CHAPTER 9

EFFECT OF CARAMBOLA POMACE FORTIFIED MIX FRUIT BEVERAGE POWDER ON THE SERUM CHOLESTEROL AND GLUCOSE LEVELS AND ON THE FUNCTIONS OF TWO LIVER ENZYMES IN SPRAGUE DAWLEY RAT

9.1. Introduction

A diet rich in dietary fibre helps to decrease glycaemic index in diabetics. [1] Dietary fibre also reduces cholesterol biosynthesis, and decreases the absorption of lipid, cholesterol, and bile acid [2] as well as helps to maintain healthy bowel function. However, the effectiveness and functional properties of dietary fibre generally depends on its composition, source and preparation. [3] Chau and Huang [4] has reported that insoluble fibres derived from the fruits and vegetable by-products often possess desired physicochemical properties as well as associated bioactive compounds, and thus can be beneficial for health when incorporated in the diet. Studies on the functional properties of dietary fibre from different food sources in rats found a lowering effect on plasma lipids and glycemic response. [5, 6] Moharib and El-Batran [7] reported that dietary fibre from Egyptian grape leaves (*Vitis vinifera*) had hypoglycaemic effect in diabetic rats.

The consumption of dietary fibre rich foods from the functional food segment has witnessed a rapid rise with increase in the awareness among the consumers regarding the role it can play in controlling and preventing many degenerative diseases. This has led to the development of newer products with enhanced functionality derived from unconventional and newer dietary fibre sources. One example of such a dietary fibre source is the pomace derived from carambola fruit.

Carambola or star fruit (*Averrohoa carambola*) is a juicy tropical fruit used in the beverage industries for making juice concentrate and refreshing drinks. But these industries also contribute to the production of tons of pomace as by-product. The fruit waste is the major source of many bioactive compounds like dietary fibre, phenolic compounds, carotenoids, and essential oils (from peels), etc. The carambola pomace is reported to be rich in dietary fibre. [8] Up to 80 g/100g of dietary fibre is present in pomace of carambola with insoluble dietary fibre as the predominant fibre fraction (58.2 g/100g) as reported by Chau et al. [9] The swelling properties, water and oil holding properties of the carambola fibre are relatively higher than that of cellulose. Similarly, the cholesterol and glucose lowering effect of dietary fibre derived from carambola

pomace in hamster blood was reported by Chau et al. [10] Although number of animal studies on the health promoting properties of carambola dietary fibre have been carried out and reported, there are only a few studies on the effectiveness of the cholesterol and glucose lowering properties of the dietary fibre that has been incorporated or fortified into a complex food system e.g. breakfast cereals, or biscuits etc. No *in vivo* studies have been reported regarding the health promoting properties of the carambola pomace after fortification into a fruit beverage.

Generally, for the in *vivo* study, rat model is used as the digestive system of rat and human is almost similar as far as location and function are concerned. Both are mammals and are omnivorous in their food habit. They possess the three main part of the digestive system: salivary glands, the oral cavity, and the abdominal cavity. However, rat consists of a bile duct (ductus choledochus) formed of several tubes from the liver in place of a gall bladder. Therefore, use of rat models for in vivo study is prevalent compared to other laboratory animals. If a study requires large volume of blood, the use of Sprague Dawley rat is preferred over the other rats and mice. Moreover, Sprague Dawley rats are calmer in behavior and easy to handle.

The efficacy of the health promoting properties of carambola pomace fibre fortified spray dried fruit mix fruit beverage powder in Sprague Dawley rat model was studied. The fibre rich functional beverage powder was developed as discussed in chapter 8. In this chapter, the ability of the developed beverage powder in lowering cholesterol and glucose levels and the functions of two liver enzymes viz., serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) when given to hypercholesterolemic Sprague Dawley rats are reported and discussed.

9.2. Materials and methods

The animal experiment was carried out in collaboration with Defence Research Laboratory, Tezpur, Assam. The animals were kept and maintained during the whole experiment period as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the ethical committee approval number is DoRD-Pro/TUAEC/10-56/13/Res-06.

9.2.1. Materials

All the chemicals used were supplied by Merck, India and Himedia Laboratories and the enzyme kits for the biochemical test were from Coral Clinical Systems, Tulip group, India.

9.2.2. Test diet

The test diet consists of the carambola pomace fibre fortified fruit mix fruit beverage powder mixed with specialized carbohydrate based diet with or without fat before feeding the rat. The amount of the test diet fed to the rats had been given in Table 9.1. The proximate composition of the test beverage powder has been reported in Table 8.5 (ref chapter 8). The feed mixtures were then kneaded into small balls and the rats were fed *ad libitum*. The diet was prepared daily and served freshly for prevention of deterioration of the nutritional properties.

Table 9.1. Constituents of the diet fed to the rat with addition of the test beverage powder

Constituents	Amount
Group I	
Refined wheat flour	35g
Vegetable fat	11.66 ml
Test beverage powder*	500 mg
Salt	100 mg
Group II	
Refined wheat flour	35.00g
Test beverage powder*	500 mg
Salt	100 mg

*500 mg per kg of body weight

9.2.3. Animal experiment conditions

Six weeks old male rats were used in the present study. Animals were acclimatized for 7 days at room temperature (24°C±2°C) with 12 h light-dark cycle before the commencement of the experiments. Total nine numbers of rats with three numbers in each group were used for the study.

9.2.4. Induction of high cholesterol condition in the rat

The rats were divided into three group's viz., control (given normal rat chow without test diet), Group I. In the Group I and Group II rats, high serum cholesterol condition was induced before feeding of the beverage powder mixed along with the carbohydrate rich diet was started. The rats of Groups I and II were given wheat flour diet with cholesterol powder (10 mg/kg body weight) and were screened for increase in serum cholesterol level. When the serum cholesterol level was above the base value level (±8 mL/dL), the rats were given their designated diets for 9 weeks (Table 9.1).

9.2.5. Administration of diet with beverage powder and dosage determination

The rats were given the beverage powder with their test diet at 500 mg/kg of body weight. The dosage in rat per kg body weight can be converted to human equivalent dose (HED) taking into consideration the body surface area (m^2). This was calculated based on the method given by Center for Drug Evaluation and Research (CDER), U.S. Food and Drug Administration. [11] Briefly, to convert the dose used in a rat to a dose based on surface area for humans, the *Km* factor (6) for a rat is multiplied with mg per kg of dose in rat and then divided by the *Km* factor (37) for a 60 kg adult human (Table 9.2).[12, 13]

$$\text{HED} = \frac{\textit{Dosage in rat} \times K_m \textit{ factor for rat}}{K_m \textit{ factor for human}} \qquad \textbf{Eq. 9.1}$$

Where, Km is factor that indicates surface area to weight ratios

After calculation, it was found that at 500 mg/kg body weight as dose of the test beverage powder containing 9.29% fibre (ref chapter 8, Table 8.5) when given to the rat, the human equivalent dose for an adult weighing average 60 Kg will be 7530 mg per 60 kg when expressed in terms of total fibre consumed. Therefore, 500 mg/kg body weight of test diet in rat will be equivalent to 7530 mg fibre in a 60 kg human.

For analysis purpose, blood from rat (max.1mL) was drawn from the retro-orbital sinus of eye at an interval of 15 days. At the end of the experimental period the rats were sacrificed and the liver, heart, and caecum were collected and weighed. The caecum was analysed for short chain fatty acid (SCFA) composition by gas chromatography mass spectrophotometer (GCMS) following appropriate protocol. The aorta and heart tissues were studied for any histopathological changes. The carcass of the animals was destroyed by incineration.

Table 9.2. Conversion of animal dosage to human equivalent dose (HED) based on body surface area (BSA)

Species	Weight (kg)	BSA (m^2)	*Km* factor
Human Adult	60	1.6	37
Human Child	20	0.8	25
Rat	0.15	0.025	6
Hamster	0.08	0.02	5
Mouse	0.02	0.007	3
Baboon	12	0.6	20
Dog	10	0.5	20
Monkey	3	0.24	12
Rabbit	1.8	0.15	12
Guinea pig	0.4	0.05	8

Values based on data from FDA Draft Guidelines. [12]

9.2.6. Determination of the serum biochemistry

Blood for analyzing the serum biochemistry was collected in serum tubes devoid of anticoagulant and allowed to clot at room temperature for 45 min. The clotted blood samples were centrifuged at 3000 rpm for 10 min and then the supernatant or serum was collected. The obtained serum was analysed for the parameters viz. low-density lipoprotein (LDL), high-density lipoprotein (HDL), glucose (GLUC), triglycerides (TGL), serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) using commercially available biochemical enzyme kits (Coral clinical systems) and were measured in an autoanalyser (Coralyzer-100, Tulip Diagnostics Pvt Ltd, Goa, India). Total cholesterol was calculated using Friedewald's formula. [14]

$$TC = HDL + LDL + (TGL/5) \qquad \textbf{Eq. 9.2}$$

Where, TC is total cholesterol; HDL is high density lipoprotein; LDL is low density lipoprotein; and TGL is triglycerides

9.2.7. Changes in body weight of the two test groups

The two groups of animals were monitored on weekly basis for any changes in their body weight during the period when they were on the test diet.

9.2.8. Histopathology of the heart and aorta tissues

The heart ventricle and aorta tissues of the control and of test rats were fixed, processed and studied for histopathological changes and compared. The tissue of heart and aorta were fixed in 10% formalin. All the tissue samples were then washed in xylene and after that dehydrated in an alcohol gradient from 78-100%. The dehydrated tissue sample was then embedded in paraffin and was sectioned into 4-6 mm thickness using a tissue microtome. The paraffin was removed, washed in xylene and again the tissues were rehydrated with 100-78% ethanol gradient series and stained with hematoxylin and eosin. The final obtained sections were studied under the microscope at 40 x and compared with that of the control group.

9.2.9. Identification of short chain fatty acids (SCFA) in the ceacal matter of the two test groups by GCMS

Extraction of sample

Ceacal samples were weighed and suspended in 0.5% phosphoric acid (0.1g sample: 0.5% phosphoric acid) and frozen at −20°C immediately after collection. [15] For the experiment, the samples were thawed and the suspensions were homogenized by vortexing for about 2 min and centrifuged for 10 min at 17,949 × g. Each milliliter of water supernatant was extracted with 1 mL of ethyl acetate for 2 min and centrifuged for 10min at 17,949 ×g. Organic extracts were stored at −20°C.

GCMS conditions

The GCMS (Perkin Elmer, India) coupled with a mass spectrometer was used. The GC was fitted with a standard 5% polysiloxane stationary phase column (30 m, 0.25 mm id, 0.25 µm film thicknesses) and helium was used as the carrier gas at 1 mL/min. Injection was made in split mode (30 mL/min) with an injection volume of 1 µL and an injector temperature of 250°C.

The column temperature was initially 90°C, then increased to 150°C at 15°C/min, to 170°C at 5°C/min, and finally to 250°C at 20°C/min and kept at this temperature for 2 min. Solvent delay was 3.5 min. The detector was operated in electron impact ionization mode (electron energy 70 eV), scanning the 50–500 *m/z* range. The temperature of the ion source, quadrupole, and interface were 230, 150, and 280°C, respectively. The NIST library was followed for the identification of the SCFAs. [16]

9.2.10. Statistical analysis

All experimental results are reported as mean ± standard deviation of mean (S.E.M) using SPSS version 11.5. The data were statistically analyzed by Duncan's multiple range test at $p \leq 0.05$ significant level.

9.3. Results and discussion

9.3.1. Serum biochemistry

The serum biochemical analysis of the two test groups for 9 weeks experimental period showed significant variations. As seen in Table 9.3, all the test parameters were found to decrease from 7th week in Group I. A fluctuation in all the parameters was observed between 1st and 7th week. Similarly, in Group II (Table 9.3) also a decrease in values was observed. However, the fluctuation in levels between 1st and 7th week were not that high as seen in Group I. This could be attributed to complex animal physiology and its reaction to the given diet. Compared to Group I, at the 9th week, the effectiveness of the test diet was found to be more prominent and significant in Group II. This could be due to absence of fat in the diet. But it must be noted that even in the presence of saturated fat (Group I), the test diet was able to lower the cholesterol and glucose levels as well as the SGOT and SGPT enzyme activities.

Dietary fibre mainly, the insoluble fibre has tendency to bind cholesterol and bile acids in the intestinal lumen, which decreases the cholesterol absorption, increases its catabolism into bile acids and increases the bile excretion in the faeces. Decrease in the cholesterol level triggers the LDL receptors which results in decrease of the LDL cholesterol level in the blood.[17, 18] Another possible reason might be the reduced hepatic synthesis of cholesterol.[2, 19] Chau and Huang [20] had reported the hypocholesterolemic effect of insoluble fibre rich fraction derived from *Passiflora edulis* seed *in vivo*.

Moreover, dietary fibre has good cation exchange properties which might destabilize, entrap, and disintegrate the micelles that are formed in the intestine and thus negatively impacts their diffusion or absorption leading to a reduced lipid and cholesterol absorption. [21] The glucose lowering effect could be due to direct adsorption of the glucose molecules by the fibre in the intestine. In addition to that, fibre has a tendency to form gel around the food particles in presence of water which renders the food particles sometimes inaccessible to the digestive enzymes and slows the absorption rate of glucose as well as lipid in the blood stream. [7]

SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys, whereas, SGPT enzyme is mainly found in the liver. As liver plays a major role in the various metabolic processes often the activity levels of SGOT and SGPT are used as a marker for liver health and function. Under diseased conditions such as myocardial infarction, hepatic diseases, pancreatitis and other diseased conditions usually their activity increases. In the present study, the SGOT and SGPT activity in the rats were found to decrease with time when fed on the test diets. This suggests that, the fibre present in the test diets had hepatoprotective effect.

Therefore, the serum anaylsis results imply that the test diets fed to Group I and Group II rats had both glucose and cholesterol lowering effects and also improved the liver functions.

Table 9. 3. Serum biochemistry of Group I and Group II experimental rats

Tests	Week 1	Week 3	Week 5	Week 7	Week 9
Group I					
LDL (mg/dL)	20.20±0.12^{b}	15.00±0.19^{a}	22.50±0.23^{c}	14.10±0.13^{a}	15.25±0.19^{a}
HDL (mg/dL)	49.40±0.17^{d}	21.10±0.09^{c}	63.40±0.28^{e}	17.80±0.17^{b}	13.00±0.11^{a}
CHOL (mg/dL)*	95.24±0.22^{d}	72.24±0.11^{c}	151.90±0.31	54.82±0.09^{b}	39.17±0.21^{a}
TGL (mg/dL)	128.20±0.11^{c}	180.70±0.18^{d}	330.60±0.12^{e}	94.60±0.35^{b}	54.60±0.28^{a}
GLU (mg/dL)	81.00±0.19^{a}	114.00±0.15^{c}	188.0±0.17^{e}	146.00±0.21^{d}	104.00±0.25^{b}
SGPT /ALT (IU/L)	119.00±0.21^{e}	116.00±0.12^{d}	64.00±0.19^{c}	57.00±0.18^{a}	60.00±0.17^{b}
SGOT/ AST (IU/L)	204.00±0.23^{e}	163.00±0.21^{d}	96.00±0.21^{b}	101.00±0.14^{c}	85.00±0.33^{a}
Group II					
LDL (mg/dL)	20.2±0.18^{c}	18.70±0.25^{b}	17.6±0.19^{b}	11.90±0.11^{a}	10.9±0.07^{a}
HDL (mg/dL)	49.4±0.23^{c}	13.80±0.27^{a}	15.7±0.25^{b}	13.6±0.19^{a}	12.8±0.05^{a}
CHOL (mg/dL)*	96.28±0.17^{d}	46.38±0.29^{c}	42.28±0.23^{b}	34.1±0.21^{a}	31.90±0.11^{a}
TGL (mg/dL)	133.4±0.11^{c}	69.4±0.11^{b}	44.9±0.27^{a}	43.00±0.27^{a}	41.00±0.19^{a}
GLU (mg/dL)	90.00±0.09^{a}	101.00±0.17^{b}	177.00±0.13^{c}	93.00±0.25^{a}	89.70±0.16^{a}
SGPT /ALT (IU/L)	55.0±0.12^{a}	102.00±0.31^{c}	69.00±0.11^{b}	67.00±0.12^{b}	66.00±0.22^{b}
SGOT/ AST (IU/L)	145.00±0.22^{d}	123.00±0.22^{c}	107.00±0.09^{b}	110.00±0.19^{b}	89.00±0.27^{a}

*Total cholesterol was calculated using Friedewald's formula; TC= HDL +LDL+ (TGL/5)

** Means with the same letter between the columns are not significantly different at $p \leq 0.05$ by DMRT. Superscript of DMRT describes significant difference between the weeks

9.3.2. Changes in body weight

The changes in body weight (Table 9.4) in both the test groups showed a gradual increase up to 6th week. But after 6th week, no significant change in body weight was observed, indicating that the weight gain had stabilized after 6 weeks of feeding the diets.

9.3.3. Histopathology

The histopathology study (Fig. 9.1) showed no marked difference in appearance of the heart muscle and aorta tissue in the two test groups when compared with that of a healthy control rat. No prominent tissue scars or infarction were observed which are commonly observed under diseased conditions. This suggests that the diet was effective in maintaining health of the heart and aorta tissues both in absence or presence of saturated fat.

Table 9.4. Changes in body weight during experimental period

Week	Group I (g)	Group II (g)
1	205.86±0.26 ^{a}	195.96±0.19^{b}
2	212.24±0.23 ^{b}	187.56±0.21^{a}
3	216.06±0.29^{b}	186.21±0.17 ^{a}
4	214.22±0.17 ^{b}	208.58±0.10 ^{b}
5	229.08±0.15 ^{c}	212.78±0.19 ^{b}
6	246.54±0.19^{d}	241.41±0.23^{c}
7	246.07±0.21^{d}	243.60±0.24 ^{c}
8	244.19±0.31 ^{d}	241.09±0.17^{c}
9	244.03±0.12 ^{d}	240.15±0.31 ^{c}

**Results are mean±S.D of triplicate results

**Means with the same letter between the rows are not significantly different at $p \leq 0.05$ by DMRT. Superscript of DMRT describes significant difference in weight between the weeks in group I and II rats

9.3.4. SCFA by GCMS of the caecal matter

The study on the presence of short chain fatty acids in the caecal matter by GCMS revealed variation between the groups (Table 9.6 and Fig. 9.2). In Group I, iso-valeric acid, acetic acid and propionic acid were identified while, in Group II three fatty acids viz., valeric acid, propionic acid and acetic acid were found to be present. These short cahin fatty acids were produced as end products of the degradation of the fibre materials by the microflora residing in the colon by fermentation process. [22, 23] The

differences in fatty acid composition between the groups could be attributed to the different composition of diet and resultant difference in the type of microflora population present in the colon of the rats. The SCFA has beneficial effects on the intestinal health. [24] They help to stimulate the colonic circular muscle contractile response and hence, play a role in shortening the gastrointestinal tract transit time and decrease the rate of absorption of glucose and lipids. Branning and Nyman [25] had reported that, SFCA such as butyrate helps in maintaining mucosal integrity of the colon and microbial balance of the intestine. Similarly, acetate regulates mucin secretion [26] while, propionates have a positive effect on the lipid and glucose metabolism processes. [27]

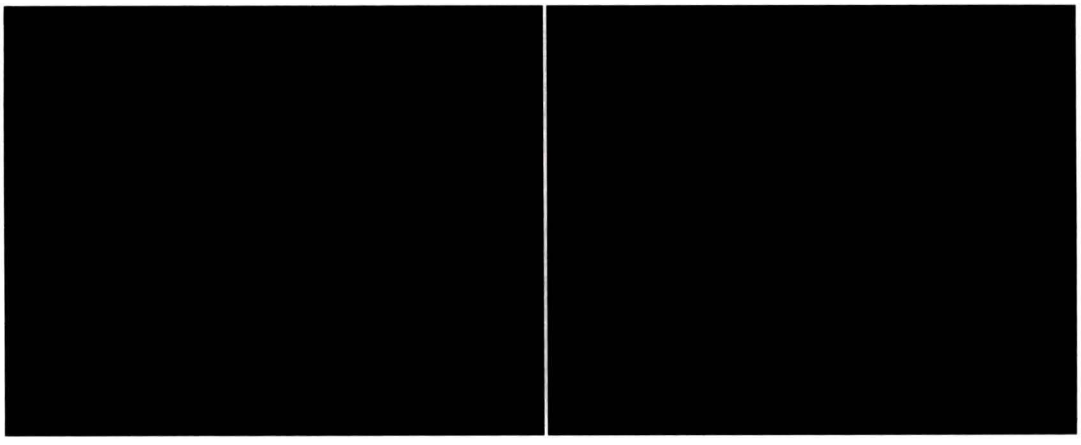
In addition to that, studies have reported that the SCFA and many other metabolites produced during microbial fermentation of the dietary fibre help in prevention of colon cancer and maintenance of overall colon health. [28] They possess anti-proliferative, anti-inflammatory and apoptotic properties. [29] Peng et al. [30] studied and reported the SCFA production in mice fed with four dietary fibers, including pectin, resistant starch (RS), fructo-oligosaccharide (FOS) and cellulose. Similarly, Frost et al. [31] opined that short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism.

Table 9.5. Short chain fatty acids identified by GCMS in caecal matter of Group I and II rats

Retention time (min)	Common name	Chemical name	Molecular weight
Group I			
5.35	Iso-valeric acid	3-methyl, 2-methyl butyl butanoic acid	172.00
15.05	Acetic acid	Chloroacetic acid, dodecyl ester	262.82
18.49	Propionic acid	Undecyl-2-enyl 2,2-dimethyl propanoic acid	254.40
Group II			
2.04	Valeric acid	Pentanoic acid, 3-methyl-,ethyl ester	144.40
2.52	Propionic acid	Propanoic acid, 2-mercapto-ethyl ester	134.19
16.80	Acetic acid	Dichloroacetic acid, 2,2-dimethylpropyl ester	198.00

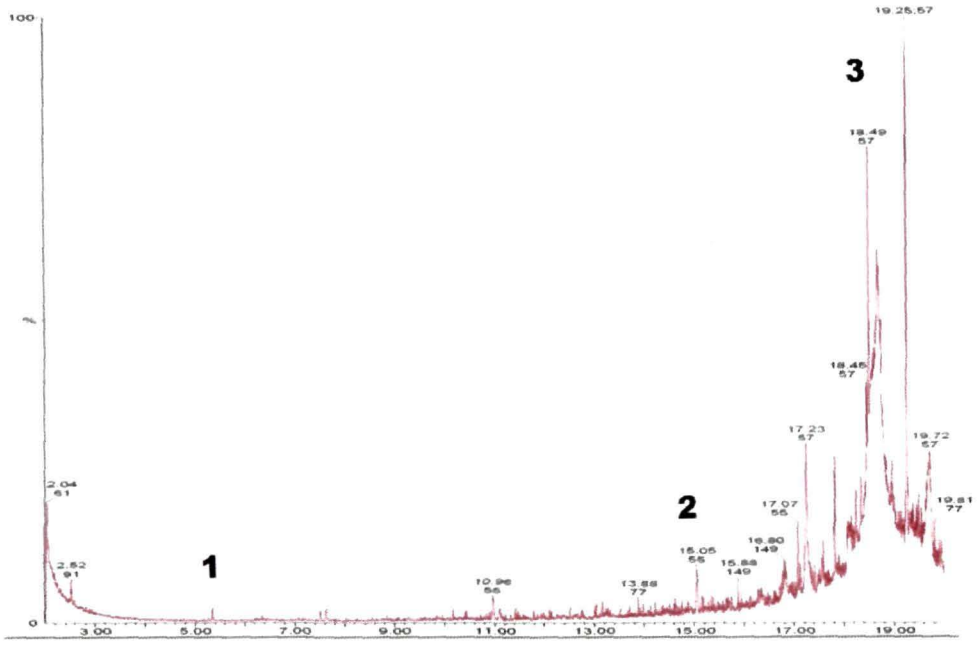
(a) Control aorta (b) Control heart

(c) GI Aorta (d) GI heart

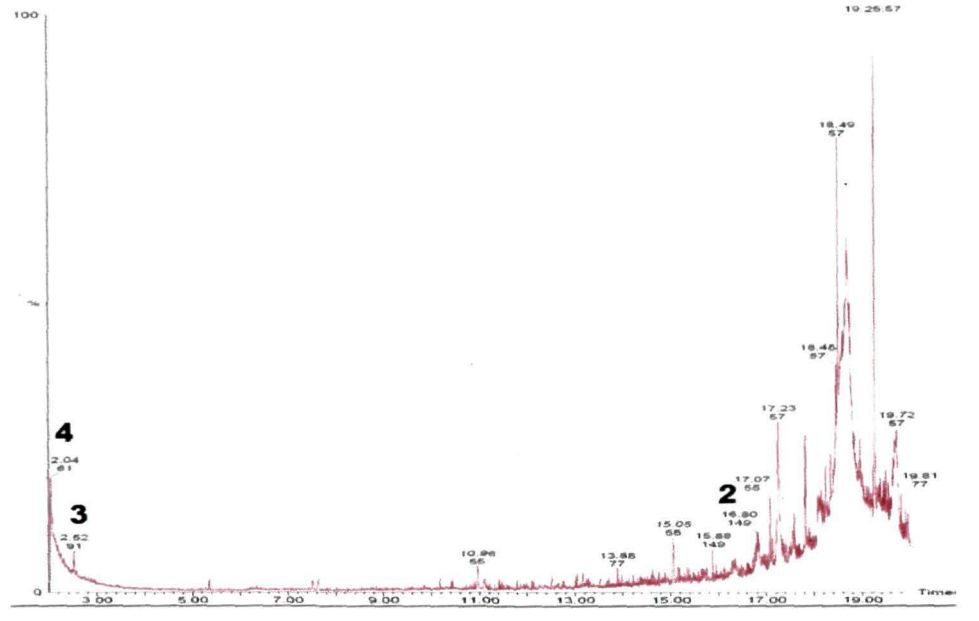


(e) GII Aorta (f) GII Heart

Fig. 9.1. Histopathological study of the aorta and heart tissues of the Sprague Dawley rat (G1 is Group I; and GII is Group II)



(a)



(b)

Fig. 9.2. GCMS spectrum of ceacal matter of (a) Group I and (b) Group II rat; 1= iso-valeric acid; 2= acetic acid; 3= propionic acid and 4= valeric acid

9.4. Conclusions

The test diet showed positive results under the given conditions in both the test groups. Decreased cholesterol, HDL, LDL, TGL, SGPT and SGOT values were obtained. Maximum decrease was obtained in Group II. Therefore, the test diet worked more effectively in absence of fat as compared to Group I where $1/3^{rd}$ of the diet contained fat. Similarly, decrease in the SGPT and SGOT in rats fed with test diet showed normal liver functions. Usually during high glucose and cholesterol conditions the liver starts to function abnormally and the activity of SGPT and SGOT increases. The results, therefore, suggests that the test diet had a positive impact on the functioning of the liver by helping in the lowering of the serum glucose and cholesterol levels. No significant increase in body weight was observed after 6^{th} week. The histopathology of the heart and aorta tissues showed no tearing or major deviation from that of the healthy control rat tissues. The analysis of the caecal matter showed presence of short chain fatty acids which are desirable for maintaining colon health. The functional beverage powder obtained may have future scope in the functional foods segment.

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