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In vitro evaluation of antihyperglycemic and anti-inflammatory potential of fermented papaya formulations

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ABSTRACT

The present study was undertaken to prepare a fermented papaya formulations *viz,* papaya leaf fermenta (PLF), ripe papaya fermenta (RPF), and unripe papaya fermenta (URPF).The aim of study was to assess in *vitro* anti-hyperglycemic& anti-inflammatory efficacy of fermented papaya formulations against L_6 myotubes and U937 cell lines. These formulations were prepared and analyzed by Gas chromatography mass spectrometry. Antihyperglycemic effects of formulations were evaluated by measuring the level of GLUT-4 in L_6 myc myotubes followed by western blotting and anti-inflammatory potential. GC-MS analysis revealed the presence of 20, 5 and 7 compounds in PLF, RPF and URPF. Some of the important phytochemicals are isocoumaran, quercetin, linolenic acid and propanoic acid. These phytochemicals are considered biologically active and have anti hyperglycemic and anti-inflammatory potential. Under *in vitro* conditions, treatment with all three fermented papaya formulations substantially increased the GLUT-4 level in L_6 cells which was associated with increased phosphorylation of AKT (Protein kinase B) (Ser-473).The formulations also showed anti-inflammatory potential by lowering TNF-α and IL-6 level in U937 monocyte cells.Results of this study displayed that fermented papaya formulations especially unripe papaya fermenta are the rich sources of bioactive compounds that can play a significant role in reducing diabetes and inflammation.

Figures : 06 References : 36 Tables : 03
KEY WORDS : Anti-inflammatory, *Carica papaya*, GLUT-4,Hyperglycemia, Interleukin.

Introduction

A chronic metabolic disorder caused due to inadequate insulin production is called diabetes mellitus. Numerous consequences, including hypertension, kidney failure, blindness, cardiovascular disease, obesity, and liver illnesses, could result from it[21]. According to International Diabetes Federation Atlas 10th edition, there is increase in diabetes globally, confirms that diabetes is a significant global challenge to the health[13].Types of diabetes include type 1, type 2 and gestational diabetes mellitus.

About 90 % of the cases of diabetes are type 2 diabetes [12] and characteristics by insulin resistance. Human body continuously produces insulin, while the body cells become resistant to its effects. Therefore, cells do not process insulin causing hyperglycemia[4,8,15]. For the influx of blood glucose, cells require glucose transporter family proteins (GLUTs). GLUT4 is a sugar transporter protein, mediate glucose uptake in the muscles and adipose tissue and continuously recycles between the plasma membrane and intracellular vessels 14,5. Insulin stimulation causes GLUT4 to be transported to the plasma membrane, which accelerates exocytosis and increases the pace at which they fuse with the membrane.

The increase of GLUT4 in plasma membrane leads to glucose uptake[35]. According to previous studies, defective GLUT4 translocation is a factor of insulin resistance and key precursor of type 2 diabetes[8,12,32]. Thus, for glucose homeostasis, GLUT4 is widely used

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TABLE-1: Phytocomponents identified in Papaya leaf Fermenta by GC-MS Analysis

Peak #	Retention time in minutes	Area	Area %	Molecular weight	Molecular formula	Compounds name
1	3.110	155046	0.24	82.1	C_5H_6O	2H-Pyran, tetrahydro-2-[(1-methylethyl) thio]-
2	3.200	512459	0.80	104.1	$C_4H_8O_3$	3,4-Furandiol, tetrahydro-, cis- **(Erythritan)**
3	3.280	2313124	3.60	114.1	$C_5H_6O_3$	2,5-Furandione, dihydro-3-methyl **(Methyl succinic anhydride)**
4	3.425	1048083	1.63	131.22	$C_7H_{17}NO$	N, N-Dimethyl-O-(1-methyl-butyl)-hydroxylamine
5	3.490	1917010	2.99	170.208	$C_9H_{14}O_3$	2-Propanol, 1-[(1-methyl-2-propynyl) oxy]-, acetate
6	3.662	1279123	1.99	120.15	C_8H_8O	Phthalan **(Isocoumaran)**
7	3.710	844431	1.32	128.13	$C_6H_8O_3$	2,5-Dimethyl-4-hydroxy-3(2H)-furanone **(Furaneol)**
8	3.805	452354	0.70	151.16	$C_8H_9NO_2$	N-Phenoxyacetamide
9	3.915	294356	0.46	112.17	$C_7H_{12}O$	3-Heptyn-1-ol
10	4.032	5011180	7.81	126.11	$C_6H_6O_3$	Maltol
11	4.188	276632	0.43	144.12	$C_6H_8O_4$	2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
12	4.994	1796651	2.80	204.26	$C_{13}H_{16}O_2$	2-Butanone, 4-hydroxy-3-methyl-
13	5.060	2066881	3.22	131.22	$C_7H_{17}NO$	N, N-Dimethyl-O-(1-methyl-butyl)-hydroxylamine
14	5.095	3754980	5.85	116.16	$C_5H_{12}N_2O$	O-Butylisourea
15	5.231	23684744	36.91	144.12	$C_6H_8O_4$	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- **(Quercetin)**
16	5.315	14959472	23.31	92.093	$C_3H_8O_3$	Glycerin
17	6.630	623499	0.97	120.15	C_8H_8O	Benzofuran, 2,3-Dihydro- **(Coumaran)**
18	9.294	1176619	1.83	150.17	$C_9H_{10}O_2$	2-Methoxy-4-vinylphenol **(4-Vinylguaiacol)**
19	13.298	1713784	2.67	165.23	$C_{10}H_{15}NO$	4-Ethoxyphenethylamine
20	17.570	287687	0.45	180.2	$C_{10}H_{12}O_3$	3', 5'-Dimethoxyacetophenone

as a key regulatory target in antidiabetic drug research. As serious side effects and economic burdens of chemical agent-based diabetic mellitus treatment strategies[8,26,29], natural products have many advantages including good patient tolerance, less side effects and low cost. Numerous herbal remedies are believed to treat diabetes mellitus by acting on multiple target areas in concert[7,20]. Recent studies have shown that phytochemicals naturally found in plants, possess antidiabetic activity[14,31,36].

Among traditional medicines *Carica papaya*(commonly known as Papaya) has demonstrated efficacy in combating a number of chronic illnesses.It has many medicinal properties like wound healing, hepatoprotective, anti-inflammatory, anti-hypertensive,treatment of gastrointestinal tract disorders, cardiac problem, sickle cell anemia and hypoglycemic activity[16,28].

Materials and Methods

Chemicals

Dulbecco Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Trypsin, Antibiotic/ Antimycotic solution and TRIZOL reagent (Gibco, USA). O-phenylenediamine dihydrochloride, Protease Inhibitor Cocktail and all other chemicals (Sigma Chemical, St. Louis, MO).Polyclonal anti-my, Monoclonal á-actinin, phospho-AKT and GLUT-4(Fine Test, Wuhan Fine Biotech Co., Ltd. China).

Preparation of various papaya fermenta

Papaya fruit purchased from market and skin (peel) and seeds were manually removed and pulp was recovered. The pulp was then crushed mechanically and diluted with distilled water 1:1 ratio (w/v)for ripe and unripe papaya whereas papaya leaves were washed with distilled water containing 0.02% PMS. Then leaves were crushed mechanically in a blender and diluted with distilled water1:10 ratio (w/v).Fermentation was done by using modified method[23].

Gas-Chromatography Mass Spectrometry Analysis

Bioactive components of fermented papaya formulations was identified by using Gas Chromatography Mas Spectrophotometer (GC-MS) triple quadrupole (GC-MS TQ 8030), Shimadzu Corp. Japan.GC was integrated with rested column (0.25 mm, 30m, Rxi. 5ms) was operated using Q3 checkup accession mode with launch time 3 min., end time 71 min, checkup speed 2500, start m/ z 40 and end m/ z 700. The ionization voltage was 70ev. original column temperature was kept at 100 ÚC, also increased linearly at 3 ÚC/ min upto 300 ÚC and held for 5 min. The temperature of injection harborage was 250 ÚC and the GC-MS interface was maintained at 300 ÚC. The sample was introduced *via* all- glass injector working in the split mode; helium carrier gas inflow rate was 1ml/ min keeping the split rate of 10:1. The identification of element was fulfilled by comparison of retention time and fragmentation pattern as well as with mass in the National Institute of Science and Technology(NIST) library.The relative percentage of phytoconstituents was expressed as percentage with peak area.

Cell culture

Rat L6 skeletal muscle cells cultured in DMEM with 10% FBS, 2ìg/ml of Blasticidin S, and 1% antibiotic/ antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin, 25 ìg/mlamphotericin B) and incubate in a 5% CO_2 at 37ÚC with humid condition..Then, confluent

TABLE-2: Phytocomponents identified in Ripe Papaya Fermenta by GC-MS Analysis

Peak #	Retention time in minutes	Molecular weight	Molecular formula	Compounds name
1	3.660	120.15	C_8H_8O	Phthalan **(Isobenzofurane)**
2	3.912	278.4	$C_{18}H_{30}O_2$	Spirohexane-5-one **(Linolenic acid)**
3	4.001	126.11	$C_6H_6O_3$	Maltol
4	4.749	318.6	$C_{15}H_{34}O_3Si_2$	1- (+) Lactic acid, tert-butyldimethylsilyl ester **(Propanoic acid)**
5	5.194	144.12	$C_6H_8O_4$	4H-Pyran-4-one, 2,3-dihydroxy-6-methyl

cells were transferred to a fresh medium with 2% fetal bovine serum in order to induce differentiation. In differentiated myotubes, experiment was performed 6-7 days after seeding[6].

Preparation of test samples

All three samples *viz,* papaya leaf fermenta (PLF), ripe papaya fermenta (RPF) and unripe papaya fermenta (URPF) were in liquid form. Samples were filter sterilized by using 0.22ìm membrane filter and desired concentrations (2%, 1%, 0.5%, 0.25% and 0.125%) were achieved using DMEM media supplemented with 1% (v/v) FBS.

Cell Viability

A 96-well plate was seeded with L_6 rat myoblast cell five thousand ($5x10^3$) cells/well, and incubated for 48 hours before sample exposures. After which the

TABLE-3: Phytocomponents identified in Unripe Papaya Fermenta by GC-MS Analysis

Peak #	Retention time in minutes	Molecular weight	Molecular formula	Compounds name
1	3.355	204.26	$C_{13}H_{16}O_2$	2 Butanone, 4-Hydroxy-3-methyl
2	3.495	180.16	$C_6H_{12}O_6$	dl—Glyceraldehyde Dimer
3	3.669	92.14	C_7H_8	1,3,5-Cycloheptatriene **(Tropilidine)**
4	3.820	190.15	$C_7H_{10}O_6$	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid **(Tannin)**
5	3.925	278.4	$C_{18}H_{30}O_2$	Spirohexan-5-one
6	4.025	126.11	$C_6H_6O_3$	Maltol
7	4.155	122.12	$C_4H_{10}O_4$	Erythritol **(Sugar alcohol)**
8	4.225	131.22	$C_7H_{17}NO$	N, N-Dimethyl-O-(1-methyl-butyl)-hydroxylamine
9	4.300	144.19	$C_8H_{13}FO$	5-Fluoro-6-methyl-5-heptene-2-one
10	4.395	92.093	$C_3H_8O_3$	Glycerin
11	4.465	156.1	$C_4H_4N_4O_3$	Pyrimidine-2,4(1H,3H)-dione,5-amino-6-nitroso
12	4.605	112.132	$C_5H_8N_2O$	(+)-3,4-Dehydroproline amide
13	4.755	318.6	$C_{15}H_{34}O_3Si_2$	1- (+)-Lactic acid, tert-butyldimethylsilyl ester
14	4.995	102.14	$C_4H_{10}N_2O$	2-Propanamine, N-methyl-N-nitroso- **(N-aminomorpholine)**
15	5.224	144.12	$C_6H_8O_4$	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy
16	5.400	325.31	$C_{15}H_{19}NO_7$	Glucosamine, N-acetyl-N-benzoyl-
17	5.500	92.093	$C_3H_8O_3$	Glycerin

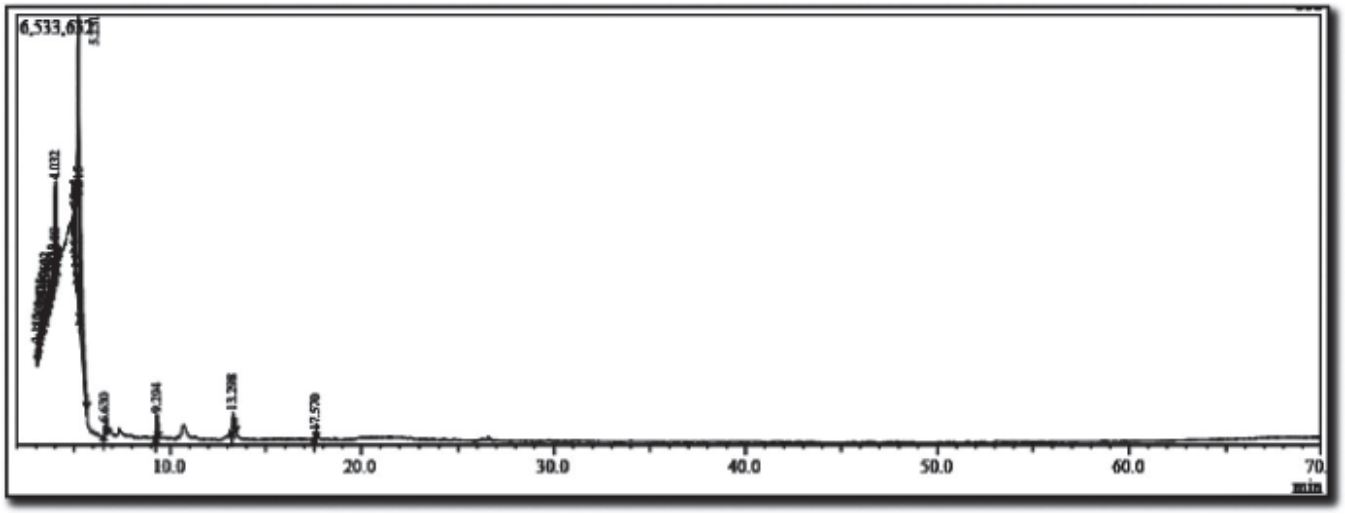

Fig. 1:GC-MS Chromatogram of Papaya Leaf Fermenta (PLF)

samples were applied to the cells. Following a 72-hour treatment period, added 5 mg/ml of MTT reagent and incubated for 4 hours at 37°C.After removing the MTT solution, added 100 ìl of isopropanol having 1N HCl to each well. Then the formazan crystals were gently shaken for 20 minutes, and the color was assessed at 570nm.

Analysis of GLUT-4 and AKT expression in Rat L6 muscle cells by Western blotting

Based on MTT assay cytotoxicity investigation, L_6 cells treated with 1% of all 3 samples *viz,* papaya leaf fermenta (PLF), ripe papaya fermenta (RPF) and unripe papaya fermenta (URPF).After treating the cells with different test fermenta's, the cells were then analyzed for AKT and GLUT-4. In 100 mm^2 tissue culture plate approx. $1x10^5$ cells/well were seeded and grown for one day. Growing cells then treated with samples for 48 hrs. After the sample treatment,cells were lysed in RIPA buffer (150 mM NaCl, 50mM Tris.Cl, 1% Triton X-100, 10% sodium deoxycholate, 0.1% SDS) and protein was extracted. Concentration of protein was determined by Bradford assay (Bradford, Sigma) and 30ìg protein from each sample was then separated by 10% polyacrylamide gel. Then these proteins were transferred onto a nitrocellulose membrane (Gbiosciences) electrophoretically at 100mA for 90 minutes. Further the membrane was blocked in PBS-T buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 and 0.1% (v/v) Tween 20) having 5% nonfat dry milk for 1hr at 37 Ú C. Then,overnight incubated the membranes at 4Ú C with the following primary antibodies: â-actin (Mouse antihuman antibody; dilution1:5000; Santacruz); pAKT (Mouse antihuman antibody; dilution 1:2000; Finetest), á-actenin (Rabbit antihuman; dilution 1:2000; Finetest) and GLUT-4 (Rabbit antihuman; dilution 1:2000;

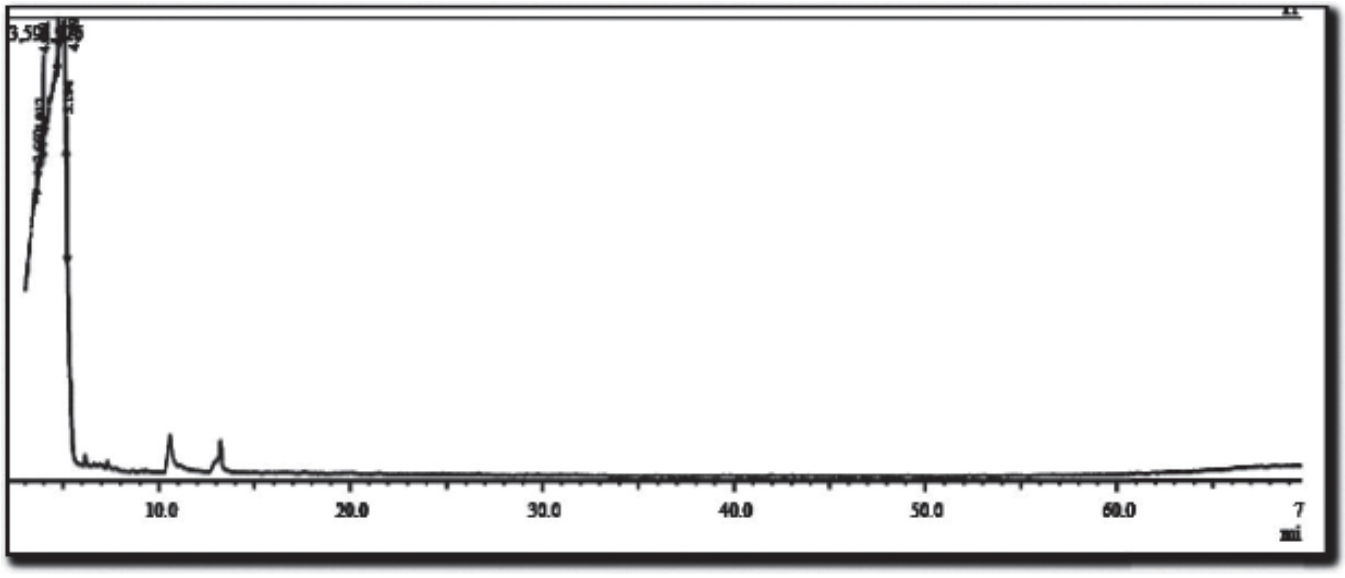

Fig. 2: GC-MS Chromatogram of Ripe Papaya Fermenta (RPF)

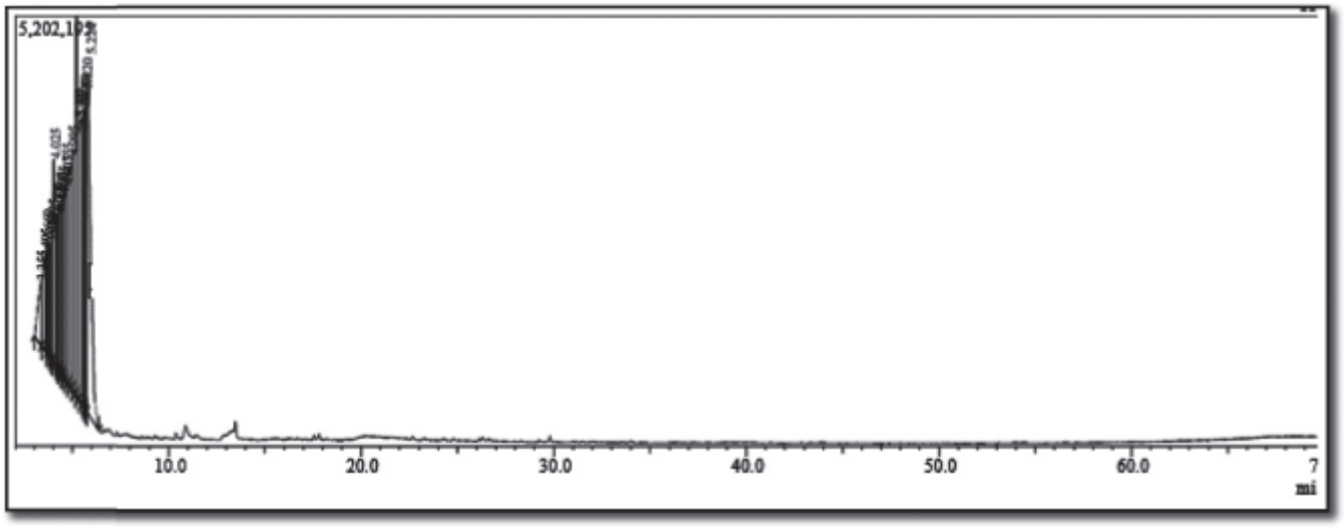

Fig. 3: GC-MS Chromatogram of Unripe Papaya Fermenta (URPF)

Finetest). â-actin used as an internal control. Then the membranes were developed using a chemiluminescence reagent(Immobilon Western Chemiluminescent HRP substrate, Merck) and immune signals were detected by LI-CORC-DiGit Chemiluminescence Western Blot Scanner. By using Image Studio™ Software, the protein band intensity was quantified.

Cytokine level

U937 (human monocyte derived) cells were cultured and maintained in 10% FBS containing RPMI medium with 100 U/ml penicillin-streptomycin. Cells were incubated at 37ÚC in 5% CO_2 and further stimulated with 100ng/ml of lipopolysaccharides (LPS) for 24 hours for the updown regulation of inflammatory cytokine genes. In order to ascertain the sample-mediated response against the cytokine secretion, culture was treated with 2% (on the basis of cytotoxicity assay)PLF, RPF, and URPF separately, for 24 hours along with media control.These Cells were further processed for RNA extraction (using TRIZOL reagent from Sigma Aldrich), cDNA conversion (Aura cDNA synthesis kits).Real time PCR was performed using (Agilent AriaMX system, using SYBR Green) for gene expression analysis for two inflammatory cytokines:(TNF-á)tumor necrosis factor and (IL-6)interleukin (Eurofins technologies).

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). All the data were evaluated by one-way analysis of variance (ANOVA) using statistical software Graphpad prism version 3. The significance of differences was determined by using one-way ANOVA followed by Dunnett's multiple comparison test. Probability values of *Pd"0.05, **Pd"0.01, ***Pd"0.001, ****Pd"0.0001 were compared with control.

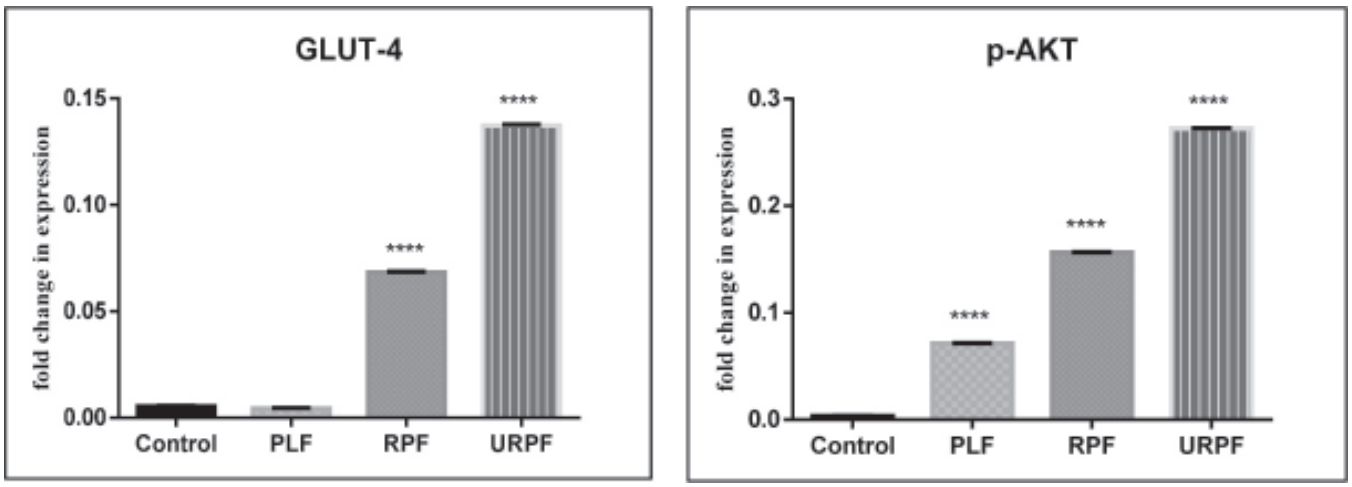

Fig. 4: Expression of GLUT-4 & p-AKT in L6 cells upon treatment with PLF, RPF and URPF respectively. Mean± SEM; ****Pd"0.0001 *vs.*control. ANOVA (F values) GLUT-4 -35955&p-AKT -121108.

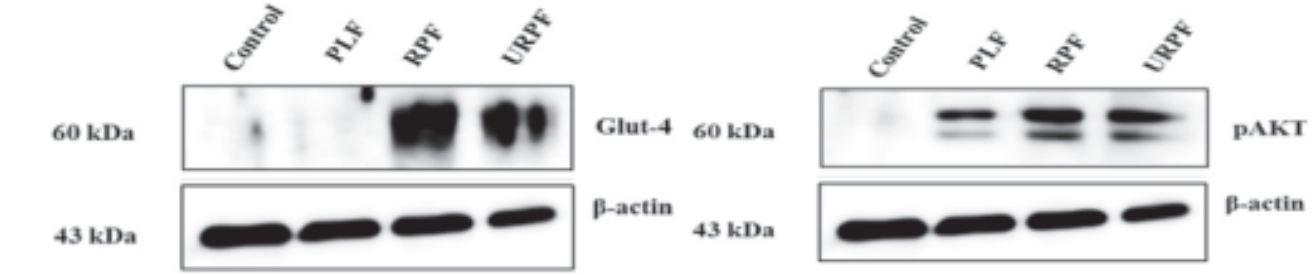

Fig. 5: Western Blot pictures of GLUT-4 and pAKT.

Results

The GC-MS chromatogram of fermented papaya formulations recorded a total 42 peaks.Bioactive compounds were recognized by their peak retention time, molecular formula and molecular weight by the NIST library. Results showed that 20, 5 and 17 compounds were identified in PLF, RPF and URPF respectively (Tables 1, 2, 3). The major phytoconstituents identified in PLF are Quercetin;Glycerin; RPF are Maltol; -(+)-Lacticacid,tert-butyldimethylsilylester and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl.URPF included Tannin and Sugar alcohol.

Impact on GLUT-4&phosphorylation of AKT:URPF had the best effect (0.137-fold increase) followed by RPF (0.068) and PLF (0.004)in GLUT-4.Among the three samples, URPF boosted pAKT levels by 0.272 folds, outperforming RPF (0.156 folds) and PLF (0.071 folds)compared to the control(Figures 4 & 5).

Effect on TNF-α & IL-6

URPF was found to have a higher reduction (21.4%) than PLF (13%) and RPF (10.8%)in TNF-á. The levels of IL-6 also reduced by PLF (12.1%) and RPF (4.8%), URPF was found to have the highest effect (18.1%) among the three products (Figure 6).

Discussion

Diabetes is a long-term metabolic illness. According to International Diabetes Federation (2021), Globally 537 million people are diabetic which is expected to increase to 643 million by 2030, 783 million by 2045[13].In low and middle-income nations, 422 million people were affected[19,33]. Now a day's diabetes is a common health problem that causes serious economic burden to nation and individuals[27].Skeletal muscles play a key role in the maintenance of glucose homeostasis[25]. Stress, diets,obesity and infection activate innate immunity which induced chronic inflammation *via* the secretion of pro-inflammatory cytokines which leads to type 2 diabetes mellitus[17].

Diabetes mellitus can be treated in a number of ways, like insulin treatment in type 1 diabetes. Sulfonylurea and DPP-IV enzymes[9]; Meglitinides: stimulate insulin secretion; Metformin[18]; Thiazolidinediones; á-glucosidaseandá-amylase inhibitors[11] are used for type 2 diabetes.

The demand for alternative treatment of diabetes with lessor no side effect was driven by the negative effects of oral hypoglycaemic medicines[3,24]. Many plants derived substances like glycosides, phenolic

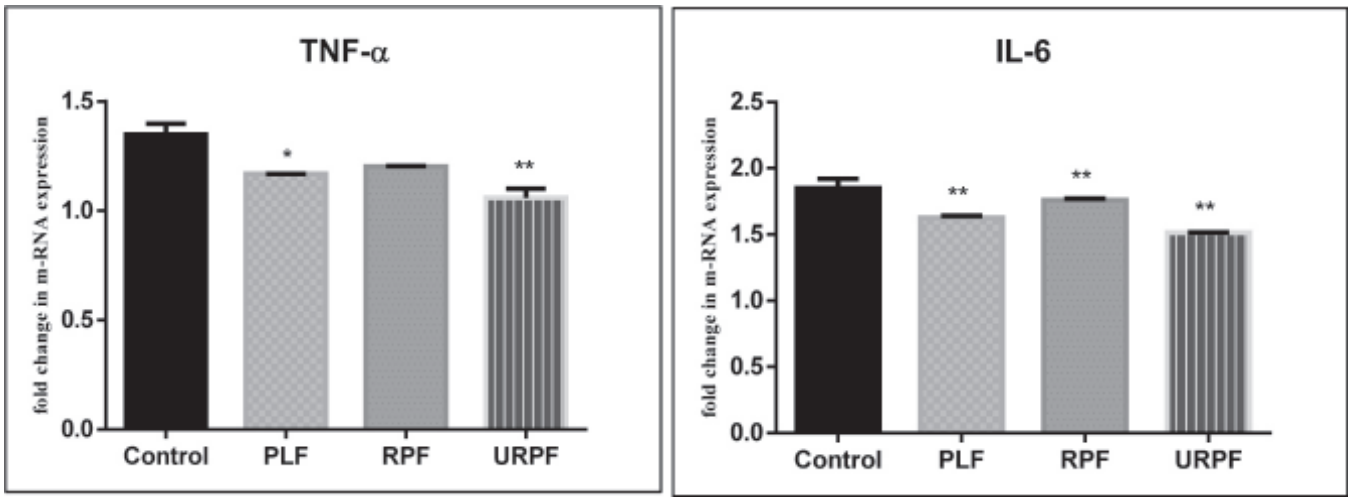

Fig. 6:Expression of TNF-α & IL-6 in U937 cells upon treatment with PLF, RPF and URPF respectively. Mean ± SEM;*Pd" 0.05;Pd" 0.01 *vs*.control. ANOVA (F values) TNF-α -13.43 & IL-6- 30.98**

compounds, alkaloids, terpenoids and flavonoids helpful in treatment of diabetes[1]. Alkaloids and saponins[22,34]; triterpenoids[10]; glycosideshave hypoglycaemic characteristics[1].

Carica papaya is well known for its ethnomedicinal properties[30] including,anti-cancerous and anti-diabetic activity[28]. We found RPF and URPF significantly enhance GLUT-4 level and activated PI-3-K/AKT signaling pathway. PI-3 kinase plays a significant role in insulin signaling pathway and regulates translocation of GLUT-4 to the cell surface. It was found that PLF, RPF and URPF increase the phosphorylation of AKT and maximum effect of URPF.

Stress, obesity and infection induced chronic inflammation *via* the secretion of pro-inflammatory cytokines lead to type 2 diabetes[17]. LPS stimulate innate immunity by regulating production of inflammatory mediators like TNF-α and IL-6[2].The results showed that the three samples, URPF was found to show higher reduction of TNF-α & IL-6 than PLF and RPF in U937 monocyte cells.

Conclusion

Our finding suggests that RPF and URPF stimulate expression of GLUT-4 in L_6 cells and highly increase URPF. PLF, RPF, and URPF also exhibit anti-inflammatory potential by lowering TNF-á and IL-6 levels. Thus, all three fermented papaya products have a beneficiary prophylactic potential that can be clinically exploited as a nutraceutical for the management of diabetes and their complications.

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