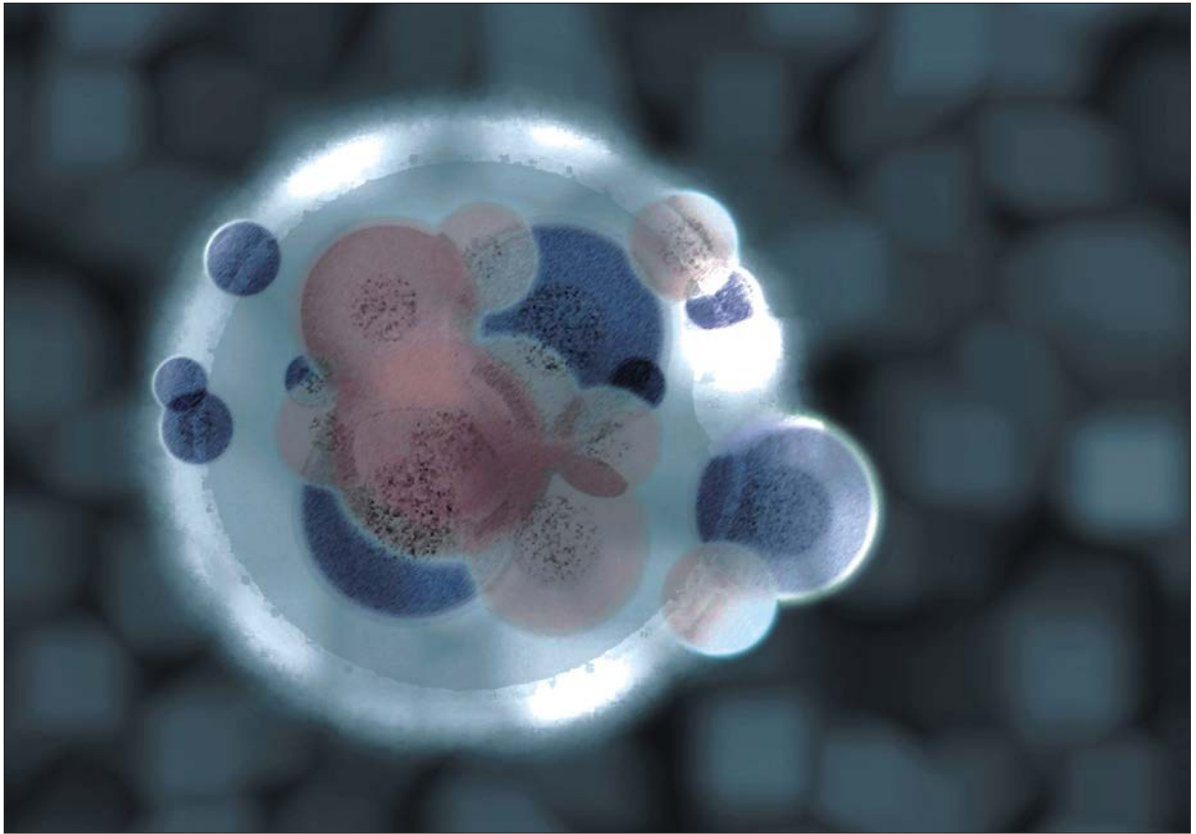

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Research Article
Evaluation of Safety and *in vitro* Mechanisms of Anti-diabetic Activity of β-caryophyllene and L-arginine

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Abstract

Background and Objective: β-caryophyllene (BCP) and L-arginine (LA) have been found to have anti-inflammatory, insulinotropic and antioxidant activity. However, a mechanistic approach regarding the combination of BCP and LA against the inflammatory processes present in diabetes mellitus has not been explored. Hence, the current study's objective was to determine the *in vitro* mechanisms of anti-oxidant, anti-inflammatory and α-glucosidase inhibitory activity of BCP, LA and their combination. **Materials and Methods:** *In vitro* cell viability (20-500 mM) was assessed using the MTT assay. Safety studies for BCP and LA were carried out for acute (2000 mg kg^{-1}) and repeated dose toxicity (300, 600, 900 mg kg^{-1}). *In vitro* anti-oxidant, anti-inflammatory and α-glucosidase activity of BCP, LA and their combination was evaluated at different concentrations (20-320 µg mL^{-1}) using the DPPH assay, H_2O_2 scavenging capacity assay, RBC membrane stabilization method and α-glucosidase inhibitory assay. **Results:** BCP+LA showed higher cell viability than BCP at 500 µg mL^{-1} (86.8 vs. 72.9%). Safety studies showed BCP+LA to be safe at 2000 mg kg^{-1}. BCP+LA was more potent than the individual agents in all the assays with >50% activity at half the concentrations of the individual agents (80, 20 and 20 µg mL^{-1}) for all the assays. The membrane stabilizing activity of BCP+LA was greater than the individual agents at 320 µg mL^{-1}. BCP, LA and BCP+LA displayed concentration-dependent increase in activity in all the assays. **Conclusion:** BCP+LA was safe and has more anti-oxidant, anti-inflammatory and α-glucosidase inhibitory activity than individual agents. These results give preliminary evidence supporting the combined use for the treatment of diabetes mellitus.

Key words: Type-2 diabetes mellitus, oxidative stress, synergistic effect of β-caryophyllene and L-arginine, α-glucosidase, anti-oxidant, anti-inflammatory

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes mellitus is a metabolic disease affecting 422 million people globally[1] with around 69.2 million patients in India itself[2]. Type-2 diabetes mellitus (T2DM) is a disorder caused due to a sedentary lifestyle and overall poor health. Recent studies have shown that T2DM is linked to inflammation of pancreatic cells which along with over-expression of cytokines, interleukin 1β and other inflammatory mediators leads to insulitis[3]. The liver, pancreatic islets and adipose tissues are especially affected by the inflammatory cascade[4] which includes changes in cytokine levels, leukocyte number and activation, increased apoptosis and tissue fibrosis[5]. Further damage is caused by intake of glucose and some macronutrients which promote inflammation and oxidative stress[6]. This oxidative stress due to reactive oxygen species (ROS) production leads to insulin resistance, β-cell dysfunction and decreased glucose tolerance[7].

Current pharmacotherapy controls T2DM due to its anti-hyperglycemic effects, often at the cost of producing some side effects. However, since life long treatment with these drugs is required for T2DM, the accumulation of such side effects often ends up proving to be costly for the patient[8]. Hence, there is an urgent requirement for new approaches to be sought, one of them being using natural agents.

Plants have been shown to contain innumerable bioactive compounds that act against multiple diseases, including diabetes that is prevalent today[9-12]. BCP is a constituent of commonly found plants like basil (*Ocimum* spp.), pepper (*Piper nigrum* L.), cinnamon (*Cinnamonum* spp.) and cloves (*Syzgium aromaticum*)[13], while LA can be obtained from protein-rich foods[14]. These foods are widely consumed across the world and hence could be beneficial in reducing hyperglycemia. The sesquiterpene BCP has been shown to possess anti-inflammatory properties that act through the cannabinoid receptor 2 (CB_2)[15] while L-arginine (LA) is known to be involved in pancreatic β-cell regeneration[16]. It is possible that combining these two separate mechanisms could lead to partial, if not complete, restoration of β-cell function, resulting in reduction of blood glucose levels. In this study, researchers evaluated the safety of BCP, LA and their combination through *in vitro* and *in vivo* methods, followed by investigation of the antioxidant, anti-inflammatory and α-glucosidase inhibitory activity. These studies can provide more insight into the mechanism of these constituents' anti-diabetic action.

MATERIALS AND METHODS

All the procedures were carried out in 5 months at SPP School of Pharmacy and Technology Management, Mumbai from January-May, 2017.

Drugs and chemicals: DPPH and BCP were procured from Sigma Aldrich (Mumbai, India), LA from Arrobiochem (Mumbai, India) and maltose, glacial acetic acid and EDTA from Fisher Scientific (Mumbai, India).

Cell lines and culture medium: Human Embryonic Kidney (HEK293) cells were purchased from National Centre for Cell Science (Pune, India) and cultured in Dulbecco's Modified Eagles Media, supplemented with 10% heat-inactivated FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin solutions. The cells were grown in 25 cm^2 tissue culture flasks (Fisher Scientific Co.) in an incubator at 37°C with 5% CO_2. After reaching 80-85% confluency, the cells were detached using mechanical scrapers, re-suspended, placed in a sterile 15 mL centrifuge tube and centrifuged at 1,000 rpm for 3 min to separate the cells. The supernatant was removed and the cells were re-suspended in fresh medium. Cell counts were performed using a hemocytometer. The cells were then subcultured to 96-well tissue culture plates, adding 5×10^4 cells per well and incubated at 37°C in 5% CO_2 for 24 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay: The MTT assay was used to determine cell viability[17]. Briefly, cells at a density of 5000 cells/well were grown in a 96-well plate and treated with different concentrations (20, 50, 100, 200 and 500 μM) of BCP, LA and BCP+LA after 2 h. After 24 h incubation, 20 μL of a 5 mg mL^{-1} MTT solution was added to each well and further incubated for 2 h. The medium was then removed and DMSO (200 μL) added to dissolve the formed formazan crystals. Absorbance at 570 nm was measured using a microplate spectrophotometer (Bioteck Instruments, USA) to determine the amount of formazan formed.

Animals: Adult male and female wistar rats (200-250 g) housed in polycarbonate cages at 25±2°C and 75±5% humidity were used. The rats were acclimatized for a week before experiment initiation. Relevant approvals were obtained from the Institutional Animal Ethics Committee (IAEC), approval number: CPCSEA/IAEC/P-14/2017.

***In vivo* safety studies:** Acute (2000 mg kg^{-1}) and repeated dose (300, 600 and 900 mg kg^{-1}) toxicity studies of BCP, LA and their combination were performed as per the relevant OECD guidelines[18,19]. Each group had 5 males and females (n = 10). Parameters mentioned in the guidelines were measured weekly. Biochemical and histopathological parameters were evaluated after 28 days. The liver, spleen, heart, kidney, pancreas and lung tissues were routinely processed, embedded in paraffin and sectioned. These sections were stained with hematoxylin and eosin dye for microscopic examinations.

Evaluation of antioxidant activity using DPPH (1,1-Dipheny-2-picryl hydrazyl): The evaluation of free radical scavenging activities of BCP and LA along with their combination was carried out using the DPPH method[20]. Different concentrations of BCP, LA and BCP+LA (20-320 µg mL^{-1}) were tested for their antioxidant activity, with the absorbance measured at 517 nm (Perkin Elmer UV-vis spectrophotometer Lambda 25, Thane, India).

Evaluation of hydrogen peroxide scavenging activity: The ability of BCP, LA and their combination to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.*[21]. Different concentrations of BCP, LA and BCP+LA (20-320 µg mL^{-1}) prepared in distilled water were mixed with a hydrogen peroxide solution (0.6 mL, 40 mM) prepared in phosphate buffer (pH 7.4). The next steps were performed as given by Ruch *et al.*[21].

Evaluation of *in vitro* anti-inflammatory activity using the red blood cell (RBC) membrane stabilization method: The extracellular activity of lysosomal enzymes released during inflammation is said to be related to acute or chronic inflammation. Non-steroidal drugs act by inhibiting these enzymes or by stabilizing the lysosomal membrane. Due to the similarities between the RBC and lysosomal membranes, the RBC membrane can be used to determine anti-inflammatory activity of compounds[22]. The method put forward by Shinde *et al.*[23] was used for the membrane stabilization study. Blood from the retro-orbital plexus of rats was collected into heparinized tubes and centrifuged at 3000 rpm followed by washing the packed RBC pellets with isosaline. A 10% (v/v) suspension of the cells was made. The reaction mixture contained 2 mL hyposaline, 1 mL phosphate buffer, 1 mL of BCP/LA/BCP+LA (20-320 µg mL^{-1}) in isosaline or the standard diclofenac and 0.5 mL of the RBC suspension to get a final volume of 4.5 mL. About 1 mL distilled water was used instead of the test sample for the control. The reaction mixtures were incubated at 37°C for 30 min followed by centrifugation at 3000 rpm for 20 min. Absorbance of the hemoglobin present in the supernatant was measured at 560 nm. Percentage membrane stabilization activity was calculated as:

$$\text{Membrane stabilization (\%)} = 1\text{-}\left(\frac{\text{OD of test sample}}{\text{OD of control}}\right)\times 100$$

Evaluation of inhibition of α-glucosidase activity: Inhibition of α-glucosidase activity was carried out by incubating 1 mL of different BCP, LA and BCP+LA concentrations (20-320 µg mL^{-1}) with 2 mL acetate buffer (pH 6.3) and 1 mL water for 10 min. The rest of the procedure was performed as per Patel *et al.*[24].

Statistical analysis: GraphPad Prism version 5.03 was used to perform statistical analyses. The difference between the groups was assessed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison *post hoc* test. The p-values less than 0.05 were considered to be significant.

RESULTS

Evaluation of cytotoxicity using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay: Figure 1 shows the cell viability of HEK293 cells when in contact with BCP, LA and BCP+LA. BCP was observed to be the most toxic at all the concentrations, while LA was the least toxic after 24 h. Combining BCP and LA seemed to reduce the cytotoxicity visible with BCP alone at all the concentrations. At the 500 µg mL^{-1} concentration, BCP+LA showed higher cell viability than BCP (86.8 vs. 72.9%).

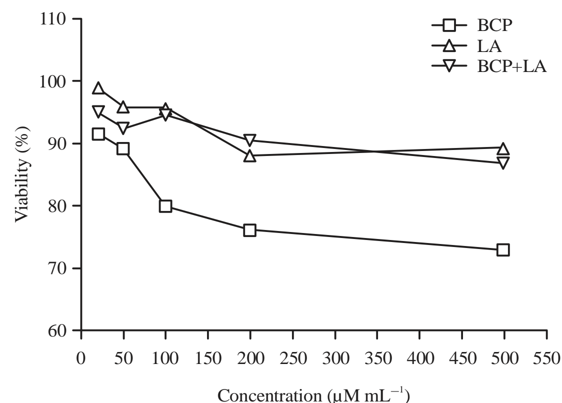


Fig. 1: Cell viability with BCP, LA and their combination using MTT assay

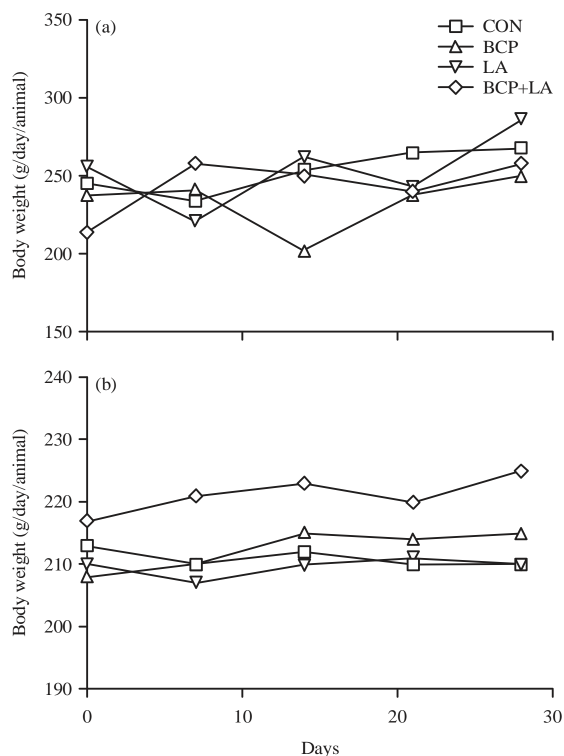


Fig. 2(a-b): Body weight during repeated dose toxicity study in (a) Males and (b) Females

***In vivo* safety studies:** BCP, LA and BCP+LA were found to be non-toxic throughout the 14 days observation period after a dose of 2000 mg kg^{-1}. Neither substance caused mortality after repeated administration of the highest dose (900 mg kg^{-1}). There were no significant differences in rat weights between the experimental and control groups (Fig. 2a, b).

Although a slight variation among males and females in terms of biochemical parameters was observed, these were found to be within range (Table 1, 2). Treatment related significant effects of the substances administered on the biochemical parameters was mostly absent. However, there were some statically significant decrease/difference in SGOT and urea were noted when control and treatment groups were compared and these changes were considered incidental and not treatment related.

None of the concentrations of BCP, LA or their combination caused any abnormal changes in hematological parameters in males and females when compared with the control (Table 3, 4). The statistically non-significant increase in WBC, RBC, HGB, HCT, lymphocytes in males after treatment were within the normal laboratory range. Increase in these values was not considered as toxicologically relevant.

Neither substance at any concentration caused abnormal changes in organ weights of the rats (Table 5, 6).

Histopathological assessment of rats dosed with 900 mg kg^{-1} BCP, LA or BCP+LA did not reveal any major abnormalities (Fig. 3). Combination of BCP and LA did not show any of the abnormality in the histopathological findings of heart (no cardiac muscle degeneration), pancreas (no infiltration and depletion of islets) and liver (no hepatocyte degeneration). Mild infiltration of inflammatory cells in the lungs and reduction of Bowman's space in the kidney was observed in the LA-treated group. However, these changes were very minimal.

Food intake was not affected due to administration of the experimental agents over the 28 days period (Fig. 4).

Evaluation of antioxidant activity using DPPH (1,1-Dipheny-2-picryl hydrazyl): This is the most widely reported method for screening of compounds with antioxidant activity. The DPPH assay is used to test the ability of compounds to act as free radical scavengers or hydrogen donors. BCP, LA and their combination (BCP+LA) had inhibitory action with the latter being the most potent (Fig. 5). BCP+LA inhibited 52.02% of the reaction at 80 μg mL^{-1}. This concentration which was much lower than the inhibition achieved by BCP and LA (55.32 and 56.93% at 160 μg mL^{-1}, respectively).

Evaluation of hydrogen peroxide scavenging activity: These results were similar to those observed in the DPPH assay. The combination BCP+LA again proved to be the most potent among the three test compounds (Fig. 6). BCP+LA scavenged 57.89% of the peroxide at 20 μg mL^{-1}, which was much lower than those of BCP and LA (55.00 and 50.53% at 160 μg mL^{-1}, respectively).

Evaluation of *in vitro* anti-inflammatory activity using the red blood cell (RBC) membrane stabilization method: BCP, LA and their combination displayed concentration-dependent increase in anti-inflammatory activity in the assay, albeit reaching around 50% activity at high concentrations (Fig. 7). While the standard drug diclofenac stabilized around 50% of the membrane around 160 μg mL^{-1}, BCP, LA and BCP+LA did so around 320 μg mL^{-1}, with BCP alone displaying the same anti-inflammatory activity as the combination at the maximal concentration evaluated (55.78%). The activity of BCP and BCP+LA was comparable to that of diclofenac.

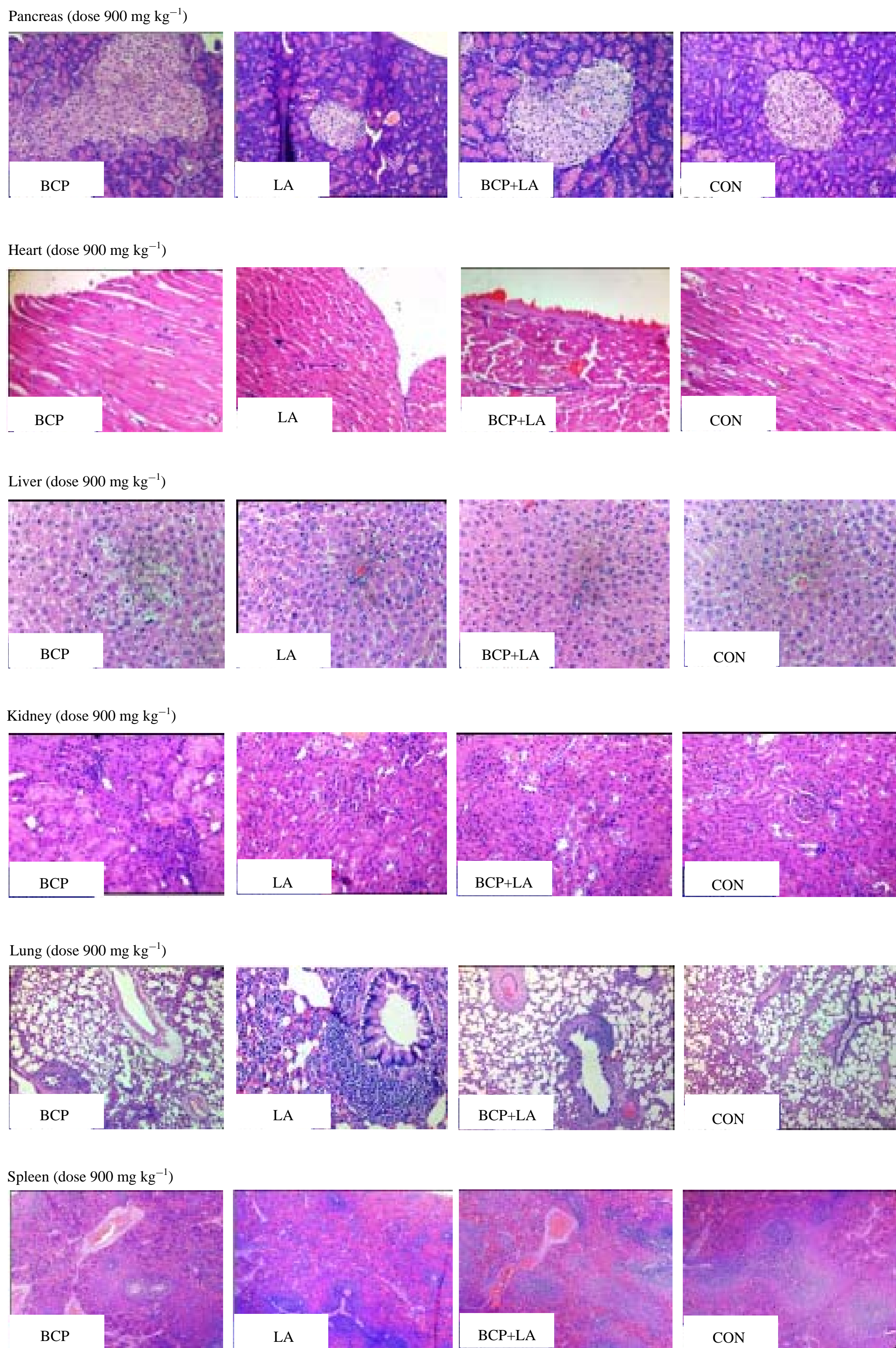


Fig. 3: Histopathological effects of BCP, LA and BCP+LA after repeated dosage

Table 1: Biochemical parameters of males at different concentrations of BCP, LA and BCP+ LA

Biochemical parameters (Males)	Control	LA-1 (300 mg kg^{-1})	LA-2 (600 mg kg^{-1})	LA-3 (900 mg kg^{-1})	B-CP-1 (300 mg kg^{-1})	B-CP-2 (600 mg kg^{-1})	B-CP-3 (900 mg kg^{-1})	LA+BCP-1 (300 mg kg^{-1})	LA+B-CP-2 (600 mg kg^{-1})	LA+B-CP-3 (900 mg kg^{-1})
TP (mg dL^{-1})	8.33±0.10	7.71±0.09	7.90±0.29	8.32±0.44	7.28±0.11	8.22±0.10	9.05±0.21	9.09±0.26	9.33±0.49	9.38±0.66
TC (mg dL^{-1})	70.70±2.01	74.57±1.9	70.00±1.84	72.20±2.01	68.87±1.08	71.27±1.33	71.67±1.62	69.60±0.92	72.73±1.27	74.53±3.51
Albumin (mg dL^{-1})	4.18±0.09	3.99±0.13	4.05±0.12	3.97±0.14	4.23±0.08	4.01±0.21	4.29±0.13	4.12±0.12	4.41±0.20	4.50±0.08
SGPT (IU L^{-1})	67.09±4.04	71.48±3.63	69.23±4.29	70.00±1.44	72.10±3.48	68.57±3.01	68.01±1.85	68.00±3.62	64.63±1.86	64.57±2.76
SGOT (IU L^{-1})	171.37±2.3	157.50±11.54	141.87±8.32	128.57±5.92	121.13±6.59	119.50±6.71	140.40±14.80	132.20±6.8	114.80±4.97	114.03±3.85
Glucose (mg dL^{-1})	99.97±0.87	101.15±1.31	100.71±3.11	104.22±4.17	104.36±2.90	98.39±5.46	101.13±1.81	105.31±5.47	107.04±6.63	96.59±5.59
Urea (mg dL^{-1})	28.39±0.93	29.78±1.89	29.83±3.24	33.33±2.47	31.28±1.03	31.25±1.41	34.89±1.66**	29.75±1.08	30.98±0.31	33.68±3.86**

**p<0.01 compared to control. All values are given as mean±SD. TP: Total protein, TC: Total cholesterol, SGPT: Serum glutamic-pyruvic transaminase, SGOT: Serum glutamic-oxaloacetic transaminase

Table 2: Biochemical parameters of females at different concentrations of BCP, LA and BCP+ LA

Biochemical parameters (Females)	Control	LA-1 (300 mg kg^{-1})	LA-2 (600 mg kg^{-1})	LA-3 (900 mg kg^{-1})	B-CP-1 (300 mg kg^{-1})	B-CP-2 (600 mg kg^{-1})	B-CP-3 (900 mg kg^{-1})	LA+BCP-1 (300 mg kg^{-1})	LA+B-CP-2 (600 mg kg^{-1})	LA+B-CP-3 (900 mg kg^{-1})
TP (mg dL^{-1})	7.96±0.28	7.02±0.71	7.44±0.42	7.92±0.35	6.84±0.32	6.82±0.49	7.64±0.37	6.95±0.59	7.76±0.42	8.21±0.38
TC (mg dL^{-1})	71.32±2.13	72.29±0.80	72.14±1.87	76.74±5.18	72.41±2.18	74.96±2.54	70.59±3.94	75.22±2.92	74.69±2.5	75.11±3.6
Albumin (mg dL^{-1})	4.30±0.30	4.24±0.51	4.68±0.28	4.55±0.32	4.57±0.28	4.15±0.41	4.41±0.26	4.04±0.08	4.17±0.15	4.02±0.19
SGPT (IU L^{-1})	69.53±2.71	66.65±2.33	73.09±3.27	71.24±3.97	65.21±1.95	67.95±2.99	66.66±3.5	67.21±1.62	66.31±3.18	67.63±3.84
SGOT (IU L^{-1})	182.40±3.06	173.54±2.93**	168.34±1.09***	157.94±2.21***	166.51±3.15***	155.79±2.60***	111.43±3.56***	142.35±3.15***	120.17±3.01***	108.95±3.29***
Glucose (mg dL^{-1})	77.27±2.18	77.34±3.19	82.18±4.62	89.19±4.42	79.89±2.11	69.38±2.62	79.74±2.96	71.48±1.64	70.31±2.93	84.21±4.04
Urea (mg dL^{-1})	38.98±1.53	34.65±1.07	33.33±2.35	36.69±3.83	31.69±1.60	35.46±2.45	32.85±1.83	33.00±2.78	37.18±1.47	32.10±1.39

**p<0.01 compared to control, ***p<0.001 compared to control. All values are given as Mean±SD. TP: Total protein, TC: Total cholesterol, SGPT: Serum glutamic-pyruvic transaminase, SGOT: Serum glutamic-oxaloacetic transaminase

Table 3: Hematological parameters of males at different concentrations of BCP, LA and BCP+LA

Hematological parameters (Male)	Control	LA-1 (300 mg kg^{-1})	LA-2 (600 mg kg^{-1})	LA-3 (900 mg kg^{-1})	B-CP-1 (300 mg kg^{-1})	B-CP-2 (600 mg kg^{-1})	B-CP-3 (900 mg kg^{-1})	LA+BCP-1 (300 mg kg^{-1})	LA+B-CP-2 (600 mg kg^{-1})	LA+B-CP-3 (900 mg kg^{-1})
WBC (10^3 cells µL^{-1})	8.26±0.38	8.80±1.61	10.43±1.69	10.47±0.97	8.33±1.79	8.27±2.76	6.57±3.41	9.63±1.95	9.23±3.33	9.33±4.60
RBC (10^3 cells µL^{-1})	8.38±0.14	8.30±0.21	7.85±0.15	8.50±0.12	7.85±0.44	8.17±0.38	7.89±0.22	8.28±0.40	8.34±0.41	8.65±0.22
HgB (g dL^{-1})	12.96±0.11	13.33±0.46	12.47±0.31	13.53±0.42	12.37±0.76	12.90±0.36	12.53±0.32	12.80±0.44	13.17±0.49	13.77±0.42
HCT (%)	42.40±0.84	43.40±1.51	41.37±0.94	44.57±0.76	41.13±2.15	43.07±1.12	41.53±1.18	42.83±1.51	43.67±1.71	45.57±0.84
MCV (fl)	52.60±1.10	52.33±1.08	52.7±0.24	52.40±0.29	52.40±0.70	52.77±1.17	52.63±0.06	51.80±1.44	52.43±2.84	52.73±0.64
MCH (pg)	15.44±0.26	16.07±0.41	15.87±0.09	15.90±0.24	15.77±0.12	15.80±0.56	15.87±0.12	15.47±0.32	15.80±0.87	15.90±0.10
MCHC (g dL^{-1})	29.74±0.55	30.70±0.22	30.13±0.09	30.33±0.42	30.07±0.55	29.97±0.38	30.20±0.17	29.87±0.25	30.13±0.06	30.23±0.45
Platelets (10^3 cells µL^{-1})	992.60±13.2	983.67±7.16	966.67±2.76	947.67±7.10	897.00±3.79	895.33±9.96	890.67±5.52	995.00±5.75	980.67±4.19	970.33±7.52
Lymphocytes (10^3 cells µL^{-1})	6.52±0.57	7.57±0.76	8.17±0.71	8.30±0.64	6.53±2.06	6.13±1.60	5.70±3.03	7.73±1.52	7.50±2.62	7.63±4.01

All values are given as Mean±SD. WBC: White blood cells, RBC: Red blood cells, HgB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration

Table 4: Hematological parameters of females at different concentrations of BCP, LA and BCP+LA

Hematological parameters (Female)	Control	LA-1 (300 mg kg^{-1})	LA-2 (600 mg kg^{-1})	LA-3 (900 mg kg^{-1})	B-CP-1 (300 mg kg^{-1})	B-CP-2 (600 mg kg^{-1})	B-CP-3 (900 mg kg^{-1})	LA+BCP-1 (300 mg kg^{-1})	LA+B-CP-2 (600 mg kg^{-1})	LA+B-CP-3 (900 mg kg^{-1})
WBC (10^3 cells µL)	8.40±0.95	8.04±0.95	8.74±0.86	8.24±1.27	8.36±0.88	8.30±0.75	8.24±0.82	8.42±1.28	8.23±1.48	7.79±1.05
RBC (10^3 cells µL^{-1})	7.61±0.63	7.73±0.83	7.54±0.74	7.64±0.98	7.37±0.72	7.60±0.88	7.45±0.61	7.11±1.09	7.70±1.43	7.52±0.97
HgB (g dL^{-1})	12.84±0.27	12.54±0.84	12.62±0.82	12.24±1.38	12.66±0.57	12.51±0.84	12.64±1.01	12.76±0.53	11.89±1.41	12.32±0.28
HCT (%)	36.26±2.98	37.01±2.80	35.49±2.54	33.28±2.03	41.98±1.27	41.39±1.81	40.70±1.38	36.49±1.89	37.81±1.91	36.24±1.85
MCV (fl)	51.06±1.19	53.80±3.79	55.38±2.60	53.44±1.91	52.79±1.49	51.98±1.34	52.07±1.28	54.44±2.14	50.06±2.41	48.00±2.31
MCH (pg)	17.17±0.55	17.85±1.03	18.16±1.06	17.25±0.81	17.28±0.97	16.90±1.37	16.64±0.61	17.22±0.22	17.71±1.70	17.18±1.50
MCHC (g dL^{-1})	32.74±0.90	34.77±1.06	34.82±1.51	33.40±1.27	32.22±0.89	31.96±1.47	32.12±1.25	31.88±0.86	36.23±2.90	35.83±1.60
Platelets (10^3 cells µL^{-1})	891.49±8.4	867.22±5.34	864.25±1.44	832.70±1.91	814.57±6.55	799.31±2.48	818.21±4.24	837.06±9.1	814.79±5.11	807.10±3.9
Lymphocytes (10^3 cells µL^{-1})	6.70±0.33	4.56±0.68	4.67±0.46	4.34±0.45	4.60±1.06	5.14±0.89	5.47±0.97	6.25±1.19	4.71±0.70	4.51±0.54

All values are given as Mean±SD. WBC: White blood cells, RBC: Red blood cells, HgB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration

Table 5: Organ weights of males at different concentrations of BCP, LA and BCP+LA

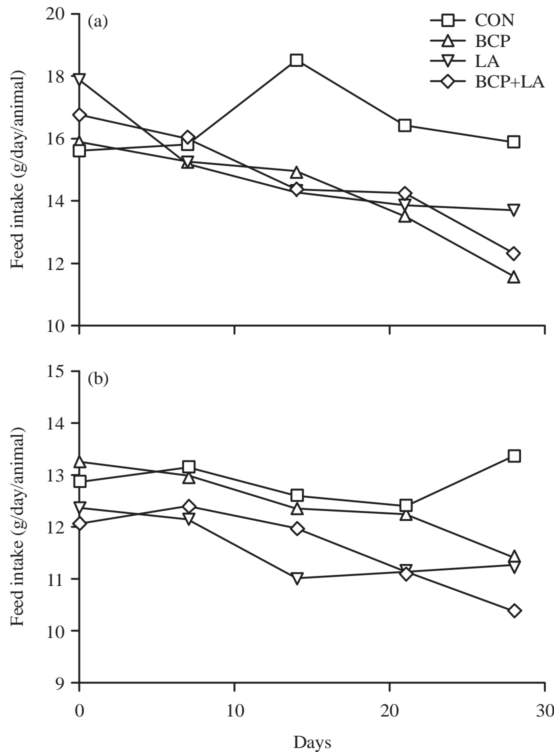
Organ	Control	L-arg (300 mg kg^{-1})	L-arg (600 mg kg^{-1})	L-arg (900 mg kg^{-1})	BCP (300 mg kg^{-1})	BCP (600 mg kg^{-1})	BCP (900 mg kg^{-1})	BCP+LA (300 mg kg^{-1})	BCP+LA (600 mg kg^{-1})	BCP+LA (900 mg kg^{-1})
Liver	10.12±0.21	10.47±0.31	11.03±0.15	11.40±0.53	10.87±0.25	11.23±0.15	11.10±0.17	11.01±0.18	11.29±0.56	11.23±0.15
Heart	0.91±0.03	0.97±0.01	0.98±0.02	0.90±0.02	0.97±0.01	0.90±0.04	0.87±0.04	0.90±0.03	0.91±0.01	0.90±0.02
Spleen	0.75±0.02	0.65±0.04	0.67±0.05	0.61±0.03	0.66±0.03	0.64±0.04	0.58±0.04	0.73±0.07	0.68±0.03	0.68±0.03
Kidney	1.86±0.2	1.99±0.01	1.90±0.06	1.84±0.05	2.00±0.14	2.01±0.08	1.85±0.04	1.85±0.03	1.79±0.01	1.78±0.03
Brain	1.70±0.03	1.86±0.02	1.71±0.07	1.78±0.04	1.77±0.05	1.77±0.03	1.75±0.05	1.76±0.02	1.73±0.03	1.72±0.02
Testes	2.78±0.03	2.90±0.05	2.78±0.17	2.69±0.04	2.82±0.03	2.88±0.07	2.83±0.05	2.71±0.03	2.73±0.06	2.86±0.12

All values are given as Mean±SD

Table 6: Organ weights of females at different concentrations of BCP, LA and BCP+ LA

Organ	Control	L-arg (300 mg kg^{-1})	L-arg (600 mg kg^{-1})	L-arg (900 mg kg^{-1})	BCP (300 mg kg^{-1})	BCP (600 mg kg^{-1})	BCP (900 mg kg^{-1})	BCP+LA (300 mg kg^{-1})	BCP+LA (600 mg kg^{-1})	BCP+LA (900 mg kg^{-1})
Liver	8.43±0.21	7.69±0.59	7.70±0.81	8.27±0.27	7.59±0.47	8.01±0.22	8.85±0.12	7.86±0.19	8.48±0.15	8.75±0.17
Heart	0.65±0.09	0.74±0.08	0.66±0.04	0.63±0.02	0.78±0.02	0.69±0.01	0.72±0.01	0.77±0.03	0.77±0.02	0.71±0.02
Spleen	0.64±0.23	0.60±0.04	0.61±0.04	0.62±0.02	0.60±0.02	0.59±0.02	0.62±0.01	0.59±0.02	0.61±0.02	0.61±0.02
Kidney	1.30±0.10	1.27±0.08	1.16±0.09	1.22±0.04	1.29±0.04	1.30±0.03	1.37±0.02	1.38±0.02	1.42±0.03	1.35±0.05
Brain	1.62±0.03	1.69±0.01	1.63±0.07	1.63±0.05	1.68±0.03	1.72±0.02	1.75±0.03	1.73±0.03	1.71±0.02	1.72±0.02
Ovary	0.08±0.02	0.06±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.08±0.01	0.07±0.01	0.07±0.01	0.07±0.01

All values are given as Mean±SD



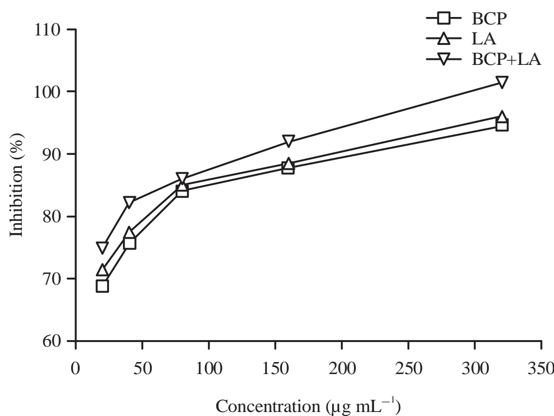


Fig. 4(a-b): Feed intake of after 900 mg kg^{-1} repeated dose of BCP, LA or BCP+LA in (a) Males and (b) Females

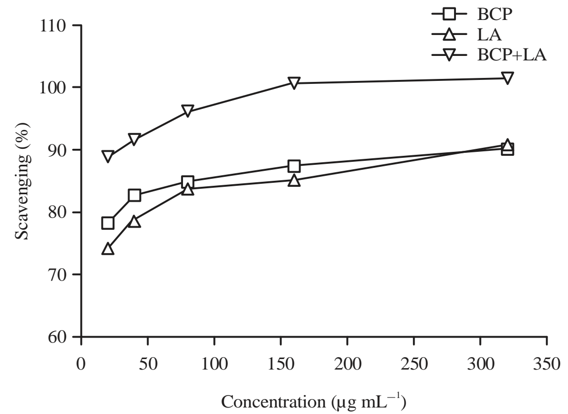


Fig. 6: Antioxidant activity of BCP, LA and their combination using H_2O_2 assay

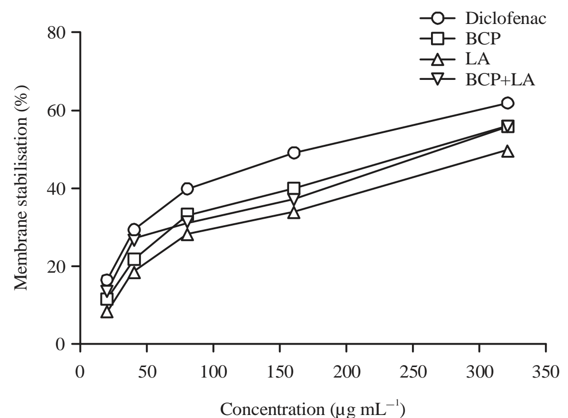


Fig. 7: Anti-inflammatory activity of BCP, LA and their combination using the RBC membrane stabilization assay

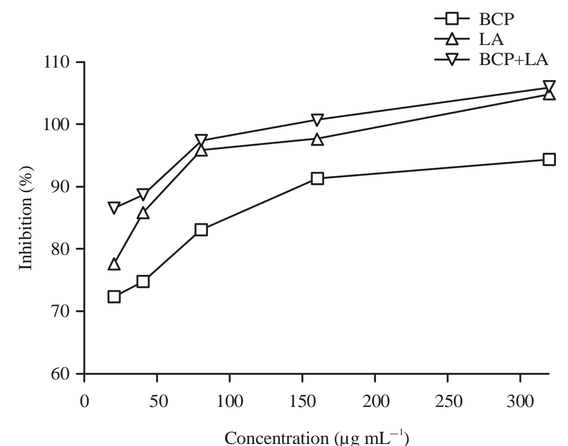


Fig. 8: Inhibition of α-glucosidase by BCP, LA and their combination

Evaluation of inhibition of α-glucosidase activity: All the three compounds inhibited enzyme activity in the assay (Fig. 8). The results showed that LA was more potent than BCP, with LA inhibiting around 50% of the enzyme at half the concentration of BCP (40 vs. 80 μg mL^{-1}). However, the combination BCP+LA was the most potent among the three since it was able to inhibit 53.28% enzyme activity at 20 μg mL^{-1}.

DISCUSSION

This study evaluated the safety as well as the antioxidant, anti-inflammatory and α-glucosidase inhibitory activity of BCP, LA and their combination to provide insight into the mechanisms by which these compounds could display anti-diabetic action. Acute and repeated toxicity studies as per OECD guidelines[18,19] showed that the combination elevated SGOT and urea levels. The combination of BCP and LA was found to be more potent than the individual agents in all the assays except the membrane stabilization assay, in which BCP's and the combination's potency were found to be equivalent.

Diabetes is widely prevalent across the globe and is also one of the major causes of mortality[1]. In India, it was the 7th highest cause of death in 2015, an increase of 34.8% from 2005[25]. Despite the significant burden posed by this disease, we have not been able to cure it. Current clinical management measures like change in lifestyle and pharmacologic management are useful[8]. However, lifelong polypharmacy contributes to the economic as well as clinical burden due to issues like medication errors, accumulation of side effects and noncompliance[8,26]. Therefore, it makes sense to utilize an alternative approach and explore natural substances as anti-diabetic agents, as was done in the current study.

BCP is a sesquiterpene acting on the CB_2 receptor and has been shown to have anti-diabetic action. Basha and Sankaranarayanan[27] have shown that oral administration of BCP for 45 days was able to reduce glucose levels as well as elevate insulin levels in streptozotocin-induced diabetic rats. This insulinotropic action could be due to CB_2 receptor activation as has been proven for trans-Caryophyllene[28]. This mechanism has also been reported to increase fatty acid oxidation by stimulating sirtuin 1 deacetylase activity, which through the peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) pathway, increases transcription of fatty acid oxidation enzymes[28]. Furthermore, BCP elevated glycolytic and lipogenic enzymes and at the same time reduced gluconeogenic enzymes, thus decreasing the overall glucose levels[27]. Additionally, BCP plays an important role in protecting glycoprotein components which could be damaged to high glucose levels[29].

LA is a naturally existing semi-essential amino acid and is the precursor of the vasodilator nitric oxide (NO). At the pre-clinical stage, LA has been shown to reduce hyperglycemia, triglycerides and lipid levels[30] and also inhibit the polyol pathway which decreases sorbitol accumulation in tissues and downstream ROS production, thus preventing oxidative stress and inflammation[31]. It is also known to induce glucagon-like peptide 1 (GLP-1) release from the intestines which then acts on GLP-1 receptor present on pancreatic β-cells to release insulin in response to elevated glucose levels[32]. Evaluation of LA clinically has also resulted in favorable results. Oral LA administration was shown to increase insulin sensitivity and β-cell function[33], with the latter visible as LA increased insulin levels compared with controls[34]. With respect to the current study results, both BCP and LA displayed antioxidant activity and anti-inflammatory activity which was in line with earlier research. The H_2O_2 assay in fact clearly displays that BCP's antioxidant capacity was potentiated after combining with LA. This potentiation could be possible since BCP is known to offset the damage caused by hyperglycemia mediated oxidative stress by elevating the levels of antioxidant enzymes while simultaneously reducing lipid peroxidative markers in both plasma and pancreatic tissue[35], whereas, LA increases the total serum antioxidant capacity[36]. BCP also reduces levels of the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6[33] which damage pancreatic cells. To achieve complete glycemic control, it makes sense to utilize different approaches. One of them is to reduce the absorption of carbohydrates in the intestines through the action of α-glucosidase inhibitors. α-glucosidase is one of the enzymes present in the intestine along the brush-border surface membrane and is involved in converting oligosaccharides and disaccharides to monosaccharides, which are then absorbed. Delaying carbohydrate absorption in this manner also decreases secondary post prandial glucose peaks[24]. LA was found to be more effective in inhibiting the enzyme compared with BCP, with the combination being the most potent. Although exclusively BCP has not been evaluated for α-glucosidase inhibitory activity, plants containing this sesquiterpene have been noted to have this activity[37].

CONCLUSION

This study shows that combination of β-caryophyllene and L-arginine is safe and has more antioxidant, anti-inflammatory and α-glucosidase inhibitory activity than individual agents. Utilizing this combination could be beneficial in reducing the inflammation that accompanies diabetes as multiple targets can be impacted. Further *in vivo* research needs to be conducted to properly assess the efficacy of this combination.

SIGNIFICANCE STATEMENT

This study discovers the possible synergistic effect of β-caryophyllene and L-arginine combination that can be beneficial for the treatment of type II diabetes mellitus. This study will help the researchers to look further into using natural agents for treatment of T2DM, rather than synthetic drugs which often have adverse effects.

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REFERENCES

1. WHO., 2017. Diabetes. Fact Sheet/November 2017, World Health Organization, Geneva, Switzerland. http://www.who.int/mediacentre/factsheets/fs312/en/
2. WHO., 2017. World health day 2016: Diabetes. World Health Organization, South-East Asia Regional Office, New Delhi, India.
3. Cieslak, M., A. Wojtczak and M. Cieslak, 2015. Role of pro-inflammatory cytokines of pancreatic islets and prospects of elaboration of new methods for the diabetes treatment. Acta Biochim. Pol., 62: 15-21.
4. Shu, C.J., C. Benoist and D. Mathis, 2012. The immune system's involvement in obesity-driven type 2 diabetes. Semin. Immunol., 24: 436-442.
5. Donath, M.Y. and S.E. Shoelson, 2011. Type 2 diabetes as an inflammatory disease. Nat. Rev. Immunol., 11: 98-107.
6. Dandona, P., H. Ghanim, A. Chaudhuri, S. Dhindsa and S.S. Kim, 2010. Macronutrient intake induces oxidative and inflammatory stress: Potential relevance to atherosclerosis and insulin resistance. Exp. Mol. Med., 42: 245-253.
7. Matough, F.A., S.B. Budin, Z.A. Hamid, N. Alwahaibi and J. Mohamed, 2012. The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos Med. J., 12: 5-18.
8. Chaudhury, A., C. Duvoor, V.S.R. Dendi, S. Kraleti and A. Chada *et al*., 2017. Clinical review of antidiabetic drugs: Implications for type 2 diabetes mellitus management. Front. Endocrinol., Vol. 8. 10.3389/fendo.2017.00006.
9. Kaur, G. and C. Meena, 2012. Amelioration of obesity, glucose intolerance and oxidative stress in high-fat diet and low-dose streptozotocin-induced diabetic rats by combination consisting of curcumin with piperine and quercetin. ISRN Pharmacol., 10.5402/2012/957283.
10. Kaur, G., V. Wani, A. Dave and P. Jadhav, 2015. Effect of *Lagenaria siceraria* and *Trigonella foenum graecum* on lipid absorption and excretion for modulation of lipid profile. Sci. Int., 3: 18-24.
11. Madrigal-Santillan, E., E. Madrigal-Bujaidar, I. Alvarez-Gonzalez, M.T. Sumaya-Martinez and J. Gutierrez-Salinas *et al*., 2014. Review of natural products with hepatoprotective effects. World J. Gastroenterol., 20: 14787-14804.
12. Li, L., X. Zhou, N. Li, M. Sun, J. Lv and Z. Xu, 2015. Herbal drugs against cardiovascular disease: Traditional medicine and modern development. Drug Discov. Today, 20: 1074-1086.
13. Fidyt, K., A. Fiedorowicz, L. Strzadala and A. Szumny, 2016. *β*-caryophyllene and *β*-caryophyllene oxide-natural compounds of anticancer and analgesic properties. Cancer Med., 5: 3007-3017.
14. King, D.E., A.G. Mainous III and M.E. Geesey, 2008. Variation in L-arginine intake follow demographics and lifestyle factors that may impact cardiovascular disease risk. Nutr. Res., 28: 21-24.
15. Klauke, A.L., I. Racz, B. Pradier, A. Markert, A.M. Zimmer, J. Gertsch and A. Zimmer, 2014. The cannabinoid CB_2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. Eur. Neuropsychopharmacol., 24: 608-620.
16. Salil, G., K.G. Nevin and T. Rajamohan, 2012. Arginine-rich coconut kernel diet influences nitric oxide synthase activity in alloxandiabetic rats. J. Sci. Food Agric., 92: 1903-1908.
17. Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.
18. OECD., 2001. OECD guideline for testing of chemicals acute oral toxicity: Acute toxic class method. OECD 423, Organization for Economic Cooperation and Development (OECD), Paris, France, December 17, 2001, pp: 1-14.
19. OECD., 2001. OECD guideline for testing of chemicals: Acute oral toxicity-fixed dose procedure. OECD/OCDE 420, Organization for Economic Cooperation and Development (OECD), Paris, France, December 17, 2001.
20. Mehta, A., G. Kaur and M. Chintamaneni, 2012. Piperine and quercetin enhances antioxidant and hepatoprotective effect of curcumin in paracetamol induced oxidative stress. Int. J. Pharmacol., 8: 101-107.
21. Ruch, R.J., S.J. Cheng and J.E. Klaunig, 1989. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis, 10: 1003-1008.
22. Joseph, J., A.R. Bindhu and N.A. Aleykutty, 2013. *In vitro* and *in vivo* anti-inflammatory activity of *Clerodendrum paniculatum* Linn. leaves. Indian J. Pharmaceut. Sci., 75: 376-379.

23. Shinde, U.A., A.S. Phadke, A.M. Nair, A.A. Mungantiwar, V.J. Dikshit and M.N. Saraf, 1999. Membrane stabilizing activity-a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodara* wood oil. Fitoterapia, 70: 251-257.
24. Patel, A.G., G. Kaur and C. Meena, 2011. α-Glucosidase inhibitory activity of curcumin and its comparison with combinatorial extract consisting of curcumin with piperine and quercetin. Pharmacologyonline, 3: 796-801.
25. IHME., 2016. India. Institute for Health Metrics and Evaluation (IHME), Seattle, WA., USA. http://www.healthdata.org/india
26. McAdam-Marx C., 2013. Economic implications of type 2 diabetes management. Am. J. Managed Care, 19: S143-S148.
27. Basha, R.H. and C. Sankaranarayanan, 2014. β-Caryophyllene, a natural sesquiterpene, modulates carbohydrate metabolism in streptozotocin-induced diabetic rats. Acta Histochem., 116: 1469-1479.
28. Zheng, X., T. Sun and X. Wang, 2013. Activation of type 2 cannabinoid receptors (CB2R) promotes fatty acid oxidation through the SIRT1/PGC-1α pathway. Biochem. Biophys. Res. Commun., 436: 377-381.
29. Basha, R.H. and C. Sankaranarayanan, 2015. Protective role of β-caryophyllene, a sesquiterpene lactone on plasma and tissue glycoprotein components in streptozotocin-induced hyperglycemic rats. J. Acute Med., 5: 9-14.
30. Mendez, J.D. and F. Balderas, 2001. Regulation of hyperglycemia and dyslipidemia by exogenous L-arginine in diabetic rats. Biochimie, 83: 453-458.
31. West, M.B., K.V. Ramana, K. Kaiserova, S.K. Srivastava and A. Bhatnagar, 2008. L-arginine prevents metabolic effects of high glucose in diabetic mice. FEBS Lett., 582: 2609-2614.
32. Clemmensen, C., S. Smajilovic, E.P. Smith, S.C. Woods and H. Brauner-Osborne *et al*., 2013. Oral L-arginine stimulates GLP-1 secretion to improve glucose tolerance in male mice. Endocrinology, 154: 3978-3983.
33. Monti, L.D., E. Setola, P.C.G. Lucotti, M.M. Marrocco-Trischitta and M. Comola *et al*., 2012. Effect of a long-term oral L-arginine supplementation on glucose metabolism: A randomized, double-blind, placebo-controlled trial. Diabetes Obesity Metab., 14: 893-900.
34. Ozbek, M., M. Erdogan, M. Karadeniz, S. Cetinkalp and A.G. Ozgen *et al*., 2009. Evaluation of beta cell dysfunction by mixed meal tolerance test and oral L-arginine in patients with newly diagnosed type 2 diabetes mellitus. Exp. Clin. Endocrinol. Diabetes, 117: 573-576.
35. Basha, R.H. and C. Sankaranarayanan, 2016. β-Caryophyllene, a natural sesquiterpene lactone attenuates hyperglycemia mediated oxidative and inflammatory stress in experimental diabetic rats. Chemico-Biol. Interact., 245: 50-58.
36. Fazelian, S., M. Hoseini, N. Namazi, J. Heshmati, M.S. Kish, M. Mirfatahi and A.S.S. Olia, 2014. Effects of L-arginine supplementation on antioxidant status and body composition in obese patients with pre-diabetes: A randomized controlled clinical trial. Adv. Pharmaceut. Bull., 4: 449-454.
37. Adefegha, S.A., T.A. Olasehinde and G. Oboh, 2017. Essential oil composition, antioxidant, antidiabetic and antihypertensive properties of two *Afromomum* species. J. Oleo Sci., 66: 51-63.