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Assessment of phytochemical constituents, fatty acids profile and *in vitro* antioxidant activity in soapnut shell powder

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ABSTRACT

Sapindus mukorossi, commonly called as soapnut, is an extremely valuable medicinal plant which possesses several biological and pharmacological properties. The present study was carried out to determine the phytochemical composition, saponin content, fatty acid profiles and *in vitro* assessment of antioxidant potential of soapnut shell powder (SSP) as a phytogenic feed additive for livestock and poultry feeding. The DM, CP, EE, CF, TA, Ca and P levels in SSP were 95.16, 5.01, 1.78, 2.21, 7.49, 0.24 and 0.41%, respectively. Total phenol (TP), non tannin phenols (NTP), total tannin (TT), condensed tannin (CT) and hydrolysable tannin (HT) levels in SSP were 1.43, 0.60, 0.83, 0.36 and 0.47%, respectively. The crude saponin extracted from SSP was 28.4% (DMB). SSP was composed of 68.8% saturated and 31.2% unsaturated fatty acids, respectively. The total antioxidant activity at the concentration 250 µg/ml of SSP was 57.16%. Thus, it could be concluded that SSP had high antioxidant activity which may be beneficial for livestock and poultry feeding.

Key words: Antioxidant activity, Fatty acids, Phytochemical, Saponins, Soapnut shell powder, Tannins

North West Himalayan Region (NWHR) is very rich in medicinal plant/herbs, which are being traded naturally as an alternate to traditional agriculture. These medicinal plants have active principles that produce a definite physiological action on both livestock and poultry. The important bioactive constituents of plants are alkaloids, tannins, flavonoids, phenolic compounds and saponins. Due to their medicinal property, these herbs are regularly being used as feed additives in both livestock and poultry feed (Kumar *et al.* 2017). *Sapindus mukorossi* is one such plant belonging to family Sapindaceae, and widespread in the Himalayan range. It is commonly called as reetha, soapnut, soapberry, washnut, dodan and doadni (Sonawane and Sonawane 2015). Due to its medicinal values (Dobhal *et al.* 2007), it is also used in Indian Ayurvedic medicine since decades. The dried fruit of reetha is most valuable part of the plant.

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With adequate agricultural practices, an adult tree can produce about 35–40 kg of fruits per year (Poudel 2011). The fruit appears in the month of July–August and ripens by November-December (Sonawane and Sonawane 2015). Its fleshy portion contains saponin of high surface activity (Yang *et al.* 2010), which is a good substitute for washing soap and is as such used in preparation of quality shampoos and detergents, etc. The dry fruit contains about 11.5% saponin, 10% carbohydrate and the seeds contain 45.4% oil and 31% protein (Kamra *et al.* 2006). Heng *et al.* (2015) reported that yield of total sapindus saponins (TSS) from the pericarp was 33.41%.

Saponins are important constituents of nutraceuticals and functional foods (Guclu-Ustundag and Mazza 2007) and are credited with a number of bioactivities like anti-inflammatory, antimicrobial, immunostimulant, hypocholesterolaemic, anticarcinogenic, antioxidant and hemolytic activities (Francis *et al.* 2002, Chwalek *et al.* 2006, Guclu-Ustundag and Mazza 2007). Saponins exhibits potent hemolytic activity (Gauthier *et al.* 2009) and this property is being used for their detection in thin layer chromatograms (Muetzel *et al.* 2003). Several researchers have tried saponin containing plants and/or its extract in livestock and poultry feeding but the results were very variable with sources of saponin, dose level, species of animal and age of animals (Patra and Saxena 2010). Research related to soapnut shell powder supplementation in livestock and poultry diet is very limited. Thus, the present study was conducted to explore

the possibility of using SSP as phytogenic feed additive for livestock and poultry feeding by investigating phytochemical composition, saponin content, fatty acid profiles and *in vitro* assessment of antioxidant potential of soapnut shell powder (SSP).

MATERIALS AND METHODS

Procurement of soapnut fruits and preparation of SSP: Soapnut fruits were purchased from local market of Palampur, District-Kangra, Himachal Pradesh, India. The fruits were dried at room temperature followed by manual separation of seed. Collected soapnut shells were dried at room temperature and grinded in the mixer to a uniform particle size and stored in an air tight container for further analysis purpose.

Analysis of SSP: The representative sample of test material (SSP) used in the study was analyzed for the following parameters.

Proximate analysis: The representative samples of SSP were analyzed for proximate (AOAC 2000) and mineral composition mainly calcium (Ca) (Talapatra *et al.* 1940) and phosphorus (P) (Spectrophotometeric method).

Estimation of phenolics: Total phenols (TP) and non-tannin phenols (NTP) were estimated by Folin-Ciocalteau method in combination with polyvinyl polypyrrolidone (PVPP), with tannic acid as a reference standard (Makkar 2003). The condensed tannin (CT) was estimated using butanol-HCl method (Porter *et al.* 1986).

Extraction of tannins: About 200 mg of dried (finely ground) SSP was taken in a conical flask of approximately 250 ml capacity. The pigments and fats present in the sample were removed by adding 20 ml of diethyl ether containing 1% acetic acid. The mixture was then shaken well and left undisturbed for 20 min. The diethyl ether was decanted following addition of 10 ml 70% aqueous acetone to the residue. Finally the flask was shaken for 2 h in the orbital shaker (30ºC, 130 rpm). The contents was then filtered and stored at refrigerated temperature (4ºC) for further analysis.

Total phenol estimation (*TP*)*:* The aliquots of the extract were taken and their volumes were made to 1 ml with distilled water. 0.5 ml of Folin-Ciocalteu reagent and 2.5 ml of Na_2CO_3 was added to the extract. The contents were mixed properly and kept at room temperature for 40 min. After 40 min, the absorbance was noted at 725 nm spectrophotometrically. The absorbance values were compared with the standard curve which was drawn with the help of standard tannic acid treated in similar fashion.

Non tannin phenols estimation (*NTP*)*:* About 100 mg of polyvinyl polypyrrolidone (PVPP) was weighed and transferred to the clean test tube containing 1 ml of each tannin extract and distilled water. After that the tubes were kept at 4°C for 15 min. and then vortexed for few seconds so that the contents get mixed well and centrifuged at 1500 rpm. This supernatant contained only phenols other than tannin was used as the sample to estimate NTP, by the similar procedure followed to estimate the total phenolics.

Condensed tannin estimation (*CT*)*:* About 0.50 ml of the tannin extract was pipetted in a clean test tube containing 3.0 ml of the butanol-HCl reagent and 0.1 ml of the ferric reagent. The tube was vortexed for few seconds and kept in a water bath at 97 to 100°C for 60 min after covering them with a glass marble. The tubes were cooled to room temperature and absorbance was recorded at 550 nm. Condensed tannins (%DMB) as leucocyanidin equivalent were calculated by the formula:

$$\text{Condensed tannins (\% DMB)} = \frac{(\text{A550 nm} \times 78.26 \times \text{Dilution factor})}{(\text{\% DM})}$$

Total tannin (*TT*) = Total phenol (TP) – Non tannin phenol (NTP)

Hydrolysable tannin (*HT*) = Total tannin (TT) – Condensed tannin (CT)

Estimation of total antioxidant activity (*TAOA*)

Extraction: Dried ground sample of SSP (1 g) was taken in 250 ml of conical flasks in triplicates and added with 50 ml of methanol in each conical flask. The flasks were then shaken for 3 h on an orbital shaker (30ºC, 130 rpm). After that, the contents were centrifuged at 5000 rpm for 20 min. The supernatant were collected in test tubes and stored at refrigeration temperature (4°C).

In vitro *antioxidant assay:* Antioxidant activity of sample was estimated by DPPH anti-oxidant method described by Sharma and Bhat (2009).

Stock solutions of DPPH was prepared in methanol as above and methanol buffered with acetic acid buffer (0.1 M, pH 5.5), respectively. Buffered methanol was prepared by mixing 40 ml of 0.1 M acetate buffer (pH 5.5) with 60 ml methanol. The reaction tubes, in triplicates, were wrapped in aluminum foil and kept at 30ºC for 30 min in dark. All measurements were done under dim light at 517 nm using spectrophotometer and calculation was done by % inhibition or % oxygen scavenging capacity of DPPH radical by using the following formula.

$$\% \text{ inhibition} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Extraction of saponin: Saponin was isolated by modified protocol of Sharma *et al.* (2012). SSP sample was dried at 70°C and 100 g of dried sample was taken in duplicate and defatted with 1L n-hexane using Soxhlet apparatus for 5 h. 50 g of defatted sample was extracted thrice with 100 ml of methanol:water (1:1). The extracts were pooled and the solvent was removed *in-vacuo.* The remaining aqueous portion was partitioned three times using equal volume of n-butanol. Then the butanol layers were pooled and the solvent was removed *in-vacuo*. The residue left was expressed on % DMB. This residue was used as saponins enriched extract for further tests of saponin.

Tests for the presence saponin in SSP: Crude saponin (20–35% sapogenin) (Sigma-Aldrich, New Delhi, India) was used as a standard against test material, i.e. SSP saponin.

Foam test: About 12.5 mg standard and test material were taken in 250 ml measuring cylinders in quadruplets,

separately. Distilled water (87.50 ml) was added in all the measuring cylinders. After that, measuring cylinders were shaken vigorously for about 30 times by closing the mouth of cylinder with stopper. After shaking, stopper was removed and mouth of the cylinder was covered with aluminum foil. Observations were recorded at different intervals of time.

Test for hemolytic activity of saponin: A stock solution of saponin containing 5 mg/ml was prepared in methyl alcohol solvent, for both tests material and standard. Then aliquots of different concentration of 25 to 375 µl from stock solution were taken in duplicates in hemolytic glass test tubes. After that, these tubes were kept in incubation at 37°C for overnight to evaporate the solvent. Hemolytic activity of soapnut saponin was evaluated on sheep red blood cells (erythrocytes). RBC suspension (3%) was prepared following dilution with phosphate buffer saline (pH 7.4). Fresh 3% RBC suspension (200 µl) was added to each tube. Tubes were mixed gently and left undisturbed. Observations were recorded at two different intervals of time, i.e. after one hour and after overnight incubation. Interpretation of results was made on the basis of change in the intensity of colour in test tubes.

TLC analysis of saponin: TLC analysis of saponin was performed as per protocol described by Sharma *et al.* (2012). It was carried out on both aluminium and glass plates coated with silica gel 60 (Merck KGaA Darmstadt, Germany). A 10 µl, 20 µl and 25 µl aliquots of the solution of the standard saponins and the extracts of soapnut shell was applied and the plates were developed in the solvent system n-butanol: water: acetic acid (84: 14: 7) to a distance of 15 cm from the origin (Kerem *et al.* 2005). The developed plates were air dried (Stahl 1969) and results were studied.

Detection of chromatograms

By immersing in suspension of sheep erythrocytes: Fresh 3% (v/v) sheep RBC suspension in PBS (pH 7.0) was prepared. The RBC suspension was taken in a glass tray followed by immersion of the developed TLC plate for 20 sec in it. The plate was taken out of the suspension and held vertically for 30 sec. Clear white spots against a pink background were appeared. Again the plate was immersed in PBS for 30 sec to remove excess blood on the plate surface and held vertically for 30 min for complete drying. A good contrast on TLC plate between the background and the spots developed during this period indicating presence of saponin. The detection limit was determined by applying a series of concentrations of each of the samples.

By spraying with ethanol: sulphuric acid (*90:10*)*:* TLC plate after development and drying was sprayed with freshly prepared solution of ethanol: sulphuric acid (90:10). After that they were heated at 110°C for 10 min in hot air oven (Kerem *et al.* 2005).

Fatty acid profile of SSP: Fatty acid profiling in SSP was done by direct FAME synthesis method described by O'fallon *et al.* (2007).

Direct FAME synthesis: Test material was solvent extracted with petroleum ether in order to extract all the fatty acids present in it. The ether extract so obtained was transferred into a 15 ml airtight centrifuge tube to which 1.0 ml of the C13: 0 (Tridecanoic acid) internal standard (0.5 mg of C13: 0/ml of methanol), 0.7 ml of 10N KOH in water and 5.3 ml of methanol was added and vortexed for 30–50 sec. The tubes were incubated in a water bath at 55°C for 1.5 h with vigorous hand-shaking for 5 sec at every 20 min so that the reagents can properly permeate, dissolve and hydrolyze the fat. After incubation, the samples were removed from water bath and cooled to room temperature under cold tap water and 0.58 ml of 24N H_2SO_4 was added in all test tubes. The tubes were mixed by vortexing with precipitated K_2SO_4 and were incubated again in a water bath at 55°C for 1.5 h with vigorous hand-shaking for 5 sec at every 20 min. After FAME synthesis, the tubes were cooled under cold tap water. n-hexane (3 ml) was added and the tube was vortexed for 5 min. on a vortex machine. The tubes were centrifuged at 3000 rpm for 5 min in a centrifuge and the n-hexane layer, containing the FAME, was placed into a GC vial. The vial was capped and placed at –20°C until further GC analysis.

RESULTS AND DISCUSSION

Chemical composition of SSP: The proximate composition, phytochemical composition and antioxidant activity of SSP are summarized in Table 1 and 2. The DM, CP, EE, CF, TA, Ca and P level in SSP was 95.16, 5.01, 1.78, 2.21, 7.49, 0.24 and 0.41%, respectively. The TP, NTP, TT, CT and HT level in SSP was 1.43, 0.60, 0.83, 0.36 and 0.47%, respectively. The crude saponin extracted from SSP was 28.4% (DMB). The total antioxidant activity at the concentration 250 µg/ml of SSP was 57.16%. Tanaka *et al.* (1996) and Kamra *et al.* (2006) reported that soapnut contained about 10.1 and 11.5% crude saponin (% DMB), respectively. A yield of 16.3% crude saponin (% DMB) with the ethanolic extract of fruit pulps of *S. mukorossi* was reported by Du *et al.* (2014). Heng *et al.* (2015) reported that yield of total sapindus saponin (TSS) from the pericarp was 33.41%. Both *Yucca schidigera* and Quillaja bark

Table 1. Proximate composition in SSP (%DMB)

Ingredient	DM	CP	EE	CF	TA	Ca	P
SSP	95.16	5.01	1.78	2.21	7.49	0.24	0.41

DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fibre; TA, total ash; Ca, calcium; P, phosphorus.

Table 2. Phytochemical composition and antioxidant activity of SSP (% DMB)

Particular	TP	NTP	TT	CT	HT	Crude saponin	% inhibition of DPPH radicals
SSP	1.43	0.60	0.83	0.36	0.47	28.4	57.16

TP, total phenols; NTP, non-tannin phenols; TT, total tannins; CT, condensed tannins; HT, hydrolysable tannins.

saponins are commercially available products that have been used as feed additives, reported to have only 10% (Oleszek *et al.* 2001) and 9–10% (San Martin and Briones 1999) crude saponin, respectively on DMB. The average crude saponin (%DMB) in Camellia seed and Chlorophytum root was reported to be 15.35–21.28% (Kumar 2017, Gaurav 2015) and 7.07% (Gaurav 2015), respectively. These findings revealed that the crude saponin yield was quite higher in *Sapindus mukorossi* compared to commercially available other plant sources.

Tests for presence of saponin: The foam test, hemolytic activity test and thin layer chromatography were the qualitative tests performed to assess the presence of saponin in SSP extract.

Foam test: The amount of foam formed is presented in Table 3. The foaming capacity of SSP was higher than standard crude saponin, in all the three situations. It has been reported that saponins have strong surface-active property and can form stable foam in aqueous solution (Vincken *et al.* 2007, Kaur and Handa 2015). Due to this property, they are being used in shampoos, liquid detergents, toothpastes and beverages as emulsifier and long-lasting foaming agents (Tanaka *et al.* 1996). Excessive feeding of plants containing saponin to the ruminants lowers the surface tension of ruminal contents leading to a condition known as bloat/tympany. In this condition, huge accumulation of gas in the digesta and distension of the rumen occurs which impedes blood flow and eventually develops anorexia and respiratory failure. Hence, saponin rich forages should be fed judiciously to livestock.

Table 3. Amount of foam formed (ml) at different time interval

Particular	Amount of foam formed (ml)		
	Immediately after shaking	After 30 min	After overnight
Standard crude saponin	65.00	57.00	41.00
SSP saponin	79.00	67.67	47.00
% variation in foam production in SSP saponin from standard	21.54	18.72	14.63

Table 4. Fatty acid profile of soapnut shell powder

Fatty acid	Carbon number	Amount (%)
Butyric acid	C 4: 0	2.5
Caproic acid	C 6: 0	2.9
Caprylic acid	C 8: 0	0.7
Capric acid	C 10: 0	0.6
Undecanic acid	C 11: 0	0.5
Lauric acid	C 12: 0	0.1
Tridecanic acid	C 13: 0	16.7
Myristic acid	C 14: 0	10.0
Myristoleic acid	C 14: 1	0.1
Pentadecanoic acid	C 15: 0	0.4
Palmitic acid	C 16: 0	21.7
Palmitoleic acid	C 16: 1	0.3
Heptadecanoic acid	C 17: 0	0.3
Heptadecenoic acid	C 17: 1	1.8
Stearic acid	C 18: 0	11.6
Elaidic acid	C 18: 1 (n9t)	0.7
Oleic acid	C 18: 1 (n9c)	15.9
Linoleic acid	C 18: 2 (n6c)	3.9
γ-Linolenic acid	C 18: 3 (n6)	2.1
Linolenic acid	C 18: 3 (n3)	3.3
Nervonic acid	C 24: 1	0.9
Eicosatrienoic acid	C 20: 3 (n3)	1.5
Arachidonic acid	C 20: 4 (n6)	0.3
Docosadienoic acid	C 22: 2	0.3
Lignoceric acid	C 24: 0	0.8
Docosahexaenoic acid	C 22: 6 (n3)	0.1

Nature of omega fatty acids were mentioned in the parenthesis. c, cis form; t, trans form.

Haemolytic activity: Saponins have long been known to have a lytic action on erythrocyte membranes and this property has been used for their detection (Francis *et al.* 2002). A complete haemolysis for SSP saponin was observed at 150 µg/µl concentration while a complete haemolysis for standard saponin was seen in 25 µg/µl concentration after 1 h of addition of 3% sheep RBC suspension. Complete haemolysis in all the tubes was observed for both the samples after overnight incubation. It was in agreement with the finding of Kaur and Handa (2015), who reported that the lowest concentration showing complete haemolysis by alcoholic extract of reetha powder was 150 µg/µl. The haemolytic action of saponins may be due to the affinity of the aglycone moiety for membrane sterols, particularly cholesterol (Glauert *et al.* 1962), with which they form insoluble complexes (Bangham and Horne 1962).

TLC analysis of saponin: The appearance of saponins of standard and SSP extract in thin layer chromatogram is presented in Figs 1 and 2. Saponins gave white spots against a pink background. Due to its haemolytic property, detection

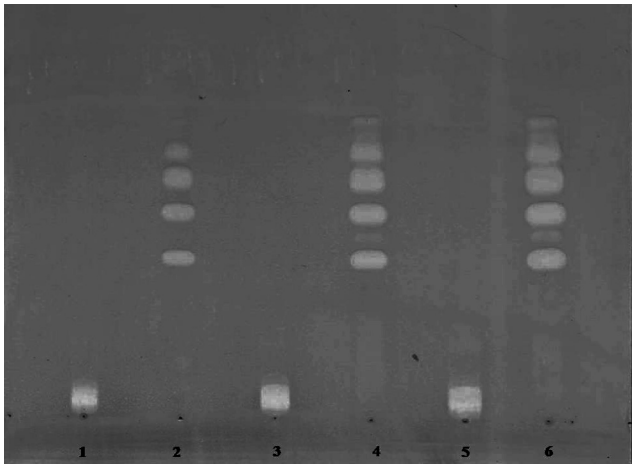

Fig. 1. TLC of Quillaja bark saponin (standard) and SSP saponin on aluminium plate coated with silica gel. Solvent system: n-butanol: water: acetic acid (84: 14: 7). Lanes 1, 3, 5: 10, 20 and 25 µl concentration of standard Quillaja bark saponin, respectively. Lanes 2, 4, 6: 10, 20 and 25 µl concentration of soapnut shell powder saponin, respectively. Detection: With haemolysis on the plate by immersion in sheep erythrocytes suspension followed by washing with PBS.

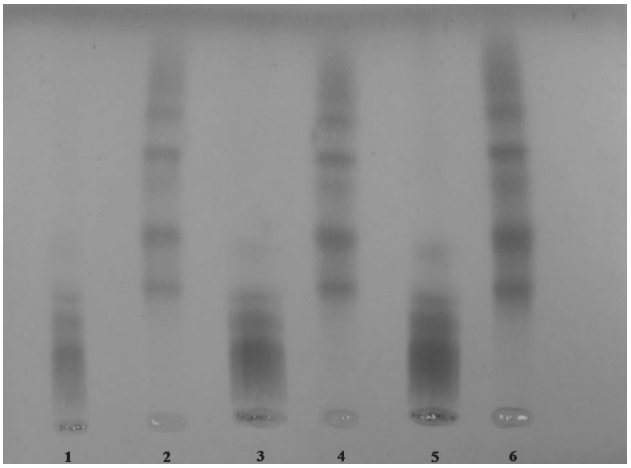

Fig. 2. TLC of Quillaja bark saponin (standard) and SSP saponin on glass plate coated with silica gel. Solvent system: n-butanol: water: acetic acid (84: 14: 7); Lanes 1, 3, 5, 10, 20 and 25 µl concentration of standard Quillaja bark saponin; lanes 2, 4, 6, 10, 20 and 25 µl concentration of soapnut shell powder saponin. Detection by spraying with ethanol: sulphuric acid (90: 10) followed by heating at 110°C for 10 min.

by haemolysis on the plate by immersion in erythrocytes suspension is more specific compared to spraying with ethanol-sulphuric acid. Furthermore, the detection by haemolysis of erythrocytes is not only specific but also the spots were more prominent and sharp (Fig. 1). The standard saponin sample was showing only one white spot that anticipate the presence of only one types of saponin moiety whereas, several white spots were shown by the soapnut sample anticipate the presence of variety of saponin moiety. The chromatogram was similar to findings of Sharma *et al.* (2012). They also reported a single white spot against pink background for standard Quillaja bark saponin and multiple white spot against pink background for soapnut saponin. These results confirmed finding of Azhar *et al.* (1994) who reported for the presence of sesquiterpenoidal glycosides with many different fatty ester of tetracyclic triterpenoids in soapnut saponin.

FAME analysis: Gas chromatogram of direct fatty acid methyl ester (FAME) synthesis from soapnut shell powder depicting fatty acid profile is presented in Table 4 and Fig. 3. Palmitic acid (C16: 0) was the major (21.7%) fatty acid in SSP. Other major FAs present in SSP were tridecanic acid (16.7%), oleic acid (15.9%), stearic acid (11.6%) and myristic acid (10.0%). SSP composed of 68.8% saturated and 31.2% unsaturated fatty acids. Research related to fatty acid profile of soapnut shell powder is scanty; however, results were available on fatty acids profile of soapnut seed oils. Interestingly, studies on the soapnut seed oils from various workers (Deka *et al.* 2014, Chhetri *et al.* 2008 and Zhang *et al.* 2017) revealed contrasting findings. Deka *et al.* (2014) reported that FAME from *S. mukorossi* seed oil consists of 4.78% of methyl palmitate (C16: 0), 58.89% of methyl oleate (C18: 1), 1.78% of methyl stearate (C18: 0), 25.93% of methyl 11-trans-eicosenoate (C18: 2) and 8.61% of methyl arachidate (C20: 0). Chhetri *et al.* (2008) reported that soapnut seed oil majorly consists of unsaturated fatty acids (85%). The fatty acid composition of soapnut seed oils was 52.63% oleic acid (18: 1), 23.84% eicosenic acid (20: 1), 7% arachidic acid (20: 1), 4.73% linoleic acid (18: 2) and 4.67% palmitic acid (16: 0). Zhang *et al.* (2017) reported that *S. mukurossi* seed oil contains 62.8% oleic acid, 22.4% eicosenoic acid, 4.6% linoleic acid and 4% palmitic acid. The SSP consists of substantial amount of both medium and long chain fatty acids and their degree of saturation will be more desirable for feeding to fattening animals. In conclusion, SSP contained desirable fatty acids profile with appreciable amount of saponin which exhibited higher *in vitro* free radical scavenging activity.

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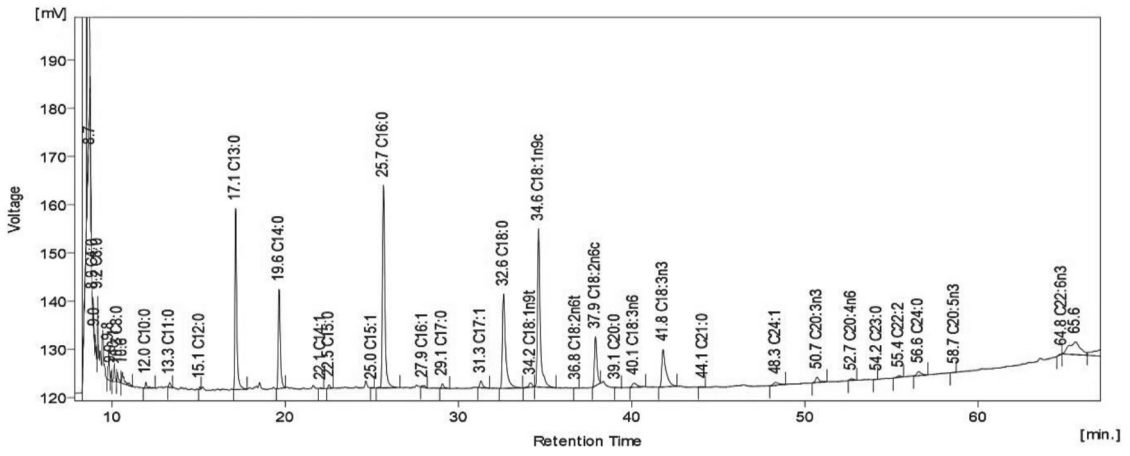

Fig. 3. Gas chromatogram of direct fatty acid methyl ester (FAME) synthesis from soapnut shell powder. Column: SP^{TM}–2560, 100 m × 0.25mm × 0.2 µm thickness; Detector: FID (DANI Master GC, Italy).

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