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Original Article

ANTAGONISTIC EFFECTS OF COMBINATION OF FLAXSEED OIL AND SPIRULINA PLATENSIS OIL ON THEIR BIOLOGICAL PROPERTIES

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ABSTRACT

Objective: To determine the composition of flaxseed oil and *Spirulina platensis* oil and to evaluate their biological activities either alone or in combination by *in-vitro* studies.

Methods: Proximate analysis of flaxseeds and *S. platensis* powder, followed by compositional analysis of oil by gas chromatography was conducted. Cytotoxic studies were performed by MTT and trypan blue dye exclusion assay. Hypoglycemic studies were assessed by glucose uptake assay using porcine diaphragm and by gluconeogenesis assay using rat liver slices. Anti-denaturation assay and HRBC membrane assay were conducted to evaluate the anti-inflammatory properties.

Results: α-linolenic acid (ALA) was the predominant component of flaxseed oil while γ-linolenic acid was found only in *S. platensis* oil. In all the experiments, *S. platensis* oil and flaxseed oil alone exhibited prominent anticancer, antidiabetic and anti-inflammatory activities. Whereas, all the above activities were reduced in the combination studies irrespective of the ratio of the mixture of oils used.

Conclusion: The results clearly suggested that the combination of *S. platensis* oil and flaxseed oil does not have any synergistic properties, indeed the combination of oils induced antagonistic effects on their biological properties.

Keywords: Flaxseed oil, Proximate analysis, Cytotoxic, Anti-inflammatory.

INTRODUCTION

The main physiological benefits of flaxseed oil are attributed to its high linoleic acid content that leads to their antioxidant potential [1] and hence was effective against several disorders such as diabetes, high blood pressure, cancer, inflammation and coronary artery diseases [2, 3]. Omega-3 fatty acids like α-linolenic acid (ALA), and its metabolites, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) inhibits different stages of mammary carcinogenesis [4-7]. Flaxseed oil and fish oil prevented lipid abnormalities in type-2 diabetes mellitus [8]. Pumpkin seeds are excellent sources of proteins, vitamins and oil [9-11], particularly ω-6 fatty acids, which possess a number of biological properties including antioxidant, anti-inflammatory and hypolipidemic activities [12]. Previous studies have shown that a combination of flaxseed and pumpkin seed mixture supplemented to diet was helpful in preventing diabetic complications in adult rats [13].

S. platensis, is a filamentous multicellular cyanobacterium belonging to the algae of the class *Cyanophyta* has a unique blend of nutrients. It is gaining importance as a potent anti-viral [14, 15], anticancer agent [16, 17] and in health improvement [18] as a nutraceutical and as a source of potential pharmaceutical agent.

Diabetes mellitus, a metabolic disorder, is turning as a major health problem. Although there are a number of drugs available for the treatment, prolong use of these drugs are found to cause adverse effects. Hence, various studies are in progress to find an alternative drug from the natural sources, which are effective in bringing down the complications of diabetes. Recently a large number of reports on *S. platensis* have proven beneficial effects on blood glucose levels and the lipid profile of type 2 diabetes mellitus subjects [19].

Inflammation is caused due to infection, oxidative stress and allergies during which the molecules like histamine, prostaglandins, bradykinin, and cytokines are released from the cells and tissues causing deleterious effects [20]. The flaxseed oil, *S. platensis* oil & sunflower oil have exhibited anti-inflammatory activities in different models [21-23].

However, no study has been reported about the effects of the combination of *S. platensis* oil and flaxseed oil mixture on their biological properties. Therefore, in the present investigation, the anticancer, antidiabetic and anti-inflammatory properties of flaxseed oil and *S. platensis* oil either alone or in combination were evaluated.

MATERIALS AND METHODS

Materials

The flaxseeds (*Linum usitatissinmum)* were procured from the local market and the *S. platensis* was purchased from Avantha Holdings Limited, Nanjangud, Mysore, Karnataka, India.

Authenticity was confirmed by the Department of Studies in Botany, Manasagangotri, Mysore. It was stored at 4 ° C and used for the experiments.

Sample preparation

The mixtures of oil samples were prepared by using 10% DMSO and following samples were used for the experiments: S_1-[Sunflower oil (SFO)], S_2-[Flaxseed oil (FSO)], S_3-[*S. platensis* oil (SPO)], S_4-[FSO: SPO (75: 25)], S_5-[FSO: SPO (50: 50)] and S_6-[FSO: SPO (25: 75)]

Experimental animals

The adult male albino rats (120-150 g) were approved by the Institutional Animal Ethics Committee (IAEC) of the University of Mysore, [Letter no. UOM/IAEC/18/2012]. All the animals were placed in laboratory cages in an animal house maintained at temperature 23±2 °C in 24 h standard light/dark cycles. All the animals had free access to standard food pellets and filtered water.

Chemicals and reagents

Insulin was procured from Torrent pharmaceuticals LTD under license from Novo Nordisk India, GOD-POD reagent was procured from Aspen Laboratories, India, HBSS (Hank's Balanced Salt Solution) with sodium bicarbonate was procured from Himedia,

India. Bovine serum albumin, tris buffer, linoleic acid, lipoxidase were purchased from SRL, India. Diclofenac sodium was purchased from Cipla pharmaceuticals, India. All solvents and other chemicals used in the studies were of analytical grade and purchased from SDFCL, Mumbai, India.

Proximate analysis

The moisture content was determined by drying 2±0.1 g of sample (flaxseed/Spirulina) at 105±1 ˚C for 40 h and the total ash was determined by incineration of the samples weighing 2±0.1 g at 550 ˚C for 48 h [24]. The crude fibre content was determined by drying 2±0.1 g of the sample [25]. Analysis of total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) was carried out [26]. Total lipids of flaxseed and *S. platensis* samples were determined [27, 28]. Crude protein was determined by Kjeldahl protein units and the protein was calculated as nitrogen (%) × 6.25 [29]. Carbohydrate was determined by the difference between 100 and the sum of the percentages of all the other components. All proximate determinations were done in triplicates.

The fatty acid methyl esters (FAMEs) were prepared from the extracted lipids by the esterification reaction according to the method described by Christi [30]. Gas chromatography was performed using GC-2010 (Gas Chromatography, Shimadzu, Japan) [30]. Each fatty acid component was expressed as mass of fatty acid in 100 g of oil.

Cell lines and culture

Human chronic myelogenous cell lines (CM cells) were purchased from National Centre for Cell Science, Pune, India. Cells were grown in RPM1 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg of streptomycin/ml and incubated at 37 ˚C in a humidified atmosphere with 5% CO_2.

MTT assay

The cytotoxic effects of the different oil samples (S_1-S_6) were assessed against the CM leukemic cells ($5x10^5$ cells) using 3-(4, 5 dimethyl-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) assay [31]. The test samples are dissolved in DMSO and treated with different concentrations of oil samples (20, 40 and 80 mg/ml respectively). Cells in the control wells received the same volume of medium containing DMSO. After 48 h treatment, cells were harvested and incubated with MTT (0.5 µg/ml) for 4 h at 37 ˚C in 96 well plate. The blue MTT formazan precipitate formed in the viable cells is solubilized by the addition of 70 µl DMSO. The suspension is placed in microvibrator for 5 min and absorbance was measured at 540 nm using multimode reader (Varioskan Flash Multimode, Thermo scientific, USA). The experiment was performed in triplicate and repeated at least for three times.

Trypan blue dye exclusion assay

To study the growth suppressive effects of the oil samples (S_1-S_6), $0.5X10^5$ CM leukemic cells/ml were plated in a 24 well plate (Corning, USA.) in 1 ml of complete medium [31]. The cells were treated with various concentrations of the oil samples (20, 40 and 80 mg/ml respectively). DMSO treated cells were used as control. Cells were harvested after 48 h and stained with 0.4% trypan blue and calculated using a hemocytometer for viable cells. Experiments were done in triplicates and the percentage of growth inhibition by different samples at different concentrations was plotted against time (48 h).

Antidiabetic studies

Glucose uptake assay by porcine diaphragm

Glucose uptake assay was performed using a porcine diaphragm [32] with the few modifications. Porcine diaphragm was obtained from a slaughter house and washed many times using ice cold saline to remove the blood stains. Then the diaphragm was used to investigate the inhibitory effects on the glucose uptake process by oil samples (S_1-S_6) at 20 and 40 mg/ml concentrations. Diaphragm weighing around 100-150 mg was suspended in a 24 well culture plate containing saline. 0.2% glucose was added to each well to initiate the reaction. DMSO treated plates served as control. 0.4 units of insulin were used in each well to enhance the glucose uptake by the diaphragm and the volume was made up to 2 ml with saline. Plates were incubated for 30 min at 37˚ C in an atmosphere of 100% O_2 with shaking. The quantity of glucose formed in the culture plate was assayed using the GOD-POD method.

***In-vitro* gluconeogenesis assay in isolated rat liver slices**

The antidiabetic properties of the different oil samples (S_1-S_6) were studied by the *in-vitro* gluconeogenesis assay [33]. Adult male albino rats were fasted for overnight and were killed by cervical dislocation. The liver was excised and washed in ice cold saline and stored on ice. Oil samples (S_1-S_6) were dissolved in DMSO at different concentrations (5-20 mg/ml) and were transferred to different wells in a 24 well plates containing Hank's Balanced Salt Solution (HBSS). Sodium pyruvate (10 mmol/l pyruvate) prepared in HBSS was added to the 24 well plates. Liver slices were cut as described [34] with the few modifications. The slices were weighed using a digital balance. The weights of tissue slices were between 100 and 150 mg and are added to plates containing HBSS medium and pyruvate with oil samples at different concentrations. DMSO treated plates served as control and insulin (1 mmol/l) was taken as the standard. The culture plates were incubated at ambient temperature (27 ˚C) for up to 60 min. Aliquots were taken from the plates at 0, 30 and 60 min. The quantity of glucose formed in the culture plate was assayed using the GOD-POD method as identified under.

Glucose estimation by GOD-POD method

Glucose in the culture plates was assayed by the GOD-POD assay kit protocol. Briefly, 50 µl of the incubated medium was transferred to a 96 well ELISA plate. The GOD-POD color reagent (200 µl) was added to each well. The color was developed in the dark at 37 ˚C for 30 min and then the optical density was evaluated at 505 nm and the percentage production of glucose was estimated by applying the convention:

$$\%\ \text{production of glucose} = \frac{(\text{glucose in DMSO control} - \text{glucose in sample})}{(\text{glucose in DMSO control})} \times 100$$

***In-vitro* anti-inflammatory activities**

HRBC (Human red blood cell) membrane stabilization assay

The HRBC membrane stabilization assay was carried out [35] with slight modifications. The blood was collected from a healthy donor, who was not taken NSAIDS for 2 w prior to the experiment. The collection of blood was carried out as per the ICMR guidelines Govt. of India (vide O. M. No 19015/53/1997-IH Pt). The equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) was added to the blood sample and centrifuged at 2500rpm. The obtained packed cells were washed with isosaline and 10% suspension was made by using the isosaline. Diclofenac (50µg/ml and 100µg/ml) was used as a positive control. The different concentrations of oil samples S_1-S_6 (10-40 mg/ml) are taken and to each concentration, 1 ml of phosphate buffer, 2 ml of hypo saline & 0.5 ml of packed cells were added. Then the test tubes were incubated for 20 min at 37 ˚C and centrifuged at 3000 rpm for 15 min. The supernatant containing hemoglobin was estimated at 560 nm spectrophotometrically. The percentage of hemolytic or HRBC protection was calculated by using the following formula:

$$\text{Percent inhibition} = \frac{(\text{control} - \text{test})}{(\text{control})} \times 100$$

Anti-denaturation activity

The protein anti-denaturation assay was carried out [36] with the few modifications. The reaction mixture contains 1 ml of 0.2% of bovine serum albumin in tris buffer saline (pH 6.8) and oil samples S_1-S_6 in different concentrations (10-40 mg/ml). The control contains 1 ml of 50 µl methanol and 1 ml bovine serum albumin. Diclofenac (50 µg/ml & 100 µg/ml) is used as a positive control. Then the tubes were kept in a water bath at 72 ˚C for 5 min and cooled. The tubes were centrifuged at 3000rpm for 10 min. The absorbance of the supernatant was determined by spectrophotometer at 660 nm. The percentage of denaturation was calculated by using the following formula:

$$\text{Percent inhibition} = \frac{(\text{control} - \text{test})}{(\text{control})} \times 100$$

Statistical analysis

The data obtained were analyzed using graph pad software prism 5.1 and excel software. The data were expressed as mean±standard deviation and all experiments were compared with control and performed in triplicates.

RESULT S AND DISCUSSION

The results of the proximate analysis are provided in the table 1 and 2. Chemical composition of the flaxseed and *S. platensis* such as moisture, total ash, total dietary fibre, crude fibre and total lipids is depicted in the table 1. The fatty acid profile of the flaxseed and *S. platensis* oils is presented in percentages and are detailed in the table 2.

Table 1: Chemical composition of ground flaxseed and *S. platensis* powder

S. No.	Parameters	% composition	
		Ground flaxseed	*S. platensis* powder
1	Moisture	6.85±0.0575	11.94±0.0809
2	Total ash	4.39±0.0010	6.60±0.8019
3	Total dietary fibre	14.77±0.5299	0.72±0.0349
4	Crude fibre	6.42±0.1335	0.33±0.0424
5	Total lipid	27.39±2.4534	4.51±0.3112
6	Crude protein	15.22±0.1316	53.52±0.2607
7	Total carbohydrate	31.4±3.1961	22.72±1.5204

Table 2: Fatty acid profile of flaxseed oil and *S. platensis* oil

S. No.	Fatty acids	% composition	
		Flaxseed oil	*S. platensis* oil
1	Palmitic acid	6.7	58.1
2	Palmitoleic acid	0.1	1.7
3	Stearic acid	7.6	6.9
4	Oleic acid	22.8	14.4
5	Linoleic acid	12.5	13.7
6	α-Linolenic acid	49.6	0.4
7	γ-Linolenic acid	-	5.1
8	Arachidic acid	0.5	-
9	Behenic acid	0.2	-

Table 3: Cytotoxic studies of oil samples (S_1-S_6) on CM cells showing IC_{50} values

	CM Cells (IC_{50} values in mg/ml)	
Samples	MTT assay	Trypan blue assay
S1	44.44±0.33	45.97±0.35
S2	42.83±0.29	46.46±0.32
S3	50.29±0.41	44.75±0.29
S4	130.1±0.77	115.0±0.71
S5	92.96±0.55	99.95±0.63
S6	98.24±0.59	123.5±0.68

mean±SD of triplicates. SD: Standard deviation

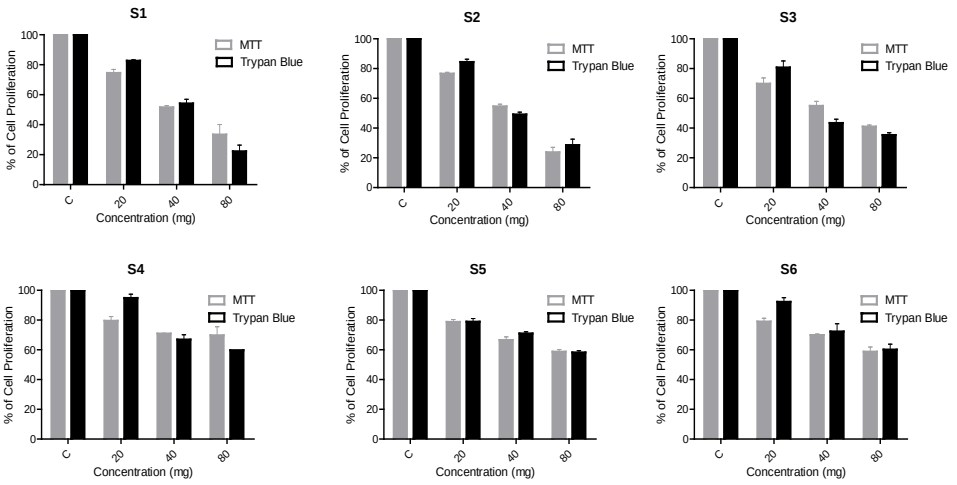

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Fig. 1: Antiproliferative effects of oil samples (S_1-S_6) at 20, 40 and 80 mg/ml concentrations against Human CM leukemic cells in MTT assay and trypan blue assay after 48 h treatment. C stands for DMSO treated vehicle control

From the results, it was observed that the percentage of dietary fibre, total lipid and total carbohydrate content was high in flaxseeds compared to *S. platensis*. In similar manner higher concentrations of total ash, moisture content and crude protein were observed in *S. platensis* sample.

The values obtained in our investigations were comparable to the previous studies [37, 38]. In the flaxseed oil the percentage of α-linoleic acid was high (49.6). In contrast, the percentage of palmitic acid was higher in *S. platensis* oil (51.1). γ-linoleic acid was exclusively present in *S. platensis* oil. Whereas, arachidic and behenic acid was found only in flaxseed oil. The high polyunsaturated fatty acids (PUFAs) content makes flaxseed oil and *S. platensis* oil as important food additives.

The data on cytotoxic studies of these oil samples (S_1-S_6) in terms of IC_{50} values are presented in the table-3. All the oil samples (S_1-S_6) were evaluated for *in-vitro* cytotoxicity against CM cancer cell lines by MTT assay. Each oil sample was tested in triplicates. The data obtained after 48 h of treatment by MTT assay showed that oil sample S_2 has maximum growth inhibitory activity and found to be more effective, followed by S_1 and S_3 respectively. The growth suppressing activities of S_4, S_5 and S_6 noticeably decreased when compared to S_1, S_2 and S_3 oil samples (fig. 1).

The trypan blue dye exclusion assay exhibited the significant reduction in cell viability after treatment with different oil samples compared to the control. The results of trypan blue assay were comparable with that of MTT assay. The data obtained after 48 h treatments revealed that oil sample S_3 has maximum growth inhibitory activity and found to be more potent and the effect decreased in S_1 and S_2 oil samples. However, in combination studies the growth suppressing activities of S_4, S_5 and S_6 samples significantly decreased when compared with oil samples alone (fig. 1).

The efficacy of the drugs for an anticancer treatment can be measured by their ability to suppress the proliferation of cancer cells. MTT is a reliable method to assess the proliferative rate [39]. Cytotoxic properties of flaxseed oil and *S. platensis* oil was revealed by MTT and trypan blue assay. The growth suppressing activity was decreased when the cell lines are treated with combinations of oils in different proportions when compared to the cytotoxic activities of oil samples alone. Thus the study indicates that the antiproliferative potency of *S. platensis* oil or flaxseed oil alone is high and decreases considerably in combination treatments.

Table 4: Glucose uptake studies of oil samples (S_1-S_6) using porcine diaphragm

Sample Description	Glucose uptake at 20 mg sample (S_1-S_6) (mg/g/30 min)		Glucose uptake at 40 mg sample (S_1-S_6) (mg/g/30 min)	
	Without insulin	With insulin	Without insulin	With insulin
S_1	27.68±0.22	29.56±0.23	28.35±0.26	29.90±0.31
S_2	24.92±0.19	29.51±0.26	25.60±0.24	29.46±0.27
S_3	24.83±0.21	29.90±0.24	26.13±0.25	30.00±0.29
S_4	22.60±0.19	26.28±0.22	23.62±0.22	26.61±0.22
S_5	22.31±0.18	27.24±0.23	22.99±0.22	27.39±0.24
S_6	24.10±0.21	26.76±0.25	24.95±0.24	27.00±0.25
Control	22.12±0.17	25.94±0.24	22.56±0.21	26.038±0.21

mean±SD of triplicates. SD: Standard deviation

The *in-vitro* hypoglycemic activities of the oil samples were measured by the glucose uptake assay using porcine diaphragm. In the presence of insulin (0.4 units), porcine diaphragm showed increased uptake from 22.12±0.17 mg to 25.94±0.24 mg of glucose/g/30 min in the control well. The glucose uptake by the diaphragm at 20 mg/ml concentrations of the S_1 oil sample was found to be 27.68±0.22 mg. In the presence of insulin, the uptake was further increased to 29.56±0.23 mg of glucose/g/30 min. Similarly, the diaphragm incubated with different oil samples (S_1-S_6) at 20 & 40 mg/ml concentrations showed an increase in the uptake of glucose, which was further increased in the presence of insulin in the medium (table 4).

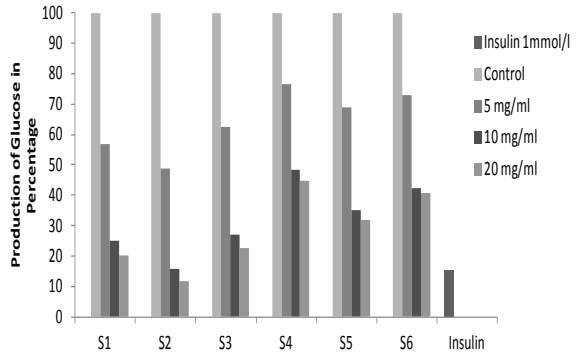

Fig. 2: The hypoglycemic effects of insulin and different oil samples (S_1-S_6) on gluconeogenesis in rat liver slices showing glucose production in percentage

The diaphragm is a striated muscle tissue and is the most important tissue for the disposal of glucose in the animal. *In-vitro* glucose uptake assay using porcine diaphragm showed increased utilization of glucose by different oil samples. The glucose uptake was notably increased in S_1, S_2 and S_3 oil treatments compared to S_4, S_5 and S_6, reflecting the decreased hypoglycemic effects in the combination studies.

Different oil samples (S_1-S_6) were screened for hypoglycemic effects by gluconeogenesis assay using rat liver slices (fig 2). 0.145 units of insulin (1 mmol/l) inhibited gluconeogenesis and showed only 15% productions of glucose with respect to the production of glucose in DMSO treated control plates. Oil sample S_1 showed 56.74, 25.09 and 20.28% of glucose production in 5, 10 and 20 mg/ml concentrations respectively. Similarly, oil sample S_2 showed 48.74, 15.70 and 11.70% of glucose production in 5, 10 and 20 mg/ml concentrations, respectively, and found to be more effective among the oil samples. Sample S_3 also exhibited an effective inhibition of gluconeogenesis and showed 62.42, 27.0 and 22.52% production of glucose at 5, 10 and 20 mg/ml concentrations respectively. Oil samples S_4, S_5 and S_6 showed 44.66, 31.93 and 40.76% production of glucose at 20 mg/ml.

Blocking the gluconeogenesis process in hepatocytes is a promising approach for an antidiabetic drug [40]. Though the oil samples (S_1, S_2 and S_3) showed prominent inhibition in gluconeogenesis, the oils in combination (S_4, S_5 and S_6) were found to be less effective in inhibiting gluconeogenesis. The result suggests the antagonistic effects of the combination studies that caused the decline in the efficacy when compared to the gluconeogenesis inhibiting ability of the individual oil treatments.

The study was to examine the synergistic effects of different oil samples (S_1-S_6) against inflammation and other associated diseases. From the experimental data obtained in HRBC membrane stabilization assay, flaxseed oil and *S. platensis* oil at 40 mg/ml concentrations exhibited an inhibition of 72.11% and 75.89%, respectively. The inhibitory effects were decreased in the

combination studies and the samples S_4, S_5 and S_6 at 40 mg/ml concentrations showed 58.37, 64.35 and 37.12% of inhibition respectively (fig 3).

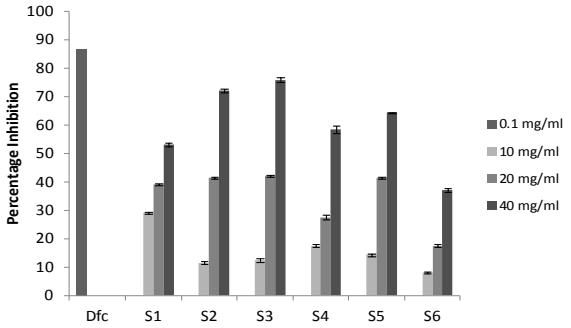

Fig. 3: The anti-inflammatory effects of different oil samples (S_1-S_6) by HRBC membrane stabilization assay showing percentage of inhibition

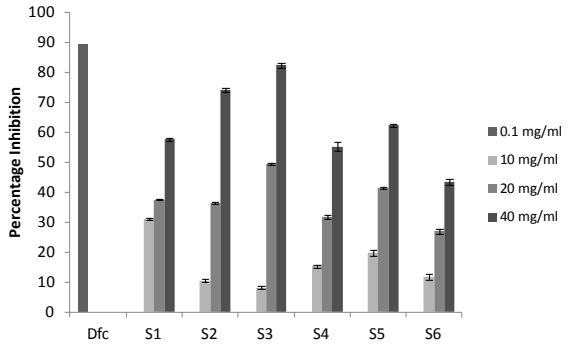

Fig. 4: The anti-inflammatory effects of different oil samples (S_1-S_6) by anti-denaturation activity showing percentage inhibition

In the protein denaturation assay, the flaxseed oil and *S. platensis* oil at 40 mg/ml concentration showed 74.11% and 82.35% of inhibitory activity, respectively. Whereas, in the samples S_4, S_5 and S_6, at 40 mg/ml concentration, the inhibitory activity decreased and exhibited an inhibition of 55.29, 62.35 and 43.52% respectively (fig. 4)

In protein anti denaturation asssay, the heat induced protein (albumin) denaturation has been studied. The heat induced protein denaturation involves delayed type III hypersensitivity and arthritis like diseases. The heat induced hemolytic study (HRBC membrane stabilization assay) has been carried out by using HRBC. The heat causes damage to the membrane and leads to the release of serum proteins and fluids into the tissues which increase the membrane permeability that leads to inflammation. The anti-inflammatory activity of flaxseed oil was attributed to the presence of alpha linolenic acid. Whereas, *S. platensis* oil contains gamma linolenic acid, which may be involved in the possible anti-inflammatory activity. The oils studied in different combinations (S_4, S_5 and S_6) does not show any synergistic effects against inflammation in both the assays. This may be due to antagonistic behavior of oils in combination that causes a decline in the biological potentiality.

CONCLUSION

Flaxseed oil and *S. platensis* oil was assessed individually and in different proportions for the *in-vitro* antiproliferative, hypoglycemic and anti-inflammatory properties. Compositional analysis of oils showed that they are rich in omega-3 and omega-6 fatty acids. The present findings suggest that oils alone have predominant biological effects and the efficiency decreases considerably in combination studies. From these observations, it can be concluded that, the blending of flaxseed oil and *S. platensis* oil will antagonize the overall biological properties and thus provide an experimental evidence that, the combination of oils does not show any synergistic effects. Hence, the combination of certain type of oils is not preferable for the dietary purposes which forms the basis of the principles of Ayurveda, the Indian system of medicine.

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CONFLICT OF INTERESTS

The authors have declared no conflict of interest

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