carried to check for crystal deposits in renal tissues by using a polarized microscope after fixation and staining the specimens [12] The obtained results were collected for analysis and assessment. Significance was set at $P<0.05$.

RESULTS

The results of this study revealed significant elevation in the levels of BUN, both serum creatinine and K^+ with significant reduction of serum Na^+ levels in the control group as compared to the levels of pre-induction state [Table 1].

Table 1. Mean BUN, S. creatinine, K+, Na+ and urine Ca+2 levels of the studied animals measured before induction of NC

Analyte	Measured levels (mmol/L)
BUN	4.0±0.07
S. creatinine	65.0±8.9
S. K+	3.3±0.8
S. Na+	160.0±4.0
Urine Ca^{+2}	2.05±0.07

The results of Zea Mays extract (group 2) showed significant reduction of BUN levels (5.2 ± 0.08 *vs.* 7.3 ± 0.2) mmol/L, serum creatinine (85.9 ± 0.2 *vs.* 97.3 ± 0.5) mmol/L with $P<0.05$, and insignificant reduction in serum K^+ (4.0±0.02*vs* 4.2±0.05) mmol/l with significant decrease in serum Na^+ levels (137 ± 0.2 *vs.* 142.16 ± 0.7) mmol/L with $P< 0.05$ in comparison to the control group after one day and ten days when results became more evident [Table 2-3].

Table 2. Mean BUN and Serum creatinine levels of the studied groups measured after induction of NC

Group	BUN (mmol/L)			Creatinine (mmol/L)		
Agent/Dose	After 1 day	After 5 day	After 10 day	After 1 day	After 5 day	After 10 day
Oxalic acid 333mg/kg	7.3±0.2	7.9±1.1	9.6±0.2	97.3±0.5	99.5±0.8	100.3±1.2
Corn silk 1gm/kg/day	5.2 ± 0.08	5.1±0.3	5.0± 0.03	85.9± 0.2	81.7±0.3	77.7± 0.09

Table 3. Mean Serum K^+ and Serum Na^+ levels of the studied groups measured after induction of NC

Group	Serum K^+ (mmol/L)			Serum Na^+ (mmol/L)		
Agent / Dose	After 1 day	After 5 day	After 10 day	After 1 day	After 5 day	After 10 day
Oxalic acid 333mg/kg	4.2± 0.055	1±0.24	5.7± 0.08	142.16 ±0.7	139.3±1.0	137.6 ± 0.4
Corn silk 1gm/kg/day	4.0± 0.023	9±0.1	3.8± 0.08	137 ± 0.2	136.1±0.7	134.3±0.4

Table 4. Mean urine Ca^{+2} levels of the studied groups measured 10 days after induction of NC

Group	Urine Ca^{+2} levels (mmol/L)	Crystals in urine examination
- (negative control)	2.05±0.07	fewamorphous urate
1 (Oxalic acid)	4.11±0.03	amorphous urate, uric ac stellar phosphate, calcium oxalate id
2 (Oxalic acid + Corn silk)	5.06±0.08amorphous urate	

Examination of urine samples at the end of the experiment (10 days) showed that there was a significant elevating effect of corn silk in urinary Ca^{+2} excretion than normal values [12] and a significant reduction of its concentration in renal parenchyma after histological examination of renal tissue samples [Table 4] [Figures 1-2].

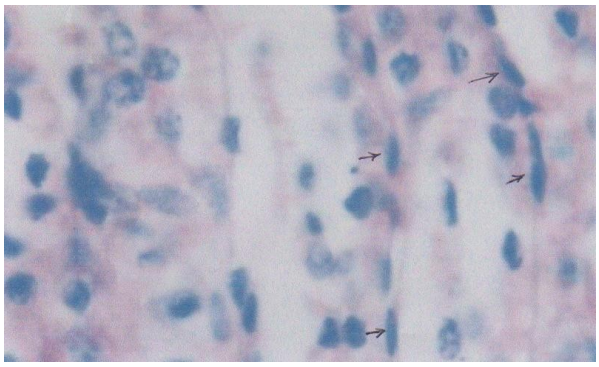

Figure 1.heavy calcium oxalate crystals deposition in the renal tissues of rabbits receiving oxalic acid. X100

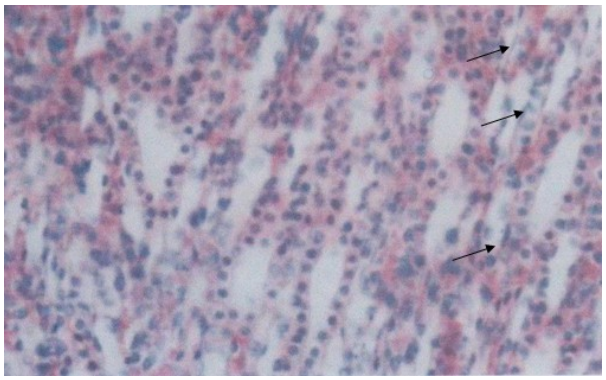

Figure 2. Mild calcium oxalate crystals deposition in the renal tissues of rabbits receiving oxalic acid and Corn silk. X100

DISCUSSION

Oxalic acid (a highly oxidizing and strong Ca^{+2} chelator) was used for induction of NC using a single large dose (333mg/kg) [11] In this model of NC, there was a significant elevation in BUN and serum creatinine levels observed after induction of Nephrocalcinosis [Tables 2 and 3]. The idea to use some herbals like corn silk is to evaluate its effect in improvement of NC. Corn silk contains many active ingredients like flavonoides, volatile oil, saponins, and allantoin besides some minerals like calcium, potassium, magnesium and sodium. It produced a significant lowering effect in BUN and serum creatinine levels with an increase of urinary Ca^{+2} levels than normal values [12] and a reduction of its concentration in renal tissue [Figures 1-3]. Because of its high concentration of potassium, it becomes a powerful diuretic, which encourages the body to flush out toxins by increasing urinationand Ca^{+2} excretion [13] Unlike other diuretics, the high level of potassium offsets the potassium loss normally caused with increased urination when in use [14]

CONCLUSION

Zea mays has a significant diuretic and attenuating effect in reducing calcium deposits from renal tissues.

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Original Research

Comparative study of the analgesic activity of two Iraqi medicinal plants, *Ruta graveolens* and *Matricaria chamomilla* extracts

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Keywords: Ruta graveolens, Matricaria chamomilla, analgesic activity

Abstract

Aims: The study was performed to compare the analgesic activity of different fractions of the extracts of *Ruta graveolens* and *Matricaria chamomilla*.
Materials and Methods: The plant materials were extracted with 70% ethanol, petroleum ether, ethyl acetate and n-butanol. The ethyl acetate and n-butanol fractions of each plant were evaporated to dryness and analyzed by HPLC. The analgesic activity of these extracts was evaluated using writhing reflex test and compared with that produced by a standard drug (Diclofenac sodium).
Results: Flavonoids were found in all fractions of both plants (i.e ethyl acetate and n-butanol), while trace of alkaloids were found in the ethyl acetate fraction of *Ruta*. The prepared extracts showed better analgesic activity than the standard drug; when compared with each other, *Matricaria* extracts showed better analgesic activity compared to *Ruta* extracts.
Conclusion: There is similar efficacy of chamomile and common rue as analgesic agents.

INTRODUCTION

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of diseases and clinical disorders, and relatively little knowledge available about their exact effective doses and mode of action [1]. *Ruta graveolens* (common rue) F: Rutaceae is a flavonoid containing medicinal plant which has been traditionally used as a sedative and to relieve menstrual and gastrointestinal disorders [2,3]. Also it shows hypotensive [4,5], antifertility [6-8], analgesic and anti-inflammatory effects [9,10]. The plant grows in the Balkans as far as siedengebirge, upper and central Italy and is cultivated elsewhere [3]. The roots and aerial parts of Rue plant contain more than 120 compounds that belong to different classes of natural products such as alkaloids (skimianine, arborine and graveolene), coumarins (herinarin and umbelliferon, essential oil, flavonoids (rutin glycoside I and its aglycone quercetin II) and furoquinoline [4]. The main uses of rue are to relieve gouty and rheumatic pains and to treat nervous heart problems [5]. Coumarin derivatives and alkaloids are spasmolytic; the furocoumarines (psoralens) are responsible for hepatotoxicity and photo-sensitization, while alkaloids and polyphenols are responsible for the anti-inflammatory activity [6,7]. Also rue was found to have cytotoxic effect [8]. Chamomile, also known as German chamomile, consists of the fresh or dried flower heads of *Matricaria chamomilla* F: compositae. It is indigenous to Europe and northwest Asia, naturalized in North America. The flowers contain volatile oil, flavonoids (apigenin III, luteolin and chamaemeloside) and coumarins. It has anti-inflammatory, antineoplastic, antioxidant, antibacterial, and spasmolytic properties [9]. Chamomile has

purported anti inflammatory effects but there are no published clinical trials supporting the findings in experimental animals. Chamomile contains many chemical constituents such as flavonoids epigenin and luteolin, which posses anti inflammatory properties [10,11] with well defined selective COX-2 inhibitory activity [12] and contains a pain relieving effect [13]. The present study was designed to compare the analgesic activity of different extracts from the aerial parts of *Ruta graveolens* and the flower parts of *Matricaria chamomilla* cultivated from the Baghdad governorate area.

MARERIALS AND METHODS

Plant Material

Both the flowers of *Matricaria chamomilla* and the aerial parts of *Ruta graveolens* were collected from Al-Graiaat area in Baghdad city and authenticated by Dr. Ibrahim Salih, College of Pharmacy, University of Karbala and voucher samples were kept in the department of Pharmacognosy and Medicinal Plants, College of Pharmacy, University of Baghdad.

Preparation of Plant Extracts

Twenty grams of the dried flowers of *Matricaria* and 20 gm of the dried aerial part of *Ruta* were macerated separately with 350 ml of 70% ethanol for 24 hours; after filtration, the extracts were concentrated under vacuum to about 10-15 ml, and then partitioned with petroleum ether, ethyl acetate and n-butanol respectively using 50 ml x 3 for each fraction. The ethyl acetate fractions were dried over anhydrous sodium sulfate, filtered and evaporated to dryness, while the n-butanol fractions were evaporated to dryness under reduced pressure.

Phytochemical Investigations

Preliminary investigations for the chemical constituents of the extracts were done using ammonia vapor, ethanolic KOH, Meyer's and Dragendorff's reagents. Utilizing HPLC separation technique (Waters, USA), the ethyl acetate and n-butanol fractions of both plants were analyzed for their flavonoids contents using a system consisted of methanol:water 90:10 as a mobile phase with a flow rate 0.5% ml/min, and wave length 280nm, C_{18} 25cm column.

Evaluation of the analgesic activity of plant extracts

Thirty six mice weighing 18-22 gm of both sexes are used to evaluate the analgesic activity of the prepared extracts using writhing test [13]. The animals were housed in the animal house, College of Pharmacy, University of Baghdad at controlled temperature and humidity; they are fed standard animal chow and left free for drinking water *ad libitum*. The animals were allocated into six groups (each include 6 mice) and treated as follow: first group injected with vehicle i.p. and saved as control; 2nd group injected with 20mg/kg Diclofenac sodium i.p and saved as standard drug comparator; the other 4 groups were injected with 25mg/kg i.p. of the prepared extracts of the two plants respectively. Twenty minutes after administration of the vehicle, diclofenac and the plant extract, 0.2 ml of 1% acetic acid was injected intraperitonially to each animal; the number of writhes for each animal was calculated.

Statistical Methods:

The data were analyzed using Statistical Package for Social Science (SPSS version 15.0). The results were expressed as mean±SD; then the differences in writhes score among groups were calculated and statistically evaluated using unpaired Student's *t*-test and multiple-way ANOVA; values with $P<0.05$ are considered significantly different.

Table 1. Writhing scores of the analgesic activity of the extracts compared to diclofenac and control.

Treatment Group	Number of writhes
Control (n=6)	67.0 ± 29.3 [a]
Diclofenac (n=6)	36.3 ± 7.8 [*b]
Chamomile Ethyl acetate extract (n=6)	9.2 ± 5.9 [*c]
Chamomile *n*-Butanol extract (n=6)	1.7 ± 0.8 [*d]
Rue Ethyl acetate extract (n=6)	4.5 ± 5.1 [*d]
Rue *n*-Butanol extract (n=6)	11.2 ± 8.3 [*c]

Data are expressed as mean±SD; n=number of animals per group; values with non-identical superscripts (a,b,c,d) represent significant difference among groups (P<0.05); *significantly different compared to control group (P<0.05).

RESULTS

Preliminary investigations revealed the presence of flavonoids in all fractions of both plants (i.e ethyl acetate and n-butanol) and the presence of trace of alkaloids in the ethyl acetate fraction of Ruta. The HPLC peaks of standard rutin and quercetin are shown in figures 1 and 2 respectively. HPLC of ethyl acetate fraction of Ruta (Figure 3) showed higher quercetin amount than rutin while the HPLC chromatogram of n-butanol fraction of Ruta, while figure 4 showed higher rutin amount than quercetin. On the other hand, the HPLC chromatogram of ethyl acetate fraction of matricaria (Figure 5) showed higher rutin amount than quercetin and a major unidentified flavonoid with t_R 2.363 minute while n-butanol extract of matricaria (Figure 6) showed rutin as a major constituent and very small amount of quercetin. The results of the writhing

score after the injecting the extracts and the standard comparator (diclofenac) are shown in table 1. The analgesic activity produced by diclofenac and the four types of extracts are significantly greater compared to the control group. Meanwhile, the four extracts showed analgesic activity which is significantly higher compared to diclofenac. When the analgesic activity produced by the four extracts were compared, the data demonstrated that chamomile ethyl acetate extract and Rue n-butaol extract showed comparable analgesic activity when compared with each other, and both of them demonstrated higher analgesic activity than both the chamomile n-butaol extract and Rue ethyl acetate extract; the later two extracts also found to produce comparable analgesic activity (P>0.05).

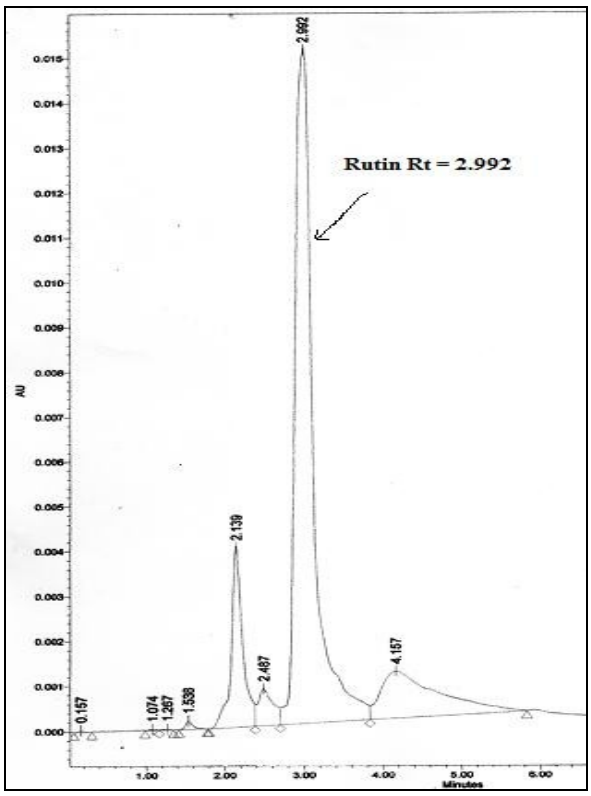

Figure 1. HPLC Chromatogram of Standard Rutin.

DISCUSSION

Significant analgesic activity was observed with all four fractions and the extracts were more effective than diclofenac sodium. The use of higher dose (50 mg/kg) of the extracts abolished the writhes completely, especially in the extracts of Matricaria; however, the use of 50mg/ml dose was discontinued because the treated animals died, which may be due to respiratory depression attributed to the sedative effect of these plants despite that the LD_{50} of the total extract injected intraperitoneally in mice being 563 mg/kg [14,15]. Dose-response study is required to determine the

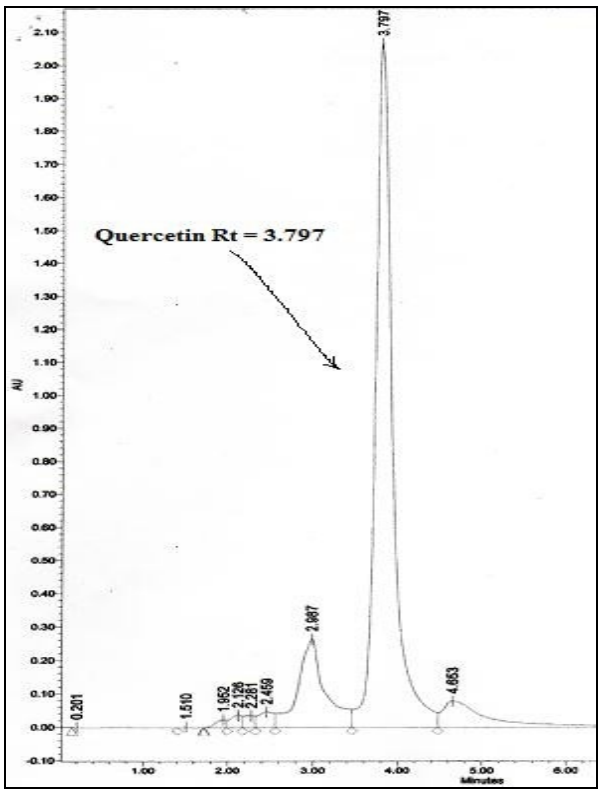

Figure 2. HPLC chromatogram of standard Quercetin

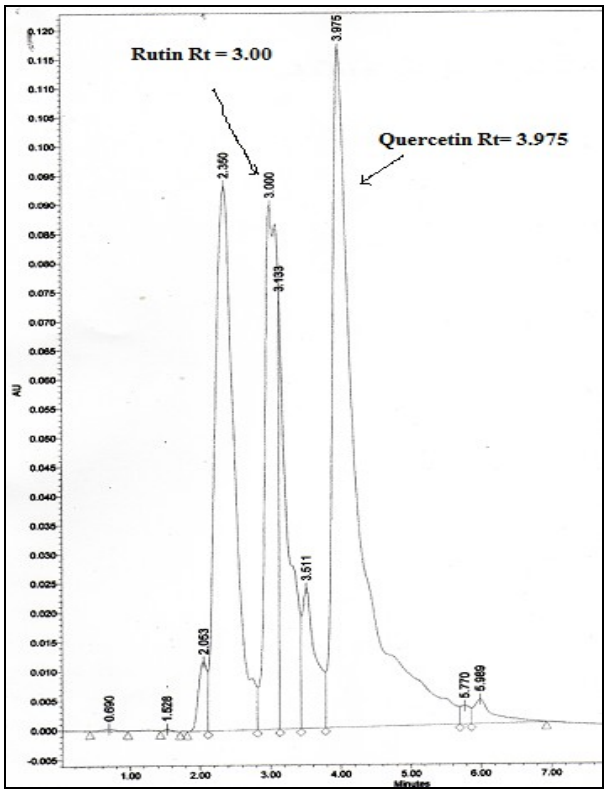

Figure 3. HPLC chromatogram of Ethyl Acetate fraction of Ruta extract

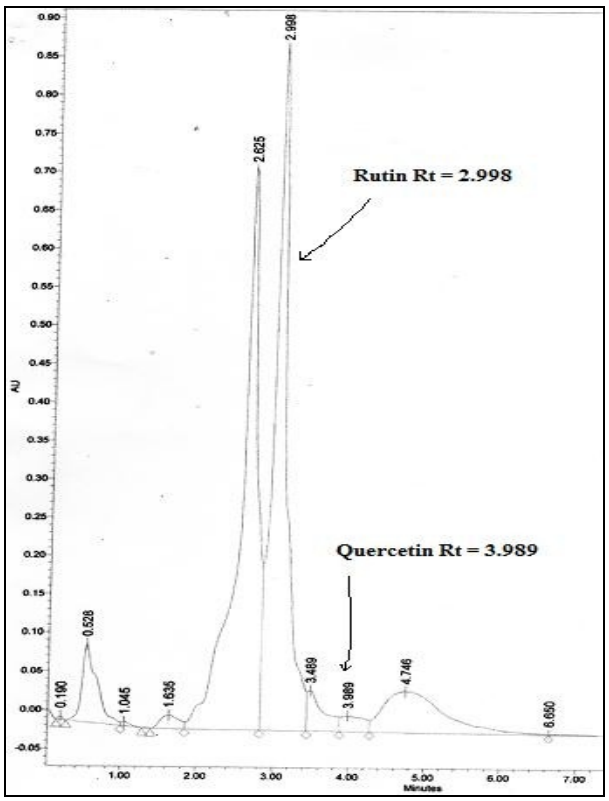

Figure 4. HPLC chromatogram of the n-butanol fraction of Ruta extract.

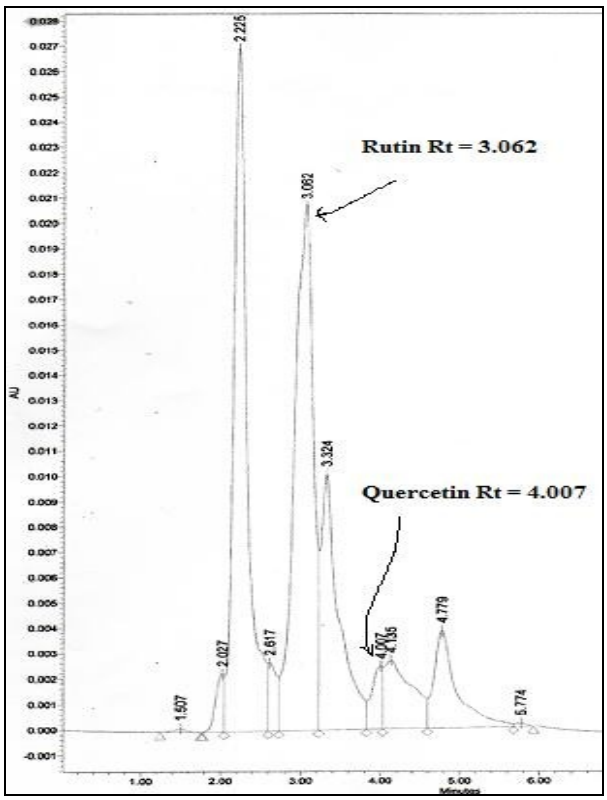

Figure 6. HPLC chromatogram of the n-butanol fraction of Marticaria extract.

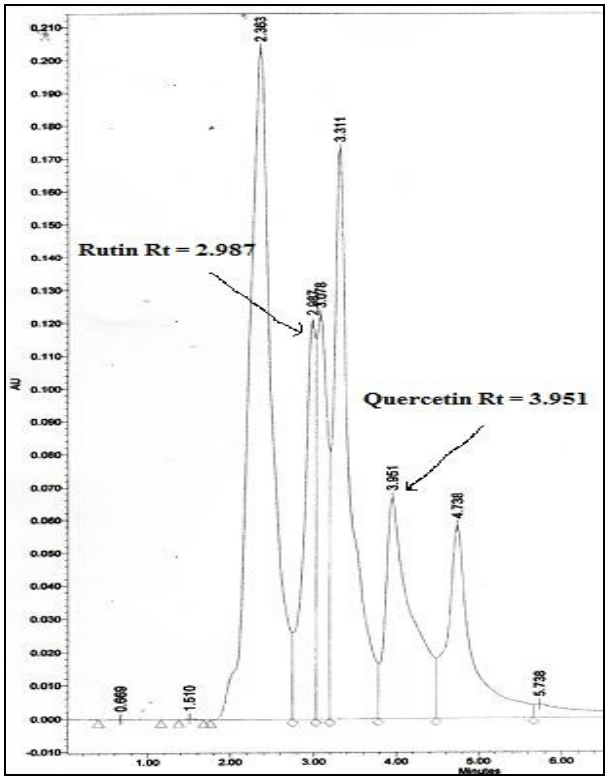

Figure 5. HPLC chromatogram of the Ethyl Acetate fraction of Marticaria extract

optimal doses with low side effects and toxicity. The mechanism of anti-nociceptive activity of chamomile in male and female animals is not fully understood [13]. The *n*-butanol extract of chamomile produced significantly less writhes than the ethyl acetate extract, this indicates that the compounds present in the *n*-butanol extract of chamomile have better analgesic activity than those present in the ethyl acetate fraction. The ethyl acetate extract of rue showed better analgesic activity than that observed with the ethyl acetate fraction of the chamomile extract; this indicates that Rue has a better analgesic activity than chamomile. Further phytochemical studies are required to separate and characterize the active constituent(s) responsible for this effect. The highly significant anti-inflammatory activity of common rue may be due to the interference with the mediators of inflammation such as histamine, serotonin and prostaglandin [16], and may therefore be responsible for its analgesic activity. In another study, the anti-inflammatory effect of polyphenols and alkaloid fractions of Rue was evaluated and found to be higher than that observed with the standard drug diclofenac. The results demonstrated the potential beneficiary effect of the isolated polyphenolic and alkaloid fractions of common rue in acute and chronic models of inflammation in rats [16]. An active compound was isolated from the methanolic extract of

rue and in a dose of 40 mg/kg this compound inhibits the inducible nitric oxide synthase and interleukin-lβ gene expression significantly in endotoxin-induced inflammatory model of BALB/c mice. The low level of nitric oxide production was also observed in the sera of treated mice. Also the plant extract and the isolated active compound blocked the lipopolysaccharide-induced activation of NF-κB through the prevention of inhibitor-κB degradation; the purified compound also showed antioxidant activity [14]. These anti-inflammatory mechanisms may be responsible for the analgesic activity of the common rue.

CONCLUSION

The present data indicates the efficacy of chamomile and common rue as efficient analgesic agents which may be attributed to their high contents of flavonoids.

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Original Research

Design and characterisation of matrix tablets of highly water soluble drug

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Keywords: Tramadol HCL, Controlled Release Tablets, Direct Compression Method and Melt Granulation Technique.

Abstract
Tramadol HCL is a centrally acting opioid analgesic. Although the drug has a higher plasma half life, the steady state plasma concentration is not achieved with frequent dosing of q.i.d at 6 hour intervals. Therefore, the objective of the present work was to formulate a 100mg strength Tramadol matrix tablets to extend the drug release and thus decrease the dosing frequency and achieve steady state plasma concentration. Initially, preformulation studies were carried out to rule out any incompatibility between the drug and the chosen polymer(s) after exposing physical mixtures of the drug and the polymer(s) to 40°C/75% RH for three months. A suitable method was developed for drug estimation at 271nm by a UV double beam spectrophotometer. Next, various batches of tablets were designed using different polymers such as Ethylcellulose, Carnauba wax, HPMC-K100M, Carbopol-974P and Kollidon-SR. Direct compression technique was used except for the formulation containing carnauba wax for which melt granulation was done followed by compression. Formulations F-1 to F-15 contained single polymers in increasing concentrations in drug:polymer ratios of 1:1, 1:2 and 1:3 where it was observed that the drug release extended with increasing polymer concentrations. Carbopol-974P extended drug release better followed by HPMC-K100M and Carnauba wax compared to other polymers. A combination of these polymers was also used at various ratios to get formulations F-16 to F-20 and observed that the polymer combinations controlled drug release better. The type of fillers like lactose and microcrystalline cellulose had no effect on the physiochemical characters as well as on the drug release profiles. The in vitro release data from the best formulation fitted well in Higuchi as well as Peppas model, the 'n' value, which confirmed that the release mechanism shifted from initial dissolution to later extended diffusion in which both diffusion and erosion governed the drug release.

INTRODUCTION

Oral drug delivery continues to rise in popularity as formulation scientists look for ways to control drug release and improve patient convenience. However, developing oral controlled release tablets for highly water soluble drugs with a constant release rate has always been a challenge to pharmaceutical technologist. Among various dosage forms, matrix tablets are widely accepted for oral controlled release (CR) as they are simple and easy to formulate. The Matrix system is the release system, which prolongs and controls the release of the drug that is dissolved or dispersed[1]. Tramadol HCL is a centrally acting opioid analgesic useful in the treatment of fibromyalgia pain, myofascial pain, chronic knee pain, chronic back pain, chronic hip pain and osteoarthritis pain. It is highly soluble in water [2]. The half life of Tramadol Hcl was 5.5 to 7 hours. Although the drug has a higher plasma half life, the steady state plasma concentration

is not achieved with frequent dosing of q.i.d at 6 hour intervals. In recent years, considerable attention has been focused on various polymers in the design of oral controlled drug delivery systems because of their flexibility to obtain a desirable drug release profile, their cost-effectiveness, and broad regulatory acceptance [3]. Controlled drug delivery aims at enhanced bioavailability, minimized side effects and increased therapeutic effectiveness [4]. Therefore, the objective of the present investigation was to design matrix tablets to control the drug release of a highly water soluble drug like Tramadol HCL using various single and combination polymers and thus decreasing the dosing frequency to achieve steady state plasma concentration.

MATERIALS AND METHODS

Chemicals

Tramadol HCL, Ethylcellulose, Carnauba wax, Hydroxypropylmethylcellulose-K100M, Carbopol-974P and Kollidon-SR were gifted by Matrix laboratories Ltd., Hyderabad, India. All other materials used were of analytical grade and were obtained from S.D. Fine Chemicals Limited, Mumbai, India.

Experimental Protocol

Drug-excipients Compatibility Studies

The drug-excipients compatibility study was initiated for Tramadol HCL at accelerated conditions for 3 months with different polymers Ethylcellulose, Carnauba wax (CW), Hydroxymethylcellulose-K100M (HPMC), Carbopol-974P and Kollidon-SR individually and in combination. The drug and the polymers were sifted through sieve number 40 separately. Then the drug and the polymers were weighed individually and mixed well to form a uniform mixture. The drug and polymers physical mixtures were placed in separate glass vials and sealed with aluminum foil. These vials were placed at 40°C/75%RH in stability chamber. At suitable time intervals, the samples were analyzed for physical changes and drug content [5].

Analytical Method Development for Drug Estimation

A spectrum of the working standards was obtained by scanning standard solution of 10ppm in 0.1 N HCL from 200-400nm by UV double beam spectrophotometer (Model: Shimadzu, UV-1800) against the reagent blank to fix absorption maxima. The $\lambda_{max}$was 271nm. Hence all further investigations were carried out at the same wavelength. After conducting some trials, the concentration range, which obeyed Beer's law was between 0 - 100µg/ml.

Design of Oral CR Tablets

Various batches of Tramadol HCL CR tablets were formulated using various polymers such as Ethylcellulose, CW, HPMC, Carbopol-974P and Kollidon-SR individually as well as in combination at various drug:polymer ratios 1:1, 1:2 and 1:3. Direct compression and melt granulation methods were used as the manufacturing techniques. Microcrystalline cellulose and lactose monohydrate were used as fillers, whereas Aerosil was used as lubricant in all the formulations. All the tablets were punched using round biconcave punches in a single rotary compression machine (Cadmach, Mumbai., India).

In case of direct compression technique, the drug and the excipients were sifted through sieve number 40 separately. Required quantities of all the ingredients were weighed individually and were mixed well to form a uniform blend in a double cone blender (VJ Instruments, India) and the blend was finally punched.

In case of melt granulation technique [4], CW was melted with continuous stirring in a glass beaker by heating at 75˚C to which drug was added with continuous stirring. Then, the molten mass was allowed to cool down and solidify. The above mass was ground and screened through sieve number 40 (equivalent to 595- microns) to obtain granules. After lubrication, the blend was punched.

Evaluation of the CR Tablets

Physicochemical Characterization of Tablets

The properties of the controlled release tablets, such as hardness, weight variation, and drug content uniformity were determined as per British Pharmacopoeia. Briefly, for each batch, hardness was determined by using the Pfizer hardness tester. Weight variation of tablets was determined as per official procedure for 20 randomly selected tablets. Uniformity of drug content was determined by using a UV double beam spectrophotometer. 10 samples were randomly selected and crushed in a mortar; drug equivalent to 100 mg was weighed into 100 mL volumetric flasks containing a small amount of 0.1N HCL buffer and then made up to the mark after thorough mixing. The samples were then filtered through a 0.45 µm filter, and after suitable dilutions were analyzed spectrophotometrically the drug content was determined from the calibration curve [6], [7].

In Vitro Dissolution Studies

The drug release profiles of the formulated tablets were studied using a USP Type-I dissolution apparatus (Model: Electrolab, Mumbai, India) in 900ml of 0.1N HCl buffer solution as the dissolution medium. The temperature of the medium was maintained at 37±

0.5°C, and the stirring speed was set at 75 rpm. Aliquot samples were withdrawn every 1 hour and after suitable dilution of filtrate with 0.1N HCl buffer, absorbance was measured spectrophotometrically at 271nm. The amount of the drug released was determined from the calibration curve [8]. The volume of the sample withdrawn each time was replaced with the same volume of fresh 0.1N HCl buffer solution. The studies were carried out in triplicate and mean value plotted versus time with standard error of mean, indicating the reproducibility of the results.

Release Kinetics

Drug release data from the most satisfactory formulation was fitted to various mathematical models viz., Korsmeyer-Peppas, Zero-order, Higuchi release models and first-order release models for describing the release mechanism from the formulations.

RESULTS AND DISCUSSIONS

Drug-excipients Compatibility Studies

All the Physical mixtures of the drug with polymers from PM-1 to PM-7 were checked at the end of 1st and 3rd month for any physical changes and drug content. PM-1 to PM-5 contained drug and single polymers in the ratio 1:3 and in the case of PM-6 and PM-7 containing a mixture of polymers the ratio was 1:1.5:1.5 as indicated in Table 1. It was observed that there was no change in color or physical appearance of all the mixtures except for a slight lump formation in PM-5, which could be due to moisture absorption by Kollidon-SR [9]. As indicated in Table 1, the drug content was within limits according to official guidelines in all the physical mixtures at the chosen time intervals [5].

Analytical Method Development for Drug Estimation

The calibration curve constructed for the drug in 0.1N HCl at 271nm had a regression coefficient of 0.9994. Since there was compatibility between the drug and the chosen polymers, An analytical method could be developed for drug estimation and formulation studies were carried out as next step.

Formulation Development of Tramadol HCl CR Matrix Tablets

For the fabrication of once-a-day ER tablet formulation of Tramadol HCL, various polymers such as Ethylcellulose, CW, HPMC-K100M, Carbopol-974P and Kollidon-SR were tried individually as well as in combination as indicated in Table 2 to investigate the influence of type of matrixing agent on the drug release and to compare the dissolution profiles of the various formulations.

The dose of Tramadol HCl in all the formulations F-1 to F-22 was 100mg. The method of manufacturing process followed was direct compression for all the formulation except for the formulation F-20 for which melt granulation followed by direct compression technique was used. The diameter of the punch used was based on the total tablet weight. All the formulations contained Aerosil at 2% level.

At the out set, CR formulations F-1 to F-15 were prepared by using single polymers. In the formulations F-1 to F-3, F-4 to F-6, F-7 to F-9, F-10 to F-12 and F-13 to F-15, the polymers used were Ethocel, CW, HPMC-K100M, Carbopol-974P and Kollidon-SR respectively and the concentration of the polymers used increased in drug:polymer ratio of 1:1, 1:2 and 1:3 respectively in all the formulations. In the formulations F-1, F-4, F-7, F-10 and F-13, in which the average total tablet weight was 200mg, the punch size used was 8mm. If the average total tablet weight was 300mg as in the formulations F-2, F-5, F-8, F-11 and F-14, the punch size used was 9mm, and if the average total tablet weight was 400mg as in the formulations F-3, F-6, F-9, F-12 and F-15, the punch size used was 10mm.

CR formulations F-16 to F-22 were prepared by using a combination of two polymers as indicated in Table 2. CR formulations F-16 and F-17 were prepared using a mixture of polymers HPMC-K100M and Carbopol-974P in the ratio of 1:2 and 1:1, respectively. The punch size used for the formulations F-16 and F-17 was 11mm and 12mm respectively, whose average total weights were 400mg and 500mg, respectively.

CR formulations F-18 and F-19 were prepared by using a mixture of CW and Carbopol-974P in the ratio of 1:2 and 1:1, respectively. Formulation F-20 was the same as formulation F-19 as indicated in Table 2, except that the method of preparation of formulation F-20 was by melt granulation technique to compare if the method of preparation had any effect on the physiochemical characters and in vitro release patterns [10]. The punch sizes used for formulation F-18 was 11mm and in case of formulation F-19 and F-20 it was 12mm. The average total weights of formulation F-18, F-19 and F-20 were 400mg, 500mg and 500mg, respectively. When Carbopol was present in the formulation as a matrixing agent, a bigger punch size was used as its bulk density is higher [11].

To study the effect of fillers like Microcrystalline cellulose and Lactose monohydrate on the physiochemical characters and in vitro drug release properties, formulations F-21 and F-22 were formulated by incorporating them at 50mg, respectively. The average total weights of formulation F-21 and F-22 was the same i.e. 550mg. The punch size used for both the formulations was 12mm.

Table 1. Physico-Chemical Characterization of Drug-Polymer Mixture

PM	Drug:Polymer(s) Ratios						Physical Appearance			Drug Content (%) ± S.D.		
	Drug	EC	CW	HPMC-K100M	Carbopol 974P	Kollidon SR	Initial	After 1 month	After 3 months	Initial	After 1 month	After 3 months
PM-1	1	3	-	-	-	-	WCP	WCP	WCP	100.3 ± 0.47	100.2 ± 0.31	100.0 ± 0.52
PM-2	1		3	-	-	-	CWP	CWP	CWP	98.5 ± 0.86	98.5 ± 0.76	98.5 ± 0.73
PM-3	1	-	-	3	-	-	WCP	WCP	WCP	98.9 ± 0.91	98.9 ± 0.63	98.6 ± 0.52
PM-4	1	-	-	-	3	-	WCP	WCP	WCP	101.2 ±0.83	101.2 ± 0.63	101.1 ± 0.49
PM-5	1	-	-	-	-	3	WCP	WCL	WCL	99.3 ± 0.17	99.2 ± 0.53	99.1 ± 0.15
PM-6	1	-	-	1.5	1.5		WCP	WCP	WCP	100.8 ± 0.29	100.7 ± 0.66	100.5 ± 0.81
PM-7	1	-	-	-	1.5	1.5	WCP	WCP	WCP	99.5 ± 0.63	99.3 ± 0.51	99.2 ± 0.28

PM-Physical mixture, EC-Ethylcellulose, WCP-White Coloured Powder, CWP-Creamish White Powder, WCL- White Coloured Lump.

Table 2. Formulation Development of Tramadol HCl CR Matrix Tablets

FC	Ingredients (mg)								Total Weight
	Tramadol HCl	EC	CW	HPMC K100M	Carbopol 974P	Kollidon SR	MCC	LM	
F-1	100	100	-	-	-	-	-	-	200
F-2	100	200	-	-	-	-	-	-	300
F-3	100	300	-	-	-	-	-	-	400
F-4	100	-	100	-	-	-	-	-	200
F-5	100	-	200	-	-	-	-	-	300
F-6	100	-	300	-	-	-	-	-	400
F-7	100	-	-	100	-	-	-	-	200
F-8	100	-	-	200	-	-	-	-	300
F-9	100	-	-	300	-	-	-	-	400
F-10	100	-	-	-	100	-	-	-	200
F-11	100	-	-	-	200	-	-	-	300
F-12	100	-	-	-	300	-	-	-	400
F-13	100	-	-	-	-	100	-	-	200
F-14	100	-	-	-	-	200	-	-	300
F-15	100	-	-	-	-	300	-	-	400
F-16	100	-	-	100	200	-	-	-	400
F-17	100	-	-	200	200	-	-	-	500
F-18	100	-	100	-	200	-	-	-	400
F-19	100	-	200	-	200	-	-	-	500
F-20	100	-	200	-	200	-	-	-	500
F-21	100	-	-	200	200	-	50	-	550
F-22	100	-	-	200	200	-	-	50	550

FC – Formulation Code, EC – Ethylcellulose, MCC – Microcrystalline cellulose, LM – Lactose Monohydrate.

Table 3. Physicochemical Characterization of formulated Tramadol HCl Matrix Tablets

FC	Appearance	Hardness* (Kp) ± S.D.	Wt Variation (%) ± S.D.	Drug Content (%) ± S.D.
F- 1	S	12.7 ± 0.05	±1	100.5 ± 0.35
F- 2	S	13.2 ± 0.12	±2	99.2 ± 0.57
F- 3	S	13.4 ± 0.07	±1	100.1 ± 0.64
F- 4	S	12.6 ± 0.16	±1	99.4 ± 0.57
F- 5	S	12.7 ± 0.11	±1	98.4 ± 3.1
F- 6	S	13.5 ± 0.05	±1	99.3 ± 0.49
F- 7	S	13.1 ± 0.14	±1	100.6 ± 0.28
F- 8	S	13.1 ± 0.09	±1	99.1 ± 0.64
F- 9	S	13.0 ± 0.11	±2	98.9 ± 0.78
F- 10	S	12.9 ± 0.08	±1	100.5 ± 0.35
F- 11	S	12.8 ± 0.16	±2	101.8 ± 0.14
F- 12	S	13.5 ± 0.09	±1	99.7 ± 0.21
F- 13	S	12.6 ± 0.11	±1	99.2 ± 0.57
F- 14	S	13.2 ± 0.12	±1	100.8 ± 0.14
F- 15	S	13.0 ± 0.05	±2	99.6 ± 0.28
F- 16	S	12.7 ± 0.07	±1	98.7 ± 0.49
F- 17	S	13.0 ± 0.09	±2	102.9 ± 0.17
F- 18	S	13.2 ± 0.15	±2	99.6 ± 0.28
F- 19	S	13.0 ± 0.13	±1	98.8 ± 0.85
F-20	S	13.3 ± 0.09	±3	100.7 ± 0.21
F-21	S	13.1 ± 0.12	±2	99.5 ± 0.35
F-22	S	13.4 ± 0.11	±1	101.8 ± 0.14

FC - Formulation code; S-Satisfactory; * Average of three findings

Hardness, Weight Variation and Drug Content of the Formulated Tablets

The tablet hardness of all the formulations F-1 to F-22 ranged from 12.6 ± 0.16Kp to 13.0 ± 0.09Kp as indicated in Table 3. It was observed that there was not much difference in the hardness of all the formulations.The percentage weight variation of the formulated tablets F-1 to F-22 ranged from±1% to ±3% as indicated in Table 3, which was within the given official specifications. The percentage drug content of the formulations F-1 to F-22 ranged from 98.4%±3.1 to 102.1%± 0.17 as indicated in Table 3, which was within the official specifications. Since all the developed formulations F1 to F22 satisfied all the required physiochemical parameters, in vitro dissolution studies were conducted on all the formulations.

Release profile and Mechanism

The in vitro drug release studies from all the formulated matrix tablets F-1 to F-22 were conducted for a period of 12 hours. Of the formulations F-1 to F-3, containing Ethyl cellulose as the drug retardant, formulation F-1 released almost 100% of the drug with in 1 hour and F-2 released 100% of drug within 2 hours, whereas formulation F-3 released 100% of the drug in 6 hours as indicated in Figure: 1.

Out of the formulations F-4 to F-6 containing CW as the drug retardant, formulation F-4 released 100% of the drug in 4 hours, whereas formulation F-5 released

in 8 hours and formulation F-6 released in 10 hours as indicated in Figure: 1.

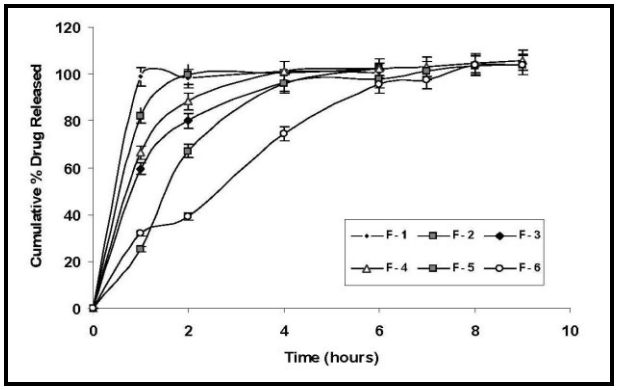

Figure 1. Comparison of in vitro drug release profiles of tramadol HC1 from the formulations F-1 to F-6

In the formulations F-7 to F-9 containing HPMC-K100M as the drug retardant, formulation F-7 released 100% of the drug within 6 hours, whereas formulation F-8 released in 10 hours and formulation F-9 released only 93.8% ± 0.56 of the drug in 12 hours as indicated in Figure: 2.

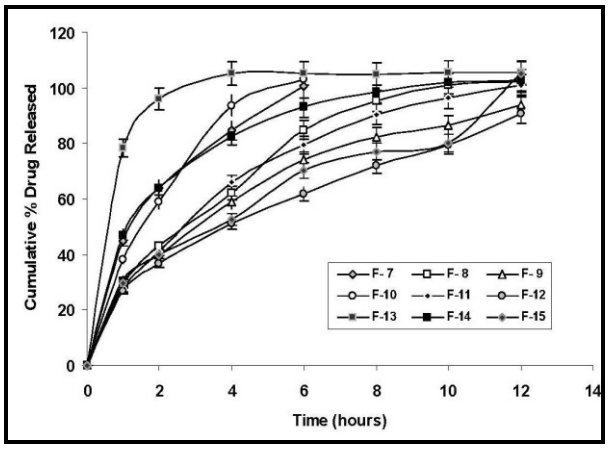

Figure 2. Comparison of in vitro drug release profiles of tramadol HC1 from the formulations F-7 to F-15

Among the formulations F-10 to F-12 containing Carbopol-974P as the drug retardant, formulation F-10 released 93.7% ± 1.2 of the drug within 4hrs and 100% of drug in 6 hours, whereas formulation F-11 released 96.3% ± 0.73 of the drug within 10 hours and 100% the drug in 12 hours and formulation F-12 released only 90.8% of the drug in 12 hours as indicated in Figure: 2.

In the formulations F-13 to F-15 containing Kollidon-SR as the drug retardant, formulation F-13 released 100% of drug within 4 hours, whereas formulation F-14 controlled the release to 10 hours and formulation F-15 controlled the release further and released 100% of the drug in 12 hours as indicated in Figure: 3. Burst release was observed in the first hour in these formulations.

It was observed that the increasing amount of polymer in all the above formulations from 1:1, 1:2 to 1:3 imparted a significant control over the drug release [12, 13] and thus controlled the duration of drug release.

From the above observations, it was noticed that among the formulations F-1 to F-15 only formulation F-12 containing Carbopol-974P as the matrix forming polymer could extend the duration of drug release to the maximum at a higher drug polymer ratio of 1:3 followed by HPMC-K100M and CW. Therefore, it was decided to take a combination of these polymers to further control the drug release of highly soluble Tramadol HCl.

In the formulations F-16 and F-17 containing a combination of polymers Carbopol-974P and HPMC-K100M, formulation F-16 released 82.1% ± 3.4 of the drug in 12 hours, whereas formulation F-17 released only 77.6% ± 1.03 of the drug in 12 hours as indicated in Figure: 3. When the quantity of the drug:polymers ratio increased from 1:1:2 to 1:2:2, as in formulations F-16 and F-17 respectively, the duration of drug release was also controlled.

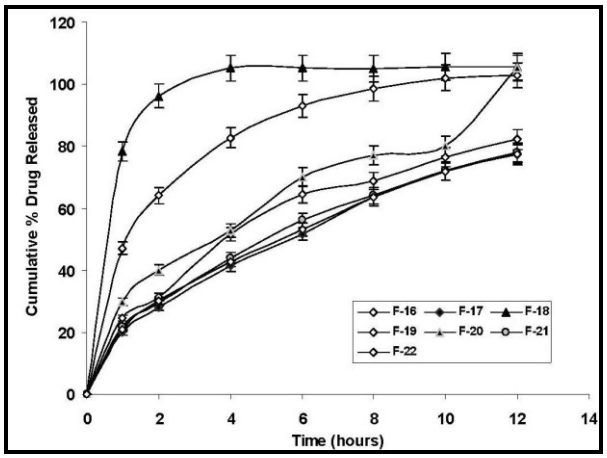

Figure 2. Comparison of in vitro drug release profiles of tramadol HC1 from the formulations F-7 to F-15

In the formulations F-18 to F-20 containing a combination of polymers Carbopol-974P and CW, formulation F-18 released 89.8 % ± 0.95 of the drug in 12 hours and formulation F-19 liberated 85.1 % ± 0.17 of the drug in 12 hours. Formulation F-20 released

82.8% ± 0.26 of the drug at the end of 12 hours as indicated in Figure: 3, which was almost the same as that of formulation F-19. Therefore, it was concluded that there was no significant effect of method of preparation on drug release pattern.

From the above in vitro drug release studies on the formulations F-16 to F-20, it was observed that formulations containing a combination of polymers better controlled the drug release of highly water soluble Tramadol HCl than formulations F-1 to F-15, which contained single polymers.

Out of the formulations F-16 to F-20, formulation F-17, containing a combination of polymers HPMC-K100M and Carbopol-974P in the ratio 1:1 was a better retard drug release compared to other formulations. Therefore, formulation F-17 was chosen as the most satisfactory formulation and therefore, further studies were carried out the same way.

Table 4. In vitro release Kinetics of the most satisfactory formulation F-17

Formulation	Zero order (R^2)	First order (R^2)	Higuchi (R^2)	Hixson-Crowell (R^2)	Korsmeyer-Peppas	
					(n)	(R^2)
F-17	0.984	0.9145	0.997	0.9444	0.5608	0.9983

Formulations F-21 and F-22 were the same as formulation F-17 except that they contained microcrystalline cellulose and lactose monohydrate fillers. Both the formulations released almost 77% of the drug at the end of 12 hours in the in vitro dissolution studies, which was the same as that of the formulation F-17 as indicated in Figure-3. Therefore, it was concluded that the fillers did not have any effect even on the drug release pattern from the formulation like that of physiochemical characters.

The in vitro Tramadol HCl release data from the most satisfactory formulation F-17 was fitted to various kinetic equations and the mechanism of drug release was studied from the R^2 values obtained as indicated in Table 4. The data fit with higher values in Higuchi as well as Peppas' model [14], [15]. The 'n' value was 0.5608, which confirmed that the formulation followed non-Fickian diffusion kinetics (anomalous transport), i.e. the release is ruled by both diffusion of the drug and dissolution of the polymer. In this case, the release mechanism shifted from initial dissolution to later extended diffusion in which both diffusion and erosion governed the drug release.

CONCLUSION

Thus, the most satisfactory formulation F-17 satisfied the physiochemical parameters and in vitro drug release profile requirements for an oral controlled release tablet formulation of the highly water soluble drug Tramadol HCl.

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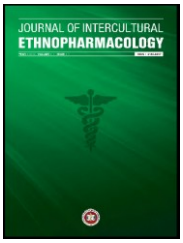

Original Research

Use of aqueous extract of corn silk in the treatment of urinary tract infection

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Abstract

Aim: Worldwide about 150 million people were diagnosed with UTI each year. The increasing incidence of antibiotic resistance among bacterial pathogens necessitates medicinal plants as an alternate therapy in the management of UTI. Corn silk has been widely used as a folk medicine in Iraq. The aim of this study is to evaluate the clinical advantages of using the aqueous extract of corn silk in Iraqi patients with UTI.

Method: This study was carried out on 42 patients of both sexes with age of 29.91±10.57 (mean ± SD); who attend to outpatient clinic in Alkindy College of Medicine, Baghdad, Iraq, over the period from October 2011-March 2012; UTI is confirmed by defined symptoms together with laboratory results. Manifestation of UTI were checked clinically include suprapubic pain, urgency, frequency and dysuria; each symptom scored as follow: 0(none), 1 (mild), 2(moderate), 3(severe) and 4(very severe). All of the patients were followed up after 5 days, 10days and 20 days from starting course of treatment with aqueous extract of corn silk.

Results: Administration of aqueous extract of corn silk to UTI patients result in significant $P \leq 0.05$ decreases in UTI symptoms after 5 days, 10 days and 20 days from starting the treatment compared to baseline values. The symptom score also showed significant reduction $P \leq 0.05$ reduction after 5, 10 and 20 days from starting the treatment.

Conclusion: administration of aqueous extract of corn silk significantly reduce the symptoms in patient with UTI in addition to reduction in the values of pus cells, RBCs, and Crystals, without any reported side effect which indicate its efficacy and safety

INTRODUCTION

Urinary tract infection (UTI) is the second most common infections presentation in community practice; worldwide about 150 million people were diagnosed with UTI each year [1]. For better management and prognosis, it is mandatory to investigate the possible site of infection, occurrence of re-infection, or relapse, treatment failure in addition to pathogenesis and risk factors [2]. UTI may involve only the lower urinary tract or may involve both the upper and lower tract. The term "cystitis" has been used to describe lower UTI, which is characterized by a syndrome involving dysuria, frequency, urgency and suprapubic pain [3]. Diagnosis of UTI often requires laboratory examination of a urine sample in addition to clinical evaluation. For patient with symptomatic UTI empiric antimicrobial treatment is generally recommended while culture and sensitivity test are pending. Pyuria is an expected accompaniment of significant bacteruria. The absence of pyuria is considered useful in excluding UTI [4]. The increasing prevalence of antimicrobial resistance is a major health problem; many bacterial species including *E.coli* are showing an increasing resistance to antibiotics. Multidrug resistance among *E.coli* isolates have been reported from many parts of world, and these rates of resistance to antibiotics differ from region to region [5]. It has been shown that the choice of drugs in the treatment of UTI is quite narrow today due to the wide scale resistance that the common UTI pathogens show to drugs which have been used previously. Many drugs which are considered as effective against uropathogens are now rarely prescribed as empirical therapy in areas where resistance rate to these antibiotics is high [6]. The increasing incidence of

antibiotic resistance among bacterial pathogens necessitates medicinal plants as an alternate therapy in the management of UTI [7].

Medicinal plants have been acknowledged as potential sources of new compound of therapeutic values as a source for drug design and development [8]. Throughout the world there are several reports for the use of herbal treatment of UTI; one of these most common herbal agents is corn silk [9].

Corn silk (*Zea mays L.*) is an outer thread like part of corn; is an abundant and in expensive by-product of maize cultivation; corn silk contains alkaloids, vitamins, saponins, proteins, carbohydrates, Na, K, Mg, and Ca salts; fixed and volatile oils, steroids such as sitosterl and stigmasterol; tannins and flavonoids [10]. There have been many reports on the biological activities of corn silk constituents. Corn silk has been used in many parts of the world for the treatment of edema, cystitis, gout, kidney stones, nephritis, and prostitis [11]. It has been reported that consumption of corn silk has no adverse effects and it is safe for humans [12].

To the best of our knowledge, no scientific reports on the use of corn silk in the management of UTI were so far available in Iraq. Therefore, the aim of this study is to evaluate the clinical advantages of using the aqueous extract of corn silk in Iraqi patients with UTI.

MATERIALS AND METHODS

This Prospective randomized clinical trial was carried out on 42 patients (16 male, 26 female) with age of 29.91±10.57 (mean±SD); who attend to outpatient clinic in Alkindy College of Medicine, Baghdad, Iraq, over the period from October 2011-March 2012; the study was approved by scientific and ethical comittee in Alkindy College of Medicine ,University of Baghdad; an informed consent was taken from all patients. The inclusion criteria were: adult patients with chronic recurrent UTI who develop at least one UTI in the last 6 months; patients included in this study should not take antibiotic for any reason for at least one month before starting the study. The exclusion criteria were: pregnancy, patient with chronic diseases like ischemic heart diseases and diabetes, and congenital genitourinary problems.

For all patients, UTI is confirmed by defined symptoms together with laboratory results. Routine examination of urine is utilized, the microscopic examination is done for the presence of RBCs, pus cells, and crystals; a 10ml sample is sufficient for conducting the test [13]. Blood urea and serum creatinine tests were done according to standard methods [14, 15] to monitor the renal function during the course of treatment. Manifestation of UTI were checked clinically include suprapubic pain, urgency,frequency and dysuria [16]; each symptom scored as follow: 0 (none), 1 (mild), 2 (moderate), 3 (severe) and 4 (very severe), for these criteria the sum symptom score was calculated and recorded as out of 16 in each time interval, patients completed these symptom scores similar to those used in other UTI trials [17]. All of the patients were followed up after 5days, 10days and 20 days from starting course of treatment with aqueous extract of corn silk.

Corn silk extract preparation

Corn silk was obtained from HATRA HERBS, Baghdad, Iraq; and it is approved by Ministry of Health – Medicinal Plant Center-Baghdad, Iraq. Aqueous extraction was performed by adding 100ml of boiling water to 8gm of corn silk for 15 minutes; wait for the solution to become cold; the extract taken in divided doses over 24 hours.

Minitab software package was utilized for statistical analysis; results expressed as mean±SD; paired student t-test was used to evaluate significant changes; $P \leq 0.05$ considered to be significant.

RESULTS

The incidence of UTI symptoms obtained by this study were shown in (Table 1); 78.57% of patients have had suprapubic pain, 83.33% of UTI patients suffered from both urgency and frequency urination, while 76.19% of patients have had dysuria. Administration of aqueous extract of corn silk to UTI patients result in significant $P \leq 0.05$ decrease in UTI symptoms after 5 days, 10 days and 20 days from starting the treatment compared to baseline values.

The symptom score also showed significant reduction after 5, 10 and 20 days from starting the treatment ($P \leq 0.05$), (Table 2). Figure 1 showed that the sum symptom score decreased significantly $P \leq 0.05$ by 322.4% after 5days compared to baseline value; in addition to that, sum symptom score decreased significantly $P \leq 0.05$ after 10 and 20 days to 0.75 and 0.19 respectively compared to baseline value which is 12.8.The above results clearly indicating the beneficial effect of aqueous extract of corn silk in reducing the symptoms in patient with UTI.

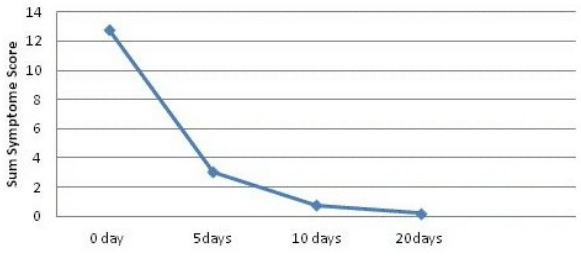

Figure 1. Time course change of Sum symptom score in UTI patients

Table 1. Time course incidence of UTI symptoms

Time(days)	Suprapubic pain	Urgency	Frequency	Dysuria
0	33 (78.57%)	35 (83.33%)	35 (83.33%)	32 (76.19%)
5	5 (11.9%)	10 (23.8%)	11 (26.19%)	6 (14.28%)
10	1 (2.38%)	2 (4.76%)	3 (7.14%)	2 (4.76%)
20	0 (0.0%)	0 (0.0%)	1 (2.38%)	1 (2.38%)

Results represent percent of total, * significant change P ≤0.05.

Table 2. Time course change in UTI symptoms score

Time(days)	Suprapubic pain	Urgency	Frequency	Dysuria	Total
0	3.12	3.32	3.32	3.04	12.8
5	0.47	0.95	1.04	0.57	3.03*
10	0.095	0.19	0.28	0.19	0.73*
20	0.0	0.0	0.095	0.095	0.19*

* Significant change P ≤0.05

Table 3. Time course change in routine urine examination values.

Time(days)	Pus cells	RBCs	Crystals
0	32 (76.19%)	24 (57.14%)	30 (71.42%)
5	10 (23.8%)*	8 (19.04%)*	15 (35.71%)*
10	3 (7.14%)*	2 (4.76%)*	4 (9.52%)*
20	2 (4.76%)*	0.0 (0.0%)*	1 (2.38%)*

Results represent percent of total, * significant change P ≤0.05.

Table 4. Time course change in renal function test of UTI patients.

Time(days)	Bl.Ureammol/L	S.Cr. μmol/L
0	4.46±0.21	72.3±3.14
5	4.1± 0.3	66± 5.2
10	3.5± 0.22	48± 2.7
20	3.2± 0.41	41± 3.5

Results represent mean ± SD

On the other hand, the routine examination of urine revealed a significant P≤0.05 improvement after administration of aqueous extract of corn silk , Table 3 clearly showed the significant reduction in the values of pus cells, RBCs and crystals after 5,10 and 20 days after starting the treatment; again these results confirm the beneficial effect of aqueous extract of corn silk in patient with UTI.

Table 4 showed that blood urea and serum creatinine were not altered significantly in patients with UTI neither at the baseline nor after starting the treatment course; at the same time, no any side effect was reported during the course of treatment, indicating the safety of aqueous extract of corn silk.

DISCUSSION

The use of herbal medicine in the treatment of disease in general and specially UTI is not a new approach; it has been reported that the Ebers papyrus from ancient Egypt recommended herbal treatment to ameliorate urinary symptoms without providing insight into pathological mechanisms [18].

Urinary tract infection (UTI), acute and chronic, can be effectively treated with herbal medicine. It has been shown that, there are two strategies which are essential in utilizing herbal medicine; the choice of herb depending on its herbal action, and the appropriate therapeutic dosing strategies that will determine the effectiveness of herbal treatment and prevent the need to intervene with antibiotics [19].

It has been found that medicinal plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids; these metabolites have been found *in vitro* to have antimicrobial properties [20]. Interest in medicinal plants has increased in recent years; this interest has lead to the discovery of new biologically active molecules and the adoption of crude extracts of plants for self-medication by the general public [21]. Many plants have been evaluated not only for their inherent antimicrobial activity, but also for their action as a resistance-modifying agent [22].

It has been believed that certain medicinal plants can prevent the recurrence of UTI; examples of such are cranberry juice [23], garlic [24], and others [25]. The mechanisms of action are believed to include stimulation of the immune response, change in urinary PH [26], and prevention of growth and adhesion of pathogens [27, 28]. In addition to that, recent works documented that early severe inflammatory response to uropathogenic microbials predispose to chronic and recurrent UTI [29]; and that many medicinal plants have been used in the treatment of UTI because of their anti-inflammatory effect [30]. All the above mentioned mechanisms may be exist as a potential

explanation for the results obtained in this study; although diuretic and uricosuric properties have traditionally been attributed to corn silk [31].

In conclusion, our results showed that administration of aqueous extract of corn silk significantly reduce the symptoms in patient with UTI in addition to reduction in the values of routine urine examination like pus cells, RBCs, and Crystals, without any reported side effect which indicate its efficacy and safety.

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Original Research

α-Glucosidase and α-amylase inhibitory activities of *Mukia maderaspatana* (L) Roem

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Abstract

Aim:The present study is to evaluate the invitro antidiabetic activity of Mukia maderaspatana ethanolic extract and fractions by α-glucosidase and α -amylase inhibitory activity.

Methods:In order to evaluate if the extraction procedure could influence the activity, we decided to extract with ethanol and fractions with chloroform and n-butanol of Mukia maderaspatana and test each of them with enzyme α-glucosidase and α-amylase inhibitory activity .

Results:Mukia maderaspatana ethanolic extract and fractions showed dose dependent inhibition of α-glucosidase and α-amylase enzyme and exhibited lower inhibitory activity than acarbose.

Conclusion:The study revealed the antidiabetic potential and could be helpful to develop medicinal preparations and nutraceuticals and functional foods for diabetes.

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secreted insulin. Such a deficiency results in increased blood glucose level, which in turn can damage many of the body's systems, including blood vessels and nerves [1].

One of the therapeutic approaches is to decrease the postprandial hyperglycemia by retarding absorption of glucose by inhibition of carbohydrate-hydrolyzing enzymes, such as α-amylase and α-glucosidase [2]. From this point of view, many efforts have been made to search for more effective and safe inhibitors of α-glucosidase and α-amylase from natural materials to develop physiological functional food to treat diabetes [3].

Many traditional plant-driven treatments for diabetes have been reported in India but only a small number of these have received scientific and medical evaluation to assess their efficacy. On the basis of ethnomedical/tribal information *M. maderaspatana* is used to treat and prevent diabetes. However, the studies on antidiabetic effects of *M. maderaspatana* were not focused on the enzyme inhibitory activity of the extract and fractions. The present study is designed to study the *in vitro* antidiabetic activity of *M. maderaspatana* ethanolic extract and to understand how the extract and fractions acts against α-glucosidase and α-amylase.

MATERIALS AND METHODS

Materials

The entire plant of *M. maderaspatana* was collected from the forests of Doddabetta in Nilgiris. The plant species was identified and authenticated by Botanists from Government Botanical garden, Ooty.The voucher specimen was deposited in the herbarium of the Department of Pharmacognosy, JSS College of Pharmacy, Ooty. Porcine pancreatic amylase, α glucosidase from Bakers yeast, p-nitrophenyl-a-d-glucopyranoside and dinitrosalicylic acid were purchased from Sigma chemicals.

Preparation of crude extract and fractions

100 g powdered sample of various extracts were weighed and soaked in 250 ml of 95% ethanol in a separating funnel for 24 hours, with intermittent shaking. The plant extract was then collected and filtered through Whatman No. 1 filter paper. The extract was concentrated at 50 ^{0}C using vacuum rotatory evaporator and then air-dried. The dried powder was stored at 40 ^{0}C in an airtight bottle. The extract was fractionated with chloroform and n-butanol and all were used for invitro antidiabetic studies.

α-Amylase inhibition activity

The α-amylase inhibitory activity of the *M. maderaspatana* ethanolic extract and fractions (chloroform and n-butanol) were determined [4]. A total of 250 µl of sample and 125 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 6 mM NaCl) containing α-amylase solution (0.5 mg/ml) was incubated at 25 ◦C for 10 min. After preincubation, 250 µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 6 mM NaCl) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25 ◦C for 10 min. The reaction was stopped with 0.5 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 5 ml of distilled water, and absorbance was measured at 540 nm. Acarbose was used as the positive control. The α-amylase inhibitory activity was calculated as follows:

$$\textbf{Inhibition (\%)} = (1 - A_{samp} / A_{cont}) \times 100$$

where A_{samp} and A_{cont} were defined as absorbance of the sample and the control respectively.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory of activity *M. maderaspatana* ethanolic extracts and fractions (chloroform and n-butanol) were determined [5]. A mixture of 50 µl of sample and 100 µl of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1 U/ml) was incubated in 96 well plates at 25ºC for 10 min. After preincubation, 50 µl of 5 mM pNPG solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25 ◦C for 5 min. Before and after incubation, absorbance was recorded at 405 nm by microplate reader. Acarbose was used as the positive control. The α-glucosidase inhibitory activity was expressed as inhibition percent and was calculated as follows:

$$\textbf{Inhibition (\%)} = (1 - A_{samp} / A_{cont}) \times 100$$

where A_{samp} and A_{cont} were defined as absorbance of the sample and the control respectively.

RESULTS

Pancreatic α-amylase is a key enzyme in the digestive system and catalyzes the initial step in the hydrolysis of starch, which is a principal source of glucose in the diet. The *M. maderaspatana* ethanolic extract and fractions (chloroform and n-butanol) showed dose dependent inhibition of the α-amylase enzyme (IC_{50} =35-45µg/ml). As observed, the extract and fractions exhibited lower α-amylase inhibitory activity, compared with that of acarbose, which showed potent inhibition of α –amylase.

The *M. maderaspatana* ethanolic extract and fractions (chloroform and n-butanol) showed dose dependent inhibition of the α-glucosidaseglucosidase enzyme(IC_{50} =45-51µg/ml). As observed, the extract and fractions exhibited lower α-glucosidaseglucosidase inhibitory activity, compared with that of acarbose, which showed potent inhibition of α –glucosidase.

The inhibitory activities of extract and fractions of *M. maderaspatana* on enzyme inhibitory activity is given in Table 1.

DISCUSSION

The treatment goal of diabetes patients is to maintain near normal levels of glycemic control, in both the fasting and postprandial states. Many natural resources have been investigated with respect to suppression of glucose production from carbohydrates in the gut or glucose absorption from the intestine [6].

Table 1. α –amylase and α –Glucosidase inhibitory activity of M. maderaspatana

Groups	Treatment	α –amylase (IC 50) µg/ml	α – Glucosidase (IC 50) µg/ml
1	EEMM	35.52±0.66	45.92±1.73
2	CFMM	39.13±0.67	51.16±1.10
3	BFMM	40.85± 1.19	47.32±1.02
4	ACARBOSE	11.84±0.19	13.39±0.11

Values are expressed as Mean ± SEM (n=3)

EEMM – Ethanolic extract of *M. maderaspatana*

CFMM – Chloroform fraction of *M. maderaspatana*

BFMM – n-butanol fraction of *M. maderaspatana*

Pancreatic α-amylase is a key enzyme in the digestive system and catalyzes the initial step in the hydrolysis of starch, which is a principal source of glucose in the diet. α-GlucosidaseGlucosidase, a key enzyme for carbohydrate digestion, has been recognized as a therapeutic target for modulation of postprandial hyperglycemia, which is the earliest metabolic abnormality to occur in type 2 DM. α-amylase catalyzes the hydrolysis of a-1, 4-glycosidic linkages of starch, glycogen and various oligosaccharides and α –glucosidase further breaks down the disaccharides into simpler sugars, readily available for the intestinal absorption. The inhibition of their activity, in the digestive tract of humans, is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes [7]. Therefore, effective and nontoxic inhibitors of α -amylase and α -glucosidase have long been sought.

In this study we have investigated the antidiabetic potential of the *Mukia maderaspatana*, which is used in traditional ayurvedic medicine for the treatment of several diseases [8]. This valuable herb was not previously investigated for its *in vitro* antidiabetic activity. However, our study clearly established the antidiabetic potential of *M. maderaspatana*, and revealed that the active principles responsible may be flavonoids, terpenes and phenolic compound.

Flavonoids, like antioxidants, may prevent the progressive impairment of pancreatic beta-cell function due to oxidative stress and may thus reduce the occurrence of type 2 diabetes [8].

Although, in the present study, the enzyme inhibitory activity of these extract and fractions were assayed *in vitro*, the results from this work should be relevant to the human body. In addition to α-amylase and α glucosidase inhibitory activities, these phytoconstituents are also reported to have several other biological activities including anti-bacterial, anti-oxidative, anti-cancer etc. [9]. This supportive evidence further increases the medicinal importance of this *M. maderaspatana* indicating that herb is not only beneficial for diabetes but may also be useful to a number of other human health complications.

CONCLUSION

This study investigated the potential antidiabetic activity of the *M. maderaspatana*, focusing on the inhibitory effects on α-glucosidase and α-amylase. Further isolation of active principles would be helpful to explain the pharmacological mechanism and also develop medicinal preparations, nutraceuticals or functional foods for diabetes and related symptoms.

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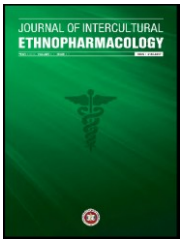

Original Research

Total phenolic, flavonoids and tannin contents in different extracts of *Artemisia absinthium*

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Keywords: A. absinthium, total phenolic content, tannin, aqueous, ethanolic extract

Abstract

The *A. absinthium*iscommonly is commonly known as wormwood having antipyretic, antimicrobial, antifungal, diuretic and anti-inflammatory properties. Natural bioactive compounds like phenols and flavonoids are the important secondary metabolites in plant posses high scavenging ability of free radical and reactive oxygen species produced in mammals. To maximize these agents in the extract different solvents viz. aqueous, ethanolic and chloroform are used for the extraction procedure (among these different extractions). Current study was aimed to determine the levels of total phenolic, flavonoids and tannin contents. Observations suggested that ethanolic extract has significantly high ($P<0.05$) concentration of flavonoids, phenolic and tannin contents as compared to aqueous and chloroform extracts. Therefore, ethanolic extract of *A. absinthium* has greater potential to scavenge free radicals/ ROS and can produce more beneficial effects as compared to aqueous and chloroform extracts.

INTRODUCTION

The genus *Artemisia* L. is a member of family Asteraceae and comprises of more than 200 species, found throughout the northern half of the world. In India, it grows in Kashmir valley and is locally known as 'Tethwen'. Plant is used in indigenous traditional systems of medicine as a vermifuge, an insecticide, an antipyretic [1], antimicrobial [2], antifungal [3], diuretic and as an antispasmodic in animals [4]. It is a rich source of terpenes, antioxidant phenolics, flavonoids and other biologically-active compounds. In modern medicine these compounds have been investigated for their anthelmintic and antioxidant activities in parasitized animals by neutralizing the free radicals and toxins formed in their blood, boost their immune system, and help fighting gastrointestinal parasites [5].

Natural bioactive compounds like phenols and flavonoids are the important secondary metabolites in plants having intrinsic properties that affect appearance, taste, odor and oxidative stability of plant based foods. These compounds also posses biological properties like antioxidant, anti-aging, anti-carcinogen, protection from cardiovascular, immune/autoimmune diseases and brain dysfunctions viz. Parkinson's, Alzheimer's, Huntington's diseases, etc [6,7]. Therapeutic potential of *A. absinthium* extract is directly related to total phenolic and flavonoids contents. These active metabolites especially from herbs are the interest subject of research, but their extraction as part of phytochemical or biological investigations presents specific challenges that must be addressed throughout the solvent extraction process. Therefore, present study was aimed to investigate the

levels of phenolic, flavonoids and tannin contents in different extracts prepared using aqueous, ethanolic and chloroform solvents.

MATERIALS AND METHODS

Chemicals and plant materials: The aerial parts of *Artemisia absinthium* was purchased from Agro Food Processing Emporium, Peerbagh, Srinagar, India. All parts were cleaned of adulterants and air-dried under shade at a well ventilated place. The plant material was pulverised to powder form with a mixer grinder. The 100 g of powder was soaked in 500 mL of aqueous, 95% ethanol and chloroform solvents to exhaustion (~120 h). The extraction was carried out in a percolator by a combination of maceration and percolation at room temperature. The filtrates were collected through a piece of porous cloth. Removal of the solvents at temperatures, below 60^0C for aqueous and 40^0C for ethanol and chloroform, under reduced pressure and a rotation speed of 20 rpm in vacuum rotary evaporator yield the respective extract. The extracts were scrapped off, transferred to an air tight container and stored in a freezer at -20^0C till subsequent uses. Different chemicals viz. Quercetin (Sigma Aldrich, USA), Gallic acid (SD Fine Chem Ltd Mumbai, India), Folin-Ciocalteu reagent (Central Drug House, New Delhi, India) and other chemicals used for the analysis are analytical grade.

Determination of Total Phenolic Contents: 1% of plant extract solution was prepared in methanol and the amount of total phenolic contents in extracts was determined by the methods of Savitree*et al* [8]. In brief, 0.5ml of each sample was taken into test tube and mixed with 2.5 ml of a 10 fold dilute Folin-Ciocalteu reagent and 2ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature. Then the absorbance was read at 760nm spectrometrically (U-1800, Hitachi, Japan) against Gallic acid as a standard (Concentration of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol). The Folin-Ciocalteu reagent is sensitive to reducing compounds including polyphenols, thereby producing a blue color upon reaction. All determination was performed in triplicate and the total phenolic content was expressed as mg/g gallic acid equivalents (GAE).

Determination of total flavonoids contents: The total flavonoids content of each plant extract was estimated as per Zhishen*et al* [9]. In-brief, each sample (1.0mL) was mixed with 4ml of distilled water and subsequently with 0.30 mL of a $NaNO_2$ solution (10%). After 5 min, 0.30 mL of an $AlCl_3$ solution (10%) was added followed by 2.0 mL of NaOH solution (1%). Immediately, after thorough mixing the absorbance was measured at 510 nm versus the blank. Standard curve of quercetin was prepared (0-12mg/mL) and the results are expressed as quercetin equivalents (mg quercetin/gm dried extract).

Determination of tannin contents: Tannin content in each sample was determined using insolublepolyvinyl-polypirrolidone (PVPP) as describedby Makkar*et al* [10]. Briefly, 1.0 mL of extract dissolved in methanol (1%), was mixed with 100 mg PVPP, vortexed, left for 15 min at 4°C and then centrifuged for 10 min at 3,000 rpm. In the clear supernatant the non-tannin phenolics were determined in the way similar to the total phenolics content [11]. Tannin content was calculated as a difference between total phenolic and non-tannin phenolic content in the extract.

Statistical analysis: The determinations were conducted in triplicate and results were expressed as mean ± standard error. Statistical analyses were done by one-way ANOVA followed by Dunnet's test with $P < 0.05$ as a limit of significance.

RESULTS

Standard curve prepared was used for the determination of total phenolic content and flavonoids using different concentrations of Gallic acid and quercetin respectively. Tannin content was calculated as a difference between total phenolics and non-tannin phenolic content. The total phenolics, flavonoids, tannin and non-tannin content in different extracts of*A. absinthium* have been presented in table 1. Observation shows that the total phenolic content is highest in the ethanolic extract (43.04 ± 0.57mg of GAE/g of extract) fallowed by aqueous and significantly lower ($P<0.05$) in the chloroform extract (28.34 ± 2.39 mg of GAE/g of extract). Similarly concentration of flavonoids is significantly high ($P<0.05$) in ethanolic extract as compared to aqueous and chloroform extracts. However, the concentration of tannin content is significantly lower ($P<0.05$) in chloroform extract (22.62± 2.45 mg of GAE/g of extract) as compared to aqueous and ethanolic extracts.

DISCUSSION

The WHO survey indicated that about 70–80% of the world's populations rely on non-conventional medicine, mainly of herbal source, for their primary healthcare [12]. These medicinal plants are rich sources for naturally occurring antioxidants especially phenolic and flavonoids contents. These agents have ability to scavenge free radicals, super oxide and hydroxyl radicals, etc thus they enhance immunity and antioxidant defense of the body [13]. Dietary

supplementation of these compounds reduces the oxidative damage to cell membrane lipid, protein and nucleic acid due strong quenching property of free radicals [14].

For acceptance of medicinal plants into scientific medicine, it is necessary that their effectiveness and safety be evaluated and confirmed through active ingredient testing. To maximize the extractive capability of phenolic and flavonoids components from plant material is considerably depended on the type of solvent. Highest content of phenolic, flavonoids and tannin in ethanolic extract in comparison to other solvents used, make this organic solvent (ethanol) an ideal and selective to extract a great number of bioactive phenolic compounds. Similarly, Mohammedi [15] also reported that hydro-alcoholic mixtures are suitable to extract different bioactive phenolic compounds from *Tamarixaphylla.*

Table 1. The total phenolic, flavonoids, tannin and non-tannin contents present in different extracts of *A. absinthium*

Parameters	Aqueous extract	Ethanolic extract	Chloroform extract
Total Phenolic Content (mg of GAE/g of extract)	40.00 ^{a} ± 2.11	43.04 ^{a} ± 0.57	28.34 ^{b} ± 2.39
Total Flavonoids Content (mg Quercetin/g extract)	550.53^{a} ± 45.93	1108.15^{b} ± 48.78	667.40^{a} ± 51.26
Non-tannin Content (mg of GAE/g of extract)	7.09 ^{a} ± 0.24	6.52 ^{a} ± 0.81	2.45 ^{b} ± 0.24
Tannin Content (mg of GAE/g of extract)	30.44 ^{a} ± 1.08	36.91 ^{a} ± 1.24	22.62 ^{b} ± 2.45

Values are expressed as mean ± SE of three replicates. The different superscripted (a, b) values have significantly differ ($P<0.05$) from the other extract in same row.

Tannins are generally defined as naturally occurring polyphenolic compounds of high molecular weight to form complexes with the proteins. Tannins are important source of protein in animals but unfortunately the amounts of tannins that they contain vary widely and largely unpredictably, and their effects on animals range from beneficial to toxicity and death [16]. The toxic or anti-nutritional effects tend to occur in times of stress when a very large proportion of the diet having high concentration of tannins. Thus consumption of foods naturally having antioxidant activity is the most efficient way of combating such tissue injuries, undesired transformations and preventing health risks [17]. In present study the ethanolic extract have high concentration of flavonoids and phenolic concentration. Therefore, ethanolic extract of *A. absinthium* have greater potential to reduce or scavenge free radicals or produces more beneficial effects as compared to other extracts.

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Original Research

Phytochemical analysis and *in vivo* anti-diarrhoeal potentials of *Dialium guineense* (wild) stem bark extract

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Abstract
This study was undertaken to evaluate the in vivo anti-diarrhoeal activity of methanolic stem bark extract of *Dialium guineense* used traditionally as remedy for gastrointestinal disorder in South-Western Nigeria. The effect of the extract at oral doses of 50-200mg/kg body weight on the castor oil-induced diarrhoea, gastrointestinal motility (charcoal meal) and castor oil-induced intestinal fluid accumulation (enteropooling) were examined in rats. The extract employed produced a dose-dependent and significant ($P<0.05$) reduction (32.30 - 80.81%) in the watery nature and frequency of faecal droppings over 4hours, while loperamide gave 85.81% reduction. On gastrointestinal motility and enteropoolings, the extract also dose-dependently reduced ($P<0.05$) the small intestinal transit of charcoal meal (28.90 - 45.54%) and intestinal fluid volume (46.27 - 73.88%) in a manner comparable to 5mg/kg each of atropine (58.20% motility time inhibition) and loperamide (76.12% enteropooling inhibition). The acute toxicity and lethality studies on the extract revealed an oral LD_{50} value greater than 5000mg/kg in mice. The phytochemicals detected in the extract were cardiac glycosides, tannins, phlobatannins, saponins, terpenoids, resins, steroids/triterpenes, alkaloids, flavonoids, reducing sugars and carbohydrates.The findings from this study suggest that methanolic extract of *D. guineense* stem bark possesses anti-diarrhoeal property, probably by anti-secretory mechanism and thus, substantiate the traditional application of the bark extract in the treatment of diarrhoea in Nigeria.

INTRODUCTION

Diarrhoea is a major disease of especially children (under five years) with 3-5 billion cases occurring annually, and approximately 5 million deaths resulting thereby [1,2]. Infected children usually experience clinical manifestations such as increased frequency of bowel sound and movement, abdominal pain and watery stool leading to rapid dehydration and death if treatment is not given [2]. Despite the intervention of government agencies and international organizations to curtail the trend the incidence of diarrhoeal diseases still remains high. According to W.H.O. estimates for 1998, about 7.1 million deaths were caused by diarrhoea [3].

In recent times, emphasis has been focused on the use of oral rehydration solution (ORS) as a replacement therapy to replenish the lost fluid and electrolytes in diarrhoeic cases, but the oral rehydration solution (ORS) formulae supplemented with cooked rice powder was more effective than ORS treatment [4]. In addition, the World Health Organization (WHO) has constituted a diarrhea disease control program aimed at the holistic approach to include all aspects of traditional medicinal practices, evaluation of health education and preventive approaches [5]. In many African countries there is the common practice application of herbal drugs in the management of various gastrointestinal disorders including diarrhea, because it is generally believed that herbs are efficacious, readily available, accessible, safe and easy to prepare. Since the World Health Organization (WHO) currently encourages

studies for the treatment and prevention of diarrhoeal diseases depending on traditional medical practices [6], it is therefore pertinent to intensify research into several of medicinal plants claimed to be effective in the management of gastrointestinal ailment like diarrhoea.

One of such medicinal plant is *Dialium guineense* Wild (Fabaceae), commonly called black velvet or velvet tarimand (English), Icheku (Ibo, Eastern Nigeria), Awin (Yoruba, Western Nigeria), Tamarinier noir (French) [7]. It is a woody plant that occurs in the rain forest region of West Africa and can grow up to 10-20m. The tree possess densely, hairy leafy crown, smooth greyish bark and whitish flowers which bears densely velvet black fruits that are more or less circular and flattened enclosing dry, brownish, sweet acidic edible pulp [8]. In Nigeria, the tree flowers from September to October and fruits from October to January [9].

The ripe fruits of the plant are chewed among some women in southeast Nigeria to improve lactation and check genital infection [10]. Among the Esan tribe of Edo State in Nigeria, the twig or bark is chewed for oral hygiene and stomach ache [11]. The genus *Dialium* comprises five species in West Tropical Africa but *D. guineense, D. dinklagel* and *D. packyphyllum* are represented in Nigeria [12]. Traditionally, Dialium guineense leaves and stem bark are used as remedies for diarrhoea, severe cough, bronchitis, wound, stomachaches, malaria fever, jaundice, antiulcer and haemorrhoids [13].

Scientifically, the molluscicidal activity of the fruits and leaves of *D. guineense* have been reported [14]. The antimalarial properties of the aerial parts have also been reported [13]. Recently, the methanolic leaf and stem bark extracts were reported to possess analgesic and anti-vibrio potentials [7, 15]. The phytochemicals identified in the methanolic leaf extracts of the plant were tannins, alkaloids, flavonoids, saponins, steroids and cardiac glycosides [15].

Despite these array of documented reported of *D guineense,* currently available literature revealed that there is paucity of information on the scientific proof to buttress the acclaimed potentials of this plant as traditional remedy for diarrhea. The present study was therefore undertaken to investigate the phytoconstituent and anti-diarrhoeal activity of the methanolic stem bark extract of *D. guineense* with the aim of establishing the pharmacological basis for its folkloric use as treatment for gastrointestinal disorder including diarrhea.

MATERIALS AND METHODS

Plant collection and Identification

Fresh stem bark was collected from *Dialium guineense* tree found in the forest of Amai town, Delta State. Sample (fruits, leaves, and bark) of the plant was used to identify it according to the morphological illustrations as described by [16] and authenticated taxonomically at the Department of Biological Sciences, Novena University, courtesy of Prof. J. M. O. Eze (Botanist), where voucher specimens were kept for future reference purpose.

Preparation of plant extract

The collected stem bark of *Dialium guineense* was chopped into small pieces with a knife, air- dried on the laboratory workbench for 14days. The dried sample was reduced to smaller bits with mortar and pestle and further reduced to fine particles with an Electric blender. 400g of the powdered sample was exhaustively extracted with 80% methanol using a Soxhlet extractor apparatus. The liquid extract obtained was filtered concentrated to dryness using rotary evaporator. A dark brownish mass of weight 22.3gw/w was obtained, and then stored in desiccators. Prior to the commencement of each study, fresh solutions of the extracts were prepared to obtain working concentrations of 50, 100 and 200mg/kg/body weight.

Phytochemical screening of stem bark Extract

The stem bark extract of *D guineens*e were tested for the presence of phytochemical compounds using the standard procedures described by [17, 18].

Animals

Swiss albino mice of (25-34g) and Wistar Albino rats (190-246g) of either sex used for the experiments were obtained from the Animal House of the Faculty of Health Sciences Delta State University, Abraka, Delta State, Nigeria. They were housed in standardized environmental conditions (25 ± 1.5^{o}C, relative humidity 75 – 80%, 12h light/ 12h dark cycle) and maintained on standard animal pellets and water *ad libitum*. This research was carried out in accordance with the rules governing the use of laboratory animals as accepted internationally (NIH pub. No 85-23, 1985).

Drugs

Normal saline (Dana Nig. Ltd), Activated charcoal (Kochlight Laboratories ltd, England), Atropine sulphate (Fugisawa USA, Inc.), Loperamide (Janssen Chemica, Belgium) Castor oil (Bell Sons and Co., Druggist Ltd, Southport, England)

Acute toxicity test

The method previously described by [19] was adopted using thirteen Swiss albino mice (25-34g). In the first phase, three increasing doses (10 - 5000mg/kg) of the aqueous root extract were administered orally to three groups each containing three mice. In the second phase, more specific doses were administered to four groups each containing one mouse. The median lethal dose (LD_{50}) value was determined as the geometric mean of the highest non-lethal dose and the lowest lethal dose of

which there is 1/1 and 0/1 survival [19].

Effect of extract on Castor oil induced diarrhea

The method described by [20], with slight modification, was adopted. Thirty rats (190-246g) of either sex were fasted for 12 h prior to the commencement of the study and were randomly divided into five groups each containing six rats. The rats were fasted for 12 hours prior to the commencement of the experiment and were randomly divided into five groups of five mice each. The first group was administered 5ml normal saline, group two was administered loperamide (5mg/kg), while group three, four and five were administered 50, 100, and 200mg/kg respectively, of the methanolic stem bark extract orally. An hour later, 1 ml castor oil was administered to each mouse orally. An hour later, 1 ml castor oil was administered to each mouse orally. The animals were housed singly in cages lined with transparent paper. Thereafter, they were observed for four hours for the presence of characteristic diarrhoeal droppings. The absence of which was considered as protection from diarrhea.

Effect of Extract on gastrointestinal transit (charcoal meal study)

The effect of *D. guineens*e leaf extract on gastrointestinal transit (motility) was investigated in rats according to the methods of [21]. Thirty rats (190-246g) of either sex were fasted for 12 h before the commencement of the experiment and were randomly allocated into five groups of six rats each. The first group and second groups were administered orally with normal saline (5ml) and atropine sulphate (5 mg/kg body weight) respectively. The third, fourth and fifth groups were administered 50, 100 and 200mg/kg of methanolic stem bark extract respectively using same route. 1ml of marker (10% charcoal suspension in 5% gum acacia) was administered orally 1 h after castor oil treatment. The animals were sacrificed after 1 h and the distance traveled by charcoal meal from the pylorus was measured and expressed as percentage of the total length of the intestine from the pylorus to caecum.

Effect of Extract on Castor oil-induced enteropooling (Intestinal fluid accumulation)

Effect of methanolic leaf extract of *D. guineens*e on castor oil-induced enteropooling was done according to the method of [22]. Thirty albino Wistar rats (190-246g) of either sex were fasted for 12 h before the commencement of the experiment and were randomly allocated into five groups of six rats each. Group one was treated with 5ml of normal saline orally. The second group received loperamide (5mg/kg bodyweight, orally), while the last three groups received the extract at doses 50, 100 and 200mg/kg body weight by oral intubation, 1 hour before the oral administration of castor oil. One hour later, the animals were sacrificed by cervical dislocation; the small intestine was removed after tying the ends with thread and weighed. The intestinal contents were collected by being milked into a graduated tube and their volumes were measured. The intestine was reweighed and the differences between full and empty intestines were calculated.

The results on gastrointestinal transit time were expressed as mean ± S.E.M. Differences between means were analyzed using one-way analysis of variance (ANOVA).Values of $P < 0.05$ were considered statistically significant.

RESULTS

Phytochemical analysis

Phytochemical analysis of the crude extract confirm the following secondary metabolites; cardiac glycosides, tannins, phlobatannins, saponins, terpenoids, resins, steroids/triterpenes, alkaloids, flavonoids, reducing sugars and carbohydrates. Anthraquinones and phenol were not detected (Table 1)

Acute toxicity test

The oral administration of the doses of 10 - 5000mg/kg body weight showed no signs of toxicity in the mice within and after 24 h. Apart from sedation and weakness, no deaths were recorded even at the highest dose of 5000mg/kg body weight, indicating that the LD_{50} was greater than 5000mg/kg.

Table 1. Phytochemical constituents of the methanolic stem bark extract of *D. guineens*e

Phytochemical Constituent	Stem bark
Carbohydrate	+
Reducing sugars	+
Tannins	+
Phlobatannins	+
Flavonoids	+
Saponins	+
Cardiac glycosides	+
Steroids/Triterpenes	+
Alkaloids	+
Resins	+
Anthraquinones	-
Phenols	-

+ = Present, - = Absent

Effect of *D. guineense* methanolic stem bark extract on castor oil-induced diarrhoea

The methanolic stem bark extract produced a dose-dependent decrease in the number of faecal matter passed by the animals. The effects of the extract (50-200mg/kg) treated group were significant ($P<0.05$) when compared with normal saline (control) group, however there was no significant difference ($P<0.05$) in the

percentage protection (68.24 and 80.81%) against castor oil induced diarrhea at doses of 100 and 200mg/kg body weight when compared with that of loperamide (85.81%), the standard anti-diarrhoel agent (Table 2). There was no protection from diarrhoea in the animals treated with normal control group.

Effect of *D. guineense* methanolic stem bark extract on intestinal transit

The effects of extract on gastrointestinal transit revealed that the methanolic stem bark extract of *D. guineens*e caused a significant inhibition in the propulsion of charcoal meal through the gastrointestinal tract when compare to the control group, though in a dose dependent manner (Table 3) Significant percentage inhibition ($P<0.05$) comparable to that of the standard drug (atropine) were only observed at doses of 100 and 200mg/kg body weight, though atropine treated group caused better decrease in the propulsive movement and the intestinal length travelled by charcoal meal (Table 3).

Effect of *D. guineense* methanolic stem bark extract on castor oil-induced enteropooling

The methanolic stem bark extract of *D. guineense* was found to possess significant ($P<0.05$) anti-enteropooling activity when compared with the control and standard drug treatment (Table 4). Extract doses (50-200mg/kg) were able to significantly decreased intestinal fluid volume of from 1.34ml to 0.72ml 0.43ml and 0.35ml respectively in the mice, which are equivalent 46.27%, 67.91% and 73.88% inhibition of enteropooling.

DISCUSSION

Diarrhea induced by castor oil, occurs when there is hydrolysis of the oil by intestinal lipases resulting in the release of ricinoleic acid which causes irritation on gastric mucosa leading to release of endogenous prostaglandins which induce changes in mucosa fluid, electrolytes transport and diarrhoea [24, 24]. The mechanisms proposed to explain the diarrhoeal effect of castor oil are inhibition of intestinal Na^{+} K^{+s} ATPase activity, thus reducing normal fluid absorption, activation of adenylate cyclase or mucosal cAMP mediated active secretion, stimulation of prostaglandin formation, stimulation of platelet activating factor and nitric oxide [25-28]. The peak effect of the extract was similar to loperamide, which is at present one of the most efficacious and widely employed anti-diarrheal drug. Loperamide drugs have the ability to antagonize diarrhea induced by castor oil, prostaglandin or cholera toxin [28]. The pharmacological effect of the loperamide is believed to be due to its anti-motility and anti-secretary activity [30]. This probably suggests that the extract of *D. guineense* exerts anti-diarrhoeal effect through similar mechanism (loperamide-like effect). Similar finding was reported by [31] in their study on the anti-diarrhoel activity of the stem bark extract of *Ceiba pentandra* (Bombacaceae).

Table 2. Effect of methanol stem bark extract of D. guineense on castor oil induced diarrhea in rat

Group/Treatment	Dose (mg/kg)	Number of Wet faeces in 4h	% inhibition
Normal saline	10	7.40 ± 0.21	0
Loperamide	5	1.05 ± 0.67	85.81**
Extract	50	5.01 ± 0.50	32.30*
Extract	100	2.35 ± 0.67	68.24**
Extract	200	1.42 ± 0.56	80.81**

Values are mean ± SEM, n = 6 *significant as compared to control $P< 0.05$, **significant as compared to standard drug $P<0.05$

Table 3. Effect of Methanolic stem bark extract of D. guineense on charcoal transit time in rat

Group/Treatment	Dose (mg/kg)	Distance traveled by charcoal meal (cm)	% inhibition
Normal saline	10	77.16 ± 0.59	-
Atropine	5	32.25 ± 0.10	58.20**
Extract	50	54.86 ± 0.89	28.90*
Extract	100	51.41 ± 1.01	33.37**
Extract	200	42.02 ± 0.99	45.54**

Values are mean ± SEM, n = 6 *significant as compared to control $P< 0.05$, **significant as compared to standard drug $P<0.05$

Table 4. Effect of methanolic stem bark extract of D. guineense on castor oil-induced enteropooling in rat

Treatment	Dose (mg/kg)	Fluid volume (ml)	% inhibition
Normal saline	10	1.34 ± 0.15	-
Loperamide	5	0.32 ± 0.02	76.12a*
Extract	50	0.72 ± 0.18	46.27a*
Extract	100	0.43 ± 0.66	67.91a*
Extract	200	0.35 ± 0.19	73.88a*

Values are mean ± SEM, n = 6 *significant as compared to control $P< 0.05$, [a]significant as compared to standard drug $P<0.05$

The methanolic stem bark extract of *D. guineense* also significantly reduced gastrointestinal propulsion as observed by the decrease in transit motility of charcoal meal. This probably makes it beneficial as a preventive agent. Antidiarrhoeal treatment in patient is achieved through the objective of the therapy which includes increasing resistance to flow (segmental contraction and decrease propulsion) and increased mucosal absorption or decreasing secretion [32]. This suggests the ability of the plant to alter normal peristaltic movement and hence decrease the movement of materials in the intestinal tract allowing greater time for absorption. This observation agrees with similar reports which have established reduction in gastric motility as being the mechanism by which many anti-diarrhoeal agents act [2, 33-35]. The anti-diarrhoeal activity of atropine drug is probably due to its anti-cholinergic effect [36].

In the fluid accumulation test, the extract significantly reduced volume of intestinal content (enteropooling). This may promote reabsorption of materials in the intestine due to decrease propulsion of material in the intestinal tract, and the extract might have probably exerted its anti-diarrhoeal action by anti-secretory mechanism. This indicates that methanolic stem bark extract possess anti-enteropooling effect and thus suggest that the extract could be equally effective in prevention and curing of diarrhea.

The phytochemical analysis of the methanolic stem bark extract of *D. guineense* revealed the presence of reducing sugars, flavonoids, alkaloids, saponins, tannins, steroids/ terpenes and resins. Identification of these compounds is in agreement with the phytochemical compounds in the leaf extract of the same plant [15], though other bioactive ingredients such as reducing sugars, carbohydrate, resins, terpenes, and phlobatannins were detected in the stem bark. Previous studies have shown that anti-dysenteric and anti-diarrheal properties of plants are due to tannins, alkaloids, saponins, flavonoids, sterol, triterpenes and reducing sugars [37-41]. The anti-diarrhoeal activity of the stem bark extract of *D. guineense* is most likely due to the presence of one or more of these bioactive principles present in the extract.

CONCLUSION

The findings from this study suggests that the methanolic stem bark extract of *D. guineense* possess pharmacologically active substances with antidiarrheal significant anti-diarrhoeal properties based on the fact that its ability to reduce the number of diarrhoea stool, delay gastrointestinal motility and enteropooling in the intestinal tract of the experimental animals. This has however justified the application of this plant in the treatment of gastro-intestinal disorder by traditional healers. Further research is required to fractionate and purify the extract to thoroughly understand the mechanisms responsible for the anti-diarrhoeal activity observed.

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Original Research

Role of Cyperus rotundus oil in decreasing hair growth

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Keywords: Androgenic hair, Cyperus rotundus oil, Decrease hair growth, Hirsutism.

Abstract

Background: There is a lack of value of Egyptian Cyperus rotundus essential oil in the treatment of Androgenic hair.

Aim: To evaluate the efficacy and safety of application of Egyptian Cyperus rotundus essential oil in comparison to 0.9% saline on androgenic hair.

Methods: Ninety one female patients with Androgenic hair (hirsutism and axillary hair) completed the study. They were randomly assigned to two groups: group I (active group) (n=47) and group II (control group) (n=44). Patients used topical Cyperus rotundus essential oil for six months and were evaluated on the 6th month.

Results: The topical Cyperus rotundus oil was significantly more effective ($p<0.05$) than the placebo without side effects. This result was proven by three assessment methods; difference in hair count, independent observer assessment and patients' self assessment.

Conclusion: The topical Egyptian Cyperus rotundus essential oil is an effective method in treating moderate degrees of hirsutism and axillary hair. But without affecting serum testosterone. This study is the first report on using Cyperus rotundus essential oil for decreasing hair growth

INTRODUCTION

Growth of sexual hair (androgenic hair) is entirely dependent on the presence of androgen [1-5]. Before puberty, hair in androgen-sensitive follicles is vellus (small, straight, and fair). In response to the increased levels of androgens at puberty, vellus follicles in specific areas develop into terminal hairs (larger, curlier, and darker, hence more visible), becoming sexual-hair follicles; higher androgen levels are required for the growth of a beard than for the growth of pubic and axillary hair [1-5].

Hirsutism results from an interaction between the androgen level and the sensitivity of the hair follicle to androgen. Severity of hirsutism does not correlate well with the level of androgen, because the response of the androgen-dependent follicle to androgen excess varies considerably within and among persons. Some women with excess androgen have no skin manifestations, or they may have seborrhea, acne, or androgenic alopecia without hirsutism [6]. It is a distressing condition for a lot of women, and leads to significant psychological distress that supports a multibillion-dollar effort to reverse this condition [7].

The available allopathic medicines for treating this condition are electro-epilation, LASER (Light Amplification by the Stimulated Emission of Radiation) for hair removal, Intense Pulsed Light, Eflornithine, oral anti-androgens, oral contraceptives and finasteride [8,9]. Nowadays, the new approaches have been tried to develop the herbal formulations for curing this condition and to avoid harmful side effects of the allopathic system. Egypt is famous for the herbal medicine since ancient times [10]. It is still a confident way of remedy among Bedouins and folk communities. Although in traditional systems of medicine, there are reports of many plants and herbal formulations for decreasing hair growth, the lack of sound scientific backing and information limits their use [11, 12]

Egyptian Cyperus rotundus (Cyperaceae) is a well-known functional food and traditional herbal medicine.

From the plant profile of C. rotundus, it possess: antioxidant and anti-apoptotic activities [13], hypoglycemic activity [14], protein oxidation protection [15, 16], 5α–reductase inhibitor [17], antibacterial and anti-fungal as biological activity [18-21], anti-platelet effects [22] and anti-inflammatory activity [23]. Also, patients with irritable bowel syndrome benefit from two weeks treatment by receiving a capsule containing powder of a crude plant with [24].

Phytochemical studies have shown that the major chemical components of this herb are essential oils, flavonoids, terpenoids, mono-and sesquiterpenes [25-26]. The plant contains the following chemical constituents-Cyprotene (1), cypera-2, 4-diene (2), a-copaene (3), cyperene (4), aselinene (5), rotundene (6), valencene (7), ylanga-2, 4- diene (8), g-gurjunene (9), trans-calamenene (10), d-cadinene (11), g-calacorene (12), epi-a-selinene (13), a-muurolene (14), g-muurolene (15), cadalene (16), nootkatene (17) by comparison with a spectral library established under identical experimental conditions [27], cyperotundone (18) [28], mustakone (19), cyperol (20) [29], isocyperol (21) [30] and a-cyperone (22) [31-32].

In this study, we have evaluated the effect of topical application of Cyperus rotundus essential oil on hairy skin.

MATERIALS AND METHODS

Plant Materials

Tubers of Egyptian *C. rotundus* were taken from the National Research Centre Farm of Aromatic and Medicinal Plants.

Preparation of the Essential oil:

The oils were prepared by hydrodistillation of the underground parts (tubers) of *C. rotundus*. The prepared oils were kept refrigerated until usage. Percentage yield was determined according to the Egyptian Pharmacopoeia [33].

Patients

This single blinded, placebo-controlled study was carried out on 91 patients who had moderate degree of hirsutism & axillae (androgen dependent areas) and were between the ages of 19 to 63 years. Active group contained of 20 axillae and 27 hirsute [stage 3 (2cases), stage 2(18 cases), stage 1(7 cases) Ferriman – Gallwey]. Volunteers and patients were recruited from the outpatient clinic of the dermatology department, in University Hospital of Suez Canal University, at the period from July 2011 to February 2012. The study was carried out in accordance with the guidelines of the Helsinki Declaration, and was performed after obtaining the informed consent from all patients.

Patients participate in this study were randomly distributed to 2 groups:

1- Group (I) active group: contained of 47 patients who treated with oil applied on the tested area twice daily.

2- Group (II) placebo group: contained of 44 patients who treated with a 0.9% saline applied also twice daily.

The inclusion criteria were as follow:

A- Normal volunteers who are seeking removal of axillary hair.

B- Patients with hirsutism who:

1. Matched with the diagnostic criteria of hirsutism according to the 2006 guidelines of the Alberta Medical Association.

2. Had no other dermatological disease.

3. Had no other systemic disease.

Patients like breast feeding women, pregnant women or patients already receiving treatment for decreasing hair growth were excluded from the study.

Full history was taken from each patient in addition to a general and systemic examination. Serum testosterone and ovarian ultrasound were done to all hirsute patients. Ferriman-Galloway Score applied to all hirsute patients. Patients were asked to remove hair their usual way (sugaring and threading) every 3 weeks. After each session of hair removing, patient put ¼ ml of the oil/saline on the tested area and rubbed for 2 minutes until absorption. Then they put ¼ ml of oil/saline twice daily after washing and cleansed the test area till the end of study. Oil/saline was given to patients in similar containers without any labels.

Assessment:

After 1 month of the last hair removing session, three methods were used to assess the results: hair count in a defined area of the skin, independent observer assessment of global photo-graphs and patients' self assessment. At each visit (every week) digital photos were taken for the tested area at standard distance and light conditions. At the tested area, counting (for white and black hair) was done in the beginning of the 1st month and the 6th month with the help of a hand magnified lens (x10) in a circle 1cm diameter.

Reproducibility of this area was assured by using a cardboard target area template, which had multiple holes, each of 1 cm diameters. The first hole was 2 cm away from the beginning of the strip, and the rest of the holes were 1cm apart from each other. The beginning of the strip was centralized at the sternal notch, then run horizontal with the arm abducted in 90^{o} to meet the

axilla at midline. For the chin, the strip ran from the mandibular angle along with the arch to the chin. Every patient had his marked and fixed hole, which was determined at the 1st visit.

Three professional independent observers examined the photos which had been taken at the 1st month and the 6th month. The mean value of all three assessment methods was taken. Assessment of improvement was done using a score from -1= regression compared to the base line, 0= no change, 1= minimal (< 20 %), 2 = mild (20% - 39 %), 3 = moderate (40% - 59%), 4= good (60% - 79%) and 5 = excellent (80% - 100%).

At the 6th month the patients were asked to fill a self-assessment questionnaire to evaluate the regression degree of area covering and hair quality (rate of hair growth, hair thickness, its color and brightness). The results of the patients' opinions for the area covered were scored as follows: -1 = worse, 0= no change, 1= mild <25%, 2 = moderate (25%-49%), 3 good = (50%-74%) and 4 = excellent (>75%) improvement. As for hair quality, each parameter was given a score of -1= worse compared to the base line, 0= no change, +1= improved.

At each visit the patients were asked about local side effects (irritation, dis-pigmentation, and folliculitis). They also were asked about the smell, color and greasiness of oil.

Statistical analysis of data was done using SPSS (statistical program for social science) version 12. Receiver operating characteristic (ROC) curve was used to determine a cutoff value for a clinical test.

RESULTS:

Demography: 91 out of 100 females with unwanted hair, completed the study (group I, n=47) and group II (n=44). Two cases in the active group and six cases in the control group were lost to follow up. Also, one case discontinued the study as she could not tolerate the smell of oil. The mean age of all included cases was 35.96 ± 11.5 years (range 19-63 years) (graph 1). The studied group contained 20 axillae and 27 hirsutes [stages 2 (1case), stage1 (3 cases) Ferriman – Gallwey and rest cured]. There was no significant difference before and after treatment in serum testosterone level and ultrasonic picture of ovaries between the two groups (table 1).

Hair Count Assessment: The mean of baseline hair count in a circle of 1 cm diameter on the skin of active treated group was 23.3 ± 10.9 (table 2). There was no significant difference between the two groups with respect to age and site. Hair Count Assessment: At month 6 of the study, mean hair count in the (group II) increased by 24.7 ± 6.2 hairs, while hair counts in the (group I) decreased by 1.7 ± 2.1 hairs, the difference was statistically significant ($p < 0.001$) (graph 2).

Independent observer evaluation of global photographs: 32 (68.1 %) cases out of 47, 14 cases (29.8%), 1case (2.1%) in the active group have excellent, good and moderate improving respectively, and 24 (54.5%) out of 44 and 20 (45.5%) in the placebo group were reported as no change and worse respectively. The active group showed significantly greater improvement than the placebo group ($p<0.05$) (table 3).

Patients' self-assessment: Regarding the decrease of hair density or skin covering which was assessed by the patients, the active group showed significantly greater improvement than the placebo group ($p<0.05$). The patients who considered their decrease in hair growth obvious (decrease more than 50%) were 97.9% and 0% in group I and II respectively (table 4).

Change of hair quality: A significant decrease in the rate of hair growth ($p=0.001$) and increase in hair growth was noticed by the patients in the active and placebo group respectively. The active group reported a significant decrease of hair thickness ($p=0.001$), color, and brightness ($p=0.001$) compared to the placebo group (table 5).

Only 24 patients in cthe ontrol group and 20 patients in the active treated group had white hair. All cases in later groups show a significant decrease in white hair count, quality and density ($p=0.001$) (table 2).

ROC curve analysis shows that hair count before treatment ≤ 21 can be used as predictor for complete cure after continuing treatment for 6 months (graph 3).

The observer assessment and patients' self assessment were concordant.

Safety & tolerability:

C. rotundus oil was used previously as oral and topical therapy in animals and humans without any side effects [13-24]. The only side effects which noted during the study was the smell. No cases were reported to have infection, folliculitis, dis-pigmentation, itching or irritation.

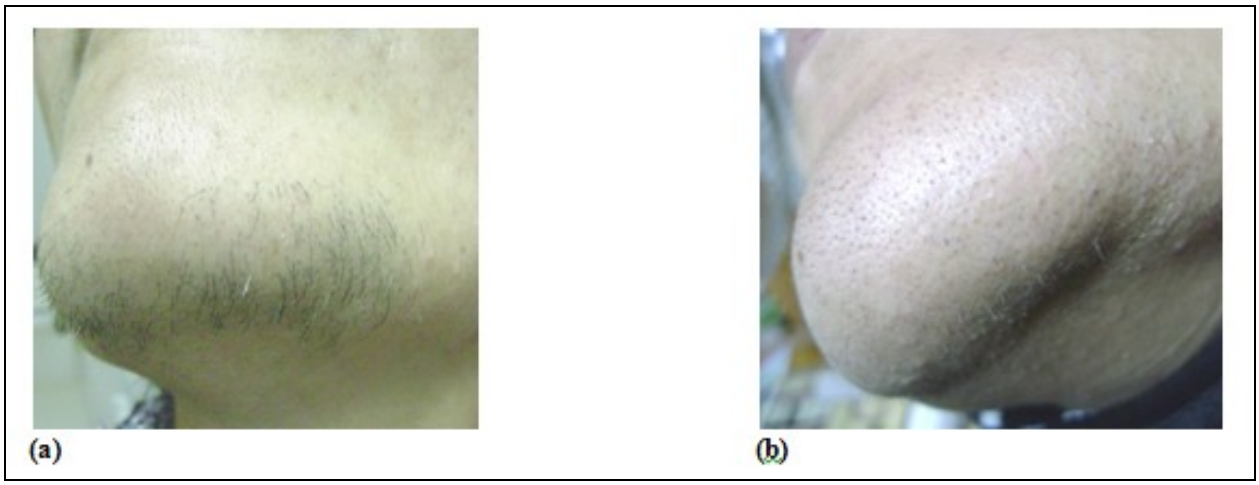


Fig. 1. a) Group I before and b) 6 months after start of treatment.

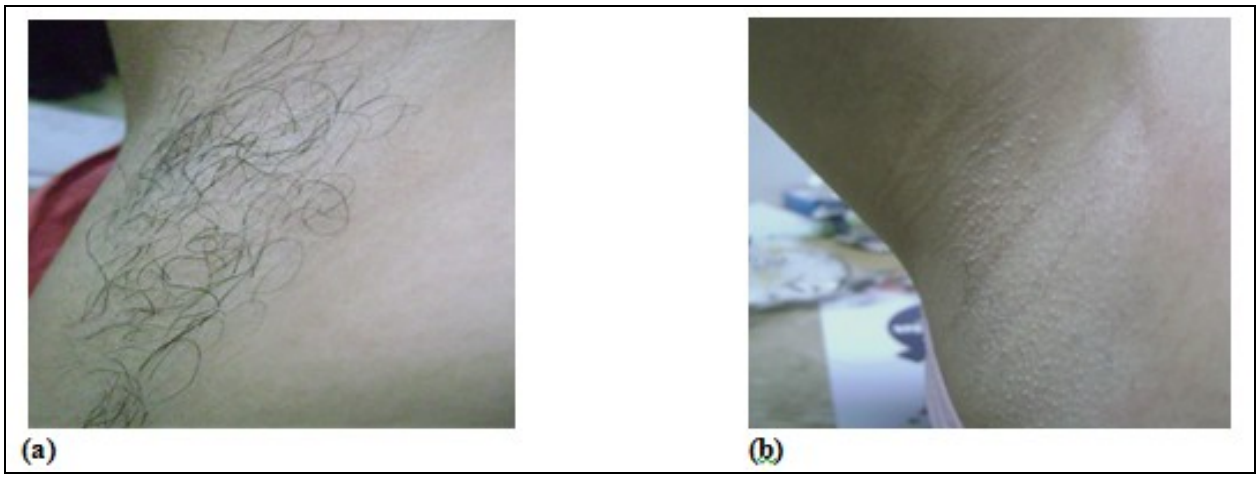


Fig. 2. a) Group I before and b) 6 months after start of treatment**.**

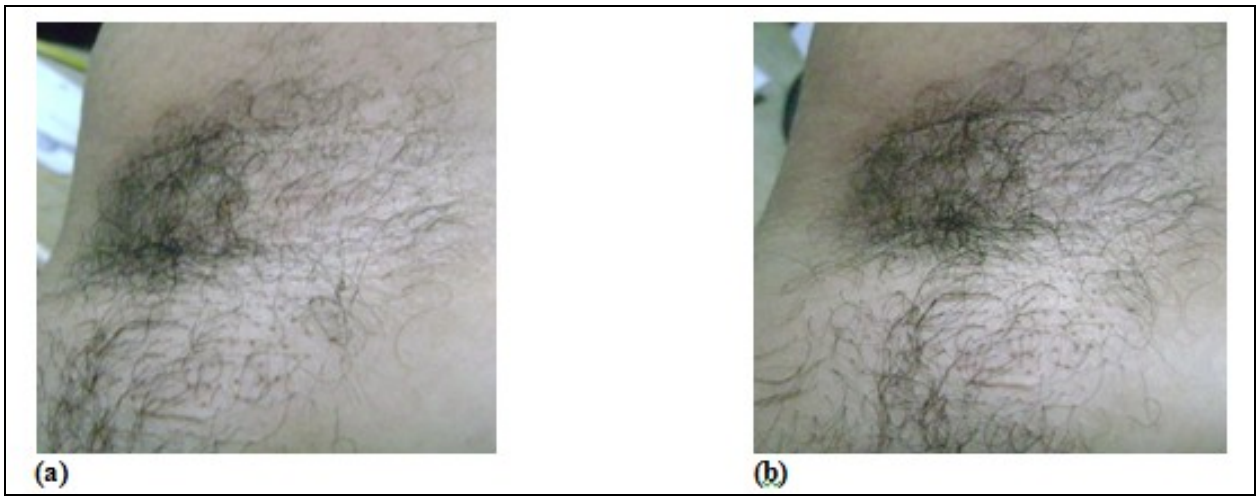
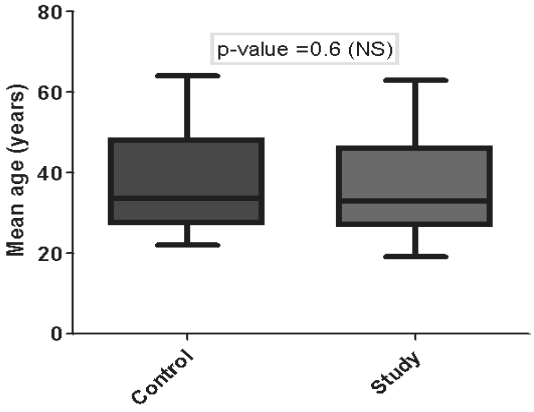
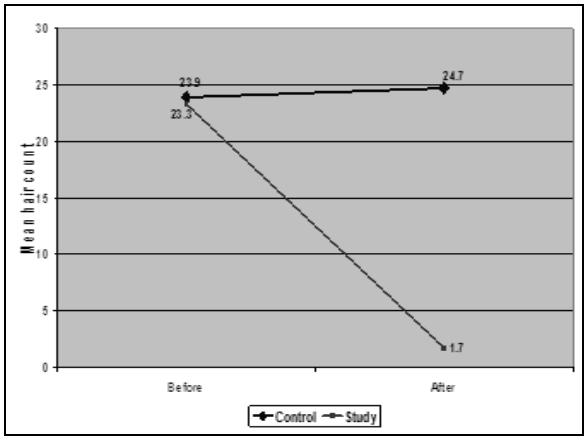


Fig. 3. a) Group II before and b) 6 months after start of treatment.



Graph 1. Mean age among both groups



Graph 2. Hair count before and after treatment in both groups

Table 1. Demographic data of hirsute in two groups

			Study (n=27)		Control (n=27)	
			Before treatment	**After treatment**	**Before treatment**	**After treatment**
Hirsutism	**Ferriman – Gallwey score**	**Stage 0**	0 (0%)	23 (85.1%)	0 (0%)	0 (0%)
		Stage 1	7 (26%)	3 (11.1%)	8 (29.6%)	8 (29.6%)
		Stage 2	18 (66.6%)	1 (3.8%)	18 (66.6%)	18 (66.6%)
		Stage 3	2 (7.4%)	0 (0%)	1 (3.8%)	1 (3.8%)
	Testosterone	**Normal**	22 (81.5%)	22 (81.5%)	24 (88.9%)	24 (88.9%)
		High	5 (18.5%)	5 (18.5%)	3 (11.1%)	3 (11.1%)
	Overian u/s	**Normal**	16 (59.2%)	16 (59.2%)	18 (66.6%)	18 (66.6%)
		Mild	10 (37%)	10 (37%)	9 (33.4%)	9 (33.4%)
		Moderate	1 (3.8%)	1 (3.8%)	0 (0%)	0 (0%)
		Severe	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 2. Hair count and white hair count in two groups

		Control (n=44)	Study (n=47)	p-value
Hair count	Before treatment	23.9 ± 6.7	23.3 ± 10.9	0.9 (NS)
	After treatment	24.7 ± 6.2	1.7 ± 2.1#	0.001*
White hair Count & denisty**	Increase	12 (50%)	0 (0%)	0.001*
	No change	12 (50%)	0 (0%)	0.001*
	Decrease	0 (0%)	20 (100%)	0.001*

**only 24 patients in control group and 20 patients in study group have white hair
*Statistically significant difference
NS: no statistically significant difference

Table 3. Independent observer assessment

		Control (n=44)	Study (n=47)	p-value
Level of improvement *(assessed by doctors)*	Increase hair growth	20 (45.5%)	0 (0%)	0.001*
	No change	24 (54.5%)	0 (0%)	0.001*
	Minor (≤20%)	0 (0%)	0 (0%)	-
	Mild (20 – 39%)	0 (0%)	0 (0%)	-
	Moderate (40 – 59%)	0 (0%)	1 (2.1%)	0.9 (NS)
	Good (60 – 79%)	0 (0%)	14 (29.8%)	0.003*
	Excellent (80 – 100%)	0 (0%)	32 (68.1%)	0.001*

*Statistically significant difference
NS: no statistically significant difference

Table 4. Patients' self assessment of area covering

		Control (n=44)	Study (n=47)	p-value
Area covering *(assessed by patients)*	Increase covering	26 (59.1%)	0 (0%)	0.001*
	No change	18 (40.9%)	0 (0%)	0.001*
	Mild decrease (<25%)	0 (0%)	0 (0%)	-
	Moderate decrease (25 - 49%)	0 (0%)	1 (2.1%)	0.9 (NS)
	Good decrease (50 - 75%)	0 (0%)	14 (29.8%)	0.003*
	Excellent (> 75%)	0 (0%)	32 (68.1%)	0.001*

*Statistically significant difference
NS: no statistically significant difference

Table 5. Patients' self assessment of hair quality

		Control (n=44)	Study (n=47)	p-value
Rate of hair growth	Increase	26 (59.1%)	0 (0%)	0.001*
	No change	18 (40.9%)	0 (0%)	0.001*
	Decrease	0 (0%)	47 (100%)	0.001*
Color and brightness	Increase	26 (59.1%)	0 (0%)	0.001*
	No change	18 (40.9%)	0 (0%)	0.001*
	Decrease	0 (0%)	47 (100%)	0.001*
Hair thickness	Increase	16 (36.4%)	0 (0%)	0.001*
	No change	28 (63.6%)	0 (0%)	0.001*
	Decrease	0 (0%)	47 (100%)	0.001*

*Statistically significant difference
NS: no statistically significant difference

Area under the curve = 83%

Standard error = 0.07

95% CI = 0.7 – 0.9

P-value = 0.001* (Statistically significant)

Hair count before treatment ≤ 21 can be used as predictor for complete cure after treatment with the following characteristics

Sensitivity = 72% 95% CI = 53.3 – 86.2

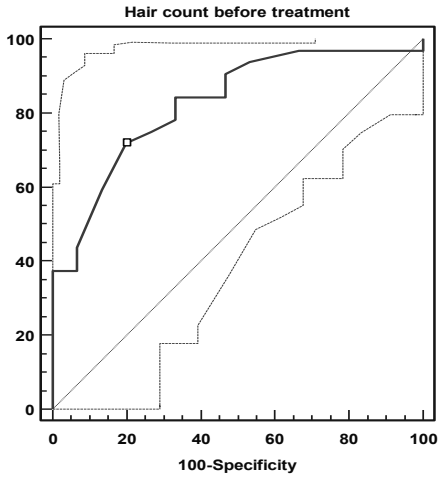
Specificity = 80% 95% CI = 51.9 – 95.4

Positive predictive value = 88.5%

Negative predictive value = 57.1%

Positive likelihood ratio = 3.59

Negative likelihood ratio = 0.3



Graph 3. ROC curve analysis to detect cut off value of hair count before treatment to predict cure:

DISCUSSION

In the present study *C. rotundus* oil used as topical treatment twice per day for six months. *C. rotundus* essential oil preparation was found to be significantly more effective than the placebo in hirsute females with (mustache & chin) or in axillary hair in normal females. Three assessment methods were employed: difference in hair count, independent observer and lastly patients' self assessment of the condition before and 6 months after initiation of treatment.

The growth of sexual hair is entirely dependent on the presence of androgen (Androgenic hair) [1-5]. Flavonoids have an estrogenic activity [34]. Flavonoids and lignans (which are constituents of many plants) could inhibit 5 alpha-reductase and 17 beta-hydroxysteroid dehydrogenase [17]. Flavonoids are found in many herbs such as *C. rotundus* [25, 26, 35], which inhibit 5α–reductase enzyme [17]. But, topical usage of oil did not affect the serum level of testosterone or ovarian ultrasonic pictures.

Removing white hair showed a great challenge in the

previous decades, because of its irresponsiveness to LASER or IPL. Hair coloring is an efficient and feasible technique that can be combined with IPL to eliminate white facial hair [37]. Also Melanin-encapsulated liposomal spray in combination with diode laser treatment showed a significant higher efficacy in the treatment of white and blond hair [37]. In the present study, there was a significant decrease of white hair by *C. rotundus* oil which is an easy, cheap, and harmless treatment.

In the present study, independent observer assessment and patients' self assessment were high compared to results of hair count. Changes in the apparent skin covering is not only due to decrease in the number of hair, but also due to thinning of hair, color, and shine, which the patients reported to decrease greatly.

Laser is the most expensive method, followed by IPL, Eflornithine HCl 13.9% cream then electrolysis [38]. In this study, *C. rotundus* oil cost is about 1 dollar per month, which was the cheapest choice. Side effects reported were minimal. No cases reported infection, irritation, itching or dis-pigmentation.

This study which involved new treatment, raises a lot of questions which will generate a lot of future research.

CONCLUSION

C. rotundus essential oil has anti-androgenic activity on androgenic hair; this may be due to flavonoids. But without affecting serum testosterone. It is an effective method in treating moderate degrees of Androgenic hair (hirsutism and axillary hair), further studies using *C. rotundus* essential oil are recommended.

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Original Research

GCMS analysis and antimicrobial action of latex of *Euphorbia caducifolia*

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Abstract

Euphorbia caducifolia is conspicuously and regularly represented in the flora of the Rajasthan state. Latex of *E. caducifolia* is used to cure skin infections, cutaneous eruption, leucoderma and applied to cuts and wounds for speedy healing.

The GCMS analysis of fraction isolated from latex showed presence of methyl palmitate, 5,9-heptadecadienoate, methyl 11 octadecenoate, methyl octadecenoate and 3,7,11,15-tetramethyl-2-hexadecene-l-ol. Isolated fraction of *E. caducifolia* (IFEC) and latex of *E. caducifolia* (ECL) were tested against *S. aureus, M. luteus, B. subtilis, E. coli, S. typhi, A. niger* and *C. albicans*. IFEC was found to be more effective against fungal species, and MIC was found to be 150 µg/ml against *A. niger*.

INTRODUCTION

Ethnomedicinal plants are one of the most important sources of finding new therapeutic agents. Some of the most outstanding medicines have been developed from ethnomedicinal plants [1]. Rich flora of the Thar Desert has many unexplored and underutilized ethnomedicinal plants. One such plant *Euphorbia caducifolia* is conspicuously and regularly represented in the flora of the Rajasthan state. This plant is common throughout the state on rocky habitat [2]. *Euphorbia caducifolia* is considered poisonous and not used as food or fodder except for the juicy leaves, which can be consumed as a vegetable. Latex is present in great abundance in the entire plant. The latex content appears to be the highest compared to that of the entire desert flora in this area. The latex of *Euphorbia caducifolia* (ECL) is considered to have medicinal value and used by shepherds and local inhabitants for treating bleeding wounds caused by accidental injury. ECL is also used to cure the skin infections, leukoderma, earache and to expel guinea worms [3].

Latex of many euphorbia species such as *Euphorbia abyssinica gmel* and *Euphorbia antiquorum* have been reported to have antimicrobial property [4-5]. No report has been published regarding biological activity of latex of *Euphorbia caducifolia.* this prompted us to investigate the phytoconstituent present in latex by using GCMS and to perform antimicrobial screenings of latex as well as an isolated fraction.

MATERIAL AND METHOD

Plant material

The *Euphorbia caducifolia* was collected from Jodhpur, India and was identified by Taxonomist of Botanical survey of India, Jodhpur. The voucher specimen (MGEC) was deposited for future reference.

Latex was collected by making incisions on the stems of the plant.

Preparation of sample for GCMS

Latex (2kg) was obtained from Euphorbia caducifolia and kept at a low temperature. The latex was extracted with ether, chloroform and methanol by liquid-liquid extraction. Ether extract was further purified by column chromatography using acetonitrile and methanol (1:1); the terpenoid mixture so obtained (7.5%) was subjected to GCMS and antimicrobial activity along with fresh latex.

GCMS analysis

The GCMS analysis was carried out at Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Madras using a JEOL GCMATE II Gas Chromatograph coupled to a mass detector. Electron impact mode was used with ionization voltage of 70eV.

Identification of Phytoconstituents

The identification and interpretation on mass-spectrum GC-MS was conducted using the database of National Institute Standard and Technology NIST Ver.2.0 MS, AOCS Lipid Library, Mass. library and research papers.

Test Microorganisms

Microbial strains obtained from Institute of Microbial Technology, Chandigarh, India were used. Microbial strains includes: Gram-positive bacteria- *Staphylcoccus aureus* MTCC-96, *Micrococcus luteus* MTCC-106, *Bacillus subtilis* MTCC-441, Gram-negative bacteria- *Escherichia coli* MTCC-443, *Salmonella typhi* MTCC - 734 and fungi *Aspergillus niger* MTCC- 282 and *Candida albicans* MTCC-227. Bacterial cultures were prepared by transferring two to three colonies into a tube containing 20 ml of nutrient broth and grown overnight at 37°C. The turbidity of the culture was adjusted with sterile saline solution to match 0.5 Mc Farland standard 10^8 colony forming units/ml (CFU/ml)

Well-in agar method

Anti-bacterial activity of latex (ECL) and isolated fraction (IFEC) was tested by a modified well-in agar method [6]. The inoculum suspension was spread uniformly over the agar plates using a sterile glass rod spreader to get uniform distribution of bacteria. Subsequently, using a sterile borer, a well of 0.7 cm diameter was made in the inoculated media. Addition of 0.2 ml of each extract was aseptically filled into the well. Later, the plates were placed at room temperature for an hour to allow diffusion of extract into the agar. Then the plates were incubated for 24 hours at 37°C. The results were recorded by measuring the diameter of the inhibition zone at the end of 24 hours.

Determination of MIC by serial dilution technique

Anti-bacterial activity of ECL and IFEC was carried out by broth micro-dilution method. Serial dilutions of the test fractions, isolated compounds and reference drugs were prepared in DMSO to attain a final concentration of 1 mg/ml. Further progressive dilutions with Mueller-Hinton agar were performed to obtain the required concentrations of 1, 2, 4, 16, 31.25, 62.5, 125, 250 and 500 µg/ml. The tubes were inoculated with 10^8 cfu/ml (colony forming unit/ml) of each microorganism and incubated at 37°C for 18 hours. To ensure whether solvent had any effect on the bacterial growth, a respective parallel control was performed. Minimum inhibitory concentration (MIC) of the fractions was determined. Ciprofloxacin and fluconazole were used as standards to compare the antibacterial activity of the fractions of the plant.

RESULTS

GCMS analysis

Gas chromatogram of the isolated fraction is presented as Figure-1. The mass spectra of all major peaks shown in gas chromatogram were analyzed and seven compounds were identified.

The identified compound are presented in Table 1 according to their retention time, compounds identified include 3-Oxo-25, 26, 27-trisnor (5a, 13a,14b,17a) lanost-8-en-24-al, methyl palmitate, 5,9-heptadecadienoate, methyl 11 octadecenoate, methyl octadecenoate, 3,7,11,15-tetramethyl- 2-hexadecene-l-ol (Phytol) and lanost-8-en-24-al.

Antimicrobial study

Table 2 shows the antimicrobial effect of the isolated fraction of *Euphorbia caducifolia* (IFEC) and latex of *Euphorbia caducifolia* (ECL), there were significant differences in their activities depending on the microorganism tested. Diameters of inhibition zones ranged between 8.1 and 20.5 mm. *C. albicans* and *A. niger* were the most susceptible microbes by IFEC showed with inhibition zones of 18.3 and 20.5 mm, respectively.

Minimum inhibitory concentration (MIC) of IFEC and ECL against all tested microorganisms is shown in Table 3. The MIC of ECL against *S. aureus, M. luteus, B. subtilis, E. coli* and *S. typhi were* found to be 458, 450, 475, 525 and 500 µg/ml respectively. MIC against *A. niger* and *C. albicans* were found to be 237 and 225 µg/ml. IFEC was found to be more effective against fungal species, MIC was found to be 150 µg/ml against *A. niger* and maximum MIC 275 µg/ml against *S. typhi*.

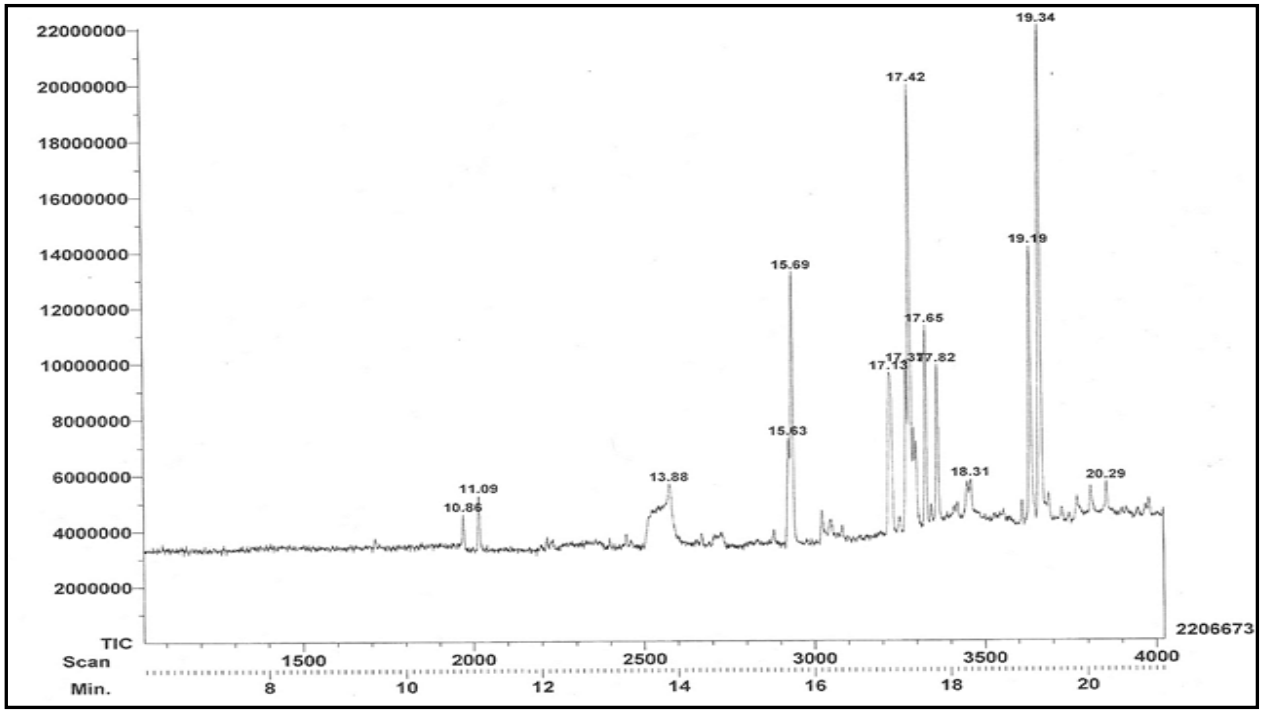

Figure 1. GCMS chromatogram of isolated fraction of latex of Euphorbia caducifolia

Table 1. Results of analysis of mass spectra obtained by GCMS analysis of isolated fraction of latex of *Euphorbia caducifolia*

S. No.	RT	M+	Compound
1.	10.86	398	3-Oxo-25,26,27-trisnor(5a,13a,14b,17a)lanost-8-en-24-al
2.	15.69	270	Methyl Palmitate
3.	17.13	280	5,9-heptadecadienoate
4.	17.42	296	Methyl 11 octadecenoate
5.	17.65	298	Methyl octadecenoate
6.	19.19	278	3,7,11,15-tetramethyl- 2-hexadecene-l-ol (Phytol)
7.	20.29	442	Lanost-8-en-24-al

RT(Retention Time) M+ (Molecular ion peak)

Table 2. Preliminary antimicrobial activity of latex of *Euphorbia caducifolia* and isolates fraction against bacterial and fungal species.

Micro-organisms	ECL	IFEC 500 µg/ml	Ciprofloxacin, 60 µg/ml	Flucanazole 100 µg/ml
Staphylcoccus aureus	9.3 ± 0.21	16.2 ± 0.22	23.1 ± 0.58	-
Micrococcus luteus	9.2 ± 0.31	14.4 ± 0.33	21.8 ± 0.64	-
Bacillus subtilis	8.1 ± 0.25	14.5 ± 0.26	24.4 ± 0.42	-
Escherichia coli	9.4 ± 0.24	15.1 ± 0.41	21.6 ± 0.34	-
Salmonella typhi	8.2 ± 0.33	16.4 ± 0.51	26.4 ± 0.21	-
Aspergillus niger	18.7 ± 0.52	20.5 ± 0.23	-	22.5 ± 0.26
Candida albicans	15.6 ± 0.41	18.3 ± 0.32	-	18.4 ± 0.35

Values are the mean ± SEM of six replicates and inhibition zone including the diameter of the bore (7 mm).

Table 3. Minimum inhibitory concentration of latex of *Euphorbia caducifolia* and isolates fraction against bacterial and fungal species.

Micro-organisms	ECL (micrograms)	IFEC (micrograms)
Staphylcoccus aureus	458	262
Micrococcus luteus	450	212
Bacillus subtilis	475	187
Escherichia coli	525	225
Salmonella typhi	500	275
Aspergillus niger	237	150
Candida albicans	225	175

MIC (Minimum inhibitory concentration)

DISCUSSION

Euphorbia species have been reported for antibacterial and anti-fungal activities. Crude extracts of *Euphorbia hirta* were found to be effective against gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae. Shigella dysentriae, Salmonella typhi* and *Proteus mirabilis* [7]. *Euphorbia thymifolia* is active against *Bacillus Subtilis, Staphylococcus Aureus* and *Escherichia coli* [8] and *Euphorbia segetalis* also possess antiviral and antimicrobial properties [9]. In the present study, latex of *Euphorbia caducifolia* showed antibacterial and anti-fungal activities against the tested bacteria and fungal strains.

The diverse composition of the latex, which includes toxic compounds as well as other interesting and potentially bioactive molecules such as diterpenes and triterpenes could be responsible for antibacterial and anti-fungal activity [10-11].

IFEC found to have a lower MIC value than ECL, and is indicative of activity of terpenes. Furthermore, a number of studies have been reported on the antibacterial and anti-fungal activity of terpenes of natural origin [12]. The possible mechanism of antimicrobial activity of terpenes is by reducing the synthesis of ergosterol, a specific fungal cell membrane component [13]. The inhibition of synthesis ergosterol causes defective cell wall formation and leakage of cellular contents. Terpenes increase the permeability of bacterial and mammalian cell by inserting themselves into the lipid layer of the cell membrane and thus influencing the selective permeability of the cell to foreign substances [14].

CONCLUSIONS

It could be concluded that the results observed are in line with the therapeutic use of the plant in traditional medicine. Latex and compounds isolated from latex of Euphorbia caducifolia have potential antimicrobial effects. The study of ECL and separated compound for wound healing potential is underway.

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