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Original Article

Evaluation of antidiabetic antihyperlipidemic and pancreatic regeneration, potential of aerial parts of *Clitoria ternatea*

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ABSTRACT

The aim of the present study was to investigate the pancreatic regeneration potential of of diferent fractions of the ethanol extract *Clitoria ternatea* L., Fabaceae. The antidiabetic and antihyperlipidemic potential was evaluated in streptozotocin-induced diabetic rats and correlated with its *in vivo* and *in vitro* antioxidant activity. The extract and its fractions were initially screened for acute and sub-chronic antidiabetic activity in the dose range of 100-200 mg/kg. The most potent extract and fractions were further evaluated for pancreatic β-cells regeneration activity along with antioxidant and antihyperlipidemic activity. The polyphenolic, flavonoid and flavanone contents were assessed and correlated with its antidiabetic activity. The most significant pancreatic regeneration activity, antidiabetic and antihyperlipidemic activity and was shown by ethanol extract and butanol soluble fraction at a dose level of 200 mg/kg, while rutin was found to be least potent. In conclusion, pancreatic regeneration studies of ethanol extract treated rats show nesidioblastosis. It is also suggested that the factors causing regeneration are present within the pancreas. The newly generated islets may have formed from the ductal precursor cells and reduced oxidative stress helps in restoration of β-cell function.

Introduction

Diabetes mellitus (DM) is a major endocrine disorder, affecting approximately 5% of the world's population. Oxidative stress is one of the main factors associated to the development of glucose toxicity in diabetes since it causes decrease in levels of two critical transcription factors, PDX-1 and MafA. These normally bind to the insulin promoter and stimulate insulin gene transcription, decreased levels of these two proteins cause decrease in insulin promoter activity, insulin gene expression and insulin secretion (Robertson and Harmon, 2006).

Many of oral antidiabetic drugs provide only symptomatic relief and do not actively treat diabetes. An alternative strategy for diabetes treatment is the use of various plant extracts and herbal biomolecules, for their pancreas regeneration properties. Detailed investigations of the mechanism of action of these biomolecule have revealed that some of them allow the regeneration of β-cells, thus causing a reversal of diabetes in human and non-human subjects. For example, chard extract (*Beta vulgaris* L. var. *cicla*) has been used as a hypoglycaemic agent by DM patients in Turkey (Bolkent et al., 2000) and it has been documented that the number of

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β-cells and secretory granules increase after treatment with this herbal extract. Similarly various phytoconstituent such as epicatechin, rutin, quercetin, nymphayol and flavonoid extracts from *Pterocarpus marsupium* have shown to possess β-cells regeneration capacity (Chakravarthy et al., 1980;1981; Limbert et al., 2008; Subash-Babu et al., 2009). Flavonoids represent the most common and widely distributed group of plant phenolics (Harborne, 1986) and are abundant in foods; quercetin and rutin are the flavonoids most abundantly consumed (Nakamura et al., 2000).

Clitoria ternatea L., Fabaceae, is a highly nutritious legume used a livestock forage plant in many countries (Gomez and Kalamani, 2003; Hall, 1992) and is one of the most widely used herbal medicines in India. Phytochemical investigations of *C. ternatea* revealed the presence of flavonoid glycosides such as rutin, delphidin, kaempferol, quercetin and malvidin, and it has been documented that its leaves contain δ-lactone of 2-methyl-4-hydroxy-*n*-pentacosanoic acid. This plant is commonly used in the Ayurvedic medicine, as a memory enhancer, nootropic, anxiolytic, antidepressant, tranquilizing and sedative agent. Its extracts possess a wide range of pharmacological activities including antimicrobial, antipyretic, anti-inflammatory, antiasthmatic, hepatoprotective analgesic, diuretic, local anesthetic, antidiabetic, insecticidal, blood platelet aggregation-inhibiting and for use as a vascular smooth muscle relaxing properties (Kalyan et al., 2011; Nithianantham et al., 2013; Taur and Patil, 2011; Mukherjee et al., 2008)

Aqueous and ethanol extracts of roots, leaves and flowers have been previously studied for its antidiabetic potential, the mechanism of action and effect of flavonoid rich fraction of plant on pancreatic regeneration property was not evaluated keeping the above information in view (Daisy et al., 2009; Mukherjee et al., 2008).

The present study aimed to investigate the pancreatic regeneration potential of the different fractions of the ethanol extracts, in comparison to the flavonoid rutin. The antidiabetic and antihyperlipidemic potentials were also evaluated in streptozotocin-induced diabetic rats and correlated with its antioxidant activity and pancreatic regeneration activity.

Materials and methods

Animals

Male Swiss albino rats of Sprague-Dawley strain (175-200 g) were obtained from our institute's animal housing facility. The animals were fed a standard pellet diet and water was administered *ad libitum*. They were maintained in a controlled laboratory environment (22 ± 5 °C, 12 h of light/dark cycle). All experimental protocols were approved by the Institutional Animal Ethical Committee (23/2009/CPCSEA).

Plant material, preparation of extract and its fractionation

The aerial parts of *Clitoria ternatea* L., Fabaceae, were collected locally and authenticated by the Department of Botany, R. T. M. Nagpur University Campus, Nagpur. A voucher specimen was deposited in the Herbarium of the Department of Botany, with collection number RA 9778. The aerial parts were dried under shade and pulverized into a coarse powder. The powdered crude material was defatted with petroleum ether and then extracted with ethanol. The ethanol extract (CTAE) was concentrated in a rotary vacuum evaporator to yield a dark brown mass (yield 20.78% w/w). For fractionation, the ethanol extract was subjected to liquid-liquid partition with chloroform to yield a chloroform soluble fraction (CTACS; yield: 18.92% w/w) and a chloroform insoluble fraction. The chloroform insoluble fraction was further fractionated with *n*-butanol to yield a *n*-butanol soluble fraction (CTABS; yield: 55.83% w/w) and a *n*-butanol insoluble fraction (CTABIS; yield: 25.25% w/w). The ethanol extracts (CTAE) and these three broad fractions *i.e.* CTACS, CTABS and CTABIS were subjected to phytochemical and pharmacological screening.

Phytochemical screening

The CTAE extract and its broad fractions were screened for the presence of tannins, saponins, unsaturated sterols, triterpenes, alkaloids, flavonoids, lactones/esters, protein/amino acids, carbohydrates and/or glycosides, using thin layer chromatography (TLC) (Stahl, 1969). Thin layer plates pre-coated with silica gel G (Merck, 0.25 mm thickness) were used. Development was carried out with different solvent systems such as ethyl acetate: methanol: water (100:13.5:10, v/v/v), ethyl acetate:formic acid:acetic acid:water (100:11:11:26, v/v/v/v), toluene:methanol (80:20, v/v), toluene:ethyl acetate:formic acid (78:18:4, v/v/v) and ethyl acetate:methanol:water:acetic acid (65:15:15:10, v/v/v/v). After development of chromatogram, the plates were dried and sprayed with Dragendorff's, $AlCl^3$, hydroxylamine-ferric chloride solution, ninhydrin and antimony trichloride for the detection of alkaloids, flavonoids, lactones/esters, protein/amino acids, unsaturated sterols and triterpenes respectively. While detection of anthraquinones, saponins, tannins, carbohydrate and/or glycosides used KOH, anisaldehyde-sulphuric acid reagent, ferric chloride and naphthoresorcinol reagent respectively. Visualization was carried out under visible and UV light (λ 366 nm). CTAE extract and its fractions were also quantified for presence of important secondary metabolites such as total polyphenol, flavonoid and flavonone compounds, using following spectroscopic methods.

Determination of total polyphenol contents (TP), flavonoids (TFA) and total flavanones (TFO)

Total polyphenol content was measured using the Folin-Ciocalteu colorimetric method (Singlenton et al., 1999). Gallic acid was used as a reference for standard curve construction. The results were expressed as mg of gallic acid equivalents (GAE)/g of extract. Flavonoid content was determined by the aluminium chloride method (Stanojević et al., 2009). Rutin was used as a reference for standard curve construction. The results were expressed as mg of rutin equivalents (RE)/g of extract. For determination of flavanones the modified 2,4-dinitrophenylhydrazine method was used (Nagy and Grancai, 1996). Naringin was used as a reference standard and

results were expressed as mg of naringin equivalents (NE)/g of extract. All determinations were carried out in triplicate.

Quantitative estimation of rutin by HPTLC (Gupta et al., 2006)

Crude extracts and its fractions were standardized in relation to their rutin content using HPTLC . Ten microliter of test and standard solutions were applied on a pre-coated silica gel G 60 F^{254} TLC plate (E. Merck). Chromatogram was developed in ethyl acetate:formic acid:ethanol:water (100:11:11:26) (Marston and Hostettmann, 2006), visualized at 366 nm and compared against rutin standard for quantification using area under the curve method.

In vitro antioxidant studies

The antioxidant activities of rutin, ethanol extract and its fractions were assessed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method (Sharma and Bhat, 2009) and nitric oxide (NO) radical scavenging method described previously (Tsikas, 2007). Ascorbic acid was used as a reference standard and all determinations were made in triplicates.

Acute toxicity studies

Rats were divided into test and control groups (n = 6). The test group was given an increasing oral dose (1, 3 and 5 g/kg) of extract and its fractions. Rats were allowed food and water *ad libitum* and were kept under regular observation for symptoms of mortality and behavioural changes for a 48 h period (Dixon, 1965; OECD, 2000).

Induction of experimental diabetes

Diabetes was induced by sub-cutaneous (*s.c.*) injection of streptozotocin (STZ) in a single dose of 45 mg/kg body weight. STZ was dissolved in freshly prepared 0.01 M citrate buffer (pH 4.5). Animals were allowed to drink 5% glucose solution overnight to overcome the drug induced hypoglycemia. Twenty four hours after the injection, blood was withdrawn from overnight fasting animals and blood glucose level was assessed by a glucometer. Fasting animals (8 h) with blood glucose levels above 200 mg/dl were selected for the experiment as diabetic rats. Control animals were administered normal saline alone (Banskota et al., 2006).

Experimental design

Acute antidiabetic studies

The diabetic rats fasted overnight and were divided randomly into eleven groups (I–XI) of six rats each (n = 6) as follows: Group I, received vehicle (distilled water 10 ml/kg, *p.o.*); Group II, received insulin (4 IU/kg, *s.c.*); Group III received rutin (100 mg/kg, *p.o.*); Group IV-XI received CTAE extract and its fractions in dose range of 100 and 200 mg/kg administered orally using oral syringe. After administration, antidiabetic activity was assessed by withdrawing blood samples at 0, 1, 3, 5 and 24 h respectively. Results were reported as mg/dl (Itankar et al., 2011).

Sub-chronic antidiabetic studies

Rats were divided into six groups of six rats each (n = 6). Group I served as diabetic untreated control. Group II served as standard and was treated with 4 IU/kg/day insulin (Torrent Pharmaceuticals Ltd., India). Group III received rutin (100 mg/kg, *p.o.*). Group IV-VI received 200 mg/kg of CTAE extract; CTACS fraction and CTABS fraction, respectively, orally per day for 21 days. Fasting blood glucose levels (8 h fasting) and body weight were measured on day 0, 7, 14 and 21 of the study (Salahuddin and Jalalpure, 2010). Finally, on day 21, blood was collected from retro-orbital plexus, the rats were sacrificed, and pancreas removed, cleaned and washed in ice-cold normal saline for biochemical and histopathological study.

Pancreatic regeneration study

Tissue processing

Rats were sacrificed and pancreas removed were cleared of fat and lymph nodes, weighed and fixed for 1 h in 10% formalin and dehydrated by graded alcohol. The tissue was embedded in paraffin. Each gland was sectioned serially (4 µm in thickness) on a rotary microtome (Shandon, S325, England) along its length to avoid any bias due to changes in islet distribution or cell composition. For each pancreas, ten sections were randomly chosen at a fixed interval through the block (every 35th section), a procedure shown to ensure the selected sections are representative of the whole pancreas (Movassat et al., 1997), and stained with haematoxylin and eosin (H and E) for histological examination (Ahmed et al., 2010).

Histopathological and morphometric analysis

For morphometric analysis, the mean area (μm^2) of individual islets, perimeter of individual islet as a measure of islet size, and number of islet per section was determined on H and E-stained section of each group on a Motic digital microscope equipped with Motic Image plus 2.0 software (Motic, China). To determine the cross-sectional area of focal tissue, sections were systematically sampled using an image analysis system. Slides were examined in 1.0 x 1.5-mm increments, resulting in the assessment of 70 ± 5 fields per slide and 82 ± 5% of the total pancreatic section. Individual islets were quantified by capturing each field and, computer assisted, hand tracing focal areas within the captured images. The thresholding option was used to quantify the cross sectional areas occupied by tissue and to subtract unstained, unoccupied, areas (white space). The magnification was calibrated using a calibration grid micrograph. Apoptotic cells were photographed from these samples. To evaluate the distribution of islet sizes, islets were classified arbitrarily as small (perimeter < 500 µm), medium (perimeter between 500-1000 µm), large (perimeter between 1000-1500 µm), extra large (perimeter between 1500-2000 µm) and extra-extra large (perimeter between 2000-2500 µm).

Estimation of biochemical parameters

The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH) and lipid peroxidase (LPO) were assessed in erythrocyte and pancreas by methods described previously (Jamall and Crispin Smith, 1985). Serum triacylglycerides (TG), total cholesterol (TC) and high-density

lipoproteins (HDL) level was also estimated (Muller et al., 1977) using standard kits obtained from Span Diagnostics, India. Serum low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) level was calculated by using the following formula:

VLDL = TG/5

LDL = TC – (HDL + VLDL)

Acute toxicity studies

Rats were divided into test and control groups (n = 5). The test group was given an increasing oral dose (1, 3 and 5 g/kg) of TPLE and its fractions. The rats were allowed food and water *ad libitum* and were kept under regular observation for symptoms of mortality and behavioral changes over a period of 48 h (Dixon, 1965; OECD, 2000).

Statistical analysis

All the data concerning acute and sub-chronic antidiabetic study, *in vivo* antioxidant activity, antihyperlipidemic activity, body weight, and morphometric analysis were expressed as mean ± SD. Statistical differences between the treatments and the controls were tested by two-way analysis of variance (ANOVA) followed by Bonferroni posts-test using the "GraphPad-Prism" (version 5.02) statistical analysis software. A difference in the mean values of $p < 0.05$ or less was considered statistically significant.

Results

Phytochemical screening

The phytochemical screening of CTAE extract revealed the presence of alkaloids, phenolic compounds, tannins, flavonoids, carbohydrates, sterols, terpenoids and proteins. CTACS fraction showed the presence of tannins, triterpenoids and flavonoids; CTABS fraction proved presence of tannins and flavonoids; and CTABIS fraction contained alkaloids, carbohydrates, proteins, flavonoids, and tannins.

Acute toxicity studies

There was no mortality observed at doses up to 2 g/kg (*p.o.*) in rats. During observation, the animals exhibited decreased mobility but no signs of convulsions or loss of writhing reflex. This result indicates that CTA extract and its broad fraction have a low toxicity profile.

Determination of total polyphenol, flavonoid and flavanones

Total polyphenol content (mg/g) determined by Folin-Ciocalteu colorimetric method was 24.24 ± 0.01, 6.51 ± 0.01, 48.06 ± 0.05, and 5.77 ± 0.01 (GAE mg/g of extract) for CTAE, CTACS, CTABS and CTABIS respectively. Polyphenol content was determined by a linear regression equation of gallic acid and expressed as GAE of extract (y = 0.009x + 0.028, $r^2 = 0.996$). The flavonoid content determined by aluminium chloride method was found to be 3.28 ± 0.02, 2.92 ± 0.01, 8.89 ± 0.41 and 2.64 ± 0.02 RE mg/g of extracts for CTAE, CTACS, CTABS and CTABIS respectively. Flavonoid content was determined from linear regression equation of rutin (y = 0.014x - 0.017, $r^2 = 0.996$). The flavanone content determined by 2,4-dinitrophenylhydrazine method was found to be 1.12 ± 0.02, 0.186 ± 0.01,and 2.89 ± 0.06 and 0.056 ± 0.09 NE mg/g of extract for CTAE, CTACS, CTABS and CTABIS respectively. Flavanone content was determined from linear regression equation of naringin (y = 0.156x + 0.564, $r^2 = 0.976$).

As suggested by Chang et al. (2002) the flavones, flavonol and isoflavones formed complexes only with aluminium chloride, while flavanone strongly reacted only with 2,4-dinitrophenylhydrazine, so the contents determined by the two methods were added up to obtain the total flavonoid content.

The flavonoid and flavanone content represent only 13.53% (w/w) and 4.62 % (w/w) of the TP in CTAE extract respectively and a similar pattern was observed in all its fractions, suggesting that the extracts are very complex and contain many other polyphenols such as flavanones, isoflavones, phenolic acids and tannins, and the degree of polymerization of the polyphenols present in the samples is high. Degree of polymerization can be estimated by the ratio between the TP and TFA contents (Souza et al., 2008). The highest polymerization is observed in CTAE fraction and it varies from 7.39, 2.9, 5.41 and 2.18 for CTAE, CTACS, CTABS and CTABIS respectively. Thus the fractionation of ethanol extract CTAE with different solvents resulted in segregation of important secondary metabolites *i.e.* steroids. Moderately hydrophobic polyphenols were separated in CTACS fraction, while flavonoids and majority of polyphenols were enriched in CTABS fraction.

Quantitative estimation of rutin

Rutin extract content and its fraction was found to be 0.15% w/w, 0.32% and 0.08% w/w in CTAE, CTABS and CTABIS respectively. These results demonstrate that fraction of the extract resulted in enrichment of flavonoids in *n*-butanol fraction.

In vitro *antioxidant studies*

To understand the mechanism of antioxidant capacity, different analytical methods varying in their oxidation initiators and targets were used. Antioxidant capacity of an extract greatly depends on its composition and the methodology used, for example the oxidant and the oxidisable substrate used in the measurement (Cao and Prior, 1998). Apart from this, the characteristic of a particular test reaction can influence the analysis outcome. Some tests are preferential towards hydrophilic or hydrophobic compounds, whilst others are insensitive in this matter.

The antioxidant activity of the rutin extract and its fractions were observed in decreasing order of rutin > CTABS > CTACS > CTAE > CTABIS having IC_{50} (µg/ml) value of 1.26 ± 0.01, 6.05 ± 0.02, 21.95 ± 0.04, 36.79 ± 0.03, 67.14 ± 0.01 and 19.79 ± 1.01, 169.63 ± 1.09, 182.25 ± 3.98, 254.47 ± 2.01, 324.52 ± 6.25 in DPPH and NO scavenging method, respectively.

Antihyperglycemic effect

Acute toxicity studies revealed the non-toxic nature of CTAE extract and its fractions. There was no lethality or toxic reaction observed up to a dose level of 2 g/kg, *p.o.* The CTAE extract, its fractions, and rutin, have shown to induce a significant reduction in blood glucose levels in an acute antidiabetic study (Table 1). The insulin (short-acting soluble human insulin) at dose level of 4 IU/kg showed 80.98% reduction in plasma glucose after 24h (570.5 ± 9.12 mg/dl at 0 h to 108.53 ± 8.50 mg/dl; $p < 0.001$). Amongst the extract and its fraction CTAE extract has reduced elevated blood glucose level to 66.0% dose level of 200 mg/kg, *p.o.* after 24 h (541.25 ± 14.26 mg/dl at 0 h to 184.0 ± 7.87 mg/dl; $p < 0.001$), while comparing with the blood glucose at 0 h. Oral administration of rutin also significantly reduced 50.19% blood glucose level after 24 h (528 ± 10.61 mg/dl at 0 h to 263 ± 6.78 mg/dl; $p < 0.001$). The 66.0% reduction in blood glucose level exhibited by CTAE extract at 200 mg/kg *p.o.* at 24 h seemed to distribute into chloroform (CTACS), and *n*-butanol (CTABS) soluble fractions in 55.23 and 46.14% respectively. However, CTABIS fraction did not show any antidiabetic activity in both the selected doses. None of the extract fractions was as potent antidiabetic as insulin. Thus, it is evident from the above study that higher antidiabetic potential of CTAE extract than its fraction may be because of synergistic effect of constituent's presents in it, which was distributed in its fraction.

In a sub-chronic antidiabetic study, it was observed that prior to the extract administration there were no significant differences between the fasting blood glucose levels of the diabetic groups of animals (Fig. 1). However, after three weeks, the fasting blood glucose levels of the treated rats were significantly lower than the diabetic controls. In contrast, the blood glucose level of the untreated diabetic rat remained

Table 1
Effect of *Clitoria ternatea* aerial parts extract and its fraction in acute antidiabetic study.

	Time (h)				
Treatment	**0**	**1**	**3**	**5**	**24**
Diabetic Control	553.75 ± 17.97	550 ± 21.6	530.75 ± 7.80	512.75 ± 9.43	473.25 ± 9.07
Insulin (4 IU/kg, s.c.)	570.5 ± 9.12[c]	383 ± 12.99[c]	346 ± 17.66[c]	261.75 ± 16.34[c]	108.5 ± 8.50[c]
Rutin (100mg/kg; *p.o.*)	528 ± 10.61[b]	457.75 ± 10.37[c]	419.75 ± 3.59[c]	365.5 ± 4.04[c]	263 ± 6.78[c]
CTAE (100 mg/kg, *p.o.*)	543.5 ± 15.77	451.75 ± 2.75[c]	408.25 ± 3.30[c]	358.75 ± 4.78[c]	280.25 ± 5.56[c]
CTAE (200 mg/kg, *p.o.*)	541.25 ± 14.26	461.75.5 ± 4.43[c]	387.75 ± 9.26[c]	254.5 ± 9.11[c]	184.0 ± 7.87[c]
CTACS (200 mg/kg, *p.o.*)	533.25 ± 9.39	455.75 ± 5.56[c]	419 ± 4.96[b]	350.75 ± 10.4[b]	238.75 ± 8.52[b]
CTABS (100 mg/kg, *p.o.*)	525 ± 8.40	482 ± 7.83[c]	458.5 ± 7	402.5 ± 6.80[b]	316 ± 5.36[a]
CTABS (200 mg/kg, *p.o.*)	524 ± 6.38	467.5 ± 6.14[b]	430.75 ± 10.07[b]	372 ± 6.78[b]	282.25 ± 9.57[b]
CTABIS (100 mg/kg, *p.o.*)	531.75 ± 10.78	525 ± 10.63	502.0 ± 9.53[a]	483 ± 4.32[a]	461 ± 4.44
CTABIS (200 mg/kg, *p.o.*)	540 ± 10.36	518 ± 11.40[a]	497 ± 2.38	473.02 ± 6.78	459.5 ± 8.42[a]

CTAE, *Clitoria ternatea* aerial part ethanol extract; CTACS, chloroform soluble fraction of ethanol extract; CTABS, *n*-butanol soluble fraction of ethanol extract.
[a]The data are expressed in mean ± S.D. $n = 6$ in each group.
[b]Represents statistical significance vs. Diabetic control ($p < 0.01$).
[c]Represents statistical significance vs. Diabetic control ($p < 0.001$).

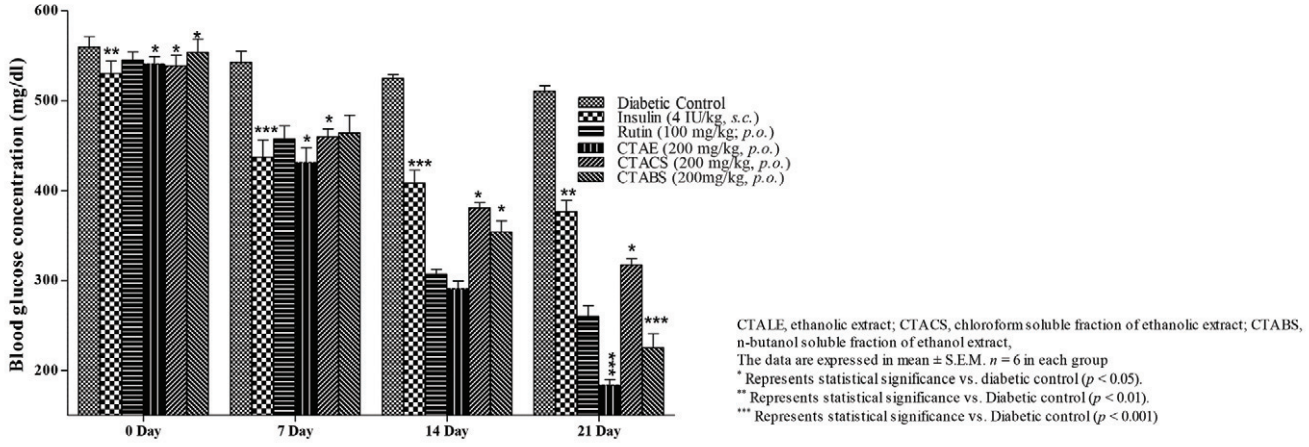

Fig. 1 - Effect of *Clitoria ternatea* aerial parts extract and selected fraction in sub-chronic antidiabetic study.

elevated throughout the experimental period. The most prominent antidiabetic activity was observed within the CTAE and CTABS fractions (66.02 and 59.32%; compared with its blood glucose level at day zero) at dose level of 200 mg/kg on day 21 of treatment (Table 2). However rutin also decreased the fasting (8 h) blood glucose level to 52.34% compared to diabetic rats at day zero. Results obtained from this experiment reveal that, CTAE extract and its fractions show antidiabetic activity in decreasing order of CTAE > CTABS > rutin > CTACS.

Histological examination, morphometric analysis of islet

In STZ-diabetic rats, the islet is considerably reduced and shrunken. There is destruction of some β-cells with central hyalinization; a few cells showed pyknotic nuclei and total cell number decreased. The degree of atrophy, hydropic degeneration, necrosis, hyalinization, or fibrosis was checked in H & e stained sections. Many of the islets were degranulated and high infiltration of T-cells was observed on treatment with STZ (Fig. 2B).

The average number of islet and average islet area per square µm of pancreatic samples was determined by a computer assisted measurement Morphometric analysis has confirmed that treatment resulted in pancreatic regeneration. The size distribution pattern reflected that normal group animals islets were larger in size (2000-2500 µm perimeter; n = 6) however the larger islet subset were also evident in diabetic control group nevertheless these are mostly degranulated and having central hyalinization with T-cell infiltration (Fig.2. A-B). Similarly, the average number of islets (0.87 ± 0.02 × 10-4 vs. 5.1 ± 0.16 × 10-4 in the control group) was 82.98% less abundant and islet density (2.89 ± 0.09 vs. 3.29 ± 0.24 per sq. µm of pancreas in normal rats) was 91.23% less in per sq. µm of pancreas compared to normal rats. On the other hand, average area of islet increased 1.52 fold (769.9 ± 12.9 vs. 1167.8 ± 23.7 in diabetic control; $p < 0.05$) in diabetic rats than normal rats. Whereas insulin and rutin administration for 21 days also resulted in the increase of islets less than 500 µm in size representative of islet regeneration. Comparing with the diabetic group, the average number of islet per sq. µm of pancreas increased by 3.22 and 4.72 fold in the insulin and rutin group respectively. On the other hand, compared to the diabetic control, islet density improved by 173.95% in insulin group (5.01 ± 0.16 × 10-4 vs. 2.8 ± 0.02 × 10-4 in diabetic control) and 240.28% in rutin treated group (0.69 ± 0.09 vs. 0.29 ± 0.09 in diabetic controls) (Fig. 2 A-D). Thus, from the previous study (sub-chronic antidiabetic study) and morphometric analysis it was evident that rutin possesses pancreatic regeneration potential and these results are in agreement with previous studies (Guz et al., 2001)

Treatments with CTAE fraction and CTABS extract resulted in improvement in β-cell granulation and reduced necrosis, hyalinization, or fibrosis of islets compared to diabetic control rats. However, treatments with CTACS fraction for 21 days resulted in partial improvement in pancreatic tissue integrity. The number of T-infiltrated and degranulated islet cells were

Table 2
Effect of *Clitoria ternatea* aerial parts extract and its selected fractions on islet size and distribution pattern in STZ-induced diabetic rats after treatment for 21 days.

	Treatment						
Distribution of Islet size (µm; perimeter)	**Distribution of islet as per size**						
	Normal Control		**Diabetic Control**	**Insulin (4 IU/kg s.c.)**	**Rutin (100 mg/kg; *p.o.*)**	**CTAE (200 mg/kg; *p.o.*)**	**CTACS (200 mg/kg; *p.o.*)**
< 500	2 ± 0.15	0	6 ± 0.15	16.6 ± 0.23	17.2 ± 1.29	10 ± 1.2[a]	12.46 ± 0.58
500 - 1000	2 ± 0.3	0	4 ± 0.12	3 ± 0.18[b]	4 ± 0.13[a]	1 ± 0.17	2 ± 0.24
1000 - 1500	4 ± 0	1 ± 0.21	2 ± 0.6	2 ± 0.52	3 ± 0.05	1 ± 0.82	1.18 ± 0
1500 - 2000	4 ± 0.01	4 ± 0.08	0	1 ± 0.02	1 ± 0.02[a]	0	2 ± 0.2
2000 - 2500	5 ± 0.21	4 ± 0.001	2 ± 0.11	0	0.35 ± 0.01	0	[0]
Average no. of islet per sq. µm of pancreas (x 10^{-3})	0.51 ± 0.016[b]	0.0868 ± 0.0021	0.28 ± 0.002	0.41 ± 0.08[a]	0.712 ± 0.022[b]	0.43 ± 0.001[b]	0.625 ± 0.085[b]
Average size of islet (µm^2 x 10^{-3})	769.9 ± 12.9[a]	1167.8 ± 23.7[c]	845.4 ± 49.8[c]	328.5 ± 15.2[a]	296.8 ± 15.3[a]		
3[a]	162.4 ± 18.5[a]	239.2 ± 12.6[b]					
Average Islet area per sq. µm of pancreas	3.285 ± 0.24[b]	0.288 ± 0.186	0.501 ± 0.012	0.692 ± 0.09[a]	0.9235 ± 0.03[a]	0.512 ± 0.09[a]	0.806 ± 0.003[a]

CTAE, ethanol extract; CTACS, chloroform soluble fraction of ethanol extract; CTABS, *n*-butanol soluble fraction of ethanol extract.
The data are expressed in mean ± S.E.M. n = 6 in each group.
The perimeter (µm) of islet size in 10 sheets of sections/rats was measured. The data are expressed in mean ± S.D n = 6 in each group.
[a]$p < 0.05$.
[b]$p < 0.01$.
[c]$p < 0.001$.

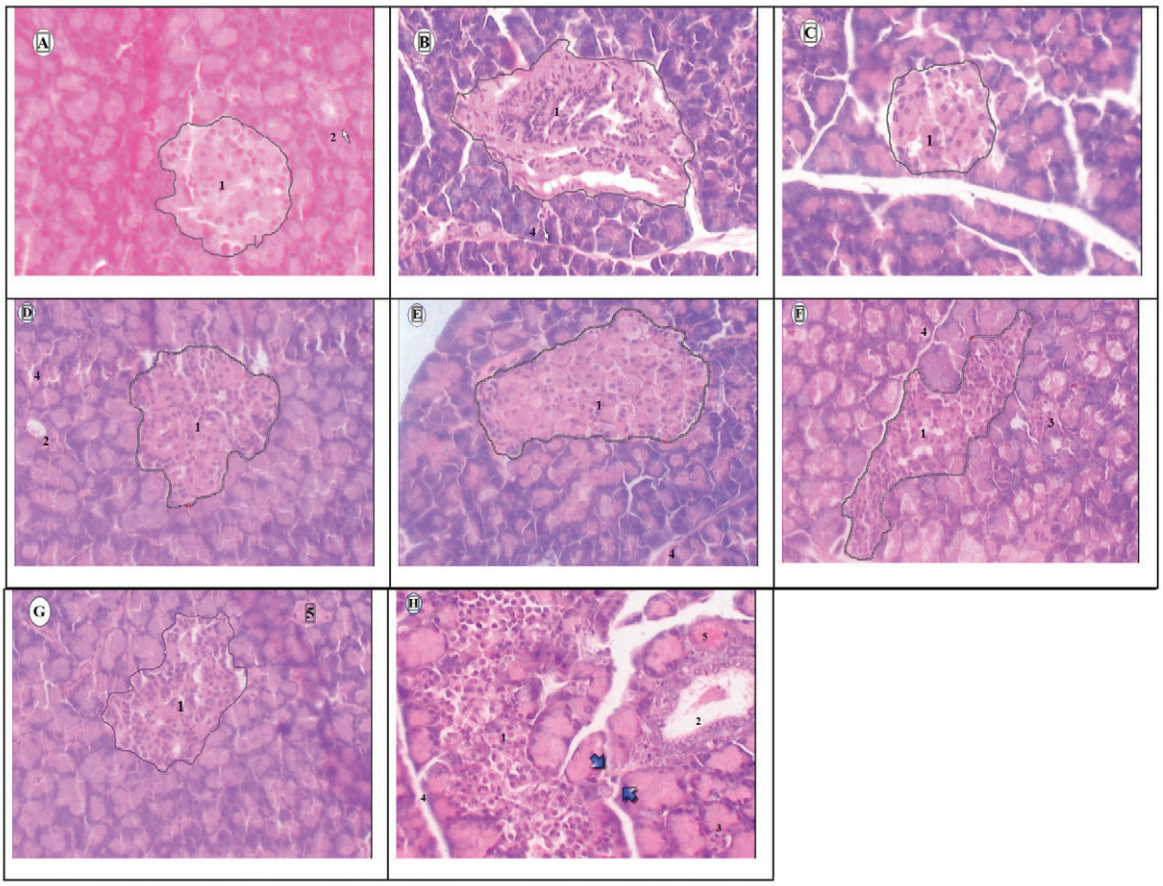

Fig. 2 - Hematoxylin and eosin stained histopathological sections of pancreas after 21 days treatment (A) Normal control: Native architecture of islet and pancreatic tissue (B) Diabetic control: Islet of Langerhans displaying degenerative & necrotic changes in diabetic control rats (C) Insulin group: Islet of Langerhans displaying degenerative changes and less β cell granulation compare to normal rats (D) Rutin group: Islet of Langerhans displaying increase in size with degenerative changes compared to normal control group . (E) CTAE group: Islet of Langerhans displaying increase in size and increased granulation compared to diabetic control group, islet architecture similar to normal control group (F) CTACS group: Enlarged islet of Langerhans with light degenerative few changes in (G) CTABS group: Newly formed islet of Langerhans with degeneration (H) Showing the regeneration of neo islets from the intralobular duct. 1, Newly formed islet.2, Intralobular duct from which the islet sprouted. 3, Glandular Acinus. 4, Intralobular connective tissue septa. 5, Blood vessels Blue Arrow. Connection between the duct and the islet.

reduced in CTACS fraction compared to diabetic control group. Morphometric analysis confirmed that treatment has resulted in pancreatic regeneration. The size distribution pattern has revealed the presence of higher number of smaller islets (≤ 500 µm in perimeter) in CTAE fraction (17.2 ± 1.29; $p < 0.001$ resp.) compared to rutin treated, normal and diabetic control group, thus indicating the formation of neo-islets. Similarly, CTACS and CTABS fractions resulted in the increase of islets numbers, however it is less pronounced than in the rutin treated group.

The average number of islet increased in CTAE and CTABS by 8.20 and 7.20 fold (0.87 ± 0.02 vs. 7.12 ± 0.22 and 6.25 ± 0.85 in CTAE and CTABS group, respectively) compared to the diabetic control group, and these effects were more pronounced than insulin and rutin treated groups. Similarly, a 320.66 % and 279.86 % increase in the average islet area per sq.µm was observed in CTAE and CTABS treated groups compared to diabetic control after treatment for 21 days respectively. However, the average islet area of CTAE and CTABS treated group was only 38.55% and 31.06% of islet area of normal control group. Treatment with CTACS fraction had a very marginal improvement in islet size area and distribution pattern compared to the insulin group, but it was less effective than rutin treated group. The pancreatic regeneration potential exhibited by rutin treatment was less pronounced than that of the CTAE fraction (Table 2).

In vivo *antioxidant activities in erythrocytes and pancreas*

The *in vivo* antioxidant enzyme activity in the erythrocytes and pancreas of normal and diabetic groups were assessed. In general, normal groups maintained higher enzyme levels of SOD, CAT, GSH and lower LPO levels than diabetic groups, (Sarkhail et al., 2007). Daily administration of extract and its

Table 3
Effect of aerial parts of *Clitoria ternatea* and leaves of *Tephrosia purpurea* extract and their fractions on lipid profile of diabetic rats.

Treatment	TC	TG	HDL	LDL	VLDL
Normal Control	76.1 ± 3.51	35.94 ± 3.24	27.27 ± 4.72	41.65 ± 2.04	7.19 ± 0.65
Diabetic Control	155.33 ± 2.40	156.67 ± 1.67	13.71 ± 0.38	110.29 ± 2.97	31.33 ± 0.33
Insulin (4IU/kg, *s.c.*)	74.2 ± 2.62[c]	43.48 ± 1.38[c]	27.05 ± 0.25[c]	38.45 ± 2.61[c]	8.70 ± 0.28[c]
Rutin (100 mg/kg, *p.o.*)	84.25 ± 2.6[c]	60.76 ± 0.28[c]	19.03 ± 1.03	53.07 ± 3.05[c]	12.15 ± 0.06[c]
CTAE (200mg/kg, *p.o.*)	77.23 ± 1.43[c]	42.65 ± 1.33[c]	29.6 ± 2.43[c]	39.10 ± 1.90[c]	8.53 ± 0.27[c]
CTACS (200 mg/kg, *p.o.*)	81.49 ± 0.64[c]	57.17 ± 2.50[c]	20.32 ± 0.53	49.74 ± 1.56[c]	11.43 ± 0.50[c]
CTABS (200mg/kg, *p.o.*)	79.27 ± 2.67[c]	55.93 ± 3.68[c]	20.82 ± 1.9[a]	47.27 ± 3.70[c]	11.18 ± 0.74[c]

The data are expressed in mean ± S.E.M. $n = 6$ in each group.
[a]Represents statistical significance vs. diabetic control ($p < 0.05$).
[b]Represents statistical significance vs. diabetic control ($p < 0.01$).
[c]Represents statistical significance vs. diabetic control ($p < 0.01$).

fractions were effective in fully recovering the enzyme activity, as level detected in normal group animals. Treatment with rutin, CTAE, CTACS and insulin significantly increased levels of SOD, CAT and GSH and decreased elevated LPO levels near to normal in both erythrocytes and pancreas in diabetic groups (Table 3).

Therefore, the extract and or fractions showing antidiabetic potential by reducing the production of ROS by inhibiting auto-oxidation of glucose elicit *in-vivo* antioxidant potential.

Effect on body weight and lipid profile

There was no significant intra-group variation in the basal body weight of the rats at day zero. However, at the end of 21 days of treatment the body weight of the rats in the normal group, insulin group, rutin, CTAE, CTACS and CTABS treated group, increased significantly by 22.5%, 18.83%, 16.11%, 17.83%, 11.17% and 16.41% respectively; whereas the body weight of diabetic control group decreased by 22.5%.

In the present study, diabetic rats treated with rutin, CTAE extract and its fractions showed an increase in body weight as compared to the diabetic control, which may be due to its protective effect in controlling muscle wasting (reversal of gluconeogenesis). The most potent reduction in elevated TC, TG, and LDL was observed within the insulin group and CTAE extract. Treatment with CTAE extract for 21 days resulted in 50.28%, 72.78% and 64.55% reduction in plasma LDL, TG and total cholesterol, while 215.90% increase in HDL level in comparison to diabetic control. Similarly, TC: HDL ratio and LDL: HDL ratios were significantly improved by -76.76%, -83.27% in CTAE treated group, as compared to diabetic control. Treatment with CTACS and CTABS fraction have shown a moderate decrease of elevated TC, TG and LDL levels, on the other hand rutin a naturally occurring bioflavonoid shows a lower efficacy in management of lipid profile in comparison to selected fractions of *C. ternatea* and its extract (Table 3).

Discussion

The most potent antioxidant activity was proven by rutin in both the assays. However, the higher antioxidant potency of extract and its fractions in DPPH assay in comparison to the NO assay can be explained as both DPPH and NO assays measure the free radical-scavenging activity but DPPH is carried out in an organic environment (an alcohol for example), while NO assay requires a buffered aqueous solution. Although the NO assay is carried out in an aqueous buffer, the nitric oxide molecule itself is a lipophilic species, hence it has a higher compatibility to such compounds that could both disperse well in the buffer and interact with the free radical. Amongst the extract and its fractions, CTABS fraction showed the highest antioxidant potential in both assays. These results are in agreement with its chemical content, as fractionation of extract resulted in enrichment of polyphenol and flavonoid components in the CTABS fraction, which as a resolut showed a higher antioxidant activity than CTAE extract. These findings suggested that there could be a correlation between the higher content of total polyphenol, flavonoid contents and its antioxidant potential.

Antidiabetic studies reveal that, CTAE extract and its fractions have sub-chronic antidiabetic activity in decreasing order CTAE > CTACS > CTABS > Rutin. The higher potential of CTACS fraction over CTABS extract, which contains higher amount of polyphenol and flavonoids, suggests that fractionation increases the degree of polymerization and segregation of secondary metabolites such as sterols, terpenoids and complex polyphenols that may results in increase antidiabetic potential. In contrast to all the fraction and extract in sub-chronic antidiabetic study, the lower antidiabetic potential of well-known bioflavonoid, rutin, may be caused of synergistic effects with different secondary metabolites present in the fraction and extract.

Previous studies have shown that there is a close relationship between the increase of free radicals, blood glucose and lipid peroxidation (LPO) in diabetes progression (Reddy et al., 2005). Increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors.

Enzymatic antioxidants such as SOD and CAT are considered as primary enzymes since they are involved in the direct elimination of reactive oxygen species (ROS) (Arulselvan and Subramanian, 2007). SOD is an important defense enzyme, it scavenges O_2- anions from H_2O_2 and hence diminishes the toxic effects of this and other free radicals derived from secondary reactions. CAT is a hemoprotein, which catalyzes the reduction of hydrogen peroxides and is known to be involved in detoxification of H_2O_2 concentrations *in vivo*.

Furthermore, in our study the activity of SOD, CAT and GSH decreased in the diabetes group as reported earlier (Gokce and Haznedaroglu, 2008) which could be due to involvement in the elimination of ROS generated by STZ. Treatment of diabetes with the extract and fractions reversed the activity of these enzymatic antioxidants, which might be due to the involvement in decreased oxidative stress as evidenced by the decrease of LPO. These findings suggest that CTAE induces *in vivo* antioxidant activity by attenuating the lipid peroxidation caused by various forms of free radicals; in this way may affect lipid profile in diabetes. The *in vivo* antioxidant activity of the rutin, extract and its fractions were observed in decreasing order of CTAE > CTACS > CTABS > rutin. The *in vivo* antioxidant pattern observed is inverse from its *in vitro* antioxidant pattern. This can be explained as the elevated blood glucose level in diabetes, not only generates ROS but also attenuates anti-oxidative mechanisms by scavenging enzymes and antioxidant substances. Since the glucose toxicity causes abnormal fatty acid metabolism, as auto-oxidation of glyceraldehyde, which generates hydrogen peroxide and a ketoaldehydes and this can lead to chronic oxidative damage (Shahat et al., 2004). The decrease in body weight associated to diabetes mellitus has been attributed to the gluconeogenesis leading to increased muscle wasting and loss of tissue proteins (Shirwaikar et al., 2005). Thus, the ability of CTAE extract and its fractions to effectively control the increase in blood glucose levels in the diabetic group of rats and significantly increasing the body weight may be attributed to its antihyperglycemic activity.

The most commonly observed lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia. A marked increase in total cholesterol and a decrease in HDL cholesterol have been observed in diabetic control rats. Insulin deficiency results in failure to activate lipoprotein lipase thereby causing hypertriglyceridemia. In diabetes LDL and VLDL carry cholesterol to the peripheral tissues where it is deposited, whereas HDL transports cholesterol from peripheral tissues to the liver and thus aids its excretion. Hence the increase in LDL and VLDL is atherogenic. There was a significant control of serum lipids level in CTAE-treated diabetic rats.

Hence, the ability of CTAE extract and its fractions to effectively control the increase in blood glucose in the diabetic group and significantly increase the body weight may be attributed to its antihyperglycemic activity. Thus from the above results, it is observed that the efficiency of lipid level reductions is associated with its potential to treat hyperglycaemia. This observation is further supported by histopathological studies of CTAE extract treated rats; there is evidence of nesidioblastosis, as can be seen by the intact ductal connection between the islet and associated duct in the pancreas of CTAE-treated rat (Fig. 2F). This results are in agreement with previous published data showing reversal of diabetic mice by formation of new islets from ductal cells (Kodama et al., 2003).

The notion that recruitment of β cells from a precursor pool may remain active in adulthood is supported by the observations that even in the adult rat pancreas some islets are still closely connected to ducts. Moreover, it was recently demonstrated that adult β-cell de novo formation from ductal cells is inducible. It is evident that the regenerating pancreas has the potential to induce islet neogenesis in the STZ-induced diabetic animals. These newly formed islets were then seen to grow into larger mature islets, probably by islet β-cell replication. The increase in islets size both in insulin and diabetic control groups, is a result of continuously increasing functional demand to which the residual β-cells are exposed and in order to compensate for their decreased cell number (Wang et al., 1996). Rutin, by its ability to scavenge free radicals and to inhibit lipid peroxidation, prevents streptozotocin-induced oxidative stress and protects β-cells resulting in increased insulin secretion and decreased blood glucose levels. In this context, research by Vessal et al. (2003) have shown that quercetin, the aglycone form of rutin, decreased blood glucose concentration and increased insulin release in streptozotocin-induced diabetic rats. Coskun et al. (2005) have also reported that, in streptozotocin-induced diabetic rats, quercetin protected pancreatic β-cells integrity by decreasing oxidative stress. Increased insulin levels could also be due to the stimulatory effect of rutin, thereby potentiating the existing β-cells of the islets of Langerhans in diabetic rats. Hii & Howell (1984;1985)reported an increased number of pancreatic islets in quercetin treated animals. It can be concluded that CTAE extract possess most potent antidibetic and significant *in-vivo* antioxidant activity by virtue of its capacity to cause pancreatic regeneration. The near normoglycemia achieved by the CTAE extract is either due to the formation of neo-islets rich in β-cells granulation or by the replication or expansion of the existing residual islets. These neo-islets formed by CTAE stimulation resulted in a decrease of fasting blood glucose level by increased insulin secretion, which also prevents atrophy by opposing to ubiquitin-proteasome pathway. This in turn helps in pancreatic regeneration, decrease in oxidative stress decrease in diabetes induced elevated lipid levels and decrease level of SOD, GSH and CAT enzyme activity.

It is evident from the study that *C. ternatea* exhibited significant pancreatic regeneration activity in STZ-induced diabetic rats. CTAE extract & its fractions proved relevant for the improvement in parameters like body weight & lipid profile and as a consecuence, their use might be of value in the treatment of diabetes.

Authorship

PRV (PhD student) contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the data and draft of the paper. PRI designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. SKA contributed to critical reading of the manuscript and running *in vitro* antioxidant activity. All the authors have read the final manuscript and approved for submission.

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