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Full Length Research Paper

Formulation and evaluation of captopril floating matrix tablets based on gas formation

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Captopril has a short elimination half-life and is stable at pH 1.2 and as the pH increases; the drug becomes unstable and undergoes a degradation reaction. The purpose of this study was to develop a gastroretentive controlled release drug delivery system with swelling and floating properties. Seventeen tablet formulations were designed using hydroxyl propyl methyl cellulose (HPMC) K4M, Carbomer 934, Eudragit RS PO as release retarding polymer(s), lactose or Avicel PH 102 as a filler and sodium bicarbonate as a gas former by direct compression. Tablets were evaluated for various physical parameters, floating properties, swelling ability and drug release characteristics in 12 h. Based on the release kinetics, all formulations best fitted the Higuchi, Hixson Crowell model and non-Fickian as the mechanism of drug release. Statistical analyses of data revealed that formulation containing HPMC K4M (42%, w/w), $NaHCO_3$ (8%, w/w) and Avicel PH 102 (32.35%, w/w) was the promising system exhibiting excellent floating properties and sustained drug release (12 h) characteristics.

Key words: Floating drug delivery system, captopril, HPMC K4M, Carbomer 934, Eudragit RS PO.

INTRODUCTION

The drug bioavailability of pharmaceutical dosage forms is influenced by various factors. One of the important factors is the gastric residence time (GRT) of these dosage forms. Variable and short gastric emptying time can result in an incomplete release of drug and diminished efficacy of the administered dose. Floating drug delivery system (FDDS) is one of the gastroretentive dosage forms that could prolong GRT to obtain sufficient drug bioavailability (Sungthongjeen et al., 2008; Strubing et al., 2008a,b; Gambhire et al., 2007).

FDDS is desirable for drugs with an absorption window in the stomach or in the upper small intestine such as furosemide and theophylline. It is also useful for drugs that act locally in the proximal part of GI tract such as antibiotic administration for *Helicobacter pylori* eradication in the treatment of peptic ulcer, for drugs that exhibit poor solubility in the intestinal tract such as diazepam and verapamil HCl, and for drugs that are unstable in the intestinal fluid such as Captopril (Sungthongjeen et al., 2008; Gambhire et al., 2007; Singh and kim, 2000).

Captopril, (1-[(2S)-3-mercapto-2-methyl propionyl]-1-proline), an angiotensin-converting enzyme inhibitor, has been used widely for the treatment of hypertension and congestive heart failure (Brunton et al. 2005). The drug is freely water soluble and has half-life elimination after an oral dose of 2h (Sweetman et al., 2002). It is stable at pH 1.2 and as the pH increase; the drug becomes unstable and undergoes a degradation reaction. Thus, captopril is a candidate for the development of FDDS. Various pharmaceutical approaches for the controlled-release preparation of captopril, including biodegradable microparticles, osmotic pump tablets and hard gelatin capsules, have been reported (Dandagi et al., 2006; Efentakis and Vlachou, 2000; Xu et al., 2006). The effect

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of compaction pressure on floating behavior of captopril tablets have been studied (Jimenez et al., 2008). In another study, bilayer floating tablets of captopril have been reported (Ziyaur et al., 2006). In the present study, the details of formulation development and evaluation of gas forming floating tablets of captopril using hydroxyl propyl methyl cellulose (HPMC K4M), Carbomer 934, Eudragit RSPO as release-retarding polymer(s) are described.

EXPERIMENTAL

Materials

Captopril (Farmhispania, Spain), HPMC K4M (Seppic, France), Carbomer 934(Corel Pharma Chem, India), Eudragit RS PO (Evonik, Germany), Avicel PH 102 (Blanver, Brazil), Lactose (Blanver, Brazil), Magnesium stearate (Merck, Germany) and Sodium bicarbonate (Merck, Germany) were used as obtained.

Methods

Preparation of captopril floating tablets

Tablets containing 50 mg captopril were prepared, according to the design shown in Table 1, by direct compression. The respective powders, namely captopril, release-retarding polymer(s) (HPMC K4M, Carbomer 934 and Eudragit RS PO alone or in combination with each other), a gas-forming agent ($NaHCO_3$) and a filler (Lactose or Avicel PH 102) were passed through sieve no. 40, separately. Mixing of powders was carried out using a pestle and mortar for 10 min. Magnesium stearate was passed through sieve no. 60 and then added to the mixed powders. Mixing was continued for another 3 min. Finally, 300 mg of each mixture were weighed and fed manually into the die of a single punch tabletting machine (Korsch), equipped with concave punches (10.0 mm), to produce tablets adjusted at a hardness of 5 to 7 kg/cm². The hardness of the tablets was measured using a hardness tester (Erweka TBH 30 GMD, Germany).

In vitro evaluation of the prepared tablets

Tablet weight variation, tablet thickness and tablet friability test were carried out according to USP [31] and BP [21], respectively.

Drug content uniformity

Ten tablets were individually weighed and crushed. A quantity of powder equivalent to the mass of one tablet (300 mg) was dispersed in 100 ml of 0.1 N HCl. The solution was filtered through a cellulose acetate membrane (45 µm). The drug content was determined by UV spectroscopy (UV-1650 PC Double beam spectrometer, Shimadzu, Kyoto, Japan) at a wavelength of 205 nm after a suitable dilution with 0.1 N HCl (Tadros, 2010).

Tablet floating behavior

A tablet was placed in a glass beaker, containing 200 ml of 0.1 N HCl, maintained in a water bath at 37 ± 0.5°C. The floating lag time ‘‘the time between tablet introduction and its buoyancy” and total floating duration ‘‘the time during which tablet remains buoyant” were recorded (Rosa et al., 1994).

Tablet swelling ability

The swelling behavior of the tablets was determined, in triplicate, according to the method described by Dorozynski et al. (2004). Briefly, a tablet was weighed (W_1) and placed in a glass beaker, containing 200 ml of 0.1 N HCl, maintained in a water bath at 37 ± 0.5°C. At regular time intervals, the tablet was removed and the excess surface liquid was carefully removed by a filter paper (Patel et al., 2009). The swollen tablet was then reweighed (W_2). The swelling index (SI) was calculated using the formula as follows:

$$SI = (W_2-W_1) / W_1$$

Drug release studies

Drug release studies of the prepared floating tablets were performed, in triplicate, using apparatus 2 (Erweka DT 800, Germany) at 37 ± 0.5°C and 50 rpm. The tablets were placed into 900 ml of 0.1 N HCl solution (pH 1.2). Aliquots of 5 ml were withdrawn from the dissolution apparatus at different time intervals and filtered through a cellulose acetate membrane (0.45 µm). The drug content was determined spectrophotometrically at a wavelength of 205 nm, as mentioned earlier. At each time of withdrawal, 5 ml of fresh medium was replaced into the dissolution flask. The resulting data were analyzed by using the software Statistical Package for Social Sciences 19.0 (SPSS Inc., Chicago, USA) applying one way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Physicochemical characteristics of tablets

To avoid processing variables, all batches were produced under similar conditions. The hardness of the tablets was between 5 and 7 kg/cm² and all formulations had friability less than 1%. Average mass variation was 300.36 ± 0.35 mg, mean thickness was 4.22 ± 0.19 mm and the content uniformity of the tablets was 101.69 ± 0.91%. All formulations, except formulation containing Eudragit RS PO alone with poor compressability, showed acceptable physicochemical properties.

In vitro buoyancy

Floating dosage forms could be floated due to an intrinsic density lower than gastric content, which is reported as 1.004 to 1.010 g/cm³ or due to the formation of a gaseous phase inside the system after contact with gastric fluid (Elkheshen et al., 2004). This attribute allows them to remain afloat on the surface of the gastric content for a longer period of time without affecting the rate of emptying. For evaluation, the effects of the amount of sodium bicarbonate C series were prepared. The formulation C_1, prepared without sodium bicarbonate, did not show any sign of floating. Therefore, sodium bicarbonate was essential in order to float the tablet. To study the effect of sodium bicarbonate amount on floating lag time, A_2, C_2

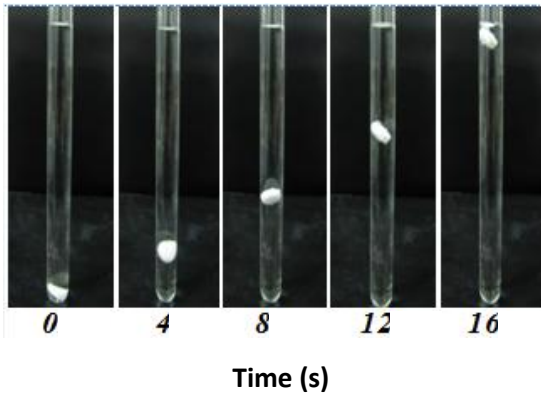

Figure 1. Floating behavior of the best floating effervescent captopril formulation (C_3).

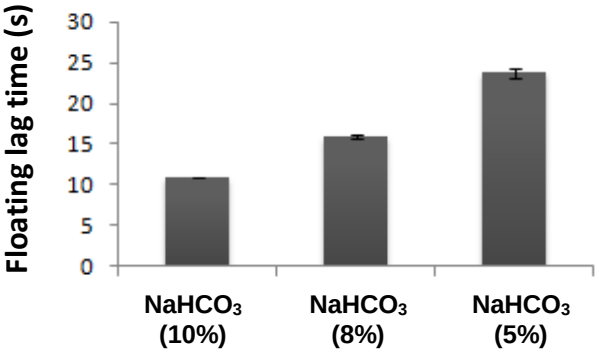

Figure 2. The effect of amount of $NaHCO_3$ on floating Lag time of matrix type captopril tablets (mean ± SD, n = 3).

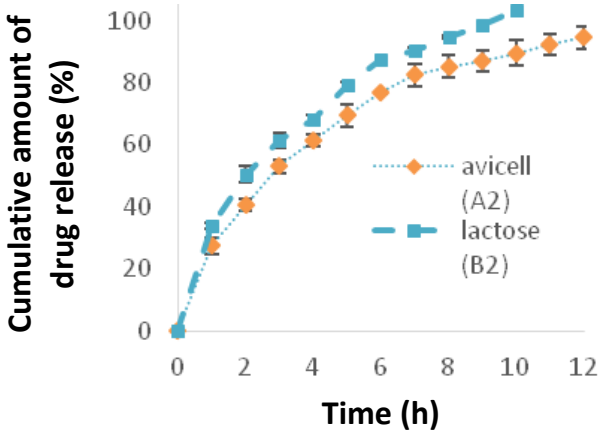

Figure 3. The effect of filler on drug release pattern.

and C_3 formulations were prepared. It was found that as the amount of sodium bicarbonate increases, the floating lag time decreases (Figure 2), but it did not have any effect on floating duration. The type of polymer and filler affected the floating lag time and floating duration, as shown

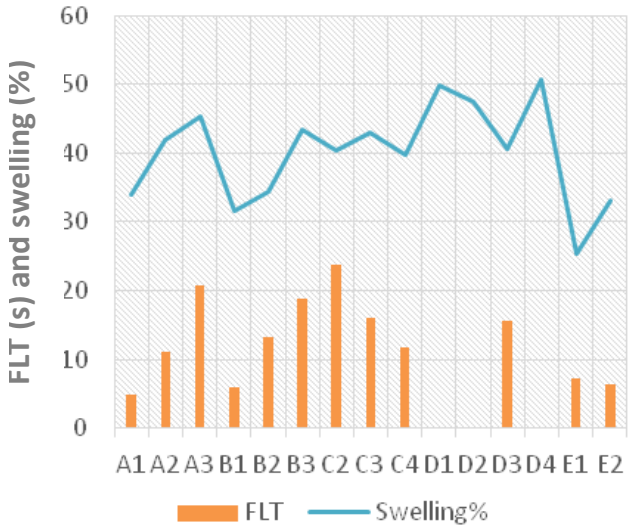

Figure 4. Floating lag time (S) and swelling percent of all formulations.

noticeable that the matrices containing Carbomer 934 (more than 25 mg) did not have any floating lag time, while increasing HPMC K4M increased floating lag time. The *in vitro* behavior of the best formulation is as shown in Figure 1.

Swelling indices

Hydrophilic matrices in contact with water swell and increase their volume due to water diffusion through the matrix. The polymer chains continue the hydration process and the matrix gain more water. The increasing water content dilutes the matrix until a disentanglement concentration is attained. At this point, the polymer molecules are released from the matrix, diffusing to the bulk of the dissolution medium. Then, the matrix volume decreases slowly, because of polymer dissolution. Polymeric matrices experience simultaneously swelling and polymer dissolution and diffusion. The hydration ability of the formulation is important, because it influences tablet buoyancy and drug release kinetics. The test medium uptake by prepared matrices depends on the type and amount of polymer. Higher polymer contents increase the tortuosity and the length of matrices delaying its entire hydration, as shown in Table 2 and Figure 4.

The high affinity of Carbomer to the test medium causes high swelling ability. Hydrophilic groups in Eudragit RS PO are less than other polymers, so the formulation containing it had the lowest swelling.

In vitro drug release studies

Ideally, a sustained release formulation should release the required quantity of drug with predetermined kinetics

Table 1. The composition, in milligrams, of the investigated captopril gas forming floating tablets.

F	Drug	HPMC K4M	Carbomer 934	Eudragit RS PO	Avicel PH 102	Lactose	$NaHCO_3$	Mg Stearate
A1	50	90	-	-	127	-	30	3
A2	50	120	-	-	97	-	30	3
A3	50	150	-	-	67	-	30	3
B1	50	90	-	-	-	127	30	3
B2	50	120	-	-	-	97	30	3
B3	50	150	-	-	-	67	30	3
C1	50	126	-	-	106	-	0	3
C2	50	126	-	-	106	-	15	3
C3	50	126	-	-	97	-	24	3
C4	50	126	-	-	-	97	24	3
D1	50	88.20	37.80	-	97	-	24	3
D2	50	100.80	25.20	-	97	-	24	3
D3	50	113.40	12.60	-	97	-	24	3
D4	50	-	126	-	97	-	24	3
E1	50	63	-	63	97	-	24	3
E2	50	88.20	-	37.80	97	-	24	3
E3	50	-	-	126	97	-	24	3

Table 2. Floating and swelling properties of the prepared captopril effervescent floating tablets*.

F	Floating lag time (s)	Floating duration (h)	Swelling percent	Swelling index
A1	4.92±0.28	>12	34.01±0.07	0.3401±0.0007
A2	11.04±0.13	>12	41.86±0.35	0.4186±0.0035
A3	20.77±0.61	>12	45.37±0.08	0.4537±0.0008
B1	6.07±0.30	10	31.62±0.65	0.3162±0.0065
B2	13.22±0.53	12	34.37±0.62	0.3437±0.0062
B3	18.79±0.41	12	43.35±0.20	0.4335±0.0020
C2	23.75±0.87	>12	40.49±0.25	0.4049±0.0025
C3	15.96±0.62	>12	42.98±0.49	0.4298±0.0049
C4	11.87±0.44	12	39.83±0.26	0.3983±0.0026
D1	0	>12	49.75±0.43	0.4975±0.0043
D2	0	>12	47.49±0.47	0.4749±0.0047
D3	15.53±2.11	>12	40.70±0.27	0.4070±0.0027
D4	0	3	50.64±0.18	0.5064±0.0018
E1	7.17±0.64	>12	25.32±0.42	0.2532±0.0042
E2	6.34±0.33	>12	33.14±0.27	0.3314±0.0027
E3	-	-	-	-

*Mean ± SD, n = 3.

in order to maintain effective drug plasma concentration. To achieve this, the delivery system should be formulated so that it releases the drug in a predetermined and reproducible manner. The release of captopril from GRDDS was analyzed by plotting the cumulative percent drug released against time (Figure 5). It is worth to note that, a burst effect was observed with all formulations. This could be due to the fact that the gel layer, which controls the drug release rate, needs some time to become effective. The rapid drug dissolution from the surface of the tablets could be another possible explanation. Kulkarni and Bhatia (2009) suggested that the resulting gel-like networks surrounding these matrices, upon contact with aqueous media, would produce strong surface barriers that would effectively reduce the burst drug release. This effect significantly reduced when the concentration of HPMC K4M increased or when Carbomer 934 is used as a polymer in combination with HPMC K4M.

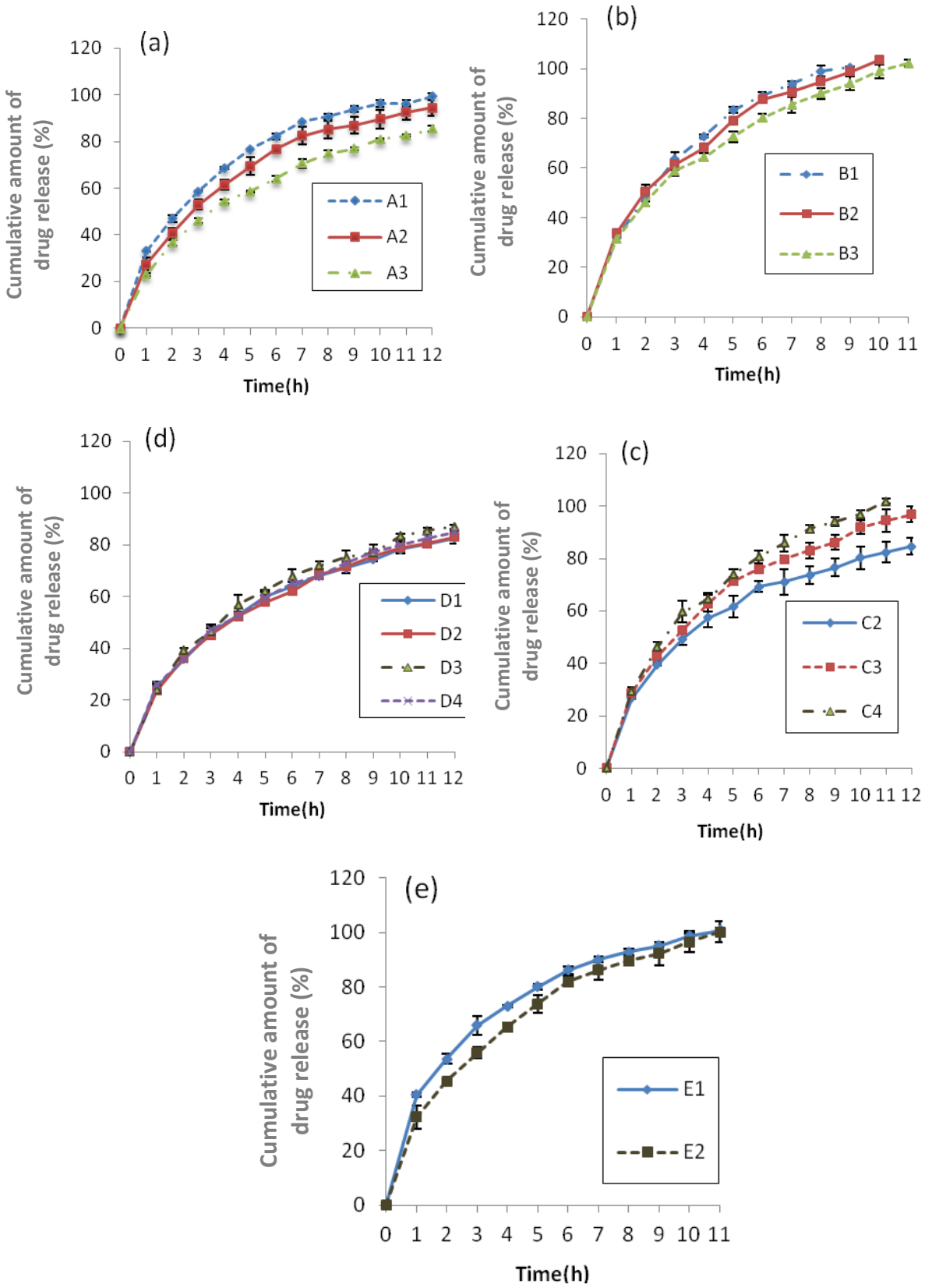

Figure 5. The effect of various polymers, lactose and Avicel PH 102 on the release of captopril from floating effervescent tablets: (a) HPMC K4M and Avicel PH 102, (b) HPMC K4M and Lactose, c) HPMC K4M and different amount of $NaHCO_3$, (d) HPMC K4M and Carbomer 934, (e) HPMC K4M and Eudragit RS PO (mean ± SD, n = 3).

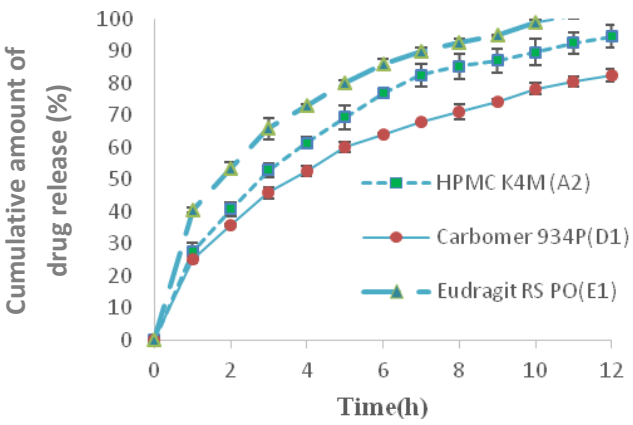

Figure 6. Comparison of drug release from gas forming tablets with different kinds of polymer.

K4M. On the other hand, the presence of carbon dioxide bubbles, produced after reaction of sodium bicarbonate with the acidic dissolution medium, decrease the drug release rate.

Siepmann and Peppas (2001) suggested that the drug release from HPMC matrices is sequentially governed as follows: (1) at the beginning, steep water concentration gradients are formed at the polymer/water interface resulting in water imbibition into the matrix; (2) due to the imbibition of water, HPMC swells resulting in dramatic changes of polymer and drug concentrations and increasing dimensions of the system; (3) upon contact with water, the drug dissolves and diffuses out of the device due to concentration gradients; (4) with increasing water content, the diffusion coefficient of the drug increases substantially. When the amount of HPMC K4M increased, drug release rate decreased ($p < 0.05$) (Figure 5 (a and b).

The drug release rate from matrices that contained Carbomer 934 and HPMC K4M, in combination, in comparison with HPMC K4M alone significantly reduced ($p < 0.05$) and it was independent of concentration of Carbomer 934. Yao et al. (2011) suggested that the hydrogen bonds between the –COOH group of the Carbomer and –OH group of the HPMC are more stable than those between the –OH groups of water and HPMC, and the hydrophobicity of the HPMC chains in presence of Carbomer increases, so drug release rate decreases (Figure 5d).

The drug release rate from matrices that contained Eudragit RS PO and HPMC K4M, in combination, in comparison with HPMC K4M alone significantly increased ($p < 0.05$). Hydrophilic groups that exist in polymer structure play an important role in drug release rate and swelling ability. The number of hydrophilic group in Eudragit RS PO is less than HPMC K4M, so by increasing the amount of Eudragit RS PO drug release rate increases and swelling ability decreases (Figure 5e). The drug release rate in matrices with lactose as filler was faster than that contained in Avicel PH 102 ($p < 0.05$) (Figure 3). Lactose is water soluble filler, so entrance of water to polymeric network is easier than formula that contained water insoluble Avicel PH 102 (Figure 5b). Moreover, the increased amount of sodium bicarbonate caused a large amount of gas evolution, which in turn resulted in pore formation, which led to rapid hydration of the polymer matrix and thereby to rapid drug release ($p < 0.05$) (Figure 5c).

In the whole, drug release from Eudragit RS PO was faster than HPMC K4M and it was faster than Carbomer 934P (Figure 6).

Drug release kinetics

The release profile of the optimized formula (C_3) fitted best to the Hixson-Crowell (R^2 = 0.992 and n = 0.518), indicating non-Fickian diffusion or anomalous transport, with release by diffusion and swelling (combination of diffusion and erosion-controlled release).

Conclusion

This study was conducted to develop an effervescent floating drug delivery system using HPMC K4M, Carbomer 934 and Eudragit RS PO in different concentrations. Optimized formulation C_3 showed an excellent buoyant ability and a suitable drug release pattern. This could be advantageous in terms of increased bioavailability of captopril. The developed gastroretentive drug delivery system provides advantages of ease of preparation and sustained drug release for 12 h.

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Full Length Research Paper

Flavonoid and saponin rich fractions of kiwi roots (*Actinidia arguta* (Sieb.et Zucc.) Planch) with antinociceptive and anti-inflammatory effects

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The roots of kiwi (*Actinidia arguta* (Sieb.et Zucc.) Planch) have medicinal uses as anti-tumour, antinociceptive and anti-inflammatory agents. We aimed to evaluate the antinociceptive and anti-inflammatory effects of the major fractions of kiwi roots. The ethanolic extract of the plant roots was partitioned using a liquid–liquid extraction procedure to give five major fractions. Following phytochemical screening of isolated fractions, the total extract and each fraction were evaluated for their antinociception and anti-inflammatory effects using acetic acid, hot plate test, formalin and carrageenan-induced paw edema tests, respectively. The results indicated that the total extract, ethyl acetate fraction (EAF) and n-butanol fraction (BF) exhibited significant inhibition of acetic acid-induced writhing, and both phases of the formalin-induced pain response increased in time of response to thermal stimulation in hot plate test and exhibited significant dose-related inhibition of carrageenan induced paw oedema volumes when compared with the control group. It can be concluded that the flavonoid and saponin content of kiwi roots can be responsible for antinociception and anti-inflammatory effects of the plant, respectively.

Key words: *Actinidia arguta*, anti-inflammatory, antinociceptive, flavonoid, saponin.

INTRODUCTION

Actinidia arguta (Sieb.et Zucc.) Planch belongs to Actinidia genus in the family of Actinidiaceae, which is a large deciduous vine. The genus *Actinidia* contains around 54 species. The plant is chiefly distributed in the mountains of South China. Some species are also found in Siberia, Japan, Indochina, Malaysia, Indonesia (Rosemary et al., 1994), New Zealand (Kyoung and Hong, 2009; Robert, 1983).

As known to all, the fruits of *A. arguta* (Sieb.et Zucc.) Planch (kiwi fruit) are edible, but interestingly, the roots of kiwi, which are called Tengligen usually, are used to treat tumors of the alimentary canal widely in traditional Chinese medicine, such as gastric cancer, esophagus cancer, liver cancer etc. (Song et al., 2001; Zhong et al., 2004; Liang et al., 2007). Pharmacological research indicated that the extract of Tengligen could inhibit the carcinoma of gastric cells (Wei et al., 2005; Li et al., 2004; Fu et al., 2011). Zhang et al. (2007) and Guo et al. (2011) reported that the extracts from Tengligen by n-butyl alcohol had good inhibitory effect on human carcinoma of esophagus cells (Eca-109). Sun et al. (2011) have reported that the extracts from Tengligen by ethyl acetate could induce the apoptosis of EC109 cell in a dose-and time-dependent manner *in vitro*. At the same time, the extracts could down regulate the expression of Bcl-2, up regulate the expression of Bax protein level and increase intercellular calcium which promotes apoptosis. Lou et al. (2009) notified that the fraction extracted by

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chloroform displayed potent activity against hepatocellular carcinoma with Bel-7402 cells and the results were confirmed in murine hepatocellular carcinoma H22 and human Bel-7402 xenograft. The results of recent study showed that the extracts could inhibit the proliferation of A549 cells during the G_0-G_1 period and significantly decrease the cell ratio of S stage (Wang et al., 2010).

To the best of our knowledge, there was no study on the evaluation of antinociceptive and anti-inflammatory effects of the major fractions of kiwi roots so far. Therefore, the present study was aimed to evaluate the antinociceptive and anti-inflammatory effects of fractions of ethanol extract of kiwi roots. The antinociceptive effect was examined against chemically and thermally induced nociceptive pain in mice, using the acetic acid, hot plate test and formalin methods. In addition, the anti-inflammatory effect was investigated by utilizing the carrageenan induced paw edema method in mice.

MATERIALS AND METHODS

The roots of *A. arguta* (Sieb.et Zucc.) Planch was collected in Changbai Mountains, Jilin Province, China on March, 2012, and identified by Dr. Yue-Chun Sun, Life Science and Technology College, Heilongjiang Bayi Agricultural University, Daqing, China. Ibuprofen powder (Tianjin Shike Pharmaceutical Co. Ltd., China) and carrageenan (type I, Sigma Co., UK) were of pharmaceutical grade. The other chemicals and reagents were of analytical grade. An amount of 5 kg of dried kiwi roots was extracted with ethanol applying heat refluxing method. The obtained extract was evaporated under vacuum to give a viscose mass. Then, an amount of 525 g of the extract was suspended in 1000 ml of distilled water and was partitioned sequentially with cyclohexane (5 × 500 ml), chloroform (5 × 500 ml), ethyl acetate (5 × 500 ml), n-butanol (5 × 500 ml) at room temperature. Totally, six major fractions were collected and concentrated under vacuum and stored at -20°C until pharmacological tests.

Phytochemical screening

The total extract of the kiwi roots and each of the fractions were screened to investigate the presence of saponins, flavonoids, alkaloids and terpenoids.

Animals

Wistar rats (age, 8 to 12 weeks; weight, 180 to 200 g) and imprinting control region (ICR) mice (age, 2 to 3 weeks; weight, 18 to 22 g) of either sex were used for the experiments. Animals were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd. and used once only. The animals were kept in standard laboratory conditions (relative humidity: 55 to 60%, room temperature 25 ± 2°C, 12 h light/dark cycle) and had free access to standard diet and water *ad libitum* during the experiment. The animals were acclimatized to the laboratory environment for a period of 7 days prior to performing the experiments.

Acetic acid induced writhing response

The acetic acid-induced writhing test was carried out using the reported technique (Pinheiro et al., 2010; Mariana et al., 2012). The mice were pre-treated with positive drug and test drugs for 3 days, half hour after final administration 0.6% acetic acid (0.1 ml/10 g) by intraperitoneal injection to mice. The number of writhing movements during the next 15 min was recorded. The number of writhes in each treated group was compared with control group which received only the saline. The inhibition rate of writhes was calculated thus:

$$[(Mean_{control} – Mean_{test}) / Mean_{control}] \times 100$$

In the writhing test, mice were randomly divided into eight groups. They are control group with i.g. isometrical physiological saline, the test drug groups at the dosage of 50 mg/kg of five major fractions and total extract (200 mg/kg) in the experiments with i.g. The mice of positive drug group were conducted with i.g. ibuprofen (50 mg/kg) to compare the results obtained.

Hot plate test

The method is an adaptation of that described by Eddy and Leimbach (1953) and Ramzi et al. (2012). In the test, female mice were placed in a 24 cm diameter glass cylinder on a heated metal plate maintained at 55 ± 1°C. Animals were habituated twice to the hot plate in advance. Response was defined as licking or biting of a paw, or jumping. The time in seconds between the placing of the animal on the platform and reaction was recorded as the response latency. The mice exhibiting latency time greater than 30 s or less than 5 s were excluded. Animals were divided into eight groups of ten mice each and pretreated with oral dose of total extract (200 mg/kg), 50 mg/kg of five major fractions, respectively. Ibuprofen (50 mg/kg) was used as the standard drug. Mice were tested at 30, 60, 90 and 120 min after oral administration of the extracts and ibuprofen.

Formalin induced nociception

The procedure was similar to the method described by Hunskaar and Hole (1987) with some modifications done by Gomes et al. (2007). Animals were divided into eight groups of ten mice each and pretreated with oral dose of five major fractions (50 mg/kg, respectively) and total extract (200 mg/kg). Ibuprofen (50 mg/kg) was used as the standard drug. The mice were pre-treated with positive drug and test drugs for 3 days, half hour after final administration 20 µl of 5% v/v formalin was injected subcutaneously into the right hind paw of mice. The time that animals spent on licking or biting responses of the injected paw was taken as an indicator of pain response. On the basis of the response pattern described by Mariana et al. (2012), responses were measured for 5 min after formalin injection (early phase, neurogenic pain response) and 15 to 30 min after formalin injection (later phase, inflammatory pain response).

Carrageenan induced paw edema

Carrageenan-induced hind paw edema model was used for determination of anti-inflammatory effect (Winter et al., 1962; Zhu et al., 2012; Ali et al., 2012). Rats were orally treated with the saline, five major fractions (50 mg/kg, respectively), total extract (200 mg/kg) and ibuprofen (50 mg/kg), 30 min prior to injection of 1% carrageenan (0.1 ml) in the right hind paw sub-plantar region of each mouse. Hind paw volumes were measured using the plethysmograph at 0.5, 1, 2 and 3 h intervals after injection of the phlogistic agent. Percent of inhibition was calculated according to the hind paw volumes.

Table 1. The result of phytochemical screening of total extract and separated fractions from Kiwi roots.

Sample	Saponin	Flavonoid	Alkaloid	Terpenoids
Total extract	+++	+++	-	++
CYF[a]	-	-	-	++
CHF[b]	-	-	-	-
EAF[c]	+	+++	-	-
BF[d]	+++	+	-	-
Aqueous fraction	+	+	-	-

[a]Cyclohexane fraction; [b]Chloroform fraction; [c]Ethyl acetate fraction; [d]n-butanol fraction; +++: high content; ++: medium content; +: low content; -: no content (content was evaluated as the sediment or the intensity of color).

Table 2. The antinociceptive effect of total extract and major fractions separated from kiwi roots in acetic acid-induced nociception.

Group	Dose (mg/kg)	Number of writhing (per 15 min)	Inhibition (%)
Control	-	56.27±2.03	-
Ibuprofen	50	10.48***±2.46	81.37***
CYF[a]	50	54.12±2.49	3.82
CHF[b]	50	52.27±2.12	7.11
EAF[c]	50	12.59***±2.26	77.63***
BF[d]	50	16.41***±2.57	70.84***
Aqueous fraction	50	53.43±2.64	5.47
Total extract	200	11.73***±2.87	79.15***

[a]Cyclohexane fraction; [b]Chloroform fraction; [c]Ethyl acetate fraction; [d]n-butanol fraction; *$p < 0.05$ significantly different from control; **$p < 0.01$ significantly different from control and ***$p < 0.001$ significantly different from control.

Statistical analysis

The experimental data was expressed as mean ± standard error of mean (SEM). The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Tukey's t-test. The differences with $p < 0.01$ were considered statistically significant, $p < 0.001$ were considered highly significant.

RESULTS

Fractionation and phytochemical screening

The yield of extraction was measured about 42.7%, from which five major fractions were separated. As shown in Table 1, in phytochemical screening, the fractions of ethyl acetate and n-butanol exhibited strong positive reaction for flavonoids and saponins, respectively.

Effect of kiwi roots on acetic acid induced writhing

In the acetic acid-induced writhing mice (Table 2), the total extract, ethyl acetate fraction (EAF), n-butanol fraction (BF), at the doses used, exhibited a significant analgesic effect after oral administration in mice submitted to acetic acid-induced writhing when compared with control group ($p < 0.001$). In addition, the highest analgesic activity observed with the total extract (200 mg/kg) was lower than the analgesic activity of ibuprofen (50 mg/kg). The maximal inhibition of the nociceptive response (81.37%) was achieved by the ibuprofen at a dose of 50 mg/kg. However, the results presented in Table 2 showed that no significant analgesic effects were generated at all doses of CYF (Cyclohexane fraction), CHF (Chloroform fraction) and aqueous fraction.

Effect of kiwi roots on the nociception in hot plate test in mice

In the hot plate test, the results presented in Figure 1 show that the total extract, EAF, BF, ibuprofen produced a significant increase in the response time in the heated plate experiment from 30 to 120 min, respectively ($p < 0.001$). At the same time, the antinociceptive response observed with the total extract, EAF, BF was considerably more pronounced than that obtained with ibuprofen at all the tested doses from 60 to 120 min. Nevertheless, the analgesic activity observed with the total extract, EAF, BF was lower than the analgesic activity of ibuprofen from 0 to 30 min.

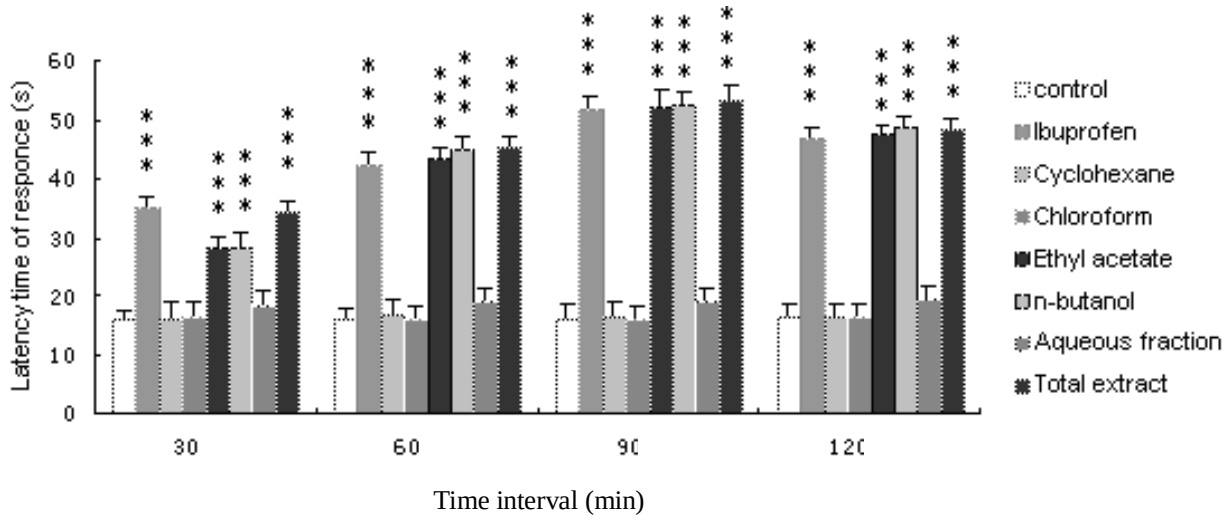

Figure 1. The antinociceptive effect of total extract and major fractions separated from kiwi roots in hot plate test. The effect has been calculated on the basis of latency time of response. Each point is the mean ± SEM of ten animals. *p < 0.05 ,**p < 0.01 ,***p < 0.001 in comparison to normal saline group.

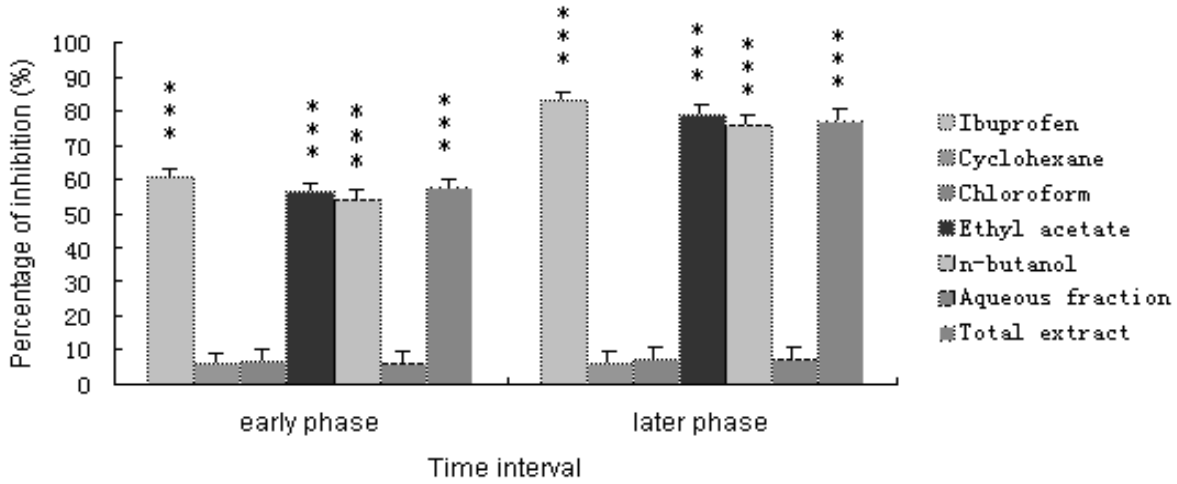

Figure 2. The antinociceptive effect of total extract and major fractions separated from kiwi roots in formalin-induced nociception. The effect has been calculated on the basis of percentage of pain inhibition. Each point is the mean ± SEM of ten animals. *p < 0.05 ,**p < 0.01 ,***p < 0.001 in comparison to normal saline group.

Effect of kiwi roots on formalin induced pain

As indicated in Figure 2, treatment with total extract (200 mg/kg), EAF (50 mg/kg), BF (50 mg/kg) and ibuprofen (50 mg/kg) significantly increased the percentage of pain inhibition in early phase and later phase of formalin test (p < 0.001). Moreover, in both early and late phases of experiment, the percentage of pain inhibition of total extract, EAF and BF was as good as ibuprofen. However, as shown in Figure 2, the percentage of pain inhibition of total extract, EAF, BF, and ibuprofen was more in the later phase than in the early phase.

Carrageenan induced paw edema

In carrageenan-induced hind paw edema experiment, as shown in Table 3, the examined samples demonstrated a significant anti-inflammatory activity at all tested doses in comparison with control group 3 h after carrageenan administration (p < 0.001). Amongst the examined samples, the total extract (200 mg/kg), EAF (50 mg/kg), BF (50 mg/kg) and ibuprofen (50 mg/kg) could significantly induce a reduction in paw edema from 1 to 3 h of experiment in animals (p < 0.05, p < 0.01, p < 0.001). The total extract exhibited the highest inhibition of paw edema

Table 3. The antinociceptive effect of total extract and major fractions separated from kiwi roots in carrageenan-induced hind paw edema.

Group	Percent of inhibition (%)			
	0.5 h	1 h	2 h	3 h
Ibuprofen (50 mg/kg)	8.21±1.68	43*.73±2.51	71.79**±2.49	96.61***±2.16
CYF[a] (50 mg/kg)	3.15±2.62	19.82±2.85	36.82±2.57	45.07*±3.93
CHF[b] (50 mg/kg)	4.41±2.31	16.58±2.03	45.73*±2.71	49.79*±2.17
EAF[c] (50 mg/kg)	16.57±1.87	46.45*±1.73	78.96**±2.63	95.31***±2.82
BF[d] (50 mg/kg)	17.15±2.69	44.82*±2.36	74.89**8±2.27	93.63***±2.93
AF[e] (50 mg/kg)	11.47±2.52	19.87±2.93	45.25*±3.09	54.46*±2.97
Total extract (200 mg/kg)	15.36±1.82	42.76*±2.09	79.74**±2.94	97.62***±1.88

[a]Cyclohexane fraction; [b]Chloroform fraction; [c]Ethyl acetate fraction; [d]n-butanol fraction; [e]Aqueous fraction; *p < 0.05 significantly different from control; **p < 0.01 significantly different from control; ***p < 0.001 significantly different from control.

at 3 h in comparison with the control group (p < 0.001).

DISCUSSION

This study investigated the antinociceptive and anti-inflammatory effects of kiwi roots in three analgesic models: acetic acid-induced writhing model, hot plate test and formalin-induced licking model for assessing antinociceptive effect as well as carrageenan-induced hind paw edema model for assessing anti-inflammatory.

The acetic acid-induced writhing reaction in mice has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents, and is described as a typical model for visceral inflammatory pain (Dickenson and Besson, 1997). The hot plate test, which utilizes thermal stimulus to induce pain, is frequently used to evaluate centrally mediated antinociceptive effect (Su et al., 2011). The acetic acid-induced writhes and hot plate test methods have been regarded as useful techniques of evaluating the peripherally and centrally acting analgesic drugs, respectively (Eddy and Leimback, 1953; Koster et al., 1959). For this reason, the acetic acid-induced writhes in mice and hot plate test were selected to observe the analgesic response in this study. In order to explore the pathway of analgesic activity of extract of kiwi roots, ibuprofen was selected as the reference drug. The results indicated that the total extract, EAF and BF exhibited a significant analgesic effect which significantly inhibited the number of writhes and increased in time of response (latency) to chemical stimulation and thermal stimulation in comparison with the control group (p < 0.001) (Table 2 and Figure 1). In a word, the results of the study show that at all dose levels used, the total extract, EAF and BF significantly reduced acetic acid-induced writhes which suggests that its analgesic effects could be peripherally mediated. The increase in the reaction time, by the extract to the thermal stimulus in the hot plate test indicates that the total extract, EAF and BF also possess a central analgesic effect.

The formalin-induced nociception is a well-described model for evaluating the mechanism of pain and analgesia (Hunskaar and Hole, 1987). The study has shown that the total extract, EAF and BF can inhibit both phases of formalin-induced pain with a more potent effect on the later than the early phase (Figure 2). Considering the inhibitory property of the total extract, EAF and BF on the formalin test, we might suggest that the analgesic activity of the extract could be dependent on central and peripheral sites of action. The conclusion is in good agreement with the conclusion reported by Shibata et al. (1989). Taken together, the ability of the total extract, EAF and BF to suppress pain perception might be mediated via peripheral and central pathways of pain perception.

The carrageenan test is highly sensitive to non-steroidal anti-inflammatory drugs and has long been accepted as a useful phlogistic tool for investigating new anti-inflammatory drugs (Just et al., 1998). Carrageenan-induced inflammation is useful in detecting orally active anti-inflammatory agents (Willoughby and DiRosa, 1972) and therefore has a significant predictive value for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation (Mossa et al., 1995). The results obtained show that the extract possesses anti-inflammatory activity (Table 3).

Previous investigations have reported that saponins, flavonoids, phenylpropanoids, quinones and steroids compounds have been separated and structurally identified from kiwi roots, among them are ursolic acid, oleanolic acid, succinic acid, quercetin, β-sitosterol, isoscopoletin, aesculetin, fraxetin, esculetin, umbelliferone, vanillic acid, protocatechuic acid, vanillic acid 4-O-β-D-glucopyranoside, carotenoids, lutein, and 5, 7-dihydroxychromone, etc. Flavonoids, saponins, tannins, phenolic compounds, and glycosides have all been associated with various degrees of anti-inflammatory and analgesic activities (Garcia et al., 1973; Hosseinzadeh and

Younesi, 2002; Wang et al., 2008; Thirugnanasambantham et al., 2007; Viana et al., 1998; Chang and Case, 2005; Yutaka et al., 1992; Yutaka et al., 1994; Lai and Xu, 2007; Lahlou et al., 2001; Cassano et al., 2006; Xu et al., 2010; Anne-Marie et al., 2002; Fu et al., 2010; Qian et al., 1999). The result indicated that the mechanism of antinociceptive and anti-inflammatory effect of extract of kiwi roots may be related to flavonoids and saponins.

However, the pharmacological studies have focused mainly on crude extracts, and many of the constituents responsible for different pharmacological activities remain unknown. Therefore, the antinociceptive and anti-inflammatory effects observed in this study are perhaps due to the activity(ies) of one or a combination of some of the identified classes of compounds. More studies are needed to prove clinical efficacy and reveal the exact mechanism of action.

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Full Length Research Paper

Flaxseed and quercetin improves anti-inflammatory cytokines level and insulin sensitivity in animal model of metabolic syndrome fructose-fed rats

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The purpose of this study was to assess the beneficial effect of quercetin, flaxseed and/or in combination as synergetic, in an animal model of metabolic syndrome (MtS), high fructose (HF)-fed rats. Fifty male Sprague-Dawley rats, 3 months old, weighing between 110 to 120 g were randomly divided into 5 groups. Rats were given drinking water (negative (-ve) control rats) or 10% fructose in drinking water (HF; fructose-fed rats) with standard chow for 8 weeks. After 4 weeks of HF feeding, rats were further divided into matched 4 subgroups. Different groups of animals (*n* = 10, each group) were administered 10% HF (5 mg/kg, +ve control), flaxseed (F; 50 mg/kg), quercetin (Q; 50 mg/kg), flaxseed + quercetin, (FQ; 25 mg/kg each), respectively. All ingredients were given orally, once daily and subsequently for 4 weeks. Serum glucose, insulin, lipids profile, leptin, and adiponectin were estimated. After 4 weeks of feeding, a significant increase in blood glucose level was observed in HF fed rats compared to normal rats, but this was significantly decreased after administration of F, Q and FQ. The serum insulin level in HF fed rats was significantly decreased after administration of F and FQ groups. Significantly, higher concentrations of triacylglycerols (TG), total cholesterol and low density lipoprotein cholesterol (LDL- C) were observed in HF fed rats, and these increases were lower after administration of F, Q and FQ. There was a significant increase in serum high density lipoprotein cholesterol (HDL-C) in FQ group. The increase of serum leptin level was decreased significantly in F, Q and FQ groups. Whereas, the reduction of serum adiponectin level in HF fed rats was increased in F, Q and FQ groups. These data suggests that protective effect of flaxseed and quercetin consumption as functional foods could be less risky for people with decreased insulin sensitivity and increased oxidative stress, such as those with the metabolic syndrome or type 2 diabetes.

Key words: Flaxseed, protective effect, quercetin, insulin sensitivity, metabolic syndrome, type 2 diabetes.

INTRODUCTION

The metabolic syndrome (MetS) is a constellation of risk factors, including impaired fasting glucose, hypertension, central adiposity, predisposing to higher risks of oxidative stress, type 2 diabetes and atherosclerotic cardiovascular disease (CVD) (Park et al., 2007; Grattagliano et al., 2008; Chen et al., 2008; Ishizaka et al., 2009). The etiopathology of the metabolic syndrome has not yet been fully elucidated. Recent studies have highlighted

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the involvement of a pro-inflammatory state that induces insulin resistance and leads to clinical and biochemical manifestations of the metabolic
syndrome (Horiuchi and Mogi, 2011).

Obesity/insulin resistance is associated with metabolic syndrome, which plays a pivotal role in cardiovascular risk. The mechanisms that link obesity, insulin resistance, and endothelial dysfunction are numerous and complex (Steinberg et al., 1996). Increase in visceral fat, usually involved in obesity, leads to an imbalanced production of metabolic products, hormones, and adipocytokines including tumor necrosis factor-α (TNF-α), free fatty acids (FFAs) or adiponectin which causes decreased insulin sensitivity in skeletal muscle and liver, and impairs endothelial function through direct or indirect mechanisms.

Insulin itself acts as cytokine at sufficiently high concentrations, and this may underlie vascular damage and dysfunction in human and animal studies (Absher et al., 1997, 1999). There are a number of recognized cytokines that are related to obesity, metabolic syndrome and cardiovascular disease, including adipocyte-related peptide adiponectin and inflammatory marker, interleukin-1b (IL-1b) (Dinarello, 1998, 2005; Huypens, 2007). Researchers observed that obesity is inversely correlated with adiponectin, a marker of anti-inflammation (Brooks et al., 2007). Similarly, IL-1b is a mediator of systemic pro-inflammatory pathways and may provide an index of the inflammatory processes that are known to accompany atherosclerosis.

The Mediterranean diet which include a high intake of plant food content, such as vegetables, legumes, and fruits, have been directly associated with the prevention of obesity, type 2 diabetes, and other cardiovascular risk factors (Estruch et al., 2006). The protective effect of plant foods that contain flavonoids, polyphenolic compounds against chronic pathologies such as, obesity, diabetes and cardiovascular disease mortality, is reported by many workers (Knekt et al., 2002; Mink et al., 2007).

Quercetin (3,3',4',5,7- pentahydroxyflavone) is one of the most widely used flavonol in human dietary sources (Hertog et al., 1993). The intervention trials with quercetin in human subjects is an effort in the development of dietary supplements with a higher dose, which might prove useful for the prevention or treatment of functional alterations clustered in the metabolic syndrome (Middleton et al., 2000; Duarte et al., 2002; Comalada et al., 2006; Rivera et al., 2008). Other human studies, however, failed to confirm these effects (Williamson and Manac, 2005).

Flaxseed is a complex food containing high amounts of poly unsaturated fatty acids (PUFA), mainly a-linolenic acid (ALA), an (n-3) fatty acid, as well as soluble fiber, lignan precursors, and other substances that may have health benefits (Hall et al., 2006; Basett et al., 2009). A number of studies have shown that flax oil supplementation can reduce serum triacylglycerols and cholesterol concentrations, thus leading to reduced CVD risk (Cunnane et al., 1993; Craig et al., 1999). Furthermore, n-3 PUFA of flaxseed oil has anti-inflammatory properties that are mediated by the production of anti-inflammatory cytokines (Cohen et al., 2005). However, the effects of these foods on MtS remain unclear.

In this study, we determined the beneficial effects of quercetin, flaxseed and/or in combination as synergetic in an animal model of MtS HF fed rats. In particular, we measured various parameters related to MetS, such as hyperlipidaemia, hyperglycaemia, hyperinsulinaemia and on formation of anti-inflammatory cytokines such as leptin and adiponectin.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats were purchased from the Laboratory Animal Center (Science section and medical studies, Malaz, Riyadh, King Saud University, Saudi Arabia) and housed in plastic cages with a 12:12 h light-dark cycle, at a constant temperature of 22 to 24°C. They were given standard chow *ad libitum* for the duration of the study and allowed 1 week to adapt to the laboratory environment before experiments.

Animal model and drug administration

All the animals used in the present study were treated in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by Committee of King Saud University, College of Pharmacy (Riyadh, Saudi Arabia). Fifty male rats, 3 month old, weighing between 110 and 120 g were randomly divided into 5 groups. The initial body weights of the rats were recorded with no significant difference between control and HF-fed groups. Rats were given drinking water (negative (-ve) control rats) or 10% fructose in drinking water (HF-fed rats), with standard chow for 8 weeks (Hu et al., 2009). Fresh drinking water was replaced every 2 days. After 4 weekks fructose feeding, HF-fed rats were further divided into matched 4 subgroups. Different groups of animals (n = 10, each group) were administered; 10% fructose in drinking water (5 mg/kg, as a +ve control group), F (50 mg/kg), Q (50 mg/kg), FQ (25 mg/kg each), respectively. All ingredients were given orally once daily, subsequently for 4 weeks.

Biochemical assays

After an overnight fasting (food deprivation), rats were anesthetized and blood was withdrawn by heart puncture, in tubes protected from light, then centrifuged at 3,000 g for 10 min at 4°C. Plasma was immediately isolated, aliquoted and stored at -80°C until analyzed. Serum glucose, total cholesterol, HDL-C, and TG levels were estimated calorimetrically using kits according to United Diagnostics Industry. LDL-C was calculated using the Friedwald equation [$L = C - H - 0.16T$; where H is HDL -C, L is LDL-C, C is total cholesterol, T is TG, and k is 0.20 (mg/dl)]. Serum insulin, leptin and adiponectin were measured using enzyme linked immunosorbent assay (ELISA) kits, insulin (American Laboratory Products Company, Windham, NJ), leptin and adiponectin (AniBiotech Oy, Orgenium Laboratories Division, Vantaa, Finland).

The reading was taken using ELISA microplate reader (VERSA Max, Molecular Devices Corporation, MN, USA).

Statistical analysis

All values were expressed as mean ± Standard error (SE). Data were statistically analyzed using one way Analysis of variance (ANOVA) for multiple group comparison, followed by Student's unpaired t-test for group comparison. Significance was set at $p \leq 0.05$. Data were computed for statistical analysis by using Graph Pad Prism Software.

RESULTS

After 4 weeks of feeding, a significant increase in serum glucose and insulin levels were observed in HF fed rats compared to negative (-ve) control group. However, this increase significantly decreased after administration of F ($p \leq 0.001$), Q ($p \leq 0.05$) and FQ ($p \leq 0.001$) in serum glucose (Figure 1A). Serum insulin significantly decreased after administration of F ($p \leq 0.001$) and FQ ($p \leq 0.001$) groups, whereas no significant difference could be observed in Q group (Figure 1B). Serum lipid profiles were measured after 4 weeks of HF fed rats. Significantly higher concentrations of TG ($p \leq 0.05$), total cholesterol ($p \leq 0.001$) and LDL-C ($p \leq 0.001$) was observed in HF fed rats compared to negative (-ve) control group, but these increases were significantly lowered after administration of F ($p \leq 0.001$), Q ($p \leq 0.01$) and FQ ($p \leq 0.001$) (Figure 2A, B and C). A significant increase in serum HDL-C was observed in FQ group ($p \leq 0.001$) compared to HF fed rats (Figure 2D). Serum leptin level decreased in F ($p \leq 0.001$), Q ($p \leq 0.01$) and FQ ($p \leq 0.001$) groups (Figure 3A) whereas, serum adiponectin level increased in F ($p \leq 0.001$), Q ($p \leq 0.01$) and FQ ($p \leq 0.001$) groups (Figure 3B).

DISCUSSION

Fructose rich diet was used for the induction of diabetes, which is characterized by insulin resistance and metabolic syndrome, very much close to type 2 diabetes in human. Several studies reported that fructose feeding for long term induces diabetes associated with insulin resistance and metabolic syndrome in experimental animals (Veerapur et al., 2010; Reungjui et al., 2007). In the present study, in rats receiving HF, plasma insulin, glucose, TG, total cholesterol and LDL-C were increased significantly, whereas HDL-C was significantly decreased, compared to negative (-ve) control group. These results are consistent with several earlier reports (Thresher et al., 2000; Busserolles et al., 2002, 2003) which support that consumption of HF diet leads to the development of insulin resistance which plays a pivotal role in the pathogenic mechanism of human type 2 diabetes, and is the cause of all metabolic complications (Veerapur et al., 2010). High dietary fructose have been associated with enhanced oxidative damage in rats (Busserolles et al., 2002) and development of insulin resistance; beta-cell dysfunction, and impaired glucose tolerance (Paolisso and Giugliano, 1996; Bloch-Damti and Bashan, 2005).

In the present results of rats fed HF diet, the cluster of metabolic syndrome were reversed by administration of F, Q and/or a combination (Figure 1A and B). Some flavonoids may affect fasting glucose, insulin, and lipid profile by insulin-enhancing activity *in vitro* and may regulate the expression of genes involved in glucose uptake and insulin signaling in rats fed HF (Rivera et al., 2008). Epidemiological evidence indicated that flaxseed seemed to decrease fasting glucose, prevent the increase of HbA1c and delays the development of diabetes (Prasad, 2001). Castilla et al. (2006) found that consumption of grape juice, rich source of quercetin have improving effect on HDL-C level which was paralleled by an increase in apo A1 concentrations. This indicates that flavonoids may affect hepatic apo A1 secretion *in vivo* (Hotamisligil et al., 1995).

Flaxseed contains both n-3 fatty acids and lignans; flaxseed lignans alone had produced serum lipids in hyperlipidaemic rats (Felmlee et al., 2009). Thus, the presence of lignans could have contributed to the lipid-lowering properties of flaxseed supplementation by inhibiting fatty acid and cholesterol synthesis in the liver and hence reducing the hepatic lipid levels (Fukumitsu et al., 2008). It may be attributable; in particular, since ALA-rich flax oil can act as a better substrate for mitochondrial and peroxisomal β-oxidation, thus stimulating increased oxidation of lipids in the liver (Ide et al., 2000). Thus flaxseed consumption could be predictive of benefits for people at risk of developing cardiovascular disease.

The results of the present study revealed a significant decrease in serum leptin and increase in adiponectin levels after administration of Q ($p \leq 0.001$), F ($p \leq 0.05$) and F+Q ($p \leq 0.001$) in HF fed rats (Figure 3A and B). Quercetin has been shown to exert anti-inflammatory effects. In peripheral blood mononuclear cells, quercetin dose-dependently inhibited the gene expression and production of the pro-inflammatory cytokine TNF-α (Nair et al., 2006). The mechanism of this effect was the modulation of the NF-kB signal transduction cascade. Therefore, a high dose of quercetin might have improved the inflammatory status by decreasing the production of TNF-α in visceral adipose tissue, and increasing plasma levels of adiponectin (Rivera et al., 2008).

The serum leptin level was lowered but serum adiponectin level was increased significantly in F and F+Q treatments (Figure 3A and B). Flaxseed has recently gained popularity as a functional food. Consumption of flaxseed has been shown to lessen insulin resistance, hyperlipidemia, atherosclerosis and hypertension and

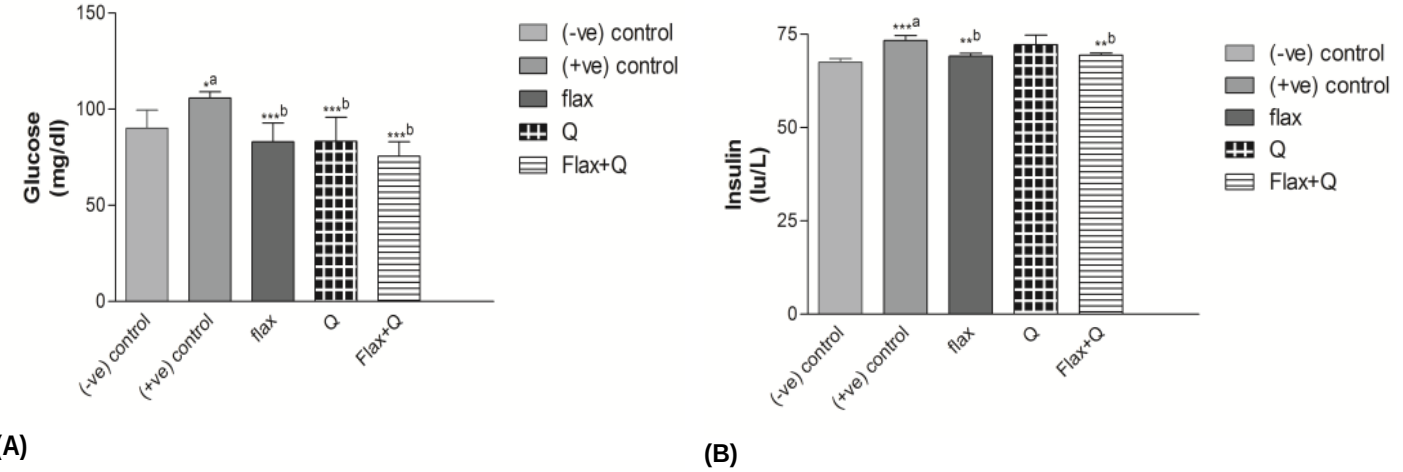

Figure 1. Serum Glucose and Insulin levels after administration of flaxseed, quercetin and in combination in fructose fed rats for 4 weeks. Values are expressed as mean ± SD. ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, a: compared to (–ve) control group, b: compared to (+ve) control group.

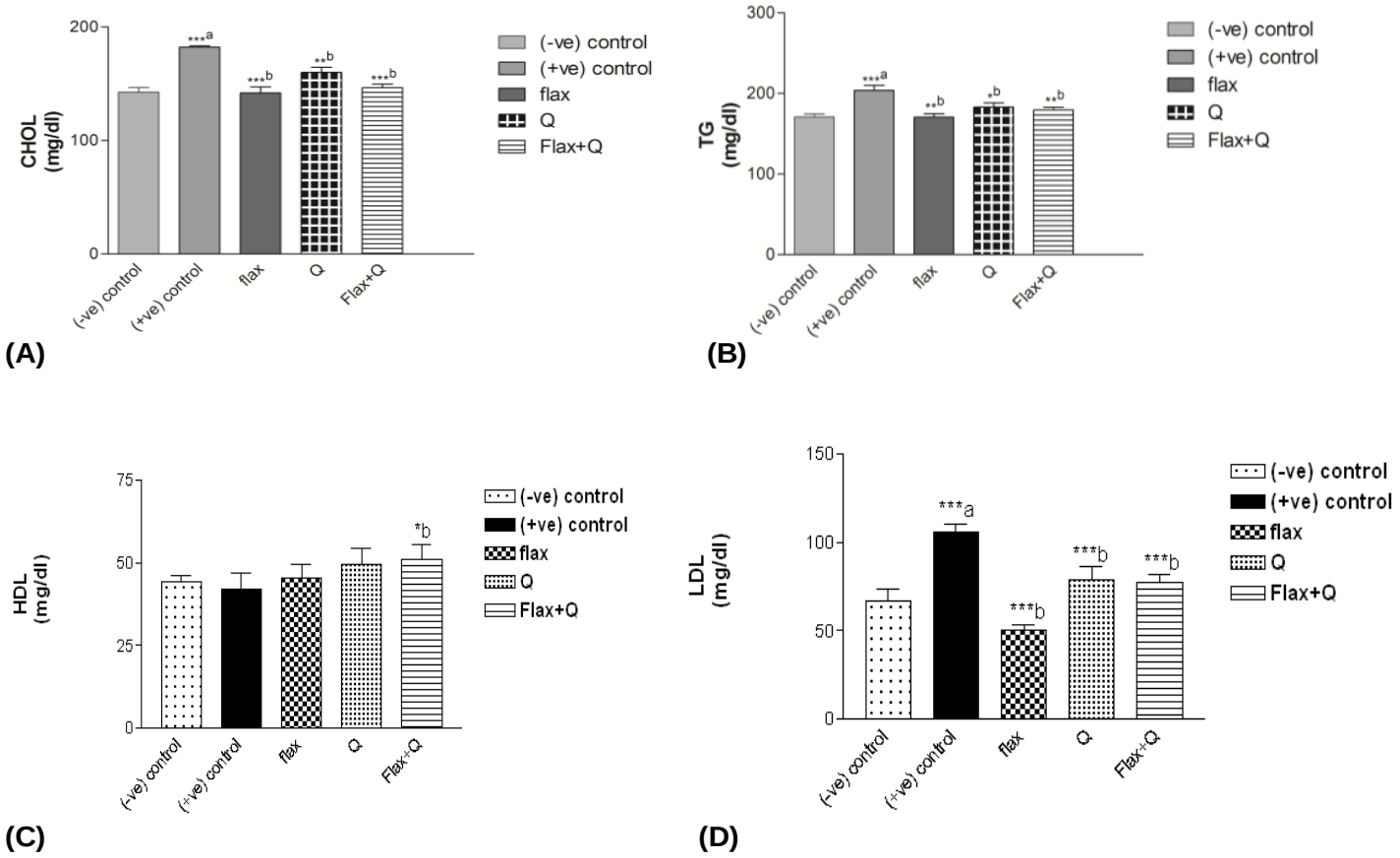

Figure 2. Serum lipid profile level after administration of flaxseed, quercetin and in combination, in fructose fed rats for 4 weeks. Values are expressed as mean ± SD. ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, a: compared to (–ve) control group, b: Compared to (+ve) control group.

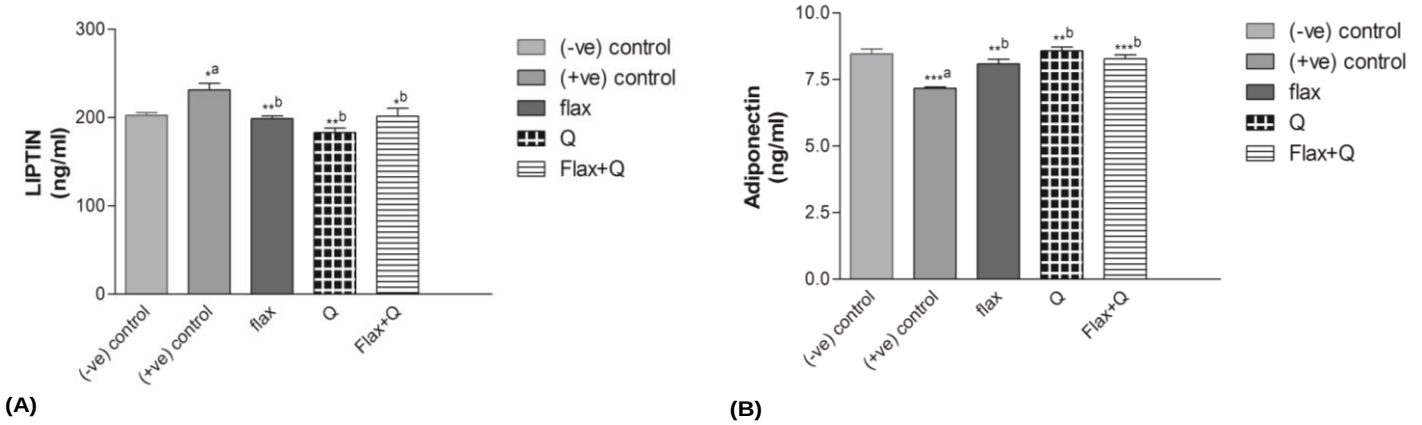

Figure 3. The leptin and adiponectin levels after administration of flaxseed, quercetin and in combination, in fructose fed rats for 4-weeks. Values are expressed as mean ± SD. ***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, a: compared to (-ve) control group, b: compared to (+ve) control group.

decrease the incidence of cardiac arrhythmias. These effects of dietary flaxseed have been attributed, in part, to the rich ALA content of flaxseed (Ander et al., 2004; Dupasquier et al., 2007). However, the mechanism to explain the induction of these effects by ALA remains elusive. ALA in adipose tissue is strongly associated with increased leptin expression and subsequent reduction of atherosclerosis. Therefore, it is suggested that flaxseed may induce its anti-atherogenic effects in part via an ALA-mediated modulation of leptin expression (McCullough et al., 2011).

Adiponectin is the most highly expressed and secreted adipokine, with beneficial effects on metabolism, inflammation, and vascular function. It plays a role in insulin sensitivity, LDL oxidation, endothelial nitric-oxide synthase (eNOS) activation, inflammation suppression and fatty acid catabolism (Halleux et al., 2001; Haluzik et al., 2004; Iacobellis et al., 2005). Thus, hypoadiponectinemia is of interest as a biomarker of both cardiovascular disease and metabolic syndrome. A study conducted on mice showed that replacing 15% of lipids with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (specifically, the replacement lipid consisted of 6% EPA and 51% DHA) improved insulin sensitivity as well as raised plasma adiponectin level, independent of food intake or adiposity. It also showed that the adiponectin gene expression was up-regulated in mature adipocytes after this intervention (Albert et al., 2005).

Conclusion

These data are encouraging and suggest that anti-inflammatory and protective effect of flaxseed and quercetin consumption as functional foods could be an important mechanism contributing to the reduced risk for people with decreased insulin sensitivity and increased oxidative stress, such as those with the metabolic syndrome or type 2 diabetes.

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Full Length Research Paper

Simultaneous determination of saikosaponins in Bupleurum Radix from different locations by high performance liquid chromatography- charged aerosol detection (HPLC-CAD) method and its immunomodulation effects on mouse splenocytes

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A high performance liquid chromatography (HPLC)-Charged Aerosol Detection (CAD) was employed to simultaneously determine saikosaponins which include saikosaponin a (SSa), saikosaponin b2 (SSb2) and saikosaponin d (SSd) in Bupleurum Radix from different locations. Meanwhile, MTT assay was used to compare the proliferation effect of SSa, SS and SSb2 on mouse splenocytes, and enzyme linked immunosorbent assay (ELISA) method was used to determined cytokines which include interleukin (IL)-4, IL-10, interfernon (IFN)-γ, TNF-α. The results show that there existed significant differences of the three saikosaponins from different locations, and SSa, SSd, and SSb2 all presented immunomodulation effects on mouse splenocytes via promoting splenocyte proliferation and regulating the balance of Th1/Th2 factors.

Key words: HPLC-CAD, Bupleurum Radix, saikosaponin a (SSa), saikosaponin b2 (SSb2), saikosaponin d (SSd).

INTRODUCTION

Bupleurum Radix, a well-known traditional Chinese medicine (TCM), was used for the treatment of common cold with fever, influenza, hepatitis, malaria and menoxenia. *Bupleurum chinense* DC. and *Bupleurum scorzoneri folium* Willd. recorded in the Chinese Pharmacopoeia are officially regarded as standard medicinal plants (The Pharmacopoeia Commission of PRC, 2010). Also, Bupleurum Radix was widely distributed in China other than Hainan province. *Bupleurum chinense* DC. grows in Northern China, and *Bupleurum scorzoneri folium* Willd. is mainly distributed in Southern China, of which 44 species, 17 varieties have been reported in China and nearly half of them are native species (Shan and She, 1979). Many compounds were found in *Bupleurum* including phytocholesterols and volatile oil, polysaccharides, saikosaponin a (SSa), saikosaponin b2 (SSb2), saikosaponin d (SSd) and so on (Shi et al, 2002). Recently, pharmacology studies indicate that the saikosaponins (SS) have many pharmacological effects such as, relieving fever, conscious sedation, anti-inflammatory, immunity regulating, antivirus, anti-liver fibrosis, anti-tumor, relieving pain, anti-biosis, protecting a liver and anti-nephritis (Zhu et al, 2002; Huang and Sun, 2010). Some studies report that SS presents specific and

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nonspecific immunity functions, and induces the aggregation of peritoneal macrophage, activates and stimulates the T lymphocyte and B lymphocyte to participate in the immunomodulation, and improves the activity of cell surface receptor on the of macrophage and changes the ultrastructure in cells so as to enhance their activities (Wei, 2005; Mohamed and Michael, 2011), and the above studies mainly concentrated on SSa and SSd, but the study about SSb2 has not been reported until now.

Therefore, it is necessary to investigate their discrimination and develop a reasonable approach to control their quality. In recent years, the employment of HPLC-UV (Li et al, 2005; Mahmood et al, 2011; Emira et al, 2011) and ELSD (Pu et al, 2010) method to determine SSa, SSb2 and SSd has been reported. But little information about the determination of saikosaponin in Bupleurum Radix using HPLC-CAD method has been published. In this study, HPLC-CAD method was first employed to simultaneously determine SSa, SSb2, SSd, the main biomarkers in Bupleurum Radix from different locations. In addition, to compare immunomodulation effects of the saikosaponins in the Bupleurum Radix, MTT assay was used to test the effects of different concentrations of SSa, SSd, and SSb2 on the mouse splenocyte proliferation, and ELISA method was applied to determine in vitro adjusting lymphocyte factors, such as IL-4, IL-10, IFN-γ, TNF-α.

MATERIALS AND METHODS

Plant material

Bupleurum was collected from thirteen different locations in Liaoning, Inner Mongolia, Jilin, Heilongjiang, Shanxi, Hebei, Anhui and so on. Voucher specimens were maintained at Liaoning University of Traditional Chinese Medicine, China. They were identified by Professor Yanjun Zhai in School of Pharmacy, Liaoning University of Traditional Chinese Medicine.

Chemicals

The standard substances, SSa, SSb2 and SSd were provided by VIKEQI biological products Co., Ltd (Sichuan, China). HPLC-grade acetonitrile was purchased from Damao chemical reagent plant (Tianjin, China) and the water used in all experiments was purified by a Milli-Q® Ultrapure Water System (Millipore, MA, USA). RPMI-1640 culture solution was purchased from Solarbio, USA, Thiazole blue (MTT) and sword bean protein A (ConA) from Sigma, USA, Red blood cell disruption solution from Solarbio, USA, and ELISA kit from R&D, USA. All other chemicals were of analytical reagent grade purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Animals

All animal studies were performed according to the Guidelines for the Care and Use of Laboratory Animals that was approved by the Committee of Ethics of Animal Experimentation of Liaoning University of TCM. BACL mice were purchased from the Experimental Animal Center of Dalian Medical University (qualified card number Liaoning 2008-0002). They were kept in an environmentally controlled breeding room for one week before the experiments and fed with standard laboratory food and water.

Instrument and chromatographic conditions

CO2 incubator was purchased from SANYO MCO-175M, Japan. Clean Benches from ZHJH-C1112B, China. Multiskan Mk3 microplate reader from Thermo, China, and NIB-100 inverted biological microscope from NOVEL China. HPLC analysis was carried out on Shimadzu LC-20A high performance liquid chromatography (LC-20 pump, SIL-20 autosampler, A CTO-20 constant temperature column oven, A CBM-20 TSC, LC Solution work station), incorporating an electrical aerosol detector (CAD). The analytes were determined at 20°C on an analytical column Waters C18 (4.6 mm × 150 mm, 5 μm). The mobile phase consisted of the solvent acetonitrile-water gradient elution (30:70-50:50). The mobile phase was passed under vacuum through a 0.45 μm membrane filter before use. The analysis was carried out at a flow rate of 1 ml/min. Injection volume was 10 μl.

Sample preparation

To the conical flask with lid, 1 g of the dried and powdered Bupleurum were extracted with 25 ml of a mixture of methanol-ammonia solution (95:5, v/v) in an ultrasonic bath for 30 min at 30°C. The supernatants were collected and then the residue of Bupleurum was washed with 20 ml methanol twice. Both of these fractions were combined and evaporated to dry. Finally, the residue was reconstituted by 10 ml methanol and supernatants were filtered with 0.45 μm membrane to obtain the filtered solution. An aliquot (10 μl) of filtrate was injected into the HPLC system.

Method validation

Linearity, LLOQ and LOD

Stock standard solutions of SSa, SSb2 and SSd were prepared with methanol. The three calibration curves in the concentration ranges of SSa, SSb2 and SSd were 0.088-8.8, 0.044-4.4 and 0.088-8.8 μg/ml, respectively. The calibration curve was constructed using six different concentrations by plotting the peak area versus the nominal concentration. LOD and LOQ were determined by stepwise dilution of the QC sample at low concentration level using a signal-to-noise ratio of 3 and 10, respectively.

Precision and accuracy

The precision and accuracy of the method were evaluated with QC samples at low, middle and high three concentrations and using five replicates on three consecutive days. The intra- and inter-assay precisions were assessed by determining the QC samples at three concentration levels of each compound. For the intra-day validation, five replicates of the QC plasma samples were analyzed on the same day, while the inter-day values were carried out over three consecutive days. The accepted criteria for each QC sample were that the precision (RSD) and accuracy (RE) should not exceed 15%, except at the LOQ where it should not exceed 20%.

Recovery

The recovery was determined by adding known amount of the standard substances of SSa, SSb2 and SSd, approximately one times the levels detected in unspiked samples prior to extraction, and

these spiked samples were prepared as described in “Sample preparation”. The total amount subtracted the content in unspiked sample was divided by the added amount of the standard substance, and the extraction recovery was calculated as percentage.

Stability

The stabilities of SSa, SSb2 and SSd in the methanol were investigated by comparing the peak area of the three saikosaponins at 3, 6 and 12 with that at 0 h, that is, the stabilities of three compounds in the solution of methanol were investigated during the storing period of 0-12 h.

Preparation of different concentration solutions of SSa, SSd and SSb2

The different concentration (Figure 2) solutions of SSa, SSd and SSb2 were respectively prepared by adding RPMI-1640 culture medium solution then filtrated through 0.22 μm millipore filtration membrane before use.

Preparation of splenocytes (Li, 2006)

BACL mice (n = 6) were soaked in 75% ethanol for 3 min after sacrifice, and the spleen was cut off under the sterile conditions, washed successively with PBS containing different concentrations of the double antibotics including penicillin and streptomycin, levigated, filtrated through 0.22 μm millipore to obtain cell suspension, added 3-5 times of red blood cell disruption solution, transferred into a centrifuge tube, shaken for 5 min at room temperature, centrifuged at 1200 rpm for 10 min, and the white precipitation was washed with PBS for twice, with culture solution for 1 time, centrifuged at 1200 rpm for 10 min after each wash. Finally, the white precipitation was respectively added the calf serum and RPMI-1640 culture solution containing double antibotics (100 U/ml), vortexed, dyed by trypan blue and then counted. The cell concentration will be adjusted based on the demand of the test before use.

MTT assay

The above spleen cells (1 × 106/ml) were seeded in each well of 96-well plates, 100 μl for each well, and maintained at 37°C with 5% CO2 under aseptic conditions for 48 h, added ConA whose final concentration in the culture solution was 5 μg/ml, and 20 μl different concentration of drugs, added with culture solution up to the volume of 200 μl, incubated for 48 h, and then MTT (20 μl/well) was added to each well, and culture was terminated after incubated at 37°C for 4 h, centrifuged at 1000 rpm for 5 min to remove the supernatant. After that, 150 μL DMSO was added in each well, shocked for 10 min, and then the plates were read using the microplate reader at a wavelength of 492 nm. All of the assays including blank control ConA(+) and negative control ConA(-) were performed at least in triplicate.

ELISA assay

The concentration of spleen cells was adjusted to 1 × 107/ml, and then the cells were seeded in each well of 24-well plates, 100 μl for each, and maintained at 37°C with 5% CO2 under aseptic conditions for 48 h. Added was ConA whose final concentration in the culture solution was 5 μg/ml) and 20 μl of the three drugs screened by above MTT assay, cultivated for 48 h, and then the supernatants were assayed to determine IL-4, IL-10, IFN-γ, TNF-α, and OD values based on the instruction of ELISA kit at 450 nm. All of the assays including blank control ConA(+) and negative control ConA(-) were performed at least in triplicate.

Statistical analysis

All results were confirmed in at least three separate experiments. Data are shown as mean ± standard deviation and were analyzed by one-factor analysis of variance. A value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

HPLC analysis

The saikosaponin is the main ingredient of Bupleurum including SSa, SSb, SSd, in which many pharmacological studies about SSa and SSd have been reported, and SSb were transformed from SSa and SSd in the extraction process (Harada, 1992) , that is, SSb1, SSb2 were respectively transformed from SSa and SSd. Therefore, the three saikosaponins are taken as the indexes for the determination except that SSb1 because which was unstable, additionally, its reference substances are not readily available.

The reflux method has been used to extract SSa and SSd, however, the contents of them is remarkably lower than that of ultrasonic, and the content of SSb2 slightly increased meaning that the transformation of saikosaponins occurred in the process of heating reflux. Therefore, the ultrasonic extraction was applied for sample preparation because the contents of SSa and SSd are higher than that of reflux method. In addition, a mixture of methanol-ammonia solution (95:5, v/v) was used to extract the sample because it can avoid saponins transformated (Fu et al., 2010).

Our attempts to use the method with isocratic elution for the determination of SSa, SSb2 and SSd were unsuccessful. The gradient elution method was therefore used for the separation of them. The maximum absorption determined in this experiment of SSa and SSd is at 204 nm and SSb2 is at 254 nm, respectively, in which 204 nm were close to the end absorption presenting low sensitivity, baseline drift when Bupleurum saponins were detected by HPLC with UV detection (Li et al., 2005) and ELSD detection (Pu et al., 2003). Therefore, an HPLC-CAD method was used to determine SSa, SSb2 and SSd to obtain a good separation, symmetric peaks and little interference chromatogram. Figure 1 shows typical chromatograms of the standard substances (A), and the SSa, SSb2 and SSd in Bupleurum Radix (B). The retention times of SSa, SSb2 and SSd were approximately 19.3, 21.0 and 30.9 min, respectively, and the total chromatographic run time was 40 min.

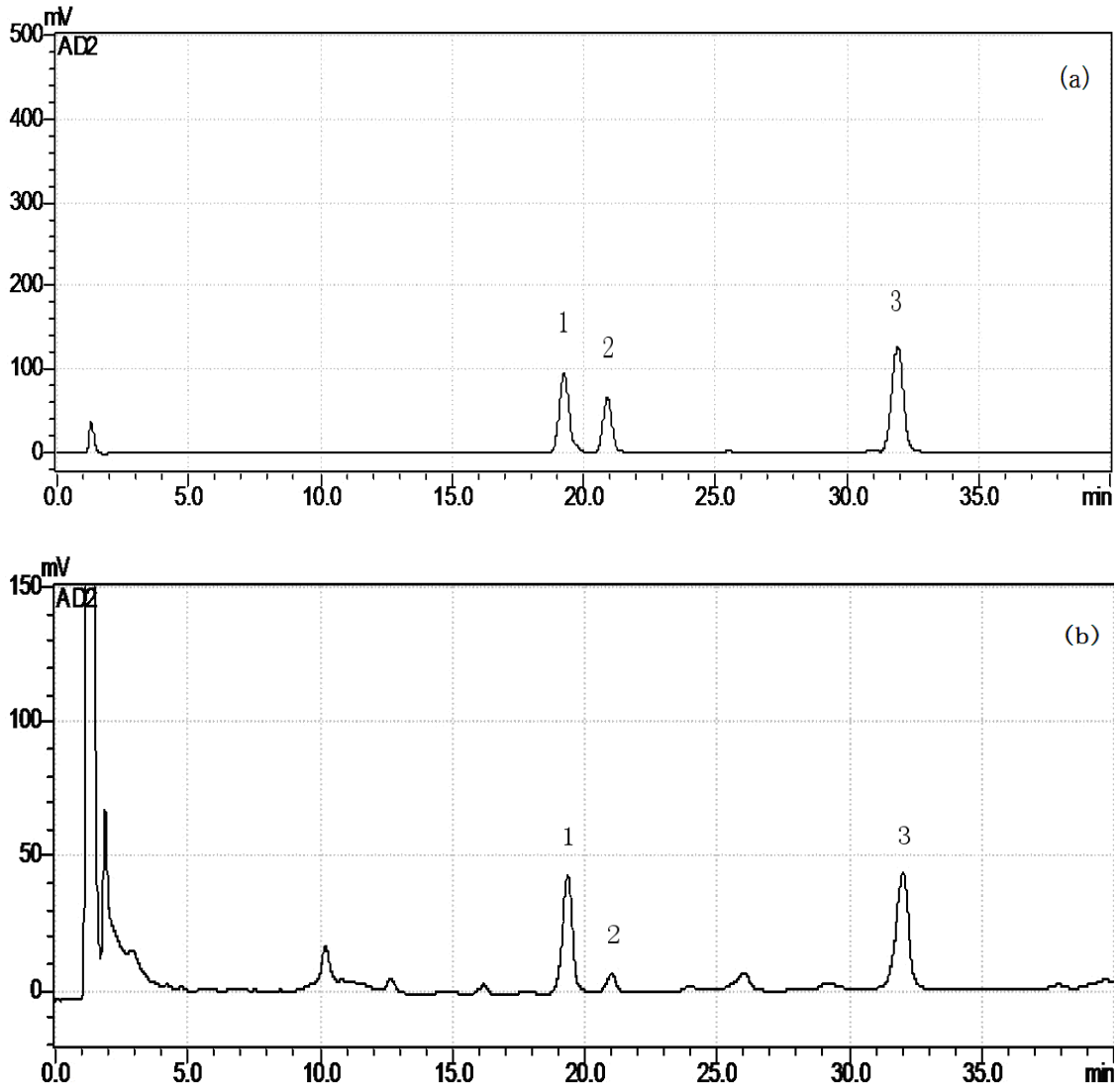

Figure 1. Representative chromatograms of SSa, SSb2 and SSd (A) and the Bupleurum Radix (B). Peak1: SSa, Peak 2: SSb2 and Peak3:SSd.

Method validation

Linearity, LLOQ and LOD

The evaluation of the linearity was performed with a six-point calibration curve over the concentration range of 0.088-8.8, 0.044-4.4 and 0.088-8.8 µg/ml for SSa, SSb2 and SSd, respectively. The calibration curve was constructed using six different concentrations by plotting the peak area versus the nominal concentration. The regression equations, coefficients and results of LOD and LOQ are shown in Table 1. Precision and accuracy of LOD and LOQ were within 15% after repeated analysis

Precision and accuracy

The summaries of intra- and inter-day precision/accuracy at low, medium, and high concentrations of each analytes in plasma are listed in Table 2 indicating that the procedures described above were satisfactory with respect to both accuracy and precision.

Recovery

For a validation of the extraction recoveries of the three compounds, the analysis for each biomarker was carried out in six replicates. The results show that the mean extraction recoveries were acceptable ranged from 95.92% to 99.69%, suggesting that there was negligible loss during the leaves extraction.

Stability

The stock solutions of three compounds were found to be stable at room temperature over the time range of 0 to 12 h.

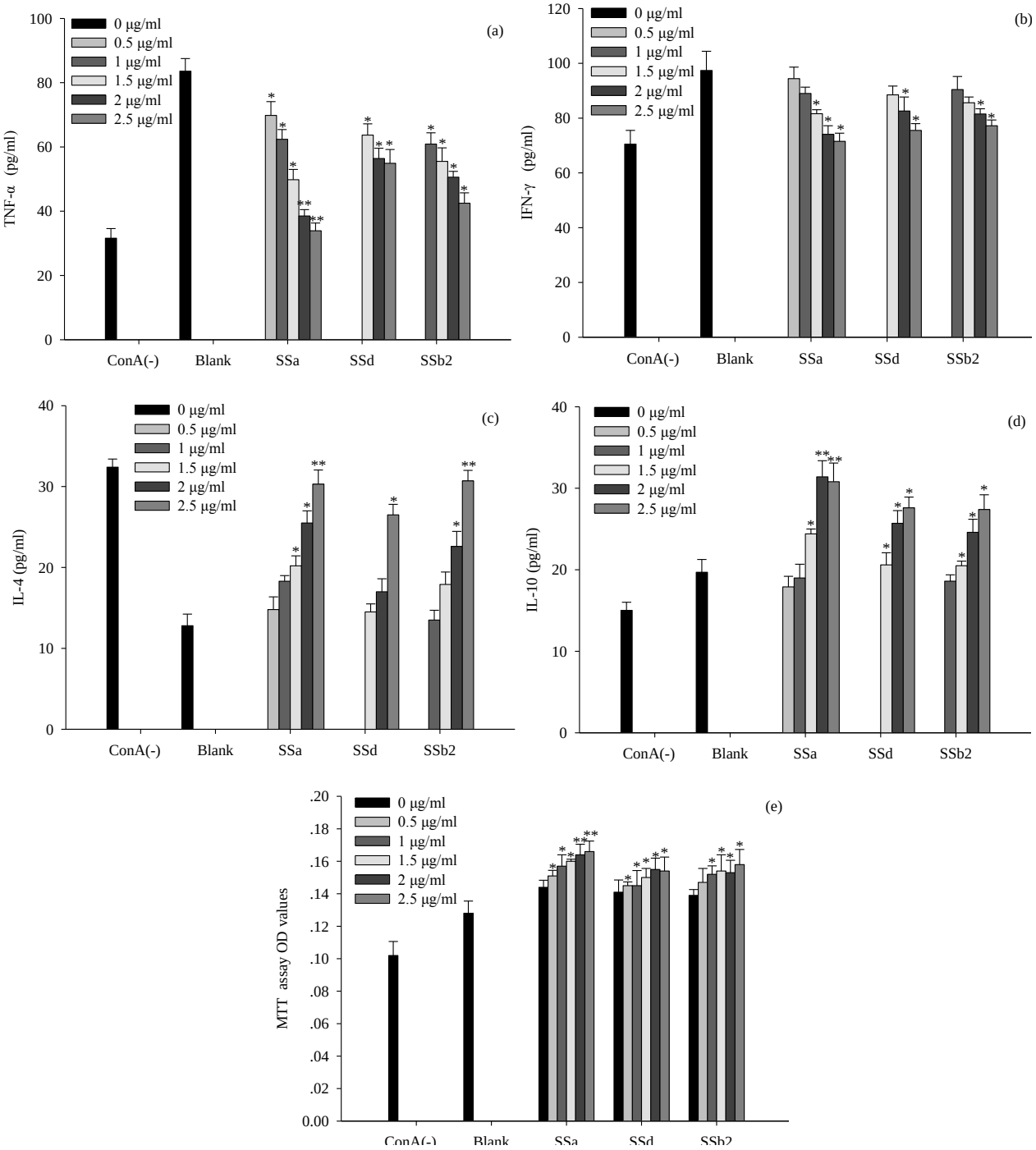

Figure 2. SS effects on the cytokines (a) TNF-α (b) IFN-γ (c) IL-4 (d) IL-10 and on splenocyte proliferation (e). * $p < 0.05$, ** $p < 0.01$, compared with blank group.

The stabilities of three compounds in the solution of methanol were investigated during the storing period of 0-12 h, RSD (%) of the area for three compounds were 4.59, 3.25 and 3.55%, respectively, suggesting that they were stable after the samples were treated as “Sample preparation” under the experimental conditions of the regularly analytical procedure.

Simultaneous determination of three saikosaponins

HPLC incorporating CAD method was employed to simultaneously determine three biomarkers in Bupleurum Radix from different locations. The dried and powdered leaves were treated as the “Sample preparation”. Table 3 shows the variations of three compounds in Bupleurum

Table1. Results of linearity, LOD, LOQ for SSa, SSb_2 and SSd.

Parameter	Regression equation	Coefficient (r)	Range of linearity (μg/ml)	LOD (mg/l)	LOQ (mg/l)
SSa	Y=526487.2X + 86905.0	0.9991	0.088-8.8	1.06	3.54
SSb_2	Y=352021.9X + 67742.5	0.9990	0.044-4.4	0.604	2.01
SSd	Y=784478.2X + 131142.3	0.9985	0.088-8.8	0.371	1.24

Table 2. Contents of SSa, SSb_2 and SSd at different locations (n = 5).

Parameter	Added C (mg/ml)	Intra-day			Inter-day		
		Found C (mg/ml)	RSD (%)	RE (%)	Found C (mg/ml)	RSD (%)	RE (%)
SSa	0.176	0.171±0.004	2.61	2.92	0.160±0.004	2.21	4.14
	3.52	3.58±0.082	2.29	1.73	3.545±0.076	2.13	0.71
	7.04	6.97±0.15	2.13	1.08	6.97±0.163	2.34	0.96
SSb_2	0.088	0.081±0.005	6.10	8.64	0.087±0.005	5.45	1.14
	1.76	1.71±0.10	6.07	2.98	1.71±0.085	4.97	2.80
	3.52	3.58±0.15	4.17	1.62	3.59±0.125	3.48	2.06
SSd	0.176	0.169±0.005	3.13	4.14	0.170±0.004	2.37	3.53
	3.52	3.55±0.09	2.74	0.79	3.66±0.096	2.62	3.88
	7.04	6.99±0.15	2.18	0.74	7.02±0.164	2.33	0.30

Table 3. Precision and accuracy for the determination.

Location	Content (mg/g)		
	SSa	SSb_2	SSd
Hebei	3.14±0.27	0.142±0.012	3.24±0.25
Anguo of Hebei	4.09±0.35	0.183±0.009	4.10±0.56
Cultivation in Liaoning	7.25±0.68	0.142±0.013	8.86±0.78
Wild growing in Liaoning	4.08±0.52	0.542±0.049	4.19±0.21
Shandong	2.32±0.18	0.422±0.053	3.07±0.46
Jilin	3.46±0.45	0.0781±0.098	4.55±0.39
Hubei	3.93±0.29	0.327±0.028	9.45±1.3
Cultivation in Inner Mongolia	4.58±0.37	0.419±0.056	11.8±1.09
Wild growing in Inner Mongolia	3.40±0.22	0.331±0.045	7.54±0.78
Gansu	3.77±0.33	0.242±0.019	7.79±0.84
Anhui	3.56±0.21	0.434±0.033	7.43±0.55
Shanxi	5.12±0.66	0.486±0.027	11.5±0.97
Heilongjiang	3.85±0.43	0.289±0.008	5.99±0.64

Radix from different locations, meaning that it existed a large difference in the quality of 13 batches from different Bupleurum Radix, and the commercially available herbs basically met to the standard of Chinese Pharmacopoeia (>3 mg/g). The contents of SSa and SSd were the highest respectively from Liaoning and Shanxi Cultivation. Conversely, the lowest of SSa and SSd occurred, respectively, in Hebei and Shandong. The results indicated that Bupleurum cultivars of Liaoning and Inner Mongolia have remarkable advantages, and this study provides a reference for the rationally exploiting and developing medicinal resources.

Assay on *in vitro* immunomodulation of saikosaponins

To judge and compare the immunomodulation effects of SSa, SSd and SSb2, MTT method was firstly applied to screen the immunocompetence of the saikosaponin via determining the influence of different concentrations of SSa, SSd and SSb2 on mouse splenocytes. Secondly, the in vitro immunomodulation factors, such as IL-4, IL-10, IFN-γ, TNF-α were detected by ELISA method. After achieving the effective concentration, each dosed group could enhance the transformative capacity of T lymphocyte induced by the ConA, and stimulated the proliferation of splenic lymphocyte, presented immuno-modulation effects and good dose-effect relationship. At the same time, in vitro study showed that SS showed an adjusted effects on the cytokines released by T lymphocyte. In addition, the results indicated that SSb2 also had certain immunomodulation effect which existed no distinct differences compared to that of SSa but superior to that of SSd (Figure 2).

Under normally physiological conditions, Th1 cells and Th2 cells in an organism present dynamic balance which will evoke pathological reaction once losing the balance, and this is also called Th1/Th2 excursion which happen in many diseases such as microbial infection, tumor, autoimmunity disease, allergic reaction and transplant rejection (Xing and Zhang, 2002). Th1 and Th2 cells respectively, secrete IFN-γ and IL-4, and therefore discriminate of Th1 and Th2 cell must rely on the excretion of IFN-γ and IL-4. TNF-α being one of the members of tumor necrosis factor family, which play an important action in inflammatory reaction and apoptosis, is an important proinflammatory and immunomodulatory factors excreted dominantly from monocyte-macrophages and activated T lymphocyte, and this cause an extensive tissue damage. IL-10 is also called the inhibiting factor of cytokine, a multieffect factor presenting strong immunomodulation effects. Therefore, in the study, IL-4, IL-10, TNF-α and IFN-γ being the indices were selected to investigate the effect of SS on the contents of proinflammatory factors and anti-inflammatory factors of mouse splenosytes stimulated by ConA.

The immunomodulation effects of SS maybe adjust the abnormal function of cytokine network to allow Th1/Th2 recovering to the normal state. In our study, the contents of TNF-α、IFN-γ and IL-10 in the blank group were all higher than that in ConA (-) group, IL-4 in the blank lower than that in ConA group. The contents of TNF-α、IFN-γ in each dosed group were lower than that in the blank group, but still higher than those in ConA (-) group. When stimulated by ConA, the proinflammatory factor excreted by mouse splenocytes increased, SS could improve the above phenomenon and also showed some dose-effect relationship. In summary, SS can modulate the balance of Th1/Th2 factors and presents the immunomodulation effects via decreasing TNF-α、IFN-γ excretion and increasing IL-4, IL-10, furthermore, there was no remarkable difference of immunomodulation effects among SSb2, SSa and SSd.

Conclusion

A simple HPLC-CAD method was developed for the simultaneous determination the SSa, SSb2 and SSd in Bupleurum Radix from different locations. Meanwhile, the immunomodulation effects of the multi-saikosaponin in Bupleurum Radix were investigated. The results show that there existed significantly content differences in the three saikosaponins from different locations; and SSa, SSd and SSb2 all presented immunomodulation effects on mouse splenocytes.

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Full Length Research Paper

The contribution of disease and drug related factors to non-compliance with directly observed treatment short-course among tuberculosis patients

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Non-compliance with anti-tuberculosis therapy has been cited as a major barrier to the control of TB. There are different factors responsible for the non-compliance of TB. The cross sectional prospective participants in the study were interviewed by using a set of questionnaire. TB patients were enrolled at DOTS Centre of LRS Institute of Tuberculosis and Respiratory Disease, New Delhi, India. Patients who discontinued and interrupted the treatment for more than 2 months were categorized as non-compliance cases. T-test was used for comparing the means of control and case groups. The default rate of Delhi LRS-RNTCP defined area was 3.53%. 60% of non-compliance occurred in category II patients. Maximum patients had 3+ initial bacillary load (42.5%). 27.5**% patients had positive influence on non-compliance in response to problem created by TB. Patients who suffered from adverse drug reaction and toxicity of drug contributed the highest rate (40%); and 22.5% had no role in non-compliance of tuberculosis patients. Drug related factors were major factors involved in non-compliance with tuberculosis treatment. Thus, we recommend that ADR monitoring and least ADR active drug be used.**

Key words: Tuberculosis, non-compliance, multiple drug resistance, adverse drug reactions, anti-tuberculosis drugs.

INTRODUCTION

Tuberculosis (TB) affects nearly 1/3rd of the world's population, which is more than any other infectious diseases. Among infected individuals, approximately eight million develop active TB and almost two million of these die from this disease. The incidence of tuberculosis has steadily increased and is responsible for 26% of all avoidable deaths of adults in the developing world (Tripathi et al., 2004; Erhabor et al., 2000; The Global Tuberculosis Epidemic, 2010). Many tuberculosis epidemiologists regard obtaining high compliance levels in the population under treatment as even more important to a community's welfare as finding new cases. Poor compliance with treatment leads to a major impediment to effective tuberculosis chemotherapy worldwide, and is one of the major causes of prolonged infectivity, poor outcomes, treatment failure and drug resistance (Amoran et al., 2011). Drug resistance and obstacles to successful directly observed therapy short-course (DOTS) impede disease control. Among patients being re-treated for TB because of initial treat-ment failure, default from initial treatment, or relapse following initial treatment, drug resistance is common and re-treatment outcome is

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inferior (Dooley et al., 2011).

Several risk factors are identified as the cause of drug resistant tuberculosis, of which the three most important are: previous treatment with anti-tubercular drugs which may be inappropriate, incomplete or erratic, high prevalence of drug resistant tuberculosis in the community and contact with a patient known to have drug resistant TB. In patients with previous treatment or disease, the odds of resistant to tuberculosis were 4 to 7 times higher than for persons with no history of past treatment (Prasad, 2005). Poor case management, often because of non-adherence to treatment, has emerged as the most important factor in the resurgence of tuberculosis and the appearance of multiple drug resistance (MDR). The prolonged duration of treatment, the need for multiple drugs, socio-economic factors and drug toxicity are the main reasons for non-adherence to treatment. The currently recommended minimum duration of treatment is 6 months; even though it is much shorter than the previously recommended 12 to 24 months, it is still very long. According to World Health Organization (WHO), directly observed therapy ensures successful treatment of patients with tuberculosis (M Chan-Yeung et al., 2003). Although drug resistance could contribute to the poor response to anti-TB medications, it is unlikely to be the main factor that leads to treatment failure in our study (Samman et al., 2003).

In 2010, there was an estimated prevalence of 650,000 cases of MDR-TB and in 2008 it was estimated that there were 150,000 MDR-TB deaths annually. The number of patients enrolled for MDR-TB treatment increased to 46,000 in 2010. While more people are being treated for MDR-TB in 2010, it is just 16% of the estimated number of MDR-TB patients that needed treatment, that is, MDR-TB patients that would be identified if all newly-notified TB patients were tested for drug resistance (World Health Organization, 2011, 2012). MDR-TB is a man-made phenomenon, almost always due to inadequate therapy. Although patients' non-adherence is often thought to be the most common cause of drug resistance, many studies have shown that organizational failure of TB control programmers, lack of available drugs and clinical error are responsible for much of the MDR-TB problem existing today. Human error resulting in inadequate therapy includes incorrect drug prescription, lack of patients' education and supervision and patients' non-adherence to treatment. Common clinical errors include addition of a single drug to a failing regimen, inadequate primary regimens, failure to recognize existing drug resistance, failure to provide directly observed therapy and failure to manage non-adherence (Weyer, 2005). Non-compliance is defined as missing more than 25% of treatment in a month. It means to miss injection (daily/ intermittent) for more than one week or not collecting drugs for more than one week, which is known as defaulters; while defaulting for more than one month is known as abandoned treatment. The non-compliance group also includes patients who defaulted treatment and later retrieved it through any means such as home visits, letter, returning in own accord etc; which is known as defaulter retrieval. It also includes patients who abandoned treatment but retrieved it by bringing it back on their own accord for resumption of treatment. This is known as retrieval of abandoned treatment. Non-compliance was identified by a registry, which was regularly updated and medical records were also checked for confirmation of non-compliance. Besides well-known risk factors, the most important unresolved challenge in TB control is the treatment completion. Treatment will only be effective if the patient completes the regimen which includes a combination of drugs recommended by the physicians. Poor compliance contributes to the worsening of the TB situation by increasing incidence and initiating drug resistance. Resistance to anti-TB drugs has also emerged as an important obstacle to the control of the disease. Worldwide patients' compliance with anti-TB therapy (with an estimate as low as 40%) in developing countries remains the principal cause of treatment failure. The critical aspect of management is ensuring compliance with a full course of chemotherapy. The World Health Organization recommends at least 85% cure rate of all diagnosed TB cases. In order to achieve this cure rate, compliance needs to be in the order of 85 to 90% (Naing et al., 2001). DOTS strategy was employed for national program of tuberculosis control. Both smear positive and smear negative pulmonary tuberculosis (SPPTB, SNPTB) patients are routinely treated by six month therapeutic regimen according to NPT. Subsidizing the initial signs and symptoms of SNPTB and conversion of sputum from positive acid-fast bacillus (AFB) to negative (SPPTB) are the criteria for improvement. Lack of change in/ or worse clinical findings as well as conversion of sputum from negative to positive reflects treatment failure. Six month therapeutic regimen is a treatment of choice for smear positive patients, but for various reasons such as economic, drug toxicity, patients' compliance and availability of drugs, shorter and fewer drug combination can be employed, when resistance is not connected to anti-tuberculosis therapy like smear negative pulmonary tuberculosis (Alavi, 2009). Adverse effects diminish treatment effectiveness, because they significantly contribute to non-adherence, eventually contributing to treatment failure, relapse or the emergence of drug-resistance. Adherence to the prescribed treatment is crucial for curing patients with active TB. Because of the long treatment period, the patient should be motivated to continue treatment even when he is feeling better. Additionally, the interruption of TB treatment and the switch to anti-tuberculosis drugs, which is required in patients who do not tolerate drugs, result in a suboptimal treatment response (Tostmann et al., 2008). Monitoring the outcome of treatment is essential in order to evaluate the effectiveness of the intervention. Recommendations on how to evaluate treatment outcomes using standardized

Table 1. Default rate of TB patients.

No. of TB patients	Default patients	% of defaulted patients
566	20	3.53

categories have been issued by WHO in conjunction with the European Region of the International Union against Tuberculosis and Lung Disease (IUATLD). WHO and IUATLD use an agreed set of six possible and mutually exclusive categories of treatment outcome in high-incidence countries. These categories are cured, treatments completed, failure, death, treatment interrupted, and transfer out. Ideally, treatment outcomes in all patients should be routinely monitored by the epidemiological surveillance system. This would make it possible to recognize and amend system failures before the incidence and proportion of resistant isolates rise (Bao et al., 2007).

MATERIALS AND METHODS

The study was conducted between 10th February, 2008 to 20 July, 2009 on patients who were enrolled for TB treatment at the DOTS Centre of LRS-RNTCP defined area. This study focused on finding out the contributing factors to non-compliance with the treatment of TB. The definition of non-compliance was based on the results satisfying one or more of the following criteria:

(1) ≥ 2 consecutive weeks of therapy were missed;
(2) Treatment was prolonged >30 days against plan owing to missed doses;
(3) Incarceration by the tuberculosis control program for presenting an immediate threat to public health. Patients who missed more than 2 consecutive months of DOT were defined as having defaulted from therapy. For each case of non-compliance controls were randomly selected (using computer-generated random numbers) from cases of completed treatment.

Total patients enrolled in LRS-RNTCP defined area for treatment were 566, out of which 80 were enrolled to find out the contribution of disease and drug related factors to non-compliance with ongoing tuberculosis treatment. Out of 80 enrolled patients, 40 patients served as control (i.e. group I) & 40 patients as cases (i.e. group II). Group I received short course chemotherapy according to standard guidelines of RNTCP and was categorized as compliance under DOTS. Group II received short course chemotherapy according to RNTCP guidelines, but due to some reasons or factors, the short course chemotherapy was interrupted and patients fell under non-compliance.

The inclusion criteria for the study include: the TB patients recommended for DOTS regimen, at various DOTS centres of defined LRS- RNTCP area will be included irrespective of age and sex and patients who are treated with combination of anti-tuberculosis therapy. The exclusion criteria are mentally retarded and unconscious patients, patients who are not treated with combination of anti-tuberculosis therapy, patients who are not willing to participate and have any active or chronic disease and patients who are unable to comply. Data were collected on patient's demographic profile (age, gender, weight, height, address, marital status etc), family, social and socio-economic status, individual personality, knowledge about TB, drug toxicity, side effects etc. The questionnaires used in this study were designed to find out the role of disease and drug related factors in non-compliance with directly observed short course among tuberculosis patients. It was classified into three categories as shown in Appendix 1.

The sources of data were patients' treatment card, patients' I.D. card and individual interview from patients. Statistical analysis for finding out the factors contributing to non-compliance of DOTS amongst TB patient in cases (group II) was compared with that of the control (group I). T-test was used for comparing the means of the two groups. The number and proportions were compared with P- value.

RESULTS

During the specified period of time in different DOTS centres of LRS-RNTCP defined area, five hundred and sixty six tuberculosis patients were enrolled for treatment. The study finds out that the default rate was 3.53% among them (Table 1).

There was 27% of non compliance in category I patients, 60% in category II patients and 13% in category III patients (Figure 1).

The distribution of non-compliance according to initial bacillary load was found in this manner: the highest case of non-compliance was observed in patients with 3+ initial bacillary load (42.5%) and lowest was found in patients with 1+ initial bacillary load (17.5%) (Figure 2). Prevalence of non-compliance in patients with 2+ initial bacillary load was 22.5% and in negative patients, 17.5%.

Patients' behavior caused by TB problem was noted and it was found that 27.5% patients had positive influence on non-compliance in response to problem created by TB; 62.5% had intermediate influence on the problem created by TB as factors responsible for non-compliance with TB treatment and 10% had negative influence on the problem created by TB as factors responsible for non-compliance (Figure 3).

According to the question-and-answer session with patients, it was observed that disease related factors and drug related factors had major role in the contribution of non-compliance with TB treatments (Figure 4). Based on our finding, the disease related factors were the majority of patients who have negative influence on non-compliance with TB treatments (40%), while 35% patients had intermediate influence on disease related factors responsible for non-compliance with TB treatment and 25% patient had positive influence on non-compliance.

Drug related factors were observed as the highest contributor to non-compliance with TB treatment (Figure 5). The patients who suffered from adverse drug reaction and toxicity of drug contributed the highest rate and it was 40%. Although, 37.5% of non-compliant patients have

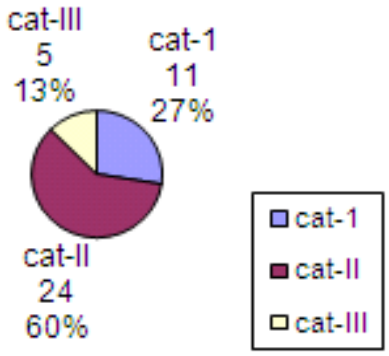

Figure 1. Category vs. non-compliance.

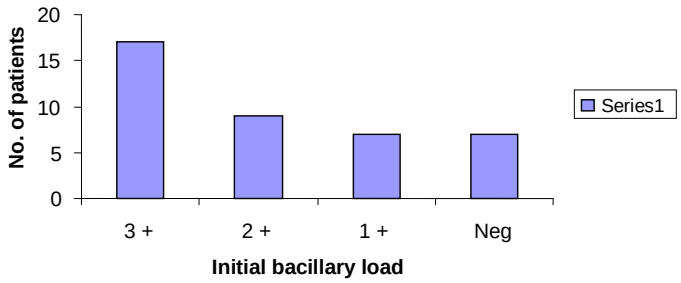

Figure 2. Initial bacillary load vs. non-compliance.

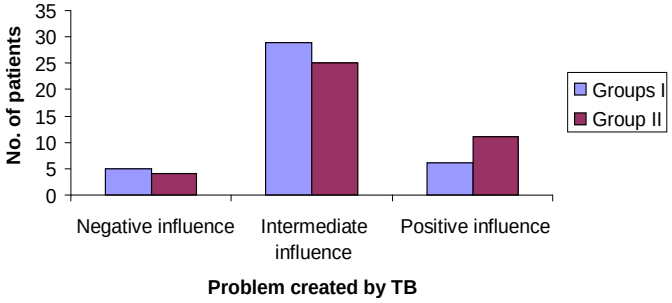

Figure 3. Problem created by TB vs. non-compliance.

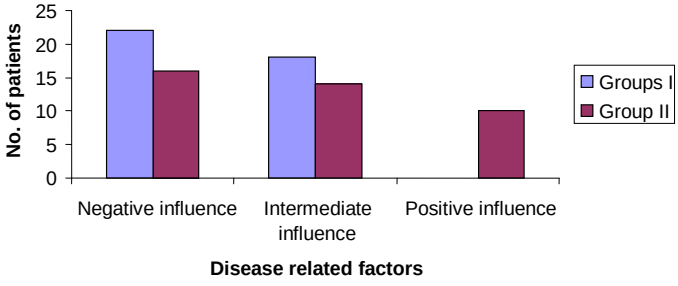

Figure 4. Disease related factors vs. non-compliance of TB treatment.

intermediate influence on the contribution to non-compliance while 22.5% had no role in non-compliance of TB patients.

DISCUSSION AND CONCLUSION

The present study revealed that the disease and drugs related factors had strong association with adherence to tuberculosis treatment. It was found that the default rate was 3.53%. Similar findings were found in RNTCP status report in 2008 where 4 to 6% were the national default rate reported. The category of non-compliant patients was studied and found that majority of non-compliant patients where from category II, which was also described by Jaggarajamma et al. (2007). The types of patients according to initial bacillary load were noted and found that non-compliance is found more in 3+ initial bacillary load patients. Similar observation was observed by Ducati et al. (2006). The study shows that the majority of patients have neither positive nor negative influence on problem created by TB; rather they have intermediate influence on problem created by TB, which is responsible for non-compliance with tuberculosis treatment; this was also observed by Naing et al. (2001). Also, we observed that the disease related factors do not have much influence on non-compliance with tuberculosis treatment; majority of patients had negative influence on the role of the disease related factors; Lertmaharit et al. (2005) had almost similar finding. Drug related factors like adverse drug reaction and toxicity contributed the highest role in non-compliance of tuberculosis. It was found that severe adverse drug reaction and toxicity have direct impact on compliance with the treatment, and that the ADR susceptible TB patients were more prone to non-adherence; this is similar to that found in the research of Jaggarajamma et al. (2007).

The treatment of TB under DOTS-RNTCP program was good and that is why the default rate of South Delhi Region, India was similar to India scenario. Although, the category of patients, initial bacillary load, problem created by TB, disease related factors did not have much influence on non-compliance to tuberculosis treatment. And the main reasons for non-compliance were drug related factors. The common problem created by anti-TB drugs are nausea, vomiting, giddiness, headache, skin rashes, tightness in chest and cough.

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Appendix 1. Questionnaires on the role of disease and drug related factors in non-compliance of directly observed short course among tuberculosis patients

(1). Problem created by TB (Knowledge about problem created by TB, the requirement of treatment and effect of non-compliance).

S/No.	Statement	Possible correct answer	Score for each item	Score obtained
1	Do you know TB decrease self esteem?	Yes No Do not know	1 -1 0	
2	Do you know TB decrease respect of self and family to society?	Yes No Do not know	1 -1 0	
3	Do you know TB can create marriage problem to a TB patient and their spouse?	Yes No Do not know	1 -1 0	
4	Do you know that TB is a major cause of child labour to financial support their family?	Yes No Do not know	1 -1 0	
5	Do you know children are sent away from school due to patients TB.	Yes No Do not know	1 -1 0	
6	Do you know women may discard from her family for her TB.	Yes No Do not know	1 -1 0	
7	Do you know TB can make a young girl infertile?	Yes No Do not know	1 -1 0	
8	Do you know TB is a cause of maternal mortality?	Yes No Do not know	1 -1 0	
9	Do you know TB is a major burden of economic development of a country as is effect the economically productive age group?	Yes No Do not know	1 -1 0	
10	Do you know patients TB is a major cause of child TB?	Yes No Do not know	1 -1 0	

The assessment of non-compliance based on above question. A higher score on the scale 0f 0 to +10 indicates negative influence to non-compliance and lower scale indicates to positive influence to non compliance. The assessment is based on following chart:

Choice	Score	Category
Positive influence	6 - 10	Most favorable personality attributes
Intermediate influence	1 - 5	Unfavorable personalities attributes
Negative influence	≤ 0	Most unfavorable personalities attributes

(2). Disease related factors.

S/No.	Statement	Possible correct answer	Score of each item	Score obtained
1	Do you know tuberculosis is a fatal disease?	Yes No Do not know	1 -1 0	
2	Do you know treatment of TB take 6-8 month?	Yes No Do not know	1 -1 0	
3	Do you know that TB is a communicable disease?	Yes No Do not know	1 -1 0	
4	Do you know Tuberculosis can occur to anybody irrespective of income?	Yes No Do not know	1 -1 0	
5	Do you know smoking habits interfere in recovery of TB?	Yes No Do not know	1 -1 0	
6	Do you know tobacco chewing habits interfere in recovery of TB?	Yes No Do not know	1 -1 0	
7	Do you know alcoholism habits interfere in recovery of TB?	Yes No Do not know	1 -1 0	
8	Do you know Drug addiction habits interfere in recovery of TB?	Yes No Do not know	1 -1 0	
9	Do you know Patient under DOTs treatment adopt contraceptive method to prevent pregnancy?	Yes No Do not know	1 -1 0	
10	Do you know a women suffering from TB continue breast-feeding her baby?	Yes No Do not know	1 -1 0	

Knowledge about TB	Score	Category
Good knowledge	6 – 10	Most favorable personality attributes
Intermediate knowledge	1 - 5	Unfavorable personalities attributes
No knowledge	≤ 0	Most unfavorable personalities attributes

(3). Drug related factors It consists of 10 questions to determine whether the drug causing side effects or adverse drug reaction. That's force the patient towards the non-compliance).

S/No.	Statement	Possible correct answer	Score of each item	Score obtained
1	Do you know you can stop medicines as it side effect appear?	Yes No	1 -1	
2	Do you feel continuous intake of drug create problem to your body?	Yes No	1 -1	
3	Did you experience gastrointestinal Disturbances (nausea, vomiting GIT Upset)?	Yes No	1 -1	
4	Did you experience liver and Biliary (hepatitis with deep jaundice, hepatitis with conjunctival jaundice) problems	Yes No	1 -1	
5	Did you experience CNS and PNS (sweating, headache, Insomnia, Paresthesia of legs, anxiety, and diabetic coma)?	Yes No	1 -1	
6	Did you experience Cutaneous reactions (rash, erythroderma, erythema, exfoliative dermatitis, urticaria, localized skin rash, pruritis, generalized hypersensitivity)?	Yes No	1 -1	
7	Did you experience Musculoskeletal (Arthralgia, long bones pain, localized joint pain, phlebitis, edema of the legs).	Yes No	1 -1	
8	Did you experience Hearing and vestibular (Tinnitus, vertigo, vertigo with loss of equilibrium).	Yes No	1 -1	
9	Did you experience Cardio Respiratory (tightness in chest, coughing, diffused chest pain, hemoptisis, angina, palpitation, total pneumothorax)	Yes No	1 -1	
10	Did you experience Hematological reactions (thrombocytopenia, leucopenia, anemia, agranulocytosis)?	Yes No	1 -1	

Choice	Score	Category
Positive influence	8 - 10	Most favorable personality attributes
Intermediate influence	4 - 7	Unfavorable personalities attributes
Negative influence	0 - 3	Most unfavorable personalities attributes

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Full Length Research Paper

Protective action of vitamin C against mutagenic effects of synthetic food color tartrazine

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The present study has been carried out to investigate the possible mutagenic effects of the synthetic food color tartrazine on mitosis, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) contents of *Allium cepa* roots and protein banding pattern of *A. cepa* seeds. The obtained results indicated that the synthetic food color tartrazine (E102) had the ability to cause different mitotic changes varying from reduction in mitotic index to the production of a large number of mitotic abnormalities. These changes appeared in varying degrees depending on the applied concentration and duration of treatment. The types of abnormalities produced were laggards, bridges, stickiness, C-metaphase and disturbed phases as well as micronuclei. The amounts of both DNA and RNA were generally decreased with increasing of most concentrations and time of treatment. At electrophoretic level, E102 induced alternations in the protein banding pattern of *A. cepa* seeds as compared with the control. These alternations were expressed as disappearance of some characteristic bands, appearance of new bands, and changes in band intensities. The administration of vitamin C was found to be very helpful in minimizing the toxic effects induced by E102.

Key words: *Allium cepa*, tartrazine, chromosome aberration, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) content, protein banding patterns, vitamin C.

INTRODUCTION

Human beings are using a variety of color additives for a long time where most of colors are of synthetic origin. Many food industries started using synthetic food dyes without knowledge of their safety. Tartrazine (E102) is a monoazo dye, popularly used as colorant in food, drugs and industrial manufacturing products intended for human consumption. The ADI for tartrazine is 7.5 mg/kg/day (Walton et al., 1999). Tartrazine is used mainly to color several foods such as breakfast cereals, chocolate chips, biscuits, ice creams, juices, sweets, jams, cereals, snack foods, canned fish and soft drinks. Generally, detailed toxicity studies on various food colors and additives products are missing (Food Reactions, 2010). The metabolite of Tartrazine can generate reactive oxygen species (ROS), which in turn, accelerate the oxidative stress (Bansal, 2005).

A variety of immunologic responses have been attributed to tartrazine including: neurobehavioral toxicity, anxiety, migraines, clinical depression, blurred vision, itching, general weakness, heat waves, feeling of suffocation, purple skin patches and sleep disturbance (FSA, 2007; Park et al., 2009). Amin et al. (2010) concluded that Tartrazine and carmosine affect adversely and alter biochemical markers in vital organs (liver and kidney) not only at higher doses but also at low doses. Allergy leading to asthma attacks, cellular damage, and interaction of food additive tartrazine with common drug products such as aspirin and ventolin were reported by various researchers leading to ban on some of the additives in Norway and Austria Food Reactions (2010). Tartrazine (E102) is still popularly used as colorant in food, drugs and many different industrial products, intended

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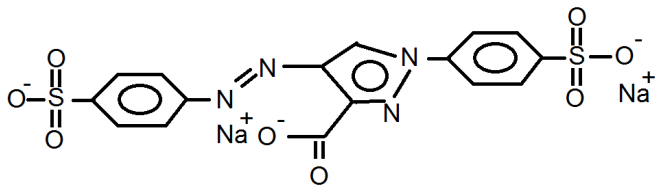

Figure 1. Chemical structure of Tartrazine

for human consumption (Mpountoukas et al., 2010). Mervat and Heba (2011) concluded that a causal link truly exists between tartrazine and inflection of hyperactivity, anxiety and depression-like behaviours in rats and points to the hazardous impacts of tartrazine on puplic health. Yonglin et al. (2011) reported that decline in the activities of catalase, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) as well as a rise in the level of monoaldehyde (MDA) was observed in the brain of tartrazine treated rats, and these changes were associated with the brain oxidative damage. The dose levels of tartrazine in the study produced a few adverse effects in learning and memory functions in treated rats. The mechanisms might be attributed to promoting lipid peroxidation products and reactive oxygen species, inhibiting endogenous antioxidant defense system and the brain tissue damage. Epidemiological evidences suggest that dyes might possess carcinogenic potential under certain circumstances (Axon et al., 2012).

Tartrazine is capable of producing free radicals, which in turn cause damage to the cellular compartment system of rat testis (Amin et al., 2010; Visweswaran and Krishnamoorthy, 2012). There has been increasing concern in recent years about the assay methods and mutagenic potential of a variety of food additives and food colors, and it is believed that such substances may present a possible hazard to man by causing gene mutations and/or chromosomal aberrations.

Vitamin C (VC) can protect indispensable molecules in the body, such as protein, lipids, carbohydrates and nucleic acids (Sanchez-Moreno et al., 2003). VC can promote the removal of oxidative DNA damage from the DNA and/or nucleotide pool, through the up regulation of repair enzymes (Arraiga-Alba et al., 2008). This inhibitory effect of VC towards a number of mutagens/carcinogens was shown by many authors in humans, animals and plants (Fahmy et al., 2008; Jennifer et al., 2009). VC is one of the leading compounds used as antioxidant. VC as a highly effective antioxidant acts as a reducing agent that can terminate free radical driven oxidation by being converted to a resonance-stabilized free radical (Ambali et al., 2011; Assia et al., 2012).

Higher plants, particularly *Vicia faba* and *Allium cepa* possess many advantages that make them ideal for use by scientists for screening and monitoring of genotoxic agents according to the standard protocol for the plant assay established by the International Program on Chemical Safety (IPCS) and the World Health Organization (WHO). Plant cells undergo mitosis and meiosis and can mutate in a manner similar to human and animal cells (Grant, 1994).

Allium test is one of the best-established test systems used in order to determine the toxicity in the laboratories. Moreover, this system is well correlated with the data obtained from eukaryotic and prokaryotic systems (Matsumoto et al., 2006). Many authors investigated the potentialities of higher plant genetic systems for monitoring and screening chemical mutagens (Konuk et al., 2007; Liman et al., 2010). Some investigations were carried out to indicate the relation between changes in mitotic and meiotic activities with changes in nucleic acid contents as a result of treatment with synthetic food colors (Giri, 1991; Tsuda et al., 2001). Electrophoretic techniques of protein have been used as a successful tool to estimate the possible mutagenic potentialities produced due to continuous and accumulative pollution by chemicals and correlate the produced variation with chromosomal aberrations caused by these pollutants (Badr, 1995; George and Ghareeb, 2001).

The present investigation was carried out to diminish the genotoxicity of the synthetic food color E102 by using the natural antioxidant compound Vitamin C. The search for new safer types of food additives, less harmful and non toxic products, have recently attracted the attention of many scientists all over the world. Thus, this work aimed to evaluate the cytotoxic effects of tartrazine and the protective effect of VC against the DNA damage induced by this food coloring agent (E102) using *A. cepa* as a biological system. For this purpose, cytological studies included mitotic index, chromosomal aberrations, as well as nucleic acids contents (DNA and RNA) and protein profiles were done.

MATERIALS AND METHODS

Plant

Bulbs and seeds of *A. cepa* (Giza 20) were supplied by the Agricultural Research Center, Giza, Egypt. They were used as experimental plants for both mitotic and biochemical analysis.

Test chemicals

Tartrazine, also known as E102, FD&C yellow No.5 was used. Tartrazine is trisodium (E)-5-oxo-1-(4-sulfonatophenyl)-4-((4-sulfonatophenyl) diazenyl)-4, 5-dihydro-1H-pyrazole-3-carboxylate. The molecular formula for tartrazine is $C_{16}H_9N_4Na_3O_9S_2$ (Figure 1). Vitamin C (L. ascorbic acid) was purchased from Memphis Co. The dose of vitamin C used in the present study was 100 mg/L.

Mitotic analysis

A. cepa bulbs were germinated in tap water, till the roots reached 2 to 3 cm in length, and then were divided into three groups. The first group was left as a control in distilled water and the second group was treated with freshly prepared test solutions of different

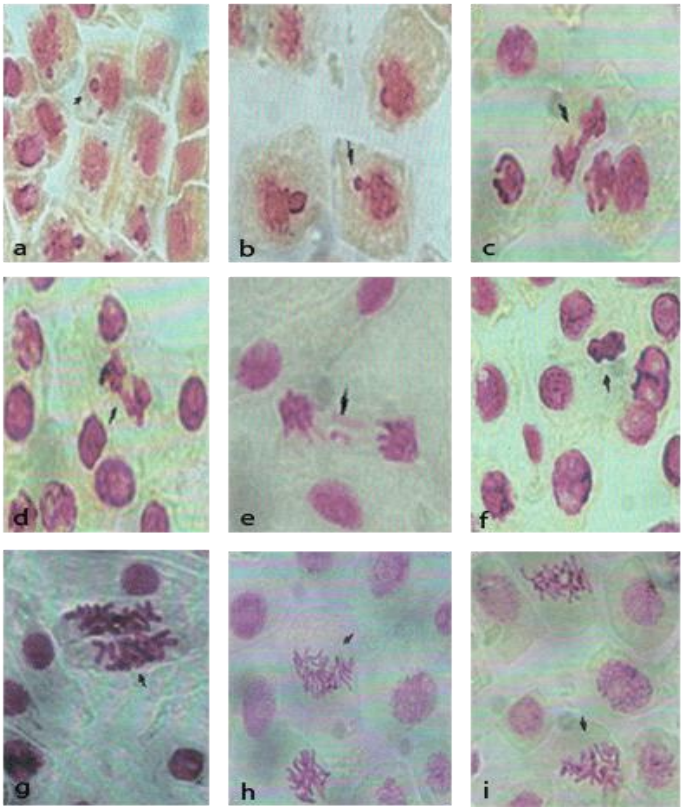

Figure 2. Some types of chromosomal abnormalities after treating *A. cepa* root tips with different concentrations of E102.
(a and b) Micronuclei; (c and d) telophase bridge; (e) lagging chromosome in telophase; (f) sticky metaphase; (g) C-metaphase; (h and i) disturbed metaphase.

concentrations of E102 for 3, 6, 12 and 24 h. E102 concentration differed according to each period. The third group was treated with the same concentrations used for 24 h by E102 alone for the same period and then treated with Vitamin C (100 mg/L) for 3 h. Following treatments, roots were detached, washed and fixed in ethanol: glacial acetic acid (3:1) for 24 h and then stored in 70% alcohol in a refrigerator until use. Cytological preparations were carried out using Faulgen squash technique according to Darlington and La-Cour (1976). Mitotic index and total abnormalities in each concentration were statistically analyzed using paired Student's t –test.

Biochemical analysis

Estimation of nucleic acids content

Roots of *A. cepa* (2 to 3 cm in length) were subjected to the same treatment mentioned before, and a method based on that of Shibko et al. (1967) was performed to estimate nucleic acid contents. RNA content was determined according to Ashwell (1957), while the method of Burton (1968) was used to determine DNA content.

SDS-PAGE of M2 seed storage protein

Characterization of protein profiles was carried out using one dimensional sodium dodecyle sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide slab gel (12.5%) was prepared according to (Laemmil, 1970). Seed samples of A. cepa previously treated with tartrazine under study were prepared for electrophoresis. For protein extraction, 0.2 ml of sample buffer was added to 0.02 g of seed meal (homogenate) and stored overnight at 4°C. Centrifugation was performed at 9000 rpm for 6 min and the supernatant was collected for analysis. Protein samples were prepared by mixing the clear supernatant with treatment buffer in 1:1 ratio, with a drop of bromophenol blue and denatured by heating at 90°C for 3 min. Equal amount of samples (20 to 30 µl) was loaded carefully through electrode buffer into the sample wells in the stacking gel layer. Wide range standard protein marker was also loaded. The apparatus was turned on and a current of 15 mA was applied till protein sample passes stacking gel, then the current was increased to 25 mA for 4 to 6 h. The bromophenol blue dye could be used to monitor the rate of migration. At the end of electrophoresis, protein bands were visualized by staining the gel with silver nitrate according to Sammons et al. (1981). The gel was removed carefully, left in the staining solution with gentle agitation until the dye penetrated the gel and then de-stained several times in de-staining solution. The gels were first photographed using a camera and documented for further analysis.

RESULTS

Concerning the effect of E102 on mitotic division of *A. cepa* root tips, it was obvious that the synthetic food color E102 had a marked reducing effect on mitotic index values. The mitotic index values were progressively decreased as the concentrations and duration of treatments were increased. The lowest value of mitotic index (MI), 1.80%, was recorded after treatment for 24 h with 0.789 mg/L as compared with the control value (4.86%). Such decrease in the MI was found to be statistically significant in most treatments (Table 1). In addition to the reduction of mitotic index, the synthetic food color caused a change in the frequencies of the different mitotic stages in all used concentrations. The results of the present study clearly demonstrated that as the concentration of E102 increased, the frequencies of both prophase and anatelophase decreased, with a corresponding increase in the percentage of metaphase. The metaphase increased gradually until it reached a maximum value of 87.92% after 3 h treatment compared with control value of 35.92%. The frequency of prophase decreased gradually until it reached a minimum value of 4.83% after 3 h treatment compared with the control value of 34.48%. This indicates that E102 affects the relative duration at each stage as compared with the control.

The synthetic food color tartrazine showed a wide range of mitotic abnormalities and their frequencies increased as the concentration of E102 and the duration of the treatment increased as compared to the control (Table 2 and Figure 2). The most common types of abnormalities observed were stickiness, C-metaphase, laggards, disturbed chromosomes, bridges and micronuclei. The percentages of mitotic abnormalities were highly significant in most of treatment with the high concentrations. Low concentrations induced either a statistically significant percentage or had non- significant effect as compared to the control. The maximum value of

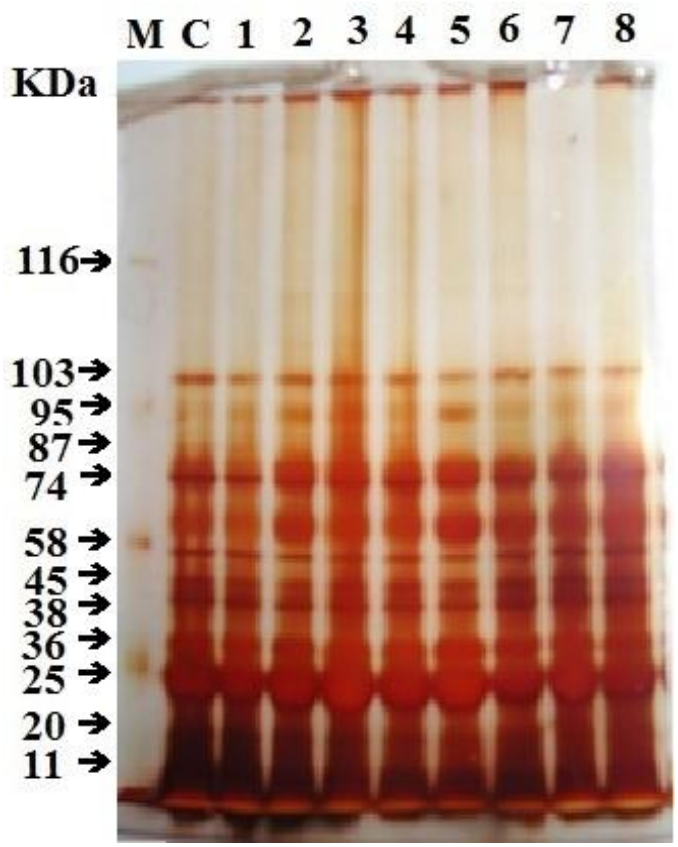

Figure 3. Electrophotograph produced by SDS-PAGE analysis of protein pattern of *A. cepa* seeds after treatment with different concentrations of the synthetic food additive color E102 for 24 h without or with further treatment by vitamin C (100 mg/L) for 3 h. M: Marker; C: Control; Lane 1: Conc. 0.097 mg/L of E102 for 24 h; Lane 2: Conc. 0.195 mg/L of E102 for 24 h; Lane 3: Conc. 0.339 mg/L of E102 for 24 h; Lane 4: Conc. 0.789 mg/L of E102 for 24 h; Lane 5: Conc. 0.097 mg/L of E102 for 24 h. + treatment with 100 mg/L of vitamin C for 3 h; Lane 6: Conc. 0.195 mg/L of E102 for 24 h. + treatment with 100 mg/L of vitamin C for 3 h; Lane 7: Conc. 0.339 mg/L of E102 for 24 h. + treatment with 100 mg/L of vitamin C for 3 h; Lane 8: Conc. 0.789 mg/L of E102 for 24 h. + treatment with 100 mg/L of vitamin C for 3 h.

the percentage of mitotic chromosomal abnormalities reached 96.44%, with the highest concentration of E102 (0.789 mg/L) for 24 h as compared with control value (1.20%). This might indicate toxic effects of E102 on the chromosomes of *A. cepa* root cells.

When different concentration of E102 were applied for 24 h and then terminated by treatment with vitamin C (100 mg/L) for 3 h (E102 + vitamin C), the results showed a marked protective effect against the mito-inhibition effect of the synthetic food color E102. The reduction of mitotic index was still observed in all treatments, but the intensity of reduction was much less in comparison to the treatment with E102 only (Table 1). The mitotic index reached 2.98% at the concentration 0.789 mg/L of E102 with vitamin C while it reached 1.80% at the same concentration without vitamin C. Vitamin C successfully reduced the effect of E102 on the percentage of chromosomal abnormalities in the roots treated with different concentrations of (E102 + VC), as compared to corresponding treatment with E102 alone. The maximum value of mitotic abnormalities was 96.44% after treatment with the highest concentration of E102 (0.789 mg/L) for 24h without vitamin C and could be reduced to 84.85% in the presence of VC (E102 for 24 h and vitamin C for 3 h).

Generally, E102 treatments resulted in a significant decrease in nucleic acid contents as compared to the control. Nucleic acid contents de-creased as the concentration of E102 and time of treatment increased as compared with the untreated roots. After vitamin C administration, only RNA contents increased in comparison with 24 h treatment with E102 alone (Table 3). The effect of synthetic food color on protein banding patterns of *A. cepa* seeds have been depicted in Table 4 and Figure 3. The total number of protein bands was 15 bands; 10 of which were common to the control and treated roots. The most visible changes in SDS-PAGE patterns were the disappearance of few bands such as: the band with molecular weight 95, 87, 74 and 11 KD found in E102 treated roots. Band with molecular weight 25 KD appeared in E102 treated roots. Over expression of bands with molecular weights 45, 20 and 25 KD was observed in E102 treatment group.

DISCUSSION

The inhibition of mitotic activity has been regarded as a common effect by numerous food colours as reported by many investigators (Palani Kumar and Panneerselvam, 2007; Shipra et al., 2008). Inhibition of mitotic division in plants has been attributed to inhibition of certain types of nuclear proteins essential in mitotic cycle and/or the inhibition of DNA synthesis (Kim and Bendixen, 1987; Lamsal et al., 2010). In addition to the reduction of mitotic index, the synthetic food color caused a change in the frequencies of the different mitotic stages with all the used concentrations. As the concentration of E102 increased, the frequencies of both prophase and anatelophase decreased, with a corresponding increase in the percentage of metaphase (Table 1). This indicates that E102 affects the relative duration of each stage, as compared with the control.

The synthetic food color E102 produced several types of chromosomal abnormalities such as stickiness, c-metaphase, laggards, disturbed chromosomes, bridges and micronuclei after all treatments used in this investigation (Table 2). These results indicate the potentiality of the investigated synthetic food color to induce mitotic irregularities that is in accordance with other studies carried out for assessing genotoxic effects of food colors or other agents (Palani Kumar and Panneerselvam, 2007;

Table 1. Total cells examined, total mitoses, percentage of mitotic phases, percentage of total abnormal mitotic phases, and mean mitotic index after treating of *A. cepa* root tip cells with different concentrations of synthetic food color E102 for 3, 6, 12 and 24 h. Two groups of root tip cells subjected to E102 concentrations used at the last period for 24 h, and one of them terminated by vitamin C (100 mg/L) for 3 h.

Time of treatment (h)	Conc. mg/L	Total cells examined	Total mitoses	Prophase		Metaphase		Ana-telophase		Mean MI%±SE
				Prophase (%)	Abnormal prophase (%)	Metaphase (%)	Abnormal Metaphase (%)	Ana-telophase (%)	Abnormal Ana-telophase (%)	
3	Control	11953	696	34.48	0.00	35.92	0.80	29.60	0.00	5.82±0.03
	0.789	15826	591	23.18	1.46	37.56	21.17	39.26	0.43	4.43±1.08
	1.560	14144	441	53.51	4.24	30.84	97.06	15.65	49.28	3.08±0.07**
	3.125	15087	467	50.75	7.59	33.19	83.87	16.06	50.67	3.10±0.05**
	6.250	11479	207	4.83	100.00	87.92	100.00	7.25	93.33	1.82±0.11**
6	Control	12640	632	36.55	3.46	25.95	9.76	37.50	2.53	5.00±0.07
	0.339	11555	520	34.62	4.44	29.81	25.16	35.58	8.65	4.50±0.39
	0.789	12843	513	25.34	1.54	37.82	46.91	36.84	10.58	4.00±0.46
	1.56	17065	505	32.67	17.58	54.46	69.45	12.87	55.38	2.96±0.03**
	3.125	14853	553	32.19	26.40	56.60	70.93	11.21	58.06	3.73±0.02**
12	Control	17713	886	27.99	0.00	39.95	3.67	32.05	0.00	5.01±0.25
	0.195	16869	639	35.21	0.89	25.35	11.11	39.44	0.00	3.80±0.14
	0.399	15402	552	33.15	3.28	42.39	25.21	24.46	2.22	3.57±0.30*
	0.789	17700	600	28.00	2.38	28.00	27.38	44.00	3.79	3.39±0.07*
	1.56	16284	326	19.94	40.00	42.94	96.43	37.12	49.59	2.01±0.05
24	Control	18864	918	27.67	0.00	31.37	2.43	40.96	1.06	4.86±0.05
	0.097	14019	308	22.73	2.86	33.44	22.33	43.83	3.70	2.19±0.40**
	0.195	15991	344	26.16	4.44	30.52	27.62	43.31	4.70	2.14±0.10**
	0.399	15051	295	25.42	4.00	30.51	57.78	44.07	23.85	1.95±0.02**
	0.789	17039	307	6.84	100.00	63.84	99.49	29.32	90.00	1.80±0.06**
24 + 3 h vitamin C	Control	12886	780	27.69	---	37.31	0.69	35.00	---	6.05±0.23
	0.097	13260	575	25.74	---	34.09	17.86	40.17	0.87	4.33±0.16*
	0.195	13100	547	27.61	---	42.41	37.07	29.98	4.88	4.24±0.30
	0.399	16768	670	22.84	---	35.07	39.57	42.09	2.13	4.00±0.09*
	0.789	21255	636	19.18	28.69	54.87	95.42	25.94	24.85	2.98±0.10**

*Significant from control at 0.05 level (t. test). **Significant from control at 0.01 level (t. test).

Shipra et al., 2008). The most common type of abnormalities observed with nearly all treatments was stickiness (Table 2). The number of sticky cells increased in all stages of mitotic division as the concentration of E102 increased during the same period.

Table 2. Frequency of different types of metaphase and ana-telophase abnormalities and mean % of abnormal mitoses after treating of *A. cepa* root tip cells with different concentrations of synthetic food color E102 for 3, 6, 12 and 24 h. Two groups of root tip cells subjected to E102 concentrations used at the last period for 24 h, and one of them terminated by vitamin C (100 mg/L) for 3 h.

Time of treatment	Conc. in mg/L	% of metaphase abnormalities							% of ana-telophase abnormalities					Mean% of abnormal Mitoses±SE
		c-m 2n	c-m 4n	Star	Dist.	Lagg.	Stick.	Total abnormality (%)	Bridge	Dist.	Lagg.	Stick.	Total abnormality (%)	
3	Control	---	---	---	0.80	---	---	0.80	---	---	---	---	---	0.29±0.12
	0.789	14.42	---	0.90	5.41	0.45	---	21.17	---	---	---	0.43	0.43	8.45±0.64
	1.56	25.74	1.47	2.21	38.24	1.47	27.94	97.06	---	5.80	1.45	39.13	49.28	39.51±0.94
	3.125	26.45	---	---	16.13	---	41.29	83.87	---	2.67	1.33	46.67	50.67	39.84±0.18**
	6.250	---	---	1.65	---	---	98.35	100.0	13.33	---	---	80.0	93.33	99.52±0.39**
6	Control	1.83	0.61	1.83	4.88	0.61	---	9.76	---	2.53	---	---	2.53	5.19±0.97
	0.339	0.65	---	0.65	17.42	1.94	4.52	25.18	1.08	4.86	1.62	1.08	8.65	12.25±0.96
	0.789	3.61	1.03	---	20.62	1.03	20.62	46.91	4.76	3.70	2.12	---	10.58	22.64±1.67*
	1.56	18.18	4.00	1.08	9.82	3.27	33.10	69.45	4.62	6.14	4.62	40.0	55.38	51.81±0.23*
	3.125	7.67	3.51	8.95	8.95	0.32	48.56	70.93	6.54	4.84	6.45	40.32	58.06	53.15±0.56*
12	Control	---	---	---	3.67	---	---	3.67	---		---	---	---	1.46±0.11
	0.195	---	---	---	11.11	---	---	11.11	---		---	---	---	3.07±0.94
	0.399	---	---	---	18.80	---	6.41	25.21	---		---	2.22	22.22	12.42±0.57
	0.789	---	---	---	27.38	---	---	27.38	1.14		---	2.65	3.79	10.01±0.23
	1.56	29.29	---	---	7.14	---	60.0	96.43	---		8.27	---	45.59	67.83±1.75**
24	Control	---	---	---	2.43	---	---	2.43	---	1.06	---	---	1.06	1.20±0.08
	0.097	1.94	---	---	20.39	---	---	22.33	---	3.70	---	---	3.70	9.95±1.20
	0.195	---	---	---	24.76	---	2.86	27.62	0.67	4.03	---	---	4.70	12.38±1.28
	0.399	55.56	---	---	---	---	2.22	57.78	11.54	0.77	---	11.54	23.85	29.12±0.72**
	0.789	8.16	---	---	2.04	---	89.29	99.49	1.11	1.11	---	87.78	90.00	96.44±0.67**
24 + 3 h vitamin C	Control	---	---	---	0.69	---	---	0.69	---	---	---	---	---	---
	0.097	---	---	---	17.35	0.51	---	17.86	---	0.87	---	---	---	0.87
	0.195	2.59	---	---	32.76	---	1.72	37.07	---	4.88	---	---	---	4.88
	0.399	7.23	---	---	32.34	---	---	39.57	---	2.13	---	---	---	2.13
	0.789	25.21	---	0.58	24.36	---	45.27	95.42	---	15.15	---	---	---	84.85

*Significant from control at 0.05 level (t. test). **Significant from control at 0.01 level (t. test). Stick: stickiness, lag: laggards, dist: disturbed chromosomes.

Liu et al. (1992) suggested that sticky chromosomes reflect highly toxic effects, usually of an irreversible type, and probably lead to cell death. Stickiness has been suggested as a type of physical adhesion involving mainly the proteinceous matrix of chromatin material (Lamsal et al., 2010). Induction of chromosomal and chromatin bridges at anaphase and telophase stages was also observed after treatment with E102.

Table 3. Percentage of DNA and RNA content after treating of *A. cepa* root tip cells with different concentrations of the synthetic food color E102 for 3, 6, 12 and 24 h. Two groups of root tip cells subjected to E102 concentrations used at the last period for 24 h, and one of them terminated by vitamin C (100 mg/L) for 3 h.

Concentration (mg/L)	DNA (%)		RNA (%)	
	mg/g	Content (%)	mg/g	Content (%)
3 h				
Control	11.47	100	3.24	100
0.789	10.67	93.03	2.86	88.27
1.560	8.80	76.72	2.48	76.54
3.125	7.46	65.04	2.10	64.81
6.250	6.13	53.44	1.71	52.78
6 h				
Control	11.20	100	3.62	100
0.339	10.13	90.45	3.05	84.25
0.789	8.80	78.57	1.90	52.49
1.56	6.67	59.55	1.71	47.24
3.125	5.60	50.00	1.33	36.74
12 h				
Control	9.86	100	3.24	100
0.195	9.85	99.90	3.43	105.86
0.399	8.27	83.87	2.10	64.81
0.789	7.20	73.02	1.33	41.05
1.56	5.87	59.53	0.35	10.80
24 h				
Control	8.80	100	2.67	100
0.097	8.27	93.98	1.90	71.16
0.195	6.94	78.86	1.33	49.81
0.399	6.67	75.80	0.76	28.46
0.789	5.60	63.64	0.76	28.46
Treatment with E102 for 24 h followed by 3 h treatment with vitamin C				
Control	9.14	100	3.47	100
0.097	8.00	87.53	3.11	89.63
0.195	6.29	68.82	2.67	76.95
0.399	5.71	62.47	2.31	66.57
0.789	5.14	56.24	2.04	58.79

Formation of bridges could be attributed to the breakage and reunion or due to the general stickiness of chromosomes (El-Khodary et al., 1990; Lamsal et al., 2010). Sifa (2005) reported that chromosome bridges might be due to chromosomal stickiness and subsequent failure of free anaphase separation. The presence of both chromosome stickiness and bridges (Table 2) supports this conclusion.

Large number of C-metaphase where chromosomes appear scattered in the cytoplasm was found after treatment with E102 (Table 2). Such type of anomaly is an indication of the action of E102 on the inhibition of spindle fiber formation by their action on microtubules which play a major role in the formation of spindle fiber (Jain and Sarbhoy, 1987). Considerable percentages of disturbed mitotic phases were induced by treatment with E102. Disturbed phases may be due to disturbance in the function of the mechanism of chromosomes movement and the orientation of these chromosomes at the equatorial plate (Shehata et al., 2000). Among aberrations that appeared frequently after treatment with E102 was lagging chromosomes at metaphase, anaphase and

Table 4. Effect of different concentrations of the synthetic food color E102 on the protein banding pattern of *A. cepa* seeds using SDS – PAGE technique, which subjected for 24 h, without or with further treatment by and vitamin C (100 mg/L) for 3 h.

Band No.	M.wt (KDa)	Band %								
		Marker	Control	E 102				E102 + Vitamin C			
				Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
1	116	+									
2	103		+	+	+	+	+	+	+	+	+
3	95	+	+	+	+	+	+	+	+	+	
4	87		+	+	+	+	+				
5	74		+	+	+	+	+				
6	66		+	+	+	+	+	+	+	+	+
7	64		+	+	+	+	+	+	+	+	+
8	58	+	+	+	+	+	+	+	+	+	+
9	52		+	+	+	+	+	+	+	+	+
10	48		+	+	+	+	+	+	+	+	+
11	45		++	++	++	++	++	++	++	++	++
12	38		+	+	+	+	+	+	+	+	+
13	36		+	+	+	+	+	+	+	+	+
14	25	++		++	++	++	++	++	++	++	++
15	20		+	++	++	++	++	++	++	++	++
16	11		+	+	+	+	+	+	+		

Specifications of lanes

Lane 1	Conc. 0.097 mg/L of E102 for 24 h
Lane 2	Conc. 0.195 mg/L of E102 for 24 h
Lane 3	Conc. 0.339 mg/L of E102 for 24 h
Lane 4	Conc. 0.789 mg/L of E102 for 24 h
Lane 5	Conc. 0.097 mg/L of E102 for 24 h + treatment with 100 mg/L of vitamin C for 3 h
Lane 6	Conc. 0.195 mg/L of E102 for 24 h + treatment with 100 mg/L of vitamin C for 3 h
Lane 7	Conc. 0.339 mg/L of E102 for 24 h + treatment with 100 mg/L of vitamin C for 3 h
Lane 8	Conc. 0.789 mg/L of E102 for 24 h + treatment with 100 mg/L of vitamin C for 3 h

telophase stages. These laggards might be distributed randomly to either pole. They might result in the formation of micronuclei (Abd-El-Salam et al., 1993a). Considerable frequencies of micronuclei were observed in the cells of mitotic division at anaphase. Micronuclei can originate either from chromosome fragments or from lagging chromosomes. In some instances, the lagging chromosomes or fragments might have either dissolved in the cytoplasm or gradually clumped and might be surrounded by nuclear membrane to form micronuclei (El-Ghamery et al., 2003). The formation of micronuclei is regarded as an induction of mutagenicity of their inducers (Abd-El-Salam et al., 1996). Multinucleated cells were recorded at few percentages.

VC is a highly effective antioxidant. It acts as a reducing agent that can terminate free radical driven oxidation by being converted to a resonance-stabilized free radical. It is well established that VC can protect indispensable molecules in the body, such as protein, lipids, carbohydrates and nucleic acids (DNA and RNA) (Schneider et al., 2001). Our results showed that administration of VC ameliorated the DNA damage and chromosome aberrations induced by E102 at all tested doses. This ameliorative effect by VC might have resulted from enhancement of detoxification pathways that converted reactive compounds to less toxic and more easily excreted products (Vijayalaxim and Venu, 1999) and/or through its VC action as a free radical scavenging agent (Chaudiere and Ferrari-Iliou, 1999). It might also be due to the formation of complex compounds with mutagens or modulation of their metabolism (Mark et al., 2008). In addition, numerous *in vitro* and *in vivo* studies have evaluated the protective effects of VC against several radical generating chemicals (Robichova et al., 2004; Arranz et al., 2007; Rudrama and Kusum, 2011).

The protective effect of VC against E102 induced genotoxicity might be due to one of the following properties of VC: antioxidant action, trapping of free radicals, formation of complex with mutagens or modulation of mutagen metabolism (Mark et al., 2008; Nancy et al., 2011). However,

the definite molecular mechanisms of antimutagenic effects or antigenotoxicity of vitamin C in *A. cepa* root meristem cells needs further investigations.

Tartrazine induces oxidative stress and DNA damages (Soheila and Zeidali, 2011). The inhibition of the nucleic acid contents could be attributed to the inhibition of DNA synthesis. Most of the treatments with E102 resulted in a progressive significant decrease in the nucleic acid contents with the increase of E102 concentration and the time of treatment, and also as compared with the untreated roots (Table 3), but still lower than the control values. The inhibition of DNA and RNA synthesis might be due to inhibition of DNA replication as suggested by Scott (1968). The inhibition of the nucleic acid contents could be attributed to inhibition of DNA synthesis. These results are in accordance with those obtained by Badr (1987). However, the reduction in DNA content could be presumably attributed to the reduction of oxidative phosphorylation that would lead to lowering adenosinetriphosphate (ATP) level in the cell (Gruenhagen and Moreland, 1971).

The present results indicated that the decrease in the rate of the cell division is also accompanied by a decrease in the DNA content of the cells. Ibrahim (1991), Tsuda et al. (2001) and Mpountoukas et al. (2010) indicated that there should be a positive correlation between the rate of cell division and DNA synthesis.

It was well concluded earlier that the synthetic food color under study caused disappearance of some bands in *A. cepa*. The vanishing of some electrophoretic bands could be attributed to the loss of the genetic materials due to fragmentation, laggards and micronuclei as found in the present study. The results of the present study are in full agreement with the earlier reports of Hassan (2000) and Soliman and Ghoneam (2004). In addition, another earlier report also clearly showed that the absence of some bands represent the deletion of their corresponding genes (Hassan, 2000). It was interesting to notice in the present study that E102 caused the appearance of new bands which were absent in untreated *A. cepa*. The appearance of new characteristic bands could be explained on the basis of mutational events at the regulatory system of an unexpected gene(s) that activated it (El-Nahas, 2000). It is well known that gene mutation and changes in gene expression are responsible for changing the banding pattern. E102 treatment induced cytological abnormalities which gave rise to changes in band intensity due to induction of gene mutation at the regulatory system. Gene expression is changed due to gene mutation (Soliman and Ghoneam, 2004). The increase in band colour intensity provide basis to represent gene duplication resulting due to cytological abnormalities (Hassan, 2000; Soliman and Ghoneam, 2004). The alternations in the electrophoretic profiles of seed proteins are indicative of the ability of E102 to alter the gene expression of the exposed cells.

There is considerable evidence that the effects of mutagenic and carcinogenic agents can be altered by many dietary constituents. VC is an essential dietary nutrient required as a co-factor for many enzymes and a very efficient antioxidant, scavenging reactive oxygen and nitrogen species and protecting cells against free radical-mediated damage. Besides exerting antioxidant influence directly, VC can promote the removal of oxidative DNA damage from the DNA and/or nucleotide pool through the up regulation of repair enzymes. Also, VC has inhibitory effect towards a number of mutagens/ carcinogens (Fahmy et al., 2008; Jennifer et al., 2009; Sanchez et al., 2003).

The supplementation of vitamin C showed to be very much helpful in minimizing the toxic effects induced by E102. Although the mutagenic potential of E102 was not significantly high, but it clearly indicates that continuous or prolonged exposure and consumption of E102 can pose a potential risk to human health. It was found that the synthetic food color E102 caused harmful effects at both cytogenetic and biochemical levels. Therefore, it is suggested to test the mutagenic potential of synthetic food colors on more intensive and extensive basis, especially on non target systems before it is recommended for wider use in foods.

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Full Length Research Paper

Chemopreventive potential of methanol extract of *Dicranopteris linearis* leaf on DMBA/croton oil-induced mouse skin carcinogenesis

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The present study was carried out to elucidate the chemopreventive potential of methanol extract of *Dicranopteris linearis* (MEDL) in a two-stage mouse skin carcinogenesis model due to the interrelated inflammation, oxidative stress and tumor promotion pathways. MEDL was prepared in a dose range of 30 to 300 mg/kg body weight. A total of 48 imprinting control region (ICR) female mice (6 to 8 weeks old) were randomly assorted into six groups. To induce skin tumor formation, a single topical application of 7,12-dimethylbenz[a]anthracene (DMBA) at 100 µg/100 µl was applied to the shaved dorsal region of mice, followed by repetitive administration of 1% croton oil, twice weekly for 15 weeks. Topical application of MEDL, 30 min prior to the croton oil application significantly reduced the tumor incidence to 12.5% in 300 mg/kg MEDL-treated group as compared to 87.5% in carcinogen control. The latency period of tumor formation was increased from sixth week in the carcinogen control to ninth and fifteenth weeks in 100 and 300 mg/kg MEDL-treated groups, respectively. The tumor burden of MEDL-treated groups (30, 100, and 300 mg/kg) were significantly lessen (5.67 ± 1.28, 5.00 ± 1.13, and 1.00 ± 0.13), as compared to carcinogen control (7.86 ± 2.37). The tumor volume was also significantly reduced from 9.00 ± 2.27 mm^3 in carcinogen control to 3.70 ± 0.96, 2.39 ± 0.54 and 0.26 ± 0.03 mm^3 in 30, 100 and 300 mg/kg MEDL-treated groups, respectively. In conclusion, the MEDL exhibited anti-carcinogenic effect in a dose-dependent manner, indicating its chemopreventive potential, which worth further study.

Key words: *Dicranopteris linearis*, leaves, methanol extract, anti-carcinogenic effect.

INTRODUCTION

Cancer is a major public health concern worldwide and the incidence of cancer continues to increase. It is now one of the leading causes of death globally. According to World Health Organization (WHO), about 7.6 million of cancer deaths were reported in 2008 and the cancer death are believed to continue rising, with an estimated 13.1 million deaths in 2030 (Siegel et al., 2011). Besides, one in four deaths due to cancer is estimated in the United State alone (Siegel et al., 2011). Moreover, the worldwide cancer burden was found to increase

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dramatically, especially in the developing countries where the contributing factors include population aging and growth, and the trend of practicing cancer-associated lifestyle such as smoking, physical inactivity, and westernized diets (Jemal et al., 2011).

Based on the statistics released by the Ministry of Health Malaysia in 2007, cancer was the third common cause of death, accounting for 11.28% of all deaths, after heart diseases and diseases of pulmonary circulation (16.49%) and septicemia (13.38%). A total of 18,219 new cancer cases were diagnosed in the same year and registered in the National Cancer Registry. Among the cancer cases, 8,123 (44.6%) cases were diagnosed in males and 10,096 (55.4%) cases for females. The age standardized incidence rates (ASR) were 85.1/100,000 in males and 94.4/100,000 in females (Ariffin and Saleha, 2011).

Under normal physiological conditions, the process of cell division or cell proliferation occurs in a control manner. This is to ensure that the body is at the state of equilibrium between proliferation and apoptosis. However, if the equilibrium is disturbed, the cells will grow and proliferate unrestrained, which finally will lead to tumor formation (Ames et al., 1993; Athar, 2002). The process of tumor development is also known as tumorigenesis. It is a multistep process involving a series of genetic and epigenetic alterations, including activation of oncogenes and inactivation of tumor-suppressor genes in a cell. These mutations accumulate in the cells and results in uncontrolled growth that eventually lead to invasion of cancerous cells into the surrounding tissue and/or finally metastasize to distant sites (Wu and Pandolfi, 2001). Although there are many different forms of cancer, the basic multistage process by which various tumors develop is similar for all cancers. The process of cellular deregulation can be caused by exposure to carcinogens, such as external exposure of body to chemicals, radiation and viruses that in turn, damage DNA and hence leads to cancer development (Greenwald and Mcdonald, 2002).

Current strategies are still not capable to effectively manage overall cancer incidence, even though efforts have been put on early cancer detection and treatment approaches. Hence, in recent years, one of the efforts in developing effective novel strategies to control cancer development is the approach of chemoprevention (Kelloff et al., 2000), a process which was first described by Sporn (1976). It is a means of cancer control where the cancer formation can be intervened in the early precancerous stages and entirely suppressed, reversed and slowed down by administration of synthetic or natural compounds (Nair et al., 1995; Lamson and Brignall, 2001; Abdullaev and Espinosa-Aguirre, 2004). An ideal chemoprevention agent should possess three important criteria first, it should be able to inhibit the development of cancer; second, it is expected to have minimal toxicity to the user; and third, it should act differentially on the premalignant or malignant cells, leaving normal cells unaffected (Lippman et al., 1998).

The most popular model used to study the anti-carcinogenic effect of extracts/compounds is the two-stage chemical-induced mouse skin carcinogenesis model. This model is a multi-factorial, multi-step process that consists of three distinct stages: initiation, promotion and progression, utilizing 7,12-dimethylbenz[a]anthracene (DMBA) and croton oil as tumor initiator and tumor promoter, respectively (Bowden et al., 1995). By separating the process of carcinogenesis mechanistically and temporally into three stages, the action of chemopreventive agent in either initiation, promotion (or premalignant progression), or malignant progression can be monitored (Yuspa, 1994).

The use of natural products started thousands of years ago and the herbal medicines are the oldest remedies known to mankind (Brown, 1980). The use of herbs as anticancer agents has a long history since traditional medicine systems (Sharma et al., 2009). Recent years, cancer prevention by natural products has gained escalating attention (Naithani et al., 2008) and undergone extensive laboratory and clinical testing due to its promising impact on human health (Cseke et al., 2006). In an effort to identify new chemopreventive agent, the current study was carried out to investigate the anticarcinogenic potential of methanol leaf extract of *Dicranopteris linearis* using a two-stage mouse skin carcinogenesis protocol.

METHODOLOGY

Plant collection

The leaves of *D. linearis* (Burm. f) Underw. were collected in September, 2011 from the roadside vicinity to Institute of Biosciences (IBS), UPM, Serdang, Selangor, Malaysia. Only the green, fresh and not shrivel leaves were collected. The young leaves and fiddleheads were all excluded. A voucher specimen was then deposited in the herbarium of IBS, with the voucher specimen number of SK 1987/11.

Preparation of MEDL

The leaves of *D. linearis* were rinsed with tap water to clean up the dirt or dusts and then air-dried at room temperature (27 ± 2°C) until dryness. The dried leaflets (pinnae) were ground into fine powder using laboratory mill (Mill Power Tech RT series, China). After that, the leaf powder was weighed and soaked in methanol in the ratio of 1:20 (w/v) for 72 h and repeated for three times. The supernatant was collected and first filtered using cotton wool, followed by Whatman no. 1 filter paper. The process was repeated three times and the last residue was air-dried and kept for reference. The filtered supernatant was concentrated until dryness in a rotary evaporator at 40°C under reduced pressure. The paste form of product was obtained and labelled as leaf methanol extract of *D. linearis* (MEDL). The stock MEDL was then stored at 4°C for future use (Zakaria et al., 2006, 2008).

Drugs and chemicals

The chemicals used in the experiment were methanol, 7,12 dimethylbenz[a]anthracene (DMBA) purchased from Sigma-Aldrich, croton oil (Sigma-Aldrich), curcumin (Sigma-Aldrich) and acetone. DMBA and croton oil served as tumor initiator and tumor promoter, respectively while curcumin was used as positive control. Acetone that was used as vehicle to dissolve all the drugs or chemicals was purchased from Mallinckrodt Chemicals (US). The tumor initiator, DMBA was prepared at the concentration of 100 µg/100 µl per mouse (Sharma et al., 2009; Chaudhary, 2011) by dissolving 100 µg of DMBA in 100 µl of acetone for each mouse. On the other hand, croton oil that serves as tumor promoter was diluted in acetone to give a concentration of 1% (w/v) (Das et al., 2005; Sharma et al., 2009; Chaudhary, 2011), based on the fact that 1% solution contains 10 mg/ml dissolved substance. Thus, 1% of croton oil was prepared by dissolving 1 mg of croton oil in 100 µl of acetone for each mouse. Curcumin was used as positive control due to its potent anti-tumor property (Park et al., 1998; Roslida et al., 2011). It was prepared at the dosage of 10 mg/kg (Roslida et al., 2011). The weight of curcumin needed to dissolve in 100 µl of acetone was calculated based on the mean body weight of Group 4 mice.

Phytochemical screening

The phytochemical screening tests were carried out based on 5.0 g of dried powder material and 100 mg of extract (organic).

Alkaloids test

Sample was macerated in chloroform followed by addition of ammoniacal chloroform. The mixture was then treated with sulphuric acid 10% and further tested with Mayer's reagent. Formation of white precipitates indicates the presence of alkaloids.

Saponins test

The methanol extract of sample was mixed with distilled water in a test tube. Formation of stable froth for at least 15 min indicates the presence of saponins.

Flavanoids test

The methanolic extract of sample was dissolved in ether and shaken in 10% ammonia solution. Formation of yellow colour in ammonia layer indicates the presence of flavonoids.

Tannins and polyphenolic compounds test

The methanolic extract of sample was mixed with 1% ferric solution. Formation of blue black colour indicates the presence of hydrolysable tannins, while brownish-green indicates that of condensed tannins.

Triterpenes/steroids test

The methanolic extract of sample was analysed using Liebermann-Buchard reagent. Formation of reddish colour indicates the presence of triterpenes and greenish colour for steroids.

Animals

The animal models used for *in vivo* study consisted of a total of 48 healthy 6 to 8 weeks old female ICR mice (Park et al., 1998), with the body weight of 15 to 25 g. The mice were divided into six groups, with eight mice per group and housed in polypropylene cage, fed with a standard laboratory pellet diet and water *ad libitum*. The animals were housed in Animal House of Faculty of Medicine and Health Sciences, at room temperature of 25 ± 4°C and alternating light and dark cycle. The animals were acclimatized for one week prior to the commencement of experiment. Three days before the experiment, all mice were dorsally shaved with electric hair clipper, with an area of 2 cm × 2 cm and 1 cm off tail (Sharma et al., 2009; Chaudhary, 2011).

Two-stage mouse skin carcinogenesis

Experimental design

All mice were randomly assigned into six groups, namely Group 1 to 6 (n = 8). The six experimental groups were composed of three treatment groups (Group 1 to 3) and three control groups (Group 4 to 6). During the promotional stage, each mouse from the three treatment groups, which are Groups 1, 2 and 3 were topically applied with 100 µl of 30, 100 and 300 mg/kg of MEDL, respectively. On the other hand, each mouse in Group 4 (positive control) was topically administered with 100 µl of curcumin at the dose of 10 mg/kg body weight of mice, while Group 5 (negative or carcinogen control) was assigned with no pre-treatment. Meanwhile, each mouse in the Group 6 (vehicle control) received acetone only throughout the experiment.

Two-stage mouse skin carcinogenesis protocol

In the *in vivo* two-stage mouse skin carcinogenesis model, the initiation phase was accomplished by a single application of sub-carcinogenic dose of mutagen that acts as tumor induction agent. This was followed one week later by repeated application of tumor promoter, twice a week, for up to 15 weeks. All the mice in all experimental groups were topically applied with 100 µg/100 µl of DMBA. After one week of waiting/retention period, the promotion phase started and lasted for 15 weeks. The three treatments groups (Group 1, 2, and 3) were topically administered with 30, 100, and 300 mg/kg of MEDL, respectively, followed by 1% croton oil at 30 min later, twice a week. For the positive control group (Group 4), the mice received 10 mg/kg of curcumin, 30 min prior to application of 1% croton oil promotion, twice a week. In contrast, mice in negative control group (Group 5) received only 1% croton oil without any pre-treatment, twice a week. Animals in vehicle control group (Group 6) were applied only with acetone (twice a week) throughout the whole experiment. All the chemicals or drugs that applied onto dorsal part of mice were at the volume of 100 µl each.

Papilloma detection

Along the 15 weeks of tumor promotion duration, the following macroscopic parameters were recorded at weekly intervals: (i) body weight of mice, (ii) latency period of tumor formation, (iii) tumor

incidence, (iv) tumor burden, and (v) tumor volume. The latency period of tumor formation was defined as the onset of first tumor in a group, expressed in terms of weeks. The percentage of tumor incidence denoted the number of mice carrying at least one tumor (Sharma et al., 2009; Chaudhary, 2011) while the tumor burden indicated the average number of tumors per tumor-bearing mouse (Sharma et al., 2009; Chaudhary, 2011). The measurements of tumor volume applied the formula of ellipsoid volume that is based on three axes (length, width and height axes) as it was the most accurate method for estimating the tumor growth (Girit et al., 2008). The experiment was terminated by the end of 15th week of tumor promotion. All the mice were scarified by cervical dislocation method (Sharma et al., 2004) followed by the skin histopathological analysis.

Morphological observation

Throughout the 15 weeks of tumor promotion, the dorsally shaved part of all mice was observed regularly for detection of any lesions and tumors development (Cibin et al., 2010). The hair of mice was shaved prior to every treatment so that the hair could not impede the absorption of chemicals onto the skin and hinder the tumor from being clearly observed during tumor size measurement, using caliper technique (Girit et al., 2008). Skin tumor was defined as reddish elevation from dorsal skin, polypoidal in shape, pedunculated, and cauliflower-like in appearance (Sharma et al., 2004). The palpable mass with a diameter greater than 1 mm and persisted at least for two consecutive weeks was considered for counting. Those skin papillomas that regressed after one week observation was excluded from data analysis (Das et al., 2005; Abel et al., 2009; Sharma et al., 2009). Since there was a couple of treatment day per week, the tumors was also observed and measured by using caliper technique (Girit et al., 2008), twice a week, to ensure the consistency of the tumors existence. Then, the two measurement readings were averaged so that only one reading was presented for each tumor. Apart from that, the body weight of each mouse was also recorded weekly for the purpose of dosage calculation in drugs preparation step and manipulation of causal factors if fluctuations of body weight occurred. Any death case in mouse should be recorded along with time of death.

Histopathological observation

The harvested skin was grossed by cross section of skin samples for both normal and tumor region. The skin samples were then kept in labelled cassettes and fixed in 10% buffered formalin for at least 24 h. Then, the fixed skin samples were sent for tissue processing in an automated tissue processor (Leica TP1020, Germany). The processed skin samples were then embedded in paraffin wax by an embedder machine (Leica EG1160, Germany) before subjected to trimming and sectioning, using a microtome (Leica RM2135, Germany) to obtain skin tissue sections with 4 µm thickness. Next, the tissue sections were fished on 40°C water bath, onto the frosted end glass slide. The slides were then stained with Hematoxylin and Eosin (H&E) stains using routine protocol in an automated slide stainer or autostainer (Sakura Tissue-Tek Prisma-E2S, Japan). Stained slides were finally mounted with DPX and viewed under biological microscope (40× and 100× magnification). Under the microscope, the histology of skin tissues was examined.

Statistical analysis

All statistical calculations were carried out with statistical package for social sciences (SPSS) software program (version 20.0). All of the values were expressed as the mean ± standard error of mean (SEM), analyzed by one-way analysis of variance (ANOVA) and significant difference among multiple means was determined using least significant difference (LSD) multiple comparison test at the level of 0.05.

RESULTS

Morphological observation

Figure 1 illustrates the representative photographs of animals of each experimental group captured at the end of study (15th week of tumor promotion). The tumors formed on the dorsal skin of mice appeared as reddish, cauliflower-like, and polypoidal in shape, ranging from 1 to 8 mm in diameter and 1 to 5 mm in height. Comparison among all the experimental groups showed that Group 5 (carcinogen control) demonstrated the highest number and largest size of tumor formation (Figure 1E), in contrast to Group 6 (vehicle control) which showed no tumor formation at all (Figure 1F). Among the MEDL-treated groups, both the tumor number and tumor size were reduced in Group 1, which has been treated with MEDL at 30 mg/kg body weight (Figure 1A), compared to Group 2, which has been treated with MEDL at 100 mg/kg body weight (Figure 1B), and almost null in Group 3, which has been treated with MEDL at 300 mg/kg body weight (Figure 1C). Throughout the 15 weeks of tumor promotion period, the number and body weight of mice, latency period of tumor formation, percentage of tumor incidence, tumor burden and tumor volume were recorded at weekly interval, which are summarized in Table 1.

Body weight

The body weight of mice was statistically analyzed for group factor, at the first week of tumor promotion (initial body weight) and fifteenth week of tumor promotion (final body weight). Results show that there was no significant different ($p > 0.05$) among experimental groups for both initial and final body weight. Figure 2 shows a net increment in body weight from week 1 to 15 of tumor promotion for all the experimental groups

Gross morphological observation

The onset of first tumor ranged from sixth to fifteenth week for different experimental groups (Figure 3). The first tumor appeared simultaneously at week 6 of tumor promotion period, for Group 1, which has been treated with MEDL at 30 mg/kg body weight and Group 5 (carcinogen control). The latency period of tumor formation were then increased from week 8 for Group 4 (positive control) to week 9 for Group 2, which has been

Table 1. Effect of methanol extract *of Dicranopteris linearis*(MEDL) leaf on two-stage mouse skin carcinogenesis at the promotion stage. The terms ‗initial' and ‗final' indicate the first and fifteenth week of tumor promotion, respectively.

Group	No. of animal		Body weight (g)		Tumor latency period (weeks)	Tumor incidence (%)	Tumor burden	Tumor volume (mm^3)
	Initial	Final	Initial	Final				
30 mg/kg MEDL	8	8	25.00 ± 0.87^{A}	29.50 ± 1.27^{A}	6	75.0^{B}	$5.67\pm1.28^{a,b,C}$	$3.70\pm0.96^{b,B}$
100 mg/kg MEDL	8	8	23.88 ± 0.88^{A}	30.00 ± 0.60^{A}	9	62.5^{B}	$5.00\pm1.13^{b,B}$	$2.39\pm0.54^{b,A,B}$
300 mg/kg MEDL	8	8	26.00 ± 1.02^{A}	32.38 ± 1.00^{A}	15	$12.5^{a,b,A}$	$1.00\pm0.13^{a,b,A}$	$0.26\pm0.03^{b,A}$
Positive control	8	8	25.25 ± 0.59^{A}	32.13 ± 0.92^{A}	8	75.0^{B}	$4.83\pm1.02^{b,A,B}$	$3.06\pm0.78^{b,A,B}$
Carcinogen control	8	8	25.13 ± 1.09^{A}	30.25 ± 0.98^{A}	6	87.5^{B}	$7.86\pm2.37^{a,D}$	$9.00\pm2.27^{a,C}$
Vehicle control	8	8	24.13 ± 1.00^{A}	30.38 ± 1.27^{A}	-	$0.0^{a,b,A}$	$0.00\pm0.00^{a,b,A}$	$0.00\pm0.00^{b,A}$

Values are expressed as mean ± S.E.M, except latency period of tumor formation and tumor incidence. ap < 0.05: significantly different from the positive control (Group 4). bp < 0.05: significantly different from the carcinogen control (Group 5). A,B,C,Dp < 0.05: comparison among all the experimental groups. The values followed by the same superscript (capital letter) are not significantly different (p > 0.05). Percentage of tumor incidence was calculated as ration of number of tumor-bearing mice in a group/number of mice in a group × 100. Tumor burden was calculated by dividing total number of tumor in a group by number of tumor-bearing mice in a group. Tumor volume was calculated using the formula V = π/6 × length × width × height.

treated with MEDL at 100 mg/kg body weight, and further increased to week 15 for Group 3, which has been treated with MEDL at 300 mg/kg body weight.

The percentage of tumor incidence was recorded at weekly interval and displayed in Figure 4, where it indicates the percentage of tumor-bearing mice in a group. The highest incidence of tumor formation was recorded in Group 5 (carcinogen control) (87.5%), followed by 75.0% observed in both the Group 4 (positive control) and Group 1 (MEDL treatment at 30 mg/kg body weight). The percentage of tumor incidence was further reduced to 62.5% in Group 2 (MEDL treatment at 100 mg/kg body weight) and the lowest percentage of tumor incidence was found in Group 3 (MEDL treatment at 300 mg/kg body weight) (12.5%) with only one mouse showing tumor. Group 6 (vehicle control) showed no tumor formation at all throughout the experiment.

The statistical analysis of percentage of tumor incidence (Figure 5) indicates that only Group 3, which has been treated with MEDL at 300 mg/kg body weight, showed significantly decrease ($p < 0.05$) in tumor incidence, as compared to other experimental groups, including both positive and carcinogen controls. The percentage of tumor incidence was not significantly different ($p > 0.05$) among positive control (75.0%), carcinogen control (87.5%), Group 1, treated with MEDL at 30 mg/kg body weight (75.0%), and Group 2, treated with MEDL at 100 mg/kg body weight (62.5%).

Tumor burden was also recorded at weekly interval (Figure 6) and is defined as the average number of tumors formed on a tumor-bearing mouse. Group 5 (carcinogen control) documented the highest number of tumor per tumor-bearing mice (7.86 ± 2.37), followed by Group 1, treated with MEDL at 30 mg/kg body weight (5.67 ± 1.28), and then Group 2, treated with MEDL at 100 mg/kg body weight (5.00 ± 1.13). Group 4 (positive control) recorded tumor burden of 4.83 ± 1.02, while the least tumor burden was found in Group 3 with MEDL at 300 mg/kg body weight (1.00 ± 0.13). Group 6 (vehicle control) showed no tumor formation at all throughout the experiment.

The statistical analysis of tumor volume reflected that all the tumor volumes were significantly smaller ($p < 0.05$) in experimental groups, as compared to carcinogen control. When comparing with positive control, only Group 3 treated with MEDL at 300 mg/kg body weight demonstrated significantly decrease ($p < 0.05$) in tumor volume. Besides, when comparing among the MEDL-treated groups, the tumor volume in Group 3 is significantly lesser ($p < 0.05$) than Group 1, which has been treated with MEDL at 30 mg/kg body weight, as well as Group 2, which has been treated with MEDL at 100 mg/kg body weight. There was no significant difference ($p>0.05$) between Group 1 and 2.

In summary, Group 5 (carcinogen control) showed the lowest latency period of tumor formation (week 6), greatest tumor incidence (87.5%), highest tumor burden (7.86 ± 2.37) and largest tumor volume (9.00 ± 2.27 mm^3). In contrast, Group 3,

Table 2. Phytochemical constituents in methanol crude extracts and powder of *Dicranopteris linearis*.

Sample	*Alkaloids	**Saponins	***Flavonoids	***Tannins and polyphenolic compounds	***Triterpenes	***Steroids
D.linearis extract	-	3+	2+	2+Condensed tannins detected	3+	3+
D.linearis powder	-	3+	1+	2+Condensed tannins detected	-	2+

*1+ negligible amount of precipitate; 2+ weak precipitate; 3+ strong precipitate. **1+ 1 to 2 cm froth; 2+ 2 to 3 cm froth; 3+ > 3 cm froth. ***1+ weak colour; 2+ mild colour; 3+ strong colour.

the highest dose of MEDL-treated group (300 mg/kg body weight) demonstrated the highest latency period of tumor formation (week 15), lowest tumor burden (12.5%), lowest tumor burden (1.00 ± 0.13) and smallest tumor volume (0.26 ± 0.03 mm^3).

Histopathological analysis

The effect of leaf methanol extract of MEDL was further assessed by histopathological studies after 15 weeks of tumor promotion. Microscopic exami-nation of the dorsal skin of mice has been carried out. The histopathological features observed in the dorsal skin tissues of mice in control and treatment groups are depicted in Figures 1 to 5.

The histological studies on vehicle control mice showed normal skin structure (Figure 7). The skin epidermis seen in acetone-treated mouse skin was approximately a single layer of basal cells overlaid by flattened squamous cells. Keratin was found in the stratum corneum. Hair follicles and sebaceous glands can be found in dermis layer. The skin tissues from carcinogen control demonstrated severe papillomatosis with severe hyperkeratosis (thickening of the stratum corneum) and severe acanthosis (thickening of the stratum spinosum) of the epidermis (Figure 8). In general, the hyperplastic lesions with intact epidermis indicated that all the papillomas formed were benign in nature. On the other hand, the papilloma formed in positive control was smaller, as compared to carcinogen control. Histological features observed in positive control were characterized by mild papillomatosis with mild hyperkeratosis and mild acanthosis of epidermis (Figure 9).

When comparing the cutanenous histological chracteristics among MEDL-treated groups, Group 1 (MEDL treatment at 30 mg/kg body weight) displayed a moderate papillomatosis with moderate hyperkeratosis and moderate acanthosis of epidermis (Figure 10), whereas Group 2 (MEDL treatment at 100 mg/kg body weight) showed mild papillomatosis with mild hyperkeratosis and mild acanthosis of epidermis (Figure 11). On the other hand, Group 3 (MEDL treatment at 300 mg/kg body weight) exhibited only mild hyperplasia of epidermis, with no papillomatosis observed (Figure 12).

Phytochemical tests

Phytochemicals screening of *D. linearis* was done in two forms which are powdered *D. linearis* and *D. linearis* extract to detect the presence of alkaloids, saponins, flavonoids, tannins and polyphenolic compounds, triterpenes and steroids as shown in Table 2. Both sample showed no presence of alkaloids but high amount of saponins. Moderate amount of flavonoids were present in *D. linearis* extract and low amount in the powder. Tannins and polyphenolic compounds were moderately found in both *D. linearis* extract and *D. linearis* powder. In comparison to *D. linearis* extract, *D. linearis* powder has no presence of triterpenes at all. Steroids were found in both sample extracts containing the higher amount while powder only contained a moderate amount.

DISCUSSION

The effort of screening natural products which can inhibit or reverse the process of carcinogenesis and thereby turn out into promising chemopreventive agent which gained increased attention (Craig, 1997; Kellen, 1999; Nishino et al., 2005).

According to Park et al. (1998), the inflammatory, oxidative stress and tumor promotion pathways are interrelated. Thus, a compound that displays anti-inflammatory and/or antioxidative properties is expected to act as an anti-tumor promoter as well (Park et al., 1998). This formed the basis of the current study to investigate the chemopreventive potential of *D. linearis* in the promotion stage of DMBA/croton oil-induced mouse skin carcinogenesis. In DMBA/croton oil-induced mouse skin carcinogenesis protocol, topical applications of DMBA and croton oil was able to induce skin papilloma formation through the process of chronic inflammation, reactive oxygen species (ROS) overproduction and oxidative stress-related DNA damage in skin (Das and Bhattacharya, 2004).

The proposition of an inflammation-cancer connection has arisen since the 19th century where the role of inflammation in carcinogenesis has gained more evidence (Balkwill and Mantovani, 2001). Numerous laboratory and population-based studies have proposed that some

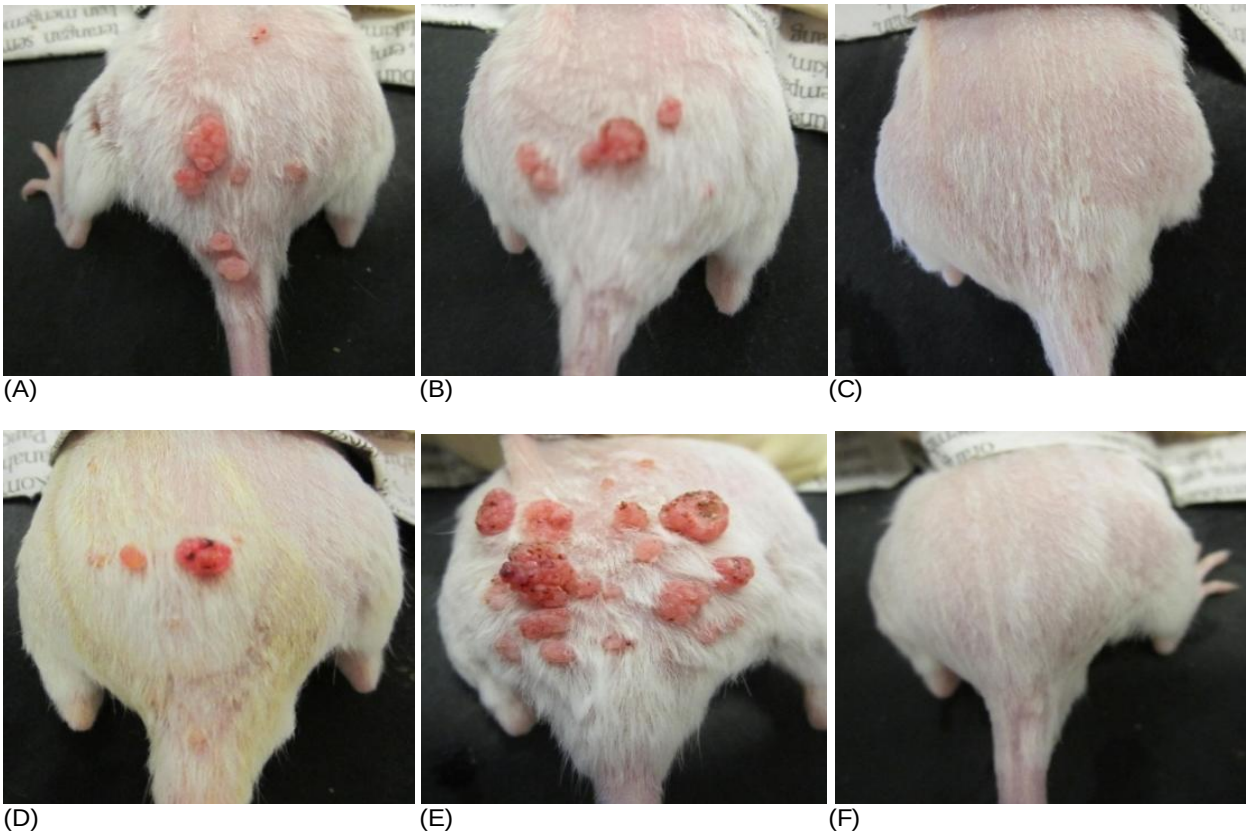


Figure 1. Representative photographs for an animal from each experimental group captured at the end of study (15 weeks). (A) MEDL treatment at 30 mg/kg; (B) MEDL treatment at 100 mg/kg; (C) MEDL treatment at 300 mg/kg; (D) positive control, curcumin treatment at 10 mg/kg; (E) carcinogen control, application of 1% croton oil only at the promotion stage, without any pre-treatment; (F) vehicle control, application of acetone only, throughout the experiment.

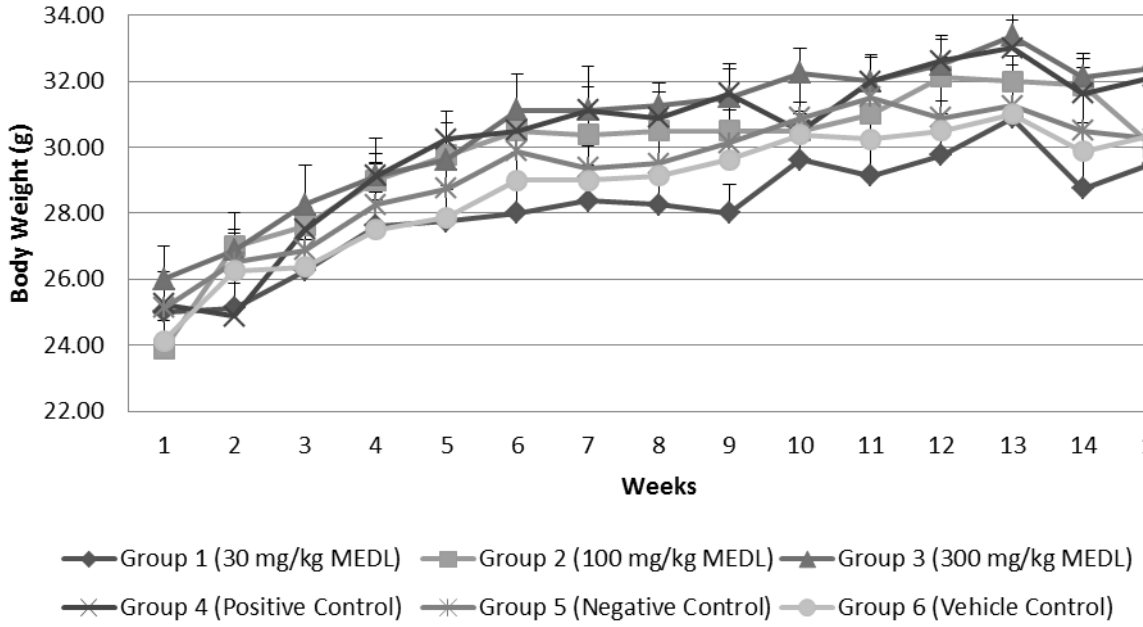


Figure 2. Effect of methanol extract *of Dicranopteris linearis*(MEDL) leaf on body weight of mice.

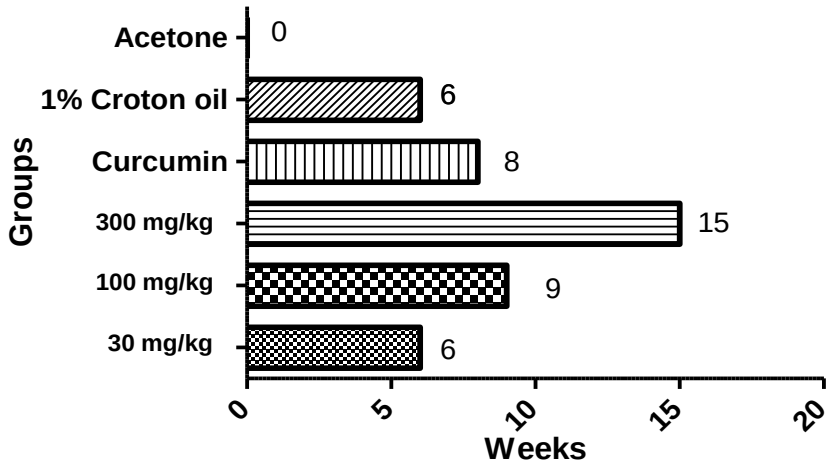


Figure 3. Effect of methanol extract of *Dicranopteris linearis* (MEDL) leaf on latency period of tumor formation in mice. Group 1 (MEDL at 30 mg/kg body weight); Group 2 (MEDL at 100 mg/kg body weight); Group 3, (MEDL at 300 mg/kg body weight); Group 4 (positive control); Group 5 (carcinogen control); and Group 6 (vehicle control).

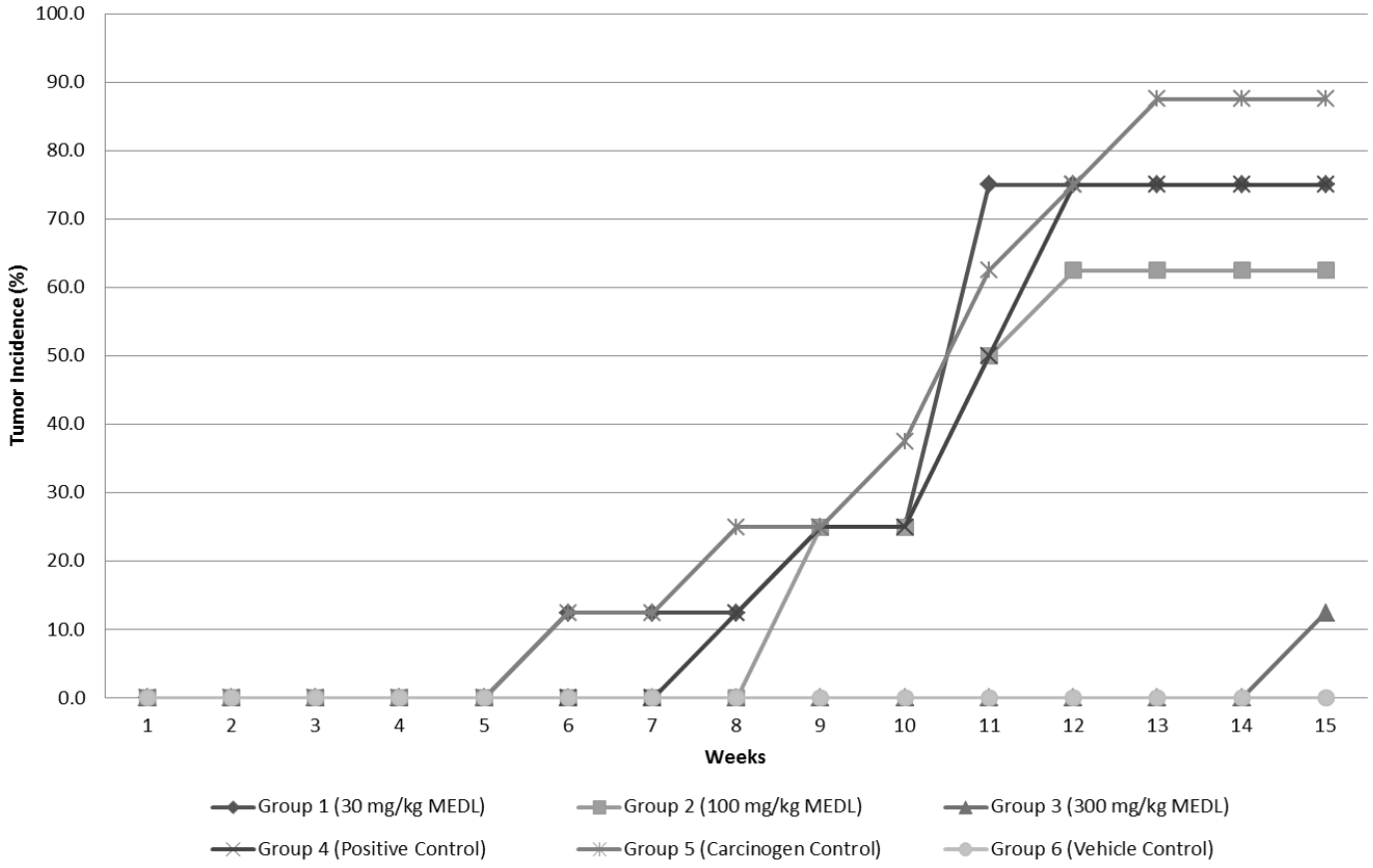


Figure 4. Effect of methanol extract *of Dicranopteris linearis*(MEDL)leaf on percentage of tumor incidence in mice. At the end of the study, highest tumor incidence was reported in carcinogen control group (87.5%), followed by positive control and Group 1 (75.0%), and then Group 2 (62.5%) whereby the lowest percentage of tumor incidence has been seen and in Group 3 (12.5%). Vehicle control showed no tumor formation at all throughout the experiment.

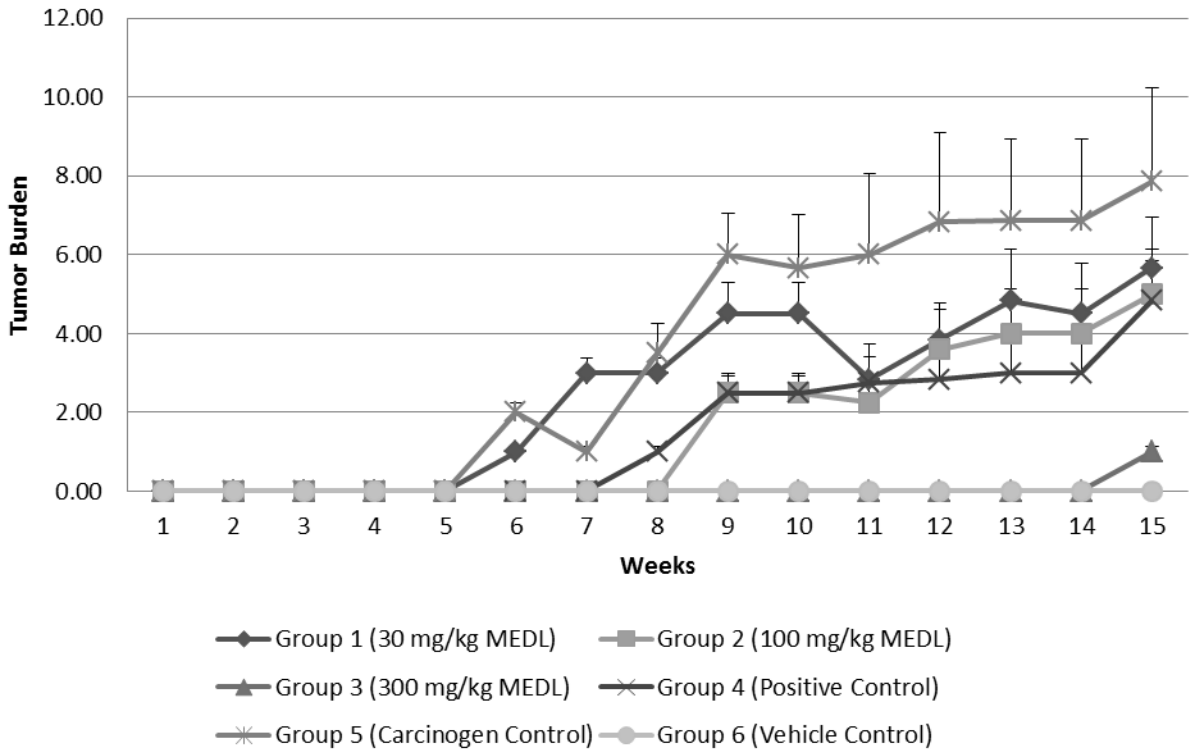


Figure 5. Effect of methanol extract *of Dicranopteris linearis*(MEDL)leaf on tumor burden.

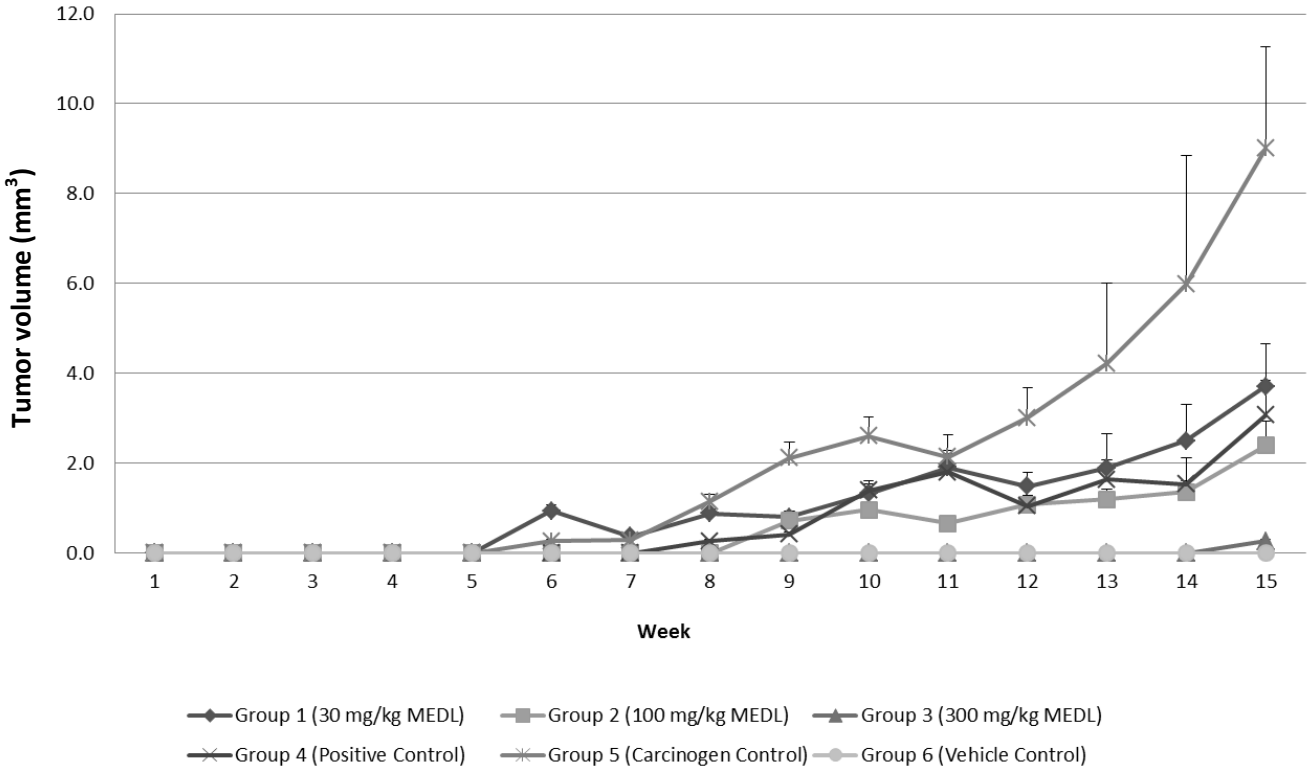


Figure 6. Effect of methanol extract *of Dicranopteris linearis*(MEDL) leaf on tumor volume in mice.

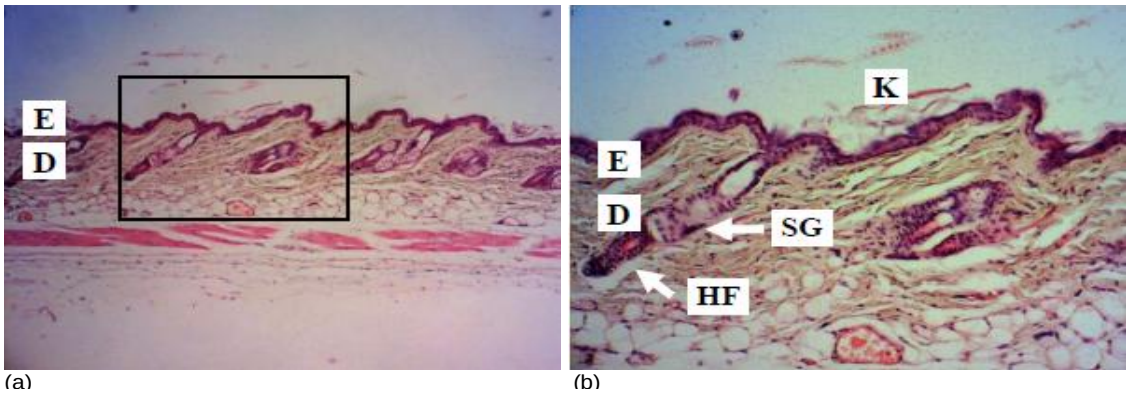


Figure 7. Representative microphotograph taken from H&E stained mouse skin of Group 6, vehicle control (acetone-treated group). (i) Normal skin structure can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows approximately a single layer cell thick of epidermis (E) overlaid by keratin (K). Hair follicles (HF) and sebaceous glands (SG) can be observed in dermis (D) (magnification 100×).

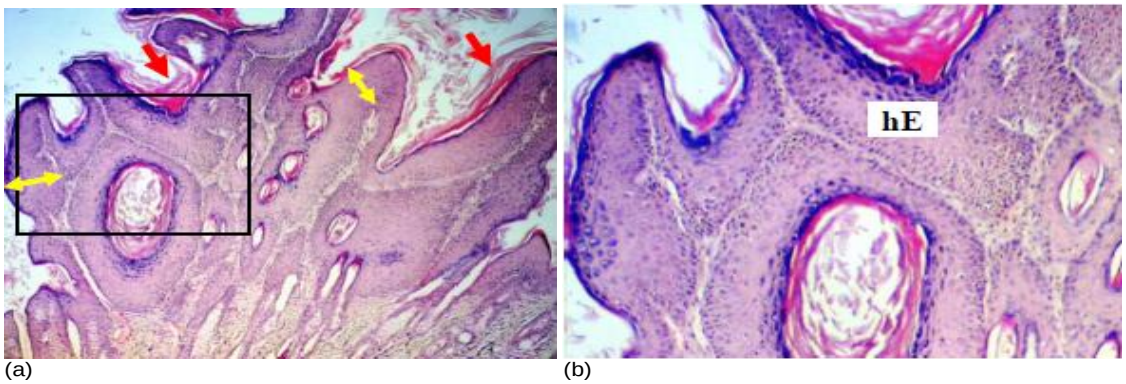


Figure 8. Representative microphotograph taken from H&E stained mouse skin of Group 5, carcinogen control (DMBA/croton oil-treated group). (i) Severe papillomatosis with severe hyperkeratosis (red arrow) and severe acanthosis (double-pointed yellow arrow) of epidermis can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows no evidence of invasion. The hyperplastic epidermis (hE) was well-defined and intact (magnification 100×).

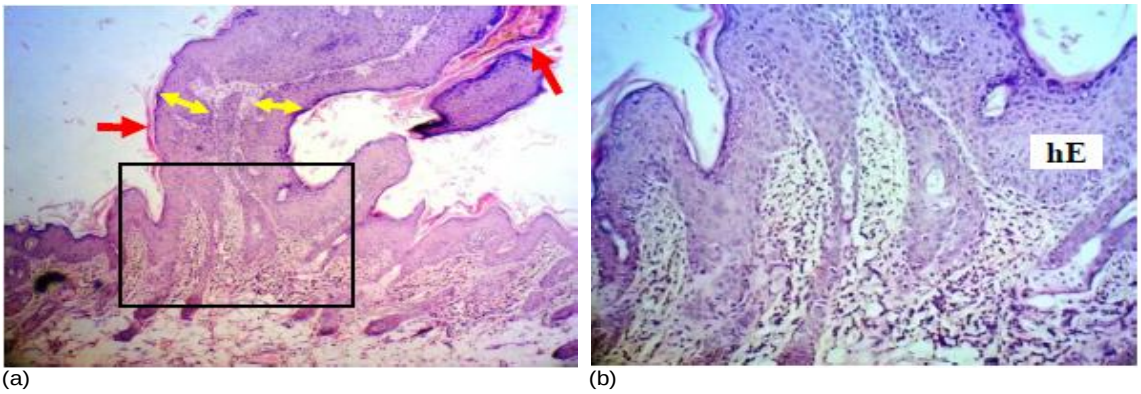
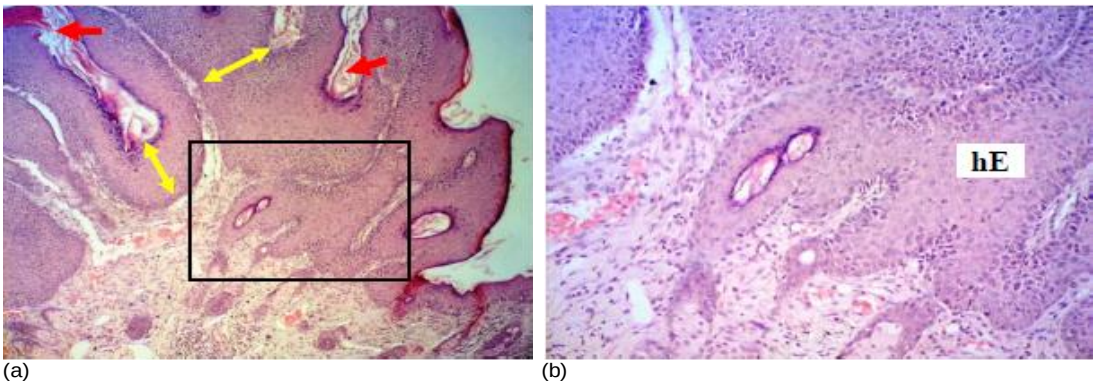
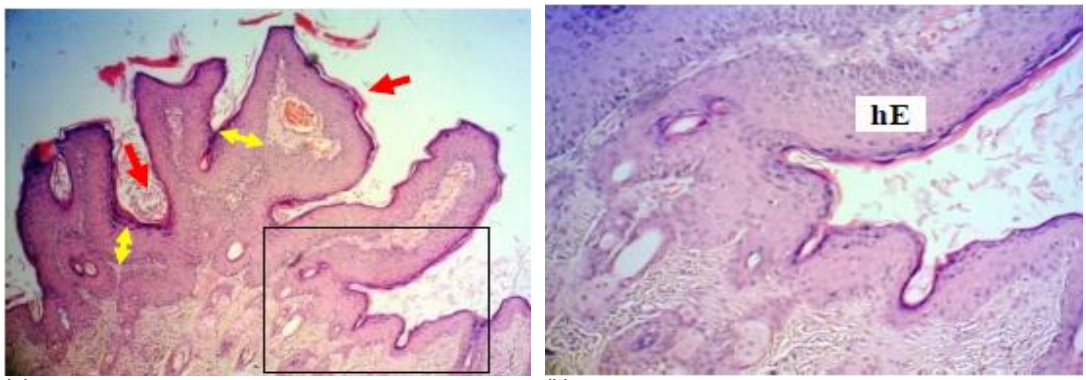


Figure 9. Representative microphotograpgh from positive control, Group 4 (curcumin treatment at 10 mg/kg body weight). (i) Mild papillomatosis with mild hyperkeratosis (red arrow) and mild acanthosis (double-pointed yellow arrow) of epidermis can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows no evidence of invasion. The hyperplastic epidermis (hE) was well-defined and intact (magnification 100×).



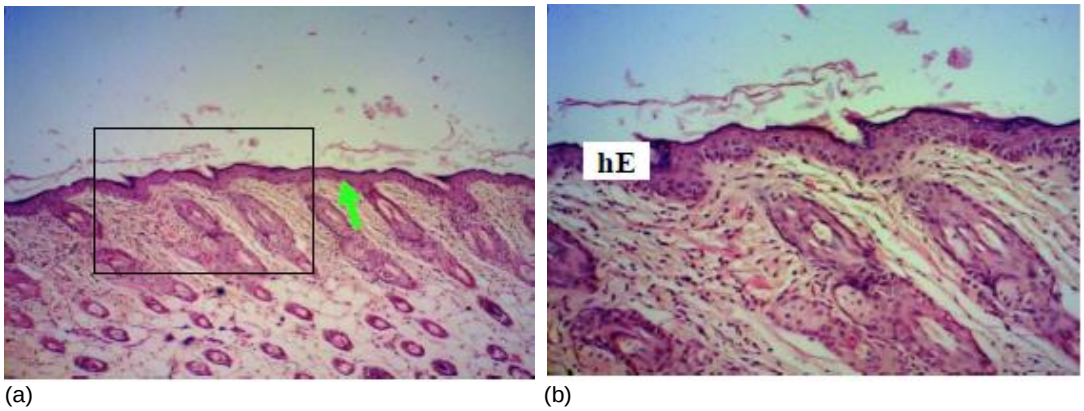
(a) (b)

Figure 10. Representative microphotograph taken from H&E stained mouse skin of Group 1 (MEDL treatment at 30 mg/kg body weight). (i) Moderate papillomatosis with moderate hyperkeratosis (red arrow) and moderate acanthosis (double-pointed yellow arrow) of epidermis can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows no evidence of invasion. The hyperplastic epidermis (hE) was well-defined and intact. (magnification 100×).



(a) (b)

Figure 11. Representative microphotograph taken from H&E stained mouse skin of Group 2 (MEDL treatment at 100 mg/kg body weight). (i) Mild papillomatosis with mild hyperkeratosis (red arrow) and mild acanthosis (double-pointed yellow arrow) of epidermis can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows no evidence of invasion. The hyperplastic epidermis (hE) was well-defined and intact (magnification 100×).



(a) (b)

Figure 12. Representative microphotograph taken from H&E stained mouse skin of Group 3 (MEDL treatment at 300 mg/kg body weight). (i) Mild hyperplasia of epidermis (green arrow) can be seen (magnification 40×). (ii) Magnified of boxed region in microphotograph (i) shows thickening of epidermis. The hyperplastic epidermis (hE) was well-defined and intact with the absence of characteris of invasion (magnification 100×).

malignancies evolved from tissues that are severely damaged by chronic inflammation (O'Byrne and Dalgleish, 2001; Itzkowitz and Yio, 2004; Kundu and Surh, 2008). Various pro-inflammatory mediators (cytokines, cyclooxygenase 2, prostaglandins, nitric oxide and et cetera) can contribute to carcinogenesis through the mechanisms of:

(1) induction of chromosomal instability, (2) alterations in epigenetic events and error in the subsequent gene expression, (3) enhancement of cell proliferation, (4) evasion from apoptosis, (5) stimulation of angiogenesis, (6) invasion through basement membrane, and (7) promoting the metastatic movement (Perwez Hussain and Harris, 2007; Kundu and Surh, 2008; Colotta et al., 2009; Porta et al., 2009). Previous studies done by Zakaria et al. (2006, 2008) showed that *D. linearis* possess positive anti-inflammatory effect, thus suggesting the possibility that *D. linearis*-based extracts might contribute to the action of chemoprevention, by interrupting any of the inflammation mechanisms that lead to tumor formation.

The role of ROS-mediated oxidative stress in pathogenesis of cancer has been well documented (Ray and Husain, 2002). Oxidative stress created by free radicals often causes DNA damage such as DNA bases mutation, single- and double-strand breaks, DNA cross-linking and chromosomal breakage and rearrangement (Ames et al., 1993). Previous experiments have shown that a variety of plants exert chemoprotective effect by interrupting the tumor promotion stage (Javed et al., 1998; Zhao et al., 1999), the stage where the role of ROS is mostly emphasized (Huang et al., 1997). It is therefore implied that agents that can reduce the generation of free radicals *in vivo* including *D. linearis* may be presumed as a chemopreventive agent (Huang et al., 1992).

Previous phytochemical screening done on *D. linearis* showed the presence of flavonoids (Zakaria et al., 2006, 2008). The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including free radical scavenging, modifying enzymes that activate or detoxify carcinogens, and inhibiting the induction of the transcription factor activator protein-1 (AP-1) activity by tumor promoters (Canivenc-Lavier et al., 1996; Shih et al., 2000; Steenkamp et al., 2013). Therefore, it is possible that these flavonoids are responsible for the *D. linearis* anticarcinogenesis.

In the present study, the chemopreventive potential of *D. linearis* was evaluated *in vivo* by monitoring the latency period of tumor formation, percentage of tumor incidence, tumor burden, tumor volume as well as microscopic examination by observing histological changes on the dorsal skin of mice. The body weight of mice was also monitored weekly to obtain information of growth condition of mice. At end of the study, the number of mice survived was maintained at eight, which is same as the initial number. The body weight of mice also showed a net increment. In addition, all the mice were free from any toxicity symptoms. Therefore, the mice were assumed to be tolerable to all the doses of methanol extract of MEDL used, even at the highest dose (300 mg/kg).

Statistical analysis indicated that there is significant ($p < 0.05$) positive effect in all the parameters measured when comparing the three doses (30, 100 and 300 mg/kg body weight) of MEDL-treated groups with the carcinogen control (DMBA/croton oil-treated group), particularly at the highest dose (300 mg/kg body weight). The results also showed that MEDL exhibits its activity in a dose-dependent manner in all the parameter measured. The latency period of tumor formation was increased from week 6 to week 9 and to week 15 in MEDL-treated groups at 30, 100 and 300 mg/kg body weight, respectively. In addition, the histopathological examinations showed that MEDL was able to suppress the skin tumor formation with less extent of histological changes observed in MEDL-treated group at 300 mg/kg body weight, as compared to 30 mg/kg body weight. Further histopathological examination suggested that all the skin tumors formed was begin tumors. This finding is parallel to the studies done by Abel et al. (2009) which suggested that papilloma was expected to be converted to squamous cell carcinoma after 20 weeks of tumor promotion. It has been reported that 52 weeks are needed to achieve the maximum number of malignancy conversion (Abel et al., 2009).

In this study also, positive control mice that have been treated with curcumin at 10 mg/kg body weight showed significant ($p < 0.05$) reduction in the tumor burden and tumor volume as compared to carcinogen control. However, the percentage of tumor incidence was not significantly ($p < 0.05$) different from the carcinogen control. This similar result was observed in a study conducted by Limtrakul et al. (1997). They reported the difference in the number of animals developing tumors was not statistically significant between 1% curcumin diet-fed group versus control diet-fed group, though the number and size of tumors developed in the curcumin-treated group were significantly lower (Limtrakul et al., 1997). They agreed with the previous study which claimed that 2% turmeric diet significantly suppressed DMBA-induced skin tumors in mice (Azuine and Bhide, 1992). Thus, one of the determining factors of high incidence of tumor formation in positive control might be due to the low concentration of curcumin used in the study.

Flavonoids which also act as antioxidants have the potential of inhibiting carcinogenesis. Apart from inhibiting angiogenesis, there are also some flavonoids stated as to be potent inhibitors of cell proliferation such as fisetin, apigenin, and luteolin (Nijveldt et al., 2001). Flavonoids play a role in cancer prevention. Identified mechanisms of action includes carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation,

inhibition of angiogenesis, antioxidation and reversal of multidrug resistance or a combination of these mechanisms. It is demonstrated that flavanoids are able to inhibit carcinogenesis *in vitro* and substantial evidence indicates that they can also do so *in vivo*. This carcinogenesis inhibition may occur by affecting the molecular events in the initiation, promotion, and progression stages. Flavonoids could inhibit tumor initiation as well as tumor progression through animal studies and investigations using different cellular models (Ren et al., 2003).

Tannins which are in the category of polyphenolics have been found to reduce the carcinogenic activity of a number of carcinogens. These anticarcinogenic and antimutagenic potential has been related to their antioxidative property that is known important in protecting cellular oxidative damage including lipid peroxidation (Bhattacharya, 2012). Phenolic compounds can be found in plants consumed by humans (Selim et al., 2013). There is still lack of evidence in proving that dietary phenols are anticarcinogens but it is believed to do so due to their antioxidative properties. Inhibition of tumorigenesis by plant polyphenols has been described in many publications. Polyphenols that are effective when given during the post-initiation period, that is by inhibiting tumor promotion and progression, are believed to be more useful in preventing cancer in humans than are polyphenols, which are effective only when given before and during the carcinogen treatment. Although extensive studies on the inhibition of carcinogenesis by dietary polyphenols had been done, the molecular mechanisms of action and their applicability to human cancer prevention are unclear. Recently, *in vitro* studies have been published on the modulation of oncogenes, tumor suppressor genes, cell cycle, apoptosis, angiogenesis, and related signal transduction pathways by polyphenols (Yang et al., 2001). Tea polyphenols has the ability to inhibit the level of activated carcinogens, oxidative stress-induced cellular damage, carcinogen-DNA adduct formation, and possibly the initiation of carcinogenesis, the inhibition of post-initiation events. Numerous mechanisms for the action of tea polyphenols include inhibition of MAP kinases and the PI3K/AKT pathway, inhibition of NFκB- and AP-1-mediated transcription, inhibition of growth factor-mediated signalling, inhibition of aberrant arachidonic acid metabolism and other activities (Yang et al., 2009).

Saponins are in a diverse group of plant glycosides showing soap like frothing with water. High amount of saponins present in MEDL has contributed to anticarcinogenic activity exhibited by this study (Bhattacharya, 2012). They possess surface-active characteristics that are due to the amphiphilic nature of their chemical structure and proposed mechanisms of anticarcinogenic properties of saponins include direct cytotoxicity (Rao and Sung, 1995).

Phytochemical investigations of various triterpenes were found to possess several pharmacological properties, including anti-inflammatory, anti-tumor, antioxidative and anti-diabetes effects (Barkatullah et al., 2013). The triterpenes-rich fraction and several isolated triterpenes showed the inhibitory effect on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation and Epstein-Barr virus early antigen (EBV-EA) activation induced by TPA in mice. It was also reported that the major triterpenes, ursane types, such as corosolic acid (CA) and ursolic acid (UA), and oleanane types, such as maslinic acid (MA) and oleanolic acid (OA) were present. These major triterpenes showed anti-proliferative activities against gastric cancer cells (NCI-N87), colorectal cancer (HCT15), cervical cancer (HeLa), glioblastoma (U291, U373 and T98G) and colon cancer (HT29) cell lines (Uto et al., 2013).

Conclusion

The methanol extract of MEDL leaves exhibited some level of inhibition of tumor promotion in a dose-dependent manner in DMBA-initiated and croton oil-promoted mice skin carcinogenesis protocol, with the highest dose (300 mg/kg body weight) showing the greatest activity. This suggested that *D. linearis* is a potential chemopreventive agent.

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Full Length Research Paper

Evaluating the *in silico* activity of bioactive compound iressa, tarceva and capsaicin against epidermal growth factor receptor tyrosine kinase

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Epidermal growth factor receptor (EGFR) protein tyrosine kinases (PTKs) are known for their role in cancer. Lapatinib drug have been reported to be the molecules of interest, with potent anticancer activity and they act by binding to adenosine triphosphate (ATP) site of protein kinases. ATP binding site of protein kinases provides an extensive opportunity to design newer analogs. Here we aimed to do the molecular docking studies for the potent anti-cancer drugs iressa, tarceva and capsaicin against the breast cancer treatment. The estimated free energy of binding and inhibition constant are highly differed with each drugs compared to the current market available drugs and bioactive compounds. Our results strongly suggest that the bioactive compound capsaicin activity would be comparable with the commercially available cancer drug. Further study indicates that *in silico* method would be an important tool for the drug design and development against cancer.

Key words: Epidermal growth factor receptor (EGFR), inhibitors, docking studies, protein tyrosine kinases (PTKs), breast cancer.

INTRODUCTION

The development of tyrosine kinase inhibitors has become an active area of research in pharmaceutical science. Epidermal growth factor receptor (EGFR) that plays a vital role as a regulator of cell growth is one of the intensely studied tyrosine kinase targets of inhibitors (Carpenter and Cohen, 1990; Yarden and Sliwkowski, 2001; Cohen et al., 1982; Yarden and Schlessinger, 1982, 1987). EGFR is over expressed in numerous tumors, including those derived from brain, bladder, lungs, breast, colon, neck and head. EGFR hyper activation has also been involved in other diseases including, psoriasis polycystic kidney disease and asthma (Albuschat et al., 2004; Bridges et al., 1996; Ma et al., 2005). Since the hyper activation of EGFR has been associated with these diseases, inhibitor of EGFR has potential therapeutic value and it has been extensively studied in the pharmaceutical industry. One could not, however, confirm that the compounds designed would always possess good inhibitory activity to EGFR, while experimental assessments of inhibitory activity of these compounds are

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compounds are time-consuming and expensive. Consequently, it is of interest to develop a prediction method for biological activities before the synthesis. Many of the tyrosine kinase enzymes are involved in cellular signaling pathways and regulate key cell functions such as proliferation, differentiation, anti-apoptotic signaling and neurite outgrowth. Unregulated activation of these enzymes, through mechanisms such as point mutations or over expression, can lead to a large percentage of clinical cancers (Yarden and Sliwkowski., 2001; Thompson et al., 2011). The importance of tyrosine kinase enzymes in health and disease is further under-scored by the existence of aberrations in tyrosine kinase enzymes signal occurring in inflammatory diseases and diabetes. Inhibitors of tyrosine kinase as a new kind of effective anticancer drug are important mediators of cellular signal transduction that affects growth factors and oncogenes on cell proliferation (Saloman et al., 2000; Harris et al., 1989). In the present investigation, we have taken two commercially available compounds as well as select a bioactive compound for docking analysis.

MATERIALS AND METHODS

EGFR crystal structure selection

Various different X-ray crystal structures were available in the Protein Data Bank server. Using model evaluation method that involved analysis of stereochemistry and overall quality factor distribution identified the best crystallographic structure of EGFR kinase protein. The stereochemistry of protein structure quality and verification in both side and main chain was checked by employing WHAT_CHECK, PROCHECK, ERRAT, VERYFY_3D and PROVE. Following this server helps to check the stereochemical quality of a protein structure by analyzing residue-by-residue geometry and overall structure geometry and checking of many sterochemical parameters of the residues in the protein model. It also analyzes the statistics of non-bonded interactions between different atom types and plots calculated by a comparison with statistics from highly refined structures, determines the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class based on its location and environment (alpha, beta, loop, polar, nonpolar etc), calculates the volumes of atoms in macromolecules using an algorithm which treats the atoms like hard spheres and calculates a statistical Z-score deviation for the model from highly resolved (2.0 Å).

Ligand structure and pharmacophore preparation

There are eleven commercial and bioactive compounds available as drugs for breast cancers. Information about them were downloaded from pubchem (http://pubchem.ncbi.nlm.nih.gov/). All the compounds were prepared by using LigX module in Molecular Operation Environment (MOE) (2009) version. LigX is a strong collection of conducting interactive ligands modification and energy minimization in the active site of a flexible receptor. The verification of drug ligand step will isolate all such molecules. The chosen drug ligand were identified to be treated as though they were part of the receptor. The LigX/protonate application was used to add hydrogen atoms according to the ionization state of the current atom. The heavy atoms are then fixed and a brief energy minimization is conducted to refine the positions of the added hydrogen atoms. The protonate 3D application is used to add hydrogen atoms. The ligands preparation was based on geometry optimization method and charge calculation method, a pH of 7.6 was used. Pharmacophore function group predictions were predicted from LigandScout, this also analyzed the collection of chemical features that characterize a specific mode of interaction of a drug in the active site of the macromolecule in 3D space. Examples of chemical features include charge interactions, hydrogen bonds and hydrophobic areas. It also includes unfavorable steric interactions which can be the optimization of a potential drug, which also analyzes pharmacophoric elements. Also we analysed hydrogen bond donor, hydrogen bond acceptor, positive ionizable area, negative ionizable area, hydrophobic interactions, aromatic ring, metal binding feature and excluded the volume.

Protein structure preparation

One of the best x-ray crystal structure was selected for the docking simulations and prepared using ‘protein preparation module’ (Berman et al., 2000; Berman, 2008; Hooft et al., 1996). The hydrogen atoms were added and unwanted water molecules were removed from the target protein; EGFR partial charges and atom types were assigned by using MOE. Automatically, type of information was connected and assigned using element and coordinate information. Hydrogen and refine/relax structures was added using AMBER '89, '94, MMFF94 or Engh-Huber parameters augmented with an implicit solvent model Energy minimization which was done until the average Root-Mean-Square Deviation (RMSD)of non-hydrogen atoms was reached

Binding site prediction

The active sites were predicted by using MOE. An alpha shape algorithm is used to determine potential active sites in 3D protein structures (Westbrook et al., 2005). We analysed active site of a receptor, protein surface calculations and of course molecular docking used to search for favorable binding configurations between one or more small, flexible ligands and a protein target. These are most likely to contribute to tight protein-ligand binding. Typically, scoring functions emphasize favorable hydrophobic, ionic and hydrogen bond contacts. By using this information, we detected the candidate of protein-ligand binding sites using a fast geometric algorithm, based on Edelsbrunner's alpha shapes. EGFR binding site on a macromolecular structure is ranked according to its accessible hydrophobic contact surface and active sites analysis to identify polar, hydrophobic, acidic and basic residues. Visualize solvent exposed ligand atoms and residues in close contact with ligand atoms as well as side chain and backbone acceptor.

Molecular docking method

The bioactive compound and the other known drugs were docked into the active site of the receptor (PDB ID: 1XKK) protein docking server (http://www.dockingserver.com/web). This server is based on AutoDock version 4 (Schames et al., 2004). This application was employed for accurate ligand geometry optimization, energy minimization, charge calculation, docking calculation and protein-ligand complex representation. It is shown that more accurate partial charge calculation and as a consequence, more accurate docking can be achieved by using quantum chemical methods. For docking calculations, quantum chemical partial charge calculation as a routine was only used for ligands and also, the use of application MOPAC2009 allowed fast partial charge calculation of

Table 1. Docking results in between EGFR (epidermal growth factor receptor kinase) and commercially available.

Compound	Est. Free Enery of Binding (Kcal/mol)	Est. Inhibition constant. Ki (µM)	Vdw+Hbond+desolv energy (Kcal/mol)	Total Interamolecular energy (Kcal/mol)	Interact surface
Iressa (drug)	-8.79	358.75	-11.33	-11.24	981.316
Lapatinib (drug)	-8.33	779.60	-11.78	-11.78	1201.519
Capsaicin	-6.84	9.62	-8.85	-8.95	796.202

proteins by quantum mechanical semi-empirical methods.

RESULTS AND DISCUSSION

First we validated the protein structure generated from our modeling study. For that purpose for the first step, the model protein x-ray crystal structures from Brookhaven Protein Data Bank was extracted (Wolber and Langer, 2005). Further, we used the following programme such as ERRAT, VERYFY_3D, PROCHECK, PROVE and WHAT_CHECK to validate and find the best model. Specifically, the following software and online tools such as SMART Server (EMBL) were used for domain identification, FISH Server for domain analysis and SAVES Server for procheck, to verify 3D and error checking.

Our results suggest that the protein 1XKK crystal structure was good enough to proceed for further docking analysis based on stereochemistry and overall quality factor among six EGFR crystal structures. ERRAT is a best protein structure verification algorithm that was especially well suited for evaluating the progress of crystallographic model building and refinement. This program was worked by analyzing the statistics of non-bonded interactions between different types of atoms. A single output plot was produced that gives the value of the error function versus position of a 9-residue sliding window. By comparison with statistics from highly refined structures, the error values have been calibrated to give confidence limits. This is extremely useful in making decisions about the reliability. After confirming the quality of the modeled structured, we carried out the molecular docking analysis with different bioactive compounds with our model. Our model structures of EGFR do not have any substrate or co-crystallized ligand, so the binding site was predicted by using MOE Site-finder. The site-finder generates information on the character of binding sites using novel search and analysis facilities, and provides site points for ligand binding.

Based on the docking result, it was found that phytochemical of the bioactive compound of capsaicin possess a high docking score of estimated free energy of binding (-6.84 Kcal/mol) and good estimated inhibition constant, Ki (9.62 µM), other bioactive compound, such as lapatinib and iressa drug showed similar docking score of binding free energy (-8.033 and -8.79 Kcal/mol, respectively) with good interactions, respectively (Table 1). The phytochemical of bioactive compounds lapatinib, capsaicin and iressa were predicted by pharmacophore (Figure 1).

Based on the docking score, Hydrogen Bond Acceptor (HBA) and Hydrogen Bond Donor (HBD) bonding and energy, our results suggest that the natural derivatives of capsaicin showed better scoring function than the commercially available drugs like lapatinib and iressa (Figure 2). Despite the intensive efforts and substantial advances that have occurred through focusing on improving treatments, cancer is still a leading cause of death worldwide. Our data shows that phytochemicals with precisely defined best target identification can be linked to successful drug discovery. The MOE molecular modeling software was used to search for three-dimensional shape complementarity and to examine the common elements of ligands with a reference molecule. Similar chemicals are identified and then 'docked' into the target protein by computer, enabling detailed protein-ligand interactions to be obtained. All these three approaches require the computational processes of docking and scoring using known and hypothetical drug targets on a protein, coupled with databases of virtual chemical compounds. The molecular docking will determine the binding interactions between two molecules either protein to protein or protein to ligand.

Once a compound is docked, it is then scored using mathematical models. Scoring estimates the chemical interactions, such as binding strength and energy state, between the ligand and protein to assist in the in ranking the efficacy of the compound being scored (Nagarajan et al., 2009; Perez-Sanchez and Wenzel, 2011; Oi et al., 2010). From these approaches, several potential candidate phytochemicals that directly interact with target proteins can be identified in future for the development of effective drug against cancer. Bioinformatics approach would help and provide an enhanced approach to personalized cancer drug development.

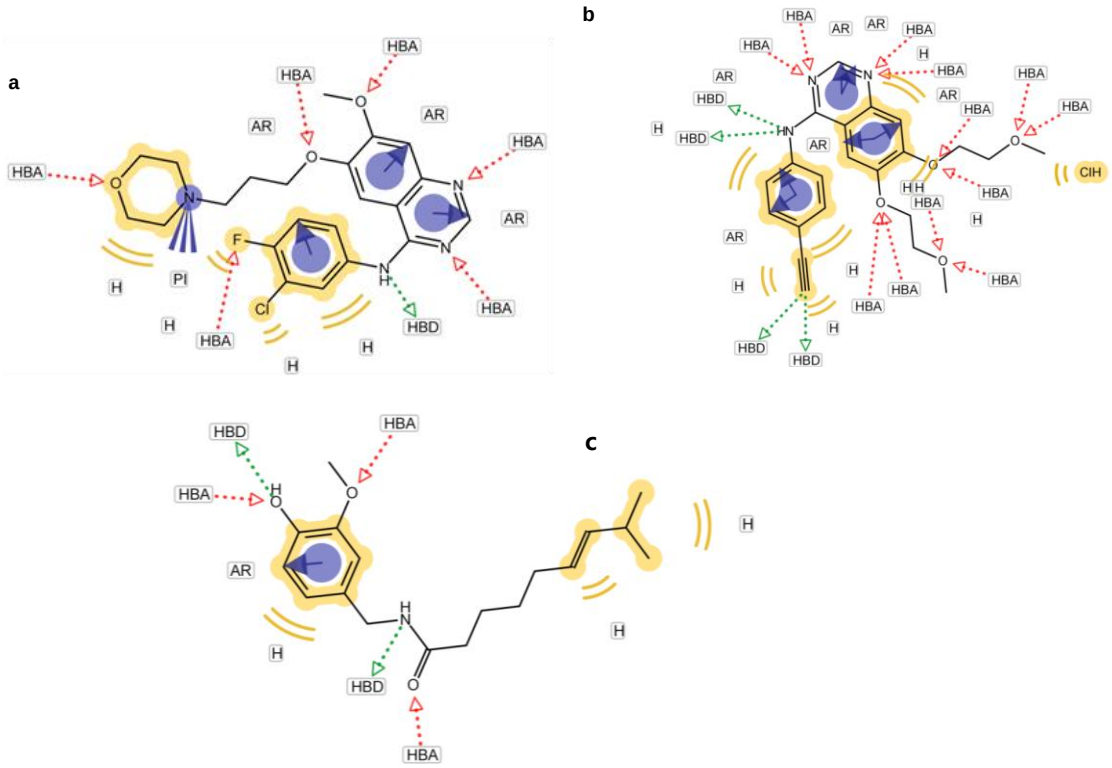

Figure 1. Pharmacophore structure prediction of drugs and bioactive compounds. A) Iressa, B) Tarceva, C) Capsaicin.

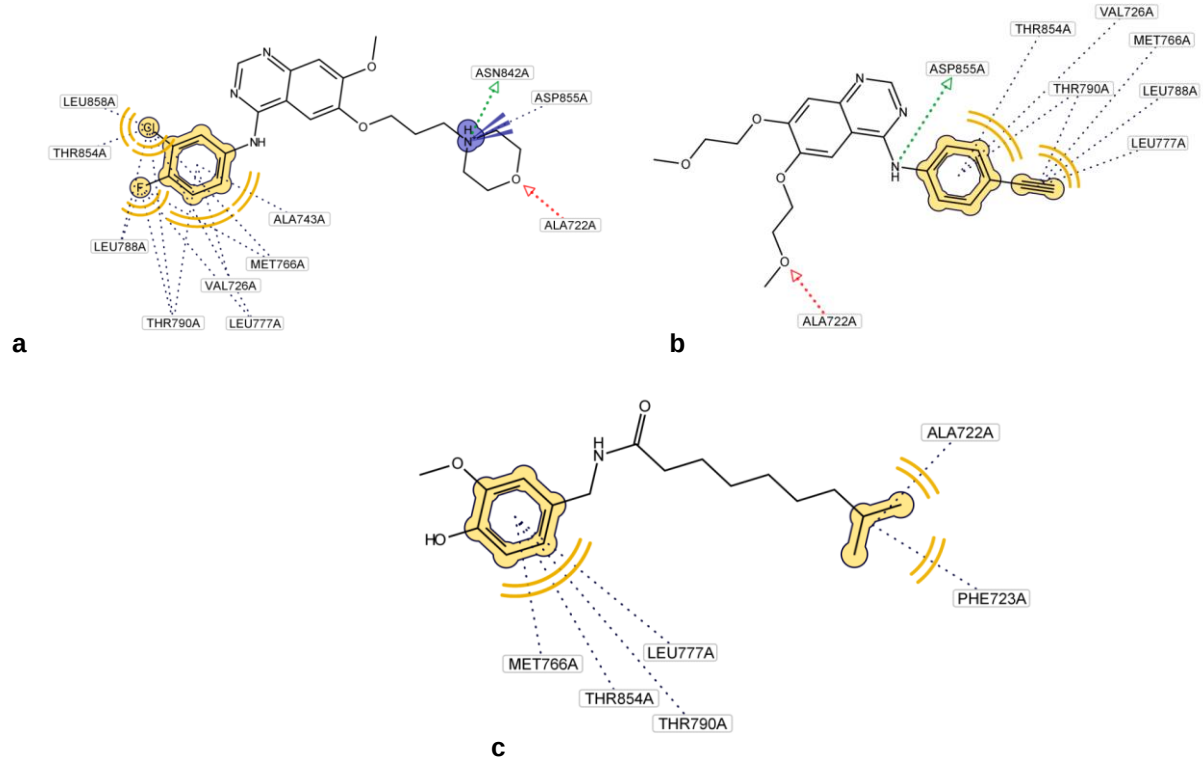

Figure 2. Active binding site and interacts residues in both drugs and bioactive compounds. (A) Iressa, (B) tarceva and (C) capsaicin.

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Full Length Research Paper

Enhanced selectivity approach for fast analysis of enalaprilat, lisinopril and benazepril in pharmaceutical dosage forms and spiked human plasma by ion chromatography

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A simple and highly selective method for determination of enalaprilat (ENT), lisinopril (LSP) and benazepril hydrochloride (BZP) in pharmaceutical dosage forms as well as spiked human plasma by ion chromatography with UV detection has been developed. ENT and LSP are compounds with little inherent hydrophobicity that make their analysis using HPLC essentially sophisticated necessitating either complex extraction procedures or especial detection methods. The method proposed is based upon ionization of those two drugs in basic medium to be well retained and readily resolved as dicarboxylic acid anions using IonPac AS11 (13 µm particle size, 4 × 250 mm, Dionex) anion exchange column adopting a very simple clean-up procedure via a single-step protein precipitation with UV detection at 215 nm. The three drugs were successfully determined in pharmaceutical dosage forms as well as in spiked human plasma with tyrosine as internal standard with a recovery approaching 100%. Adopting this proposed procedure, the analytes produce well shaped peaks with good linear relationship over the investigated concentration ranges and values of (r) higher than 0.998 for all drugs. ion level of200 ng ml^{-1} of the three drugs, therefore being satisfactory for their purposed analysis. The method was validated with respect to specificity, recovery, accuracy, precision and linearity. Moreover, the method could be applied to the determination of the drugs in pharmaceutical preparations. The method was validated with respect to specificity, recovery, accuracy, precision and linearity and the method proved applicable for routine fast analysis of the studied drugs.

Key words: Benazepril, enalaprilat, lisinopril, ion chromatography, pharmaceutical, plasma.

INTRODUCTION

Angiotensin converting enzyme (ACE) inhibitors have achieved widespread usage in the treatment of cardiovascular and renal diseases. ACE inhibitors alter the balance between the vasoconstrictive, salt-retentive, and hypertrophic properties of angiotensin II and the vasodilatory and natriuretic properties of bradykinin and alter the metabolism of a number of other vasoactive substances. They are especially important because they have been shown to prevent early death resulting from hypertension, heart failure or heart attacks (Brown et al., 1998).

Enalaprilat (ENT), lisinopril (LSP) and benazepril hydrochloride (BZP) are important members of this group that is nowadays being massively administrated for a variety of cardiovascular and renal disorders increasing the need for more convenient methods for their investigation and monitoring in their dosage forms and biological fluids. A wide range of methods were reported in the literature for their analysis in pharmaceutical formulations and biological fluids. These include spectrophotometric (Belal et al., 2000; Bonazzi et al., 1997; El-Emam et al., 2004; El-Gindy et al., 2001; El- Yazbi et al., 1999),

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spectrofluorimetric (Aktas et al., 2003; El-Gindy et al., 2001; El-Yazbi et al., 1999), bioanalytical (Shepley et al., 1988; Yuan et al., 1996), voltametric (Belal et al., 2001), high performance liquid chromatographic (HPLC) followed by spectrophotometric detection (Abou-Basha et al., 2003; Bonazzi et al., 1997; Cirilli and La Torre, 1998; El-Gindy et al., 2001; Wang et al., 2007) or spectrofluorimetric detection (El-Emam et al., 2004; Sagirli and Ersoy, 2004) and capillary electrophoresis (Gotti et al., 2000) methods. Gas (Leis et al., 1995; Xiao et al., 2005) and liquid (Huang et al., 2006; Kousoulos et al., 2005; Leis et al., 1999; Lu et al., 2009) chromatography–mass spectrometry methods were widely applied to ACE inhibitors and their metabolites determination in biological fluids due to their high sensitivity and selectivity.

Although HPLC is a commonly used method in bioanalytical laboratories (Ahmad et al., 2011; Khan et al., 2011; Nama et al., 2011; Prakash et al., 2011), it is difficult to develop an HPLC method for ENT and LSP pharmacokinetic application, due to their low plasma concentration and endogenous interference.

EXPERIMENTAL

Regents and materials

Benazepril HCl, benazeprilat, enalaprilat, and lisinopril were all purchased from China's National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol was obtained from Tedia Company (Fairfield, USA). Analytical reagent grade sodium hydroxide was obtained from Sinopharm Chemical Reagent Co., Ltd (China). Acetic acid (HAC) was delivered from Jinlu Chemicals (Shanghai, China). Ammonia solution was obtained from Jinsha Chemicals (Shantou, China). Tyrosine internal standard (I.S.) was bought from Kang DaAmino Acids Co. (Shanghai, China). Ultra-pure water was prepared by the Millipore Milli-Q Academic water-purification system (Molsheim, France). Drug products were bought from the local market. Drug-free human plasma was supplied by the Blood Bank of Zhejiang Province. Waters Oasis® (3 cc/60 mg, 30 µm) HLB (hydrophilic–lipophilic balanced) reversed-phase sorbent solid phase extraction cartridges (Milford, USA) was used to isolate the drugs from plasma samples.

Preparation of drugs stock solutions, calibration standards, quality-control samples and other reagents

Standard stock solutions of each analyte and the IS were prepared separately as 20 mg% in distilled water and stored at 4°C for a maximum of two weeks. Working solutions were prepared by appropriate dilution of stock solutions using distilled water and stored at 4°C. Calibration standards in the concentration range from 0.2 to 4 µg ml^{-1} for the three drugs were prepared by spiking 0.45 ml of blank plasma with 50 µl of the standard drugs mixture solution. QC samples were prepared in bulk at the concentration of 0.2, 1 and 4 µg/ml and stored at -20°C. 50 mM HAC and ammonia solution were prepared by appropriate dilution of the calculated volumes into water

Sample preparation

Drug formulations

A powdered sample of each drug tablets was extracted for 15 min by ultrasonication using distilled water and then completed to volume. For ENT injection, an accurately measured volume was directly diluted with deionized distilled water. The content of the drug samples was calculated by referring to its linear regression equation obtained from analysis of their respective standards.

Analysis of spiked human plasma samples

Adopting protein precipitation procedure: Aliquots of 500 µl plasma samples were mixed with 25 µl IS solution (10 µg ml^{-1}) followed by 475 µl methanol being added. The mixture was vortex mixed for about 30 s before being centrifuged at 10000 G for about 20 min. Then, an aliquot of 800 µl of the clear supernatant were pipetted and was evaporated under vacuum in a stream of nitrogen at about 40°C and the residue was dissolved in 200 µl water. The sample was filtered through a 0.22 um syringe filter and an aliquot of 20 µl were directly injected to the system.

Adopting solid phase extraction (SPE) procedure: To each 500 µl plasma samples, 50 µl IS solution (2.5 µg ml^{-1}) and 550 µl 50 mM HAC were added in a 4 ml polypropylene tube. The mixtures were vortex-mixed for 30 s, and then loaded to the preconditioned SPE cartridge. The cartridge was preconditioned by washing with 2 ml water, 2 ml methanol then another 1 ml water and finally 1 ml 50 mM HAC. After loading the sample, the cartridge was washed by 1 to 2 ml 50 mM HAC then 1 ml 20% methanol in 50 mM HAC. The sample was then eluted using 1 ml 10% 50 mM ammonia in methanol. The collected eluate was evaporated under vacuum in a stream of nitrogen at about 40°C and the residue was dissolved in 125 µl water. The sample was filtered through a 0.22 um syringe filter and an aliquot of 20 µl were directly injected to the system.

Chromatographic system

The chromatographic system was Dionex Ultimate 3000 series (USA) equipped with a quaternary pump, a temperature controlled auto injector. The Ultimate 3000 quad-wavelength ultraviolet detector was set at 215 nm for all drugs. The separation was performed at ambient temperature on a Dionex IonPac AG11 guard column (13 µm particle size, 4 × 50 mm) and a Dionex IonPac AS11 analytical column (13 µm particle size, 4 × 250 mm). The data collection and analysis were run with Dionex Chromeleon Client Software (USA). The mobile phase consists of 30 mM NaOH, methanol and water (50:36:14) in isocratic mode at a flow rate of 1.0 ml/min.

Assay validation

The extraction recoveries of each drug and I.S. were determined by comparing the peak area of extracted spiked samples to those of standard solutions at same concentration. Calibration curves were constructed with plasma standards spiked with 0.2, 0.5, 1, 2 and 4 µg/ml of each drug. During the method validation, calibration standards were independently prepared and measured on 3 consecutive days. The intra-day and inter-day precision and accuracy were determined based on Quintuplicate measurements of QC samples at low, middle and high concentrations (0.2, 1 and 4 µg mL^{-1}). All samples were spiked with the drug standards on Day 1, and then analyzed on five different days. The drugs concentrations

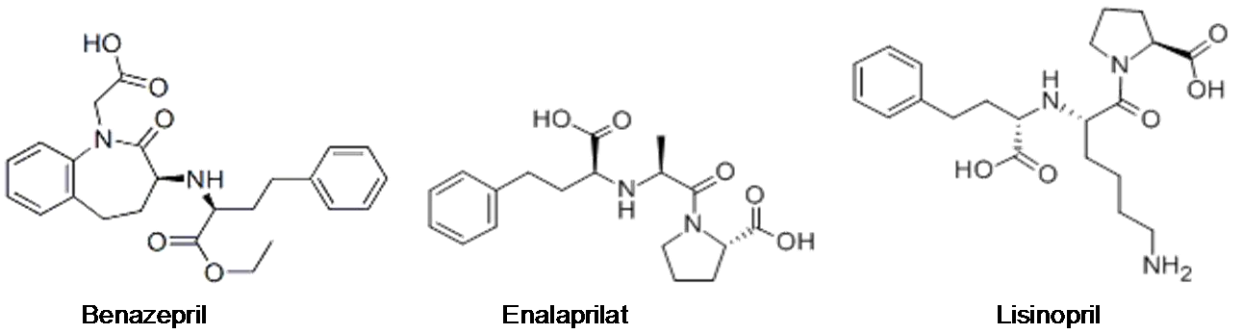

Figure 1. Chemical structures of the studied drugs.

in QC samples were calculated from the linear regression equation obtained on the same day.

RESULTS AND DISCUSSION

LSP per se and ENT as a metabolite of the prodrug enalapril are amphoteric in nature having both acid carboxylic and basic amino group functionalities in their structure (Figure 1). The pKa of their carboxylic moieties lying in the range of 2 to 4 (Lemke and Williams, 2008) indicate significantly ionizable acidic functions. In alkaline pH, they behave as dicarboxylic acids with substantial anionic nature that keep them highly retained on anionic exchange columns giving the chance for their selective retention and subsequent analysis. The previously mentioned drugs were sufficiently polar to be eluted in the first part of the chromatograms in most published HPLC methods even with a small percentage of organic modifiers. This makes their analysis a point of sophistication due to their subject of interference from endogenous components in relatively complex matrix and their significant loss from most like plasma with a very simple protein precipitation clean up procedure using methanol. Alternatively and for more elegant chromatograms, Water Oasis HLB sorbent cartridges were used for sample purification. The unique outcome of using this sorbent for extracting those relatively polar compounds is the promising recovery values as well as enhanced extraction selectivity with consequent optimum resolution. The procedure was so simple to be applied for their routine analysis in less than 5 min.

Development of the IC method

The IonPac AS11 Analytical Columns are specifically designed to resolve a large number of inorganic anions and organic acid anions from a single sample injection in one run using hydroxide eluent systems. AS11 columns are stable between pH 0 and 14 and are compatible with eluents containing 0 to 100% organic solvents (Dionex Reference Library ver. 33 (CD-ROM), 2008). At moderately high pH values, the amine functionality of the studied drugs is deprotonated and the carboxyl group is ionized and free for electrostatic interaction with available positively charged ionizable sites (alkanol quaternary ammonium groups) of the column stationary phase resin. Thus, NaOH solution was chosen as mobile phase providing the optimal experimental conditions in terms of pH and solution compatibility for acceptable resolution via anion-exchange chromatography and satisfactory detection. In order to evaluate the effect of the hydroxide concentration on the retention times, a standard mixture of the drugs was eluted isocratically with mobile phase containing various concentrations of NaOH. Only, ENT and LSP were eluted as broad markedly tailed peaks and their retention time as expected being decreased with increasing hydroxide concentration. Therefore, methanol was added to the mobile phase in order to modify the hydrophobic interactions of the analytes giving more symmetric Gaussian shaped peaks necessary for reproducible retention and optimum quantitation. Only 10% was found enough for that purpose; however, 36% was used to permit elution of BZP as a well resolved peak in a reasonable time together with the other drug peaks. BZP is mainly retained according to its high hydrophobic properties; nevertheless its anionic characters are also considered as could be indicated by the inability of 50 or even higher percentages of methanol in water mixtures to affect its elution from the column without a specific anion eluent like hydroxide. By performing the chromatographic separation using 30 mM NaOH, methanol, water (50:36:14) as mobile phase, the selected drugs were separated within less than 5 min with a good S/N ratio considering the column efficiency and UV detector reliability. The brilliant advantage lying in choosing AS11 column is its strong anion exchange capacity in accordance with very low hydrophobicity enhancing selectivity and affecting superior resolution of anionic drugs from hydrophobic and neutral compounds. A typical chromatogram obtained with an IonPac AS11 analytical column and UV detection at 215 nm for the selected analytes together with tyrosine (I.S.) is shown in Figure 2.

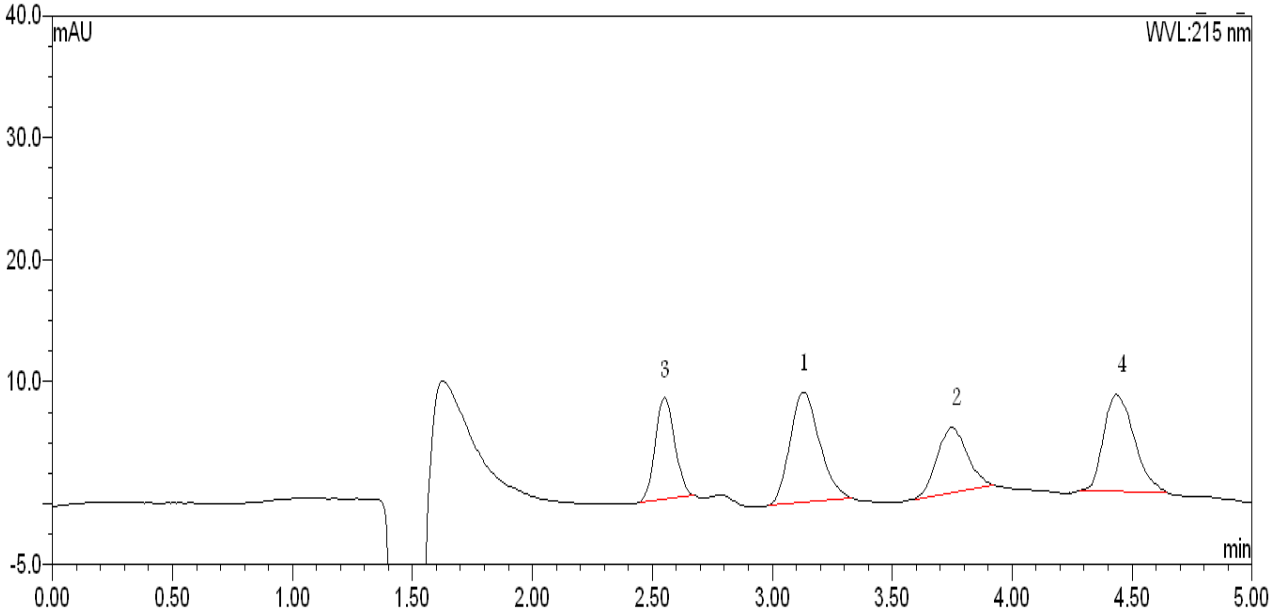

Figure 2. Representative typical IC-UV chromatogram of a combined standards solution, 2 µg mL^{-1} each; I.S. 1 µg mL^{-1}.BZP, 1; ENT, 2; LSP, 3; I.S., 4.

Applications

The developed IC method was applied for the determination of the studied drugs in their commercial tablets and results were found in good agreement with the claimed content. Concentrations of the drugs were calculated by referring to the calibration equations obtained for pure standards shown in Table 1. Results for the analysis of different tablet dosage forms are shown in Table 1. The chromatograms obtained with BZP and LSP tablets as well as ENT injection were shown in Figure 3. On the other hand, the method can differentiate intact BZP dosage forms from degraded ones. Benazeprilat (BZT), the main degradation product of BZP (Gana et al., 2002), was not eluted within at least 10 min under the proposed experimental conditions. This should be explained on the basis of more anionic features of the degraded product with respect to intact one due to one additional carboxyl group being produced during degradation pathway. This could be a great advantage for quality control and drug evaluation protocols. Unfortunately, Diketopiperazines of ENT and LSP as their main degradation products were not available to be tried in order to investigate the capability of the method to evaluate the drugs stability in their dosage forms.

Analysis of spiked human plasma was also successfully conducted. Calibration graphs were constructed for the studied drugs in spiked human plasma samples according to the proposed procedures detailed under the experimental section. Results are summarized in Table 2. Adopting the protein precipitation procedure, lower concentrations were also tried and better sensitivity and detection limits for the drugs in plasma samples could be achieved by reconstituting drug residues after extraction and evaporation in lower injection volumes such as 100 µl but for more convenience and better baseline 200 µl was chosen (Figure 4). In addition, adopting the same procedure, some endogenous compounds in plasma interfered with first eluted drug LSP, however due to the great difference in their inherent anionic characters, the drug could be easily resolved from these components by decreasing the percentage of NaOH used in the mobile phase from 50 to 25% (Figure 5). Still for other drugs, 50% NaOH was preferred for faster, sharper, easier and more accurately quantified peaks. Using 25% NaOH, there was interference with tyrosine peak while that of ENT became a little bit broader.

However, the three drugs can be determined simultaneously adopting the HLB SPE extraction procedure with even much better quantification limits. Oasis HLB is a Hydrophilic-Lipophilic-Balanced, water- wettable, reversed-phase sorbent. It is made from a specific ratio of two monomers, hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. It provides superior reversed-phase capacity with a neutral polar “hook” for enhanced retention of polar analytes (Waters Corporation, 2008). This extraordinary retention of polar compounds, compared to traditional silica-based SPE sorbents like C18 gives rise to optimal recovery values as well as much better confident sample clean-up as a result of an extra washing step using 20% methanol solution. This washing step is prohibited using C18 SPE sorbents due to serious loss of sample with little or even no drug recovery. A comparison between the drugs recovery from extraction

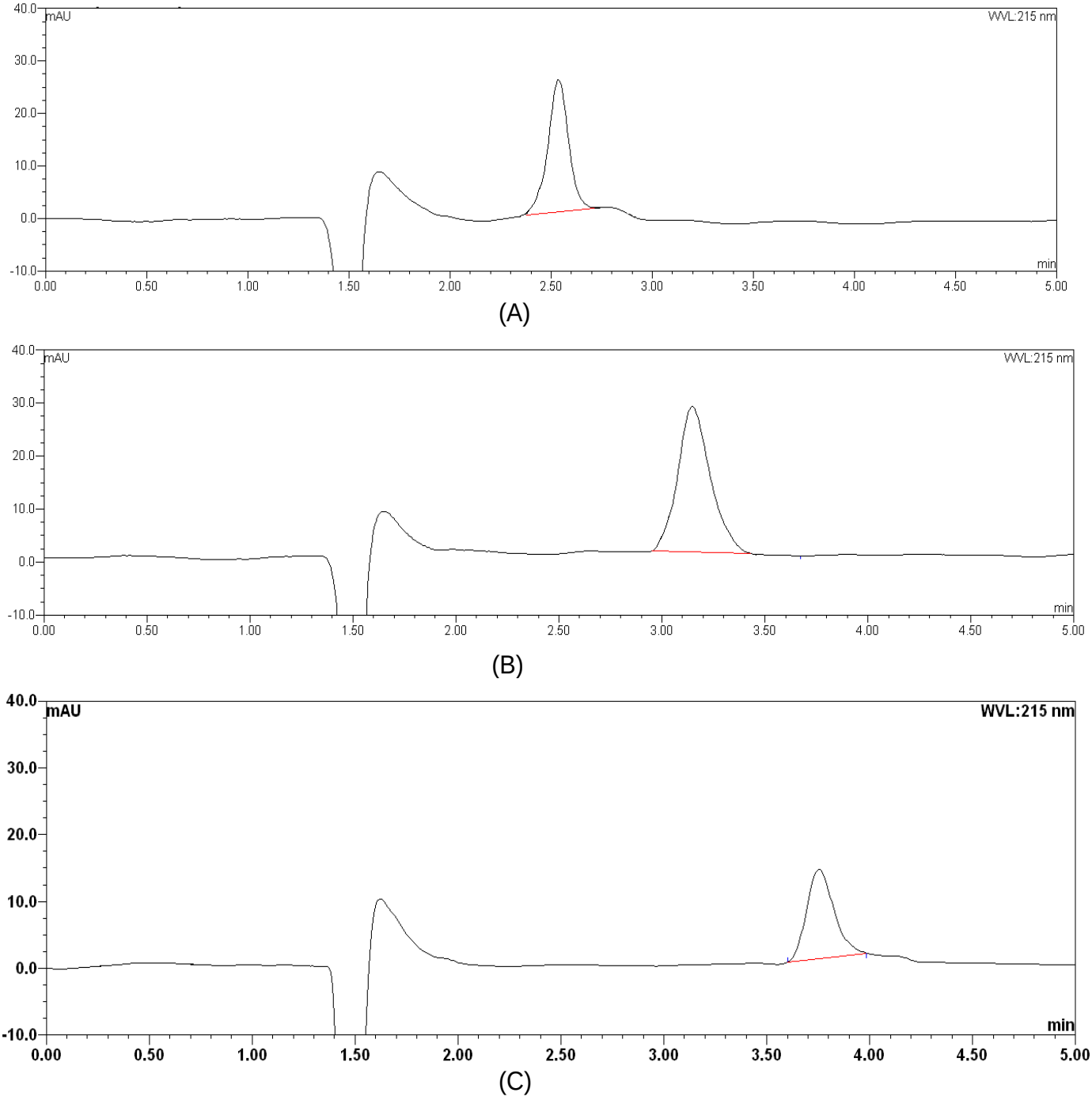

Figure 3. Representative typical IC-UV chromatograms of 8 µg ml^{-1} LSP (A) and BZP (B) in tablets and 5 µg ml^{-1} ENT (C) in injection dosage forms.

extraction using HLB and C18 cartridges is shown in Figure 6. These outcomes, in addition to obviating the need for complex derivatization steps have a distinct impact on the method sensitivity with even much less quantification limits. The chromatogram obtained using waters Oasis HLB SPE cartridge was shown in Figure 7.

Assay validation

Specificity and recovery

Representative chromatograms of blank plasma samples as well as their spiked parallels were shown in Figures 4,

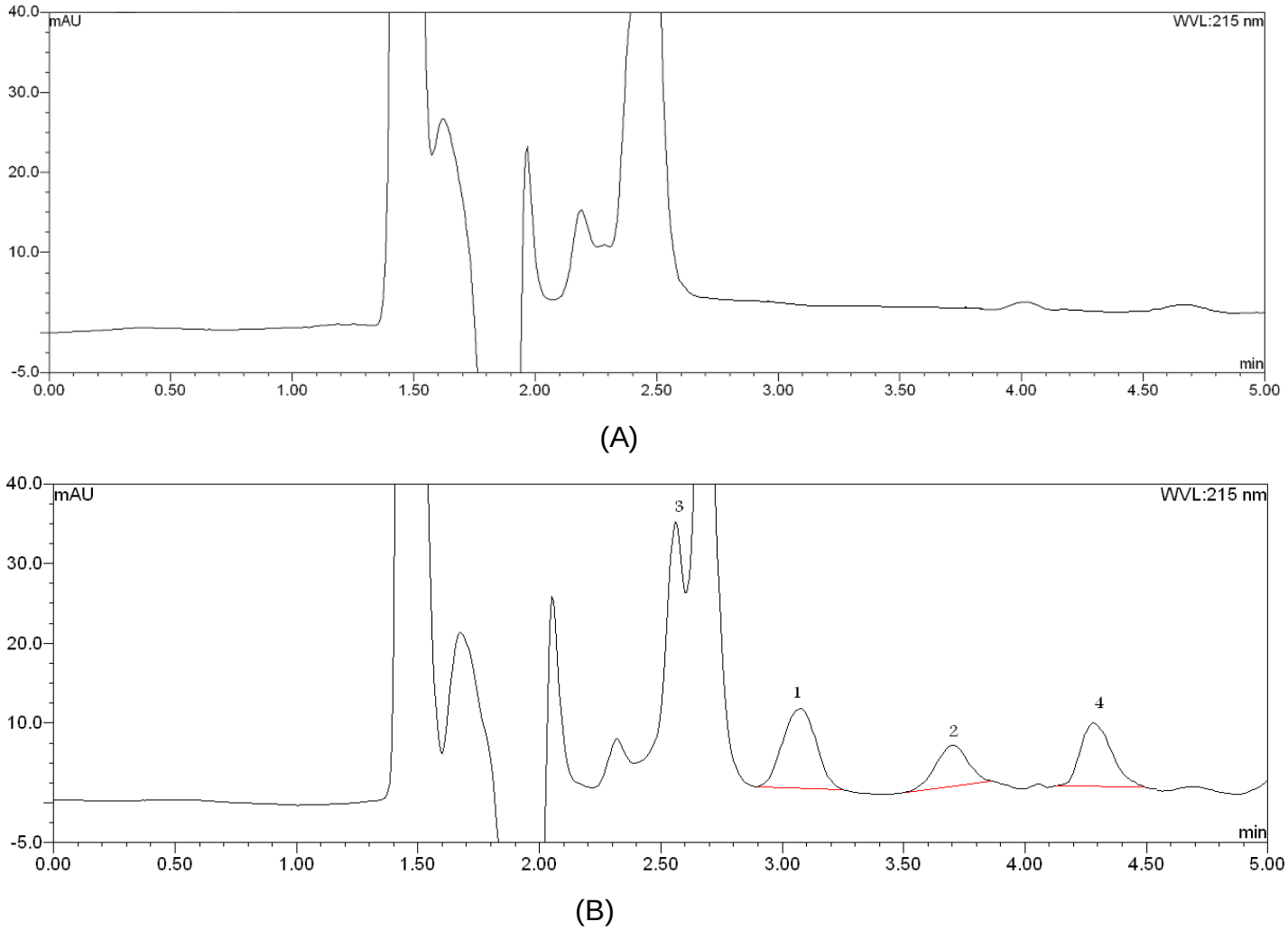

Figure 4. Representative typical IC-UV chromatograms of a blank human plasma sample from a healthy human volunteer (A) and that spiked with a combined Drugs standard mixture solution, 1 µg mL^{-1} each, (B) prepared according to the protein precipitation procedure. Mobile phase composition: 30 mM NaOH, methanol and water (50:36:14). BZP, 1; ENT, 2; LSP, 3; I.S., 4.

Calibration graphs

Regression parameters for drug standard mixtures including the slopes intercepts and regression coefficients are summarized for all the analyzed drugs in Table 1. These regression parameters were used for calculation of drugs concentrations in their pharmaceutical tablets.

For the analysis of the studied drugs in plasma samples, an internal standard was necessary to compensate for any variability of the extraction recoveries during sample preparation. Calibration equations were obtained by plotting each drug peak area to IS ratio vs. the concentration of each drug. In all cases, the intercepts were not significantly different from zero and correlation coefficients were greater than 0.998. Lower limits of detection (LOD) and quantification (LOQ) were also calculated from the obtained chromatograms for a signal-to-noise ratio (S/N) of 3 and 10 (n=8), respectively. Results are shown in Table 2. This analytical performance in terms of detection limits is generally sufficient for analysis of LSP and BZP peak plasma levels after a single oral dose of 20 mg or ENT after its IV injection, especially if some procedure of enhancing the sensitivity like post extraction sample evaporation was applied. The method was superior to other methods applying extensive derivatization techniques with more advantages of intact dug detection and enhanced drug selectivity and resolution. Another advantage is that the linear ranges showed good correlation coefficients (that is, >0.998) giving rise to excellent accuracy results for all drugs, this was referred to the super signal stability of the UV detectors if compared with other amperometric sensors or photometric detectors after pre-or post-column derivatization.

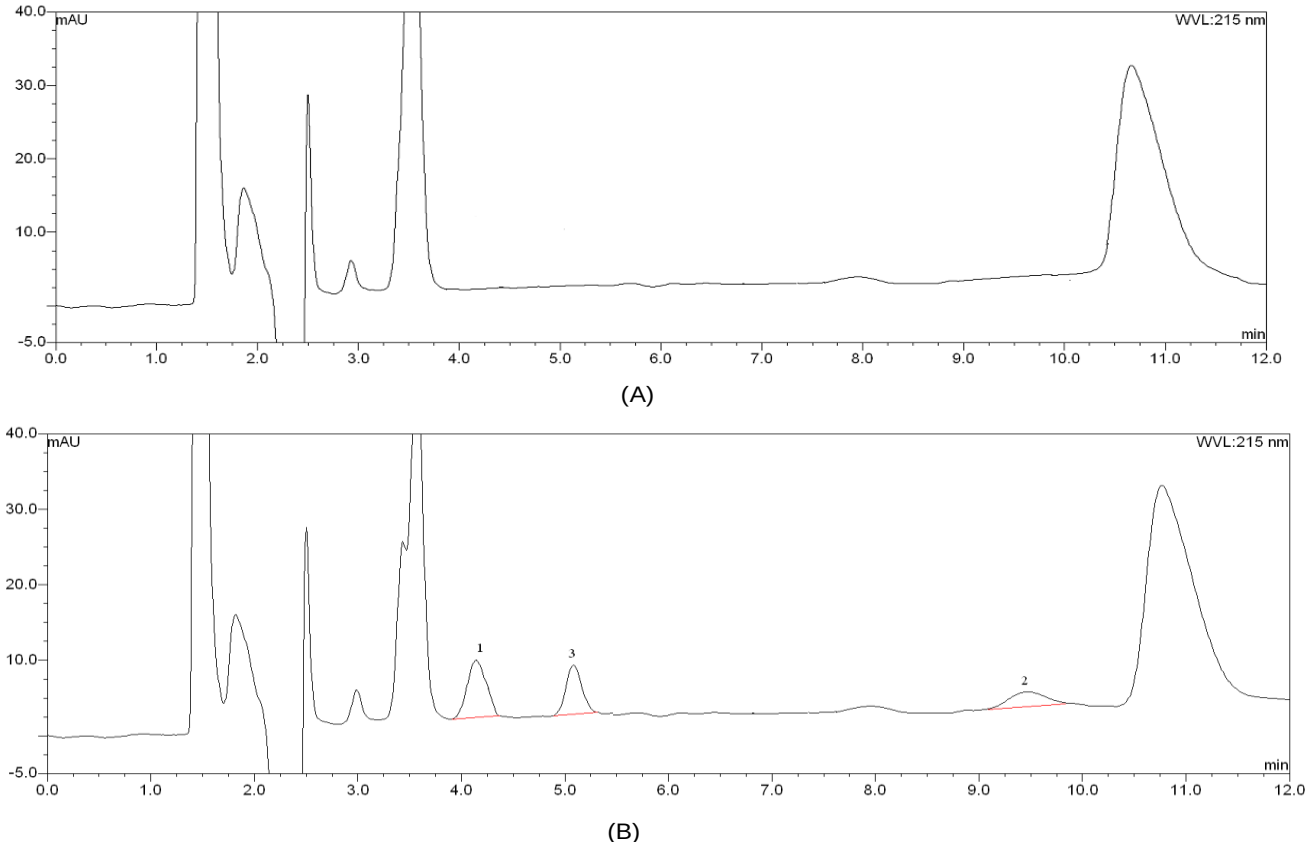

Figure 5. Representative typical IC-UV chromatograms of a blank human plasma sample from a healthy human volunteer (A) and that spiked with a combined drugs standard mixture solution, 1 µg ml^{-1} each, (B) prepared according to the protein precipitation procedure. Mobile phase composition: 30 mM NaOH, methanol and water (25:36:39). BZP, 1; ENT, 2; LSP, 3; I.S., 4.

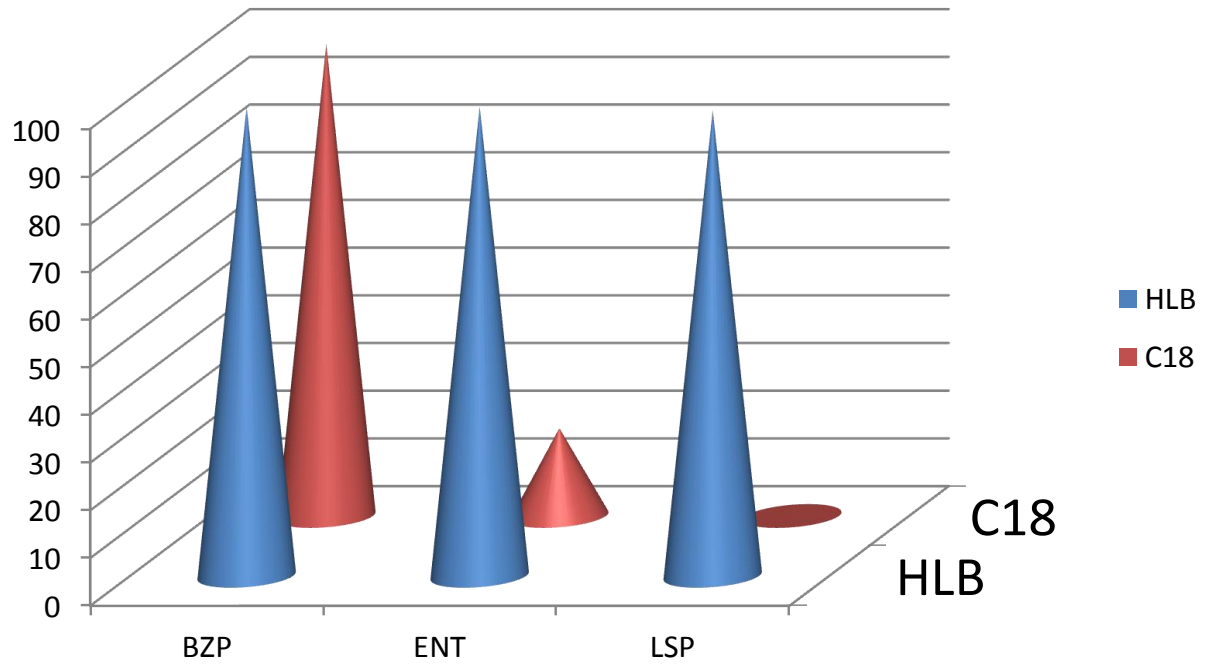

Figure 6. Comparison of the studied drugs recoveries from SPE extraction using (A) Oasis HLB sorbent, (B) C18 silica sorbent (300 mg/1 ml 40 µm particle size, Tianjin Fuji Science and Technology Co., Ltd, China) concentration = 1 µg ml^{-1} each.

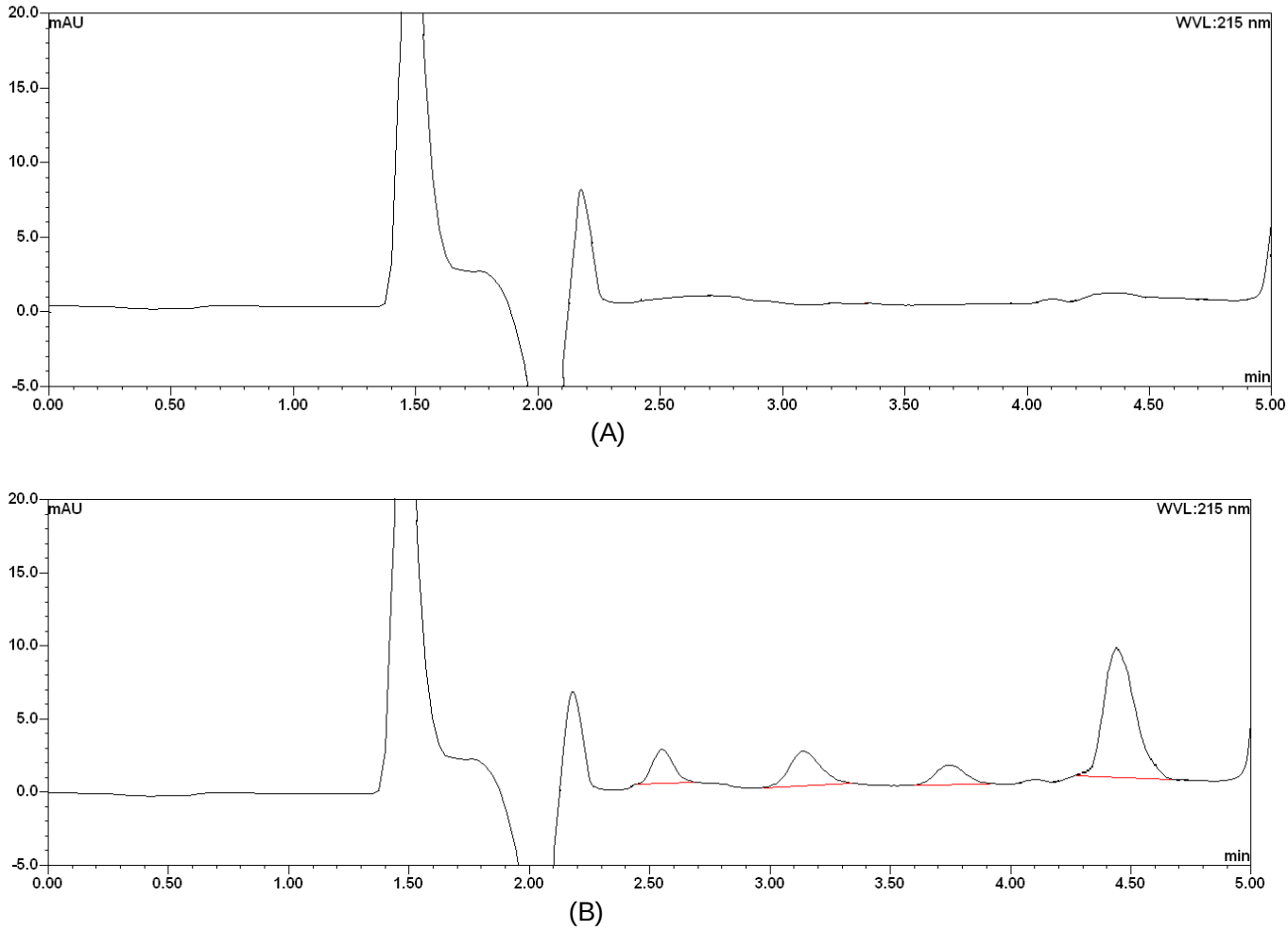

Figure 7. Representative typical IC-UV chromatograms of a blank human plasma sample from a healthy human volunteer; (A) and that spiked with a combined drugs standard mixture solution, 100 ng ml^{-1} each, (B) prepared according to the SPE procedure. Mobile phase composition: 30 mM NaOH:methanol:water (50:36:14) (other chromatographic conditions as described in the text). BZP, 1; ENT, 2; LSP, 3; I.S., 4.

Table 1. Collective calibration data for analysis of the studied drugs in their pharmaceutical formulations by the proposed method

Drug	Regression equation [a]		Correlation coefficient	Linear range (µg ml^{-1})	LOD [b] (ng mL^{-1})	LOQ [c] (ng mL^{-1})	Tablets	
	b	a					% Recovery[d]	% RSD
BZP	0.6626	0.0010	0.9998	0.12 - 10.00	35	120	99.65[e]	1.81
ENT	0.4007	-0.0033	0.9997	0.18 - 10.00	55	180	102.05[f]	2.01
LSP	0.4111	-0.0075	0.9995	0.16 - 10.00	50	160	101.01[g]	1.63

[a], With respect to A = bC + a, where C is the concentration in (µg ml^{-1}), A is the peak area, a is the intercept and b is the slope. [b], Lower limit of detection. [c], Lower limit of quantification. [d], The data are expressed as percentage of the claimed content and are average of 6 determinations for three concentration levels 2, 5 and 10 ug ml^{-1}. [e], Beijing Nuohua Pharmaceutical Limited Corporation under license from Novartis, Switzerland. (Batch no. X1338). [f], Changzhou Pharmaceutical Factory Co., Ltd., China. (Batch no. 09032311). [g] Livzon Group New Beijiang Pharmaceutical Co., LTD., China. (Batch no. 0908009).

Precision and accuracy

The intra- and inter-day precision and accuracy of the developed method were evaluated with five replicates of samples at concentration of 0.2, 1 and 4 µg/ml, and on five different days. The precisions ranged from 2.89 to 7.63% RSD for the three studied drugs at the three concentration levels which should be satisfactory to

Table 2. Results for the analysis of the studied drugs in their spiked human plasma samples.

Drug [a]	Concentration (µg ml $^{-1}$)	Protein precipitation procedure					HLB SPE extraction procedure				
		Accuracy (%) ± SD [b]	Precision R.S.D. (%)		LOD [e] (ng ml^{-1})	LOQ [f] (ng ml^{-1})	Accuracy (%)	Precision R.S.D. (%)		LOD (ng ml^{-1})	LOQ (ng ml^{-1})
			Intra-day [c]	Inter-day [d]				Intra-day	Inter-day		
BZP	0.2	102.52 ± 4.02	4.72	6.51	20	60	101.73 ± 4.42	4.89	6.73	10	30
	1	101.63 ± 3.88	3.82	5.47			99.21 ± 3.77	4.51	6.23		
	4	99.28 ± 3.52	3.09	4.75			101.25 ± 3.56	3.01	4.3		
ENT	0.2	96.34 ± 4.82	5.21	7.62	35	100	97.91 ± 4.89	4.94	7.53	18	50
	1	98.44 ± 3.72	4.06	6.36			98.23 ± 3.67	4.36	6.11		
	4	101.43 ± 3.60	3.55	4.30			100.79 ± 3.07	3.66	4.68		
LSP	0.2	97.09 ± 4.35	5.03	7.01	30	90	98.05 ± 3.87	4.94	6.33	15	45
	1	102.01 ± 4.09	3.92	5.65			99.63 ± 3.71	3.69	5.36		
	4	100.69 ± 3.21	2.89	4.67			100.41 ± 2.99	2.94	4.56		

[a], Results shown for a selected quality control concentrations of 0.2, 1 and 4 µg ml $^{-1}$ for all the studied drugs. [b], Calculated as mean % recovery (n=5); [c,d], Expressed as relative standard deviation (RSD) (n=5) on the same day and five different days, respectively. [e], Calculated on the basis of a signal to noise ratio of 3. [f], Calculated on the basis of a signal to noise ratio of 10

determine the drugs in the sample matrix. The method proved to be sufficiently accurate with mean percentage recovery in the range of 96.34 to 102.52 at the three concentration levels for all the studied drugs. The drugs concentrations in QC samples were calculated from the linear regression equation obtained on the same day. Results of analysis are shown in Table 2.

Conclusion

The method proposed in this work permits selective confident measurements of BZP, ENT and LSP in pharmaceutical formulations as well as spiked human plasma with a very simple clean up procedure without any serious interference from other endogenous matrix components. The fundamental advantage for the procedure lies in its ability to enhance drugs retention allowing a confident analysis in complex matrix with highly simple, convenient chromatographic procedure within less than 5 min making it an easy decision about applying the method for routine analysis in large pharmaceutical companies or medical laboratories. The suitability of the proposed procedure for analysis of the studied drugs in biological fluids with a simple technique and equipment is a great outcome. Drug concentrations matching their pharmacokinetic blood levels can be efficiently conducted making the method a good alternative for many published methods.

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Full Length Research Paper

Formation of polymeric films containing supersaturated levonorgestrel contraceptive drug by transdermal metered dose aerosol

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Due to the benefits of transdermal sprays, formulation of levonorgestrel as a transdermal metered dose aerosol was studied in this work. Simultaneously, super-saturation was evaluated as an effective method to increase the transfer of active ingredients to the skin. By using anti-nucleant polymers and solvent evaporation after spraying, we could form films containing supersaturated drug after spraying the formulation and inhibiting crystallization. Film formation and visual properties of films were investigated. The kinetics of weight loss and super saturation after film formation was calculated and finally, the rate of drug release from cellulose acetate membrane was assessed. In the formulations containing higher amounts of polyvinyl pyrrolidone (PVP) and 4 mg of levonorgestrel, 84.31% of drug was released during 4 h. While, in formulation with 2 mg drug, the maximum 95.79% of the drug was released during that time. According to these results, this kind of transdermal metered dose aerosol could be suggested as a suitable form for delivery of levonorgestrel through the skin.

Key words: Transdermal delivery, levonorgestrel, topical aerosol, supersaturation.

INTRODUCTION

Transdermal delivery is generally considered as a noninvasive and acceptable route of drug administration that provides continuous penetration of drugs through the intact skin (Fan et al., 2008; Chen et al., 2010). In this drug delivery approach, number of physical, chemical and biochemical methods have been suggested to improve the transportation of drug through the skin. All these attempts are aimed to reduce the barrier properties of stratum corneum or increase the diffusion properties of the drug (Raghavan et al., 2000; Wokovich et al., 2006).

Enhancing permeation into the skin via supersaturation of the drug has previously been shown to improve the efficiency of topical drug release (Raghavan et al., 2001a; Valenta and Auner, 2004; Moser et al., 2001). Supersaturation is achieved when a compound is solubilised at a concentration which is greater than the saturated equilibrium solubility. The increased concentration of drug in a vehicle above saturation leads to a greater thermodynamic activity which proportionally increases the rate at which the drug can pass through the skin (Reid et al., 2008; Jones et al., 2009). It has been shown that metered dose aerosol (MDA) formulations can be applied for formation of topical films by solvent evaporation to induce supersaturation of therapeutic agents (Lulla et al., 2004). Supersaturation in these transient systems is driven by evaporation of the propellant during dose actuation and disappearance of the co-solvent once the dose has reached the skin. Although these

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these types of systems are considered to have high levels of organic solvents, due to their highly volatile nature and short time spent on the surface of the skin, they are not thought to pose an irritation risk (Jones et al., 2009).

Transdermal delivery of estrogens and progestogens for contraception and hormone replacement therapy has been evaluated in numerous researches (Agrawal and Pruthi, 2011; Burkman, 2007; Raynaud, 2005). The use of long acting implants and patches are currently two conventional methods that are available in the pharmaceutical markets for this indication (Chrisman et al., 2006; Mansour et al., 2011; Toole et al., 2002; Caruso, 2003). In this way, application of topical metered dose aerosols to provide greater transdermal dosing flexibility and overcome some of the dosing limitations of current transdermal delivery dosage forms has been proposed in the recent years (Fraser et al., 2007).

The aim of this study was to investigate topical formulations that enhance release of levonorgestrel as a contraceptive drug from polymeric films via transient supersaturation and to determine the degree of supersaturation for this system after application. Levonorgestrel is a synthetic derivative of the progesterone hormone. It is thought to prevent pregnancy in three different ways, depending on the stage of the menstrual cycle at which unprotected intercourse occurs: it suppresses ovulation, inhibits the fertilization of any egg already released, and may also cause changes to the endometrium to prevent a fertilized egg implanting (Schindler et al., 2003). Metered dose aerosols that contained levonorgestrel, hydroxyfluoroalkane as propellant, ethanol as volatile cosolvent and a film forming polymer was developed to deliver drug. Efficacy of poly vinyl pyrrolidone (PVP) and hydroxy propyl methyl cellulose (HPMC) to form films containing supersaturated drug were compared and attempts were made to determine the differential scanning (DS) by testing drug release from the formulations through regenerated cellulose membranes.

MATERIALS AND METHODS

Levonorgestrel was kindly gifted by Iran hormone Co. (Iran). Absolute ethanol (99.7 to 100%) was purchased from Bidestan (Iran). HPMC (methocel E5) was from Colorcon (England) and PVP K30 was purchased from Fluka (Switzerland). 1,1,1,2-tetrafluoroethane (HFA 134a) propellant was kindly donated by INEOS Fluor (UK). Tween 80 was supplied by Merck (Germany). Regenerated cellulose membrane (RCM) (2000 Da molecular weight cut-off) was purchased from Spectrum (USA).

Formulation and preparation of metered dose aerosol

A solution was prepared by mixing film former polymer (PVP or HPMC), the drug substance and pure ethanol and was left to stir overnight to allow the polymer to solvate. An aliquot of the solution was dispensed into an aerosol container (Bespak Europe Ltd, UK). A 63 µl valve was immediately crimped onto each container and the canister was filled with HFA 134a through the valve using a 3,4-methylenedioxyamphetamine (MDA) filler (Pamasol Willi Mader AG, Switzerland) until the desired weight was obtained. The compositions of a series of formulations are shown in Table 1. The concentration of levonorgestrel was adjusted to 33 µg per puff. The concentration of pure ethanol was fixed to a maximum of 10.0% (w/w), to minimize the effect on the vapor pressure and consequently on the release velocity of the liquefied propellant throughout the orifice. To evaluate film forming of polymers, sprays were applied onto a plastic plate and the films were characterized after evaporation of solvents.

Optical microscopy

The morphology of produced films was studied with an optical microscope (Euromex, Netherlands) equipped with a camera system (Sony, Japan). The samples were prepared by spraying the formulations into glass plates and were observed by 40× lens and then took photographs by the camera.

Measurement of evaporation rate and degree of saturation

Thirty actuations from every metered dose aerosol were applied to an aluminum stage on an analytical balance (Sartorius, Germany) and monitored for weight loss after application. Weight of the formulation was plotted against time and the rate of solvent evaporation was calculated using a line of best fit over at least three time points. The final weight of the film was compared to the weight at a set time point to calculate the weight of the remaining ethanol at that time, and this was used to determine the concentration of drug, as described previously by Jones et al. (2009). By comparing this value with the saturated solubility of the drug in ethanol, degree of saturation was obtained using equation 1:

$$DS = \frac{WD_{App} / (WF_t - WF_{Final})}{C_{SS}}$$

Where WD_{App} (mg) was the weight of the drug applied, WF_t (g) was the weight of the formulation at the time point t, and WF_{Final} (g) was the final weight of the formulation after 4 h. This gave a concentration (mg of drug/g of solvent) at time t, which was then divided by the saturated solubility concentration of the drug in the solvent, C_{SS} (mg/g). If the concentration at time t was greater than the saturated solubility, then the formulation was classified as supersaturated. The degree of saturation was plotted against time to assess degree of saturation kinetics over the time of the experiment.

Drug release studies

The *in vitro* drug release was evaluated using United States Pharmacopeia (USP) 23 dissolution test apparatus 5 (paddle over disk). It was performed using a dissolution tester (Erweka, Switzerland) and the dissolution medium comprised 500 ml

Table 1. Composition of formulations for preparation of transdermal metered dose aerosols.

Formulation	Polymer type	Amount of polymer (%)	Amount of drug (mg)
F_1	PVP K30	3	1
F_2	PVP K30	3	1.5
F_3	PVP K30	3	2
F_4	PVP K30	3	2.5
F_5	PVP K30	4	1
F_6	PVP K30	4	1.5
F_7	PVP K30	4	2
F_8	PVP K30	4	2.5
F_9	HPMC	0.10	1
F_{10}	HPMC	0.10	1.5
F_{11}	HPMC	0.10	2
F_{12}	HPMC	0.10	2.5
F_{13}	HPMC	0.20	1
F_{14}	HPMC	0.20	1.5
F_{15}	HPMC	0.20	2
F_{16}	HPMC	0.20	2.5

of 5 microgram poly sorbate 80/g water maintained at a temperature of 35.0°C and a paddle rotation speed of 50 rev/min. For sample preparation, firstly, the synthetic cellulose membrane was soaked in deionized water. Then 5 puffs of aerosol were applied to the disk surface and the film surface was covered with membrane and was sealed. The disk containing the sample was submerged into the dissolution medium. Five milliliters of sample were collected at predetermined time intervals over 4 h. The drug concentration was measured by an ultra violet (UV) spectrophotometer (Spekol, Germany) according to USP monograph for levonorgestrel.

Differential scanning calorimetery (DSC)

Thermal behavior of raw materials and the selected films containing active substance were studied quantitatively and qualitatively by differential scanning calorimetry (DSC 204 F1, Netzsch, Germany). The samples (7 to 12 mg) were accurately weighed into standard aluminum pans and sealed. Thermograms were recorded during heating and cooling runs at a scan rate of 10°C min^{-1} between 25 and 300°C.

RESULTS AND DISCUSSION

Supersaturation of a drug in a topical formulation is one approach by which skin penetration enhancement can be achieved without the use of exogenous chemical enhancers and expensive complicated technologies. In a supersaturated state, the saturation solubility of the compound in its formulation exceeds the equilibrium solubility and the driving force for diffusion is elevated and therefore a higher flux across the skin membrane can be achieved. However, such a system must remain physically stable, and minimal crystallization of the drug should occur during the permeation process. The addition of anti-nucleant polymers to supersaturated formulations prevents crystal nucleation or growth and thus maintains the elevated thermodynamic activity. Also, anti-nucleant polymers can extend the time of supersaturated solution stability and also facilitate the generation of higher levels of supersaturation. Addition of PVP and HPMC to supersaturated solutions has been reported to be effective in crystal growth inhibition of drugs (Megrab et al., 1995).

In the present study, antinucleant effects of these two polymers were investigated for formation of topical films containing supersaturated levonorgestrel. For this purpose, primarily, formulations containing levonorgestrel, film-forming polymer and ethanol were prepared and behavior of each component within the solution was assessed by visual solubility experiments. It was found that all formulations resulted in the formation of clear solutions after stirring for 24 h. However, formulations which contained relatively higher concentrations of drug or polymer than the amounts mentioned in the table did not form transparent solutions.

After addition of propellants to the solutions and preparation of metered dose aerosols, it was found that the formulations F_{11} to F_{16} containing higher levels of HPMC was not stable and the drug and polymer were precipitated in the cans. Appearance of other films was evaluated following actuation of spray onto a transparent plastic plate. Also, optical microscopy was applied for detailed evaluation of film integrity. The results showed

that all of the formulations could produce tough films with smooth surfaces. However, some fine air bubbles could be detected in all films under microscope as presented in Figure 1. In the case of F_{11} which contains HPMC films, some agglomerations was detected that could be attributed to the formation of drug nuclei after supersaturation.

The weight loss profiles of selected metered dose aerosols after application were plotted as a function of time to explore supersaturation kinetics. As shown in Figure 2, there were three obvious gradients in the weight loss profiles of PVP formulations. One immediately post dose application, a second after approximately 2 min and the third after 10 min. These three regions in the evaporation profile have previously been defined as Hydrofluoroalkane loss (first region) followed by co-solvent loss (second region) and finally hardening of the film (no weight loss in the third region) (Jones et al., 2009; Stein and Myrdal, 2006). The trend of weight loss is relatively similar in the formulations containing PVP and the difference between graphs may be related to the different weights of solid materials in the formulations. However, there is a moderate decrease in the rate of weight loss with increase of polymer in the formulations. This relatively slower rate of weight loss from F_1 to F_8 would be attributed to the proportion of ethanol to polymer. Ethanol molecules must diffuse through the polymer to reach the surface of the film and escape. Increasing the viscosity of a solution via the addition of PVP will reduce the molecular diffusion and possibly lead to ethanol depletion at the air–liquid interface and thus a reduction in evaporation rate (Aronson et al., 2004). Weight losses in the HPMC films were much faster than PVP films and the slope of graphs is very sharp (Figure 3). In these formulations, second phase of weight loss profile could not be detected and it seems that ethanol evaporation occurs in a short time. It could be related to the low concentrations of HPMC in the formulations and lower affinity of ethanol to this polymer (Kim et al., 2006).

Monitoring the evaporation of solvents enabled theoretical calculation of the degree of drug supersaturation in the films over time (Figures 4 and 5). The saturation kinetics was different as a consequence of the altered evaporation profiles and film compositions after loss of the HFA. Formulation containing PVP were supersaturated after dose actuation and during 60 min of study; while the HPMC formulations were highly supersaturated during 10 min after actuation.

Anti-nucleant polymers such as PVP and HPMC are thought to prevent crystallization through an increase insolution viscosity which slows molecular diffusion and prevents seed nucleation (Raghavan et al., 2001b). However, a chemical interaction between PVP and the drug caused by the adsorption and the orientation of the polymer at the solid/liquid interface of the crystal as it forms makes the antinucleant capability of a polymer more efficient (Megrab et al., 1995; Sekikawa et al., 1978). Although some drug nuclei were observed in the films generated by the HPMC during the time course of the experiments, the drug in the films did not necessarily return to saturated drug concentrations immediately. The quantity of drug remaining in solution is dependent upon the rate at which the drug recrystallizes and thus the potential for enhanced drug release could still exist. Although

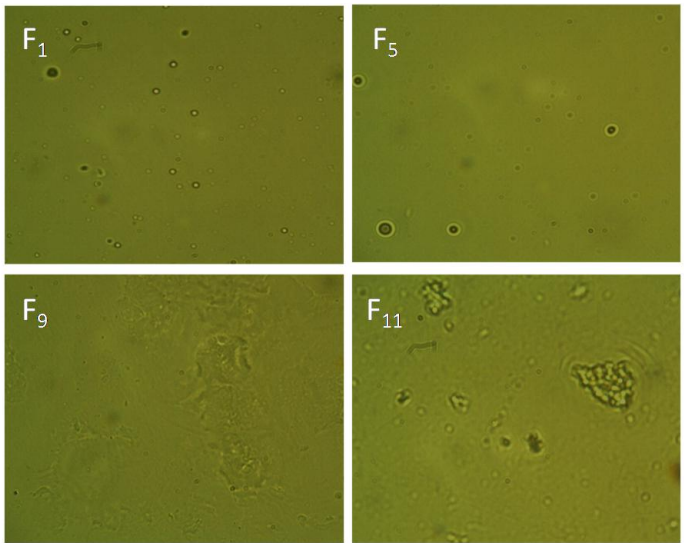

Figure 1. Optical microscopic image of some selected films.

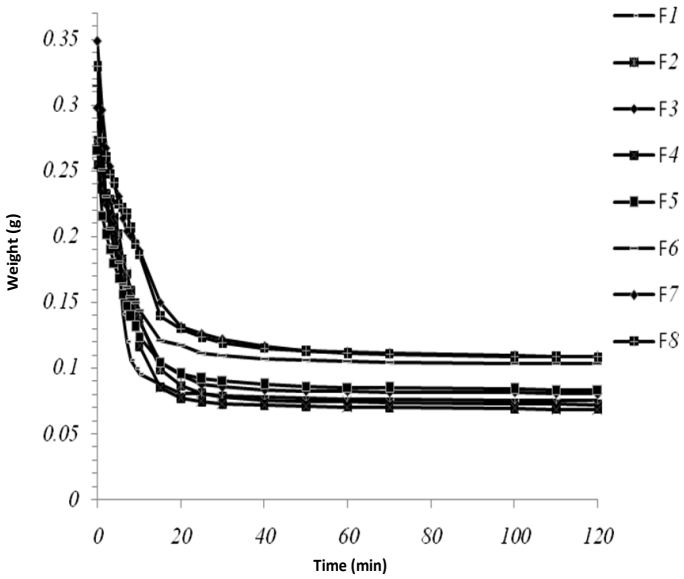

Figure 2. A comparison of weight loss of films after actuation from metered dose aerosols containing different weight ratios of PVP.

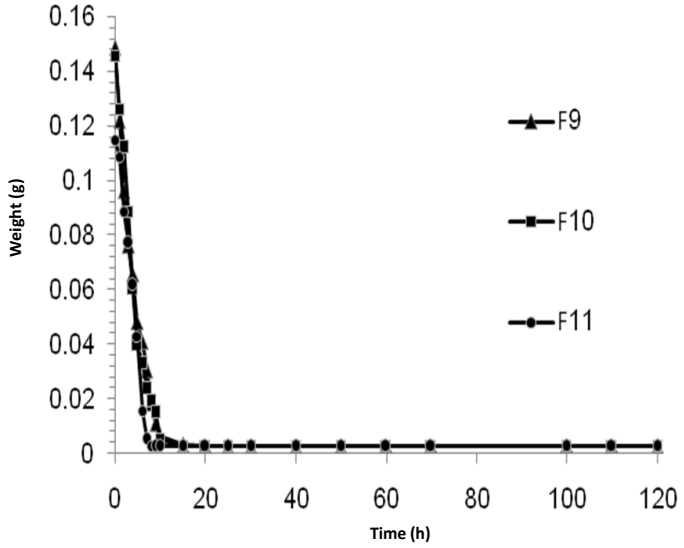

Figure 3. A comparison of weight loss of films after actuation from selected metered dose aerosols containing different weight ratios of HPMC.

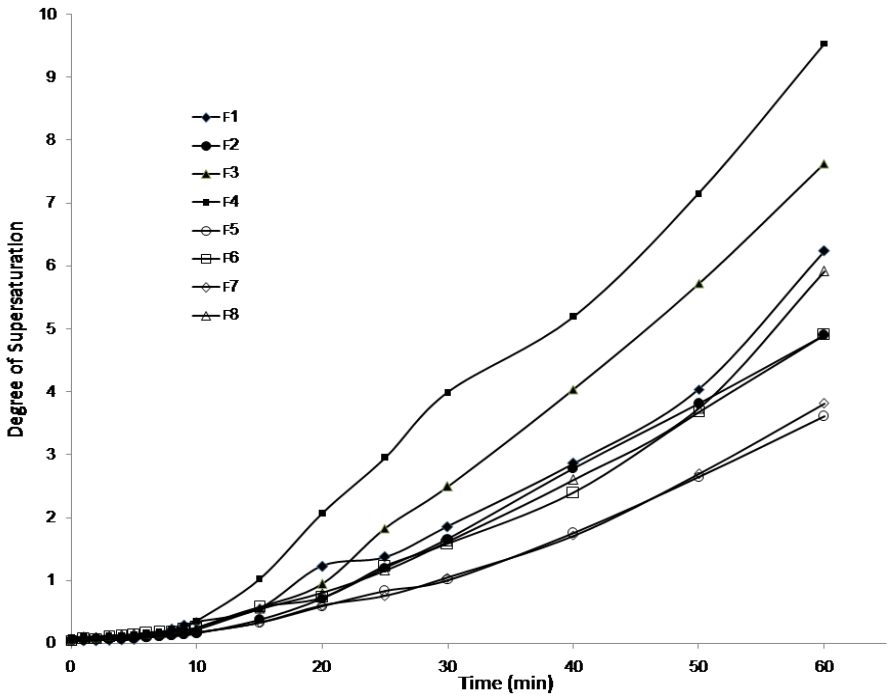

Figure 4. Theoretical degrees of saturation drug (DS) after dose delivery from metered dose aerosols containing different weight ratios of PVP.

it was critical for HPMC formulations, melting endotherm of drug was not observed in DSC thermo-grams (Figure 6). It was attributed to the difference between crystalline structures of unprocessed sample of levonorgestrel and drug nuclei in the film. Observing such an endotherm was not expected for PVP films and it was not detected.

The profiles of drug release from the films confirmed these findings and it was deduced that release of drug

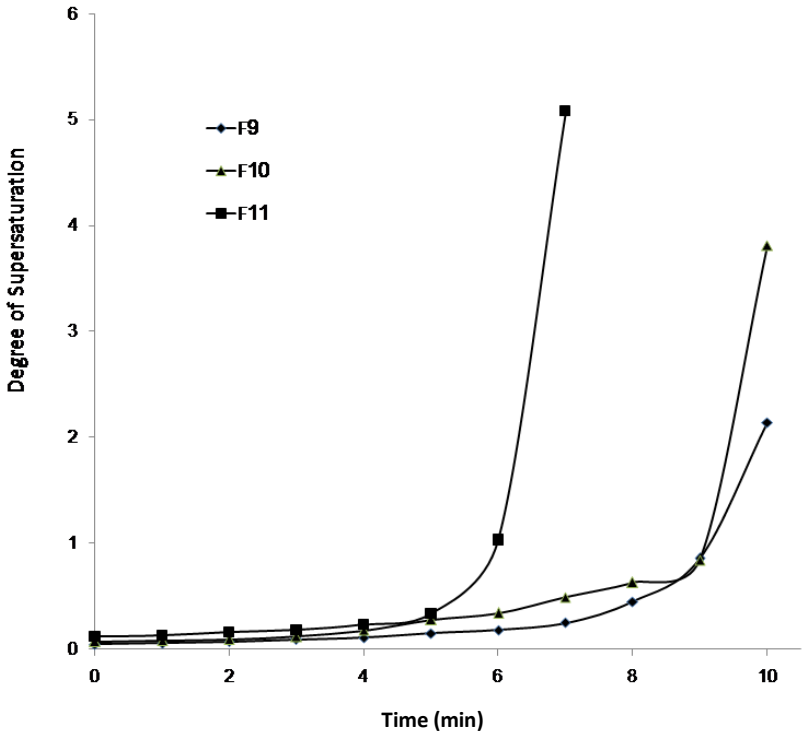

Figure 5. Theoretical degrees of saturation drug (DS) after dose delivery from selected metered dose aerosols containing different weight ratios of HPMC.

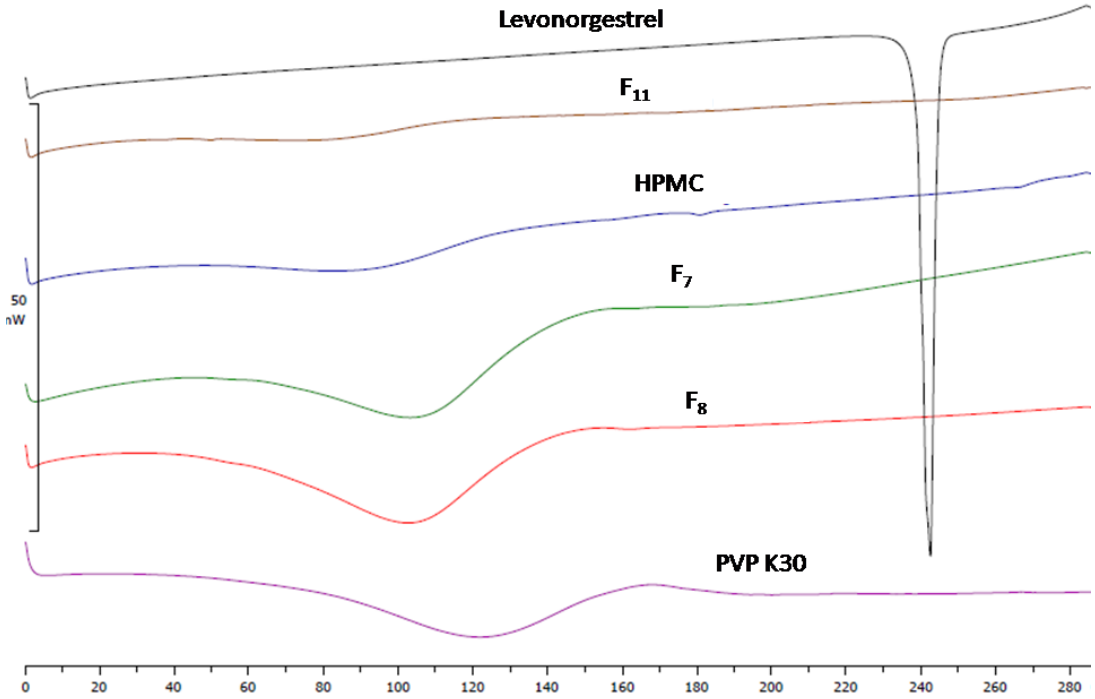

Figure 6. DSC thermograms of row materials and selected films containing PVP and HPMC and levonorgestrel.

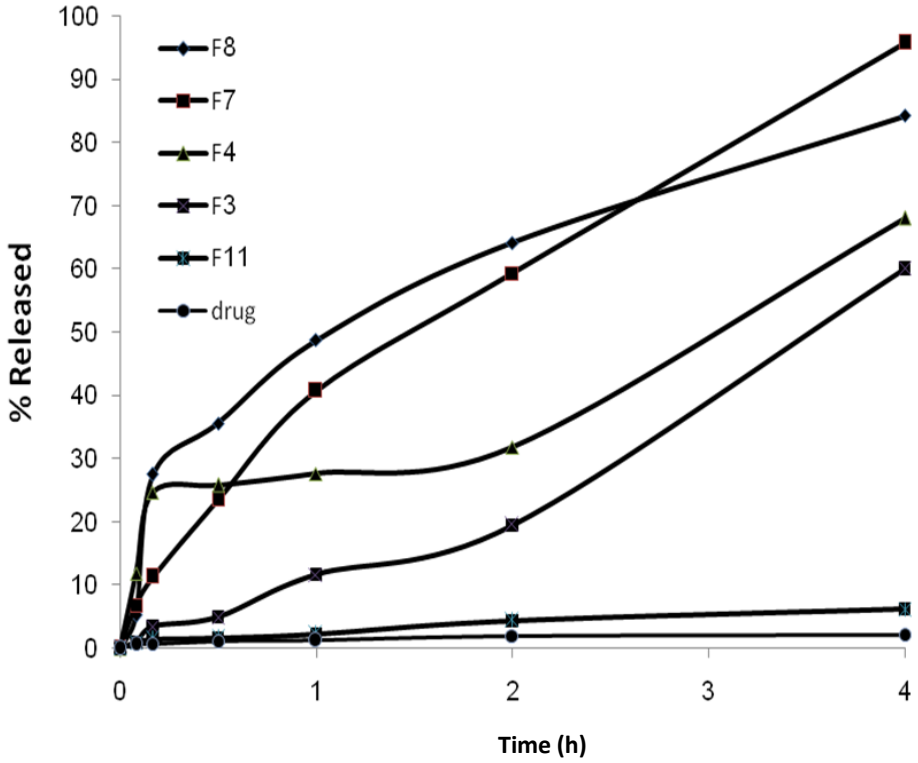

Figure 7. Profiles of drug release from unprocessed levonorgestrel and selected polymeric films containing PVP or HPMC.

from PVP films was drastically higher than both release from HPMC films and dissolution of unprocessed drug (Figure 7). In the formulations containing higher amounts of PVP and 2.5 mg of levonorgestrel (F_8), 84.31% of drug was released during 4 h while in formulation with 2 mg drug (F_7), the maximum 95.79% of the drug was released during that time. In other word, PVP could play its role as an antinucleant polymer for supersaturation of levonorgestrel, and it could probably control the release of drug by a combination of physical properties and supersaturation kinetics.

Conclusion

Transdermal metered dose aerosol could be considered as an efficient dosage form for delivery of supersaturated levonorgestrel as a potent contraceptive. This drug delivery system was specifically formulated such that the solution sprayed onto the skin was readily taken into the skin, and rapidly evaporates from the surface, thereby leaving the surface of the skin dry within less than 1 min of application. Furthermore, utilization of PVP as anti-nucleant resulted in the formation of satisfactory films capable to provide supersaturation of drug and improvement of drug release.

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Full Length Research Paper

Anti-ulcerogenic activity of unsymmetrically substituted urea derivative (1-benzyl-3-(4-methylphenyl) urea)

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Gastric ulcers are mucosal lesions that result from an imbalance between aggressive factors such as acid and pepsin, and defensive mechanisms like gastric mucous, high mucosal blood flow and high mucosal turnover rate that work towards maintenance of mucosal integrity. The aetiology of gastric ulcers is not completely understood and continuous use of anti-ulcer agents leads to many side effects. In the present study, the unsymmetrically substituted urea derivative (1-benzyl-3-(4-methylphenyl) urea) was tested for the anti ulcerogenic activity of alcohol and aspirin induced ulcer models on rats. Ulcer index was calculated by histopathological studies. The test compound at a concentration of 50 and 100 mg/kg exhibited a protective effect on ulcer-induced models in a dose dependent manner, and was comparable with the standard drug ranitidine. These findings indicate that unsymmetrically substituted urea derivative has ulcer protective activity.

Key words: Alcohol, aspirin, gastric ulcer, necrosis, stomach.

INTRODUCTION

Ulcers are the areas of degeneration and necrosis of gastro intestinal mucosa exposed to acid of the alimentary tract that is exposed to hydrochloric acid and pepsin. They occur most commonly (98 to 99%) in either the duodenum or the stomach in the ratio 4:1 (Harsh, 2009). Ulcers can occur in the stomach, where they are called gastric ulcers or they can occur in the first portion of the small intestine called duodenal ulcers. "Peptic Ulcer" is the term used to describe either or both of these two types of ulcer (Mahajan et al., 2009).

Ulcers occur due to imbalance between the protective factors and the aggressive factors (gastric mucosal integrity). The most often aggressive and protective factors in the stomach are acid pepsin secretion, mucosal barrier, blood flow, cellular regeneration, prostaglandins and epidermal growth factors. Sometimes the gastric mucosa is continuously exposed to potentially injurious agents such as pepsin, bile acids, food ingredients, bacterial products (*Helicobacter pylori*) and drugs. However, other factors such as stress, smoking, nutritional deficiency and ingestion of non steroidal anti-inflammatory drugs all can increase the incidence of gastric ulcers. It is reported that prolonged anxiety, emotional stress, haemorrhagic surgical shock, burns and trauma are known to cause severe gastric irritations (Neetesh et al., 2010).

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Test drug

Unsymmetrically substituted urea derivative (1-benzyl-3-(4-methylphenyl)urea) was used as test substance; urea derivatives had wide range of pharmacological activities. They show human immunodeficiency virus (HIV) protease inhibitor (Omar et al., 2012), anti bacterial, hypnotic, sedative (Vijey et al., 2011), anti depressant (Perveen et al., 2012), anti oxidant (Kitti et al., 2009) and anti proliferative activities (Khemkaran et al., 2011). The present study was undertaken to evaluate the anti-ulcerogenic activity of unsymmetrically substituted urea derivative by estimating the various biochemical parameters of ulcer induced models.

MATERIALS AND METHODS

Figure 1 shows urea derivative (1-benzyl-3-(4-methylphenyl)urea) structure with molecular formula of $C_{15}H_{16}NO_2$, molecular weight of 240, DMSO solubility, R_f value of 0.6 (n-hexane: ethyl acetate - 1:1), and melting point at 246 to 250°C.

Experimental animals

Wistar albino rats of either sex weighing 150 to 200 g were used in this study. Animals maintained under the standard conditions of temperature (24±2°C) and relative humidity (44±5%) with a 12:12 light:dark cycle (Gil et al., 2009; Naresh et al., 2012). Animals were given standard diet supplied by the Sainath agencies (Hyderabad) and water *ad libitum*. 24h before the experiment, animals were deprived of food but not water (Adit et al., 2009; Thirunavukkarasu et al., 2009; Bahuguna et al., 2009).

All procedures involving the animals were carried out under the Institute Animal Ethical Committee Approval (CPCSEA guidelines).

Chemicals and drugs

Ethanol, aspirin, ranitidine, sodium carboxy methyl cellulose, urea derivatives, phenolphthalein, Topfer's reagent, and sodium hydroxide were used in the study.

Acute toxicity studies

Toxicity studies of the test substance were carried out in albino mice of either sex weighing 20 to 30 g. LD_{50} test drug was found to be safe up to 1000 mg/kg peritoneally (P.O).

Anti ulcer activity

Alcohol induced ulcers

Animals were divided into five groups of six animals (Rasika et al., 2010; Suthar et al., 2007). Group I served as control received distilled water. Group II received ulcer inducing agent, that is, ethanol 99.8% (1 ml/animal, P.O). Group III received standard drug ranitidine aqueous solution (20 mg/kg, P.O). Group IV animals were administered test drug in 1% sodium carboxymethylcellulose (CMC) at the dose of 50 mg/kg (P.O) and Group V animals received test drug in 1% sodium CMC at the dose of 100 mg/kg (P.O), respectively. Standard and test groups were administered ethanol

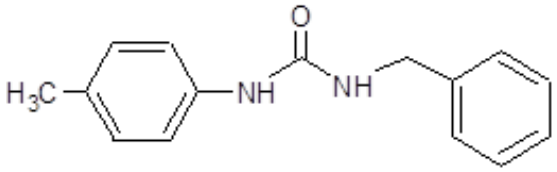

Figure 1. Urea derivative (1-benzyl-3-(4-methylphenyl)urea) structure.

99.8% in 1 ml/animal orally after the administration of the standard and test drugs, respectively. Animals were sacrificed after 1 h (Deshpande et al., 2003; Ubaka et al., 2012) by the ether anaesthesia. Stomach was removed and incised along the greater curvature. Ulcer index was determined and histopathological studies were performed.

Aspirin induced ulcers

Animals were divided into five groups of six animals. Group I served as control and received distilled water. Group II received ulcer inducing agent, that is, aspirin 200 mg/kg (P.O). Group III received standard drug ranitidine aqueous solution of 20 mg/kg (P.O). Group IV animals were administered test drug in 1% sodium CMC at the dose of 50 mg/kg (P.O) and Group V animals received test drug in 1% sodium CMC at the dose of 100 mg/kg (P.O), respectively. Standard and test groups were administered aspirin at the dose of 200 mg/kg orally after the administration of the standard and test drugs, respectively. Animals were sacrificed after 4 h by the ether anaesthesia and stomach was removed and incised along the greater curvature. Ulcer index was determined and histopathological studies were performed (Khaja et al., 2011; Papiya et al., 2008).

Ulcer index

$$UI = UN + US + UP \times 10^{-1}$$

where UI is Ulcer index, UN is the average of the number of ulcer per animal, US is the average of severity score, and UP is the percentage of animal with ulcer (Nagajananeyulu et al., 2012).

Ulcer scores

The ulcer scores are 0=No lesion, 1=1–3 Small lesions (≤10 mm length), 2=1–3 Large lesions (≥10 mm length), 3=1–3 Thickened lesions, 4=More than 3 small lesions, and 5=More than 3 large lesions, 6=More than 3 thickened lesions (Galati et al., 2001).

Percentage protection was calculated using the formula (Jhansi et al., 2010; Vinod et al., 2010):

Percentage protection = (Ulcer index) Control – (Ulcer index) Test / (Ulcer Index) Control

Histopathological studies

Gastric tissue samples from each group were fixed in 10% formalin for 24 h. The formalin fixed specimens were embedded in paraffin and section (3 to 5 µm) and stained with haematoxylin and eosin dye. The histochemical sections were evaluated by light microscopy.

Estimation of free acidity and total acidity

One millilitre of gastric juice was pipetted into a 100 ml conical flask; 2 or 3 drops of Topfer's reagent was added and titrated with 0.01 N sodium hydroxide until all traces of red colour disappears and the colour of the solution turns to yellowish orange. The volume of alkali added was noted. This volume corresponds to free acidity, then 2 or 3 drops of phenolphthalein solution was added and titration was continued until a definite red tinge appears. Again the total volume of alkali added was noted, now this volume corresponds to total acidity (Venkat et al., 2011).

Acidity = Volume of NaOH × Normality of NaOH × 100/0.1 meq/L

Statistical analysis

Statistical analysis was carried out using analysis of variance (ANOVA) by Dunnet's multiple comparison test using graph pad prism software version 5. P values < 0.05 were considered significant.

RESULTS

Acute toxicity studies of urea derivatives did not exhibit any signs of toxicity up to 1 g/kg body weight. Since no mortality of the animals was found at high dose. Hence, 50 and 100 mg/kg dose of the test drug selected for evaluation of anti-ulcer activity.

Ethanol-induced ulcer

In Table 1 and Figure 2, ulcer inhibition was evident in all treatment of the urea derivative (1-benzyl-3-(4-methylphenyl)urea) compared to the negative control. However, statistically significant ulcer inhibition (64 and 78.1%, P<0.05, P=0.001) could be seen only at doses of 50 and 100 mg/kg. The protection from ulcer was dose dependent even as ulcer was produced in all rats in this model.

Aspirin-induced ulcer

Urea derivative (1-benzyl-3-(4-methylphenyl)urea) at all the doses provided protection from ulcer and the protection was dose dependent. The urea derivative at doses of 50 and 100 mg/kg provided statistically significant protection (70 and 84.4%, P<0.05, P=0.003) when compared with the negative control (Table 2; Figure 3).

DISCUSSION

Ethanol-induced gastric ulcers serve as a common ulcerogenic agent. Ethanol is metabolized in the body and releases superoxide anion and hydroperoxy free radicals. It has been found that oxygen derived free radicals are implicated in the mechanism of acute and chronic

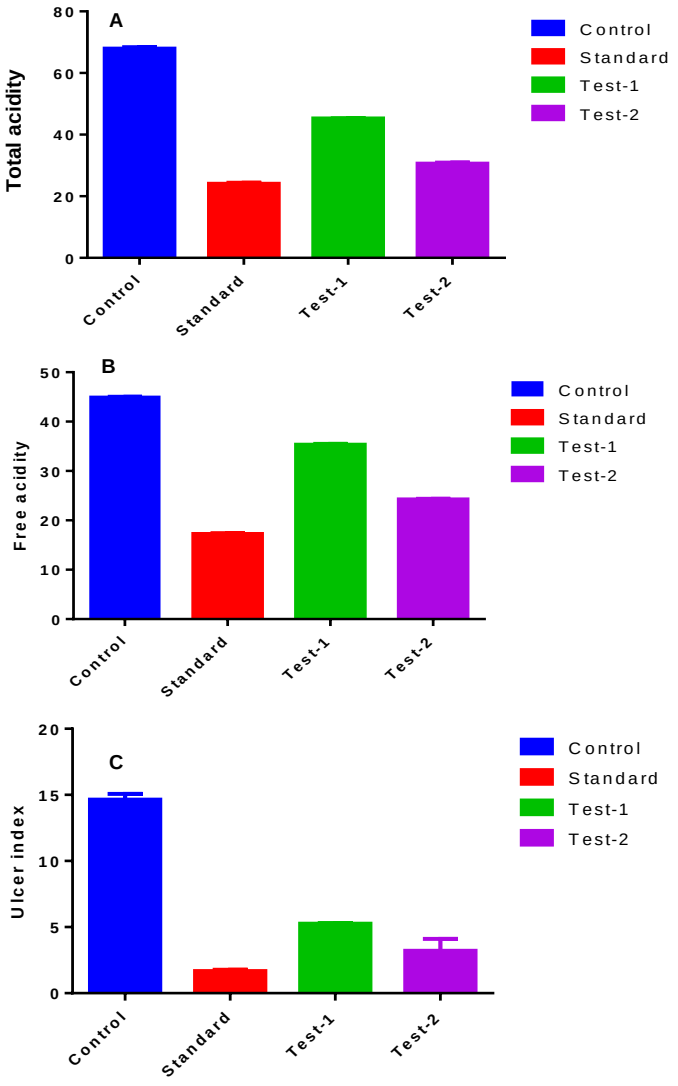

Figure 2. (A) Total acidity (alcohol induced ulcers), (B) free acidity (alcohol induced ulcers), (C) ulcer index (alcohol induced ulcers).

ulceration in the stomach. There are various mechanisms involved in the ulcer production in different experimental models. Many experimental evidences have shown that antioxidants significantly strengthen the gastric walls and protect tissue from oxidative damage. Furthermore, gastric acid secretion now accepted to play an important role in the formation of gastric ulcer (Wasman et al., 2010). Urea derivatives have significant protective effect on the gastric mucosa against ethanol challenge as shown shown by reduced values of total acidity, free acidity, and ulcer index as compared to the control group suggesting its potent cytoprotective effect. Non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin causes gastric mucosal damage by decreasing prostaglandin levels through inhibition of prostaglandin synthesis. Prostaglandins are protective agents for the gastric mucosa. They produce excess mucous and the bicarbonate ions, which

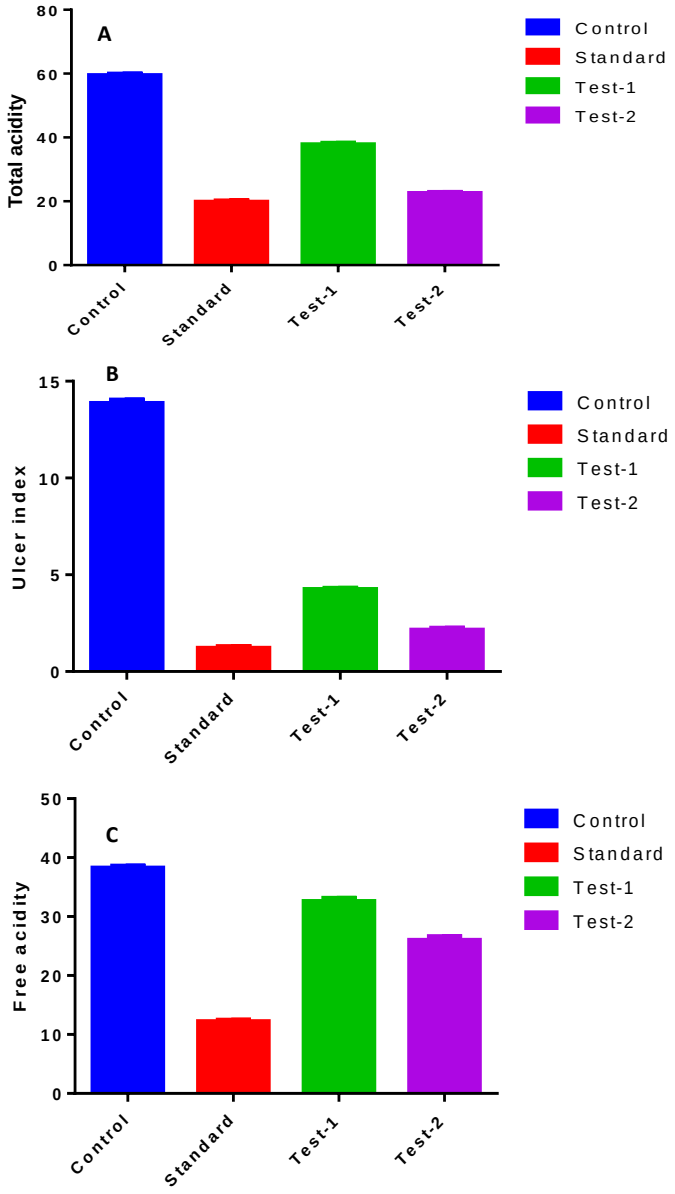

Figure 3. (A)Total acidity (aspirin induced ulcers), (B) free acidity (aspirin induced ulcers), (C) ulcer index (aspirin induced ulcers).

Table 1. Efficacy of urea derivative (Test 1& 2) in Alcohol induced ulcers.

Group	Dose (P.O)	Total acidity (meq/L)	Free acidity (meq/L)	Ulcer index (mm length)	Protection (%)
Control (Alcohol)	99.8% 1 ml/animal	393.43±4.3	272.075±0.946	14.65±0.42	-
Standard (Ranitidine)	20 mg/kg	102.3±1.16***	85.275±1.55***	1.67±0.09***	88.56
Test-1	50 mg/kg	244.92±1.17*	197.47±1.136**	5.26±0.04*	64
Test-2	100 mg/kg	186.875±0.93**	94.6±1.52***	3.2±0.912**	78.1

Data expressed mean ± S.D.(n = 6). Statistical comparison was performed by using ANOVA coupled with Dunnet's multiple comparison test.*P<0.05, **P<0.01, ***P<0.001 were consider statistically significant when compared to control group.

Table 2. Efficacy of urea derivative (Test 1 and 2) in aspirin induced ulcers.

Group	Dose (mg/kg, p.o)	Total acidity (meq/L)	Free acidity (meq/L)	Ulcer index (mm length)	Protection (%)
Control (Aspirin)	200	276.25±1.948	173.647±0.66	13.87±0.17	-
Standard (Ranitidine)	20	84.57±1.298***	74.2±1.290***	1.21±0.085***	91.2
Test-1	50	150.4±1.18*	143.425±0.573*	4.25±0.075**	69.9
Test-2	100	104.575±0.51**	84.35±0.525***	2.15±0.108***	84.4

Data expressed mean ± SEM (n = 6). Statistical comparison was performed using ANOVA coupled with Dunnet's multiple comparison test.*P<0.05, **P<0.01, ***P<0.001 were consider statistically significant when compared with the control group.

protects the gastric mucosa from ulcer inducers. Inhibitions of prostaglandin synthesis by aspirin coincide with the earlier stages of damage to the cell membrane of mucosal, parietal and endothelial cells (Donnelly et al., 1977). So, the possible mechanism of antiulcer action of urea derivative may be the reduction of the acid secretion. In this study, it was observed that urea derivative provides significant anti-ulcer activity against gastric ulcers in rats. Aspirin decreased the concentrations of all the individual carbohydrates and also the carbohydrate to protein ratio; however, a similar decrease in carbohydrate/protein ratio and of individual carbohydrates has been earlier reported in the non-dialyzable and lyophilized fractions of the mucus in aspirin-treated rats. These results tend to confirm that aspirin-like drugs cause ulceration by affecting the mucosal barrier and the carbohydrate/protein ratio of the gastric juice is a good index of the mucus barrier. The urea derivative at doses 50 and 100 mg/kg produced a significant reduction in gastric ulcer when compared with the negative control (aspirin) and with the ranitidine (standard) (Menguy et al., 1965).

Conclusion

In the present investigation, unsymmetrically substituted urea derivative (1-benzyl-3-(4-methylphenyl)urea) showed significant anti-ulcer activity at two different doses 50 and 100 mg/kg in ethanol and aspirin induced ulcer in rats. Test drug showed significant reduction in the total acidity, free acidity, and ulcer index values of treated groups. It also showed significant protection as compared to standard. However, more experimentation on human and animals and experimental analysis are required for a definitive conclusion.

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African Journal of Pharmacy and Pharmacology

Short Communication

Eugenol-rich essential oil of *Anthemis mazandranica* and its antibacterial activities

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The hydro-distilled volatile oil obtained from aerial parts of *Anthemis mazandranica* was analyzed by gas chromatography and mass spectrometry (GC-MS). Seventeen compounds were identified, representing 93.35% of the total oil composition, with eugenol (35.5%) being the main component. Oil antimicrobial activity was carried out using the disk diffusion and minimal inhibitory concentration (MIC). The best antibacterial activity was observed against Salmonella Typhi with ZI = 19 ± 0.5 mm and MIC value of 32 µg/mL.

Key words: *Anthemis mazandranica* , essential oil, antimicrobial, eugenol.

INTRODUCTION

The genus *Anthemis*, comprises 130 species widespread in the Mediterranean, South West Asia and South Africa (Kudryashev, 1932). Present in Iran are 39 species growing wild, among which 15 are endemic (Mozaffarian, 1996). From the Roman times up to now, *Anthemis* taxa have been commonly used as folk remedies, insecticides and dyes (Niko et al., 2009). Water-distilled essential oils from the leaves and flowers of *A. altissima* (L.) Var. Altissima. was analyzed by GC-MS. β-Thujone (33.7 and 19.7%, respectively) was found as the major constituent in the leaf and flower oil (Rustaiyan et al., 2004). Over the last two decades, *Anthemis* volatile compounds have received more attention (Vuckovic et al., 2006; Williams et al., 2001; Vajs et al., 1999).

MATERIALS AND METHODS

Plant material and isolation procedure

The aerial parts of *A. mazandranica* growing wild in Shiraz (Provincial capital of Fars) was collected at the flowering stage in May, 2011. Their identities were confirmed by Dr. Valiollah Mozaffarianand and a voucher specimen (no. VS-21-13) was deposited at the Herbarium of Science and Research Branch, Islamic Azad University (Tehran, Iran).

Hydrodistillation

The air-dried aerial parts (leaves, petals and stems) (100 g) were dried, powdered and the volatile fraction was isolated by hydrodistillation for 3 h using a Clevenger-type apparatus. The essential oil had a bright yellow color and yielded 0.59% w/w.

GC and GC/MS analysis

GC analysis of the oil was performed using a Shimadzu 15 A gas chromatograph equipped with flame ionization detector (FID) and a DB-5 fused silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm). Temperature program: 60°C (3 min), 60 to 220°C at 5°C/min, 220°C (5 min); injector and detector temperatures, 260°C; the carrier gas was N2 (1 ml/min). The sample was injected in split-splitless mode, using a split ratio of 1:50. The percentages of each component were reported as raw percentages without standardization. GC-MS analysis was carried out on a Hewlett-Packard 6890/5973 using an HP-5MS column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The oven temperature was as above,

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Table 1. Chemical composition of leaves and aerial parts oils of *A. mazandranica*.

Compound	RI[a]	Theoretical value	Aerial part (%)
Octane	800	800	2.28
Decane	1000	1000	3.59
α-Phellandrene	1003	1002	3.41
p-Cymene	1025	1089	2.99
limonene	1029	1024	3.45
β-Phellandrene	1030	1025	0.61
1,8-Cineole	1034	1026	4.04
Myrcenyl acetate	1327	1312	1.88
Cyclohexasiloxane, dodecamethyl	1340	1330	18.64
Eugenol	1359	1356	35.55
E-jasmone	1402	1390	1.87
β-Caryophyllene	1419	1417	6.94
Trans-α-Bergamotene	1438	1432	2.52
γ-Eudesmol	1621	1630	3.17
α-Cadinol	1654	1052	0.72
Monoterpene hydrocarbons			10.46
Oxygenated monoterpene			59.11
Sesquiterpene hydrocarbons			9.47
Oxygenated sesquiterpene			3.89
Total			91.66
Yield, w/w%			0.59

[a] Kovat's retention index, Tr: trace (< 0.05%).

interface temperature, 260°C; mass range was 40 to 300 amu; scan time, 1 s. Retention indices (RI) of compounds were determined relative to the retention times of a series of n-alkanes (C6 to C25) with linear interpolation. Identification of the oil components was done by comparison of their mass spectra with Wiley 275 GC-MS library, by comparing them with those reported in the literature and confirmed by comparison of its retention index either with those of authentic compounds or with data in the literature (Jenning and Shibamoto, 1980; Adams,1995).

Antimicrobial activity

All test microorganisms were obtained from the Persian type culture collection (PTCC), Tehran, Iran and were as follows: *Bacillus pumilus* (PTCC 1319), *Escherichia coli* (PTCC 1533), Koc*uria varians* (PTCC 1484), *Pseudomonas aeruginosa* (PTCC 1310), Salmonella Typhi (PTCC 1609), and *Listeria monocytogenes* (PTCC 1298).

Assessment of antimicrobial activity

The antibacterial activity of the A. mazandranica essential oil was screened against Gram-positive and Gram-negative bacteria, with two methods:

Disc diffusion assay

Antimicrobial tests were carried out by the disc diffusion method reported by Murray and his co-worker in 1999 (Wayne, 2006). The dried *A. mazandranica* essential oil was dissolved in Dimethyl sulfoxide (DMSO) to a final concentration of 30 mg/ml and filtered through 0.45 µm Millipore filters, using 100 µl of suspension containing 108 CFU/ml of bacteria and 104 spore/ml of fungi spread on the nutrient agar (NA) and potato dextrose agar (PD) mediums, respectively. The discs (6 mm in diameter) impregnated with 10 µl of the essential oil solution (300 µg/disc) and DMSO (as negative control) were placed on the inoculated agar. The inoculated plates were incubated for 24 h at 37°C for bacterial strains and 48 and 72 h at 30°C for mould isolates, respectively. Gentamicin (10 µg/disc) and ampicillin (5 µg/disc) were used as positive controls for bacteria. The diameters of inhibition zones were used as a measure of antimicrobial activity and each assay was repeated twice.

MIC agar dilution assay

The lowest concentration of the compounds that prevented visible growth was considered as the minimal inhibitory concentration (MIC). MIC value of the plant essential oil against standard bacterial strains was evaluated based on the agar dilution method. Appropriate amounts of the *A. mazandranica* oil were added aseptically to sterile molten Sabouraud dextrose agar (SDA) medium added with Tween 20 (0.5%, v/v) to produce the concentration range of 8 to 500 µg/ml. The resulting SDA agar solutions were immediately mixed and poured into Petri plates. The plates were spot inoculated with 5 µl (104 spore/ ml) of each fungus isolate. At the end of incubation period, the plates were evaluated for the presence or absence of growth. Ampicillin and tetracycline were used as references for gram-positive and negative bacteria, respectively. The MIC was defined as the lowest concentration of the oil needed to inhibit the growth of microorganisms. Each test was repeated at least twice.

Table 2. Antimicrobial activity of the aerial parts essential oil of *A.mazandranica*.

Microorganism	MIC (µg/ml) of *A. mazandranica*	MIC (µg/ml) of reference[a]	ZI (mm) of *A. mazandranica* [b]	ZI (mm) of reference[c]
Bacillus pumilus	128	64	11.5±0.5	16.3±0.5
Escherichia coil	128	16	10±0.1	16±0.0
Kocuria varians	64	32	13.5±0.5	17.6±0.5
Listeria monocytogenes	512	16	6.5±0.5	14.3±0.5
Pseudomonas aeroginosa	64	8	14.5±0.5	16.3±0.1
Salmonella Typhi	32	32	19.5±0.5	21.3±0.5

[a]Ampicillin, tetracycline were used as references for Gram-positive, Gram-negative bacteria, respectively. [b]ZI: Zone of Inhabitation. Including the diameter of the filter paper disc (6 mm); mean value of four experiments ± SD. Campicillin (5 µg/disc), gentamicin (10 µg/disc) were used as references for Gram-positive and negative bacteria, respectively.

RESULTS AND DISCUSSION

The oil of the aerial part contained 19 compounds with a yield of 0.59% (w/w), representing 93.35% of the total oil composition (Table 1), where main component was eugenol (35.5%). In particular, Oxygenated monoterpenes (59.11%) were the most abundant group of compounds. In this study, the antimicrobial activities of the aerial parts essential oil of *A. mazandranica* were investigated against six bacterial standard strains in laboratory situation (*in vitro*). Antimicrobial activity (inhibition zone and MIC) of the oil against standard microorganisms is shown in Table 2. The best anti-bacterial activity was observed against S. Typhi (ZI and MIC value 19 ± 0.5 mm and 32 µg/ml, respectively). From a medical point of view, the antimicrobial activity of *A. mazandranica* oil against S. Typhi is particularly interesting because of the role of this microorganism as a pathogen agent responsible for severe typhoid fever infection.

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