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Separation and potential valorization of chemical constituents of soursop seeds

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Abstract
Seeds of *Annona muricata,* by-products from the edible fruit, were assessed chemically and biologically. Using larvicidal bioassay, activity-directed fractionation of ethanolic extract led to the isolation of three known acetogenins: annonacin, murisolin and annonacinone. Their structures were established by spectroscopy experiments. Synergistic activity of these acetogenins was observed.
The low water solubility of annonacin was demonstrated to be one of the limiting factors of its larvicidal activity. The catalytic hydrogenation of annonacin afforded a mixture of diastereoisomers which is more active against *Culex quinquefasciatus* larvae than annonacin.
Micro-Kjeldahl analysis showed that residues of extraction (insoluble matter) contained 25.6% crude proteins.
Solvent partitioning of the kernel ethanolic extract yielded: oil (30.4% of kernels), mixture of acetogenins (4.2% of kernels) and mixture of carbohydrates (5.2% of kernels). The oil was weakly toxic to *Artemia salina* nauplii. Eight fatty acids were identified. Sucrose was the major carbohydrate.
Potential valorization of these components is discussed.

Keywords: *Annona muricata*, seed, oil, acetogenin, valorization, Madagascar

1. Introduction
Dealing with waste issues is one of the greatest challenges to mankind of this century. Even if agriculture wastes are already valorized (for example rice or wheat straws are used for animal feeding) their collection and transport are often difficult and expensive. On the opposite, industrial wastes obtained during food processes are already gathered and well defined physically and chemically. Their transformation could be performed if an efficient industrial ecology was set up [1, 2]. The main drawback is the relatively smaller amount of these agro-industrial wastes compared to that of other agricultural wastes. Nevertheless, this disadvantage is limited as far as the high value materials are obtained from the conversion of these wastes. As part of our research for industrial ecology, we have already described the tentative valorization of waste from mango processing [3]. In the present article we describe an attempt to evaluate the potential application of soursop seeds. Soursop, *Annona muricata* (Annonaceae) is a tropical tree [4] that grows in different localities in Madagascar. Pulp of the edible fruit is largely used for juice or jam preparation [5]. Seeds are discarded due to their toxicity. They are used in folkloric medicine for treating skin diseases [6] as cited in Le Ven [7]. In India, they are used as emetic, astringent or fish poison [8]. In Brazil, they are destinated to soil fertilization [9]. Since the isolation of uvaricin [10], the first annonaceous acetogenin, they have been mainly studied [11, 12, 13] for the research of a new class of cytotoxic drug candidates. Some authors reported pesticidal or larvicidal activity from soursop seed extract [7, 14, 15]. Soursop seeds contain fibers and proteins [16, 17] which could be useful for feeding animals. They also contain large amount of oil [18, 19, 20, 21] which is toxic due to lipophilic acetogenins [22]. These molecules exhibit a broad range of biological activities: mitochondrial inhibitor or cytotoxic [11, 12, 13, 22], pesticidal and larvicidal [8, 9]. Therefore, they may be proposed to control mosquito populations which constitute vectors of various tropical diseases in Madagascar. However, oil which contains these acetogenins, has restricted potential applications although it is used to kill head lice in India and Mexico [23]. To expand these potential applications, separation of toxic molecules from oil is useful. For this purpose, the methodology consisted of extraction with Soxhlet apparatus, followed by liquid/liquid partition, using low toxicity, more affordable and available solvents. The derived components from the treatment of soursop seeds can be valorized depending on their potential uses.

2. Materais and Methods

2.1 Plant material

Fruits of *Annona muricata* were bought at the market of Antananarivo. Seeds were provided by restaurants of Antananarivo from August 2012 to August 2013.

Pulp, peels, core and seeds of the fruits (2261 g) were separated and weighted. Seeds were dried in a well-ventilated area. Husks (37.4%) were separated from kernels (62.2%).

2.2 Solvents

Solvents (hexane, dichloromethane, ethyl acetate, ethanol and methanol) were distilled before use. Toluene (Merck), diethyl ether (Panreac Quimica) and dimethylsulfoxyde (Fisher Scientific) were analytical grade.

2.3 General analysis

Silica gel 60 Å, 230-400 mesh (Merck) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC. The spots were detected by spraying with various reagents: Kedde's reagent (acetogenins), thymol (carbohydrates), Rhodamin B (fatty acids), ninhydrin (amino acids), vanillin sulfuric acid (universal reagent) followed by heating. 1D (^{1}H, ^{13}C, DEPT) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC) NMR spectra were recorded on a Bruker Varian 400 NMR operating at 400.15/125.15 MHz using $CDCl_3$ as solvent and TMS as an internal standard. Fragments were obtained by a Micro TOF-Q Bruker Daltonics (Bremen, Germany) mass spectrometer fitted with ESI source. Positive ions mass spectra of the column eluate were recorded in the range m/z 50-2000. Nitrogen was used as the drying gas at a flow rate 4 L/min. The nebulizer temperature was set at 200 °C and the pressure was 0.6 bar.

GC/MS experiments were performed on a Focus GC chromatograph linked to a mass spectrometer (70 eV) with an electron ionization system. The carrier gas was helium with a flow rate of 50 mL/min. A DB-5MS apolar capillary column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm) was used with the following temperature program: 70 °C (2 min), 70-310 °C at 15 °C/min, and 310 °C (10 min). The injector temperature was 220 °C.

2.4 Extraction and isolation of the constituents

2.4.1 Ethanolic extracts

Maceration: The powdered seeds (600 g) were repeatedly macerated with ethanol 95° (3 L) during (24 hours x 3) at room temperature. The combined ethanol extracts were evaporated and yielded (65.9 g, 11%) EtOH extract (EM).

Soxhlet: The ground materials were extracted with EtOH 95° (700 ml) by Soxhlet apparatus (200 mL) with flow rate 5 mL/min: husks (50 g) yielded (5.4%) ethanolic extract (EH) after 16 hours; kernels (50 g) provided (40.5%) ethanolic extract (EK) and (53.8%) insoluble matter (IM) after 13 hours.

Partition of ethanolic extract

Method 1: 64.2 g of EtOH extract (EM) were partitioned successively between H_2O and C_6H_{14} to yield (58.6%) C_6H_{14} extract (A) and between H_2O and CH_2Cl_2 to afford (22.3%) CH_2Cl_2 extract (B) and (10.6%) aqueous extract(C). Fractionation was monitored by the larvicidal bioassay [24].

Method 2: The EtOH extract (EK) (50 g) was partitioned between Hexane/MeOH/Water: 50/25/25 to yield 75.2% hexane extract (A'), the MeOH/Water phase was extracted with CH_2Cl_2 and yielded 10.3% CH_2Cl_2 extract (B') and 12.7% aqueous extract (C').

Method 3: The method 2 was applied but CH_2Cl_2 was superseded by ethyl acetate. 75.5% hexane extract (A*), 13.8% ethyl acetate extract (E*) and 8.8% aqueous extract (C*) were obtained.

2.4.2 Isolation of acetogenins

The bioactive CH_2Cl_2 extract (B) (10 g) was subjected to column chromatography (elution with CH_2Cl_2/MeOH: 95/5, 90/10 and 80/20) and yielded three fractions F1 (32.8%), F2 (59.9%) and F3 (4.5%). The bioactive fraction F2 (3 g) was subjected to silica gel column chromatography and eluted with CH_2Cl_2 containing increasing amounts of MeOH (CH_2Cl_2/MeOH: 99/1 to 90/10). Three bioactive fractions were obtained: B1 which contained mainly murisolin, B2 which was a mixture of three acetogenins (murisolin, annonacinone and annonacin) and B3 which contained a bioactive mono-tetrahydrofuran γ-lactone acetogenin: annonacin (**1**) (1.33 g, 44.3% of F2 and 26.6% of B).

The CH_2Cl_2 extract (B') (3 g) was separated by column chromatography on silica gel. Elution with Hexane/EtOAc: 90/10 to 80/20 led to the isolation of three mono-tetrahydrofuran α, β-unsaturated γ-lactone acetogenins: annonacin (1) (282.3 mg), annonacinone (2) (82.8 mg) and murisolin (3) (40 mg).

These acetogenins reacted positively with Kedde's reagent and were displayed as orange spot after the treatment of the chromatoplate with vanillin sulfuric acid.

Annonacin (1)

White amorphous powder. Mp=60 °C. Identified by MS and ^{1}H, ^{13}C NMR. Rf=0.23 in CH_2Cl_2/MeOH: 95/5 (v/v). $C_{35}H_{64}O_7$; MS: m/z 619.6 $[M+Na]^+$, 597.4 $[M+H]^+$, 579.4 $[MH-H_2O]^+$, 561.5 $[MH-2H_2O]^+$, 543.5$[MH-3H_2O]^+$, 525.5 $[MH-4H_2O]^+$. NMR chemical shifts are consistent with the literature [25].

Annonacinone (2)

Needle-like crystals. Identified by MS and ^{1}H, ^{13}C NMR. Rf=0.27 in CH_2Cl_2/MeOH: 95/5. $C_{35}H_{62}O_7$; MS: m/z 617.4 $[M+Na]^+$, 595.4 $[M+H]^+$, 577.4 $[MH-H_2O]^+$, 559.4 $[MH-2H_2O]^+$, 541.4 $[MH-3H_2O]^+$, 523.4 $[MH-4H_2O]^+$. NMR chemical shifts were compared with the literature [26].

Murisolin (3)

Needle-like crystals. Identified by MS and ^{1}H, ^{13}C NMR and by comparison of data with the literature [27].

Rf=0.34 in CH_2Cl_2/MeOH: 95/5.

$C_{35}H_{64}O_6$; SM: m/z 603.4 $[M+Na]^+$, 581.4 $[M+H]^+$, 563.4 $[MH-H_2O]^+$, 545.4 $[MH-2H_2O]^+$, 527.4 $[MH-3H_2O]^+$.

Catalytic hydrogenation of annonacin

Catalytic hydrogenation of annonacin was performed in an ethyl acetate solution with 5% Pd/C at room temperature under an atmospheric pressure of H_2 and moderate stirring for 18 hours. The mixture was filtered through a column of silica gel and eluted with methanol. A mixture of diastereoisomers (94% of annonacin by weight) was obtained.

2.4.3 Isolation of sucrose

The aqueous extract (C') (523 mg) was separated by column chromatography on silica gel. Elution with CH_2Cl_2/MeOH /Water: 10/3.5/0.5 to 1/3.5/0.5 afforded sucrose (50.1 mg, 9.6%).

White crystals identified by MS and ^{1}H, ^{13}C NMR. Rf=0.26 in CH_2Cl_2/MeOH/H_2O: 5.5/3.5/0.5 (v/v/v). $C_{12}H_{22}O_{11}$; MS: m/z

365.1 $[M+Na]^+$, 203.1 (cleavage of the glycosidic bond between the two sugars); ^{13}C chemical shifts are consistent with the literature [28].

2.4.4 Seed oils
The powdered materials: husks (10 g), kernels (10 g) were separately extracted with hexane by soxhlet (10 hours, flow rate: 5 mL/min) until materials were completely defatted and yielded respectively (1.6%) (HO) and (36.8%) (KO) oil extract. Kernel oil was a pale yellow liquid.

Physical and chemical characteristics of kernel oil
Specific gravity of kernel oil was determined in triplicate and the average result reported. Chemical characteristics of kernel oil (acid value, saponification value and content of unsaponifiable matter) were determined by standardized methods [29].

Transesterification of fatty esters
Methylation of kernel oil and fatty acids of reference was performed according to Christie [30] with slight adjustments. The lipid sample (250 mg) was dissolved in toluene (3 mL) and 2% sulfuric acid in methanol (5 mL). The mixture was left overnight in a stoppered tube at 50 °C. Then, aqueous sodium chloride solution (5%, 10 mL) was added. The required methyl esters were extracted with hexane (3 x 10 mL). The hexane layer was dried over anhydrous sodium sulfate. The solution was filtered and the solvent was removed under reduced pressure in rotary evaporator. Diluted solutions were prepared before injection for GC analysis.

Analysis of fatty acid composition
The obtained fatty acid methyl esters were separated using a GC-14A chromatograph (Shimadzu) equipped with a flame ionization detector (FID). The carrier gas was nitrogen with a flow rate of 5 mL/min. A DB-5MS-UI apolar capillary column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm) was used with the following temperature program: 170 °C (1 min), 170-230 °C at 3 °C/min, 230 °C (1 min), 230-310 °C at 15 °C/min, and 300 °C (1 min).
Fatty acids were identified by equivalent length chain method [31, 32] prior to the confirmation by GC/MS analysis. Data were compared with those in MS library.

2.5 Crude protein content of kernels
After the extraction of kernel with ethanol, insoluble matter (IM) remained. Crude protein content of the latter was determined according to Kjeldahl method [33].

2.6 Biological assays
2.6.1 Feeding experiments
Fifteen swiss mice were divided into three batches. Each batch was fed with, respectively, 10 g, 5 g and 2.5 g insoluble matter (IM) mixed in their diet. The mice ate this for two days and were deprived of food during the third day. The behavior of mice was observed during the five days following their ingestion of the residues.

2.6.2 Larvicidal bioassay
Larvae of *Culex quinquefasciatus* were collected from their breeding habitat (the runoff channels in downtown Antananarivo). The larvicidal bioassay was performed according to World Health Organization protocol [24]. Solutions were prepared by dissolving extracts in ethanol. 1 mL of ethanol was used as control test. Stock solution 3.2% of CH_2Cl_2 extract (B) was prepared. 1 mL of this solution added into the vial containing 250 mL of spring water and larvae corresponded to 127.5 ppm. Different levels of concentration were prepared by diluting the stock solution with ethanol. Tested concentrations : (for CH_2Cl_2 extract (B): 1 ppm, 2 ppm, 4 ppm, 8 ppm, 16 ppm, 31.9 ppm, 63.8 ppm, and 127.5 ppm), (for annonacin: 5 ppm, 10 ppm, 20 ppm, and 40 ppm), (for the mixture of dihydro-annonacin: 5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm, 30 ppm, and 60 ppm), (for ethyl acetate extract (E*): 15 ppm, 30 ppm, 45 ppm, 60 ppm, 75 ppm, and 90 ppm).

2.6.3 Toxicity bioassay
The toxicity bioassay was performed according to Nando *et al*. [34] with slight adjustments. Stock solutions were prepared by dissolving the extracts in DMSO. Different levels of concentration were prepared by diluting the stock solution with DMSO. Each level of concentration was tested in quadruplicate. One negative control was used. Ten brine shrimp larvae of 24 hours old were transferred into each vial containing 3 mL of artificial seawater (38 g of sea salt / 1 L of spring water, pH adjusted to 8). 100 µL of solution extract were added. The volume was then adjusted to 5 mL with artificial seawater. 100 µL of DMSO were used as control test. The vials were maintained under illumination. The number of dead *Artemia salina* nauplii was recorded after 24 hours. Tested concentrations: (for annonacin: 1 ppm, 2 ppm, 3 ppm, 4 ppm, 5 ppm, 6 ppm, 10 ppm, 20 ppm, 50 ppm, and 100 ppm), (for the mixture of dihydro-annonacin: 1 ppm, 2 ppm, 3 ppm, 4 ppm, 5 ppm, and 6 ppm), (for the ethyl acetate extract (E*): 1 ppm, 2 ppm, 3 ppm, 4 ppm, 5 ppm, 6 ppm, 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, and 60 ppm), (for oils: 10 ppm, 20 ppm, 50 ppm, and 100 ppm).

2.7 Statistical analysis
Mortality data were corrected using Abbot's formula. LC_{50} and LC_{90} values were determined by log-probit regression using XLSTAT 2014 software.

3. Results and discussion
3.1 Separation of plant material
The compositions of the fruit are reported in Figure 1.

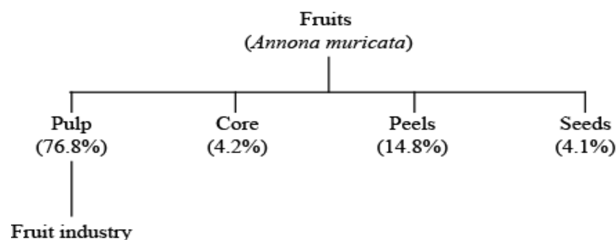

Fig 1: Fruit composition

Results slightly differed from data: 67.5% pulp, 20% peels and 4% core reported by the literature [35] as cited in Badrie and Schauss [36]. Seed content is similar to that of soursop from Nigeria [19]. In general, the average is ranging from 2.4% to 8.5% [12, 20, 35, 36]. During the drying process, the seeds had lost about 30% of their weight. Kernels and husks accounted respectively for 62.2% and 37.4% of dry seeds by weight. Literature reported that kernels represented 67% [18] to 68.55% [20]. Since the water content was evaluated at 8.9%, no drying process prior to any extraction of kernels was necessary [29].

3.2 Ethanolic extracts

Compared to maceration, the treatment with Soxhlet apparatus provided a better yield of ethanolic extract. Moreover, it required neither filtration nor renewal of solvent. It also reduced the time of extraction.

Ethanolic extract obtained from the treatment of kernels (40.5%) was more important than that obtained from husks (5.4%). This finding showed little interest to proceed to extraction of the latter.

3.3 Solvent partitioning of the ethanolic extracts

Three methods, according to solvent system, were used for the partition of kernel ethanolic extract. The binary system, hexane/water (Method 1), produced a stable emulsion that took many hours to resolve. Several extractions with hexane were necessary to obtain the apolar products. Compared with hexane/water, the ternary system: hexane/methanol/water: 50/25/25 (Method 2) reduced the formation of emulsion. It improved the separation of products. Nevertheless, the use of dichloromethane, a toxic solvent, would not be suitable in large scale. In this regard, the method 3 (Figure 2) was the best one. Ethyl acetate is less toxic than dichloromethane. Chromatographic profile of ethyl acetate extract differed from that of dichloromethane extract by the occurrence of polar products in the latter. This fact was supported by the increase of yield from 10.3% to 13.8%.

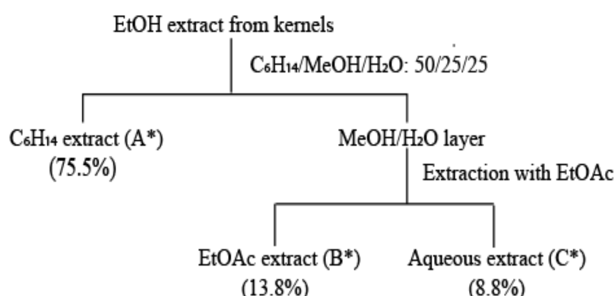

Fig 2: Solvent partitioning of the ethanolic extract

3.4 Acetogenins of soursop seeds

Three known acetogenins were isolated as main products (13.5%) from CH_2Cl_2 extract (B'): annonacin (1), annonacinone (2) and murisolin (3) (Figure 3). Annonacin was obtained with good yield (26.6% of CH_2Cl_2 extract (B)) when elution was undergone with CH_2Cl_2/MeOH. Indeed, these solvents are preferably used to extract acetogenins [37]. These acetogenins bear a tetrahydrofuran ring flanked with two hydroxyl groups with a *threo-trans-threo* relative configuration. This relative configuration is also supported by the melting point of annonacin (60 °C) which is far below 77 °C (the melting point of cis-annonacin) [12]. Based upon the investigations of Curran and co-workers [38], relative configuration of C-4 and C-34 in the hydroxybutenolide subunit was assigned. Since the difference between resonances of C-33 and C-4 is 82 ppm for annonacin (1) and 81.8 ppm for murisolin (3), the relative configuration of C-4 and C-34 is syn (4*S*, 34*S*) for the former compound whereas anti (4*R*, 34*S*) for the latter. As the difference between resonances of C-33 and C-4 is 82.1 ppm for annonacinone, the relative configuration of C-4 and C-34 cannot be assigned. However, considering the results of Rieser and co-workers [39] which assigned 4*R* and 34*S* as the absolute configuration of annonacin and annonacinone the preparation of Mosher ester derivatives of the currently isolated acetogenins would be useful.

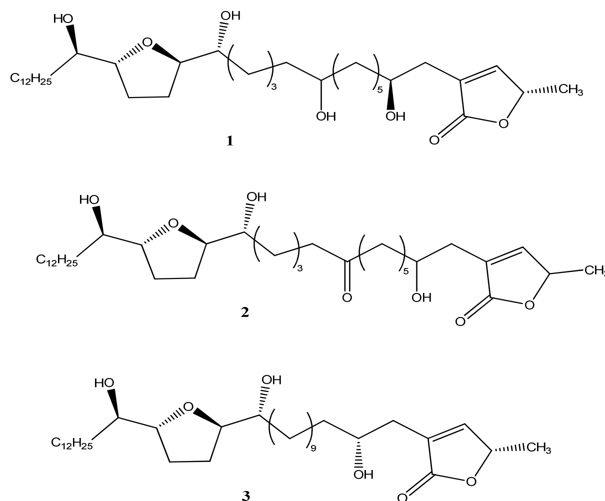

Fig 3: Acetogenins of soursop seeds: annonacin (1), annonacinone (2), murisolin (3) Isolated annonacin was hydrogenated as shown in Figure 4.

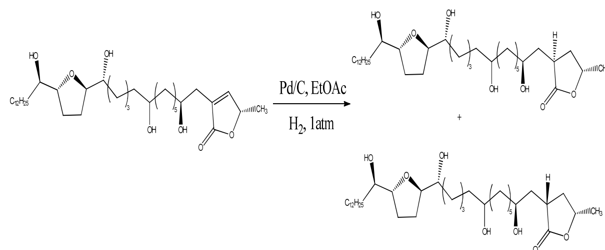

Fig 4: Hydrogenation of annonacin

The mixture obtained after hydrogenation of the isolated annonacin was analysed by TLC. After spraying the plate with vanillin sulfuric acid, followed by heating, three orange spots were displayed at Rf 0.36, 0.32 and a very intense one at 0.23 in CH_2Cl_2/MeOH: 95/5. These results suggested the existence of two isomers of the isolated annonacin since catalytic hydrogenation of a 4-hydroxylated α, β-unsaturated γ-lactone acetogenin performed with Pd/C affords a 1:1 mixture of diastereoisomers (the *cis* and the *trans* products) [40, 41]. Therefore, we hypothesized the existence of four diastereomers of dihydro-annonacin and two of them were unresolvable in the solvent system used. Main differences between the chemical shifts of annonacin and those of the mixture of dihydro-annonacin are reported in Table 1.

Table 1: Chemical shifts of hydroxybutenolide subunit of annonacin and those of the mixture of dihydro-annonacin

N°	δ ^{13}C (δ ^{1}H) ppm	
	Annonacin	Dihydro-annonacin
1	174,8	180.4
2	131.1	39.5 (2.89 ; m) / 41.7 (2.35 ; m)
3	33.2 (2.41 dd; 2.53 ; dt)	37.4 (2.47 ; 2.45 ; m)
4	69.9 (3.82 ; m)	66.7 (3.82m) / 70.5 (3.61 ; m)
33	151.9 (7.21 ; d)	25.7- 29.9 (1.19-2.6 ; m)
34	79.1 (5.08 ; q)	76.0 (4.53 ; m) / 79.7 (4.39 ; m)
35	19.2 (1.45 ; d)	21.1 (1.36 ; m) / 24.5 (1.19 ; m)

The occurrence of murisolin, annonacin and annonacinone in soursop seeds has already been reported in the literature [11, 12]. The employed method permitted the isolation of almost all molecules with such structure using solvent extraction and liquid/liquid partition. Relatively cheap and low-toxicity

solvents were used. Acetogenins could be used as starting material for chemical modification and biological evaluation. The following results concerning larvicidal properties are only one of the examples of what could be performed.

3.5 Larvicidal activity and toxicity of crude extracts and that of acetogenins

3.5.1 Larvicidal activity and toxicity of crude extracts

Dichloromethane extract (B) showed larvicidal activity with LC_{50}=74.4 ppm (95% confidence intervals: 60 ppm – 97 ppm). Three fractions were obtained from the dichloromethane extract (B). Fraction (B1) containing the murisolin, showed weak larvicidal activity. Fraction (B2) consisting of a mixture of three acetogenins (1, 2 and 3) was more active than fraction (B3) which contained annonacin. These results may indicate the synergistic action of acetogenins.

The ethyl acetate extract (E*) exhibited larvicidal activity with LC_{50}=14.5 ppm (95% confidence intervals: 12 ppm – 17 ppm) and LC_{90}=60.1 ppm (95% confidence intervals: 53 ppm – 71 ppm) as shown in Figure 5. It was about five times more effective than the dichloromethane extract (B).

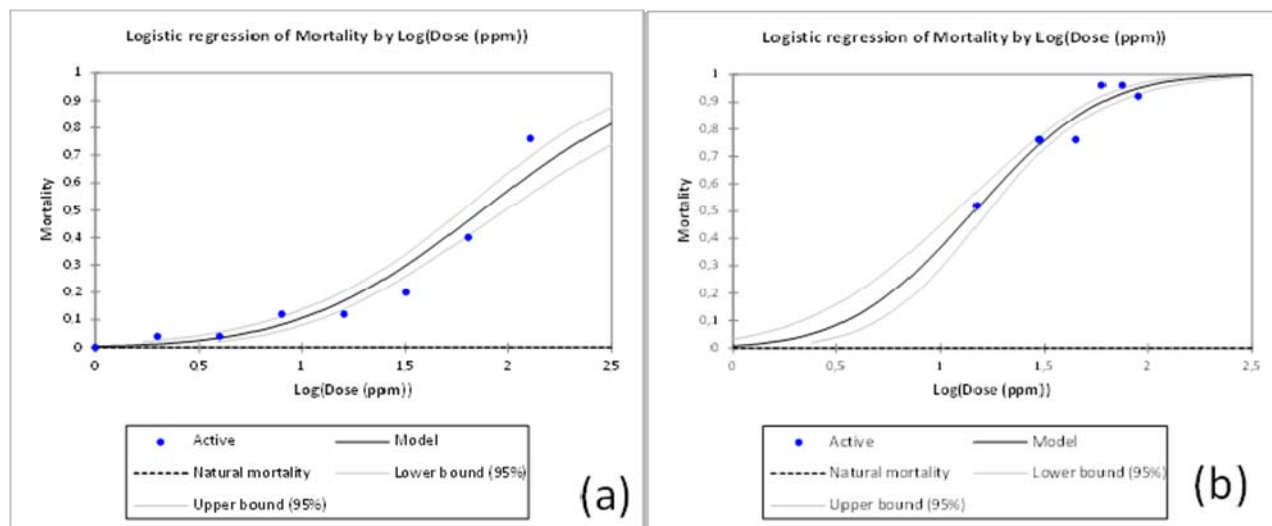

Fig 5: (a) Dose-larvicidal activity of the dichloromethane extract (B), (b) Dose-larvicidal activity of the ethyl acetate extract (E*)

Nevertheless, the ethyl acetate extract **(**E*) was also proved to be toxic to *Artemia salina* nauplii with LC_{50}=2.7 ppm (95% confidence intervals: 2.3 ppm – 3.2 ppm) which limits the application in this field. Its toxicity was five times higher than its larvicidal effect.

3.5.2. Larvicidal activity of annonacin and that of dihydro-annonacin derivatives

Annonacin was active at low concentrations. However at higher concentrations (10 to 40 ppm), the mortality rate induced by the molecule remained constant and did not exceed 50%. LC_{50} was estimated at 246 ppm.

Annonacin is lipophilic [22]. From 10 ppm, it formed a colloidal aqueous solution. Considering this fact, it is obvious that the median lethal concentration of annonacin would not be achieved. So, the low water solubility of annonacin constitutes a limiting factor of its larvicidal activity.

The mixture of dihydro-annonacin diastereoisomers exhibited larvicidal activity with LC_{50}=64.6 ppm (95% confidence intervals: 52.2 ppm – 87.5 ppm). Compared to the larvicidal activity of annonacin, that of the hydrogenated compound has increased (Figure 6).

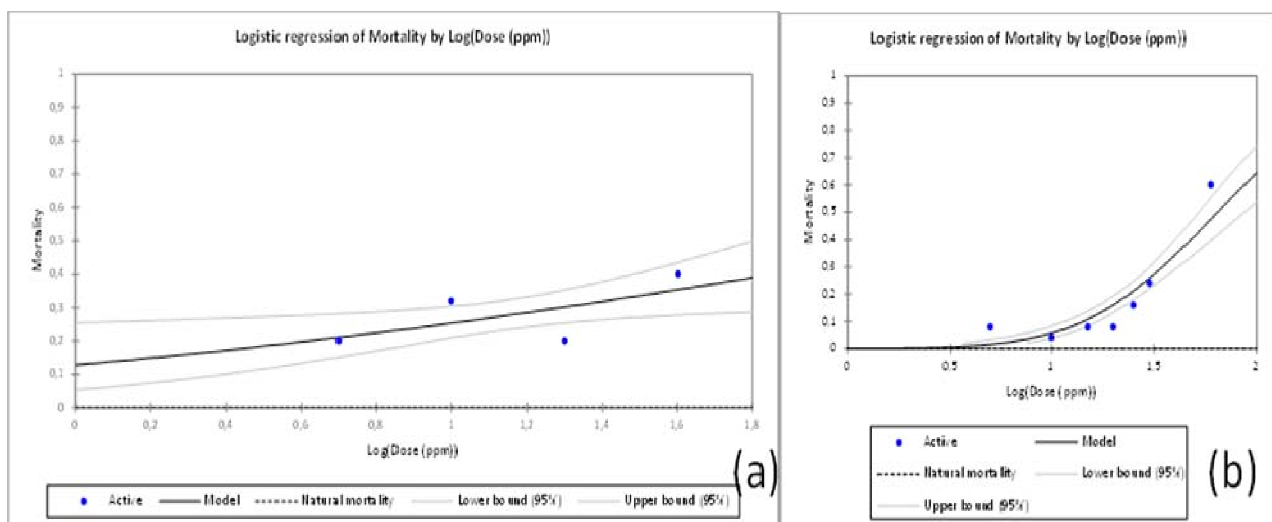

Fig 6: (a) Dose-larvicidal effect of annonacin, (b) dose-larvicidal effect of the mixture of dihydro-annonacin

This increase of activity can be explained by two phenomena: the solubility and the synergistic action. Firstly, the mixture of dihydro-annonacin diastereomers was more soluble in water than the starting molecule. Secondly, molecules acted synergistically. This fact was already noted with the fraction (B2) which contained the three acetogenins (annonacin, annonacinone and murisolin) and with the crude extract which contained mainly acetogenins.

3.5.3. Toxicity of annonacin and that of dihydro-annonacin derivatives

Annonacin exhibited potent toxicity toward *Artemia salina* nauplii with LC_{50}=1.5 ppm (95% confidence intervals: 1.2 ppm – 1.9 ppm). Previous work reported the toxicity of isomers of annonacin toward brine shrimp with LC_{50} values ranging from 2.3 ppm for *cis*-annonacin [12] to 3.3 ppm for *trans*-annonacin [42]. Therefore, the isolated annonacin, bearing *trans* relative configuration, was more toxic compared to the literature data.

The isomers of dihydro-annonacin were toxic to brine shrimp larvae with LC_{50}=0.8 ppm (95% confidence intervals: 0.5 ppm - 1.1 ppm). At low concentrations, they were slightly more toxic than annonacin. At higher concentrations, their toxicities did not show significant difference (Table 2).

Table 2: LC_{50} and LC_{90} values of annonacin and dihydro-annonacin against *Artemia salina* nauplii

Lethal dose	Annonacin	Dihydro-annonacin
LC_{50} (95% confidence intervals)	1.5 ppm (1.2 - 1.9)	0.8 ppm (0.5 - 1.1)
LC_{90} (95% confidence intervals)	11.9 ppm (9.3 - 16.5)	10.4 ppm (7.6 - 17.2)

The catalytic hydrogenation slightly increased the larvicidal activity of the molecule, but did not reduce its toxicity.

The toxicity of crude extract containing acetogenins and that of annonacin against *Artemia salina* nauplii confirms the use of *Annona muricata* seeds as fish poisons [6] and should be considered as a serious drawback.

Based upon median lethal concentrations, crude extract is more efficient than pure compound. The use of crude extract offers two main advantages: (1) it does not require isolation of pure molecule which involves high costs and (2) insect resistance is much less likely to occur. Thus, ethyl acetate crude extract may be employed as effective larvicide but its toxicity should be reduced by chemical modification.

3.6 Carbohydrates and aqueous extract of soursop seeds

Aqueous extract (C') represented 5.2% of kernels. TLC analysis of the extract revealed the occurrence of amino acids and carbohydrates. The latter was mainly composed by sucrose (9,6%) and induced the caramel texture of the aqueous extract. Sucrose was already isolated from seeds of *Annona reticulata* [43]. Carbohydrates can be used as raw materials for fermentation process or for the synthesis of nonionic surfactants such as sugar esters [44, 45] or alkylpolyglycoside.

3.7 Seed oils

Oil extracted from husks (HO) accounted for 1.6% by weight. Literature reported that husks of soursop seeds from Nigeria contained no trace of oil [16]. However, this content was much lower compared to that obtained from kernels. Since husks contain low level of lipid and acetogenins, their extraction is not worthy. Separation of kernels from husks is essential. With an ash content of 2.58% [16], husks can serve as alternative of wood-fuel.

Extraction of kernels with hexane yielded 36.5% oils (KO). Taking into account the water content of the kernels (8.9%), the oil content represents 40.4% of dry kernels by weight. This content is very high compared to the literature data: 22.1% (extracted with petroleum ether) [20], 22.57% (extracted with ether) [16]. This difference is probably due to several factors: origin, climate, variety, cultivar, the ripening stage, the harvesting period or the solvent used. The oil content of kernels of soursop seeds is lower than those of peanuts (46.57 to 53.05%) [46] but higher compared to those of some varieties of olives (18 to 23.6%) [47].

The kernel oil (A') (30.4% of kernel) obtained by liquid/liquid partition of the ethanol extract and the one extracted with hexane (KO) were compared.

3.7.1 TLC analysis of kernel oils

TLC analysis revealed the existence of acetogenins in the kernel oil (KO) extracted with hexane contrary to the other one (A') obtained by liquid/liquid partition. These acetogenins were displayed as orange spots after spraying the plate with vanillin sulfuric acid followed by heating (Figure 7).

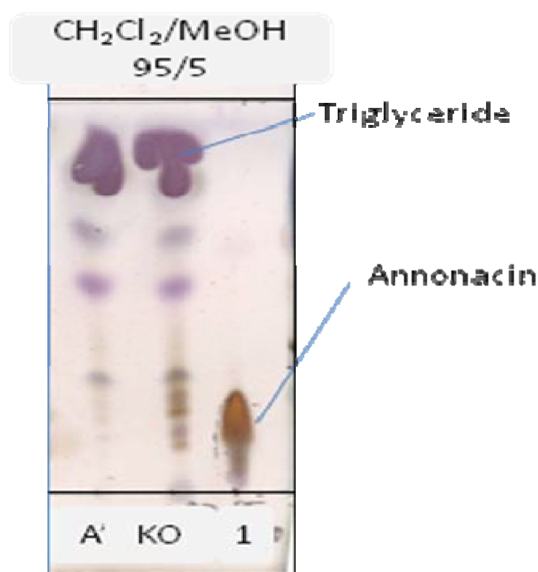

Fig 7: Chromatographic profile of the kernel oil (A') obtained by liquid/liquid partition of the ethanolic extract and that of the kernel oil (KO) extracted with hexane

Acetogenins are lipohilic. Diluted in oils, they can be extracted by hexane during the treatment of kernels. However, due to the hydroxyl groups (in annonacin, annonacinone and murisolin), they are soluble in methanol. Thus, most of them were removed from the hexane layer during the partition process using the solvent system hexane/methanol/water.

The kernel oil extracted directly with hexane contained acetogenins. It confirmed the toxicity of the oil and its folkloric use as treatment to kill head lice. Thus, this oil is not well-fitted for consumption.

3.7.2 Physical and chemical characteristics of kernel oils

The physico-chemical characteristics of the two oils obtained by the two methods are summarized in Table 3.

Table 3: Chemical and physical characteristics of the kernel oils obtained by the two methods

Physico-chemical characteristics	Kernel oil (KO) extracted with hexane	Kernel oil (A') obtained by solvent partition
Specific gravity at 25 °C	0.9136	0.9093
Acid value (mg KOH/g)	2.02	2.24
Saponification value (mg KOH/g)	215.3	221.6
Ester value (mg KOH/g)	213.28	219.36
Content of unsaponifiable matter (%)	0.4	0.39

The physico-chemical characteristics of the two oils showed no major difference. The specific gravity of the oil extracted with hexane (KO) (0.9136) was close to 0.9178 [18]. The oil obtained by liquid/liquid partition was lighter compared to that extracted with hexane. This may be explained by the absence of the acetogenins. The acid values of both oils showed no significant difference and were close to 2.29 mg KOH/g [18]. This index ranges from 0.93 [20] to 10.02 [19]. Codex alimentarius has set the maximum value of 0.6 mg KOH/g for refined oils [48]. If the kernel oil obtained by liquid/liquid partition is considered as refined one, its high level of acid value makes it unsuitable for consumption. The saponification value of both oils was superior to the data mentioned by some literatures: 117 mg KOH/g [21], 157 mg KOH/g [19], 197 mg KOH/g [18] but close to 227.48 mg KOH/g [20]. The high level of saponification value makes the kernel oil usable for specialty chemical (green gasoil, soap…).

3.7.3. Fatty acid composition of kernel oils

Fatty acid composition of the kernel oil extracted with hexane and that of obtained by liquid/liquid partition did not differ. The GC/MS spectrum of the kernel oil (KO) extracted with hexane is reported in Figure 8.

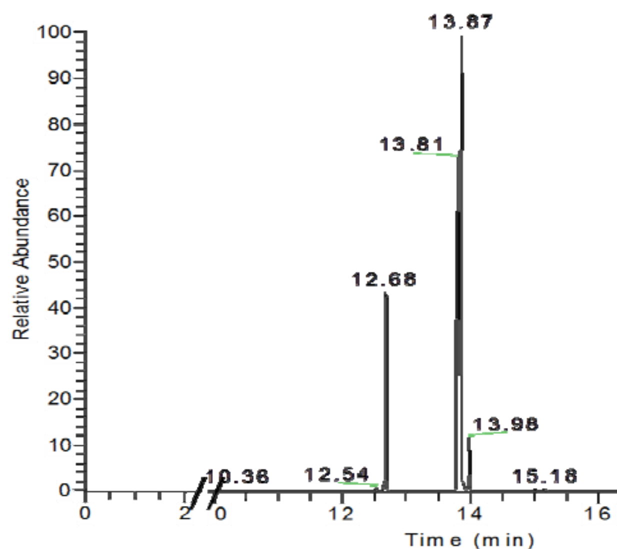

Fig 8: Separation of fatty acids in kernel oil of *Annona muricata* seeds by GC/MS. Peak assignment: tr=12.54: palmitoleic acid; tr=12.68: palmitic acid; tr=13.81: linoleic acid; tr=13.87: oleic acid; tr=13.98: stearic acid, tr=15.02: gondoic acid; tr=15.18: arachidic acid

The GC/MS analysis did not reveal the occurrence of myristic acid. This saturated fatty acid is weakly ionized by electron impact. Ravaomanarivo and co-workers [15] reported the presence of this fatty acid in soursop seeds. The monounsaturated fatty acid with 20 carbons can be gondoic or paullinic acid depending on the location of the double bond (C-11 or C-13). The previously mentioned authors designated this fatty acid as paullinic. Due to the migration of the double bond in the FAMEs, the preparation of pyrrolidide would be useful to determine this fatty acid without ambiguity.

The fatty acid composition of the kernel oils extracted with hexane and that obtained by solvent partition is reported in Table 4.

Table 4: Fatty acid composition of the kernel oils

Fatty acid		Relative percentage of fatty acid	
Noun	Symbol	Kernel oil (KO) extracted with hexane	Kernel oil (A') obtained by solvent partition
Myristic	n-14:0	0.05	0.05
Palmitoleic	9-16:1	1.30	1.29
Palmitic	n-16:0	19.65	19.77
Linoleic	9,12-18:2	35.86	35.68
Oleic	9-18:1	38.32	38.11
Stearic	n-18:0	4.05	4.19
Gondoic	11-20:1	0.23	0.33
Arachidic	n-20:0	0.55	0.58
% of saturated fatty acids		24.30	24.59
% of unsaturated fatty acids		75.70	75.41

Relative percentage of fatty acids in both oil samples did not differ. Unsaturated fatty acids predominated up to 75%. The literature reported different values: 75.45% [21], 71.93% [20] and 70.02% [18]. Oleic (38.1%) and linoleic (35.7%) were the major fatty acids. These results are consistent with the literature since the oil was classified among the oleic-linoleic acid group [21]. The main saturated fatty acid was palmitic (up to 19.7%). The value was close to 20.3% [21]. Palmitoleic acid was present in small amount and linolenic acid was not detected. These findings are consistent with the literatures which mentionned their existence in small or trace amount [15, 21]. In general, the relative percentage of each fatty acid slightly differed from that reported by literature [15, 21].

3.7.4 Toxicity of the kernel oils against Artemia salina nauplii

The kernel oil (A') obtained by liquid/liquid partition of ethanolic extract was less toxic than that extracted with hexane (KO). From 10 ppm to 50 ppm, mortality rate varied from 9% to 11%. At 100 ppm, it remained far below 50%. By contrast, the kernel oil (KO) extracted with hexane showed strong toxicity with LC_{50}=22.4 ppm (95% confidence intervals: 18.8 ppm – 26.4 ppm) and LC_{90}=84.4 ppm (95% confidence intervals: 66.1 ppm – 118.7 ppm). Logistic regression which allowed the obtention of those lethal doses is depicted in Figure 9.

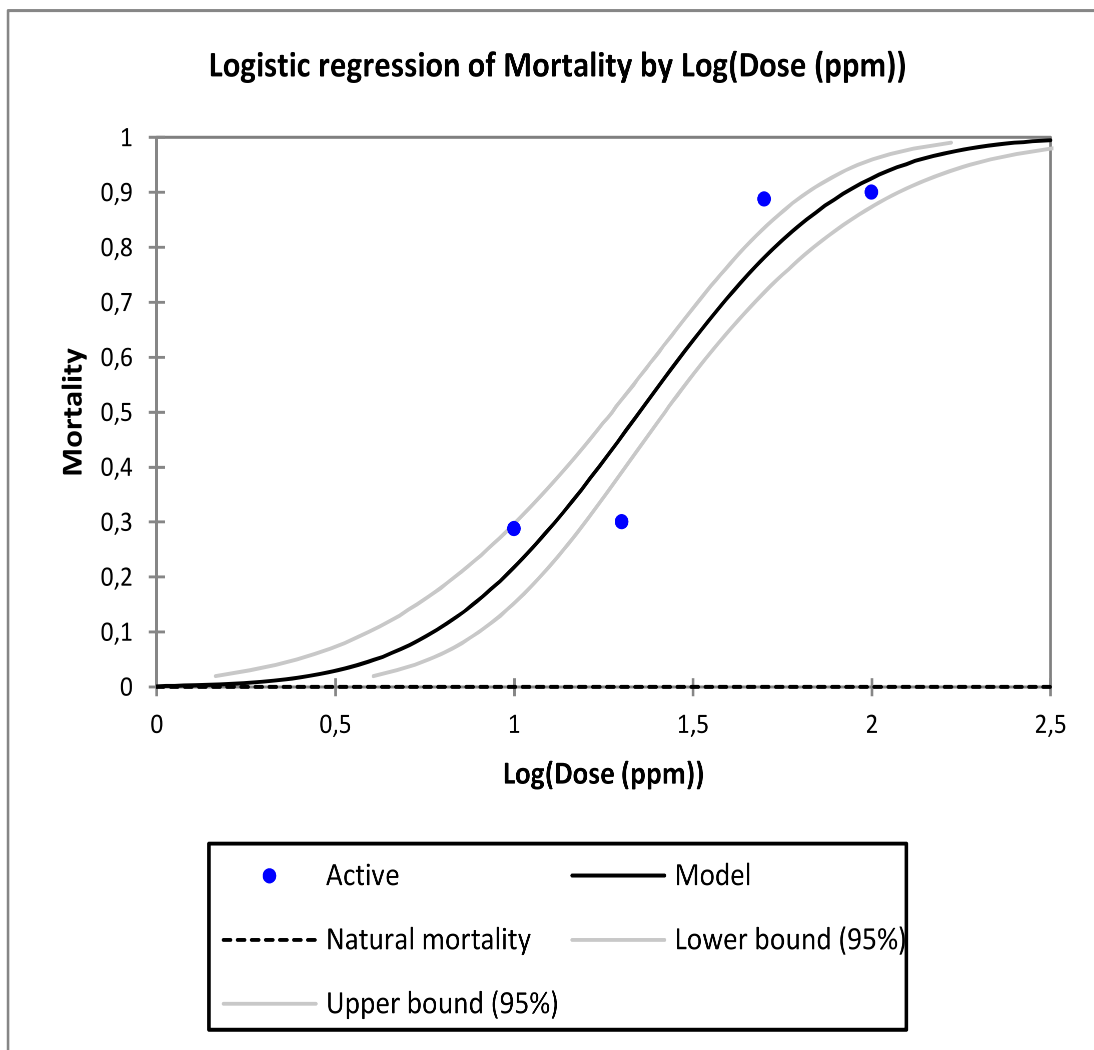

Fig 9: Dose-toxicity of the kernel oil (KO) extracted with hexane

These results confirmed the occurrence of acetogenins in the kernel oil extracted with hexane as it was noted in TLC analysis. The weak toxicity of the oil obtained by liquid/liquid partition could be induced by acetogenins in trace amount so that they were not detected in TLC analysis. For this reason, kernel oils obtained by the two methods are not suitable for consumption and their contact with living cells should be avoided as much as possible. However, they may be used in diverse areas of lipid chemistry for instance as intermediate for synthesis of surfactants [44, 45]. With high oleic content and saponification value, the kernel oil is appropriate for soap manufacturing. Methylated oil can serve as gasoil or lubricant for drilling machines. Oil has a myriad of potential applications: for treatment of wood, as industrial degreasing products, as fluxing for bitumen, as concrete form release agents or in the formulation of printing inks [49].

3.8 Residues of extraction, the insoluble matter

Insoluble matter (53.8% of kernels) remained after 13 hours of extraction of kernels with ethanol. Micro-Kjeldahl analysis of these residues showed a crude protein content of 25.6%. The average is ranging from 21.43% [20] to 27.34% [16].
The major part of these residues could be fibers. In fact, crude fiber content was estimated at 43.4% of kernels [16].
Due to these components, the residues may be considered as potential material for animal feeding.
During the feeding experiments, swiss mice tolerated residues of extraction 2.5 g, 5 g and 10 g mixed in their diet. No death was recorded. No metabolic changes or adverse effects were observed during the five days following the ingestion of the residues. Although these doses were administered once, the mice ate this for two days. Considering the period of exposure to residues and the frequency of administration, the results showed no subacute toxicity. Thus, the use of residues of extraction as animal feed is safe. However subchronic and chronic toxicity tests are necessary to assess the effects of a long-term ingestion of the residues.

4. Conclusion

Husks of soursop seeds are not worthy of extraction but they can supersede wood-fuel. The treatment of the kernels led to four components: oils, a mixture of acetogenins, a mixture of carbohydrates and residues containing fiber and crude protein up to 25%. These components are more valuable than the by-products themselves. These results showed potential valorization of soursop seeds. The methodology used was relatively simple, feasible in large scale and reproducible. Optimal yield was reached at each step of the treatment. The solvents used were weakly toxic, affordable and available in large amount. A summary of the potential valorization of soursop seeds is depicted in Figure 10.

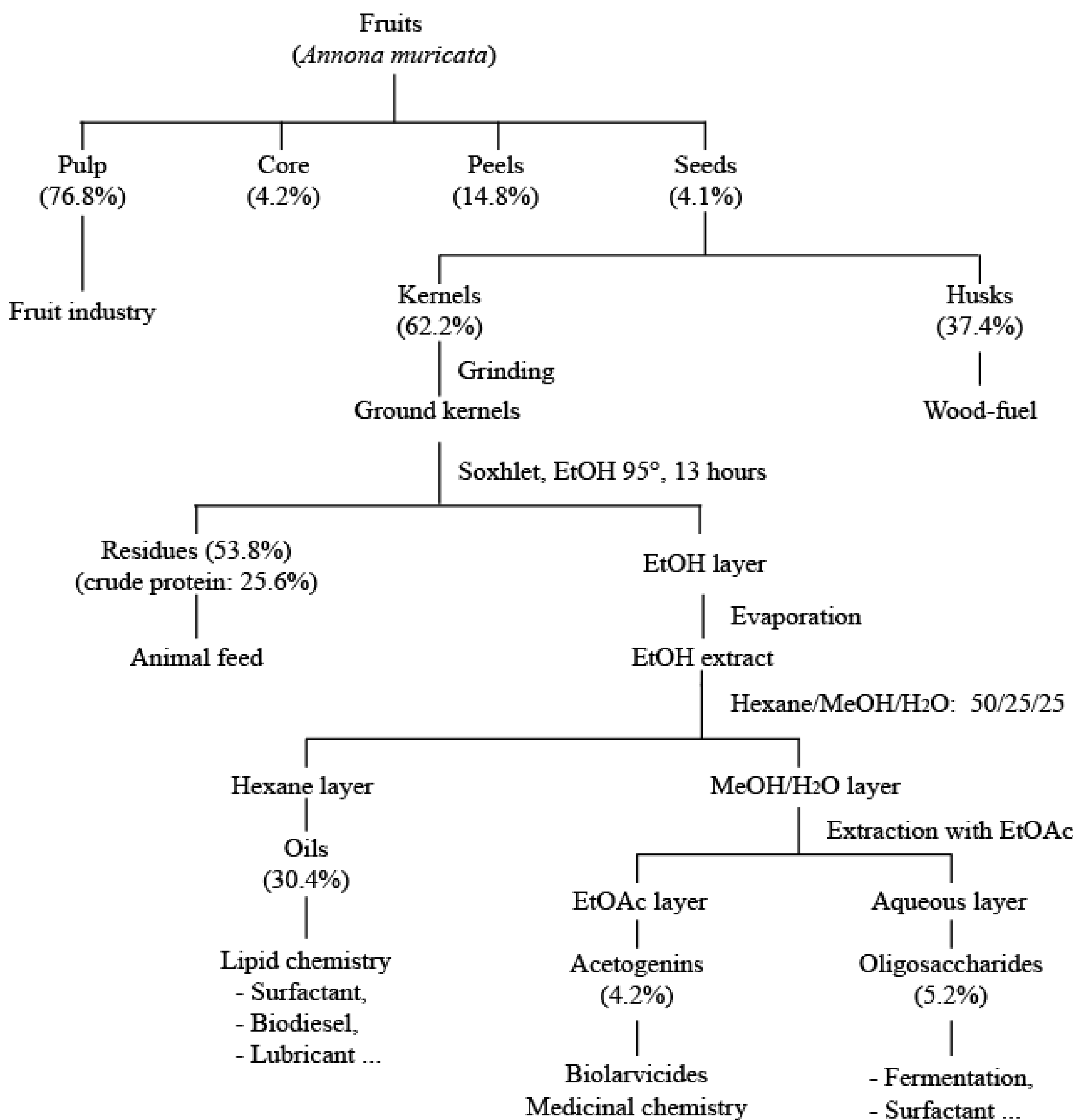

Fig 10: Method of soursop seed valorization

One kilogram of kernels can provide 304 g of oils, 42 g of mixture of acetogenins and 52 g of mixture of carbohydrates. The resultant oil was less toxic than the one extracted with hexane. It is not appropriate for dietary purpose but may be used in lipid chemistry. The mixture of acetogenins may be applied as biolarvicides but the toxicity should be reduced. Catalytic hydrogenation of annonacin, the major acetogenin of *Annona muricata*, can increase the larvicidal activity of the molecule.

The quantity of seeds depends closely on fruit biomass. Therefore, cultivation of the fruit should be promoted. Nevertheless, since atypical parkinsonism has been associated with the consumption of plants of the Annonaceae family [50], studies permitting a safe consumption is crucial. The valorization of soursop seeds is worthy and this current methodology can be applied to seeds of other species or genus of the Annonaceae family and even of other plants in general. Implementation of a pilot project is in progress. Treatment of by-products is a promising sector and is helpful to deal with waste issues.

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