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Ansell JE, Buttaro ML, Thomas VO (1997). Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 31 : 604-615

Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

Furmaga EM (1993). Pharmacist management of a hyperlipidemia clinic. Am. J. Hosp. Pharm. 50 : 91-95

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Research Articles

Full Length Research Paper

Pharmacokinetics of 600 mg loading dose of clopidogrel in patients undergoing percutaneous coronary intervention

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Clopidogrel is an oral antiplatelet drug. Loading dose of 600 mg clopidogrel was shown to improve clinical outcome in patients following percutaneous coronary intervention (PCI). Wide inter-individual variation has been detected in clopidogrel response that can be related to variation in Clopidogrel serum concentration. The aim of this study was to assess the pharmacokinetics parameters of 600 mg loading dose clopidogrel among Jordanian patients undergoing PCI. Additionally, the development of a simple and a validated High-performance liquid chromatography (HPLC) method for the quantification of clopidogrel carboxylate was described. 80 patients who received a loading of 600 mg Clopidogrel were included in our study, several blood samples were collected at different time points. Validated reverse phase HPLC method was used to determine Clopidogrel carboxylic acid metabolite. Non-compartmental analysis was used to determine peak plasma concentration (C_{max}), time to peak plasma concentration (T_{max}), elimination half-life ($t_{1/2e}$), and area under the curve (AUC). The pharmacokinetic-parameters were characterized by considerable inter-individual differences [C_{max}=24.49±11.64 µg/ml, T_{max}=2.02±1.52 h, $AUC_{0\to\infty}$= 123.17± 54.6 mg/ml.h, and t1 ∕ 2e=4.29±2.92 h]. 15% of the patients who had less than one third of the $_{Cmax}$ 8.09±2.34 µg/ml had delayed T_{max} of 4.17±1.76 h, which was not explained by standard in vitro dissolution test. Pharmacokinetic parameters of 600 mg Clopidogrel showed marked inter-individual differences. The low plasma concentrations in some of the patients and the high inter-individual variability may contribute to reported cases of resistance to Clopidogrel therapy. Further studies are needed to explain low C_{max} and delayed T_{max} values in some patients.

Key words: Clopidogrel carboxylic acid, loading dose, percutaneous coronary intervention, reverse phase High-performance liquid chromatography (HPLC), pharmacokinetics.

INTRODUCTION

Clopidogrel, a thienopyridine antiplatelet agent, is marketed worldwide as Plavix®/Iscover® and is used in the prevention of myocardial infarction, stroke and death in patients with acute coronary syndromes. In patients

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undergoing percutaneous coronary intervention (PCI), pretreatment with a loading dose of clopidogrel (300 to 600 mg) was shown to improve clinical outcome (Steinhubl et al., 2002; Lotrionte et al., 2007).

Clopidogrel is an inactive prodrug that is converted by hepatic bio-transformation via cytochrome P450 (CYP) pathway. Two sequential CYP-dependent oxidative steps are required to convert clopidogrel to its active metabolite (a thiol compound) that react with and irreversibly inhibit the platelet P_2Y_{12} ADP receptor, which is involved in platelet activation and stabilization of the platelet aggregate (Savi et al., 2000; Sugidachi et al., 2000). The active metabolite is extremely labile and unstable and it remained undetected in plasma for many years (Pereillo et al., 2002) until recently (Takahashi et al., 2008). Though, the quiet nature of the active metabolite and its determination is still not settled. The published dependable pharmacokinetic parameters of clopidogrel are related to its inactive carboxylic acid metabolite that represents approximately 85% of the total amount. Since neither the parent drug nor the active metabolite is easily detected in plasma, the quantification of carboxylic acid metabolite would be an indirect approach for studying the pharmacokinetics of clopidogrel (Caplain et al., 1999; Singh et al., 2005). High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection, have been used widely to determine the inactive metabolite of clopidogrel in the biological fluids (Bahrami et al., 2008). Recently, several liquid chromatography-tandem mass spectrometry (LC-MS-MS) methods for the study of pharmacokinetics of clopidogrel parent compound and the inactive carboxylic metabolite have also been published (Ksycinska et al., 2006). Whatever the method to detect clopidogrel parent compound or its metabolites, a high inter-individual variation has been noticed in both clopidogrel response (Muller et al., 2003; Angiolillo et al., 2005) and clopidogrel concentrations in plasma. This poor response to clopidogrel is associated with an increased risk of recurrent ischemic events (Gurbel et al., 2005). It has been suggested that the high variability in response to clopidogrel is caused by variation in pharmacokinetics parameters that could be caused by different level of activity of the activation enzymes (Heestermans et al., 2006; Brandt et al., 2007; Mega et al., 2009). The aim of this study was to investigate the pharmacokinetics of clopidogrel 600 mg loading dose in Jordanian patients undergoing PCI by measuring the inactive carboxylic acid metabolite. To the best of our knowledge, this is the first study to evaluate pharmacokinetics of 600 mg clopidogrel among relatively large number of patients undergoing PCI.

MATERIALS AND METHODS

Clopidogrel carboxylic acid was obtained from Toronto Research Chemicals in Canada. Hydrochlorothiazide (internal standard, IS) was obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and methanol were HPLC-grade and purchased from ACRŌS (Darmstadt, Germany). All other chemicals and solvents (phosphoric acid 85%, perchloric acid, sodium hydroxide) were gradient grade and used without any further purification (Merk, Darmstadt, Germany). Plavix® 75 mg tablets containing clopidogrel were obtained from Sanofi-Aventis France (batch no. 2273).

Study population

Patients who were admitted to Jordan University Hospital for catheterization and underwent PCI were eligible for the entry into this study if they were given 600 mg of clopidogrel (8 tablets of plavix®). Patients were excluded if they were known to have hepatitis B infection or carrier of respective antigen; donated blood within last 2 months; have allergic diathesis or any significant allergic disease; have gastro intestinal (GI) diseases or hepatic disease; were pregnant; have been on statin that has been altered within 14 days; have creatinine Cl < 25 ml/min; or diagnosed with heart failure with New York Heart Association (NYHA) class 4. Participants were classified into overweight (BMI ≥ 25 kg/m^2) or normal weight (BMI < 25 kg/m^2). This stratification agrees with the definition of overweight patients by the World Health Organization (Willett et al., 1999). The study was approved by local Research Ethics Committees of the Jordan University Hospital and informed consent was obtained from all participants after having been informed verbally by the medical supervisor about the need to withdraw extra blood samples for pharmacokinetic analysis. The decision to give or not to give 600 mg clopidogrel was solely the responsibility of the treating cardio-surgeon performing the PCI.

Intervention and sample collection

Whole blood samples from patients were drawn into heparinized test tubes immediately before (0) and at 20, 40, 60, 120, 240 min and 8 h after the intake of clopidogrel. Blood was centrifuged at 4000 g for 5 min and plasma samples were separated and immediately stored at -80°C until analysis. Perchloric acid (HCLO$_4$) (50 µl of 1%) was added to plasma (100 µl). Internal standard was then added (50 µl of 15.0 µg/ml hydrochlorothiazide), the mixture was vortex-mixed, then methyl-tert-butyl ether (3.0 ml) was added, vortexed for 6.0 min and then centrifuged for 10.0 min at 4,400 rounds per min (rpm). The organic layer was quantitatively transferred to another test tube and a volume of 200 µl of 50 mM sodium hydroxide (NaOH) was added and vortex-mixed for 1.0 min. After 7 min of centrifugation at 4,400 rpm, the lower layer was carefully transferred to 350 µl flat bottom insert and 25 µl of this sample was injected on LiChosphere RP-select B column (5 µm, 150 × 4.6 mm). Clopidogrel metabolite and the internal standard were separated from endogenous substances.

Chromatographic conditions

The plasma level of clopidogrel carboxylic acid were determined by reverse-phase high-performance liquid chromatographic method, where the separation achieved using isocratic mobile phase consisted of 80% of 5 mM potassium dihyrogen phosphate (KH$_2$PO$_4$) and 20% acetonitrile at pH = 3 prepared daily and degassed by passing through a 0.45 µm filter. A Dionex HPLC auto-sampler system was used and composed of the following: a constant solvent delivery system (P580); a 100 µl fixed volume injector [Rheodyne 7125; UV Detector (UVD340S)]; autosampler (ASI-100) and operated by Microsoft Windows 2000. Flow rate was 1.0 ml/min, and pressure was 70 bar. All separations were performed at   room

temperature. Detection was monitored at 220 nm.

Validation of analytical methods

Specificity/selectivity

Specificity was verified by the absence of any co-eluted peak of endogenous plasma components at the retention times of the clopidogrel carboxylic acid and the internal standard. Moreover, selectivity was affirmed by the absence of interference from commonly used drugs (aspirin, acetaminophen, ascorbic acid, caffeine, nicotine and ibuprofen).

Linearity

Standard calibration curves of 8 points (0.5, 1.0, 2.0, 5.0, 10.0, 25.0, 50.0 µg/ml) in addition to the blank sample and standard zero sample were prepared. In each day of the validation course, a calibration curve was prepared and its goodness of fit was calculated by weighted least square linear regression equation (Strutz, 2011). The sample preparation and HPLC analysis were performed as described. Calibration curves were constructed by plotting the measured peak area ratios of metabolite to the internal standard (IS) versus concentrations of standard samples, and statistical analysis was performed. The lowest standard concentration in the calibration curve is considered the lower limit of quantification (LLOQ), given that its detector's response is at least three times that of the blank.

Accuracy and precision

Accuracy and precision evaluation was held over the first three days of the validation time course employing the regression of the calibration curve that was carried out at the same day. To establish the intra-day and inter-day accuracy and precision of the method, three replicates of standard plasma solutions at three different concentrations (1.5, 20.0 and 40.0 µg/ml) were assayed per day, for two consecutive days. Four replicates of standard plasma solutions were assayed on the third day.

Recovery

The absolute recoveries were calculated for clopidogrel carboxylic acid and hydrochlorothiazide by comparing the relevant peak areas of the extracted and unextracted samples. The absolute analytical recovery of clopiogrel carboxylic acid was calculated at three different concentrations (1.5, 20.0, 40.0 µg/ml), while for the internal standard, the nominal concentration (4.5 µg/ml) was used.

Pharmacokinetic calculations

The pharmacokinetic parameters of clopidogrel carboxylic acid metabolite were estimated by standard non-compartmental methods using Kinetica^{TM} 2000 Version 4.2 (Innaphase, Philadelphia, PA, USA) computer program. The C_{max} and the T_{max} of clopidogrel carboxylic acid were taken directly from the measured data. The $AUC_{0\to t}$ was calculated from measured data points from the time of administration to the time of last quantifiable concentration (C_{last}) by linear trapezoidal rule. The following equations were utilized to calculate the remaining pharmacokinetic parameters (Gibaldi and Perrier, 1982).

$$AUC_{0\to\infty} = AUC_{0\to t} + \frac{C_{last}}{k_e}$$

$$t_{1/2e} = \frac{0.693}{-b}$$

$$AUMC_{0\to\infty} = \sum_{i=0}^{n-1} \left(\frac{t_{i+1}-t_i}{2}(C_it_i + C_{i+1}t_{i+1})) + \frac{C_{last}t_{last}}{k_e} + \frac{C_{last}}{k_e^2} \right)$$

$$\frac{Vd}{F} = \frac{D}{k_e \cdot AUC_{0\to\infty}}$$

$$MRT = \frac{AUMC_{0\to\infty}}{AUC_{0\to\infty}}$$

Where ($AUC_{0\to\infty}$) is the area under the plasma concentration-time curve extrapolated to infinity; k_e is the elimination rate constant; ($t_{1/2e}$) is the elimination half life; b is the slope of the linear regression of the Ln-transformed plasma concentration versus time in the terminal period of the plasma curve; ($AUMC_{0\to\infty}$) is the area under the first momentum curve; Vd is the apparent volume of distribution; F is bioavailability; and MRT is mean residence time.

Dissolution studies

The dissolution medium consisted of 900 ml of buffer solution, pH 2.0, prepared by dissolving 14.91 g of potassium chloride in 119.0 ml of 0.2 M hydrochloric acid and further diluted with water. The reference solution was prepared by dissolving 75.0 mg of clopidogrel working standard in 100.0 ml of methanol; 5.0 ml of this solution were diluted to 50.0 ml with the dissolution medium. The dissolution medium was thermostatically controlled at 37°C. The rotation speed of the paddles was 50 rpm. A volume of 10 ml was taken at 5, 10, 15, 20, 25, 30, 35, 40 and 60 min and analyzed in the spectrophotometer. Initially, the values obtained at 30 min were used to evaluate the differences between the samples. Based on the results of the reference and the acceptance criteria of the United States Pharmacopeia (USP) (USP, 2007), the Q-value was proposed to be 75%. This implied that the percentage of active ingredient dissolved after 30 min, for each of the six tablets examined might not be less than 80% (Q + 5%) of the theoretical clopidogrel content. If the tablets did not pass the test, another 6 units were examined. The result was satisfactory if the average of the 12 units was not less than 75% and no unit was less than 60%. If the tablets did not pass the test using these 12 units, another 12 units were tested. The batch was accepted if the average of the 24 units was not less than 75%; not more than 2 units were less than 60% and no unit was less than 50%.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS$^{©}$ software version 11.0; SPSS, Inc, Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Coefficient of variation (CV) was calculated by (SD/mean × 100). Kruskal-Wallis test was utilized to detect differences in T_{max} and $t_{1/2e}$ while analysis of the variance (ANOVA) was used to detect the differences in C_{max}, $AUC_{0\to t}$ and $AUC_{0\to\infty}$ among 3 categories.
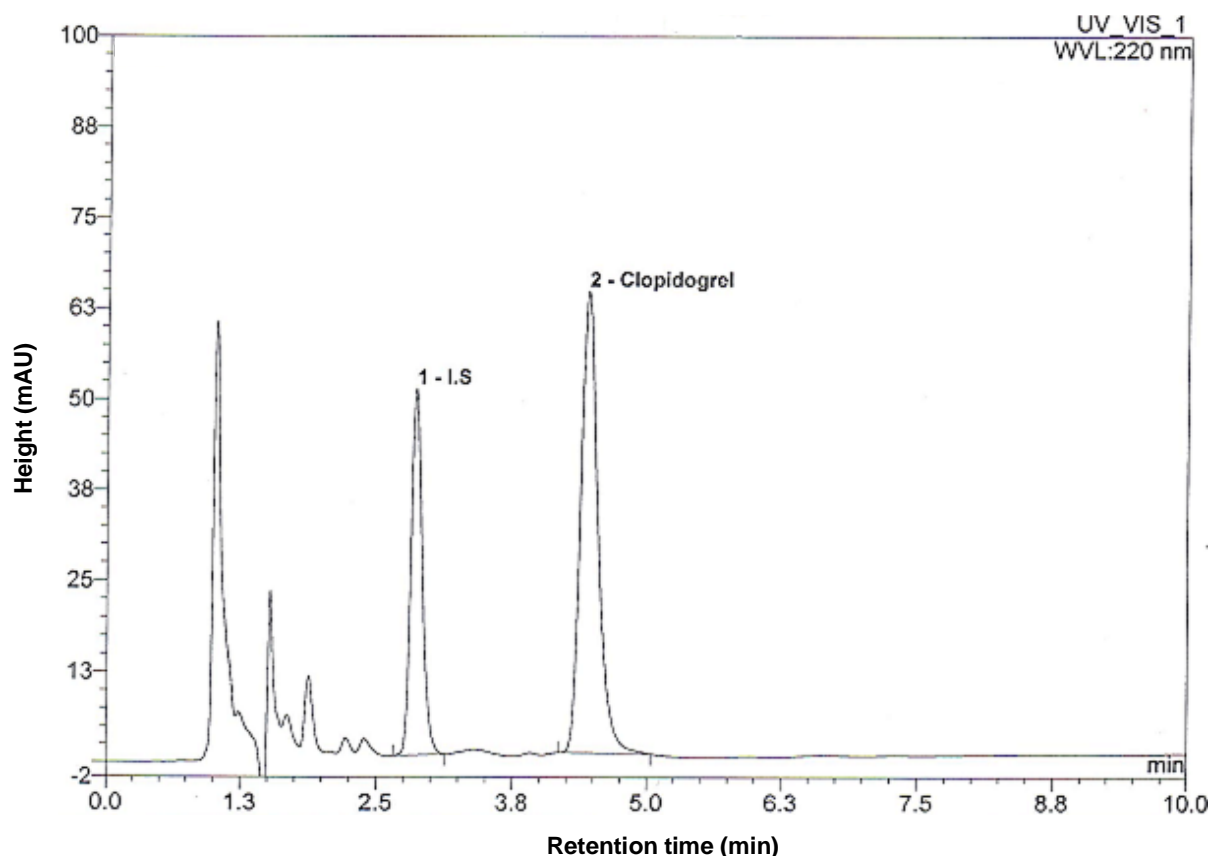
Figure 1. HPLC chromatogram of carboxylic acid metabolite of clopidogrel and internal standard (IS). Plasma sample spiked with clopidogrel carboxylate metabolite and IS; Peaks: 1, IS; 2, clopidogrel carboxylate acid metabolite.

RESULTS

Chromatographic condition

Under the chromatographic conditions described, the drug and IS were well resolved in plasma samples and eluted at 4.44 and 2.85 min, respectively. No interfering peaks of endogenous plasma components were found at the retention time of metabolite or internal standard in blank plasma (Figure 1)

Method development and validation

Linearity

A linear relationship was obtained between the peak area

ratio of metabolite and that of the internal standard versus the corresponding concentration over the range 0.5 to 50.0 μg/ml (Table 1).

Accuracy and precision

Precision and accuracy of intra-day and inter-day are presented in Table 2. Variation ranged from 3.05 to 5.97%, and accuracy ranged from 100.33 to 110.04%.

Extraction recovery

The recovery of metabolite and internal standard was determined. The mean recoveries of carboxylic acid metabolite of clopidogrel at concentrations of 1.5, 20.0

Table 1. Statistical data of calibration curves of carboxylic acid metabolite of clopidogrel in spiked human plasma.

Parameter	Clopidogrel metabolite
Range of linearity	0.5-50 µg/ml
Regression equation	Peak area ratio = 3.89×10^{-2} X + 1.48×10^{-2}
SD of slope	4.2×10^{-4}
RSD of slope	1.08%
SD of intercept	6.2×10^{-3}
Correlation coefficient	0.9997

SD: standard deviation, RSD: relative standard deviation

Table 2. Precision and accuracy of method for determination of carboxylic acid metabolite of clopidogrel in spiked plasma.

Concentration added (µg/ml)	Concentration found (mean ± SD) (µg/ml)	Precision as CV (%)	Accuracy (%)
Within-day (n = 3)			
1.5	1.62±0.06	3.95	108±4.27
20.0	20.71±1.02	4.95	103.56±5.12
40.0	40.81±2.35	5.75	102.03±5.86
Between-day (n = 10)			
1.5	1.65±0.06	3.65	110.04±4.00
20.0	20.07±1.20	5.97	100.33±5.99
40.0	41.39±1.26	3.05	103.46±3.16

and 40.0 µg/ml were 81.95, 79.2, and 81.9%, respectively with mean recovery of 80.3 ± 1.91. The mean recovery of internal standard was 77.48%.

Patients' characteristics

From a total of 100 patients who received 600 mg clopidogrel loading dose, only eighty patients were selected and accepted to take part in the study. Table 3 summarizes characteristics of recruited subjects and Table 4 summarizes their laboratory findings.

Pharmacokinetic parameters

The primary pharmacokinetic parameters of clopidogrel carboxylic acid metabolites are reported in Table 5. Figure 2a represents the average plasma concentration of clopidogrel carboxylate as it changes over time after oral administration of 600 mg of clopidogrel to patients undergoing PCI. The pharmacokinetic parameters of participants were characterized by considerably inter-individual differences [C_{max} = 24.49 ± 11.64 µg/ml (CV = 47.5%), T_{max} = 2.02 ± 1.52 h (CV = 75.2%), $AUC_{0\to\infty}$ = 123.17 ± 54.6 µg/ml × h (CV = 44.3%), and $t_{1/2e}$ = 4.29 ±

2.92 h (CV = 68%)]. The data were further subdivided into three groups based on the C_{max} value: "Lower 15%"; "Upper 15%" and the remaining data were termed "Trimmed". The three groups differed in their C_{max} (p < 0.0001, ANOVA), $AUC_{0\to t}$ (p < 0.0001, ANOVA), and $AUC_{0\to\infty}$ (p < 0.0001, ANOVA) (Table 5). The three groups did not differ with their $t_{1/2e}$ (p = 0.44, Kruskal-Wallis); AUMC (p = 0.195, Kruskal-Wallis); MRT (p = 0.736, Kruskal-Wallis); or Vd/F (p = 0.61, Kruskal-Wallis), though there was significant difference in T_{max} (p<0.0001, Kruskal-Wallis). The data indicate that the differences in C_{max} are not caused by changes in elimination rate among the three groups; rather it may be due to differences in absorption phase (extent and/or rate) (Figure 2b).

To evaluate the robustness of our findings, two other C_{max}-value dependent sub-classifications were assessed for pharmacokinetics parameters. The first one: "Lower 12.5%"; "Upper 12.5%" and the remaining data were termed "Trimmed". The second one: "Lower 17.5%"; "Upper 17.5%" and the remaining data were termed "Trimmed". In both cases, the three groups differed in their C_{max} (p < 0.0001, Kruskal-Wallis), $AUC_{0\to t}$ (p < 0.0001, Kruskal-Wallis), and $AUC_{0\to\infty}$ (p < 0.0001, Kruskal-Wallis). The three groups did not differ with their

Table 3. Demographic and clinical characteristics of recruited patients in current study as compared to published characteristics of Jordanian patients undergoing PCI.

Parameter	Number/mean (percent ± SD)	
	Current study (N=80)	Typical patients (Yousef et al., 2008) (N=159)
Age (years)	60.5 (±8.1)	57.6 (±10.2)
≥ 60	41 (52)	76 (47.8)
Gender		
Female	33 (41)	38 (24)
Body mass index (kg/m^2)	28.6 (±4.7)	28.3 (±4.3)
< 25	23 (28.8)	33 (21)
Smoking		
Current smokers	22 (27.5)	32 (20)
Quit < 12 months	3 (4)	19 (12)
Quit > 12 months	12 (15)	49 (31)
Does not smoke	43 (53.5)	59 (37)
Concomitant diseases	8 are medically free	
Hypertension	65 (81)	110 (69)
Diabetes mellitus	44 (55)	100 (63)
Dyslipidemia	32 (40)	118 (74)
Heart failure	10 (10)	25 (15.7)
Arrhythmia (include atrial fibrillation)	7 (8.5)	24 (15)
Peripheral vascular disease	4 (5)	24 (15)
Cerebrovascular accident-history	6 (7.5)	19 (11.9)
Myocardial infarction-history	12 (15)	94 (59)
Prior to admission medications history		
Aspirin	65 (81)	148 (93)
Clopidogrel	10 (12.5)	27 (17)
Beta adrenergic receptors blockers	48 (60)	78 (49)
ACEIs/ARBs	33/18 (41/22.5)	64/3 (40/2)
Diuretics	22 (27.5)	50 (31.4)
Calcium channel blockers	24 (30)	40 (25)
HMGCo-reductase inhibitors	50 (62.5)	110 (69.2)
Fibric acid derivatives	5 (6)	10 (6)
Proton pump inhibitors	23 (28.75)	16 (10)
H2 receptor blockers	16 (20)	40 (16)
Anti-diabetic agents (oral)	35 (43.75)	60 (38)
Anti-diabetic agents (injection)	25 (31)	37 (23)
Cardiac glycosides (Digoxin)	6 (7.5)	16 (10)

$t_{1/2e}$ (p = 0.21, Kruskal-Wallis) but there was significant difference in T_{max} (p < 0.001, Kruskal-Wallis). The data indicate that the differences in C_{max} are not caused by changes in elimination rate among the three groups; rather it may be due to differences in absorption phase.

Authors investigated the hypothesis that lower C_{max} and delayed T_{max} may be due to dissolution problems. To test this hypothesis, dissolution test was carried out according to published compendia of United State, with two modifications. Rather than testing one 75 mg tablet at a time, four 75 mg tablets and eight 75 mg tablets were tested together in the dissolution vessel. Additionally, the 4 tablets and 8 tablets were evaluated for dissolution once in 900 ml total dissolution medium and once in 330

Table 4. Laboratory findings of patients included in the study.

Parameter	Mean ± SD
Kidney function	
Na conc. (mEq/L)	138.8±2.9
K conc. (mEq/L)	4.3±0.42
Scr (mg/dl)	0.74±0.22
BUN (mg/dl)	16.55±6.0
Liver function	
Total protein (g/L)	6.7±0.6
Albumin (g/L)	3.9±0.4
ALT (IU/L)	22.7±12.9
AST (IU/L)	24.7±9.9
ALP (IU/L)	81.7±11.8
GGT (IU/L)	40.26±66.2
Complete blood count	
RBC (10^6/mm^3)	4.6±0.6
WBC (10^3/mm^3)	8.8±2.7
Platelets (10^3/mm^3)	232.7±61.3
Hgb (g/dl)	12.7±1.7
Other tests (mg/dl)	
Total cholesterol	165.9±44.3
LDL-cholesterol	108.8±57.6
HDL-cholesterol	35.7±8.23
Triglycerides	169±87.6

Scr: Serum creatinine; BUN: blood urea nitrogen; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phophatase; GGT: gamma-glutamyltransferase ; RBC: red blood cell; WBC: white blood cell; Hgb: hemoglobin.

ml. We were not able to utilize less than 330 ml, as the level of the dissolution medium will be lower than the paddle level. The dissolution tests were repeated in triplicates for each variation from the compendia procedure. None of the modifications on the dissolution test resulted in significant changes in dissolution behavior. In all cases, more than 90% of the nominated clopidogrel contents were dissolved in the first 15 min.

DISCUSSION

Clopidogrel is an important antiplatelet agent that has application in the primary and secondary prevention of cardiovascular complications, especially among patients unable to take aspirin. In patients who unedrgo percutaneous coronary intervention, dual antiplatelet consisting of aspirin and clopidogrel is the regimen of choice to prevent thrombotic complications (De Backer et al., 2003; Gibbons et al., 2003; Patrono et al., 2004)

This is the first report of the pharmacokinetics of 600

mg clopidogrel among Jordanians. While native Jordanians are mostly descended from people of villagers and Bedouin descent originating in the Arabian Peninsula (Lowi, 1995), ethnically, the Jordanians represent a mixed stock. Most of the population is Arab (approximately 98%), with 1% of the population is Armenian, and another 1% is Circassian. There are also small Kurd, Druze, and Chechen minorities (The Royal Hashemite Court, 2012)

Although, there was a previous study in young healthy Jordanian, this is the first to be conducted among patients undergoing PCI. The participants characteristics were comparable with published data of Jordanian patients undergoing PCI (Yousef et al., 2008). With regard to laboratory findings of recruited subjects, patients had normal or close to normal kidney function (serum creatinine, blood urea nitrogen and sodium); liver function (total proteins, albumin, aminotransferases, and alkaline phosphatase); lipid profile and complete blood count, indicating minimal if any effect on current findings (Table 4).

Very few HPLC methods were reported for the determination of carboxylic acid metabolite of clopidogrel (Singh et al., 2005; Souri et al., 2006; Bahrami et al., 2008). HPLC is the most convenient and common analytical method for therapeutic drug monitoring, and it is readily available in majority of laboratories. We reported simple isocratic reversed phase HPLC method with UV detection for the determination of inactive metabolite of clopidogrel. The statistical evaluation of the proposed HPLC method revealed its good linearity and reproducibility and led us to the conclusion that it could be used for the rapid and reliable determination of clopidogrel carboxylic metabolite in plasma in pharmacokinetic studies.

The high pharmacokinetic variability (at the level of C_{max}, AUC, T_{max} and $T_{1/2e}$) observed by us and others (Caplain et al., 1999; Taubert et al., 2004) may explain the reported inter-individual variability of ADP-induced inhibition of platelet aggregation to clopidogrel (Matetzky et al., 2004; Snoep et al., 2007). The observation is of importance as it is estimated that laboratory clopidogrel non-responsiveness can be found in 1 of 5 patients undergoing PCI (Muller et al., 2003; Snoep et al., 2007). Patients *ex vivo* labeled non-responsive are likely to be also clinically "non-responsive" with increased cardiovascular risk (Muller et al., 2003; Matetzky et al., 2004). Although there are a number of postulated causes of clopidogrel resistance phenomenon, the major underlying etiology is still unresolved (Fontana et al., 2003; Lau et al., 2004; Gurbel et al., 2005; Heestermans et al., 2006; Angiolillo et al., 2007; Snoep et al., 2007; Kim et al., 2008; Ford, 2009). It has been suggested that the high variability of clopidogrel and its metabolite may be due to differences in activation process (Angiolillo et al., 2007; Brandt et al., 2007; Kim et al., 2008; Ford,

Table 5. Pharmacokinetic parameters of clopidogrel carboxylic acid in patients after taking 600 mg clopidogrel.

Parameter	Mean ± SD			
	All (N=80)	Lower 15%[a] (N=12)	Trimmed[c] (N=56)	Upper 15%[b] (N=12)
C_{max} (μg/ml)	24.49±11.64	8.09±2.34	23.88±6.1	43.76±9.05
T_{max} (h)	2.02 ± 1.52	4.17±1.76	1.71±1.2	1.35±0.55
$t_{1/2e}$ (h)	4.29 ±2.92	3.99±0.95	4.05±2.37	4.45±3.38
AUC_{0-t} (μg/ml×h)	90.40±32.64	42.27±12.96	91.29±21.77	127.11±34.66
$AUC_{0-\infty}$ (μg ml/h)	123.17±54.60	58.09±19.36	125.54±51.22	163.89±45.07
$AUMC_{0-\infty}$ (μg/ml×h^2)	899±1186	1452±2807	754±651	1082±772
MRT (h)	6.0±2.7	5.3±1.6	5.9±2.5	6.9±4.0
Vd/F (L)	32.7±24	37±30.	32.7±24.1	29.3±19.3

[a,b] Based on C_{max} value, data were subdivided into the lower 15% and upper 15%. [c]Data excluding the lower 15% and the upper 15%.

2009).

The interindividual differences in the activity of the cytochrome P450 isoenzymes 3A4, 3A5 (Lau et al., 2003; Lau et al., 2004) and 2C19 (Brandt et al., 2007; Kim et al., 2008; Mega et al., 2009) that metabolize the inactive clopidogrel prodrug to its active thiol form were suggested to underlie the response variability. Additionally, polymorphisms of P_2Y_1 and P_2Y_{12} genes, differences in receptor expression or posttranscriptional regulation or the downstream signaling cascades are proposed to contribute to individual variations in ADP-induced response to P_2Y_{12} inhibition with clopidogrel (Fontana et al., 2003; Hechler et al., 2003; Ford, 2009).

In addition to the high variability in carboxylic acid metabolite concentrations, our results concerning pharmacokinetics parameters differed from what has been published previously (Taubert et al., 2004; von Beckerath et al., 2005). Taubert and co-authors investigated the pharmacokinetics of clopidogrel after administration of a high loading dose (600 mg) to ten fasting young healthy volunteers, and revealed higher C_{max} and $AUC_{0\rightarrow\infty}$ (C_{max}: 43 μg/ml ± 16.9; T_{max}: 1.6 ± 0.9 h; $AUC_{0\rightarrow\infty}$: 198.6 ± 52.4; $t_{1/2e}$: 1.9 ± 0.9 h) as compared to our study (Taubert et al., 2004). Nicolas von Beckerath and colleagues compared three different loading doses (300, 600, 900 mg) of already crushed tablets that were given in solution form to sixty patients with suspected or documented coronary artery disease, and their reported C_{max} (44 μg/ml) was higher than ours (von Beckerath et al., 2005). The previous two studies did not aim to assess the crushed tablets versus non crushed tablets in the same study side by side. In a recent study (Zafar et al., 2009), plasma levels of clopidogrel carboxylic acid metabolite were followed in healthy subjects who were given 300 mg clopidogrel in crushed form via nasogastric tube with 30 mL water. Two weeks later the same subjects swallowed 300 mg clopidogrel. Plasma concentrations peaked earlier after crushed delivery than

after oral intake and the median peak was 80% higher (Zafar et al., 2009). It should be noted that current study recruited patients rather than healthy volunteers who were given non-crushed and non dissolved whole eight (75 mg) tablets. Published pharmacokinetic parameters of clopidogrel administered at doses other than 600 mg were considered. Upon extrapolation to 600 mg dosing, there were similarities between C_{max} and current study C_{max} (Ksycinska et al., 2006; Souri et al., 2006; Bahrami et al., 2008).

Several studies suggested that the high variability of clopidogrel and its metabolite may be due to differences in absorption rate (Taubert et al., 2004; Taubert et al., 2006; Zafar et al., 2009). In a subset of our patients (n = 12) who had the lowest C_{max} values, they had $t_{1/2e}$ similar to the rest of the subjects but had significantly delayed T_{max}. The data indicate that the differences in C_{max} are not caused by changes in elimination process; rather it may be due to differences in absorption phase. Authors investigated the hypothesis that lower C_{max} and delayed T_{max} may be due to dissolution problems. The hypothesis was proposed for a number of reasons: Patients undergoing PCI are recommended by physicians not to drink or eat, starting from the night before the operation. This means that water content in the stomach by the time of PCI is very little, which may affect dissolution. Studies conducted on crushed tablets reported noticeably higher levels of clopidogrel carboxylic acid C_{max} (Taubert et al., 2004; von Beckerath et al., 2005; Zafar et al., 2009). To test this hypothesis, dissolution test was carried out with slight modification from published USP (USP, 2007). None of the modifications on the dissolution test resulted in significant changes in dissolution behavior. Further research has to include different ways of administering clopidogrel; as whole tablets, as crushed tablets and as crushed and dissolved tablets. Also, *in situ* disintegration and dissolution have to be evaluated.

Yet another potential explanation is the variable intestinal
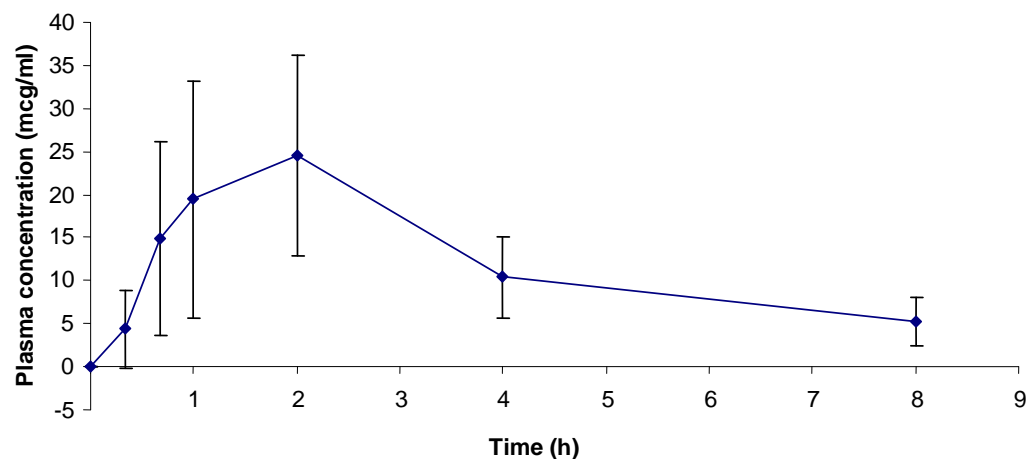
(A)
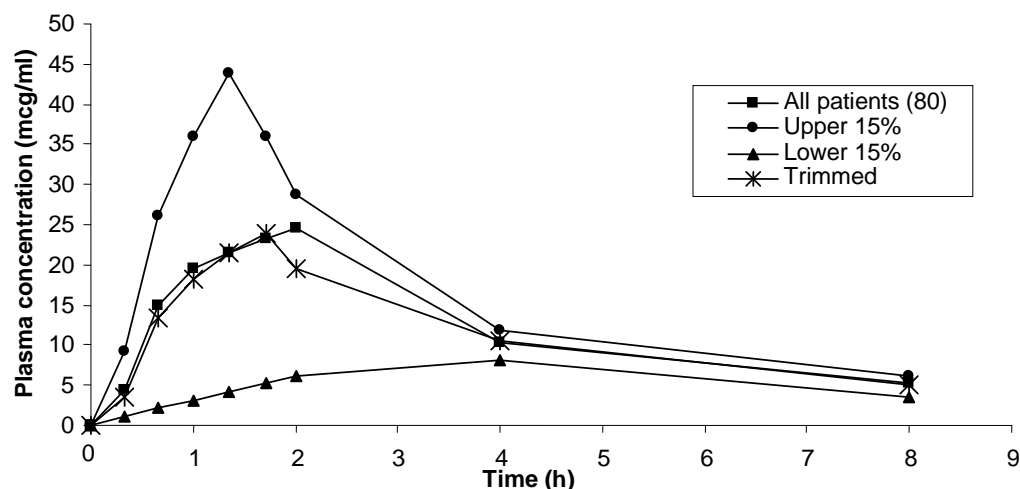
(B)

Figure 2. Average plasma drug concentration versus time profile determined after oral administration of clopidogrel (dose 600 mg). (A) Plasma concentration profile for total 80 patients; (B) plasma concentration profile for 3 subgroups (upper, lower and trimmed) compared to the 80 patients. Two additional time points were assumed (time to reach C_{max} for both trimmed (1.75 h) and upper 15% (1.35 h).

absorption mediated through P-glycoprotein (P-gp) along the intestinal mucosa. Inhibition of P-gp activity by different modulators increased the absorptive clopidogrel flux across Caco-2 monolayers. C_{max} and AUC values were lower in subjects homozygous for the *MDR1 3435T* variant compared with subjects with the *3435C/T* and *3435C/C* genotypes (Taubert et al., 2006). Other than genetic polymorphism, many factors have been found to alter P-gp expression including but not limited to food intake (Deferme et al., 2002; Zhang et al., 2009), diseases (Liu et al., 2008; Dopp et al., 2009), and drugs (Fiegenbaum et al., 2005) .

The findings of current study should be read with

caution, as the parent compound and the active metabolite were not measured. However, previous studies showed a good relationship between serum concentrations of inactive metabolite, active metabolite and parent compound (Taubert et al., 2004). Some other published bioequivalence studies also used data for carboxylic acid metabolite in their results. Additionally, the findings of this study are limited by the fact that the population being studied suffered from multiple medical problems and were on long lists of medications, all of which may influence the pharmacokinetic behavior of clopidogrel. Still, this population does reflect some certain groups of patients in community practice who are   often

prescribed clopidogrel. Moreover, the sampling time in the current study was short as it was limited to 8 h post treatment. More time is needed (24 to 48 h) to have a better estimation of terminal half-life. Unfortunately, this is not feasible in real life situation where real patients are being recruited, and most of them get discharged after much less than 24 h post stenting. It should be noted that several other studies terminated sampling by 8 h or even less (Deferme et al., 2002; Zhang et al., 2009).

ACKNOWLEDGMENTS

Abbreviations: ADP, Adenosine diphosphate; **P_2Y_{12},** platelet receptor; **C_{max},** maximum plasma concentration; **$AUC_{0\to\infty}$,** area under the "plasma concentration vs. time" curve; **BMI,** body mass index; **Hb,** hemoglobin; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **T_{max},** time to peak plasma concentration; **C_{last},** last quantifiable concentration; **$t_{1/2e}$,** elimination half life; **AUMC,** area under the first momentum curve; **MRT,** mean residence time; **Vd,** apparent volume of distribution; **Δ,** accepted margin of error; **SD,** standard deviation; **CV,** coefficient of variation.

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Full Length Research Paper

Pharmacological evaluation, molecular docking and dynamics simulation studies of salicyl alcohol nitrogen containing derivatives

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The current study was conducted to evaluate *in vivo* the anti-inflammatory, antinociceptive and antipyretic activities of salicyl alcohol nitrogen containing derivatives that are [4-(2-hydroxybenzyl) morpholin-4-iumchloride (I)], [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride (II)]. The synthetic compound I, II and standard (aspirin) were evaluated in the laboratory animal model at three different dose levels for each activity. These compounds were examined for, anti-inflammatory activity in carrageenan induced paw edema model [50, 100 and 150 mg/kg intraperitoneally (i.p)], antinociceptive properties in acetic acid induced writhing model (15, 30 and 45 mg/kg i.p), hot plate test model (30 and 45 mg/kg i.p) and antipyretic activity in Brewer's yeast induced pyrexia model (50,100 and 150 mg/kg i.p), using Swiss albino mice. Result of this study indicated that these compounds; possess dose dependent statistically significant anti-inflammatory, antinociceptive and antipyretic properties, comparable to standard aspirin. Nonetheless, these compounds did not show antinociceptive properties in hot plate test when compared with centrally acting standard analgesic (morphine), thus signifying peripheral mechanism of action in the mediation of antinociception. In order to investigate receptor-compounds interactions in terms of the binding affinity, the molecules were subjected to molecular docking simulation analysis using FRED 2.1 software that showed better binding energy of the compounds with the Cyclooxygenase X (COX)- 2 enzyme, predicting these compounds as potential COX-2 inhibitors. As in actual cellular system there was a solvent which makes the enzyme to have a dynamic movement so, molecular dynamic (MD) simulation was carried out during 200 pico seconds (ps) to better understand the binding modes of these compounds with the receptor.

Key words: [4-(2-Hydroxybenzyl) morpholin-4-ium chloride], [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride], anti-inflammatory, antinociceptive, antipyretic agents, molecular docking, MD simulation.

INTRODUCTION

Biochemical activities of drugs are clinically dependent upon their interaction with living systems. Most drugs exert their action through a complex mechanistic pathway but primarily it is the chemical skeleton which determines the drug response. Salicin (Figure 1), utilized in the treatment of acute rheumatism, was first reported by Machagan (Machagan, 1876). It is a glycoside of salicyl alcohol obtained from willow bark (*Salix alba*), chemically related to acetyl salicylic acid (aspirin) and exhibits almost similar activities in the body. Hydrolysis of salicin results

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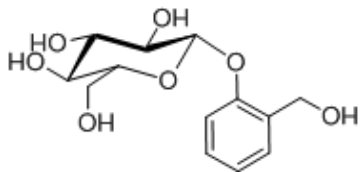
Figure 1. Salicin.
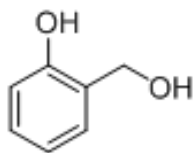
Figure 2. Salicyl alcohol.
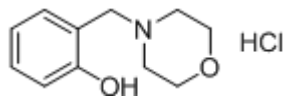
Figure 3. Compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride].
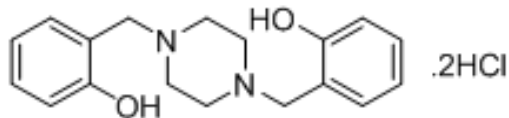
Figure 4. Compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride].

in liberation of salicyl alcohol (saligenin; 2-hydroxy methyl phenol) (Figure 2). Salicin has been reported to be useful as antipyretic pro-drug with no propensity for gastric damage (Akao et al., 2002). Saligenin which is a part of salicin, has been used for long time as a local anesthetic and has also been shown to have a local anesthetic property (Harischfelder et al., 1920; Harischfelder and Wynne, 1920). Furthermore, evidence of anesthetic and antipyretic activities of salicyl alcohol has been mentioned (Charles and Tony, 1956).

It has been reported that compounds containing piperazine moiety inhibit eicosanoid pathways (Coonrod et al., 2001) and possess different pharmacological properties (Berardi et al., 2008) including anti-inflammatory and antinociceptive activities (Jakubkien et al., 2003). In this connection, piperazine moiety is considered a critical core for novel drug design (Bali et al., 2010). Furthermore, anti-inflammatory and antinociceptive properties of heterocycles conjugated to morpholine have been also reported (Panneerselvam et al., 2009).

Additionally, it has been argued that piperazine, piperidine and morpholine ring containing compounds having nitro, oxo or chloro substitution exhibit different biological activities (Folkes et al., 2007; Wermuth and Fontaine, 2003).

To manage inflammation, pain and pyrexia, nowadays Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are commonly prescribed worldwide whereas opioids are used for severe painful conditions like cancer pain that cannot be managed by typical NSAIDs. However, it is a well recognized fact that persistent use of NSAIDs exhibit several side effects notably; ulceration, perforation, hemorrhage of gastrointestinal tract, cardiovascular disorders and renal damage (Jones et al., 2008) while opioids cause drug tolerance and physical dependence (Mayer et al., 1995). Hence, there is an explicit need for alternative drugs to opioids and classical NSAIDs that possess effective anti-inflammatory, antinociceptive and antipyretic activities still devoid of such adverse effects. In this study, we have carried out selected pharmacological evaluation and molecular docking and dynamic simulation of our already synthesized derivatives carrying salicyl alcohol attached to morpholine and piperazine nuclei are; compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] (Figure 3) and compound II [1,4-bis(2-hydroxybenzyl) piperazine-1,4-diium chloride] (Figure 4).

Keeping in view, the great importance of salicyl alcohol as a drug or a prodrug and the potential pharmacological activities of compounds obtained with morpholine and pipreazine substitutions and also their desirable solubility profile, we undertook targeted pharmacological evaluation of salicyl alcohol derivatives. In addition, to rationalize the pharmacological activities of these synthetic compounds at the molecular level, their interactions with the binding sites of COX's, were studied with molecular docking simulation approach using FRED 2.1 software.

MATERIALS AND METHODS

Equipments and chemicals

Anti-inflammatory activity was tested using digital plethysmometer (Model LE 7500 Plan lab *S.L.*), antinociceptive activity was tested using hot plate analgesiometer (Harvard apparatus, USA) and antipyretic with digital thermometer (Model CA92121, ACON Laboratories, USA). Lambda Carrageenan (Sigma, USA), acetyl-salicylic acid (Sigma, USA), glacial acetic acid (Panreac, Spain), Brewer yeast (Merck, Germany), Morphine sulphate obtained through proper channel from Punjab Drug House, Lahore Pakistan.

Animals

Mice (*Balb-c*), bred in the department of Pharmacy, University of Peshawar, animal house and bioassay laboratories, were used throughout experimental studies. Animals were housed in standard cages with free access to standard laboratory food and water available *ad libitum* except where experimental protocol restricted

to do so. All experimental procedures were carried out between 8.00 am-4.00 pm. The 12 h light and dark cycle was provided with temperature maintained at 22±2°C through a reversible air condition (AC). The animal studies were approved by Departmental ethical committee in its 3rd meeting held on June 15, 2011 and approval certificate obtained bearing number 15/EC/pharm. All the procedures were carried out in accordance with Animals Scientific Procedure Act (1986) UK.

Evaluation of anti-inflammatory activity (Carrageenan-induced paw edema model in mice)

Carrageenan-induced paw edema model was used for the determination of anti-inflammatory activity in mice as described previously (Winter et al., 1962). The animals of either sex (25-30 g) were fasted overnight with free access to water. For the experiments, the animals were randomly divided into four groups (I, II, III and IV), each group consisting of eight animals (n=8).

Group I: Control (untreated) group, received saline (intraperitoneal, i.p).
Group II: Standard treatment group (aspirin 50, 100 and 150 mg/kg kg i.p)
Group III: Test treatment group (Compound I   50, 100 and 150 mg/kg kg i.p)
Group IV: Test treatment group (Compound II   50, 100 and 150 mg/kg kg i.p)

Test samples, standard and vehicle control in their respective doses were administered intraperitoneally (i.p), 30 min prior to the injection of carrageenan. An equal volume of saline was received by saline control group. Then, each mouse was administered 0.05 ml of 1% carrageenan by subplanter injection in hind paw. A digital plethysmometer, which is a valuable instrument to quantify small volumes changes, was used to determine the anti-inflammatory activity by measuring edema (ml) before and after carrageenan injection at intervals of 1, 2, 3, 4 and 5 h. Inflammation was quantified according to the method described by Planichamy (1990).

% Inhibition = A-B/A × 100

Where, A and B indicate, increase in paw volume of control and drug-treated animals respectively.

Evaluation of antinociceptive activity

a. Acetic acid induced writhing test in mice

Albino mice (*Balb-C*), weighing 18-22 g of either sex were used in this study. Food was withdrawn from animals 2 h before the start of experiments. Writhing behavior was induced by intraperitoneal administration of 1% acetic acid (10 ml/kg).The number of writhes (abdominal muscles contraction, accompanied by an elongation of the body and hind limbs extension) occurring over a period of 20 min were counted after 5 min of administration of 1% acetic acid (Abbas et al., 2011). For the experiments, the animals were randomly divided into four groups (I, II, III and IV), each group consisting of eight animals (n=8).

Group I: Control (untreated) group, received saline (i.p).
Group II: Standard treatment group (aspirin 15, 30 and 45 mg/kg

i.p)
Group III: Test treatment group (Compound I 15, 30, and 45 mg/kg i.p)
Group IV: Test treatment group (Compound II 15, 30, and 45 mg/kg i.p)

The test samples (compound I and compound II), standard (aspirin) and the saline control were administered i.p, 30 min prior to 1% acetic acid administration. Saline control group received an equal volume of saline. Percent protection against nociception was calculated with the help of the following formula.

% Protection = (1 - Mean number of writhes of treated drug/ Mean number of writhes of control) × 100

b. Hot plate test (thermal) in mice

Albino mice (*Balb-C*), weighing 18-22 g of either sex were used in this study. Prior to the start of experimental procedure, mice were habituated to laboratory environment at least for two hours. A transparent glass cylinder was used to restrict the animal to the surface of hot plate of analgesiometer and the temperature maintained at 54.0 ± 0.1°C. Hot plate reaction time (latency to response in seconds) was observed by noting licking, flicking of hind limb or jumping from cylinder (Abbas et al., 2011). A cut-off time of 30 s was fixed so that if an animal did not respond within the prescribed time, then they could be immediately removed from the hot plate surface to avoid tissue damage. For the experiments, the animals were randomly divided into four groups (I, II, III and IV), each group consisting of eight animals (n=8).

Group I: Control (untreated) group, received saline (i.p).
Group II: Standard treatment group (Morphine 5 mg/kg i.p)
Group IV: Test treatment group (Compound I 30 and 45 mg/kg i.p)
Group V: Test treatment group (Compound II 30 and 45 mg/kg i.p)

Thirty minutes after the pretest, control, standard and test samples were administered to their respective groups intraperitonelly (i.p). An equal volume of saline was received by saline control group. The animals were tested again after thirty minutes and response was recorded at 30 and 60 min on the hot plate of analgesiometer. Antinociception was calculated using the following formula.

% Antinociception = (Test latency – control latency)/ (Cut-off time – control latency) × 100

Evaluation of antipyretic study *[Brewer's yeast induced pyrexia test in mice]*

In this study, we used albino mice (*Balb-C*), weighing 25-30 g of either sex. For the experiments, the animals were fasted overnight with free access to water. The animals were randomly divided into four groups (I, II, III and IV), each group consisting of eight animals (n=8).

Group I: Control (untreated) group, received, water for injection (i.p).
Group II: Standard treatment group (aspirin 50, 100 and 150 mg/kg i.p)
Group III: Test treatment group (Compound I 50, 100 and 150 mg/kg i.p)
Group IV: Test treatment group (Compound II 50, 100 and 150 mg/kg i.p)

Hyperpyrexia was induced by aqueous suspension of 20% Brewer's yeast (10 ml/kg body weight s.c) below the nape of neck in the back of mice (Al-Ghamdi, 2001). An equal volume of saline was received by saline control group. Changes in rectal temperature were noted after 24 h of Brewer's yeast injection at 0.5, 1.0, and 1.5 h (Barkatullah et al., 2011). For insertion, rectal probe of digital thermometer was lubricated with olive oil and maintained for thirty seconds for recording temperature. Those animals were selected for study that showed a minimum rise of at least 0.3-0.5°C rectal temperature.

Molecular docking simulation

Molecular docking study was conducted using FRED 2.1 (Khan et al., 2011). Molecular structure of the test compounds were generated by multi-conformer library. In order to get accurate results, compounds were exhaustively docked/scored to assess all possible positions for each ligand in the binding site of COX-2 (PDB ID: 3PGH). The exhaustive and extensive search of best binding mode is based on rotations of rigid and translations of all conformers inside the binding pocket defined by a default box implement in FRED. The filtered poses combine as an ensemble by omitting the poses ones, which clash with target protein. The final poses are then ranked or re-scored employing scoring functions available in FRED. In this study, Shapegauss was selected to determine the matching the shape of each ligand with the binding pocket. Default FRED protocol was employed except for the size of the searching box defining the boundaries of binding sites. In order to optimize the docking and scoring performance, we performed exhaustive docking with shapegauss using the "Optimization" mode of FRED. The "Optimization" mode includes a systematic solid body optimization of the top ranked poses from the exhaustive docking. Three different boxes were explored for enzyme. Three different simulations were carried out with an added value of 8 Å around the reference ligand.

Molecular dynamics simulation

The molecular dynamics (MD) simulation was performed using MOE-dynamic implemented in MOE-2010.11 (www.chemcomp.com). The utilized data were enzyme-ligand complexes from the docking results. Before doing MD simulation, enzyme-ligand complexes were optimized with partial charge menu and energy minimization was carried out until RMS gradient 0.05. System was subsequently solvated with TIP3P water using cubic octahedron box extending to 7Å from the system and molecular dynamics were performed after that at 300 K. MD simulation was done by choosing MMFF94x force field and NVT (N, total atom; V, volume; T, temperature) ensemble with 0.002 ps time step and sampling every 0.5 ps. The other parameter was set on default value, which was ensemble NVT and NPA algorithm for creating ensemble trajectory. The simulation observation was done by examining the enzyme-ligand complex interaction between ligand atoms and enzyme atoms with the end of simulation (200 ps).

Solubility

Our synthetic compounds were checked for solubility pattern in different solvents including water according to descriptive terminologies of solubility (Vallender, 2009).

Statistical analysis

Data were computed for statistical analysis by using Graph Pad Prism Software, version 5, for multiple comparisons by one-way analysis of variance (ANOVA) with Dunnett's 't'test. Results were considered statistically significant at $p < 0.05$.

RESULTS

Anti-inflammatory activity of aspirin (standard), compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] in   Carrageenan induced Paw edema model in mice

As depicted in Figure 5a, aspirin used as a standard, (Figure 5b) compound I and (Figure 5c) compound II, showed significant reduction in paw edema induced by carrageenan injection. One way ANOVA followed by Dunnett's post-hoc analysis revealed dose dependent decrease in paw edema. The effect was significant in standard aspirin, compound I and compound II treated groups at doses of 50 mg/kg (*p< 0.05), 100 mg/kg (**P<0.01) and 150 mg/kg (***P<0.001).

Antinociceptive activity of aspirin (standard), compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4- diium chloride] in acetic acid induced writhing test in mice

As shown in Figure 6a (aspirin), 6b (compound I) and 6c (compound II), all the treated groups tested, showed significant reduction in acetic acid induced writhes. ANOVA followed by Dunnett's post-hoc analysis of aspirin and compound II revealed significant decrease in writhes at doses 15, 30 and 45 mg/kg (***P<0.001) while compound I exhibited dose dependent decrease in writhes at doses 15, 30 mg/kg (**P<0.01) and 45 mg/kg body weight (***P<0.001).

Antinociceptive activity of morphine (standard), compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] in Hot plate test (Thermally induced) in mice

As depicted in Figure 7a and 7b , morphine used as a standard at a dose level of 5 mg/Kg, significantly increase the latency time on hot plate while compound I and II failed to increase the latency time significantly. ANOVA followed by Dunnett's post-hoc analysis of morphine revealed increase in latency time (**P<0.01, ***P<0.001) while compound I and II failed to increase the latency time in the hot plate test.
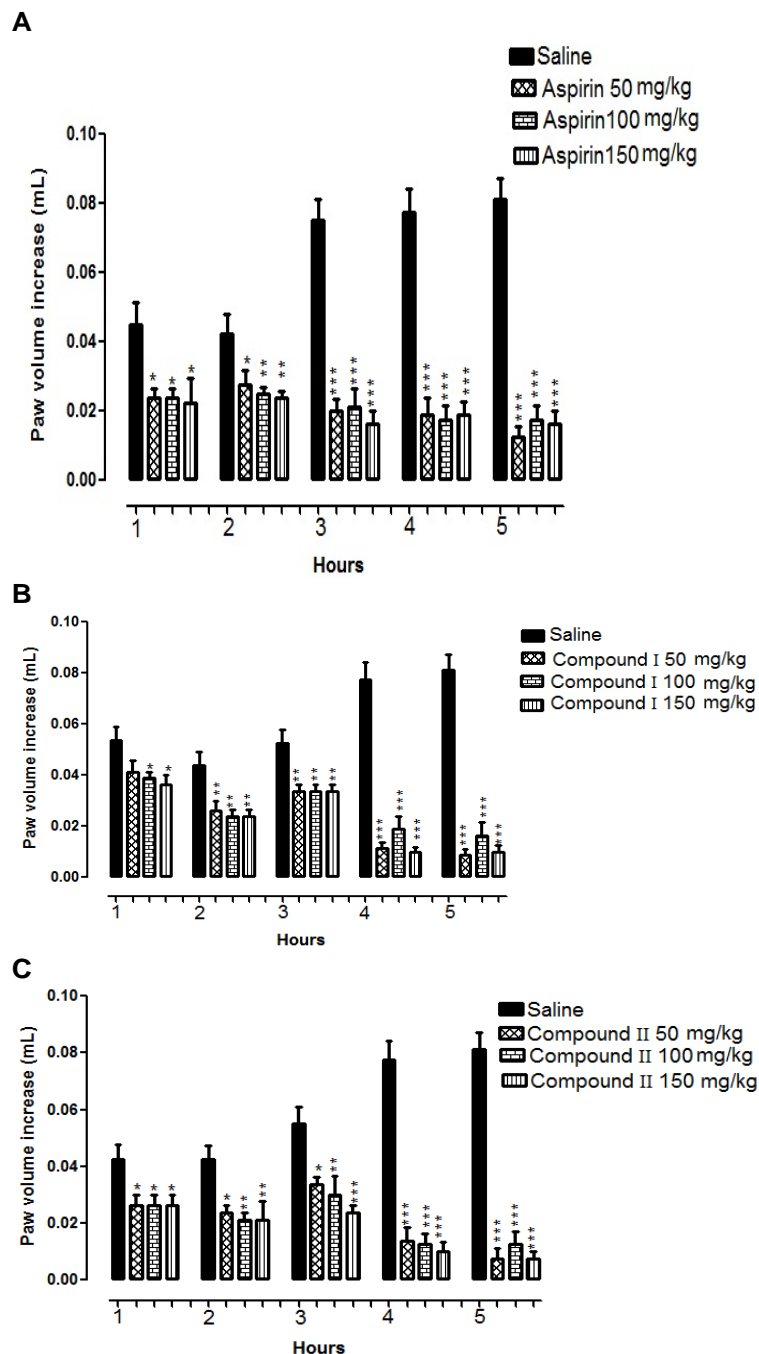
Figure 5. A, Anti-inflammatory activity of aspirin in Carrageenan induced paw edema test; B, Anti-inflammatory activity of compound I in Carrageenan induced paw edema test; C, Anti-inflammatory activity of compound II in Carrageenan induced paw edema test.

Antipyretic activity of aspirin (standard), compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] in Brewer's yeast induced pyrexia test in mice

As depicted in Figure 8a, aspirin used as a standard,

Figure 8b) compound I and (Figure 8c) compound II, showed significant reduction in hyperpyrexia induced by Brewer's yeast. ANOVA followed by Dunnett's post-hoc analysis revealed dose dependent decrease in pyrexia. The effect was significant in standard aspirin, compound I and compound II treated groups at doses of 50 mg/kg (*p< 0.05), 100 mg/kg (**P<0.01) and 150 mg/kg (***P<0.001).

A

B

C
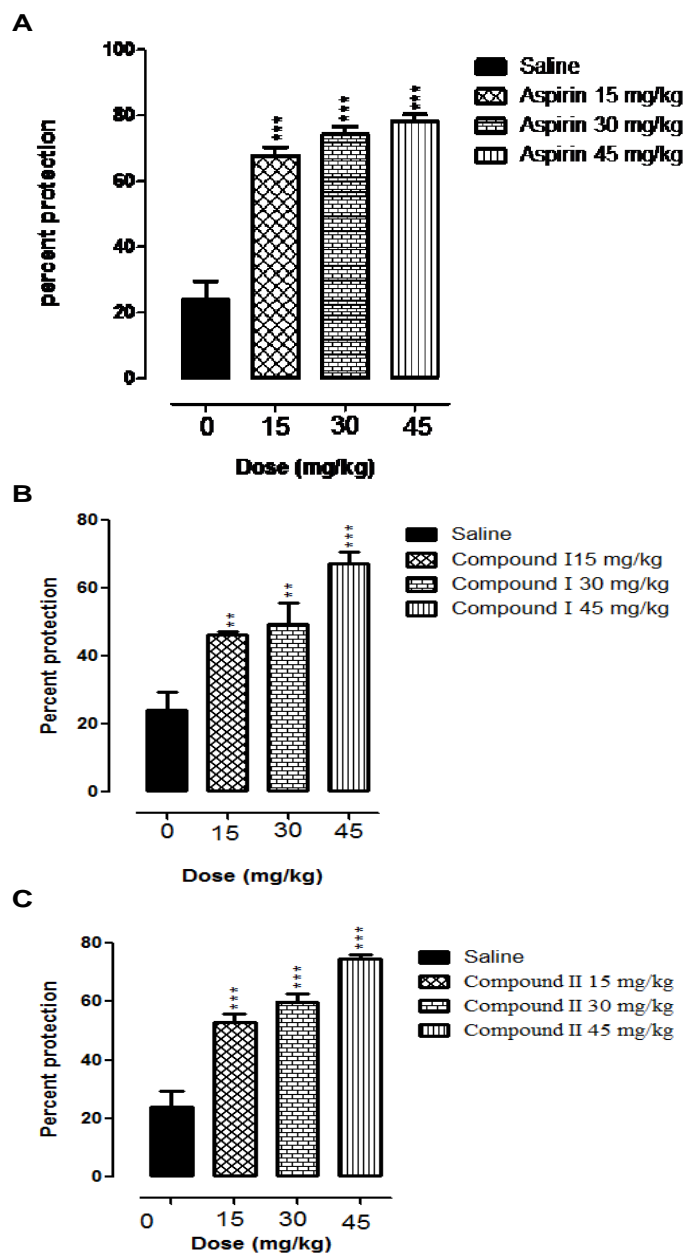
Figure 6. A, Antinociceptive activity of aspirin in acetic acid writhing test; B, Antinociceptive activity of compound I in acetic acid writhing test; C, Antinociceptive activity of compound II in acetic acid writhing test.

Molecular docking simulation

After internal validation, molecular docking simulations of the test compounds were conducted (Khan et al., 2011). Both compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] and compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride], showed considerable interactions (Figures 9 and 10) with the most important amino acid side chains (Arg120 and Tyr355), without any

A

B
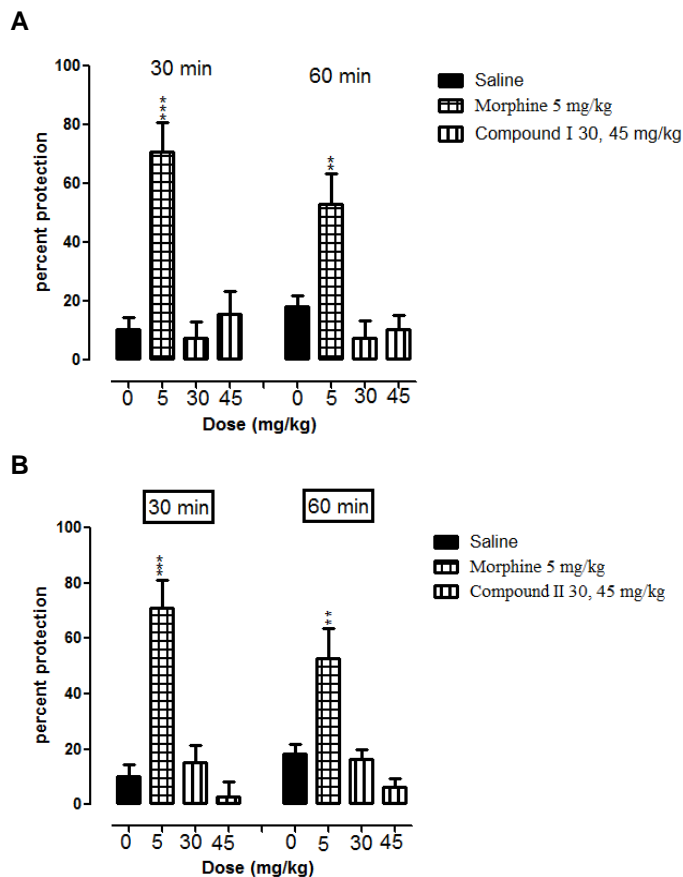
Figure 7. A, Antinociceptive activity of morphine and compound I in hot plate test; B, Antinociceptive activity of morphine and compound II in hot plate test.

steric and electrostatic clash. Their comparable binding affinities with respect to NSAID (flurbiprofen) provided a strong clue for their significant anti-inflammatory and analgesic activity. The elongated, yet flexible skeleton of compound II showed considerable molecular interactions (Figure 10) with main binding pocket of the enzyme.

Molecular dynamics simulation

Molecular dynamics simulation was carried out using enzyme-ligand complexes which were produced from docking simulation. The utilized enzyme-ligand complexes were added with partial charges, optimized and energy minimized by using energy calculation (force field MMFF94x). The applied parameters were set to MOE default value, which were ensemble NVT, constant temperature 300K and 101kPa pressure. This parameter was useful as in real experiment; it is much easier to adjust temperature. The employed NPA Algorithm was the most accurate and sensitive algorithm and it could set up the ensemble correctly.

There are several ways to analyze MD simulation

A

B

C
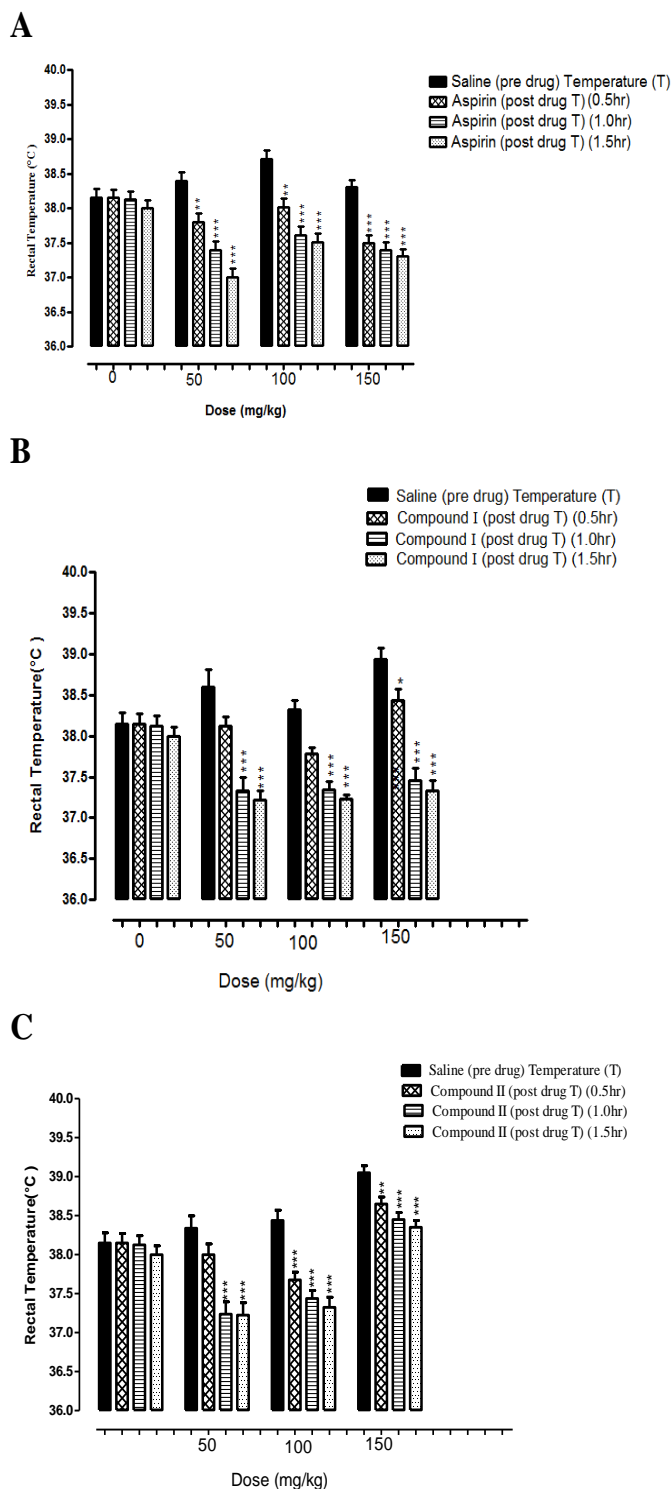
Figure 8. A, Antipyretic activity of aspirin in Brewer's yeast induced pyrexia test; B, Antipyretic activity of compound I in Brewer's yeast induced pyrexia test; C, Antipyretic activity of compound II in Brewer's yeast induced pyrexia test.

result. In this study we were concerned to review the total potential energy plot of complex conformation and ligand interaction to study the interaction between ligand and enzyme. Total potential energy plot could be used to
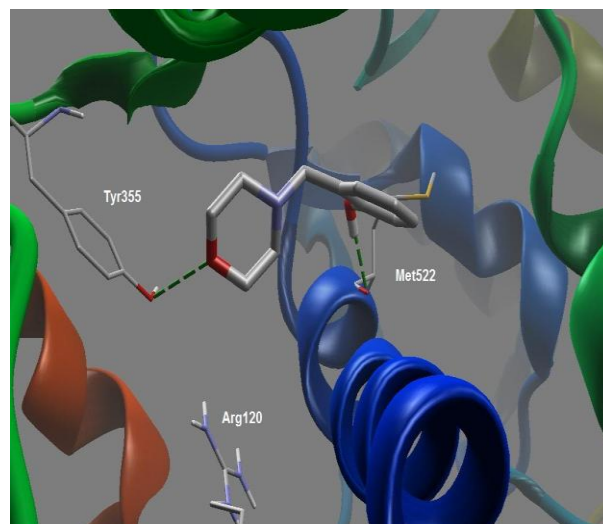
Figure 9. Molecular binding mode of Compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride] the inside main binding site of COX-2. Hydrogen bonding interactions are visible as green dotted lines. All hydrogen atoms except polar one are omitted for clarity.
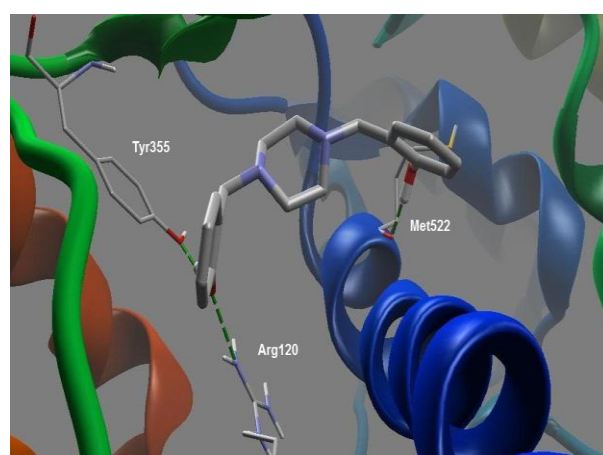
Figure 10. Molecular binding mode of compound II, [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride], the inside main binding site of COX-2. Hydrogen bonding interactions are visible as green dotted lines. All hydrogen atoms except polar one are omitted for clarity.

overview system conformational changes during simulation. Any damage to enzyme structure such as denaturation will affect total potential energy plot. As shown in Figure 12, all system gave similar plot during simulation. It means that complexion with ligand did not damage enzyme's structure. Enzyme was stable with or without ligand complex on it.

Solubility

We have checked solubility of the synthesized compounds

A

B
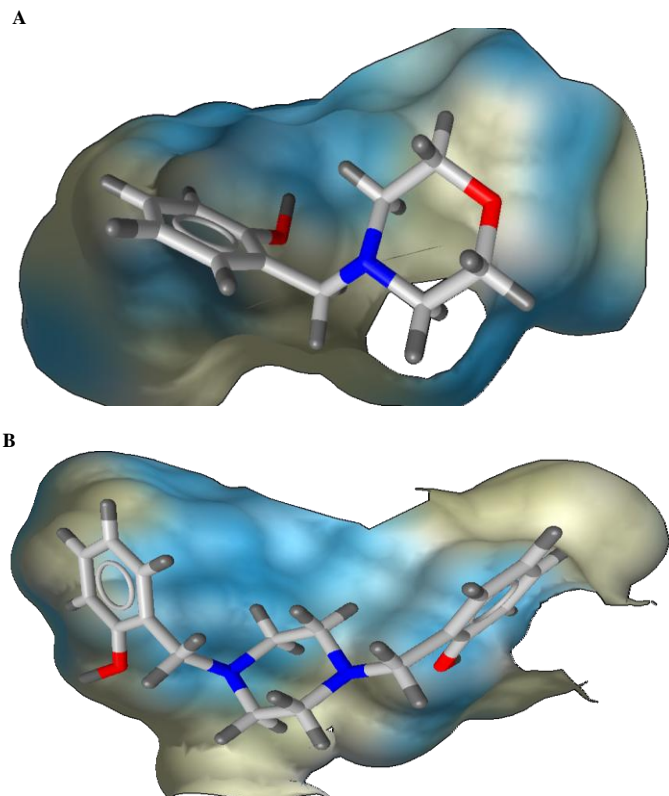
Figure 11. A and B, Hydrophobic (yellowish colored surface) and hydrophilic (blue colored surface) interactions between compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride], compound II [Compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] and COX-2.

in different solvents according to the descriptive terminologies of solubility (Vallender, 2009). Due to the presence of ammonium moieties these compounds are freely soluble in water, methanol, Dimethyl formide (DMF) and Dimethyl sulfoxide (DMSO). The pH of aqueous solution of the compounds was determined to be approximately 7 (neutral).

DISCUSSION

Across the globe, medical management of pain and inflammation is still a major challenge for drug discovery researchers and clinicians with an economic impact of billion annually (Wilhelm et al., 2009). It has been re-ported that pain, inflammation and pyrexia share common pathways in their development and mediation and often occur simultaneously. The pain receptors are activated by mechanical, chemical, thermal or other stimuli. After injury, histamine, serotonin, bradykinin, prostaglandin and other mediators are released. Histamine takes part in early stage of inflammation. Serotonin and bradykinin activates pain receptors while prostaglandins produce fever and increase pain sensation (Davies et al., 1984).

Therefore, drugs that inhibit these mediators act as analgesic, anti-inflammatory and antipyretic. The most widely accepted pathway involved in mediation of pain, inflammation and pyrexia is the cyclooxygenase pathway (Samad et al., 2002). Treatment strategies for these disorders involves inhibiting the enzymes; Cyclooxygenase-1 (COX-1), cyclooxygenase-11 (COX-11) and probably cyclooxygenase-111 (COX-111). In this respect, NSAIDs such as aspirin, flurbiprofen etc; the inhibitors of COX enzymes are in general practice for the clinical management of inflammation, pain and pyrexia (Steinmeyer, 2000). However, major serious limitation associated with chronic use of NSAIDs is the development of GIT disorders like ulceration, perforation and bleeding (Singh and Rosen Ramey, 1998), renal disorder (Bao et al., 2012), CVS disorders (David and Richard, 2005) and pulmonary distress (Camus, 1997), which arise due to their non selective inhibition of COX enzymes (Tapiero et al., 2002) and partly due to acidic nature of these drugs responsible for their local erosion properties. Among NSAIDs, aspirin is regarded a proto-typical drug and widely used despite of adverse effects.

This study aimed to evaluate *in vivo* and compare with aspirin, the derivatives bearing salicyl alcohol attached to morpholine and piprazine rings [4-(2-hydroxybenzyl) morpholin-4-ium chloride], [1,4-bis(2-hydroxybenzyl) piperazine-1,4-diium chloride] for selective pharmacological activities and molecular docking simulation studies.

Carrageenan-induced paw edema method (Winter et al., 1962) is widely used for testing anti-inflammatory activity of drugs. Inflammatory process proceed in two stages in rodents, the first stage, almost 1 h is associated with activity of biogenic amines, the second stage involves eicosanoide actions while the vasodilators peptides released in the process, maintain connection in the two stages (Vinegar et al., 1969). It has been demonstrated that the anti-edema effect of anti-inflammatory agents results due to inhibition of the cyclooxygenase (Cryer and Feldman, 1998) or lipooxygenase (De Simone et al., 2011) pathways, although such claim require further investigation. In this study, the anti-nflammatory activity of compound (I) and (II) and standard aspirin were tested in the carrageenan induced paw edema model. It was found that the com-pounds were significantly effective in reducing edema comparable to standard aspirin at doses 50, 100 and 150 mg/kg at 1, 2, 3, 4, and 5 h interval after carrageenan injection (Figure 5a, b, c). Acetic acid induced writhing and hot plate tests, were employed to study the anti-nociceptive activity of the compounds. Finding of this study indicated that both of the synthesized compounds (15, 30, 45 mg/kg) were effective in significantly ameliorating pain induced by acetic acid injected into the abdominal cavity but failed to show statistically significant effect in the thermally induced pain (Figures 6a, b, c and 7a, b). Basically, writhing test is used to evaluate both peripheral and central antinociceptive activity (Trongsakul et
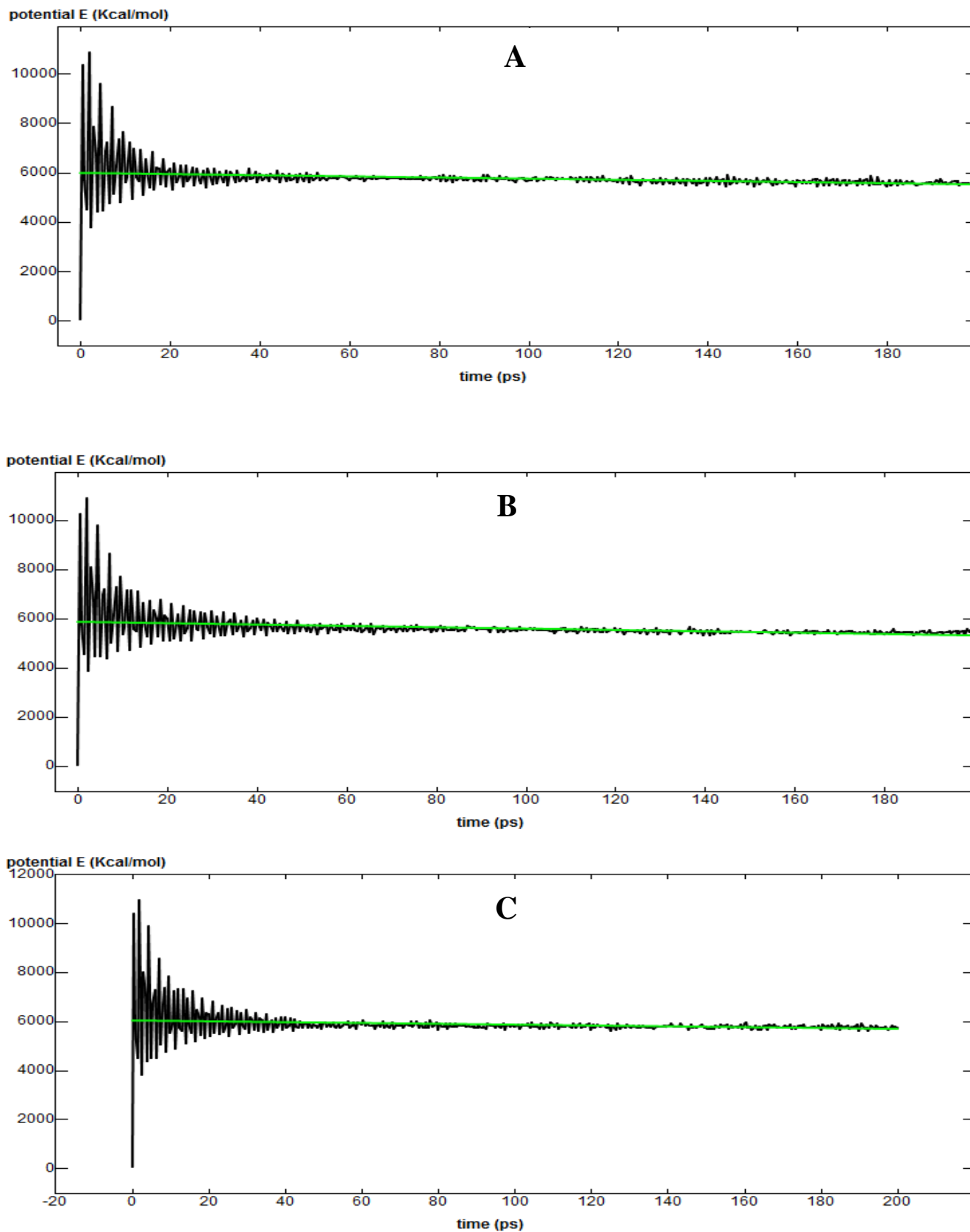
Figure 12. Total potential energy plot during MD simulation.
(A) Enzyme without ligand; (B) Enzyme complex with compound I; (C) Enzyme complex with compound II.

al., 2003).This test is very sen-sitive but it is not specific, so one cannot surely interpret that the activity is centrally or peripherally mediated (Chen et al., 1995). Accordingly, we tested our com-pounds (30, 45 mg/kg) using hot plate analgesia model that is suitable for centrally acting agents (Hosseinzadeh and Younesi, 2002). Our results clearly showed that the compounds were effective in acetic acid induced writhing test but ineffective in hot plate test thus implicating peripheral mechanisms in the antinociceptive effect like NSAIDs.

Brewer yeast induced pyrexia is considered as pathogenic and release prostaglandin to disturb the thermoregulatory center (Hullati and Sharada, 2007). In this study, we examined the antipyretic activity of these compounds using Brewer's yeast induced pyrexia model in mice. Our findings suggest significant antipyretic activity comparable to that of aspirin (Figure 8a, b, c). From our findings, it appears that these compounds may have inhibitory effect on the mediators of pyrexia in-cluding prostaglandin-biosynthesis, as prostaglandins are one of important regulators of pyrexia (Ranels and Griffin, 2003).

Accordingly, we carried out molecular modeling simulation study to see the binding mode of our synthetic compounds with cycloxygenase enzymes employing FRED 2.1 package (Khan et al., 2011). Molecular docking simulate and facilitate the study of binding of drug to a specific site of protein in the receptor of interest and thus signifying the best-fit orientation of ligand and rational drug design (Rowlinson et al., 2003). In-fact, COX-2 is one of the major enzymes involved in inflammation due to biosynthesis of prostaglandins (PGs) (Khan et al., 2011). Heme is involved in catalysis of PGs biosynthesis. Any substrate can only access the catalytic site (heme) via passing through a narrow pocket sur-rounded by the important amino acid residues especially Arg120 and Tyr355. These two amino acids play a central part in designing or virtual screening for discovery of new COX inhibitors. In this study, flurbiprofen was used as a reference and co-crystallized ligand, to validate the accuracy of binding mode and binding affinity (in terms of binding energy in Kcal/mol) of our compounds. Best scoring conformation of flurbiprofen was found very close to the experimental conformation (RMSD value: 0.453). In case of compound I [4-(2-hydroxybenzyl) morpholin-4-ium chloride], oxygen of morpholyl group (Figure 9) revealed hydrogen bonding with Tyr355 (at a distance of 3.55°A). However a weak dipole-dipole interaction was observed between the cyclic oxygen with Arg120 but no hydrogen was detected. Interestingly, the same compound I, was found in favorable contact with O (oxygen) of Met522 (2.94°A) via phenolic moiety. Surface area of the main binding pocket of COX-2 is generally polar in nature while hydrophobic regions (based on surrounding alkyl and aryl side chains) were also present. In both compounds, no unwanted hydrophobic-hydrophilic interactions were found (Figure 11; A and B), which supported our experimental activity.

The combined favorable interactions based on dipole-dipole interactions and hydrogen bonding could be the major reason behind its experimental effect on inflame-mation and peripheral pain. The elongated yet flexible skeleton of compound II [1,4-bis (2-hydroxybenzyl) piperazine-1,4-diium chloride] showed considerable molecular interactions (Figure 10) with main binding pocket of the enzyme. Phenolic groups on both ends of the compound II revealed major role in the strong binding affinity for COX-2. On outer side of the binding pocket, phenolic group showed the same role as played by the carboxylic moiety of co-crystallized flurbiprofen. Phenolic group anchored both Arg120 and Tyr355 via hydrogen bonding interactions at 2.468°A and 2.75°A, respectively. On the other hand, the second phenolic group strongly held the O (oxygen) of Met522 using hydrogen bonding at a distance of 2.60°A. However, piperazine ring played a role of spacer group without showing any polar interactions. Molecular docking simulation studies have revealed special hydrogen bonding interactions with amino acid residue of COX-2, like Arg120 and Tyr355, thus implicating prostaglandin pathway with COX activity. Techniques which are frequently considered in "rational drug designing" include structure based designing, ligand based designing and De-Novo drug design (França et al., 2006). Structure based designing is the most consistent and prevailing practice for "lead compound" than others and involves explication of three dimensional (3D) structure of the target protein or macromolecular inside or outside the cell (receptors, enzymes, ion channels, DNA, RNA etc) (Tomlinson et al., 2009). The structure of target protein can be obtained by different methods. X-ray crystallography is the most accurate and unswerving source for the structure of target protein. However crystallization of isolated and purified target protein is a restraining factor. Comparative protein modeling study involves receptors or proteins of same family are modeled by the using their protein sequences and converting the primary protein sequence structures to the 3D secondary and tertiary structures following the pattern of related protein of known X-ray structure. Molecular docking and molecular dynamic simulations (MD-simulations) approach is used for the preliminary screening of molecules to be developed as potential drugs (Alonso et al., 2006).

Molecular dynamic simulations (MD-simulations) is a well-known approach to examine structural and dynamic features (molecular motion due to intra or inter molecular interactions and external stress) of proteins. Compound to be screened is docked in the active site for evaluation of its binding mode. Scoring of various binding modes is performed by various algorithms (methods) in terms of binding energy (that is Kj/mole). Molecules with lowest binding energy are selected while others are rejected. Libraries of compounds can be screened in a day using super computers. Selected molecules are subjected to pharmacological studies including in-*vivo* (Tambunan et al., 2011).
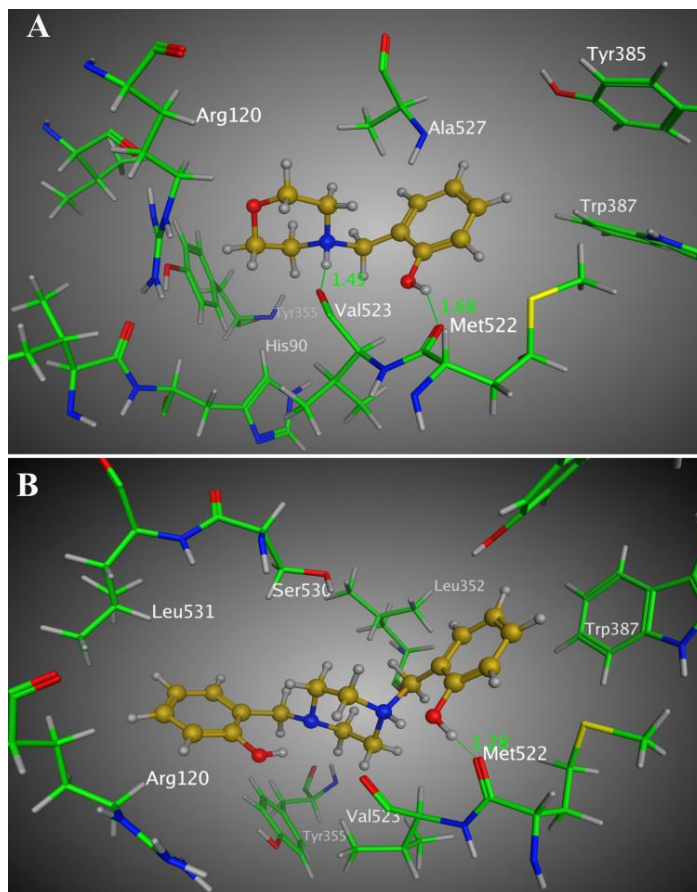
Figure 13. Ligand interaction after MD simulation COX-2 enzyme with (A) Compound I; (B) Compound II.

MD simulation, a number of possible complex conformations were being examined and we could assume that the last conformation was the best conformation. In Figure 13, the observation of MD simulation toward both ligands showed that both ligands had interaction with important active site residues of the COX-2 enzyme. We can see from Figure 13; A and B, that there were changes in ligand's orientation during MD simulation. These changes movement of enzyme.

After MD simulation phase, compound I showed two hydrogen bonds with active site residues of COX-2 enzyme (Figure 13A). Compound I bind with two important residues of COX-2 enzyme, which were Met 522 and Val 523. Compound II showed a little bit different result. Its hydrogen bond's to only one active site residue Met 522 (Figure 13B). Moreover, compounds, having ammonium moieties that are freely soluble in water and other solvents and having neutral characteristics, thus making them suitable for oral and parenteral formulations. In contrast, aspirin has low water solubility and highly acidic nature that impart it cellular erosive and ulcerogenic properties.

In summary, this study evaluates the anti-inflammatory, antinociceptive, antipyretic activities and molecular docking and dynamic studies of salicyl alcohol derivatives. Our findings indicate that these compounds possess significant antinociceptive, anti-inflammatory and antipyretics activities comparable to aspirin as supported by in-vivo animal and molecular docking and dynamic simulation studies. Moreover, compounds are freely water soluble with neutral properties thus making them suitable for oral liquid and parenteral formulations. However, further studies are warranted to find out any potential gastric ulcerogenic properties in comparison to aspirin to confirm their GIT tolerability.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Full Length Research Paper

Sunitinib enhanced the effect of combretastatin A-4 by inducing apoptosis *in vitro*

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Sunitinib is an inhibitor of angiogenesis, and CA-4 is being studied in clinical trials as a vascular disrupting agent. Various combinations involving an anti-angiogenic agent and an anti-vascular agent could cause much more effective anti-tumor responses. The present study indicated that the combination of an anti-angiogenic agent (sunitinib) and an anti-vascular agent (CA-4) exerted synergistic anti-proliferative effect against human carcinoma cells including HO-8910, HepG2 and SMMC-7721 *in vitro*. The enhanced apoptosis induced by sunitinib plus CA-4 was accompanied by greater extent of mitochondrial depolarization, caspases-3 activation and poly ADP-ribose polymerase (PARP) cleavage in HO-8910 cells. Furthermore, the induction of γ-H2AX was observed in sunitinib plus CA-4 treatment group in HO-8910 cells. Together, these data suggested that the combination of sunitinib and CA-4 could produce synergistic anti-tumor effect via DNA damage-induced apoptosis *in vitro*, and this combination might be a novel and promising therapeutic approach to the treatment of cancer.

Key words: Combretastatin A-4, sunitinib, combination therapy, apoptosis.

INTRODUCTION

Combretastatin A-4 (CA-4), a natural product isolated from the bark of a South African tree *Combretum caffrum*, has shown promising strong anti-cancer effects through inhibition of microtubule assembly and subsequent disruption of tumor blood flow (Dark et al., 1997). CA-4 phosphate (CA-4P), a water soluble pro-drug of CA-4, is rapidly dephosphorylated to the active compound CA-4 and shows reversible binding kinetics to tubulin, leading to disruption of microtubular structures (Dowlati et al., 2002). Although CA-4P is being studied in clinical trials as a vascular disrupting agent, cardiovascular toxicity and neurotoxicity are dose limiting for CA-4P (Nagaiah and Remick, 2010; Cooney et al., 2004). These severe side effects currently represent the main obstacles to broad clinical application of CA-4P (Simoni et al., 2008). Thus, it

is important to develop new anti-tumor combination therapy with lower concentration of CA-4 and more speci-ficity for tumor endothelial cells than normal endothelial cells to avoid cardiac toxicity from endothelial damage.

Tyrosine kinase inhibitors (TKIs) are rapidly being integrated into the management of a variety of malignant diseases. Sunitinib malate (Sutent) is an ATP-competitive multi-targeted TKI with efficacy against renal cell carcino-ma and gastrointestinal stromal tumor (Escudier, 2010; Bang et al., 2011). Moreover, it also has anti-cancer activity in patients with metastatic breast, hepatocellular, and ovarian cancer (Biagi et al., 2011; Koeberle et al., 2010; Mundhenke et al., 2009). Sunitinib inhibits multiple tyrosine kinases including platelet- derived growth factor receptors (PDGFR), the vascular endothelial growth

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factor receptors (VEGFR), the stem cell factor receptor c-KIT, FMS-like TK-3 receptor (FLT3), and the glial cell-line derived neurotrophic factor receptor (RET), which play a key role in both tumor angiogenesis and tumor cell proliferation (Chow and Eckhardt, 2007; Tanaka et al., 2011). However, most patients develop sunitinib resistance and progressive disease after about 1 year of treatment. Furthermore, resistance to sunitinib can be reversed by combining it with chemotherapeutic agents including cisplatin, sorafenib, docetaxel, etc (Castillo-Avila et al., 2009; Porta et al., 2011; Bergh et al., 2012).

The tumor vasculature is critical to both the survival of a solid tumor mass and its continued growth (Dark et al., 1997). Angiogenesis is a complex process that is essential for growth, invasion and metastasis of tumors (Gay and Felding-Habermann, 2011).

Two new classes of anti-cancer agents specifically target the tumor blood vessels, namely anti-angiogenic agents, which interfere with the formation of new blood vessel, and anti-vascular agents, which target the existing tumor blood vessels (Assifi and Hines, 2011). Various combinations involving an anti-angiogenic agent and an anti-vascular agent could cause much more effective and durable anti-tumor responses in various preclinical models than either type of drug used alone (Pasquier et al., 2011). Thus, we hypothesized that combining anti-angiogenic drug sunitinib with anti-vascular agent CA-4 might potentially enhance the anti-cancer therapeutic effects.

In this study, we showed for the first time that sunitinib and CA-4 in combination had substantial synergistic anti-tumor efficacy against human cancer cells *in vitro*. In addition, sunitinib greatly enhanced CA-4-mediated apoptosis in HO-8910 cells, accompanied by increased extent of mitochondrial depolarization, cleavage of poly ADP-ribose polymerase (PARP) and activation of caspase cascades. Interestingly, our results demonstrated that sunitinib plus CA-4 lead to increased levels of γ-H2AX indicating increased DNA damage in HO-8910 cells. These data suggested that the combination of sunitinib and CA-4 might be an effective therapeutic strategy to achieve synergistic activities in patients with solid tumors.

MATERIALS AND METHODS

Combretastatin A-4 was synthesized (>99% purity) at the Department of Chemical and Biochemical Engineering, Zhejiang University. Sunitinib was supplied by LC Laboratories (Woburn, MA, USA). Chemical structures of the agents are shown in Figure 1A. The primary antibodies against PARP (H250), procaspase-3 (H-277), XIAP (A-7), γ-H2AX (Ser139), and HRP-labeled secondary anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); cleaved-caspase-3 (D-175) from Cell Signaling Technology (Danvers, MA, USA); β-actin from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture

Human ovarian cancer cell line (HO-8910) and human hepato-

cellular carcinoma cell lines (HepG2, SMMC-7721) were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China); they were tested and authenticated for genotypes by DNA fingerprinting. HO-8910 and HepG2 were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, SMMC-7721 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. All the cells were maintained in a humidified atmosphere of 95% air plus 5% CO_2 at 37°C.

Experimental

Cells were seeded onto plates overnight and were divided into four groups receiving DMSO, sunitinib (4 μM), CA-4 (200 nM) or the combination for the indicated time periods. The cells were washed three times with phosphate buffered saline (PBS) and were ready for use.

Cytotoxicity assay

The anti-proliferative activity of combination treatment with sunitinib and combretastatin A-4 was measured by sulforhodamine blue (SRB) cytotoxicity assay. Briefly, cells were fixed with 10% trichloroacetic acid (TCA) solution for 1 h, wells were rinsed 5 times with tap water and then cells were stained with 0.4% SRB solution (100 μl/well) for at least 20 min at room temperature; wells were rinsed with 1% acetic acid to remove unbound dye, and were then left to air dry; the SRB dye was then solubilized by placing 100 μl of unbuffered Tris-based solution in each well, and the absorbance was measured at 515 nm using a multi-scan spectrum. The inhibition rate of cell proliferation was calculated for each well as (A515 control cells - A515 treated cells) / A515 control cells × 100%.

Analysis of apoptosis by Annexin V and propidium iodide staining

Apoptosis was quantified using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. Briefly, the cells were incubated with 5 μl Annexin V at room temperature for 15 min in the dark. Before flow cytometric analysis, 5 μl of 50 mg/ml propidium iodide (PI) stock solution was added to the samples. For each sample, 1×10^4 cells were collected and analyzed using a FACSCalibur cytometer (Becton Dickinson) and Annexin V-FITC positive cells were designed as apoptotic cells.

Analysis of apoptosis by 4'-6-Diamidino-2-phenylindole (DAPI) staining

Briefly, cells in 96-well plates treated with sunitinib, combretastatin A-4 or the combination were washed twice with PBS, and then incubated with 0.1% Triton and 0.1% DAPI. The morphology of the cells' nuclei was observed and captured using a fluorescence microscope at excitation wavelength of 350 nm and the emission data at 490 nm were collected.

Determination of mitochondrial membrane depolarization

Cells (4×10^5 well^{-1}) were exposed to sunitinib, combretastatin A-4 or the combination for 48 h, and were collected and resuspended in fresh medium containing 10 μg/ml 5,5',6,6'tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1). After incubation at 37°C for 30 min, cells were analyzed by flow cytometry.
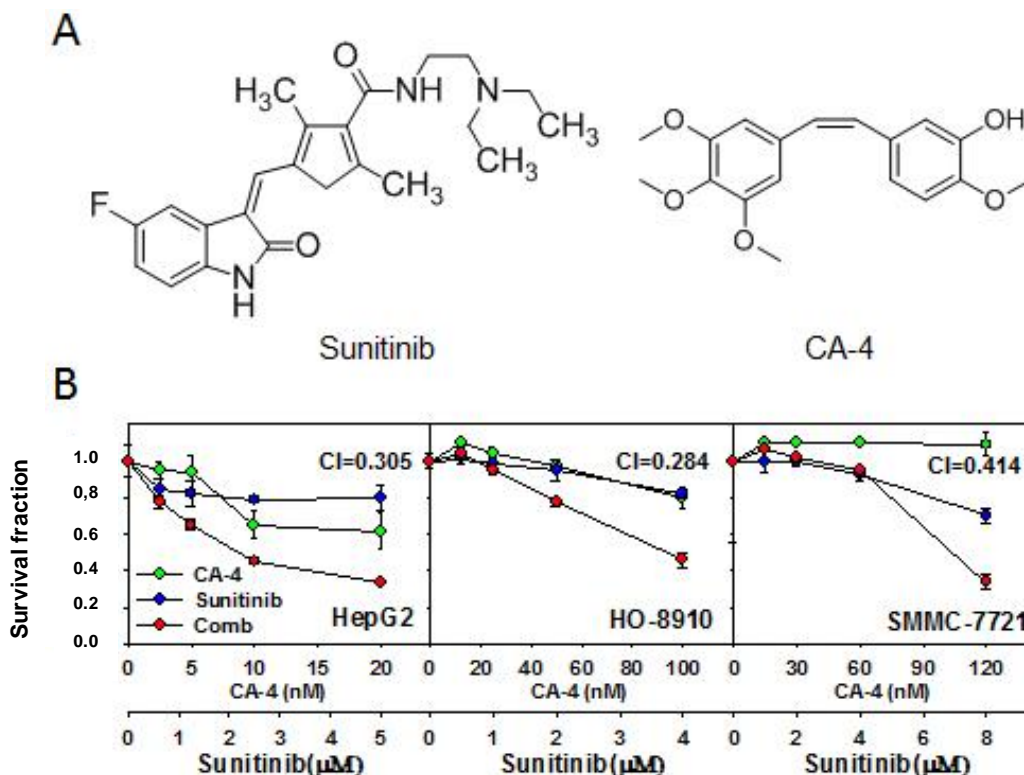
Figure 1. Combination cytotoxicity of sunitinib and CA-4. (A) Chemical structures of sunitinib and CA-4. (B) The cells were incubated with compounds for 72 h. Dose-response curves of human cancer cell lines to sunitinib, CA-4, or the combination. Each condition had 6 replicates, and error bars represent standard deviation.

Cell lysates and western blot analysis

Proteins were extracted with lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholic acid, 0.02% sodium azide, 1% NP-40, 2.0 µg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride). The lysates were centrifuged at 10,000 g for 30 min at 4°C, the supernatants were transferred to a new tube and the protein concentration was determined. Proteins were fractionated on 8 to 15% Tris-glycine gels, and then they were transferred to PVDF membrane (Millipore, Bed- ford, MA, USA) and probed with primary antibodies (dilution range 1:500 to 1:1000) followed by horseradish peroxidase-labeled secondary antibodies at 1:5000 dilution. Antibody binding was then detected with the use of a chemiluminescent substrate and visualized on autoradiography film (Liu et al., 2010).

Immunofluorescence

The HO-8910 cells were seeded onto 35 mm dishes and exposed to sunitinib, combretastatin A-4 or the combination for 48 h. Thereafter, the cells were fixed with 4% formaldehyde for 15 min. After washing with PBS, the cells were blocked with 10% serum in PBS for 10 min and incubated at 37°C for 2 h with γ-H2AX (1:200, Santa Cruz, USA). Then, the cells were washed and incubated in the dark for 1 h at 37°C with goat anti-rabbit (FITC)-conjugated antibodies (1:200, Earthox, San Francisco, CA, USA). After washing, the nuclei were counterstained with DAPI, and then cells were washed in PBS and examined using a laser-scanning confocal microscopy (Fluoview, Olympus, Tokyo).

Statistical analyses

Two tailed student's t test was used to determine the significance of differences between the various treatments. Differences were considered significant at P < 0.05. Combination index (CI) was well-accepted for quantifying drug synergism based on the multiple drug-effect equation of Chou and Talalay (1981, 1984). For *in vitro* experiments, CI values were calculated for each concentration of sunitinib, combretastatin A-4 and the combination in cell proliferation assays using Calcusyn software (Biosoft, Cambridge, United Kingdom).

RESULTS

Cytotoxicity of the sunitinib and CA-4 combination in human cancer cell lines

The sensitivities of 3 human cancer cell lines to sunitinib, CA-4, or sunitinib + CA-4 were determined at clinically achievable concentrations using SRB cytotoxicity assay. Survival curves to sunitinib, CA-4, and sunitinib combined with CA-4 are shown in Figure 1B. CI values were calculated using Calcusyn at the fixed-ratio
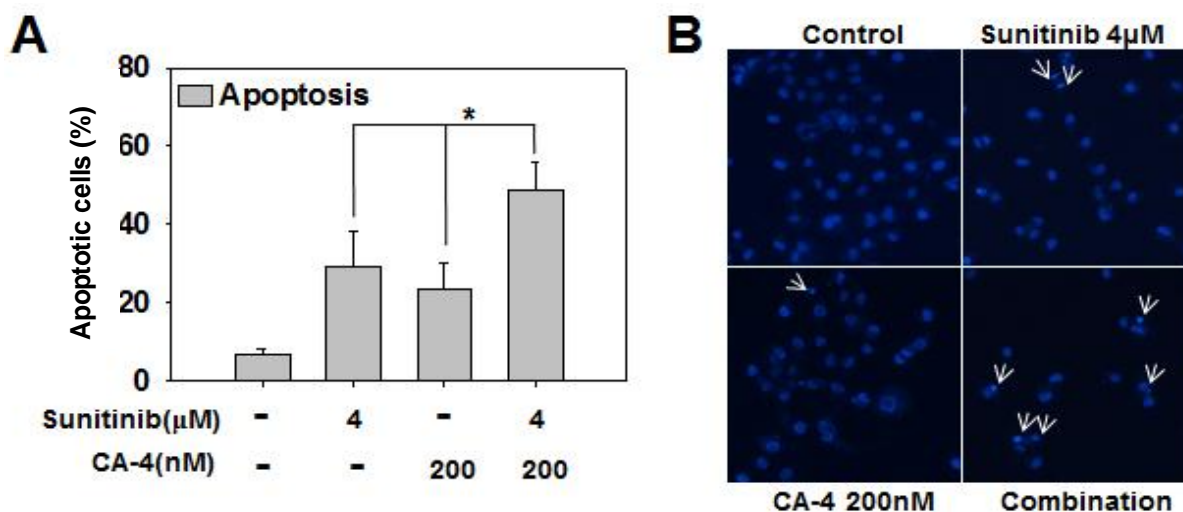
Figure 2. Sunitinib plus CA-4 caused enhanced apoptosis. (A) Annexin V-FITC/PI staining was used to evaluate the apoptosis. HO-8910 cells were treated with sunitinib (4 μM), CA-4 (200 nM) or the combination for 48 h. Total number of events analyzed for each condition was 10,000. The experiments were repeated three times, and error bars represent standard deviation. * P < 0.05 (two-sided Student t-test). (B) Sunitinib plus CA-4 induced apoptotic bodies in HO-8910 cells. Nuclear DNA was visualized by DAPI staining.

concentrations of sunitinib and CA-4 to assess combination activity. A CI less than 0.9 indicates synergism; 0.1, very strong synergism; 0.1 to 0.3, strong synergism; 0.3 to 0.7, synergism; 0.7 to 0.85, moderate synergism; 0.85 to 0.9, slight synergism; 0.9 to 1.10, additive; and more than 1.10, antagonism. CI values of three cell lines treated with sunitinib plus CA-4 were less than 0.7, indicating that sunitinib plus CA-4 showed synergy in HO-8910, HepG2 and SMMC-7721 cells (Figure 1B).

Sunitinib synergized with CA-4 to trigger apoptosis

To explore the mechanism of synergistic effects by combining sunitinib and CA-4, we first detected apoptosis by Annexin V-FITC/PI staining in HO-8910 cells that showed strongest synergistic effect in the cytotoxicity assay. As shown in Figure 2A, Annexin V-FITC/PI staining was used to characterize the apoptosis in HO-8910 cells treated with 4 μM sunitinib, 200 nM CA-4 or the combination for 48 h, the percentage of apoptotic cells was 5.91% in control cells, 19.45% with sunitinib, 15.61% with CA-4, and 46.10% in the combination treatment group. The apoptotic cells were significantly higher in the combination treatment when compared with single treatment (P < 0.05).

Furthermore, DAPI staining was also performed to visualize the apoptosis by assessing chromatin condensation. As shown in Figure 2B, 4 μM sunitinib plus 200 nM CA-4 triggered more apoptosis when compared with either treatment alone in HO-8910 cells, as indicated by the apoptotic bodies.

Sunitinib plus CA-4 induced apoptosis via mitochondrial pathway

To further confirm the combination effect of sunitinib and CA-4 on induction of the mitochondrial apoptosis pathway, we detected mitochondrial membrane potential by JC-1 staining in HO-8910 cells. HO-8910 cells were treated with 4 μM sunitinib, 200 nM CA-4 or the combination for 48 h. As demonstrated in Figure 3A, combined treatment with sunitinib and CA-4 resulted in an increased percentage of mitochondrial membrane depolarized HO-8910 cells than either single agent (42.67% in combination-treated cells, 32.34% in sunitinib-treated cells, 15.71% in CA-4-treated cells, and 4.99% in control group). In addition, sunitinib + CA-4 resulted in increased mitochondrial membrane potential than either drug alone (Figure 3B) in HO-8910 cells (P < 0.01 compared with CA-4 or P < 0.05 compared with sunitinib).

Combination therapy activated caspase cascades

Since caspase-3/PARP play a key role in initiation of cellular events during the apoptotic process, we next examined the effect of sunitinib, CA-4, and the combination on the activation of caspase-3, cleavage of PARP. As shown in Figure 4, we found that although sunitinib and CA-4 had little effect on caspase-3 and PARP, combination treatment was highly effective as it induced more significant cleavage of PARP and caspase-3 than either treatment alone. The X-linked inhibitor of apoptosis (XIAP) is the most potent caspase inhibitor in the IAP family and inhibits apoptotic cell death predominantly by preventing activation of initiator
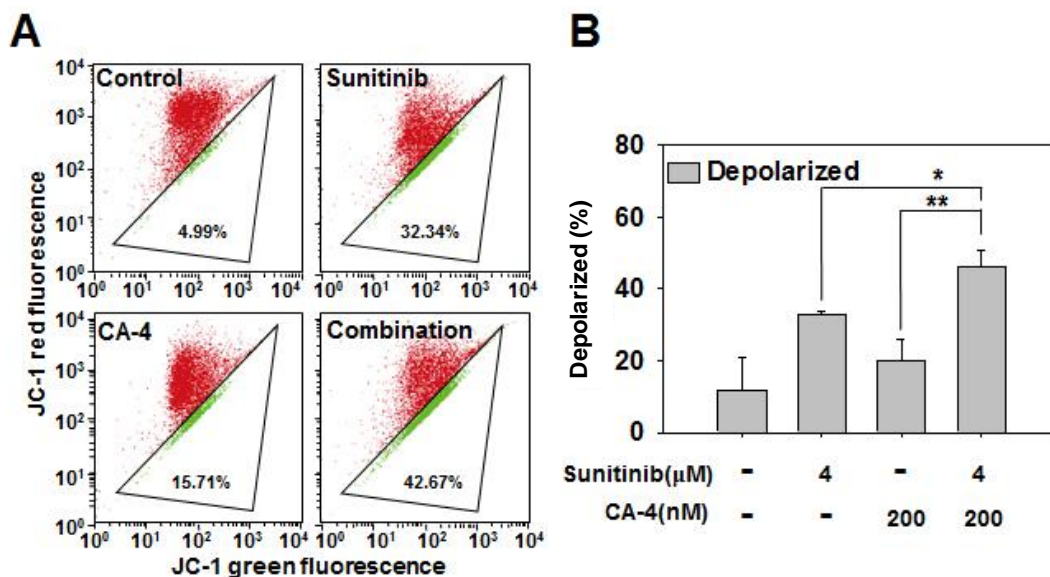
Figure 3. Sunitinib combined with CA-4 caused enhanced mitochondrial membrane depolarization. (A)and (B) The loss of ΔΨm by sunitinib, CA-4, sunitinib + CA-4 was measured by flow cytometry using the JC-1 mitochondrial probe. HO-8910 cells in 6 well plates were exposed to sunitinib (4 μM), CA-4 (200 nM) or the combination for 48 h, then, cells were incubated with JC-1 and analyzed by flow cytometry. Total number of events analyzed for each condition was 10,000. The experiments were repeated for three times, and error bars represent standard deviation. *P < 0.05, **P < 0.01 (two-sided Student t test).
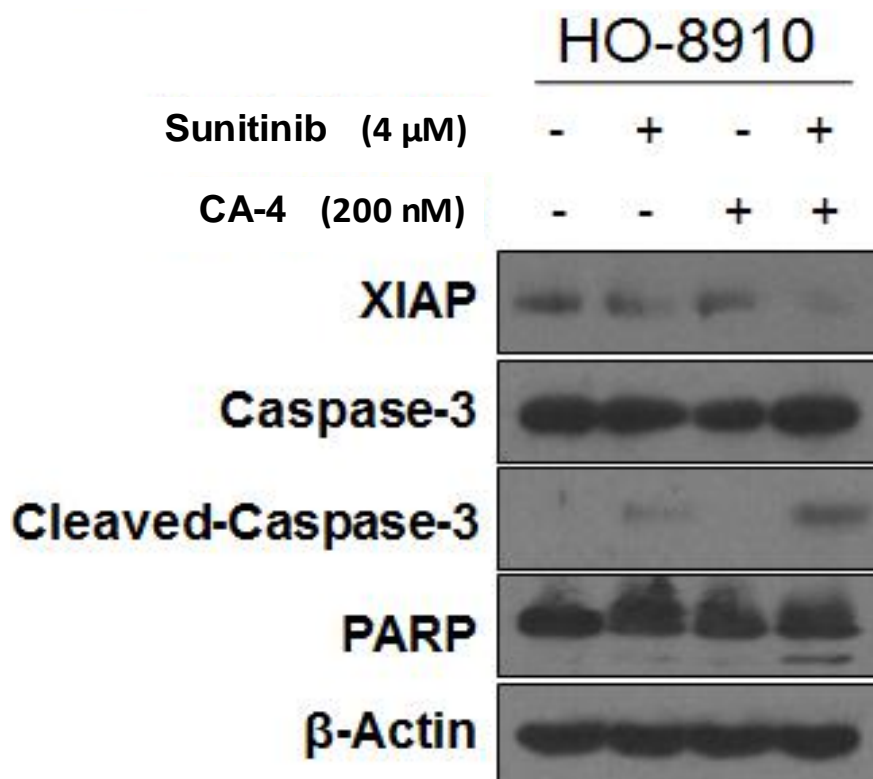
Figure 4. Sunitinib plus CA-4 caused activation of various apoptosis related proteins. HO-8910 cells were exposed to sunitinib (4 μM), CA-4 (200 nM) or the combination for 48 h, after which protein extracts were immunoblotted with specified antibodies for PARP, XIAP, caspase-3 and cleaved-caspase-3.

caspase-9 as well as effector caspases-3 and-7 (Salvesen and Duckett, 2002). We further detected the expression of XIAP in sunitinib and CA-4-treated cells. Figure 4 shows that treatment of cells with sunitinib and CA-4 significantly reduced XIAP than the either treatment alone. Taken together, these results demonstrate that the caspase cascades were activated synergistically by sunitinib and CA-4 in HO-8910 cells as indicated by increased apoptosis.

Sunitinib in combination with CA-4 induced DNA damage

The induction of phosphorylated H2AX (γ-H2AX) is a marker of DNA damage. Therefore, our aim was to determine whether sunitinib in combination with CA-4 could induce DNA damage in HO-8910 cells. As shown in Figure 5A, the induction of γ-H2AX in HO-8910 cells was observed by Western blot, only in the combination setting after 6 h, indicating that DNA may be one of the key factors in inducing cell death by combination of sunitinib with CA-4. Furthermore, using immunofluorescence (Figure 5B), the ratio of γ-H2AX-positive HO-8910 cells was significantly increased in sunitinib plus CA-4 treatment group, when compared with either treatment alone.

DISCUSSION

The clinical cancer therapy now has the potential to explore combination regimens offering improved response rates when compared with mono-chemotherapeutics. Combination cancer therapies are frequently designed to target tumor growth with diverse mechanisms to better overcome resistance (Goudar et al., 2005). The therapeutic potential of the drugs that target the tumor vascular supply is now well recognized. Two distinct groups of vascular-targeted therapies have evolved: antiangiogenic agents and vascular-disrupting approaches (Siemann et al., 2005). Angiogenesis is a fundamental event in the process of tumor growth and metastatic dissemination (Hicklin and Ellis, 2005). Sunitinib, a multitargeted tyrosine-kinase inhibitor, extends survival of patients with metastatic renal-cell carcinoma and gastrointestinal stromal tumors and inhibits angiogenesis by diminishing signaling through VEGFR1, VEGFR2 and PDGFRβ (Roskoski, 2007; Zhao et al., 2010). Inhibitors of angiogenesis interfere with new vessel formation and therefore have a preventative action, have little effect on the existing tumor blood vessels and are likely to be of particular benefit in early-stage cancer therapy (Osusky et al., 2004). Vascular-disrupting agents target the destruction of established tumor blood vessels, resulting in tumor ischemia and necrosis. CA-4 is the lead compound as vascular-

disrupting agents and is currently in Phase II/III clinical trials against a range of malignancies, in combination with conventional chemotherapeutic agents and radiotherapy (Tozer et al., 2008; Wu et al., 2009). Furthermore, CA-4 displays minimal toxicity profile at low dose, indicating its potential to be a candidate for drug combinations in the solid tumor therapy (Busk et al., 2011; Zhu et al., 2010). Since both the initiation of new vessel formation and the integrity of the existing blood vessel network are critical to a tumor's growth and survival, various combinations involving an anti-angiogenic agent and an anti-vascular agent could cause much more effective anti-tumor responses in various preclinical models (Siemann et al., 2004). Thus, combining sunitinib with CA-4 may be a logical way to potentially enhance response rates and prolong survival times for patients by targeting tumor blood vessels.

The results of the present report, for the first time, indicated that the synergistic anti-cancer effects *in vitro* achieved by sunitinib plus CA-4 were observed in human cancer cells. CI values and the significant decline in the survival of cells in combination group strongly demonstrated that sunitinib potentiated the CA-4-imposed cytotoxicity in HO-8910, HepG2 and SMMC-7721 cells. The anti-cancer efficacy by combining sunitinib and CA-4 *in vivo* may need to be further investigated. Our data showed that synergism *in vitro* achieved by the combination of sunitinib and CA-4 was accompanied with enhanced apoptosis. In addition, loss of mitochondrial membrane potential was significantly greater with sunitinib plus CA-4 than with either drug alone. Activation of caspase-3 leads to the cleavage of PARP, the presence of cleaved PARP is one of the most used diagnostic tools for the detection of apoptosis in many cell types (Narula et al., 1999). From western blot analysis, marked increases in cleaved-PARP and caspase-3 were observed in HO-8910 cells after sunitinib and CA-4 combination treatment. XIAP is a member of a novel family of inhibitor of apoptosis-inducing proteins (Porta et al., 2011). IAPs also play an important role during the apoptotic process, XIAP is the best known member of this family and correlates with resistance to chemotherapy (Kashkar, 2010). Our data showed that the synergistic effect on apoptosis obtained with the sunitinib/CA-4 co-treatment was accompanied by a large decrease in anti-apoptotic XIAP protein. The suppression of XIAP by the combination treatment resulted in a significant decrease in procaspase-3 levels, induced apoptosis, and ultimately resulted in the synergism of these two drugs.

Among the different forms of complex DNA damage, double-strand breaks (DSBs) are considered to be among the most lethal forms of DNA damage, severely compromising genomic stability (Kinner et al., 2008). H2AX moved to the center of cellular responses to DNA damage after the discovery that it becomes locally phosphorylated on Ser139, to generate γ-H2AX, in the

A

| Sunitinib (4 μM) | − | + | − | + |
| CA-4 (200 nM) | − | − | + | + |

γ-H2AX

β-Actin

B
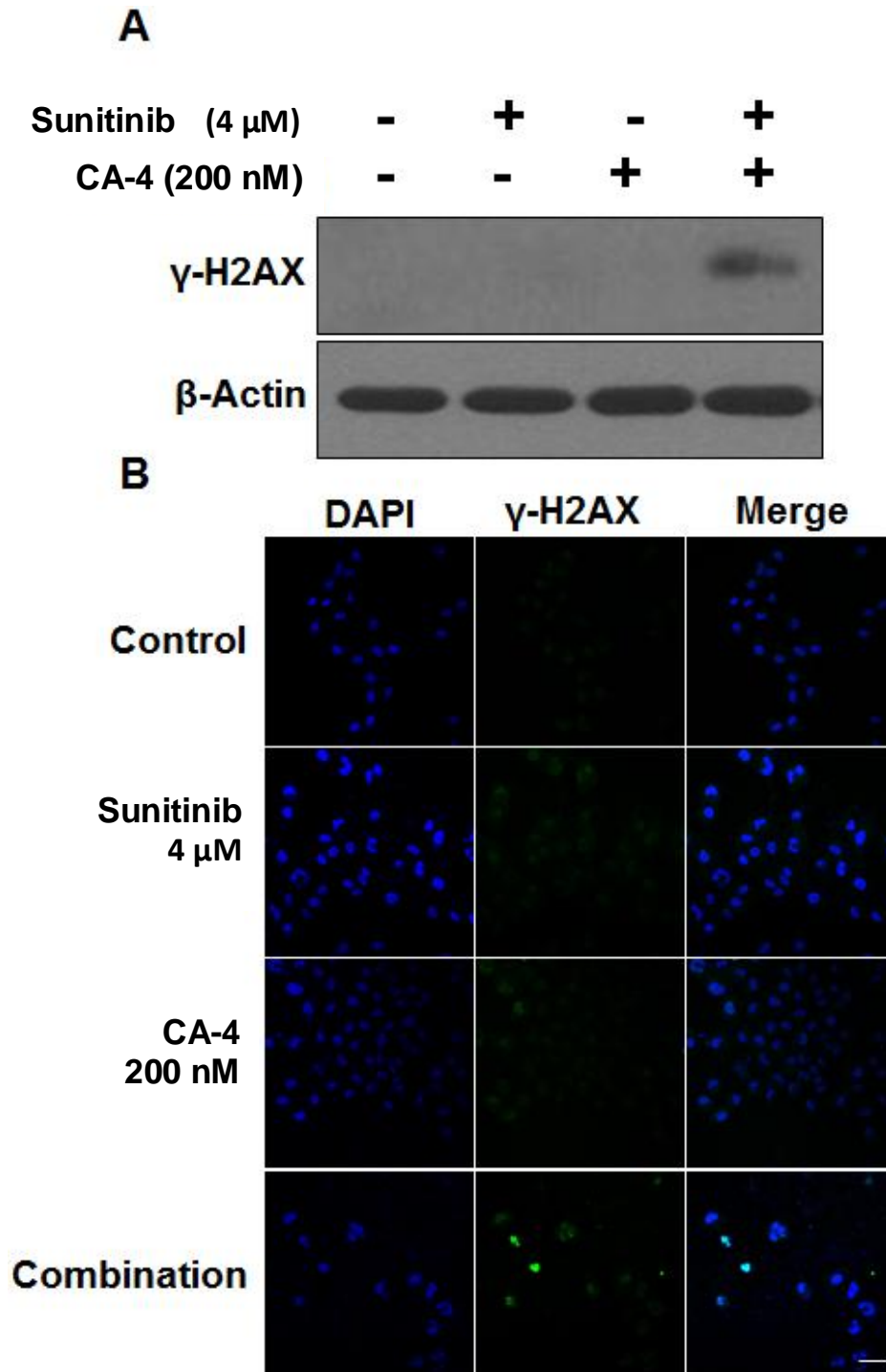
Figure 5. Sunitinib in combination with CA-4 induced DNA damage. (A) HO-8910 cells were exposed to sunitinib (4 μM), CA-4 (200 nM) or the combination for 6 h, after which protein extracts were immunoblotted using γ-H2AX. (B) HO-8910 cells were treated with sunitinib (4 μM), CA-4 (200 nM) or the combination for 6 h and imaged as in a stained with γ-H2AX immunofluorescence (green), DAPI (blue). γ-H2AX expression was indicated by a stippled appearance in the nucleus. Scale bar = 40 μm.

vicinity of DSBs (Mah et al., 2010). Quantitation of γ-H2AX is one of the earliest events in the DNA damage signaling and repair (Smart et al., 2011). To maintain genomic stability after DNA damage, multicellular organisms

activate checkpoints that induce cell cycle arrest or apoptosis (Gartner et al., 2000). A number of anti-cancer drugs exert their effect by causing DSBs and subsequent apoptosis induction, such as DNA replication inhibitors, crosslinking agents and topoisomerase inhibitors (Kawanishi and Hiraku, 2004). In our study, a clear increase in γ-H2AX expression was observed in the sunitinib + CA-4 group compared with mono-treatment groups. Furthermore, in HO-8910 cells, the number of γ-H2AX-positive cells increased in the combination treatment group. These results indicated that sunitinib plus CA-4 might induce DSBs in HO-8910 cells, thus activate apoptosis pathways, and finally result in the synergism of these two drugs.

In conclusion, we presented evidence showing better therapeutic activity of sunitinib when combined with CA-4 in vitro. The enhanced apoptosis induced by sunitinib plus CA-4 was accompanied by the greater extent of DNA damage, mitochondrial depolarization, caspases-3 activation and PARP cleavage in HO-8910 cells. Additional preclinical studies, such as anti-vascular and in vivo anti-cancer efficacy, are needed to systematically evaluate the applicability of this combination as a therapeutic strategy for cancer patients. As reported in this study, the combination of sunitinib and CA-4 might be an effective therapeutic strategy to achieve synergistic anti-tumor effects.

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Full Length Research Paper

Influence of different doses of Iopromide on renal function of elderly patients

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This study aims to investigate influences of different doses of hypisotonic non-ionic contrast agent (Iopromide) on renal function of elderly patients. A total of 30 cases of elderly patients without a history of nephrosis and with normal renal function were divided into two groups according to the different doses of received contrast agent. Influences of Iopromide on renal function were observed by detecting blood urea nitrogen (BUN), serum creatinine (Scr) and creatinine clearance rate (Ccr) at the postoperative 2nd and 5th day. There was no significant difference for BUN, Scr and Ccr levels before angiography and at the 2nd and 5th days after operation between two groups of patients ($P > 0.05$). At the postoperative 2nd day, Scr of two groups of patients slightly increased and Ccr slightly reduced. At the 5th day, they restored to the levels before angiography. Based on the results, we believe one dosage of not more than 294 ml non-ionic contrast agent Iopromide is relatively safe for elderly patients.

Key words: Non-ionic contrast agent, contrast induced nephropathy, elderly patients.

INTRODUCTION

In recent years, the incidence rate of contrast induced nephropathy (CIN) increased yearly, with wide applications of angiography and catheter intervention operation in clinic. At present, CIN has become the third largest cause of acquired acute renal injury in hospital (Finn, 2006). It is currently thought that main risk factors of CIN occurrence include age, basic kidney disease and diabetes mellitus. For patients with creatinine clearance rate less than 20 ml/min/1.73 m^2, CIN risk after receiving contrast agent reaches over 14%. By comparison, the incidence rate of patients with normal renal function is only 7.5% (Rundback et al., 2011). At the same time, the type and dosage of contrast agent are closely related to CIN occurrence (From et al., 2010). Compared with the traditional ionic contrast agent, safety of the non-ionic contrast agent greatly increases. The study of Lin and Bonventre (2005) showed that there was no significant difference for the experiment result between the homotonic non-ionic contrast agent group and the placebo

group, after the postoperative hydration therapy.

However, there are few reports on safety of using the non-ionic contrast agent for elderly patients (over 65 years old) at present. Especially on safety of using a higher-dose non-ionic contrast agent for elderly patients, the relevant literatures are very rare. Therefore, this study investigates influences of the non-ionic contrast agent on the renal function of elderly population by observing the renal function indicators before and after operation after two groups of elderly patients, respectively receive high-dose and low-dose non-ionic contrast agent Iopromide.

MATERIALS AND METHODS

Objects

A total of 30 cases of elderly patients receiving angiography, percu-taneous transluminal coronary angioplasty (PTCA) and permanent pacemaker implantation from January, 2010 to December, 2010 in

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our department were selected. Among them, 12 cases received simple coronary angiography, 14 cases received PTCA and stent implantation, 1 case received brain angiography, 2 cases received renoarteriography, and 1 case received pacemaker implantation plus right ventriculography. All 30 cases were male patients, and their ages ranged from 68 to 89 years old. The average age was 78 ± 6.1 years old. Among them, there were 29 cases of patients with coronary heart disease (including 12 cases of patients with stable angina (one case was complicated with old non-Q-wave myocardial infarction) and 17 cases of patients with unstable angina (two cases were complicated with old non-Q-wave myocardial infarction, and 6 cases were complicated with old Q-wave myocardial infarction).

Among 30 cases, 21 cases were complicated with hypertension, rather than diabetes mellitus or chronic nephrosis. As admission of all patients, both serum creatinine and creatinine clearance rate were within the normal range. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of General Hospital of Chinese PLA General Hospital. Written informed consent was obtained from all participants.

Experimental

For all 30 cases of elderly patients, hypotonic non-ionic contrast agent Iopromide (Iopromide, trade name Ultravist, manufactured by Guangdong Shering Company) was used to carry out angiography or catheter intervention operation. According to the dose of contrast agent, they were divided into the high dose group and the low dose group. In the high dose group, there were 14 cases. The dosage of contrast agent ranged from 278 to 294 ml, and the average dosage was 286 ± 6 ml. In the low dose group, there were 16 cases. The dosage of contrast agent ranged from 86 to 97 ml, and the average dosage was 92 ± 4 ml. Before and after patients received the contrast agent, hydration therapy was conducted in order to prevent CIN occurrence. For the specific method, venoclysis was started at 2 h before receiving contrast agent, and each patient was encouraged to drink more than 500 ml water. For venoclysis, 0.9% normal saline was used and the rate was 1.0 ml/kg/h. For all cases, blood was drawn, respectively before operation, and at the 2nd and 5th days after operation, to detect blood urea nitrogen (BUN) and serum creatinine (Scr). Also, the clinical common Cockcroft and Gault formula was used to calculate serum creatinine clearance rate (Ccr). For the evaluation indicators of renal injury caused by the contrast agent, compared with before angiography, Scr increased by 44 µmol/L or 25%, and creatinine clearance rate (Ccr) reduced by 25% at the postoperative 2nd day (Mehran and Nikolsky, 2006).

Statistical analysis

After collecting data, EXCEL database was established. Statistical software SPSS11.0 was used to conduct statistical analysis for variables and conduct paired t-test, F-test and correlation analysis. In case of variance nonhomogeneity, rank sum test was used. If P < 0.05, a significant difference could be observed.

RESULTS

Change of renal function of patients at 2 days after receiving contrast agent

As shown in (Table 1), compared with before angio-graphy, Scr of two groups of patients increased and Ccr

reduced on the 2nd day after receiving contrast agent, and there were significant differences for changes of Scr and Ccr (P < 0.05). In addition, there was no significant difference for the change of BUN.

Change of renal function of patients at 5 days after receiving contrast agent

As shown in (Table 1), Scr and Ccr levels of two groups of patients at the postoperative 5th day restored to the preoperative levels, and there was no significant difference for BUN, Scr and Ccr between two groups of patients (P > 0.05).

Incidence situations of contrast induced nephropathy

At the 5th day after receiving contrast agent, Scr increased values of the two groups of patients were less than 44 µmol/L. Compared with before angiography, Ccr of one case (contrast agent dose was 290 ml) within 2 days after angiography reduced by over 25%. According to the diagnosis criterion that Ccr reduced by 25% after receiving contrast agent, the incidence rate of CIN within 2 days after receiving contrast agent was 3.3%. At the 5th day, Ccr of this patient restored to the level before receiving contrast agent.

Correlation analysis

According to the correlation analysis, there was no significant correlation between contrast agent dose and Ccr change (the low dose group r = -0.34, P = 0.19; the high dose group: r = 0.16, P = 0.58).

DISCUSSION

Contrast induced nephropathy (CIN) refers to a radiology-induced acute renal function after angiography (Mehran and Nikolsky, 2006). Generally, it is regarded as the diagnosis criterion that serum creatinine (Scr) increases by 44 µmol/L or Ccr reduces by 25% within 48 h, after patients received contrast agent. In recent years, the interventional medicine is gradually popularized in China, and more and more patients receive contrast agent in examination and treatment. Correspondingly, CIN inci-dence rate also apparently increases. Studies suggest that contrast agent is the second-largest reason of causing drug toxicity-induced acute renal failure, only following aminoglycoside antibiotics, while CIN has become the third common disease causing acute renal inadequacy, currently in hospitals (Li and He, 2011).

CIN incidence not only delays hospitalization time of patients and increases medical cost, but also apparently

Table 1. Renal function changes in the two groups before and after accepting Iopromide (mean ± SD).

Kidney function index	Low dose group (n=17)			High dose group (n=14)		
	Before angiography	Two days after angiography	Five days after angiography	After angiography	Two days after angiography	Five days after angiography
BUN (mmol/L)	6.1±1.4	6.9±1.0	6.1±1.8	6.6±1.5	6.9±2.5	6.1±1.6
Scr (µmol/L)	76.4±8.4	84.5±11.2*	77.8±16.4	79.7±10.8	90.3±15.0*	80.0±20.6
Ccr (ml/min)	68.4±7.5	61.8±9.5*	67.4±14.6	69.9±9.6	62.6±10.5*	68.8±19.9

(1) Versus before angiography, *P < 0.05; (2) All time point and indexes comparison between low dose group and high dose group, P > 0.05.

increases the fatality rate of patients (Senoo et al., 2010). A majority of studies think that the contrast agent-induced acute renal function damage is reversible and patients with CIN mostly restore to the normal status within about the postoperative 7th to 10th day. In this study, after one case of patient used 290 ml Iopromide, contrast induced nephropathy occurred within 2 days. At the 5th day, re-examination of renal function showed that all indicators restored to the levels before angiography, which was in line with the conclusion of the literature (Barrett et al., 1992). However, some reports showed that after CIN occurred, 25 to 30% patients would leave over different extents of renal inadequacy. The fatality rate of patients with CIN was 34%, while that of the control group without CIN in which patients had matched age and received contrast agent was only 7% (Levy et al., 1996). There-fore, clinicians shall pay attention to CIN harm.

At present, the pathogenesis of CIN is still unclear. It is generally thought that CIN is related to direct renal toxicity of contrast agent, secondary renal hemodynamics change and renal tubular injury, and reactive oxygen species mediate these injuries (Tumlin et al., 2006; Heyman et al., 1991; Fiaccadori et al., 2004). Studies suggest that CIN incidence rate is directly related to multiple risk factors. These factors include original renal inadequacy, diabetes mellitus, high-dose or short term repeatedly-used contrast agent, contrast agent perme-ability, cardiac insufficiency, peripheral angiopathy, liver function damage and elderly population. Compared with patients without risk factors, their incidence rate of acute renal inadequacy greatly increases after angio-graphy (Sgura et al., 2010). It is worth noting that both age and dosage of contrast agent are the independent indicators among CIN risk factors.

The study of Solomon (2005) showed that for a patient with chronic renal inadequacy, as each dosage of 100 ml contrast agent was increased, CIN risk would rise by 12%. However, there is no special safety research on contrast agent for elderly patients at present. Recently, two years back, researches on hypisotonic non-ionic contrast agent safety gradually increased with populari-zation of non-ionic contrast agent application. Currently, it is widely thought in clinic that for patients with more risk factors before operation, it is better to select non-ionic

contrast agent as it will reduce the posto-perative incidence rate of CIN.

Non-ionic contrast agent has characteristics of water solubility and easy diffusion, and it is in a free state in blood. It neither binds with plasma protein and conducts dissociation, nor participates in body's metabolism, while the traditional ionic contrast agent containing Iodine, such as compound diatrzoatc meglumlne will decompose into cation and anion containing Iodine unrelated to contrast agent in solution. Therefore, its osmotic pressure is two times more than non-ionic contrast agent. Studies suggest that non-ionic contrast agent has a smaller influence to the hemodynamics of tissue and kidney than ionic contrast agent, and it has a smaller effect to renal tubule. So, kidney has a better tolerance to non-ionic contrast agent (O'Donnell et al., 2010).

The results of this study suggest that for the elderly patients without diabetes mellitus and basic renal inade-quacy, after high-dose and low-dose non-ionic contrast agent Iopromide were administered, there was no significant difference for changes of Scr and Ccr between different doses of groups, which was not in line with the reports (Vassiliu et al., 2002; Asif et al., 2003). There was a certain dose-dependent relationship for the renal toxicity of contrast agent. We think that the renal toxicity of non-ionic contrast agent possibly has a certain critical value, and in this threshold range, contrast agent dose is nonlinearly related to renal function injury. But even so, it is still one of main measure of reducing CIN to strictly control the dosage of contrast agent (Nyman et al., 2008). Therefore, for elderly patients, if a higher-dose contrast agent is required for operation, we recommend applying the non-ionic contrast agent with a lower renal toxicity. For elderly patients with better preoperative basic renal function situations, it is appropriate to use a dosage of no more than 294 ml Iopromide. But because of the cases in our study were elderly male patients, whether the conclusions are also suitable for elderly female patients still requires further research.

Meanwhile, we think that besides appropriate contrast agent type, the other cause of low CIN incidence rate after two groups of elderly patients received contrast agent lies in hydration therapy. Hydration reduces the concentration and residence time of contrast agent in

renal tubule by increasing renal blood flow to reduce the damage of contrast agent to renal tubular epithelial cells, and it is an effective means of preventing CIN (Weisbord et al., 2008; Stacul et al., 2011).

Also, it is relatively safe to use the non-ionic contrast agent within a certain dose range for elderly patients without diabetes mellitus and renal function. However, even if there are no renal function damage, diabetes mellitus and nephrosis before angiography, within 2 days after receiving the non-ionic contrast agent, Scr will change transitorily, which is worth noting by clinicians. It is necessary for preventing CIN occurrence, to take comprehensive measures by strictly mastering the indications and dosage of angiography, correctly assessing risks of patients before operation, conducting adequate hydration therapy before and after receiving contrast agent. At the same time, it is beneficial for reducing CIN incidence rate for elderly patients, to select the safer non-ionic contrast agent.

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Full Length Research Paper

Investigation of antioxidant, cytotoxic and apoptotic activities of the extracts from tubers of *Asphodelus aestivus* Brot.

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In this study, potential antioxidant activities of crude extracts from *Asphodelus aestivus* Brot. (AA) tubers were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, and cytotoxic and apoptotic activity of the extracts on MCF-7 breast cancer cells were evaluated by trypan blue exclusion assay, comet assay and hoechst 33258/propidium iodide double staining. Crude diethyl ether and ethyl acetate extracts from AA have significant DPPH scavenging activity, except methanol and water (infusion and decoction) extracts. All of the extracts have cytotoxic activity on MCF-7 cells, time- and concentration-dependently. Although all of the extracts induced significantly deoxyribonucleic acid (DNA) damage, diethyl ether and ethyl acetate extracts induced apoptosis in the middle level and the others induced apoptosis in significant level. Results of this study suggest a dose-response relationship for all extract samples. The results demonstrates that antioxidant, cytotoxic and apoptotic properties of *A. aestivus* Brot. extracts show differentiation to extract type. Crude extracts were used in this study and we do not know which chemical(s) in the extracts exhibited cytotoxic, DNA damaging and apoptotic effects. Therefore, a further chemical identification of the crude extracts and further *in vitro* and *in vivo* studies about cytotoxic and apoptotic activities are required.

Key words: *Asphodelus aestivus* Brot., antioxidant activity, cytotoxic activity, apoptosis, comet assay, MCF-7 cells.

INTRODUCTION

Over the years, humans have relied on nature for their basic needs for the production of foodstuffs, shelters, clothing, means of transportation, fertilizers, flavors and fragrances and not least, medicines (Cragg and Newman, 2003). Medicinal plants continue to play an essential role in health care; it has been estimated by the World Health Organization (WHO) that approximately 80% of the world population rely mainly on traditional medicine for their primary health care (Farnsworth et al., 1985).

Although medicinal plants have been used for a long time, their chemical contents and pharmacological effects are not well understood in most cases. The most commonly found active constituents in medicinal plants include terpenes (sesquiterpenes, diterpenes, triterpenes), alkaloids, coumarins, lignans, quinines, flavonoids, tannins, stilbenes, curcuminoids, anthroquinons, polysaccharides etc. Some of them are alleged to have antioxidant activity (Larson, 1988; Ho et al., 1994; Ng et al., 2000; Cai et al., 2004; Asparganah and Ramezanloo, 2012). Antioxidant activity is a fundamental property important for human life. Many of the biological functions, including antimutagenicity, anticarcinogenicity, antiaging, among others, may originate from this property

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(Mclarity, 1997; Niki, 1997; Yang et al., 2001; Nasri et al., 2012; Alam et al., 2012).

In recent years, there has been a growing interest to isolate new compounds from plants which are effective for cancer treatment because cancer is an important disease and the second leading cause of death worldwide. Although over 60% of currently used anticancer agents are derived in one way or the other from natural sources (Cragg et al., 1997); numerous molecules in many medicinal plants still remain to be isolated or studied in detail (Mukherjee et al., 2001; Tsai, 2001; Lee, 1999). Many naturally occurring agents show chemopreventive and chemotherapeutic (anticancer) potential in a variety of bioassay systems and animal models (Galati and O'Brien, 2004).

Asphodelus aestivus Brot. (AA) common spring-flowering geophytes encountered on the Marmara, Aegean and Mediterranean coasts of Turkey, has been utilized traditionally for culinary and medicinal purposes. The leaves of AA are commonly consumed and cooked as a vegetable dish in Turkey. In traditional medicine, the tuber and roots of this plant are used against hemorrhoids, nephritis, burns and wounds (Baytop, 1999; Tuzlacı and Eryaşar-Aymaz, 2001). Phytochemical investigations suggested that *Asphodelus* species contain anthranoids, flavonoids and triterpenes (El-Fattah et al., 1997; Adinolfi et al., 1991; Van Wyk et al., 1995) and these chemicals have various pharmacological effects. For instance, *Asphodelus ramosus* has low anti-human immunodeficiency virus (HIV) effect (Bedoya et al., 2001) and *Asphodelus microcarpus* has low cytotoxic effect on rat pheochromocytoma (PC12) and human hepatoblastoma (HepG2) cells (Ljubuncic et al., 2005).

Although with the presence of information about phytochemical contents, anti-HIV effect and cytotoxic effect of various *Asphodelus* species, no data have been reported previously on the antioxidant, cytotoxic and apoptotic activities of *A. aestivus* Brot. Therefore, our study aimed at evaluation of potential antioxidant activity and cytotoxic and apoptotic activities of ethyl acetate, methanol and water (infusion and decoction) extracts from *A. aestivus* Brot. on MCF-7 breast cancer cells.

MATERIALS AND METHODS

Chemicals

1,1-Diphenyl-2-picryl-hydrazyl radical (DPPH), rutin, dimethylsul-foxide (DMSO), Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), trypan blue, triton X-100, low melting point agarose, normal melting agarose and ethidium bromide were all purchased from Sigma Aldrich Co. (St. Louis, USA).

Plant and extractions

A. aestivus Brot. were collected in March, 2006 from the West region of Adnan Menderes University Central Campus, Aydın, Turkey, in the Eastern Mediterranean region. The botanical identification was made by Dr. Ozkan Eren. A voucher specimen

was deposited in the herbarium (AYDN 878) of the Faculty of Arts and Science, University of Adnan Menderes. Dried, ground tubers of *A. aestivus* (AA) (95 g) were extracted with a solvent series of increasing polarity (diethyl ether, petroleum ether, ethyl acetate, methanol and water). About 950 ml of solvent were added to 95 g plant material. After finishing the first soxhlet extraction (at 40°C for approximately 12 h, until the solvent became colorless) with diethyl ether and filtration, the plant material was dried and subjected sequentially to the second extraction with petroleum ether, the third extraction with ethyl acetate, and fourth extraction with methanol (Goffin et al., 2003; Lee et al., 2004; Miliauskas et al., 2004; Avcı et al., 2006). The extracts were evaporated and yielded 0.328, 0.796 and 15.178 g dried mass for diethyl ether, ethyl acetate and methanol extractions, respectively. Extracts from petroleum ether extraction was not possible.

After the methanol extraction, plant material was dried and subjected to the water (infusion) extraction. For water (infusion) extraction, 950 ml distilled water at 80°C was added to plant material for 10 min and extract was filtered. For second water extraction (decoction), 950 ml distilled water was added to 95 g dried plant material and boiled for 10 min and extract was filtered (Ljubuncic et al., 2005). Filtered extracts were lyophilized and yielded 2.069 and 2.491 g dried mass, respectively. Extracts were sealed in glass bottles and stored at -20°C until use.

Antioxidant activity

DPPH radical scavenging activity of the AA extracts

1,1-diphenyl-2-picryl-hydrazyl (DPPH) molecule that contains a stable free radical has been widely used to evaluate the radical scavenging ability of antioxidants. When DPPH reacts with an antioxidant compound that can donate hydrogen, it is reduced (Zhao et al., 2006). The free radical scavenging activity of diethyl ether, ethyl acetate, methanol and water (infusion and decoction) extracts from AA was tested by their ability to bleach the stable DPPH radical (Brand-Williams et al.,1995). One milliliter of 0.1 mM DPPH methanol solution was added to 3 ml of various concentrations (10, 25, 50, 75,100, 150, 200 and 300 µg/ml) of extracts in methanol. The mixture was shaken vigorously and left at room temperature. After 30 min, the absorbance of mixture was measured at $\lambda = 517$ nm. Tests were carried out in triplicate. Rutin (50 and 100 µg/ml), a citrus flavonoid glycoside, was used as a standard. Shimadzu PharmaSpec UV-1700 UV-Visible spectro-photometer was used. DPPH radical scavenging activity of extracts was calculated using the following equation:

Scavenging capacity (%) = 100 - [(Absorbance (Ab) of sample - Ab of blank) × 100 / Ab of control]

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

Cytotoxicity assay

Cell culture and treatments

MCF-7 breast cancer cell line was used in this study. Cells were grown and maintained in humidified incubator at 37°C and in a 5% CO_2 atmosphere. DMEM supplemented with 10% FBS, 1 mM glutamine, 1% non essential amino-acids, 100 units/ml of penicillin and 100 mg/ml of streptomycin was used as the culture medium for MCF-7 cell culture. Prior to the assay, concentrated stock solutions of extracts were prepared in DMSO and stock solutions were rediluted to the required concentrations using DMEM media. The maximum percentage of DMSO present in the wells was 0.1% (v/v),

a concentration that did not affect growth of cells. This datum was incorporated as a control element in all experiments. Cells were seeded at density of 5×10^4 cells/well into a sterile 24-well plate and allowed to adhere overnight. After 24 h incubation at 37°C under a humidified 5% CO_2 to allow cell attachment, the cells were treated with different concentrations (10, 25, 50, 75,100, 150, 200 and 300 µg/ml) of ethyl acetate, methanol and water (infusion and decoction) extracts from *A. aestivus* and incubated for 24 and 72 h under the same conditions. In order to avoid the effect of DMSO on cell proliferation and apoptosis, solvent controls were treated with similar concentrations of DMSO as used for sample preparation (in general, 0.1% DMSO). A control of growth medium was also run in parallel for each time period in cell line.

Cell viability assay

For the determination of cytotoxic activity of extracts on MCF-7 cells, viable cell numbers were determined using the trypan blue staining after the treatment (Son et al., 2003; Lee et al., 2005). In brief, a 0.4% solution of trypan blue was mixed to 50 µl of each cellular suspension during 5 min, spread onto a microscope slide and covered with a coverslip. Non-viable cells appear blue-stained. At least 300 cells were counted per concentration. Standard curves were prepared and 50% cytotoxic concentrations (CC_{50}), concentrations of extracts that caused 50% decrease in cell viability, were derived.

Determination of DNA damaging effects of the AA extracts on MCF-7 cells by alkaline comet assay

The comet assay is a very sensitive method for measuring DNA strand breaks, which has the additional unusual feature that analysis is performed on individual cells. The assay has been of value in fundamental investigations of cellular responses to DNA damage and in *in vitro* and animal studies of genotoxicity (Collins et al., 1997). Currently, comet assay is often used since it is fast, convenient and of easy application (Singh et al., 1988; Hartmann et al., 2003). DNA damaging effect of the AA extracts was determined by alkaline comet assay (Singh et al., 1988). For the comet assay, 100, 150, 200 and 300 µg/ml extract concentrations which have high cytotoxic effect, were used. Cells were seeded at density of 5×10^4 cells/well into a sterile 24-well plate and allowed to adhere overnight. After 24 h incubation at 37°C under a humidified 5% CO_2 to allow cell attachment, the cells were treated with 100, 150, 20 and 300 µg/ml concentrations of AA diethyl ether, ethyl acetate, methanol and water (infusion and decoction) extracts, and incubated for 72 h under the same conditions. Then, cells were trypsinised and centrifuged.

After centrifugation, cells were suspended in 150 µl of molten 0.5% low melting point agarose (LMPA) in PBS without calcium and magnesium. Then, 150 µl aliquots of the cell suspension were rapidly spread on three slides pre-coated with 85 µl of 1% normal melting agarose (NMA) and cover-slipped (24 × 50 mm). After the agarose was allowed to solidify for 5 min at 0°C, the cover slips were gently removed and a third layer of 75 µl LMPA was added. The slides were then placed in a tank filled with lysis solution (2.5 M NaCl, 0.1 M EDTA, pH 10, 10% DMSO and 1% Triton X-100 both freshly added) at 4°C. After 1h, the slides were removed from the lysis solution and incubated in fresh electrophoresis buffer (0.3 M NaOH and 1 M EDTA, pH > 13) for 20 min at room temperature for unwinding of DNA. Electrophoresis was then carried out at room temperature in the same electrophoresis buffer for 30 min at 0.7 V/cm and 300 mA. After electrophoresis, the slides were gently washed twice for 5 min in fresh neutralization buffer (0.4 M Tris-HCL, pH 7.59), followed by dehydration in absolute methanol.

The slides were stained with 75 µl 1× ethidium bromide and cover slipped. For each treatment concentration, 100 randomly selected cells from each of three slides (300 cells per concentration) were evaluated for DNA damage visually using a ×40 objective on a fluorescent microscope (Olympus BX 51). The DNA damage was quantified by visual classification of cells into five categories of "comets" corresponding to the tail length (Anderson et al., 1994): (i) undamaged: Type 0; (ii) low-level damage: Type I; (iii) medium-level damage: Type II; (iv) high-level damage: Type III and; (v) complete damage: Type IV. The extent of DNA damage was expressed as the mean percentage of cells with low, medium, high and complete damaged DNA, which was calculated as the sum of cells with damage from Types I, II, II and IV. From the arbitrary values assigned to the different categories (from Type 0 = 0 and Type 4 = 4), a genetic damage index (GDI) was calculated for each concentration level (Pitarque et al., 1999).

Hoechst 33258 and propidium iodide (HOPI) double staining for apoptosis determination

Apoptotic effect of the extracts (at 100, 150, 200 and 300 µg/ml concentrations) was determined by Hoechst 33258 and propidium iodide staining, which allows to distinguish between apoptosis, and necrosis was performed according to the method described by Grusch et al. (2002). MCF-7 cells (5×10^4 per ml) were seeded in 24-well plate and treated with 100, 150, 200 and 300 µg/ml concentrations of the extracts for 72 h. Hoechst 33258 (HO) and propidium iodide (PI) were added directly to the culture medium to final concentrations of 5 and 2 mg/ml, respectively. After an incubation period of 1 h at 37°C, the cells were examined under an Olympus BX 51 fluorescence microscope equipped with appropriate filters for Hoechst 33258 and PI. This method allows for a distinction between early apoptosis, late apoptosis and necrosis. Cells were counted under the microscope and the number of apoptotic cells was given as a percentage.

Statistical analysis

All experiments were performed in triplicate and analyzed by One Way analysis of variance (ANOVA) (SPSS 11.5 program). Statistically significant difference was considered at the level of $p < 0.05$.

RESULTS

DPPH radical scavenging activity of the AA extracts

DPPH radical scavenging activities of the extracts were presented in Table 1 as scavenging activity (%) and IC_{50} values. There were eight concentrations (10, 25, 50, 75, 100, 150, 200 and 300 µg/ml) in each of the extracts. Results of DPPH assay suggested that scavenging activity percentage of the AA extracts showed variation at different concentrations. Although diethyl ether and ethyl acetate extracts have significant scavenging activity, methanol and water (infusion and decoction) extracts have very low scavenging activity on DPPH radical. Therefore, IC_{50} values of methanol and water extracts could not be determined. Furthermore, diethyl ether extract was found to be more effective (IC_{50} value: 22.46 ± 0.01 µg/ml) than ethyl acetate extract with regard to DPPH radical scavenging ability (IC50 value is 188.90±0.03 µg/ml) (Table 1). However, IC50 values of

Table 1. DPPH radical scavenging activity of the *A. aestivus* Brot. tuber extracts.

Samples	Concentrations (µg/ml)	DPPH scavenging activity (%) ±SD	IC$_{50}$ values (µg/ml) ± SD
Rutin	50	89.38±0.001	7.77±0.03
Diethyl ether	10	17.74±0.003	22.46±0.01
	25	55.81±*0.001	
	50	62.26±*0.006	
	75	29.35±0.001	
	100	32.26±0.000	
	150	28.72±0.001	
	200	31.23±0.007	
	300	43.55±0.010	
Ethyl acetate	10	13.87±0.006	188.90±0.03
	25	25.81±0.001	
	50	34.84±0.007	
	75	37.74±0.007	
	100	39.68±0.005	
	150	39.78±0.009	
	200	53.23±0.002*	
	300	54.32±0.003*	
Methanol	10	10.65±0.008	-
	25	9.35±0.000	
	50	10.97±0.002	
	75	11.61±0.001	
	100	17.10±0.008	
	150	14.11±0.002	
	200	22.26±0.001	
	300	25.94±0.013	
Infusion (Water)	10	8.50±0.003	-
	25	9.29±0.002	
	50	7.31±0.002	
	75	8.69±0.007	
	100	8.30±0.009	
	150	8.49±0.004	
	200	12.85±0.006	
	300	13.87±0.011	
Decoction (Water)	10	3.95±0.006	-
	25	5.53±0.008	
	50	5.34±0.009	
	75	7.71±0.009	
	100	8.10±0.008	
	150	9.52±0.008	
	200	11.07±0.006	
	300	13.28±0.009	

*$p < 0.05$.

diethyl ether and ethyl acetate extracts are significantly higher than that of rutin (7.77±0.03 µg/ml), therefore radical scavenging activities of these extracts are lower than that of rutin.

Cytotoxic activity of the AA extracts on MCF-7 cells

Table 2 present the cytotoxic activity of AA extracts tested on MCF-7 cells as cytotoxic activity (%) and CC_{50} values. Even so, methanol and water (infusion and decoction) extracts exhibited high cytotoxic activity on MCF-7 cells, time and concentration dependently ($p < 0.05$); diethyl ether and ethyl acetate extracts have no significant influence on viability of MCF-7 cells in the ranges of applied concentrations and treatment times, so that diethyl ether and ethyl acetate extracts exhibited no cytotoxic activity at 24 h. Therefore, CC_{50} values of the diethyl ether and ethyl acetate extracts at 24 h could not be determined. Cytotoxic activity of AA extracts on MCF-7 cells after 72 h increased in the order of decoction (water) > methanol > infusion (water) > diethyl ether > ethyl acetate. Of the five extracts tested, decoction extract exhibited the greatest potency against MCF-7 cells by eliciting CC_{50} at concentrations of 48.63 µg/ml at 72 h exposure time. Higher concentrations of infusion (water), methanol, diethyl ether and ethyl acetate extracts were required to elicit the same degree of cytotoxicity.

DNA damaging effects of the AA extracts in MCF-7 cells

The percentage of damaged nuclei, genetic damage index and arbitrary units as measured in the alkaline comet assay were presented in Table 3. As presented in the table, all of the AA extracts (except for 100 µg/ml ethyl acetate extract) were found to induce significant DNA damage after 72 h of treatment compared to control groups ($p < 0.05$) (Figure 1). Although ethyl acetate extract exhibited low damaging effect at 100 µg/ml concentration on DNA of MCF-7 cells, higher concentrations of this extract were found to be significantly effective. Among the extracts, only DNA damaging effect of diethyl ether extract in MCF-7 cells was found to be < 50%, but this extract induced also significantly DNA damage in comparison with control groups ($p < 0.05$). DNA damage percentage in MCF-7 cells increased after extract treatments concentration-dependently.

Genetic damage index and arbitrary unit values also increased in extract treatment groups dose-dependently compared to control groups and reached statistically significance, mainly by the increased percentage of Type II, III and IV damages in the extract treatment groups ($p < 0.05$). DNA damaging effect of AA extracts on MCF-7 cells after 72 h increased in the order of infusion (water) > decoction (water) > ethyl acetate > methanol > diethyl ether.

Apoptotic effects of the AA extracts on MCF-7 cells

The percentage of apoptotic cells detected by the Hoechst 33258/propidium iodide double staining method after treatment with AA extracts (100, 150, 200 and 300 µg/ml) for 72 h was evaluated, and the results are shown in Table 4 as percentage of apoptotic cells and percentage of necrotic cells. As indicated in the table, diethyl ether and ethyl acetate extracts induced apoptosis slightly (9.00 to 22.66% and 8.33 to 23.00%, respectively) in MCF-7 cells at all concentrations after 72 h, while the other extracts induced significantly high apoptosis after 72 h, concentration-dependently. Although diethyl ether and ethyl acetate extracts induced apoptosis in the middle level, these values were found to be statistically significant. Apoptotic effect of AA extracts on MCF-7 cells after 72 h increased in the order of decoction (water) > infusion (water) > methanol > diethyl ether > ethyl acetate. The extracts induced also necrosis in MCF-7 cells, and percentages of necrotic cells reached statistically significant level after some extract treatments (Table 4).

Extract treatments caused morphological changes in the nuclear chromatin of the MCF-7 cells (Figure 2). Although the cells in untreated group were stained with a less bright blue color, which was homogenous by HOPI staining; the apoptotic cells in extract treatment groups were stained much brighter than control cells and showed typical apoptotic features, such as cell shrinkage, nuclear fragmentation marginalization, chromatin condensation and apoptotic bodies. Necrotic cells appeared in violet-red fluorescence without chromatin condensation and apoptotic bodies. Especially apoptotic bodies, a specific and distinct feature of apoptotic cells were found at significant levels in the extract treated cells. These results suggested that AA extracts have apoptotic effect at various levels, depending on extract type on MCF-7 cells.

DISCUSSION

Over the ages, humans use plants for various basic needs such as foodstuffs, cloth, drug etc. Currently, medicinal plants are used intensively by people for treatment of various illnesses in many countries. Natural products have long been an important source of treatments of cancer. Recently, a growing interest is present to investigate the mechanism responsible for the anticancer effects of medicinal plants and plant-based drugs (Kültür, 2007; Leong et al., 2011). Many chemo-preventive agents have been associated with anti-proliferative and apoptotic effects on cancer cells because of their high antioxidant activity, targeting signaling molecules, and preventing or protecting cells from further damage or transformation into cancer cells (Khan et al., 2007).

Free radical scavenging is a generally accepted mechanism for antioxidants (Barbaste et al., 2002), and the preferred method for evaluation of the free radicals

Table 2. Cytotoxic activity of the *A. aestivus* Brot. tuber extracts on MCF-7 cells.

Sample	Concentrations (µg/ml)	Cytotoxic activity (%) ± SD at 24 h	CC$_{50}$ value (µg/ml) ±SD at 24 h	Cytotoxic activity (%) ± SD at 72 h	CC$_{50}$ value (µg/ml) ± SD at 72 h
Control	-	0.66±0.580	-	1.33±0.577	-
Solvent control (DMSO)	0.1%	1.00±1.000	-	1.66±0.580	-
Diethyl ether	10	12.33±0.577	> 300	21.67±0.600*	188.83±1.00
	25	15.33±0.577		24.33±0.577*	
	50	21.33±0.577*		35.00±0.000*	
	75	27.67±0.600*		39.67±0.600*	
	100	31.33±0.577*		42.33±0.577*	
	150	33.00±0.000*		46.67±0.600*	
	200	39.33±0.577*		50.67±0.600*	
	300	49.00±0.000*		58.00±1.000*	
Ethyl acetate	10	15.67±0.600	> 300	20.67±0.600	215.29±0.60
	25	19.33±.,577		26.33±0.577*	
	50	22.67±0.600*		31.00±0.000*	
	75	28.33±0.577*		38.67±0.600*	
	100	34.00±0.000*		43.67±0.600*	
	150	36.33±0.577*		45.67±0.600*	
	200	39.00±1.000*		48.67±0.600*	
	300	40.67±0.600*		53.00±0.000*	
Methanol	10	21.33±0.577*	94.84±0.57	25.33±0.577*	82.06±0.53
	25	25.67±0.600*		27.33±0.577*	
	50	29.00±1.000*		41.00±1.000*	
	75	33.33±0.577*		45.67±0.600*	
	100	54.33±0.577*		61.00±1.000*	
	150	58.67±0.600*		67.67±0.600*	
	200	61.67±0.600*		71.00±0.000*	
	300	69.33±0.577*		75.33±0.577*	
Infusion (Water)	10	30.33±0.577*	185.92±0.60	32.33±0.577*	89.68±0.57
	25	33.67±0.600*		35.33±0.577*	
	50	36.00±0.000*		39.33±0.577*	
	75	38.33±0.577*		46.33±0.577*	
	100	41.33±0.577*		52.67±0.600*	
	150	45.33±0.577*		57.00±0.000*	
	200	51.67±0.600*		63.67±0.600*	
	300	55.00±1.000*		70.67±0.600*	
Decoction (Water)	10	31.00±0.000*	112.26±0.58	41.00±0.000*	48.63±0.60
	25	35.33±0.577*		44.33±0.577*	
	50	39.67±0.600*		50.33±0.577*	
	75	42.33±0.577*		54.67±0.600*	
	100	47.67±0.600*		59.00±0.000*	
	150	57.33±0.577*		61.67±0.600*	
	200	63.33±0.577*		69.00±1.000*	
	300	66.67±0.600*		76.00±1.000*	

*p <0.05

Table 3. Analysis of DNA damage as measured by comet assay in MCF-7 cells treated with *A. aestivus* Brot. tuber extracts.

Samples	Concentrations (µg/ml)	Proportion of damaged nuclei (%)				% of damaged cells	Genetic damage index (GDI)	Arbitrary units (Au)
		Type I	Type II	Type III	Type IV			
Control	-	1.00	1.33	0.00	1.00	3.33	0.10	30
Solvent control (DMSO)	0.1%	1.33	0.33	1.67	2.00	5.33	0.15	46
Diethyl ether	100	5.33	4.67	5.00	8.00	23.00*	0.62*	185
	150	5.33	6.33	8.67	11.33	31.66*	0.89*	268
	200	7.33	7.67	11.00	16.33	42.33*	1.21*	363
	300	6.33	8.00	13.00	20.33	47.66*	1.43*	428
Ethyl acetate	100	4.67	3.67	3.33	4.33	16.00	0.39	118
	150	7.00	6.33	3.67	5.00	22.00*	0.51*	152
	200	3.67	8.33	16.33	24.33	52.66*	1.67*	500
	300	3.00	3.66	20.67	38.33	65.66*	2.26*	677
Methanol	100	8.00	8.67	6.00	9.33	28.00*	0.81*	242
	150	7.33	9.33	9.00	10.67	36.33*	0.96*	287
	200	9.33	10.00	11.00	17.33	47.66*	1.32*	395
	300	11.33	9.67	13.66	25.00	59.66*	1.72*	515
Infusion (Water)	100	8.67	9.00	11.00	15.33	44.00*	1.21*	363
	150	13.00	10.67	11.33	16.66	51.66*	1.35*	405
	200	15.67	11.67	12.66	17.33	57.33*	1.46*	439
	300	16.67	13.00	15.33	19.33	64.33*	1.66*	498
Decoction (Water)	100	8.33	9.33	11.33	14.67	43.66*	1.20*	359
	150	12.33	11.00	12.33	17.00	52.66*	1.39*	418
	200	15.00	11.00	12.33	18.00	56.33*	1.46*	438
	300	17.33	11.67	13.00	20.66	62.66*	1.62*	487

*$p < 0.05$. GDI: (Type 1 + 2. Type II + 3. Type III + 4. Type IV) / (Type 0 + Type I+ Type II+ Type III+ Type IV). Source: Pitarque et al. (1999).

scavenging activities is DPPH assay (Brand-Williams et al., 1995). DPPH is a stable free radical that has been widely used as a substrate to evaluate the antioxidant activity of various samples (Blois, 1958; Jung et al., 2003). The DPPH radical scavenging assay seems to be a rapid and accurate method for assessing the antioxidant activity of plant extracts. The results are highly reproducible and comparable to other free radical scavenging methods (Gil-Izquierdo et al., 2001). The DPPH radical scavenging activity of antioxidants is thought to be due to their

Table 4. Apoptotic effects of the *A. aestivus* Brot. tuber extracts on MCF-7 cells.

Samples	Concentrations (µg/ml)	Total cells	Apoptotic cells (%) ± SD	Necrotic cells (%) ± SD
Control	-	300	1.66±0.00	-
Solvent control (DMSO)	0.1%	300	2.00±0.01	-
Diethyl ether	100	300	9.00±0.54*	3.00±0.02
	150	300	14.67±0.57*	4,66±0.03
	200	300	17.33±0.60*	3.33±0.02
	300	300	22.66±0.60*	7.66±0.05*
Ethyl acetate	100	300	8.33±0.57*	4.00±0.02
	150	300	11.66±0.50*	5.33±0.03
	200	300	19.00±0.57*	7.00±0.06
	300	300	23.00±0.60*	9.00±0.06*
Methanol	100	300	18.33±0.57*	7.00±0.05
	150	300	25.66±0.60*	9.00±0.09*
	200	300	38.00±1.00*	8.00±0.06*
	300	300	42.33±1.50*	9,66±0.10*
Infusion (Water)	100	300	26.33±0.54*	6.33±0.05
	150	300	30.66±0.60*	7.66±0.06*
	200	300	37.33±1.50*	6.00±0.05
	300	300	45.00±1.00*	9.33±0.10*
Decoction (Water)	100	300	25.66±0.50*	5.00±0.03
	150	300	35.00±0.60*	7.00±0.06
	200	300	42.33±1.00*	8.66±0.09*
	300	300	49.66±1.50*	8.00±0.10*

$^*p < 0.05$

hydrogen-donating ability.

The aim of this study was to evaluate the potential antioxidant activity and cytotoxic and apoptotic activities of different crude extracts of *A. aestivus* Brot. (AA) on MCF-7 breast cancer cell line. The different crude extracts (diethyl ether, ethyl acetate, methanol and water (infusion and decoction)) of AA were assessed for their DPPH scavenging ability. Their free radical scavenging activities were compared with the activity of well-known antioxidant and flavonoid rutin. The results of DPPH scavenging assay suggested that the ethyl acetate and methanol extracts have significant DPPH radical scavenging activity (IC_{50} values: 22.46 and 188.90 µg/ml, respectively) but diethyl ether and water (infusion and decoction) extracts have very low DPPH radical scavenging activity.

Although ethyl acetate and methanol extracts have significant DPPH radical scavenging activity, these activities seemed to be lower than that of flavonoid rutin (7.77 µg/ml) (Table 1). However, we could say that the extracts have hydrogen-donating ability and could serve as free radical inhibitors or scavengers, possibly acting as primary antioxidants. Among the diethyl ether, ethyl acetate, methanol and water (infusion and decoction) extracts, ethyl acetate extract is the most effective DPPH radical scavenger. Each herb contains generally different phenolic compounds, with different amount of antioxidant activity (Djeridane et al., 2006). The difference in antioxidant capacity of different extracts may be attributed to differences in their chemical composition such as ascorbic acid, limonoids, carotenoids, terpenoids and flavonoids. Furthermore, some of the extracts have hydrophilic and hydrophobic compounds and those samples may not work efficiently in some *in vitro* model systems (Jayaprakasha et al., 2008). Therefore, diethyl ether and water (infusion and decoction) extracts tested in this study were not efficient in DPPH assay system.

Radical scavenging activity of compounds in tested extracts was influenced also by the number and location of hydroxyl groups, glycolisation, and other substitutions.
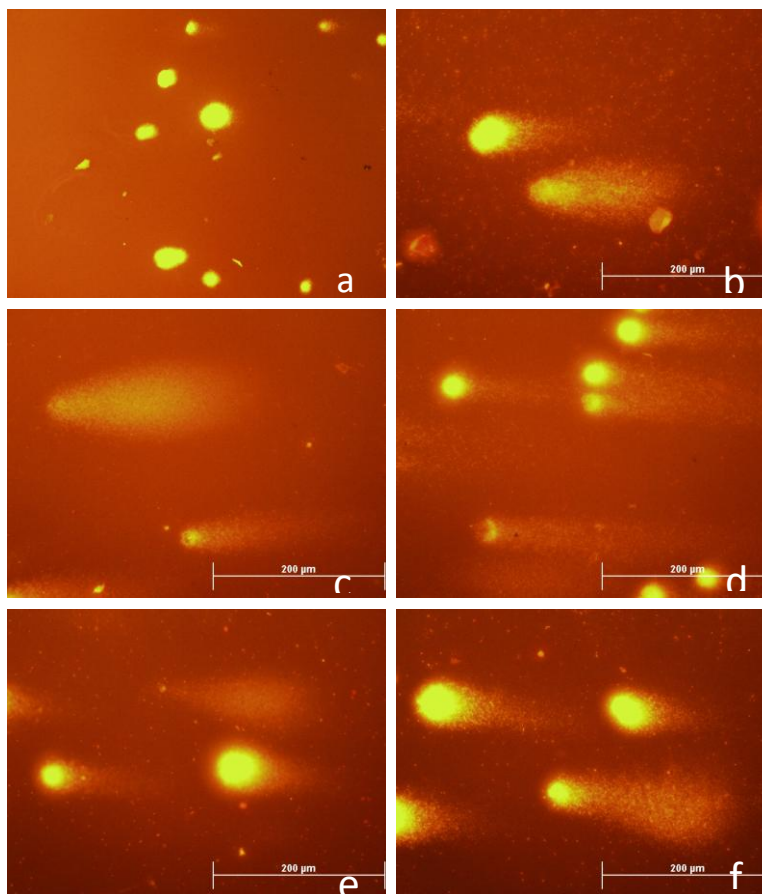
Figure 1. DNA damages after the *A. aestivus* extract treatments in the MCF-7 cells visualized with fluorescence microscopy. (a) control (undamaged); (b) 300 µg/ml diethyl ether extract treatment (Type I and Type IV); (c) 300 µg/ml ethyl acetate extract treatment (Type IV); (d) 300 µg/ml methanol extract treatment (Type III and Type IV); (e) 300 µg/ml infusion extract treatment (Type II and Type IV); (f) 300 µg/ml decoction extract treatment (Type II and Type III).

The methylation of hydroxyl groups can result in a much reduced effect on antioxidant activity (Rao et al., 2007). From chemical and kinetic viewpoints, it should be reasonable to assume that the free hydroxyl group at the C-3 position would enhance the free radical scavenging ability, since according to the reduction mechanism of free radicals, a scavenger should be capable of providing electrons or hydrogen or of receiving electron pairs, while the hydrogen bond between the 3-hydroxyl group and the 4-keto group would increase this ability (Rice-Evans et al., 1996; Decker, 1997).

Previous phytochemical investigations performed on *Asphodelus* species have resulted in the isolation of anthranoids, flavonoids and triterpenes (El-Fattah et al., 1997; Adinolfi et al., 1991; Van Wyk et al., 1995). Flavonoids have been shown to exhibit antioxidant and pro-oxidant activities, which often have been linked to their beneficial effects in cancer therapy (Birt et al., 2001). In recent years, a plant derived-bioactive sub-stance that is capable of selectively arresting cell growth in tumor cells has received considerable attention in cancer chemopreventive approaches (Galati et al., 2000; Jang et al., 2005). On the other hand, flavonoids may not act as conventional hydrogen-donating antioxidants but may exert anticancer and apoptosis inducing properties in cells, through actions at protein kinase and lipid kinase signaling pathways (Hadi et al., 2000).

Recent studies suggested that *A. ramosus* has low anti-HIV effect (Bedoya et al., 2001) and *A. microcarpus* has low cytotoxic effect on rat pheochromocytoma (PC12) and human hepatoblastoma (HepG2) cells (Ljubuncic et al., 2005). In our study, methanol and water (infusion and decoction) extracts were shown to have strong cytotoxic activity against MCF-7, and diethyl ether and ethyl acetate extracts have no significant cytotoxic activity (Table 2). However, all of the extracts showed significant DNA damaging and apoptotic activity at all concentrations after 72 h (Tables 3 and 4).
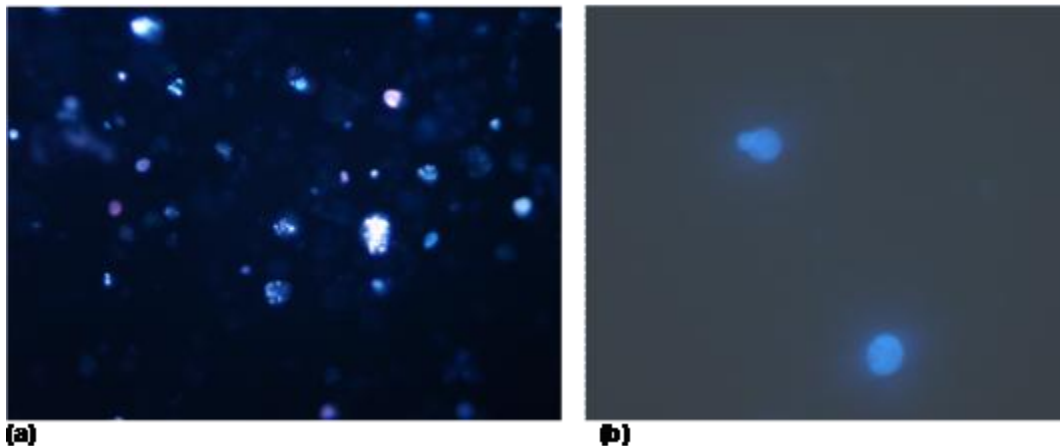
Figure 2. Apoptotic cells after the *A. aestivus* extract treatments in the MCF-7 cells visualized with fluoresce ence microscopy after HO/PI double staining. (a) control (normal) cells; (b) apoptotic cells indicated white arrow after than 300 μg/ml decoction extract treatment.

Targeting cell cycle and apoptotic pathways has emerged as an attractive approach for treatment of cancer. Apoptosis is important to eliminate undesired cells during the development and homeostasis of multicellular organisms. Normally, DNA damage increases the levels of p53 tumor suppressor protein, which transcriptionally activates the WAF1/CIP1/p21 gene whose protein product triggers cell cycle arrest to permit DNA repair (Elledge and Lee, 1995; Shao et al., 2006). However, if the repair is unsuccessful, cells commit apoptosis to safeguard the genome (Hautgraaf et al., 2006). As the HO/PI staining results showed, the extract treated MCF-7 cells clearly showed apoptotic morphology and the apoptotic cell rate significantly increased in a dose-dependent manner. At the same time, comet assay results also showed significant increase of DNA damage in extract treated MCF-7 cells, dose-dependently. Therefore, when the MCF-7 cells were treated with the high concentrations of the extracts (100, 150, 200 and 300 μg/ml), an irreparable DNA damage was possibly induced and apoptosis was initiated due to presence of phytochemicals, especially flavonoids in the extracts.

In a recent paper, Ueda et al. (2001) reported that a flavonoid, baicalin, induces apoptosis in Jurkat cells as pro-oxidant. Also, cytotoxic activity of extracts from this plant on MCF-7 cells in our study may be due to the presence of flavonoids in extracts. Also, the cause of DNA damage arising with extract treatments may be due to the presence of flavonoids that act as pro-oxidant, and they could induce apoptotic cell death, resulting in oxidetive DNA damage. It has been suggested that flavonoids can intercalate with the DNA molecule (Havsteen, 1983) and it has been shown that some flavonoids are mutagenic with pro-oxidant effects (De Carvalho et al., 2003). Flavonoids might therefore cause cytotoxic effect by inducing DNA damage, and in addition to flavonoids,

possibly also other components in the crude extracts can have dual actions (Demma et al., 2009). Previous studies suggested that plant-derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells (Marklund et al., 1982) and antitumor activity of these antioxidants is either through induction of apoptosis (Ruby et al., 1995) or by inhibition of neovascularization (Ming et al., 1998).

Necrotic cells were also observed in this study. The treatment with extracts and percentage of the necrotic cells were found statistically significant at some extract concentrations (Table 4). It is important to distinguish tissue necrosis from molecularly defined necrotic cell death. Pathologic tissue necrosis can be the end product of apoptosis, autophagic, and/or necrotic cell death. In naturally occurring tumor tissue, necrosis occurs when the death rate exceeds the ability of cells to clear dying cells and is likely to be the sum of apoptotic, autophagic, and necrotic cell death processes (Amaravadi et al., 2007).

Findings of this study revealed that constituents in ethyl acetate and diethyl ether extracts of AA have antioxidant activity, but methanol and water extracts of AA have low antioxidant activity. However, all extracts from AA induced cytotoxicity significantly, as apoptotic cell death caused DNA damage in MCF-7 breast cancer cells.

Conclusion

This is the first study about antioxidant, cytotoxic and apoptotic effects of AA tuber extracts. Result of the present study demonstrates that antioxidant, cytotoxic and apoptotic properties of AA extracts show differen-tiation to extract type. Although antioxidant activities of methanol and water extracts are not significant, all of the

extracts have significant cytotoxic and apoptotic activity. Results of this study are important because these results can contribute to researches about finding of new compounds from plants, which can be used for breast cancer treatment, but further investigations are needed to identify the active components and establish the exact mechanism of action in each extracts in order to explain their therapeutic efficacy for breast cancer.

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Full Length Research Paper

Anticonvulsant effect of *Indigofera suffruticosa* Mill: Indication of involvement of the GABAergic system

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This objective of this study was to evaluation of the anticonvulsant effect of *Indigofera suffruticosa* through the methanol extract of its leaves. Swiss albino mice were subjected to dosages of 100 and 300 mg/kg methanol extract for the study of the anticonvulsant action; the following convulsants were used: pentylenetetrazole (100 mg/kg), picrotoxin (10 mg/kg), strychnine (2 mg/kg), and pilocarpine (600 mg/kg). In the evaluation, 2 mg/kg (ip) diazepam was used as the standard drug in all experimental models and GABA$_A$ receptor antagonist Ro 15-1788. The methanol extracts presented excellent anticonvulsant activity, increasing the duration of latency for the beginning of the first seizure in all the tests. The extract in a dose of 300 mg/kg showed its anticonvulsant effect similar to diazepam. The results showed an anticonvulsant activity of *I. suffruticosa* which can be, at least partly associated with involvement of GABA-BDZ system according to effect demonstrated with Ro 15-1788.

Key words: *Indigofera suffruticosa*, anticonvulsant activity, experimental models, Ro15-1788, mice.

INTRODUCTION

The use of medicinal plants through ethnopharmacological information shows a broad spectrum of action for the pharmacological study for the various diseases that affect humans (Nasri, 2012). Various diseases affecting humans were studied, as diseases of peripheral origin, such as those affecting the central nervous system, using several experimental models (Alam et al., 2012; Elisabetsky et al., 1999; Almeida et al., 2009). Furthermore, studies phytochemicals are used in identification of possible therapeutic agents present in plants (Asgarpanah and Ramezanloo, 2012).

Epilepsy is a neurological disorder known, affecting much of the population worldwide (Engel and Pedley, 1998; McNamara, 1999). The essential component of epilepsy is the manifestation of behavioral changes,

called seizures classified according to their origins (Jones, 2002). The understanding of the pathophysiology of epilepsy has advanced considerably in recent years, especially in terms of pathophysiology and genetics (Engel and Pedley, 1998). Drug treatment also had a breakthrough with the introduction of new drugs in the 1980s, with improvement in clinical terms.

However, fell short of expectations, up to one third of the patients will continue to experience seizures or side effects related to medication, despite to appropriate the pathophysiology appropriate to receive treatment, without serious adverse side effects (Meldrum and Rogawski, 2007). Moreover, there is still a need to develop new drugs with improved efficacy and safety in patients with epilepsy (Kwan and Brodie, 2004). The herbal ethnopharmacology obtained through the information is

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a crucial step in order to identify a plant extract, which is suitable for the treatment of epilepsy (Löcher and Schmidt, 2006). *Indigofera suffruticosa* commonly known as Anil, Guatemalan indigo, Small-leaved indigo (Sierra Leone), West Indian indigo, and wild indigo is a flowering plant in the pea family Fabaceae. Anil is common to the subtropical and tropical Americas, including the Southern United States, the Caribbean, Mexico, Central America, and South America as far South as Northern Argentina (Corrêa, 1969).

These species been introduced to other parts of the world, and today has a pantropical distribution (Acevedo-Rodriguez et al., 1996).

The leaves Añil commonly used as a source for indigo dye, and if mixed with small clays can produce Maya Blue or Azul Maya, a pigment used by the Mesoamerican civilizations. *Indigofera* other species present in their characteristic feature presentation of indigo carmine, such as *Indigofera aspalathoides* M. Vahl ex DC. *arrecta* Hochst. ex A. Rich., *Indigofera articulata* Gouan, *I. tinctoria* L. In folk medicine of Brazil, the leaves are used as diuretic, antispasmodic, sedative and anti-inflammatory agent (Leite et al., 2003). Embryotoxic effects found as well as an antimicrobial activity (Leite et al., 2004a, b) and a central nervous system (CNS) depressant action using the fluid extract of *I. suffruticosa* (Alejo et al., 1996 a, b).

Methanol extract (ME) is rich in indiotine (2, 2'-bis (2, 3-dihydro-3-oxoindolilideno) which is useful for coloring of blue.

This study aims to evaluate the anticonvulsant effect of ME of dried leaves of *I. suffruticosa*.

MATERIALS AND METHODS

Plant

The leaves of *I. suffruticosa* (ME) were collected in the arid region of Pernambuco State, Brazil. The sample were identified and authenticated by Dr. Marlene Carvalho Herbarium of the Biological Sciences Center of Federal University of Pernambuco and a voucher specimen was deposited under number 23076.006792-2008-11

Animals

Adult male Swiss mice weighing 25 to 35 g were used. The animals were maintained in a standardized laboratory conditions 22°C, 40 to 50% relative humidity. The mice were maintained in a standardized dark- light cycle lights on between 06.00 and 18.00 h. Standard mice pellets and tap water were available *ad libitum* up to start of the experiments. The experiments were carried out between 08.00 am and 03:00 pm.

All the animals were carefully monitored and maintained in accordance with the ethical recommendation of the Brazilian College of Animal Experimentation (COBEA) and the National Institute of Health Guide for Care and use of laboratory animals and approved by the Ethical Committee of the Federal University of Pernambuco (UFPE) protocol number 0086207/2010-37.

Extract preparation

In obtaining the ME, 500 g of dry material used were previously ground in a forage machine. The extraction was performed using solvents of increasing polarity (hexane, ethyl acetate and methanol). First, we used the hexane at 40°C for 10 min and the solid residue was removed by filtration.

Ethyl acetate was added to the resulting solid. After 24 h of rest, the material was heated and filtered again. To the new solid separated material, the last solvent, methanol was added. After 24 h, methanol was filtered and removed on rotavapor. The material was lyophilized resulting in a yield of 25 g of dark-viscous material that was carefully sealed and stored at -20°C.

Preliminary phytochemical analysis

The aqueous, methanolic, hexane and ethyl acetate extracts of the leaves of *I. suffruticosa* obtained by infusion were analyzed by qualitative method (thin layer chromatography on silica gel/UV detection at 365 nm) for the presence of alkaloids, iridoids, saponins, carbohydrates, coumarins, flavonoids, phenol, terpenoids, and indigo carmine, sterol and essentials oils: linalool and pinene. It is extraction was performed by hydrodistillation procedure (Harborne, 1998; Matos, 1988; Galhiane et al., 2006).

Drugs

Pentylenetetrazole (PTZ), picrotoxin (Picrot), strychnine (Stry), pilocarpine (Piloc), diazepam (DZP), flumazenil (5 mg/kg), and Tween 80 (polyoxyethylene-sorbitan monolate) were purchased from Sigma (USA). In the protocol, the agents were administered intraperitoneally (ip) dose of 0.1 ml/10 g. After the experiments, the animals were sacrificed with an overdose of thiopental and sent for incineration.

Determination of the lethal dose 50% (LD50)

The determination of the LD50 was performed using doses of 100, 300, 600, 900 and 2000 mg/kg (ip) in mice by intraperitoneal route (Litchfield and Wilcoxon, 1949).

Pentylenetetrazol-induced seizures

In this study, mice used (n = 10) were treated with ME (100 and 300 mg/kg, ip) intraperitoneally in saline + 0.9% Tween 80 solution (0.2%, ip). The positive control was treated with DZP (2 mg/kg, ip). After 60 min of drug administration, the mice were treated with PTZ at a dose of 100 mg/kg (ip) and observed for the first phase of forelimb clonus and the time before the beginning of clonic seizures. The incidence of mortality was noted until 24 h after the injection of PTZ (Kaputlu and Uzbay, 1997).

The effects of the critical $GABA_A$-BZD receptor antagonist was also studied, Ro 15-1788 5 mg/kg (i.p.), the anticonvulsant activity of ME was investigated with the system involved in GABA-BDZ. One group with ten mice received Ro 15-1788 5 min before the administration of DZP (2 mg/kg, i.p.). The anticonvulsant activity of DZP and ME in mice pretreated with Ro 15-1788 were assessed and compared with the controls (File and Pellow, 1986).

Picrotoxin-induced seizures

The animals were divided into five groups (n = 10). The first group served as control and received saline (0.9%) and with one drop of
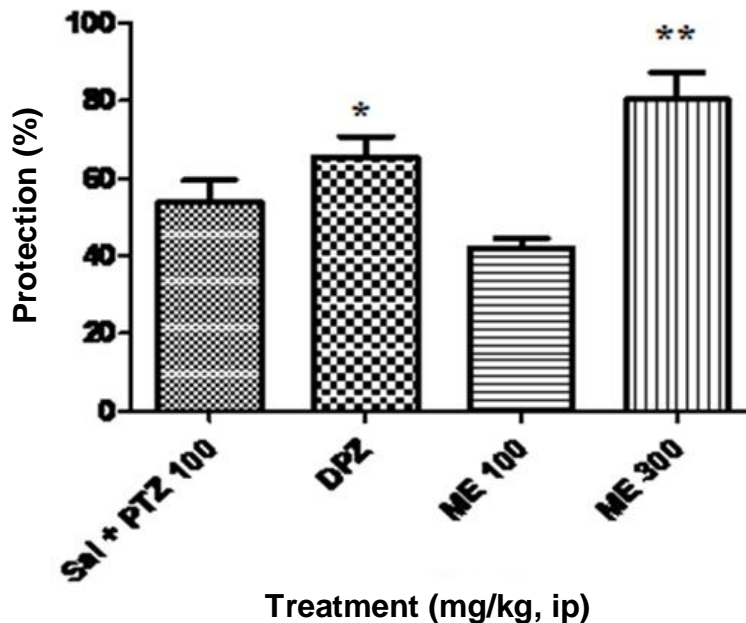
Figure 1. Pentylenetetrazol-induced convulsion effect of ME. ME inhibited generalized clonic-tonic convulsions induced by PTZ (100 mg/kg, ip) at doses of 100 and 300 mg/kg (*p<0.5), as in accordance with statistical analysis, using analysis of variance one-way (ANOVA) and followed by a post hoc Duncan's test. Saline was used in dilution of PTZ
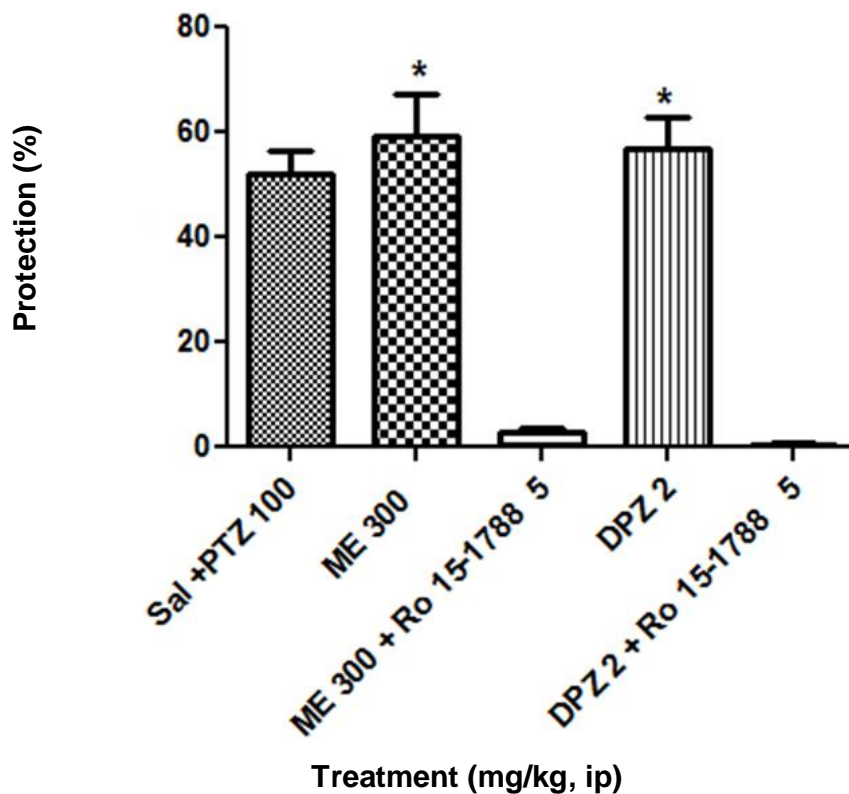
Figure 2. Effect of Pentylenetetrazol (PTZ) on ME after Ro 15-1788 suppression of seizures evaluated with the ME and DPZ. All data cited are the mean ± S.D of each group (n=10). The data was evaluated by a one-way variance analysis (ANOVA) followed by Turkey test. *p < 0.01.
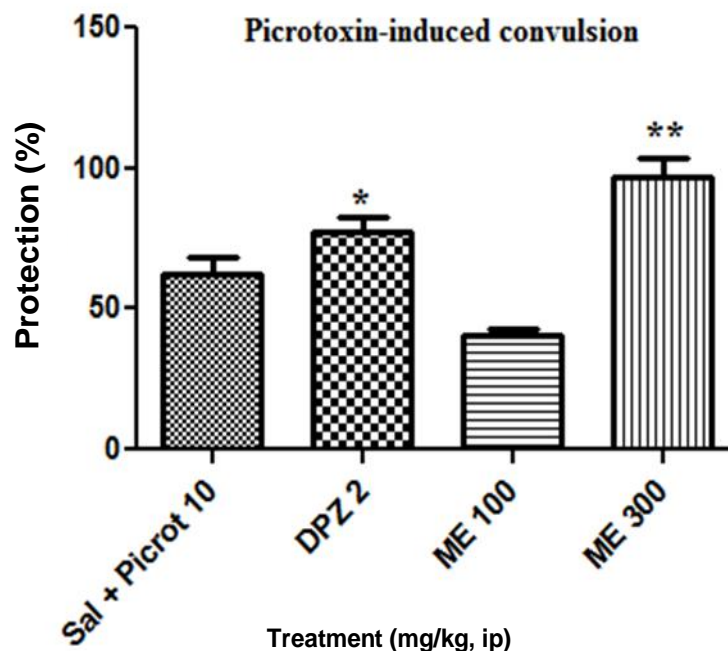
Figure 3. Effect of Picrotoxin (Pic) induced convulsion was evaluated with the ME. All data cited are the mean ± SD, of each group (n=10). The data were evaluated by a one-way variance analysis (ANOVA) followed by Turkey test. *p < 0.01.

cremophor while the second group was treated with DZP (2 mg/kg ip).

The remaining groups received a dose of ME, similar to the PTZ test. After 60 min of drug administration, the mice were treated with picrotoxin at a dose of 10 mg/kg (ip). Immediately after the injection of the convulsant drug, mice were individually placed in plastic boxes and observed for the time arrival of clonic seizures (latency), clonic seizures.

The incidence of death was not observed until 24 h after injection of picrotoxin. DZP was used as a positive control (Susan et al., 1989).

Strychnine-induced seizures

The animals were divided into five groups (n = 10). The first group served as control and received saline (0.9%) with one drop of cremophor while the second group was treated with DZP (2 mg/kg, ip). The remaining groups received a dose of ME. After 60 min of drug administration, the mice were treated with strychnine at a dose of 10 mg/kg (ip). Immediately after the injection of the convulsant drug, mice were individually placed in plastic boxes and observed for the time arrival of tonic-clonic seizures.

The incidence of death was observed until 24 h after injection of strychnine (20%). DZP was used as a positive control (Kaputlu and Uzbay, 1997).

Pilocarpine-induced seizures

Animals were divided into five groups (n = 10). The first group served as control and received saline (0.9%) with one drop of cremophor, while the second group was treated with DZP (2 mg/kg, ip).

The remaining groups received a dose of ME, similar to the PTZ test. After 60 min of drug administration, the mice were treated with

pilocarpine at a dose of 600 mg/kg (ip). Immediately after the injection of the convulsant drug, mice were individually placed in plastic boxes and observed for the time arrival of clonic seizures (latency), clonic seizures. The incidence of death was not observed until 24 h after injection of pilocarpine. DZP was used as a positive control (Graciela et al., 2009).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with post hoc Duncan's test p<0.5 considered significant. All data were expressed as mean ± standard deviation (SD) using the program Graph Pad Prism, version 5.00.

RESULTS

LD50

The extract showed a low toxicity with an LD50 of 1600 mg/kg (ip) in mice. The therapeutic dose of the extract was based on the percentage of 10 to 40% of LD50.

Identification of compounds in the ME of *I. suffruticosa*

ME showed the presence of alkaloids, flavonoids, steroids, proteins, carbohydrates, indigo carmine and essential oils (Linalool and Pinene) according to the method used.
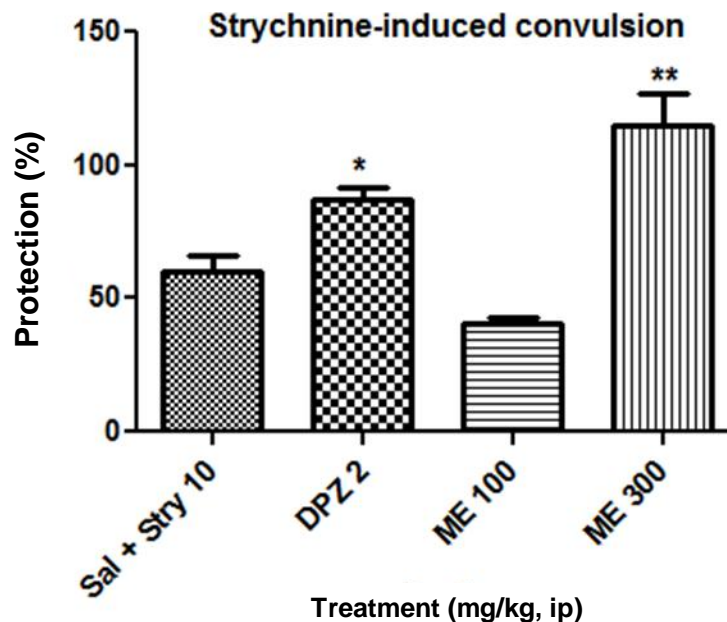
Figure 4. Effect of Strychnine (Stry) induced convulsion was evaluated with the ME. All data cited are the mean ± SD, of each group (n=10). The data were evaluated by a one-way variance analysis (ANOVA) followed by Turkey test. *p < 0.01.
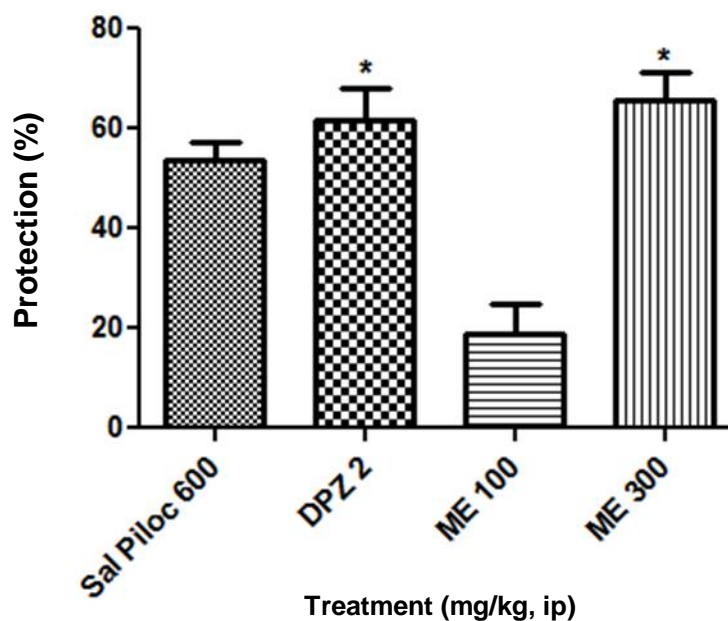
Figure 5. Effect of Pilocarpine induced convulsion was evaluated with the ME. All data cited are the mean ± SD of each group (n=10). The data were evaluated by a one-way variance analysis (ANOVA) followed by Turkey test (*p<0.01).

DISCUSSION

The results presented here show that ME exhibits an LD50 of 1600 mg/kg (ip) in mice and did not induce significant changes in individual behavioral and physiological parameters and showed a slight decrease in spontaneous locomotor activity and an increase in breathing frequency (data not shown).

In the phytochemical analysis conducted by us, ME showed the presence of alkaloids, flavonoids, steroids, proteins, carbohydrates, indigo carmine and essential oils (linalool and Pinene). The seizures induced by pentylenetetrazole acts through the chloride channel of the GABAergic system (Meldrum, 2002). ME produced protection against PTZ-induced seizures ($p<0.01$).

Picrotoxin acts as a competitive antagonist for the receptor for GABA chloride channel. Infusions of picrotoxin have stimulating effects and cause seizures. High-dose pilocarpine induced seizures in rats after systemic administration and/or intracerebral (Lechoslaw et al., 1989). The picrotoxin-induced seizure was protected by ME in doses of 100 and 300 mg/kg promoting prevention of seizures.

Strychnine-sensitive glycine receptors located chiefly in the brainstem and spinal cord is the first mediators of synaptic inhibition. ME promoted inhibition of seizures at doses of 100 and 300 mg/kg (*$p < 0.05$). Treatment with high dose of pilocarpine hydrochloride, a muscarinic cholinergic agonist, induces convulsions in rodents after intracerebral or systemic administration. The amygdale, thalamus, olfactory cortex, hippocampus, neocortex and substantia nigra are the most sensitive regions to epilepsy-related damage after seizures produced by pilocarpine (Tuski et al., 1989; Andre et al., 2009). ME caused block seizures induced by pilocarpine in doses of 100 and 300 mg/kg (*$p < 0.01$). Diazepam used on all models served as an anticonvulsant.

The identification of flavonoids and essential oils (linalool) of the ME is probably a strong indication that these compounds involved in the anticonvulsant activity of ME (Almeida et al., 2009; Ibrahim et al., 2012; Rasilingam et al., 2009; Elisabetsky, 1995 a, b, c). A significant observation was the effect of Ro 15-1788 on the PTZ and ME (300 mg/kg, ip) in mice (Albertson and Walby, 1986; Darragh et al., 1983; Susan et al., 1989). During the process seizure occurs an increase of reactive oxygen species (ROS) promoting cell damage (Packer, 1997; Evans, 2000).

Flavonoids have the ability to inhibit the formation of ROS. They are large molecules with antioxidant power, which can act in concert with other endogenous antioxidant systems in neuronal cells (Pietta, 2000). Moreover, previous studies have shown the antioxidant activity of flavonoids, in particular during induction of convulsion pilocarpine and other agents (Pietta, 2000).

The presence of linalool can contribute to the inhibition of reactive oxygen species, by inhibiting their formation in neuronal cells in addition to their probable action on the GABA receptor system.

Conclusion

The results showed that anticonvulsant activity of ME could be associated with the GABAergic system involvement in the presence of flavonoids, and linalool.

The observations were based on the literature on the action Ro 15-1788 on the GABAergic system. This study was able to increase the knowledge about the use of this plant as a CNS depressant. Probably, an influence on the GABA-BDZ receptor complex.

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Full Length Research Paper

Expression of tyrosine hydroxylase and growth-associated protein 43 in aging patients with atrial fibrillation of Xinjiang Uygur and Han nationalities

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This study explores the changes in gene and protein expression of tyrosine hydroxylase (TH) and growth-associated protein 43 (GAP43) in aging patients with atrial fibrillation of Xinjiang Uygur and Han nationalities. Real-time polymerase chain reaction (PCR) and Western blot were used to detect the gene and protein expressions of TH and GAP43 in atrial tissues of 54 patients with valvular heart disease. Comparison of the mRNA and the protein expression of GAP43 and TH between the sinus rhythm group and the atrial fibrillation group was statistically significant ($P < 0.05$); the protein expression of GAP43 and TH in patients of Xinjiang Uygur and Han nationalities in the sinus rhythm group and atrial fibrillation group was statistically significant ($P < 0.05$); the protein expression of GAP43 and TH in patients of different nationalities in the sinus rhythm group and atrial fibrillation group was not statistically significant ($P > 0.05$); the protein expression of GAP43 and TH in patients of different nationalities with different ages in the sinus rhythm group and atrial fibrillation group was not statistically significant ($P < 0.05$); only the protein expression of GAP43 in patients with different ages in the atrial fibrillation group was statistically significant ($P < 0.05$). The changes in mRNA and protein expression of TH and GAP43 played a vital role in the process of maintaining atrial fibrillation. The increase in the expression of TH and GAP43 may be one of the molecular bases of the left atrial myoelectricity remodeling of aging patients with atrial fibrillation. TH and GAP43 may be the potential therapeutic targets of atrial fibrillation.

Key words: Atrial fibrillation (AF), aging, Xinjiang Uygur, Han nationality, tyrosine hydroxylase (TH), growth associated protein (GAP43)

INTRODUCTION

Atrial fibrillation (AF) is one of the most common arrhythmia in clinical cardiac diseases, the morbidity of which is closely related with the age of the patients. AF may attack anyone, but it has high morbidity in aged individuals and extremely low morbidity in children. The

morbidity rate of AF is above 0.4% among common people. The morbidity rate rises significantly with increasing age, which reaches 6% for those who are above 65, 10% for those who are above 75, and even near 20% for those who are above 85 (Nattel et

al., 2008). The continuous existence of AF easily causes serious complications such as heart failure, intra-atrial thrombus, cerebral embolism, etc., which have a high disability and mortality rates (Fuster et al., 2001; Tsang et al., 2005). Currently, many therapeutic methods are available for the treatment of AF, including drug therapy, electrical conversion, surgical maze operation treatment, radiofrequency catheter ablation, pacemaker implanta-tion, etc. However, given that none of them is completely satisfactory, a study on the mechanism of occurrence and maintenance of AF is necessary.

In recent years, the function of neural regeneration in AF myoelectricity remodeling has gained increasing attention. Many studies found that the function change in the cardiac autonomic nerve plays an important role in inducing AF (Wijffels et al., 1995; Chen and Tan, 2007). Tyrosine hydroxylase (TH) and growth associated protein 43 (GAP43) are important factors in the regeneration and distribution of cardiac autonomic nerves. In a previous study (Horikawa-Tanami et al., 2007), TH was found to be a sign factor of the sympathetic nerve and GAP43 was found to be a sign factor of the parasympathetic nerve; TH and GAP43 could play an important role in the process of AF formation, maintenance, and recovery.

Xinjiang is located in Northwest China, where the eco-nomy is relatively backward. Most people have minority nationalities, mainly the Uyghur nationality. Given these minorities' unique customs and religious beliefs, inter-marriage is discouraged between the people of Uygur nationality and other nationalities, making the genes of Uygur nationality relatively pure. Epidemiological studies found that the prevalence of AF among Uygur people is higher than that among people of other nationalities in this region. In this study, changes in TH and GAP43 expressions in patients of Uygur and Han nationalities in Xinjiang region were detected. The study aimed to find the genetic differences between the two nationalities and provide a new theoretical basis for better AF treatment.

MATERIALS AND METHODS

Patients

The study subjects were 54 patients from in the First Affiliated Hospital of Xinjiang Medical University who suffered from valvular heart disease and needed open chest valve replacement operation from 2008 to 2011. Among the 54 cases, 28 were of Han nationality and 26 were of Uygur nationality, and 22 were male and 32 were female. Their ages ranged from 43 to 72 (46.28 ± 9.15). The 54 patients were divided into two groups: sinus rhythm group and AF group. Of the 26 cases in the sinus rhythm group, 14 were male patients and 12 were female patients, with an average age of 53.38 ± 12.74. Of the 28 cases in the AF group, 8 were male patients and 20 were female patients, with an average age of 55.29 ± 8.58. Eight cases of paroxysmal AF and 18 cases of chronic AF (AF lasting more than 6 months) were included in the AF group. All patients used cordarone (oral administration or intravenous injection) to control their ventricular rate. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of The First

Affiliated Hospital of Xinjiang Medical University. Written informed consent was obtained from all participants.

Inclusion criteria and exclusion criteria

Before valvular surgery, AF and sinus rhythm were confirmed by clinical query and dynamic electrocardiogram (ECG). The telemetry ECG was used to record and review the status of patients within 7 days after admission. AF with duration longer than 7 days and shorter than 48 h was defined as persistent AF and paroxysmal AF, respectively. In the sinus rhythm group, patients with atrial arrhythmia and heart palpitations were excluded. Coronary arteriography was performed on partial patients to exclude the possibility of coronary heart disease. All patients had no liver or kidney function damage, electrolyte disturbance, infection, hypertension, hyperthyroidism, or diabetes mellitus. Their heart functions were at grade II to III (NYHA grading).

Specimen collection and preservation

The clinical baseline data of the patients were registered, and informed consent forms were signed before the operation. Extracorporeal circulation was established during the operation, and approximately 200 mg of left auricle tissues was taken out after cardiac arrest. The left auricle tissues were immediately put into liquid nitrogen after blood and fat tissues were excluded, followed by preservation in a -80°C low-temperature refrigerator for further usage.

Real-time polymerase chain reaction (PCR)

Left auricle tissues with a weight of 100 mg were taken, and the total RNA was extracted through the Trizol one-step method (Invitrogen Company). Total RNA of 1 µg was taken and reverse transcriptased into cDNA according to the instruction of the Reverse Transcription Kit (Promega Company A3500).

Design and synthesis of the primer: primer was synthesized by TaKaRa Company (Table 1). Real-time PCR system (20 µl): speci-mens for detection were given cDNA 2-fold dilution. About 1 µl cDNA was taken using 10 µl SybrGreen qPCR Master Mix (Shanghai Ruian BioTechnologies), 1 µl upstream primer (10 µM), 1 µl downstream primer (10µM), and 7 µl ddH$_2$o. Reaction condi-tions: reaction conditions were 10 min at 95°C initial denaturation, followed by PCR circulation, 15 s at 95°C denaturation, 30 s annealing at 60°C, 20 s at 72°C extension, and 40 circulations. Reflected light signals were collected. 2-$^{\triangle\triangle}$Ct method was used to calculate the relative mRNA expression value of the target gene.

Western blotting

The total protein of the tissues was extracted according to the kit instruction (Beijing Solarbio). The content of the total protein was detected using the bicinchoninic acid (BCA) method. The total protein was separated through electrophoresis using polyacryla-mide gel (SDS-PAGE). The sample loading of each hole was 50 µg. The protein in the gel was then blotted to nitrocellulose (NC) film through galvanic transferring. After sealing and elution using 5% nonfat dry milk/Tris-buffered saline (TBS) solution, hybridization was carried out by TH-specific first resistance (Anti-rabbit IgG, Abcam Company), GAP43-specific first resistance (Anti-rabbit IgG, Abcam Company), and β-actin channel protein-specific first resistance (Anti-rabbit IgG, Wuhan Boster Company), respectively, followed by 4°C overnight incubation. Incubation for 1 h followed using specific second resistance (Goat Anti-rabbit IgG, Wuhan

Table 1. Design and synthesis of the primer.

Gene	Primer sequence	Amplified fragment length (bp)
β-actin	F:GCTACGAGCTGCCTGACG; R:TCGTGGATGCCACAGGAC	112
TH	F:TGTTCCAGTGCACCCAGTATATC; R:5'-CCAATGTCCTGCGAGAACTG	136
GAP43	F:AGAACATAGAAGCTGTAGATGAAAC; R:CCATTTCTTAGAGTTCAGGCAT	112

Boster Company) indicated by horseradish peroxidase. Diaminobenzidine (DAB) coloring was carried out. All hybridization signals were scanned quantitatively by the BIO-RAD gel imaging system.

Statistical analysis

EXCEL 2003 software was used for data collection. Statistical analysis software (SAS) JMP package was used for the statistical analysis. Mean ± standard deviation ($\bar{x} \pm s$) was used for the general description of age. Left atrial (LA), right atrium (RA), and ejection fractions (EF) were used for the sinus rhythm and AF groups. Constituent ratio and constituent rate were used for the gender and nationality constituents. T- or t`-test was used for comparing the general data in the sinus rhythm and AF groups under the premise of conducting a homogeneity test for variance. T-test was used for comparing the GAP43 and TH difference between the sinus rhythm group and the AF group, with nationality and age used as the covariant. T-test was also used for comparing the GAP43 and TH difference in the different nationalities and ages, with disease used as the covariant. The inspection level used was 0.05.

RESULTS

General information

The left atrial diameter of patients in the AF group exceeded that of the patients in the sinus rhythm group. No statistical significance was observed in the other clinical data of the two groups, such as, age, nationality, gender, ejection fraction, cardiac functional grading (NYHA Grading), etc (Table 2).

mRNA and protein expression

The mRNA and protein expressions of GAP43 and TH in the sinus rhythm group and the AF group were compared. The results showed a statistical significance (P < 0.05) in the mRNA and protein expressions of TH and GAP43 (Table 3).

GAP43 and TH protein expression

The difference in protein expression of GAP43 and TH between the sinus rhythm and AF groups was compared, with Han and Uygur nationalities used as the covariant.

The results showed a statistical significance in the GAP43 and TH protein expression of the Xinjiang Uygur and Han nationalities between the sinus rhythm and AF groups (P < 0.05) (Table 4).

Different nationalities

The difference in the GAP43 and TH protein expression between the two nationalities was compared, with different diseases used as the covariant. No statistical significance was observed in the GAP43 and TH protein expressions of the two nationalities between the sinus rhythm and AF groups (P > 0.05) (Table 5).

Different ages

The difference in protein expressions of GAP43 and TH between the sinus rhythm and AF groups was compared, with different nationalities used as the stratification factors. The results showed a statistical significance in the difference between the sinus rhythm and AF groups (P < 0.05) (Table 6).

The difference in the GAP43 and TH protein expressions in different ages between the sinus rhythm and AF groups was compared, with diseases used as the stratification factors. The results showed that only the protein expression of GAP43 in different ages in the AF group was statistically significant (P < 0.05) (Table 7).

DISCUSSION

Earlier studies indicated that stimulating the vagus nerve and acetylcholine administration could cause significant cardiac electrophysiological changes; the former could cause the shortening of the atrial refractory period, which will induce AF (Brundel et al., 2004). High-frequency electrical stimulation on the cardiac ganglionated plexi (GP) might trigger activity originating from the pulmonary veins, which could induce AF (Hauerte et al., 2001). On the basis of atrial premature stimulation, stimulating epicardial fat (which contains GP) could also lead to AF (Scherlag et al., 2005). Radiofrequency ablation of GP could reverse the changes in the atrial refractory period and eliminate the capacity of premature stimulation, which influences the superior pulmonary vein and induces

Table 2. Comparison of clinical features between two groups ($\bar{x} \pm s$).

Parameter	Sinus rhythm group (n = 26)	AF group (n = 28)	Statistic	P
Nationality, n (%)				
Han	10 (36)	18 (64)	$\chi^2 = 3.60$	0.058
Uygur	16 (62)	10 (38)		
Gender, n (%)				
Male	14 (64)	8 (36)	$\chi^2 = 3.567$	0.059
Female	12 (10)	20 (90)		
Age (years)	53.38 ± 12.74	55.29 ± 8.58	t'=-0.641	>0.05
LA (mm)	45.09 ± 7.73	57.90 ± 11.36	t= 4.807	0.000
RA (mm)	42.94 ± 8.47	45.37 ± 19.12	t'=0.611	>0.05
EF (%)	63.33 ± 11.15	61.31 ± 6.60	t'=0.802	>0.05

After test for homogeneity of variance, t` test was used for age, RA and EF comparison due to heterogeneity of variance (F_{age} = 2.2048, P_{age} = 0.0472; F_{RA} = 5.0958, P_{RA} = 0.0001, F_{EF} = 2.8541, P_{EF} = 0.0090). Homogeneity of variance was reported in LA between two groups. LA = Let atrium; RA = right atrium; EF = ejection fraction.

Table 3. The mRNA and protein expression of GAP43 and TH in two groups ($\bar{x} \pm s$).

Parameter	GAP43		TH	
	mRNA	Protein	mRNA	Protein
Sinus rhythm group (n = 26)	0.86 ± 0.23	0.28 ± 0.21	0.06 ± 0.03	0.64 ± 0.30
AF group (n = 28)	2.19 ± 0.73	0.85 ± 0.38	0.13 ± 0.05	1.03 ± 0.42
Statistic	9.163*	6.885*	6.289*	3.900*
P	<0.05	<0.05	<0.05	0.000

*Heterogeneity of variance: t` test.

Table 4. The difference of protein expression of GAP43 and TH in two groups ($\bar{x} \pm s$).

Nationality	Parameter	GAP43	TH
Han	Sinus rhythm group (n = 10)	0.26 ± 0.01	0.36 ± 0.11
	AF group (n = 18)	0.76 ± 0.35	0.87 ± 0.52
	Statistic	5.956*	4.003*
	P	0.000	<0.05
Uygur	Sinus rhythm group (n = 16)	0.29 ± 0.27	0.42 ± 0.19
	AF group (n = 10)	1.02 ± 0.40	1.05 ± 0.40
	Statistic	5.5737#	4.663*
	P	0.000	<0.05

*Heterogeneity of variance: t` test; #Homogeneity of variance: t test.

induces AF (Nakagawa et al., 2004). Further studies (Patterson et al., 2005) confirmed that the rapid discharge of the vein is the combined action result of the sympathetic and parasympathetic neurotransmitters.

Many studies showed that the functional changes in the autonomic nerves could induce AF. The study of Wijffels et al. (1995) confirmed that stimulating the vagus nerve could cause the shortening of the atrial muscle cell

Table 5. The difference of protein expression of GAP43 and TH in different nationalities in two groups ($\bar{x} \pm s$).

Group	Nationality	GAP43	TH
Sinus rhythm group	Han (n = 10)	0.26 ± 0.01	0.36 ± 0.11
	Uygur (n = 16)	0.29 ± 0.27	0.42 ± 0.19
	Statistic	0.444[#]	1.019*
	P	>0.05	>0.05
AF group	Han (n = 18)	0.76 ± 0.35	0.87 ± 0.52
	Uygur (n = 10)	1.02 ± 0.40	1.05 ± 0.40
	Statistic	1.791[#]	0.947[#]
	P	0.085	0.352

*Heterogeneity of variance: t` test; [#]Homogeneity of variance: t test.

Table 6. The difference of protein expression of GAP43 and TH in different ages in two groups ($\bar{x} \pm s$).

Age	Parameter	GAP43	TH
≤65	Sinus rhythm group (n = 22)	0.23 ± 0.46	0.51 ± 0.23
	AF group (n = 14)	0.60 ± 0.24	0.76 ± 0.21
	Statistic	5.756*	3.286[#]
	P	0.000	0.002
>65	Sinus rhythm group (n = 4)	0.53 ± 0.50	0.49 ± 0.28
	AF group (n = 14)	1.10 ± 0.34	1.18 ± 0.58
	Statistic	2.679[#]	2.268[#]
	P	0.017	0.038

*Heterogeneity of variance: t` test; [#]Homogeneity of variance: t-test.

Table 7. The difference of protein expression of GAP43 and TH in two groups in different ages ($\bar{x} \pm s$).

Group	Age	GAP43	TH
Sinus rhythm group (n=26)	≤65 years (n=22)	0.23 ± 0.46	0.51 ± 0.23
	>65 years (n=4)	0.53 ± 0.50	0.49 ± 0.28
	Statistic	1.186[#]	0.155[#]
	P	0.247	0.878
AF group (n=28)	≤65 years (n=14)	0.60 ± 0.24	0.76 ± 0.21
	>65 years (n=14)	1.10 ± 0.34	1.18 ± 0.58
	Statistic	4.495[#]	2.548*
	P	0.000	<0.05

*Heterogeneity of variance: t` test; [#]Homogeneity of variance: t test.

effective refractory period, which would induce AF. Recent studies also found that the pulmonary vein could induce AF, because of the existence of special cells with electric conduction function in the pulmonary vein. The

cells contain rich sympathetic cells, the activity of which can cause local depolarization, which induces AF (Chen and Tan, 2007). The autonomic nerve abnormal activity can also induce AF by inducing cardiac intracellular calcium overload and early after depolarization (EAD). In their studies, Burashnikov and Antzelevitch (2003) found that injecting acetylcholine into the coronary artery could shorten the action potential of the atrial muscle cell and cause rapid atrial pacemaking, which would easily induce AF. They also found that the sudden stop of AF or the increased rapid pacemaking frequency could instantly improve the tension of the atrial muscle cell, which would cause the three phases of rapid EAD of action potential and extrasystole, thus inducing AF. In their studies, Patterson et al. (2006) found that the continuous increase in relaxing period tension is an important cause of EAD. The increased intracellular calcium concentration in the relaxing period induces AF. By using optical mapping technique, Chou et al. (2005) detected that, when the atrial muscle cell membrane and pulmonary vein regeneration or the nerve automatic activity of dogs decreases the forming rate of cardiac intracellular calcium overload and EAD decreases significantly, which will promote the recovery of AF.

Current studies showed that the mechanism of AF is very complex. The autonomic nervous system (ANS) plays a vital role in triggering and maintaining AF. At present, TH and GAP43 are the main markers related to the regeneration and distribution of the cardiac autonomic nerve. TH is a rate-limiting enzyme that catalyzes the synthesis of the catecholamine neurotransmitter. TH is expressed abundantly in the sympathetic ganglia and noradrenergic neuron of the sympathetic nerve. The positive expression of TH represents the sympathetic nerve distribution in the heart. GAP43 is a fast transport cell membrane phosphate expressed in the sprouting axon growth hillock. GAP43 is distributed extensively in the ANS neurons and is closely related to neural development, regeneration of axons, and reconstruction of synapsis and neurotransmitter release. In summary, GAP43 is regarded as an inherent determinant of neuron development and regeneration. It symbolizes neural development, and it can be used to evaluate ANS growth activity.

Recent studies have expanded the further understanding of the relationship between ANS and AF. In 2001, Chang et al. (2001) established the chronic AF model by rapid atrial pacing. Immunohistochemical method was used to detect the atrium cordis, auricular appendix, eruption of atrial septum nerve, and distribution of the sympathetic nerve in dogs. They found a significant and inhomogeneous neural eruption and the over-distribution of the sympathetic nerve in the atrium cordis of dogs. The right atrium significantly exceeded the left one. Based on former studies, they proposed that the reconstruction of neural tissues could play a vital role in the triggering and maintenance of AF. Gould et al. (2006)

provided histological evidence for the reconstruction of the atrial sympathetic nerve in persistent AF patients by comparing the sinus rate and the eruption and distribution levels of the sympathetic nerve in auricular appendix tissues, further confirming that the reconstruction of the autonomic nerve partially triggers AF.

In 2009, Furukwa et al. (2009) made atrioventricular block dog models by applying the radiofrequency ablation method. Atrial enlargement, myocardial fibrosis, and atrial and pulmonary vein (PV) effective refractory period shortening significantly occurred when the sympathetic nerve was stimulated. Atrial conduction velocity accelerated eight weeks later, which did not appear when the vagus nerve was stimulated. In the radiofrequency ablation group, the triggering rate of persistent AF increased upon sympathetic nerve stimulation during sham operation. The triggering rate of persistent AF increased when the vagus nerve was stimulated. Studies indicated that the sympathetic nerve stimulation was a key factor in AF triggering in the reconstructed atria, which is different from the normal atria. The internal diameter enlargement of the left atria is regarded as an important factor in triggering and maintaining AF. Patients in the AF group had a significantly larger left atria than patients in the sinus rhythm group, which had statistical significance ($P < 0.05$). The mRNA and protein expressions of GAP43 and TH in the sinus rhythm and AF groups were compared. The results showed that the mRNA and protein expressions of TH and GAP43 were statistically significant ($P < 0.05$), which indicates that the reconstruction of neural tissues was combined with the structure reconstruction in AF patients.

The difference of protein expression of GAP43 and TH in the sinus rhythm and AF groups was also compared. The results showed a statistical significance in the protein expressions of GAP43 and TH in patients of Xinjiang Uygur and Han nationalities in the sinus rhythm and AF groups ($P < 0.05$). The difference in protein expression of GAP43 and TH in patients of different nationalities was compared. No statistical significance was observed in the protein expression of GAP43 and TH in patients of different nationalities in the sinus rhythm and AF groups. No difference was observed in the protein expressions of GAP43 and TH between Xinjiang Uygur and Han nationalities. Thus, neither the protein expression of GAP43 and TH in AF patients of Xinjiang Uygur and Han nationalities nor the distribution of the sympathetic nerve in the heart and the automatic nerve growth activity showed a nationality difference.

The difference in protein expression of GAP43 and TH in the sinus rhythm and AF groups was compared, with different nationalities used as the stratification factors. The results showed a statistical significance in the difference between the sinus rhythm and AF groups ($P < 0.05$). The difference in protein expressions of GAP43 and TH in patients with different ages was compared, with diseases used as the stratification factors. The results

showed that only the protein expression of GAP43 in patients with different ages in the AF group had statistical significance. Aging plays a vital role in the triggering and maintenance of AF.

The possible reasons why AF causes neural eruption and inhomogeneous distribution are as follows:

(1) Electrical remodeling and structure reconstruction (Miyauchi et al., 2003) during AF cause atrial enlargement, insufficient blood supply, and myocardial damage, which impair the nerve. GAP43 and β-NGF can help in neural development and neural recovery, which promote the regeneration of impaired nerves and promote the growth of the intact myocardial nerve.

(2) The atrial structure reconstruction in AF causes distribution disorder and the regeneration of nerve in the atria. ANS distribution density intensifies but not evenly. Therefore, the reconstruction of the vagus nerve is accompanied with the reconstruction of the sympathetic nerve (Sakamoto et al., 2010).

(3) AF causes myocardial ischemia. Neurohormones, such as cytokine and growth factor, which are increased in circulation, may be the cause of atrial neural development (Yang et al., 2011).

Conclusively, the reconstruction of the automatic nerve is closely related to AF. An imbalance in the automatic nerve could cause AF. Then, AF could cause the reconstruction of the automatic nerve, making AF maintenance easier. However, the exact mechanism that causes the reconstruction of the automatic nerve and electrical remodeling needs further exploration. We closely combined ANS with AF despite the fact that many blind zones are still present. The triggering and maintenance mechanism of AF will be further elucidated with in-depth studies on ANS. Reversing the reconstruction of the automatic nerve may be the new therapeutic target of AF, which will guide clinical treatment and improve the prognosis of patients with AF.

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