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Bioherbicide from *Azadirachta indica* Seed Waste: Exploitation, Efficient Extraction of Neem Oil and Allelopathic Effect on *Senna occidentalis*

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Abstract: Bioherbicides are an alternative to minimize the damage caused to the environment using agrochemicals. This study had the objective of extracting neem oil from ripe waste fruits that *Azadirachta indica* A. Juss and optimizing the process using solvents (or a mixture of solvents) with different polarities. Then, through a solid-liquid extraction system (Soxhlet), the solvents hexane, methanol/hexane (1:1), ethanol, and hexane/ethanol (1:1) were used to determine the process with the highest yield and most efficiency. The physicochemical parameters of the extracted oil (density, acidity value, iodine value, saponification value, esters value, and molecular weight) and the % of free fatty acids were determined. In addition, the allelopathic properties of the oil (0%, 2%, 3%, and 4% *m*/*v*) on septic weed *Senna occidentalis* seeds were evaluated, analyzing their growth and development parameters (germination, germination speed, hypocotyl, and radicle length). Hexane was the most efficient (4 h) in neem oil extraction, with the highest yield (43%). It also provided a better oleic and linoleic acid content (41.3% and 18.6%), similar to ethanol extraction (41.1% and 20.22%). Moreover, the allelopathic properties were more prominent for the oils extracted with hexane and hexane/ethanol. This optimized process provides an efficient alternative to obtain a natural herbicidal potential for strategically controlling harmful plants.

Keywords: allelochemicals; agro-industrial-waste; germination; hypocotyl; radicle; Soxhlet

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1. Introduction

Currently, there is a concern regarding the use of industrial waste. This is because much of the raw material is wasted during processing in the food industry. Among them, juices, pulps, fruits, shells, and seeds obtained as residues stand out. However, it has been shown that adequately processing these agro-industrial residues can contain functional ingredients with applications in the pharmaceutical, medical, and agro(industrial) areas [1].

Brazil, rich in species of a great variety of native and exotic tropical fruits with great agro-industrial potential, generates a large volume of waste daily. Unfortunately, much of this waste is discarded, causing severe environmental damage [2,3]. The residues have a high potential since they contain vitamins, minerals, and sugars and are rich in fibers and oils. Incorporating special oils in the food, chemical, or pharmaceutical industry can improve the emerging need for new sources of oil. For this reason, it is essential to look for raw materials that provide oils with a lipid profile with traditional properties that are alternatives to phytosterols or phenolic compounds that are very useful in the herbicide industry [4].

Herbicides application is the primary method of weed control; moreover, they are effective. However, the cases where herbicides are ineffective include application errors (weed growth stage, application pressure, and wrong nozzle types) and the phenomenon of herbicide resistance (HR) [5]. Furthermore, due to the environment's dispersion capacity, these products affect the soil, water, and animals and compromise human health [5,6]. Therefore, the development of alternative non-chemical tools is considered essential. One of the most efficient strategies is using bioactive compounds found in plants. When released by plants into the environment, these secondary metabolites, called allelochemicals, can cause a phytotoxic effect, stimulus, or inhibition in the development of other plants, a mechanism known as allelopathy [7,8]. Therefore, the products extracted from plants have gained a place in the market because they are ecologically correct and can reduce the environmental impacts compared to synthetic agrochemicals [9].

Rich in various chemical compounds with biological activity and a promising source of organic products, the Indian neem species (*Azadirachta indica*) is popularly known as the "tree of life" for its versatile utilization [10–12]. In agriculture, for example, its extracts are used with greater emphasis as a bioinsecticide due to azadirachtin, which causes insect repellency [13,14]. The oil from this plant has demonstrated its potential effect as a natural herbicide [10,15]. Its phytochemical components containing phenol (or conjugates) or fatty acid groups play an essential role in allelopathic activity. It has been shown that neem can prevent the development of sleeping grass (*Mimosa pudica*) and septic weed (*Senna obtusifolia*) species at the lowest application concentrations [7,16].

Despite the positive effect of inhibition from neem, few studies in the literature aim to apply, more precisely, oil as a bioherbicide. Numerous extraction methods are used to obtain vegetable oils, which generally consist of extensive procedures, but with low yield and degradation of oil compounds. In this way, it is necessary to conduct studies to optimize the process parameters, aiming at a viable and efficient extraction and obtaining maximum quality and quantity of oil. The extraction method using organic solvents has stood out as being the most common, as it has the advantage of a good yield of the lipid material [17]. In addition, this procedure is recommended due to its simple methodology, which can be used in plant and animal origin samples, providing the various bioactive compounds in the extracted product [18–21].

Due to the efficiency of their use, bioherbicides have great potential to be used in Brazil, as they are an alternative to mitigate the adverse effects of pesticide application on the environment. Weed control through allelopathy is highly biodegradable, avoiding any toxic residue in agricultural cropping systems. Therefore, this study had the objective of taking advantage of the residue of neem fruit waste and optimizing the extraction process of neem oil with solvents (or mixtures of solvents) with different polarities. In addition, the allelopathic effect of the extracted oil on the germination and development of the septic weed under laboratory conditions was evaluated.

2. Results and Discussion

2.1. Oil Yield

The extraction yield of neem oil with different solvents or solvent mixtures is reported in Table 1. The solvents used were hexane (H), hexane/methanol (1:1), ethanol, and hexane/ethanol (1:1). The variables considered for these results were the extraction time and the type of solvent. Specifically: hexane yields ranging between 31.9% (2 h)–44.4% (12 h); hexane/methanol (1:1) ranging between 22.7% (2 h)–39.6% (12 h); ethanol ranging between 22.1% (2 h)–27.6% (12 h); and with hexane/ethanol (1:1) ranging between 25.2% (2 h)–26.5% (12 h).

Table 1. Average yield (%) values of Indian neem oil extracted via Soxhlet at different times (h) and solvents. Means transformed to Ln followed by equal uppercase letters in the column and lowercase letters in the row do not differ from each other in the Tukey test at the 5% significance level (ANOVA, $n = 3$). $T_{reflux} = 60 \pm 2$ °C. H = hexane; HM = hexane/methanol; Et = ethanol; HEt = hexane/ethanol.

Variable	t (h)	Solvent			
		H	HM	Et	HEt
Yield (%)	2	31.9 ± 1.1 Ba	22.7 ± 1.1 Cb	22.1 ± 0.3 BCb	25.2 ± 1.6 Bb
	4	43.4 ± 2.6 Aa	29.5B ± 0.1 Cb	29.9 ± 2.5 Ab	26.6 ± 1.1 ABb
	6	39.0 ± 1.6 ABa	33.0 ± 0.7 ABab	22.5 ± 2.5 BCc	26.6 ± 1.1 ABbc
	8	39.8 ± 2.0 ABa	35.9 ± 0.7 Aa	19.9 ± 0.8 Cc	25.5 ± 2.4 ABb
	10	43.7 ± 2.6 Aa	39.0 ± 1.2 Aa	24.7 ± 2.8 ABCc	31.0 ± 1.1 Ab
	12	44.4 ± 1.9 Aa	39.6 ± 1.1 Aa	27.6 ± 1.5 ABb	26.5 ± 2.0 Abb

For the hexane utilization, the 2 h period showed the lowest oil yield (31.9%), and the yield improved (−40%) for longer times (4–12 h) (Table 1). Hexane is an efficient extraction solvent, mainly in seeds [22]. In addition, time and temperature play an essential role in improving oil extraction efficiency [23]. On the other hand, hexane/methanol was not as efficient at 2 h (22.7%) and 4 h (29.5%) as hexane for oil extraction. Only after 6 h did the yield increase (33–40%) significantly for this mixture of solvents (Table 1). Here, it is also verified that the extraction time improves the yield [23]. Ethanol extraction had the best yield after 4 h (29.9%). However, this solvent presented lower performance values because it was subjected to temperatures below its boiling point. On the other hand, the high rate of evaporation and effusivity and the low viscosity of the solvent at a specific temperature keep the material and the solvent in contact for a longer time. Thus, the oil extraction yield can be increased [22]. Better extraction yields were obtained when the hexane/ethanol mixture was used only after 4 h (31%). A similar result was obtained when subjected to 60 °C for 6 h in a Soxhlet extractor as described in the literature (−35%) [24]. During 2–4 h of neem oil extraction, the highest yields were given (31.9% and 43.4%, respectively), unlike the yields obtained by the other tested solvents. Nevertheless, there was no difference between the hexane and hexane/methanol solvents for the other periods, which showed their best performance for oil extraction. Similar research shows that neem oil extraction can reach its maximum yield after 5 h [25].

2.2. Physicochemical Parameters of Neem Oil

The characteristic knowledge of the physical and chemical properties of an oil or fat is essential since they are parameters that allow the analysis of the lipid composition through several specific indices of the evaluated material, in addition to being able to identify possible changes in the compounds of the oil that may have been caused by the medium that was extracted [12,26]. Therefore, neem oil was subjected to physicochemical characterization after extraction, and its properties' values are shown in Table 2.

The density of neem oil extracted with the different solvents did not show significant differences (Table 2); these values are close to the commercial one (0.90 g/cm^3) [12,21]. Similar values are shown in the literature when neem oil was extracted with hexane and ethanol solvents, which were 0.88 and 0.90 g/cm^3, respectively [20,21]. Neem oil extracted with solvent mixtures slightly differed from commercial oil ($p < 0.05$, 6.15 KOH/g) [27]. The alteration in the triacylglycerides of oil usually comes from oxidation, hydrolysis, or fermentation generated by exposure to light or high temperatures. Then, the oil structure can be broken, releasing free fatty acids expressed in oleic acid [28,29]. The iodine value calculated from the oil extracted with hexane and ethanol is within the commercial value (65–80 g I_2/100 g) [30], as well as was reported in the literature by similar methods (72.8 g I_2/100 g) [14]. On the other hand, high values were found when the oil was extracted with hexane/methanol and hexane/ethanol, indicating many unsaturation or double bonds in the extracted product [30]. The wide range of iodine values can be interpreted as the variability of ecotypes of the species of neem seeds that can present values of 33–192 g

I_2/100 g as described in the literature [11]. However, it may be due to the different types of solvents for oil extraction, as shown by this study.

Table 2. Physicochemical properties (mean ± standard deviation) of Indian neem oil extracted by different solvents (ANOVA, * $p < 0.05$, $n = 3$). H = hexane; HM = hexane/methanol (1:1); Et = ethanol; HEt = hexane/ethanol (1:1).

Properties	H	HM	Et	HEt
Density (g/cm^3)	0.87 ± 0.02	0.80 ± 0.01	0.92 ± 0.02	0.83 ± 0.02
Acid value (mg KOH/g)	6.73 ± 0.61 *	7.93 ± 0.42	6.24 ± 0.32	8.48 ± 0.51 *
Iodine value (g I_2/100g)	74.9 ± 0.55 *	131.92 ± 0.70	75.00 ± 0.26 *	150.00 ± 0.18
Saponification value (g KOH/100 g)	159.68 ± 1.33 *	194.60 ± 1.10	184.78 ± 1.44	186.35 ± 1.30
Ester value	152.95 ± 1.80 *	186.67 ± 1.42	178.54 ± 1.20	177.87 ± 1.54
Molecular weight (g/mol)	1052.10 ± 1.40	863.30 ± 1.62 *	909.18 ± 1.48	901.52 ± 1.27
Free fatty acids (% *w*/*w*)	3.38 ± 0.20	3.98 ± 0.32	3.13 ± 0.30	4.26 ± 0.26 *

The higher values of unsaturation of the neem oil fatty acids reflect the lower value of its density and, consequently, its molecular weight [31]. The saponification value quantifies the high and low molecular weight of fatty acids that make up oils and fats; the lower the molecular weight of fatty acids in the oil, the greater its saponification value [32]. The oil extracted with hexane shows a lower value (159.68 g KOH/g), differing significantly from that when using other solvents, and the commercial value (175–205 g KOH/g). Similar studies with the same extraction method and conditions found values between 167 g KOH/g and 195 g KOH/g, keeping a dependence on the type of seed [18,33]. No ester values were found in the literature for neem oil. However, it was found that values close to 170 KOH/g for copaiba oil are potentially used in medicine and the cosmetics industry [34].

2.3. Characterization of the Fatty Acid

The fatty acid composition of Indian Neem seed oil is shown in Table 3. Regardless of the solvent used in the extraction, the oil showed a wide range of saturated fatty acids. However, the unsaturated acids present the highest percentage, except for the oil extracted with hexane/methanol.

Table 3. Fatty acid composition of neem oil *Azadirachta indica* A. Juss extracted identified by GC-MS analysis. Solvent used in the extraction: H = hexane; HM = hexane/methanol (1:1); Et = ethanol; HEt = hexane/ethanol (1:1).

Fatty Acid	Area (%)			
Saturated	**H**	**HM**	**Et**	**HEt**
Palmitic acid (C16:0)	18.10	13.55	18.85	15.12
Behenic acid (C22:0)	0.14	5.42	0.21	–
Stearic acid (C18:0)	14.97	11.47	14.99	14.88
Arachidic acid (C20:0)	0.78	1.87	–	1.03
Lignoceric acid (C24:0)	–	4.74	–	–
1,1,2,2-tetramethylcyclopropane	–	13.74	0.72	3.74
Unsaturated				
Oleic acid (C18:1)	41.33	16.86	41.08	39.48
Linoleic acid (C18:2)	18.60	5.04	20.22	12.41
Total area (%)	93.92	72.69	96.07	86.66
Others	6.08	27.31	3.93	13.34

The extraction with the solvents showed in common the palmitic and stearic acids, which are the main constituents of neem oil [18,20,33]. On the other hand, oleic acid shows a more significant content in unsaturated acids for all solvents used, highlighting hexane (41.08%) and ethanol (41.08%). Previous studies showed similar results when using hexane as a solvent (41.09%) [35]. The excellent solubility or relative polarity of the

solvents hexane (nonpolar, van der Waals force, relative polarity = 0.009) and ethanol (polar: hydrogen bonding, and nonpolar portion: Van der Waals force, relative polarity = 0.654) played an essential role in extracting fatty acids [36]. The lower percentages correspond to hydrocarbons that make up neem oil [11,37].

2.4. Germination and Seedling Development Bioassays

The allelopathic effect of neem oil on *Senna occidentalis* germination was evaluated for concentrations of 0% (control), 2%, 3%, and 4%. This effect was assessed for the different extracts of neem oil extracted with hexane, hexane/methanol (1:1), ethanol, and hexane/ethanol (1:1). Neem oil showed favorable responses since it could inhibit the germination of septic weed (*Senna occidentalis*) seeds, mainly when hexane was used in its extraction. Thus, germination tests made it possible to determine the allelopathic effect of neem oil. During the germination process, water, as the primary carrier of nutrients, can carry allelopathic substances capable of inhibiting or retarding the growth or multiplication of cells, thereby totally or partially retarding germination [9,38].

Figure 1A significantly impacts septic weed germination for neem oil extracted with hexane and ethanol solvents. The oil extracted with hexane reduced germination by 64% ($p = 0.011$); also, extraction with ethanol and hexane/ethanol reduced germination up to 53% ($p < 0.001$) and 70% ($p = 0.022$), respectively. These results were observed for the concentration of 4% oil. Before that, there were no differences in their allelopathic activity. On the other hand, the same inhibitory activity was not shown in the extraction with ethanol. The germination delay was progressive with concentrations ($0 \rightarrow 4\%$) for oil extracted with hexane and ethanol (Figure 1B). Oil (hexane) delayed germination sped up to 42% with a 4% concentration. The oil (ethanol) was more efficient, delaying from 20%, 40%, and 80% ($p < 0.001$) for concentrations of 2%, 3%, and 4%, respectively. The oil (hexane/ethanol) showed similar results with germination. Nevertheless, curiously, the ethanol that managed to reduce germination up to 70% did not show activity in the delay in all comparative concentrations. The development of the septic weed was affected by the neem oil extracted with all the solvents (Figure 1C,D). When exposed to concentrations of 3% and 4% of the oil (hexane) on the septic weed, a significant decrease in radicle length of 46% and 85% ($p < 0.001$) was observed, and only for the concentration of 4%; there was a significant reduction ($p < 0.001$) of the hypocotyl (40%). The oil (hexane/methanol) caused a decrease in the radicle (75%, $p < 0.001$) independent of the increase in concentrations from $2 \rightarrow 4\%$ and only with a 4% reduction in the hypocotyl (67%, $p < 0.05$). On the other hand, the oil (ethanol) caused a delay in the development of the radicle and hypocotyl for all the concentrations evaluated from 2 to 4%. The increase in concentration was independent of the activity, achieving a decrease in these parameters of up to 50% ($p < 0.05$). Oil (hexane/ethanol) had an inhibitory effect on these septic weed development parameters. This essence caused a decrease in the size of 69% ($p = 0.012$) in the radicle when exposed to 4%. On the other hand, this oil caused a 48% ($p = 0.002$) hypocotyl decrease when exposed to 3% and 4%.

Our results show that neem oil extracted with different solvents and concentrations can inhibit septic weed germination and development. Furthermore, we observed that the allelopathic effect of neem extracts, regardless of the type of solvent used, was more prominent in plant development. Both radicle and hypocotyl slowed their development with increased extract concentration in all the tests. These findings can be corroborated with previous studies that were carried out on lettuce, wheat, alfalfa, tiara, grass, sand fescue, and *E. colonum* in low concentrations of neem oil (<4%) [15]. Similar results showed that neem oil bioactivity in plant development might be more pronounced than germination [39,40]. It has been reported that the permeability of the extracts by increasing the concentration can be more efficient and thus interrupt the plant's normal development [41]. In addition, the phytotoxic activity can inhibit cell division, which is very active from the root, inhibiting growth [42].

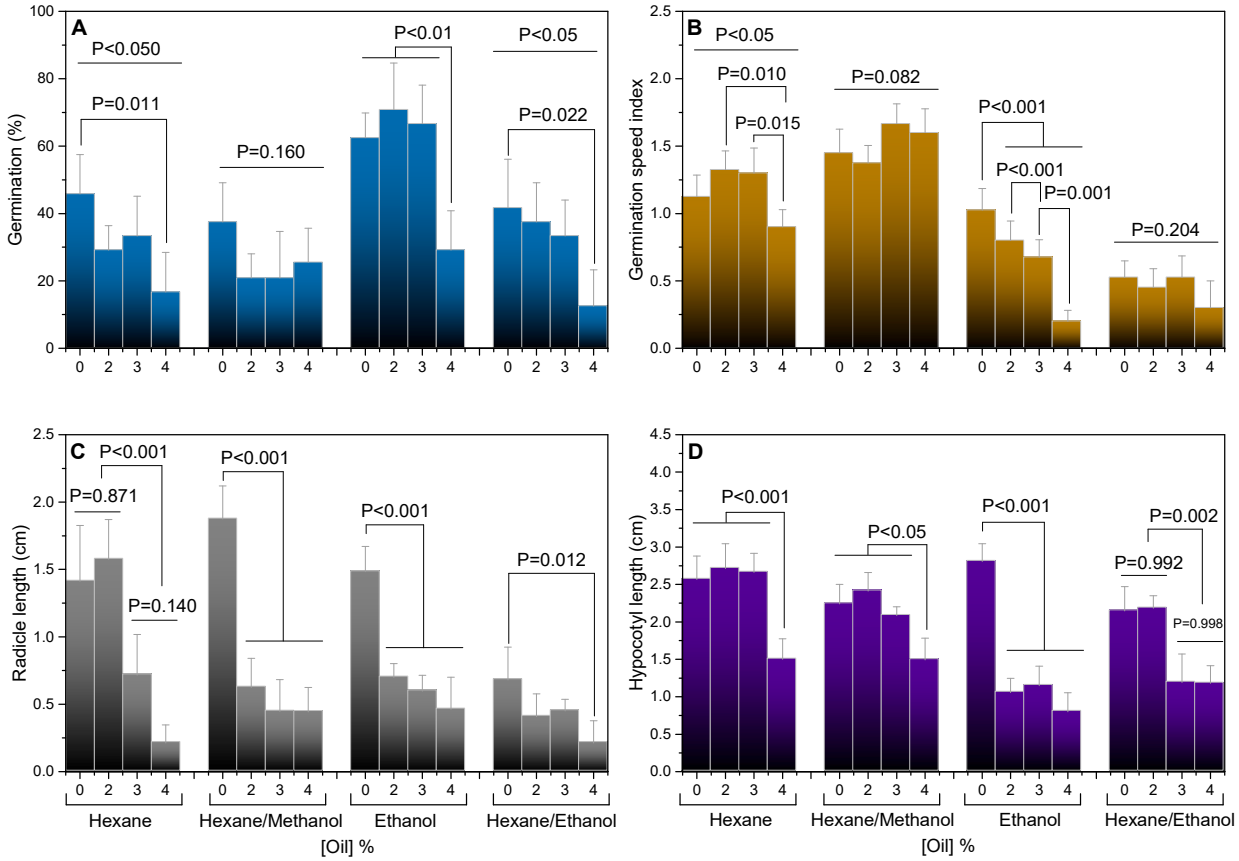

Figure 1. Allelopathic effect of germination and seedling growth of septic weed (*Senna occidentalis*) submitted to different concentrations of essential neem oil *Azadirachta indica* A. Juss. (**A**): percentage of germination (%G), (**B**): Germination Speed Index (Gs), (**C**): Radicle length (R), (**D**): Hypocotyl length (H). The results are presented as mean ± standard deviation. p values < 0.05 represent the significative differences in each group of neem oil concentrations (ANOVA, n = 3).

The multivariate analysis indicated that the original set of variables could be reduced to three new factors, which explained 94.1%, 93.5%, 95.8%, and 92.1% of the total variance for the neem oil that was extracted with the different solvents: hexane, hexane/methanol, ethanol, and hexane/ethanol, respectively (Table 4). For the oil extracted with hexane, the first main factor (F_1) explained 53.54% of the variance and highlighted that the allelopathic properties stand out for %G, R, and Gs. The second factor (F_2) explains 23.70% of the variance, indicating a greater influence on H and a negative association for %G. The third factor (F_3) explained 16.89% of the variance, and the Gs presented a positive relationship. For the oil extracted with hexane/methanol, the main factor F_1 explained 51.70% of the variance and highlighted the allelopathic properties for %G, Gs, and R. The F_2 explained 28% of the variance, representing an important relationship for H. F_3 explained 13.8%, where a positive relationship was obtained for Gs and H and negative for R. For the oil extracted with ethanol, F1 explained 47.5% of the variance, highlighting a greater influence for H and R, in addition to %G and Gs. F_2 explained 30.34% of the variance, highlighting a positive relationship for Gs, and %G and negative for R. F_3 explained 18% of the variance, showing a positive influence for %G and negative for R and H. Finally, for oil extracted with hexane/ethanol, the F_1 explained 42.73% of the variance, indicating a greater influence on the allelopathic properties for the %G, in addition to R and Gs. The F_2 explained 27.38% of the variance, indicating a positive relationship for R and H and a negative one for Gs. The F_3 explained 21.99% of the variance, showing a representative positive influence for Gs, a positive relationship for H, and a negative one for R.

Table 4. Sorted rotated factor loadings of the original variables on the four principal factors from factor analysis, employing main components analysis as the extraction procedure. Three factors were extracted considering eigenvalues higher than 1.0 (Kaiser's criteria). Only variables with loadings > 0.30 to a particular factor were associated with the respective factor. %G: germination percentage; Gs: germination speed index; R: radicle length; H: hypocotyl length.

Hexane			
	Factor 1	Factor 2	Factor 2
Variance (%)	53.54	23.70	16.89
% G	0.84	−0.43	
Gs	0.75		0.62
R	0.81		−0.52
H	0.48	0.86	
Hexane/Methanol			
	Factor 1	Factor 2	Factor 3
Variance (%)	51.70	28.04	13.80
% G	0.68		
Gs	0.67		0.34
R	0.66	0.51	−0.53
H		0.91	0.36
Ethanol			
	Factor 1	Factor 2	Factor 3
Variance (%)	47.50	30.34	18.00
% G	0.64	0.45	0.62
Gs	0.56	0.72	
R	0.70	−0.64	−0.34
H	0.83		−0.34
Hexane/Ethanol			
	Factor 1	Factor 2	Factor 3
Variance (%)	42.73	27.38	21.99
% G	0.91		
Gs	0.59	−0.31	0.72
R	0.73	0.48	−0.38
H		0.88	0.45

The allelopathic responses were recorded after ten days of exposure to the extracts. It means that the low concentrations used in this study may cause inhibition in the germination and growth of septic weed seedlings in the early stages of development. The inhibition or delay of germination was observed for one month. It may mean that the concentrations tested may still be too low to cause total inhibition and growth of the plants. These results are in harmony with previous studies that report that 5% and 10% of aqueous neem extract is sufficient to stimulate this response in the length of roots and shoots, as was shown for *Vigna radiata* and some cowpea varieties, respectively [39,40]. Other studies corroborate that neem oil can stimulate the delay in germination in low concentrations, as they showed for *Crotalaria ochroleuca* and *Senna sophera* [43]. However, low concentrations of neem oil may probably not be enough to counteract the effect of phytochemical components (flavonoids, saponins, alkaloids, tannins, and terpenes) present in *Senna occidentalis* [44]. These components can exert a protective effect that reduces the phytotoxic effects of neem oil, as shown in studies with *Zea mays*.

On the other hand, many allelochemicals at low environmental concentrations can improve the defense system by stimulating protein synthesis due to the increase in amino acids in the seedling protein [45,46]. The allelopathic effect of neem oil may be a result

of the presence of the fatty acids described in this study. In not-so-dilute concentrations, fatty acids and phenols, individually and together, can exert an allelopathic effect that can interfere with crucial metabolism enzymes [7] and cell division [47]; therefore, they can inhibit growth, as was shown for *M. spicatum* on *M. aeruginosa* [48]. In addition, it has been described that fatty acids, mainly oleic and linolenic acids, can exert allelopathic activity, interrupting the photosynthesis process [49]. Although several authors [8,50,51] hold the allelopathic activity to phenols in this pathway, it has been shown that the fatty acid content can exert a similar effect as described in this study.

3. Materials and Methods

3.1. Solvent and Reagents

All solvents (Merck, analytical grade) were redistilled before their use. Reagents were purchased from Sigma-Aldrich (MilliporeSigma, St. Louis, MO, USA). Water was deionized using a Milli-Q water purification system (18.2 MΩ cm^{-1} at 25 °C, total organic carbon $\leq$ 4.0 µg L^{-1}, Milli-Q, Millipore, MA, USA).

3.2. Experiment Location

This study was carried out in two experimental stages: (i) the oil extraction process from *Azadirachta indica* seed waste and (ii) the application of oil extracts on the septic weed *Senna Occidentalis*. They were carried out respectively at the Chemical Analysis Laboratory (LAQ) and at the Ecology Laboratory, both located at the Center for Agricultural and Environmental Sciences of the Federal University of Maranhão, Campus Universitário de Chapadinha—MA, Br 222–km 06, S/N–Boa Vista—CEP 6,550,000, with coordinates 03°44′28.7″ S and 43°18′46″ W and altitude of 107 m.

3.3. Experimental Design

The experimental design was completely randomized in a factorial scheme for the two experimental stages: (i) It consisted of 6 × 4, with six times (2, 4, 6, 8, 10, 12 h) and four solvents (hexane, hexane + methanol, ethanol, hexane + ethanol), for a total of 24 treatments and three repetitions. (ii) it consisted of 4 × 4, referring to neem oil extracted by the four solvents mentioned above, and four concentrations of oil extracts (0%, 2%, 3%, and 4%), for a total of 16 treatments and four repetitions.

3.4. Collection and Processing of Organic Waste

3.4.1. Neem oil Source

The fruit waste of Indian neem *Azadirachta indica* was obtained in the free market from Chapadinha, Maranhão, Brazil (Latitude: −3.7405, Longitude: −43.3593; 3°44′26″ South, 43°21′33″ West).

3.4.2. Seed Treatment

The fruits were collected and taken to the laboratory to remove all impurities through washing. Then, they were pulped, and their seeds were placed in paper bags for drying for 72 h in an air circulation oven at a constant temperature of 60 ± 2 °C. Subsequently, the seeds were grounded and sieved with a processor and 200 mesh sieves. For the germination and seedling development bioassay, seeds of septic weed (*Senna occidentalis*) were used. These were also harvested manually when ripe, showing good quality. After being collected, the seeds were taken to the laboratory for cleaning and classifying the best ones. Then, as a chemical method for overcoming dormancy, the seeds were immersed for 2 min in sulfuric acid [16]. After this period, the seeds were washed in running water to remove all excess acid, dried, and stored for the bioassay assembly.

3.5. Neem Oil Extraction

A Soxhlet-type extractor was used for oil extraction, exposing the sample to the heated solvent under continuous reflux and transferring the oil to the solvent [52]. In the process,

the solvent initially found in volumetric flasks, allocated under a heating plate at the bottom of the apparatus, rises in vapor to the condensers, where it liquefies. It is then transferred to the compartment where the material to be extracted is located. Upon reaching the level of the side tube, the solvent drags the oil and is released back into the flask. This process was repeated throughout the operating period. Cartridges of qualitative filter paper containing 5 g of the seed sample were prepared for this. The cartridges were taken to the extractor and coupled to the device to start the extraction. In previously weighed volumetric flasks, which remained on the hot plate, 150 mL of each solvent was placed. After extraction, the flasks with the solvent/oil mixture were taken to a rotary evaporator (Fiastom, model 802, SP, Brazil) for recovery and separation of the solvent and the oil obtained, which was quantified by weighing for the yield calculation Equation (1). The oil was packed in glass containers and stored under refrigeration temperature until its use.

$$R(\%) = \frac{EO}{W} \times 100 \quad (1)$$

where: R(%) = yield; EO = extracted oil (g); W = sample mass (g).

3.6. Physicochemical Characterization

After extracting the oil, the physical-chemical characterization was carried out to evaluate the following parameters: density (D), acid value (Av), iodine value (Iv), saponification value (I_S), ester value (Ev), molecular weight (MW), and percentage of free fatty acids (%FFA).

3.6.1. Density

The weight and volume ratio determined the oil density at 25 ± 0.5 °C. In a 5 mL volumetric flask, the oil was placed up to the meniscus and then weighed on a digital analytical balance [53].

$$D = \frac{w}{v} \quad (2)$$

where: D = density; w = sample weight in the flask (g); v = flask volume (mL).

3.6.2. Acid Value (Av)

The acid value is defined as the amount in grams of potassium hydroxide needed to neutralize the fatty acids present in the oil. The procedure consists of weighing 2 g of the oil sample in a 125 mL Erlenmeyer, adding 25 mL of the prepared ether-alcohol solution (2:1) plus two drops of 1% phenolphthalein indicator and titration with the sodium hydroxide solution (NaOH) 0.1 mol L^{-1} previously prepared and standardized, until the change from colorless to pink occurs, persisting for 30 s. With the volume spent in the titration recorded, the calculation of the acid value was determined [54].

$$Av = \frac{V * f * 5.61}{w} \quad (3)$$

where: Av = acid value (mg NaOH/g); V = volume of 0.1 mol L^{-1} NaOH solution used in the titration (mL); f = NaOH solution correction factor; w = sample weight (g).

3.6.3. Iodine Value by the Wijs (II) Method

This analysis determines the degree of unsaturation of the oil, considering that iodine reacts directly with the double bonds present. Then, it is verified that the greater the number of double bonds in the oil, the greater the iodine value.

For this, 0.25 g of oil was weighed into a 250 mL Erlenmeyer flask, and 10 mL of carbon tetrachloride (CCl_4) and 25 mL of Wijs solution were added. This solution was kept in the dark for 30 min. Then, 10 mL of potassium iodide (KI) and 100 mL of distilled water were added to the sample and titrated with 0.1 mol L^{-1} sodium thiosulphate ($Na_2S_2O_3$)

until the solution turned yellowish. Finally, 2 mL of starch indicator solution (1%) was added, and the titration continued until the solution's blue color disappeared [55].

$$\mathrm{Iv} = \frac{(\mathrm{V}' - \mathrm{V}) * \mathrm{f} * 1.27}{\mathrm{W}} \quad (4)$$

where: Iv = iodine value (I_2/100g); V′ = volume of sodium thiosulphate (0.1 mol L^{-1}) used in the blank titration (mL); V = volume of sodium thiosulphate used in the sample titration (mL); f = sodium thiosulphate solution factor (0.1 mol L^{-1}); W = sample weight (g).

3.6.4. Saponification Value (Is)

The saponification value determined the amount of high and low molecular weight fatty acids that make up the oil. Two grams of oil were placed in a 250 mL flask, then 20 mL of 4% potassium hydroxide (KOH) solution was added. Next, the sample was brought to reflux adapted in a water bath and heated to 80 °C for 30 min. After waiting for the sample to cool, it was titrated with 0.5 mol L^{-1} hydrochloric acid (HCl), using 1% phenolphthalein as an indicator [56].

$$\mathrm{Is} = \frac{\mathrm{MW} * \mathrm{V} * \mathrm{C} * \mathrm{f}}{\mathrm{W}} \quad (5)$$

where: Is = saponification value (g KOH/100 g); MW = molecular weight of KOH; V = volume of HCl used in the blank titration minus the volume of HCl used in the sample titration; C = HCl concentration; f = HCl correction factor; W = sample mass.

3.6.5. Free Fatty Acids (%FFA)

The determination of the percentage of free fatty acids present in the oil is expressed in oleic acid, and for this purpose, the value of the acid value found was divided by 1.99 [32].

3.6.6. Ester Value (Ev)

Ev was obtained through two values of acid value and saponification value, or ester value is defined as the quantity of KOH in milligrams required in the saponification of a gram of the sample minus the value used for the neutralization of two fatty acids. For the calculation of this value, the following equation was used:

$$\mathrm{Ev} = \mathrm{Is} - \mathrm{Av} \quad (6)$$

where: Ev = ester value; Is= saponification value; Av = acid value.

3.6.7. Molecular Weight (MW)

The average molecular weight of neem oil was calculated according to the methodology described [57]. After obtaining the value of the saponification value in the oil, the molecular weight was determined by the following equation:

$$\mathrm{MW} = \frac{168,000}{\mathrm{Is}} \quad (7)$$

where: MW = average molecular weight; Is = saponification value; 168,000 = constant value for the molecular weight of oils.

3.7. Characterization of Fatty Acids

Analysis of the oil's fatty acid profile was performed using gas chromatography (CG). For this, the esterification of the samples was performed before reading in the chromatograph [58,59].

Briefly, a 500 mg neem oil sample was weighed in a 50 mL volumetric flask, where 5 mL of 0.5 mol L^{-1} methanolic NaOH solution was added. The solution led to a reflux system, remaining there for 5 min. After this period, 15 mL of the esterifying reagent previously prepared (2 g of ammonium chloride + 60 mL of methanol + 3 mL of sulfuric acid refluxed

for 15 min) was added, keeping the sample under reflux for another 10 min. Then, the sample was transferred to a separatory funnel and washed with a solution containing 50 mL of distilled water and 25 mL of hexane. The aqueous phase was discarded, and a 1 µL aliquot was taken from the organic phase for GC analysis.

The sample was analyzed in a Gas Chromatograph (CG–2010, Shimadzu, Tokyo, Japan) coupled with a Mass Spectrometer (CG-MS QP2010 Ultra, Shimadzu, Tokyo, Japan). Chromatographic separation was achieved using a ZB–5HT capillary column (30 m × 0.25 mm × 0.25 µm). The carrier gas was helium, with the carrier flow at a linear speed of 30 cm s^{-1} and column flow of 1.0 mL min^{-1}. The oven was programmed at 90 °C for 3 min with a heating ramp of 5 °C min^{-1} to 180 °C. It remained for 8 min, being heated again at a rate of 10 °C min^{-1} to 230 °C, remaining up to 14 min. The injector and ion source temperatures of 250 °C and 200 °C, respectively. Split injection mode with a ratio of 1/10. Duration = 48 min.

3.8. Germination Bioassay and Seedling Development

The germination and plant development bioassays were carried out in petri dishes with a diameter of 9.0 cm, in a germination chamber of the brand Eletrolab, model EL 202 (Vila Dom Pedro II, Sao Paulo, Brazil), with a constant temperature of 25 °C and a photoperiod of 12 h, for 10 days. The dishes and the qualitative filter paper (11 µm, used to coat the dishes) used were previously sterilized at 150 °C for 2 h. In each container, 3 mL of the test solution, prepared by diluting the extracted neem oil, was added to concentrations of 2.0%, 3.0%, and 4.0% m/v. The control treatment consisted only of the application of distilled water. After evaporation of the solvent, 6 seeds and an equivalent volume of distilled water were added to each container, thus maintaining the initial concentration. Seed germination was monitored every day. The first count was performed 24 h after setting up the bioassay and later with daily counts, considering those with root extension of approximately 0.2 cm as germinated. Finally, the seedlings were carefully removed, and the radicle and hypocotyl lengths were measured with a vernier. The fresh mass was determined by weighing the seedlings on an analytical balance. Next, the seedlings were placed to dry in a forced air oven at 60 °C for 48 h and weighed again to obtain the dry mass. The inhibitory effect of oil on germination was calculated through Equation (8) to determine the percentage of germination G and Equation (9) for the germination speed index (G_s).

$$G(\%) = \frac{GS \times 100}{6} \quad (8)$$

where: G(%) = germination percentage; GS = germinated seeds.

$$Gs = \frac{G1}{N1} + \frac{G2}{N2} + \ldots \frac{Gn}{Nn} \quad (9)$$

where: Gs = germination speed index; G_1, G_2, G_n = number of seeds germinated until the nth day; N_1, N_2, and N_n = number of days to germination.

3.9. Statistical Analysis

The data were expressed as mean or mean ± standard deviation of the means for neem oil extraction yield, physicochemical proprieties, fatty acid content (%), and allelopathic effect. The Kolmogorov–Smirnov test verified the normalityand the homogeneity through Levene's test. A one-way analysis of variance (ANOVA) followed by the Tukey post hoc test was used to compare oils extracted with different solvents between yield or allelopathic properties [60,61]. All treatments were performed for each experiment in triplicate (n = 3). Data were analyzed using the SigmaStat 3.2 software (Systat Software Inc., San Jose, CA, USA) with one-way analysis of variance (ANOVA). Significant differences among the concentrations were defined as $p < 0.05$. Figures and data fitting were produced using OriginPro version 2019b (OriginLab Co., Northampton, MA, USA). Multivariate analysis was applied to complete the data set to identify links between the type of extraction solvent

with the allelopathic effects. After that, factor analysis and principal components analysis (PCA) were employed to analyze the original data set. For the factor analysis, the variables were auto-scaled (normalized) to be treated with equal importance. Variables having loadings > 0.30 to a particular factor were considered associated with the respective factor. The program used was R 4.2.3 for PCA analysis. The level of significance adopted was 5%.

4. Conclusions

Using a simple chemical treatment, neem oil has been successfully extracted from *Azadirachta* indica seeds waste. The use of a low-polar solvent improved the extraction yield. Thus, the hexane solvent presented better yield (−40%) and efficiency (4 h) in the solid-liquid extraction of neem *Azadirachta indica* oil. Increasing the polarity of the extraction solvent can affect the extraction yield without significantly altering the physicochemical properties of the extract. Hexane and ethanol and their mixture can be a good alternative for extracting neem oil rich in oleic and linoleic acid. In addition, the effect of inhibiting, retard germination, and altering the development of the septic weed *Senna occidentalis* is prominent. In general, neem seed oil has the potential as a natural herbicide as a strategy in the management of harmful plants, with particular emphasis on the management of septic weed that is relevant in the bioeconomic role.

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