

Direct preparation of fatty acid methyl esters and determination of *in vitro* antioxidant potential of lipid from fresh *Sabal causarium* Seed

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ARTICLE INFO

Article history:

Received on: 18/08/2014

Revised on: 03/10/2014

Accepted on: 27/12/2014

Available online: 28/03/2015

Key words:

Sabal causarium, direct trans-esterification, FAMES, antioxidant, essential fatty acid, Total ion chromatogram.

ABSTRACT

Classical method of sample preparation for fatty acid analysis is a complicated, rigorous, multiple-step process that is often time-consuming. However, innovation has made it possible to obtain fatty acids esters in a single step without compromise of the quality and quantity of the products obtained. Fatty acid methyl esters (FAMES) were obtained from *Sabal causarium* seed using direct trans-esterification method. The reaction was performed in a one necked glass reactor equipped with a reflux condenser. The composition of the FAMES obtained was analyzed using GC-FID/GC-MS. The FAMES was also evaluated for its antioxidant potential using DPPH assay while α -tocopherol served as a standard. From the Total ion chromatogram, a total of twelve fatty acids were identified with the dominant being linoleic acid (29.83%), an essential polyunsaturated omega-6 fatty acid. Other compounds obtained in significant yield include palmitic acid (20.75 %), dodecanoic acid (12.15 %), oleic acid, an omega-9 monounsaturated fatty acid (11.51%), 11-Octadecenoic acid (5.89 %), Ceric acid (4.97) and lignoceric acid (4.56 %). The oil had higher antioxidant potential (with IC_{50} value 0.19 ± 0.31 mg/mL) than the standard, alpha-tocopherol (with IC_{50} of 0.25 ± 0.4 (mg/mL)). The direct trans-esterification method enables the quick determination of the fatty acid profile of *Sabal causarium* seed. It is a viable method that saves time, limit use of solvents and reduce the possibility of contaminations that is associated with classical multistage procedure. The chemical composition and high antioxidant value of the oil is a strong indication of the future economical and biological relevance of the *Sabal causarium* seed oil.

INTRODUCTION

The relevance of fatty acids (FAs) in human diet and health cannot be overemphasized. The human brain is estimated to be nearly 60 percent fat (Chang *et al.*, 2009). Moreso, essential fatty acids (EFAs) usually of plant origin plays vital roles in the maintenance of optimal health and brain functions (Singh, 2005). FAs and EFAs have been demonstrated to regulate lymphocyte proliferation and metabolism (Otton *et al.*, 1998), enhance the immune system (Yaqoob *et al.*, 1998; Granato *et al.*, 2000), induce increased granulocyte macrophage-colony (Bahramian *et al.*, 2004), induced cell death in T cells, macrophage cells and also enhance autoimmunity (Zhang *et al.*, 2004; Martinsdelima *et al.*, 2006). FAs has been reported to possess antioxidant activity (Atolani *et al.*, 2012), anti-inflammatory activities (Calder, 2005) and anticancer activity (Robinson *et al.*, 2002). *Sabal causarium*, also known as Puerto Rican hat palm belongs to the family of

flowering plant called Arecaceae. It is of the subfamily Coryphoideae and the tribe Sabaleae (Roncal *et al.*, 2008). The plant is also known as *palma de escoba* in Puerto Rico (Proctor, 2005). In Puerto Rico, *Sabal causarium* grows in the subtropical moist forest zone (Ewel and Whitmore 1973). Common associates are *Cedrela odorata* L., *Delonix regia* (Bojer) Raf., *Erythrina poeppigiana* (walp.) and *Tabebuia heterophylla* (DC). *Sabal causarium* flowers in the months of April through August. The species is however not endangered (Zona, 1990). Four glycosides were initially isolated from the leaves of *Sabal causarium* (Idaka, *et al.*, 1988). Recently, a bioactive compound, A [25-ethyl,23-methyl-19-nor-24-methylene-3,4-seco-4(28)-lanosten-10, 3-olide (the first 19-nor lanostane derivatives of the 3,4-seco type with a spiro element) has also been isolated from the leave (El-Dib *et al.*, 2004). To the best of our knowledge, there is no record of the fatty acid composition of the underutilized tropical seed (of *Sabal causarium*) till date. The immense relevance of FAs in human health prompts the investigation of the antioxidant and fatty acid composition of *Sabal causarium* seed.

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EXPERIMENTAL

Plant Material

Sabal causiarum seeds were collected in a fruiting season from trees within the neighborhood of Redeemer's University, Ogun State, Nigeria. The plant was taxonomically identified by a botanist at the Herbarium of the Botany Department of the University of Lagos, Lagos, Nigeria where a voucher specimen, LUH 6063 was deposited.

Solvents and Reagents

Solvents and chemicals used which includes hexane, benzene, methanol, aluminium chloride, sulphuric acid, sodium sulphate, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and α -tocopherol were of analytical grade, and where necessary, solvents were redistilled before use.

One-step Extraction and Trans-methylation of Lipid from Plant Matrix

15 g of fresh flower sample was refluxed with 100 mL of a mixture containing Methanol: Benzene: Hexane: Aluminium chloride: Sulphuric acid in a ratio (37:20:36:5:2) for 30 minutes in a one neck-flask. The resulting mixture was allowed to cool and then filtered.

The supernatant was separated in a separating funnel and the organic layer washed with distilled water to neutrality. The organic layer was concentrated to obtain the transmethylated product: fatty acid methyl esters (FAMES) and the yield noted. The method which afforded the extraction, hydrolysis and methylation of the lipid in a single procedure is a slight modification of the method previously described (Garces and Mancha, 1993).

DPPH Free Radical Scavenging Activities

The free radical scavenging activity of the extracted fatty acid methyl esters from flower was examined *in vitro* using the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay. The spectrophotometric assay was carried out according to the method previously described (Adeosun *et al.* 2013; Atolani *et al.*, 2012). The DPPH free radical was freshly prepared at a 0.1 mM concentration in methanol and protected from light. Stock solutions of the FAMES (1mg/mL) was prepared and diluted to final concentrations of 1000, 500, 300, 200, 100, 50 and 5 μ g/mL in methanol.

1 mL of 0.1 mM DPPH methanol solution was added to solutions of the sample as well as the standard (β -tocopherol) and incubated for 30 minutes in the dark. The absorbance was determined at 517 nm. Blank experiment was also carried out to determine the absorbance of DPPH before interacting with the sample. The percentage antioxidant activity (%AA) was obtained as percent DPPH radical scavenging, which was calculated using the equation:

$$\% \text{ AA} = 100 \times [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})] / (\text{Abs}_{\text{control}})$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of the control and $\text{Abs}_{\text{sample}}$ is the absorbance of the sample at 517 nm.

The IC_{50} was determined on GraphPad Prism 3 software (San Diego, USA) through a non-linear regression analysis using the dose-response inhibition curves. IC_{50} was defined as the concentration of the sample that scavenged 50% of the DPPH free radicals. Results are presented as mean \pm standard error of the mean.

Determination of the FAMES Composition

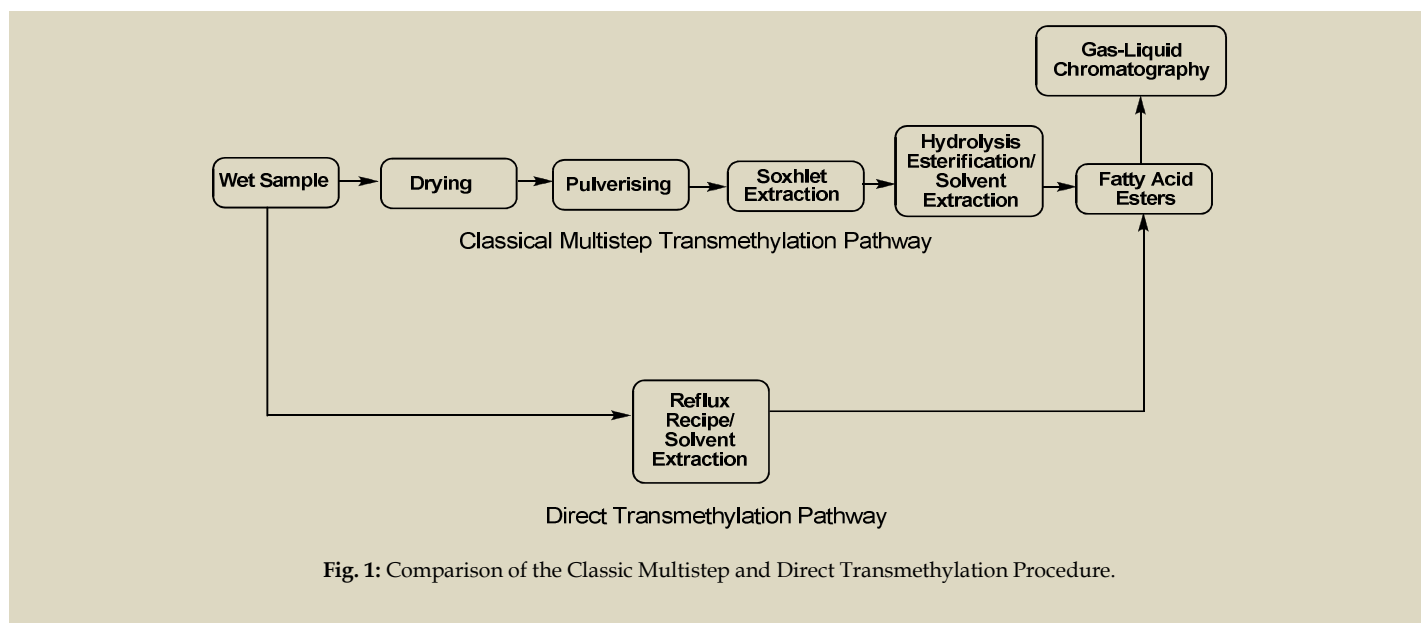
The gas chromatogram was obtained on an Agilent Technology 7890A gas chromatograph GC-FID, equipped with a fused silica capillary column HP-5MS (30 m by 0.32, 0.5 μ m film thickness) on ultra-pure helium gas and coupled to a mass selective detector (mass spectrometer). The injector and interface were operated at 250 and 380 $^{\circ}$ C respectively. The oven temperature was raised from 60 to 270 $^{\circ}$ C at a heating rate of 5 $^{\circ}$ C min^{-1} and then held isothermally. The sample was injected in a splitless mode using 7683 series Agilent Technologies injector. The percentages of the compounds were obtained by GC-FID analysis.

Mass spectrometry MS, Qualitative analyses were carried out on a MS 5975, msd Agilent Technologies operated at an ionization voltage of 70 eV over an acquisition mass range under the experimental conditions reported for GC-FID analysis. The chemical compositions of the product obtained were identified by comparison of their MS spectral with data obtained from National Institute Standard and Technology (NIST, 2008) database. The relative percentages of the constituent compounds were percentages from the GC peak areas based on the total ion chromatogram.

RESULTS AND DISCUSSION

The direct Transmethylation (DTM) adopted in this study has been reported to afford an improved recovery of total fatty acids in comparison to classic multistep method (Bligh and Dyer, 1959; Dickey *et al.*, 2002). The classical multistep method, besides consuming time also required the use of relatively large amount of solvents for the derivatization to facilitate the analysis and quantification of fatty acid methyl esters by gas chromatography. The classical method being a multiple steps procedure increases the possibility of contamination and sample losses which are significantly minimized in the DTM (Fig. 1).

The direct methylation combines extraction and transesterification into one step, thereby giving rise to a simpler and faster analysis whilst consuming less solvent (Indarti *et al.*, 2005; Meier *et al.*, 2006). The method results in a better recovery of the total fatty acid methyl ester present in the sample without bias or selectivity toward any lipid classes (Dickey *et al.*, 2002). Extraction with organic solvents is known to be majorly efficient if the plant samples are first dried and crunched (Cooney *et al.*, 2009), but the DTM eliminate the entire drying process.



Additionally, the use of conventional solvent extraction methods on samples such as fish condiment and highly pigmented plant material usually results in the recovery of many pigments and other lipid-soluble materials that contribute to the crude total lipid and thereby confound the estimate of total fatty acid content (DeKoning *et al.* 1985). It has been reported that the DTM procedure conforms to the U.S. Nutritional Labeling and Education Act (NLEA) demand (Xiao, 2010). The recipe used in this study contains hexane and benzene as solvent, sulphuric acid as for hydrolysis, aluminium chloride as catalyst and methanol for methylation. The FAMES were thus obtained in a single procedure with a yield, 0.63% of the total seed weight.

Fatty Acids Composition

The GC-MS result of the fatty acid composition of the oil obtained from the *Sabal causiarum* seed is shown in Table 1. A total of twelve fatty acids were identified from the total ion chromatogram of the GC. The ratio of the total unsaturated to saturated fatty acids is approximately 1:1. Monounsaturated accounted for about 19.2 % while polyunsaturated accounted for about 29.8 % of total fatty acids. The dominant fatty acid was Linoleic acid (C18:2) in a concentration of 29.83 %. It is the only polyunsaturated omega-6 fatty acid. Other major fatty acids include palmitic acid (20.75 %), dodecanoic acid (12.15 %), oleic acid, an omega-9 monounsaturated fatty acid (11.51%), 11-Octadecenoic acid (5.89 %), Ceric acid (4.97) and lignoceric acid (4.56 %). The most abundant FA being linoleic acid is a polyunsaturated omega-6 fatty acid which has significant importance in human diet (Singh, 2005). However, some report have suggested that certain high level of certain omega-6 fatty acids relative to certain omega-3 fatty acids may trigger the onset of disorder in human body (Okuyama *et al.*, 2006). Apart from biological relevance of the linoleic acid it is also of great

industrial application since it serves numerous oleochemical uses in pharmaceuticals, oil paints, varnishes, lubricants, cosmetics and surfactants (Beltran *et al.*, 2004).

Table 1: Fatty acids profile of *Sabal causiarum* seed oil obtained from the GC-MS.

Peak No.	Fatty Acids	Retention Time (Min)	% Yield
1	Dodecanoic acid (12:0)	8.48	12.15
2	Palmitoleic (16:1)	12.62	1.89
3	Palmitic acid (16:0)	12.92	20.75
4	Margaric acid (17:0)	13.83	1.59
5	Linoleic acid (18:2 ω-6,9)	14.55	29.83
6	Stearic acid (18:0)	14.80	5.38
7	Oleic acid (18:1 ω-9)	14.94	11.51
8	11-Octadecenoic acid (18:1)	15.02	5.89
9	Behenic acid acid (22:0)	18.13	0.99
10	Tricosanoic acid (23:0)	18.89	0.49
11	Lignoceric acid (24:0)	19.68	4.56
12	Ceric acid (26:0)	21.70	4.97
Total Saturate			50.88
Monounsaturate			19.29
Polyunsaturate			29.83

Margaric acid (1.59 %), Behenic acid (0.99 %) and tricosanoic acid (0.49 %) were found to be less dominant saturated fatty acids while palmitoleic acid, an omega-9 fatty acid was also detected at low concentration (1.89 %).

Margaric acid, though not commonly observed in natural products has been reportedly identified in the volatile of *Annona reticulata* (Pino *et al.*, 2003), and *Pourouma guianensis* (Lopes *et al.*, 1999) as well as in the hexane extract of the aerial parts of *Aristolochia acutifolia* (Palmeira *et al.*, 2001). Palmitic acid, oleic acid, linoleic acid, stearic acid, behenic acid, margaric acid and Lignoceric acid have also been reported as constituent of virgin olive and *Crambe abyssinica* seed oil (Beltran *et al.*, 2004; Lalas *et al.*, 2012).

DPPH Free Radical Scavenging Activities

The result of the *in vitro* DPPH free radical scavenging activities of the FAMES (Table 2) indicated that the antioxidant activity of the FAMES from the seed of *Sabal causiarum* with IC₅₀ of 0.19 ± 0.31 (mg/mL) was more significant than the standard, α-tocopherol with IC₅₀ of 0.25 ± 0.4 (mg/mL). This suggests a possible synergistic action of the FA present in the seed oil. Synergistic antioxidant activities of fatty acids from other plants have been reported before (Atolani *et al.*, 2012, 2013). The ability of the FAMES to scavenge the DPPH radical is an indication that the plant could be useful in slowing down the progress of oxidative stress. This therefore adds credence to the possible application of the plant in biological system.

Table 2: Result of the Antioxidant Activities.

Sample	IC ₅₀ (mg/mL)
FAMES	0.19±0.31
α-Tocopherol	0.25±0.40

Values are mean of duplicate data obtained.

CONCLUSION

Direct trans-esterification of fatty acid from the seed of *Sebal causarium* and GC-MS analysis has enabled the quick identification of the various fatty acids in the seed in a simple procedure. The one-step extraction procedure seems efficient, time saving and economical in the analysis of FAs in the seed of *Sebal causarium*. The GC-MS result indicated twelve fatty acids with the most prominent being Linoleic acid, an essential polyunsaturated fatty acid. The antioxidant activity of the oil from the underutilized tropical seed also indicated that the seed could be a viable natural renewable source of antioxidant compounds. More work would be needed to further establish the biological and economical potentials of the lesser known tropical seed.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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How to cite this article:

Olubunmi ATOLANI, Oluwaseeni ADENIYI, Oluwatimilehin O. KAYODE, Charles B. ADEOSUN. Direct Preparation of Fatty Acid methyl Esters and Determination of *in vitro* Antioxidant Potential of Lipid from Fresh *Sebal causarium* Seed. *J App Pharm Sci*, 2015; 5 (03): 024-028.