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RESEARCH ARTICLE

Vitex negundo L. oil nanoemulsion for the ecofriendly management of *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst) in stored rice

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Abstract

The widespread use of synthetic chemicals as storage protectants makes food hazardous, endangers human health and develops insect resistance. Hence, in the present study *Vitex negundo* L. oil nanoemulsion (VNO NE) was prepared to manage stored grain pests. *V. negundo* oil (VNO) had major compounds like Aromandendrene, β-caryophyllene, Squalene, 3-octen-5-yne,2,7-dimethyl-, (E)-, 5-(1-isopropenyl-4,5-dimethylbicyclo[4.3.0]nonan-5-yl)-3-methyl-2-pentenol acetate, Farnesyl bromide, 4-terpeneol and Elemol. A high-speed homogenizer was used to formulate nanoemulsions of VNO and studies on their physico-chemical and thermal stability revealed that, the optimum nanoemulsion had 5% VNO mixed at a 1:2 (w/w) ratio with tween 80 surfactant. The hydrodynamic diameter, polydispersity index and mean zeta potential of the nanoemulsion were 166.62 nm, 0.263 and -3.4 mV respectively and droplet sizes varied from 50 to 200 nm in transmission electron microscopy. Lethal dose 50 (LD_{50}) values for contact toxicity of VNO nanoemulsion (VNO NE) were 0.755 and 3.131 μL cm^{-2} against *Sitophilus oryzae* and *Tribolium castaneum* respectively which were 41.60 and 29.88% less compared to VNO. In case of fumigant toxicity, LD_{50} value of VNO NE was 322.28 μL L^{-1} against *S. oryzae* which was 26% less than that of crude oil. Highest repellency increased by 33.33 and 36.58% when treated with VNO NE in *S. oryzae* and *T. castaneum* respectively. Also significant Glutathione S-transferase enzyme inhibition activities observed in VNO NE treated insects as compared to VNO and control. Thus, VNO NE having improved efficacy and targeted delivery could contribute towards eco-friendly sustainable stored grain pest management in rice.

Keywords

Vitex negundo L. oil; GC-MS; nanoemulsion; fumigant toxicity; contact toxicity; glutathione S-transferase activity

Introduction

Storage is essential for reducing wastage and maintaining food security. Post-harvest storage loss caused by stored grain pests are a global issue since they affect both grain quality and quantity. Insect damage accounts for 2-4.2% of total storage loss (1) in India, it causes a direct and indirect loss close to Rs. 1300 crores annually (2). More than 600 species of coleopteran beetles, cause storage loss by damaging and contaminating the goods (3).

Rice, consumed by 1.6 billion people throughout the world as a primary food source of calories and proteins (4). Additionally, it is rich in carbohydrates, micro-nutrients and vitamins that are required for growth and nutrition of human being. Rice is either boiled or crushed into flour. A variety of by products like breakfast cereals, noodles and soups can be made from rice. Rice suffers enormous losses due to cosmopolitan stored grain pests like *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst). According to United Nations (UN) studies, *S. oryzae* (L.) and *T. castaneum* (Herbst) are the 2 foremost stored grain pests of rice globally having 90% damage potential within 5-6 months of infection (5). The *Sitophilus oryzae* bores a hole in the kernel and deposits her egg therein and reducing kernels to mere powder (6). The red flour beetle, *T. castaneum* Herbst (Coleoptera: Tenebrionidae) is one of the major secondary pests of stored commodities and is more destructive due to high reproduction rate (7). Insect feeding not only lessens grain weight, nutritional value and germinability but also contaminates grains, induces odor (due to noxious secretions, exuviae and feces from storage pests) that affects the grain quality and mass market by making it unsuitable for human consumption (8, 9).

From several decades, synthetic pesticides and fumigants like methyl bromide and phosphine has been used enormously to control storage pests (10). Repeated use of these chemicals, make the food toxic due to a rise in their effective concentration and the environment unsafe for humans and non-target organisms due to their hazardous residues and slow deterioration rate. Also pests began to develop resistance to these frequently used chemicals. In India and Australia, pests develop high resistance towards phosphine in many occasions that cause failure in pest control (11). Due to all these drawbacks, entomologists are shifting their focus to botanicals with a special emphasis on essential oils as grain protectants. Botanicals in form of phyto-products such as plant parts (leaf, seed, bark and root), aqueous or solvent extracts, powders and volatile oils can be used as novel and more pleasant replacements for synthetic pesticides (12). They exhibit toxicity towards large number of storage insects with minimum chance of developing resistance in pests (13). Essential oils from plant families like Acoraceae, Asteraceae, Apiaceae, Lamiaceae, Myrtaceae, Lauraceae and Rutaceae are aromatic and highly volatile present in different parts like leaves, rhizomes, fruits and bark of plant those have lethal effect on pests (14). Secondary metabolites like terpenoids, phenolics and alkaloids found in these oils contain toxicant, fumigant, antifeedant, repellant and oviposition deterrent properties that interfere with pests' biochemical, physiological and metabolic processes (15).

More than 2000 species of plants contain phytochemicals having insecticidal properties against storage pests (16). Nirgundi (*Vitex negundo* L.), a member of the Verbenaceae family, is a deciduous shrub that grows across India in wastelands, mixed open forests at an elevations of up to 1500 metres. It has 4-10 cm long, smooth, petiolate, exstipulate leaves with aromatic properties (17). Essential oil from *V. negundo* contains bioactive compounds like iridoids, iridoid glycosides, lignans, flavonoids, flavones glycosides, sterols, polyphenols and terpenoids, those are crucial for pest management (18). *V. negundo* L. leaf extracts show insecticidal properties against *S. granaries* and *T. castaneum* (19, 20). Despite meeting several requirements to be an efficient weapon, essential oil-based pesticides have a number of limitations, including water insolubility, quick degradation, susceptibility to flocculation, creaming and phase separation owing to Oswald ripening (21).

An emerging method to address these limitations is nanoemulsion formulation, where the droplet size ranged between 0.1 to 200 nm and droplets typically do not coalesce (22). Oil that has been nano-formulated has greater physical stability since, it degrades and evaporates much lesser than its normal form (23). Nano ranged particle size possesses more surface area and mobility that penetrate the insect cuticle more effectively and shows higher insecticidal activity (24). Nanoemulsions have qualities like water solubility, stability and uniform dispersion those helps in effective pest management (25). The active components of nanoemulsion spread and penetrate well in target site due to small size (26). Mostly it eliminates the requirement of high concentrations in toxicity assessments and the annoyance of side effects on organisms other than the target pests. For the preparation of nanoemulsions with a low polydispersity index and small droplet size, some techniques include high-shear blending, high-pressure homogenization and ultrasonication (27). In this study high speed homogenization technique was used to develop nanoemulsion of VNO which further characterized and tested for its insecticidal activity against major stored grain pests of rice *viz. S. oryzae* (rice weevil) and *T. castaneum* (rust red flour beetle).

Materials and Methods

Plant material

The *Vitex negundo* L. leaves were collected from Kanheipur village, Cuttack, Odisha, India (Fig. 1a)**.** The Grain Entomology Laboratory of the Crop Protection Division, ICAR-National Rice Research Institute, Cuttack, Odisha (20°45' N latitude, 85°93' E longitude and 36 m altitude) maintains a specimen copy. ICAR- NRRI comes under east and south coastal plains agroclimatic zone of odisha.

Chemicals

Polyoxyethylene sorbitan monooleate, often known as Tween 80, was bought from Merck, India, used as surfactant in nanoformulation preparation. Glutathione S-transferase (GSTs) kit was purchased from Sigma-Aldrich, Merck, India, used for GSTs assay.

Oil extraction

Hydro-distillation method was used for oil extraction in which dried and chopped leaves were boiled at 70°C for 4 h in Clevenger apparatus (Fig. 1). Due to the hot water and

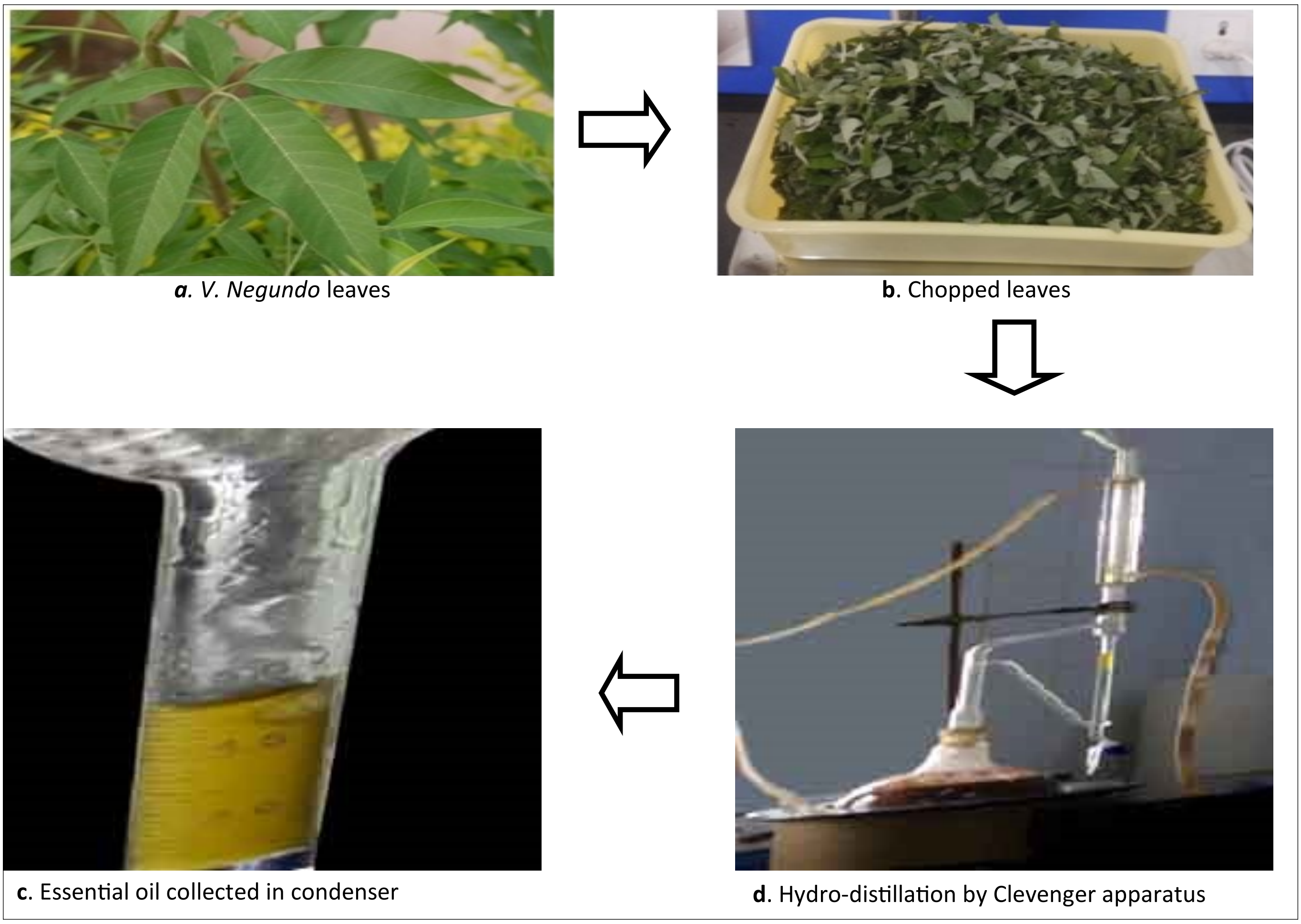

Fig. 1. Oil extraction from *V. negundo* by hydro-distillation method.

steam, essential oils escape from the oil glands of leaves. The vapour mixture of water and oil moved to cooled condenser from which oil was collected. The quantity (in g) of essential oil obtained at the end of hydro-distillation was measured. The recovery of oil was presented in %:

$$\text{Recovery of oil in percentage (\%)} = \frac{\text{Wt. of the oil (g)}}{\text{Wt. of sample (g)}} \text{ X } 100 \qquad (28)$$

Chemical characterization of Vitex negundo oil

Chemical profiling of the extracted oil was done through GC–MS/MS equipment (Shimadzu TQ8040, Shimadzu Corporation, Kyoto, Japan). The oil was diluted 100 times into hexane before injecting. The capillary column Rxi-5-Sil MS (30 m×0.25 mm, 0.25 µm) was used in the gas chromatography system. The injector's temperature was maintained at 250 °C. The temperature of the GC oven was maintained at 50 °C for 2 min, then raised to 200 °C at 5 °C min^{-1} and maintained there for 2 min, then raised to 250 °C at 10 °C min^{-1} and maintained there for 2 min and finally raised to 280 °C at 15 °C min^{-1} and maintained for 5 min. Helium with purity more than 99.99% was utilized as the carrier gas at continuous flow rate of 1.69 mL min^{-1} in the split mode with a split ratio of 25 and purge flow of 3.0 mL min^{-1}. The GC run time was 48.0 min. The ion source temperature was fixed at 200 °C and interface temperature was fixed at 250 °C, the MS was run in scan mode from m/z 40 to 500. Each chemical compound was identified by comparing the retention indices of the compounds to C8-C40 alkane standards and by comparing the MS spectra to the reference spectra in the National Institute of Standards and Technology (NIST) library (29).

Insect culture

Sitophilus oryzae (rice weevil) and *Tribolium castaneum* (rust red flour beetle) were obtained from Grain Entomology Laboratory of Crop Protection Division, National Rice Research Institute. Culture and maintenance of test insects were done under laboratory condition at 65 ± 5% RH (relative humidity) and 28 ± 2 °C throughout the study period. Rice was the feeding material for *S. oryzae* whereas, rice flour was used for *T. castaneum*. Newly emerged adults of same age insects of 7-10 days old were utilized in our experiment. For contact and fumigant toxicity, 10 numbers of insects per replication and for repellency, 20 numbers of insects per replication taken.

Preparation of nanoemulsions

Vitex negundo L. oil nano emulsion (VNO NE) was prepared by using Tween 80 as a surfactant, chosen based on the hydrophilic lipophilic balance of the VNO. A range of VNO concentrations (2.5, 5 and 7.5%) were taken to manufa-cture various VNO NEs. VNO and surfactants were mixed at different ratios on weight basis starting from 1:1 till 1:3 (w/w). The necessary amount of water was added to the mixture and it was vortexed (Maxi Mix II, Thermolyne, USA) for 2-3 min. The bulk emulsion was homogenized at 3 distinct durations (10, 15 and 20 min) and 3 different rotation speeds (10000, 15000 and 20000 revolutions per min (rpm)) using a high-speed homogenizer (IKA T25 digital ULTRA TURRAX, T 25 D S22, Germany).

Characterization of nanoemulsions

Stability study

To obtain stable VNO NE, stress tests (heating, cooling and freezing, thawing) were conducted to determine the stability of emulsion under stress (30, 31). To check phase separation, prepared nanoemulsions were centrifuged for 30 min at 3000 rpm and at 25 °C. An alternate heating and cooling stress test cycle was performed at temperatures of 40 °C and 4 °C, with each temperature being altered after 48 h. Alternate freezing and thawing stress procedures was conducted at -21 °C and 25 °C for 48 h. Both procedure was performed in triplicate and repeated twice. The VNO NEs, those succeed the stress tests were characterized further.

Dynamic Light Scattering (DLS)

Particle size analyzer (Malvern Instruments Pvt Ltd, UK) was used to determine the hydrodynamic diameter, polydispersity index (PDI) and zeta potential (ζ-potential) of VNO NEs.

Transmission Electron Microscopy (TEM)

Visualization of morphology and structure of VNO NEs were carried out using transmission electron microscopy (TEM). A drop of the nanoemulsion was kept on a carbon-coated copper grid of size 200 mesh. The grid was dried for 5 min at normal temperature followed by Infra-Red (IR) lamp drying of 2 h. Micrographs were obtained by the help of a transmission electron microscope (JEM 2100+, JEOL) working at 200 kV.

Bio-efficacy test

Contact toxicity

Contact toxicity of VNO against *S. oryzae* and *T. castaneum* was conducted (32) (Fig. 2a). Completely randomized design (CRD) was followed to conduct this experiment. Cemented petri-plates with a surface area of 64 cm^2 were used for conducting contact toxicity assay. Bio-efficacy test of individual oil was done at varying oil doses. Doses of VNO for different treatments against *S. oryzae* were 0.2, 0.4, 0.6, 0.8, 1.0, 1.5 µL cm^{-2} and control and for *T. castaneum*, doses were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 µL cm^{-2} and control. For smooth application in cemented petri-plates, different doses of VNO were diluted in soybean oil to a predetermined amount of 500 µL. In each petriplate ten adults were released along with 1 g of rice or broken rice as food. Petri plates were tightly covered with lids followed by sealing with the help of paraffin film to avoid escape of insects and pin holes were created for ventilation. Each treatment was replicated thrice and mortality was recorded at 24 and 48 h after treatment (HAT). Lethal doses (LD) were obtained using 1.5 EPA Probit Analysis Program software, where doses of treatments, total number of insects (30 per treatment) and numbers of dead insects per treatment were inputted in the probit analysis.

Fumigant toxicity

Fumigation chambers (volume 1150 mL) were used to conduct fumigant toxicity (33) (Fig. 2b). Ten adults belonging to each insect group were kept in perforated pouch with 1 g rice, tied with rubber bands and placed inside the fumigation chamber. Filter paper (Whatman No. 1) was cut into 64 cm^2 pieces and was treated with different doses of VNO (300, 400, 500 and 600 µL per litre air (µL L^{-1}) and control). Filter paper was placed at the top of the air tight fumigation chamber near the lid. Each treatment was replicated thrice and adult mortality was recorded 5 days

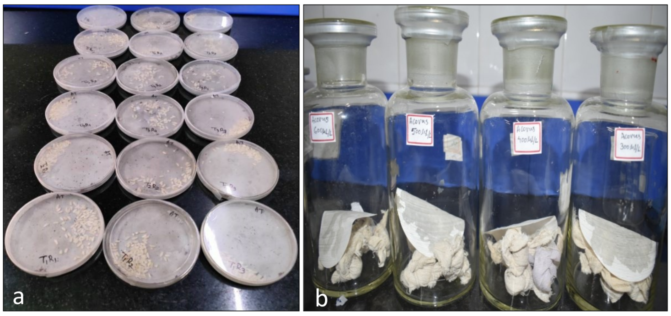

Fig. 2. Bio efficacy test. (**a**). Contact toxicity, (**b**). Fumigant toxicity

after treatment (DAT). LD_{10}, LD_{50} and LD_{90} values were obtained.

Repellancy study

Glass petri-plate of 64 cm^2 consists of a filter paper (Whatman No. 1, cut into 64 cm^2 piece) was used for repellency study (34). Different doses of VNO (1.5, 4.5 and 7.5 µL) were mixed with soybean oil to prepare VNO treatments and volume was made up to 150 µL. Filter paper was divided in to 2 equal halves in which 1 was treated with VNO treatment and other half was control i.e treated with only soybean oil (150 µL). Petri-plates were subjected to fan drying and insects were released at the middle area of filter paper. Each treatment was replicated 7 times and number of insects present in the 2 parts was recorded at 2, 6, 12 HAT. For every considered time interval, the % repellency of differe~~nt VNO~~ treatment was measured using the formula:

$$PR (\%) = \frac{Nc - Nt}{Nc + Nt} X 100 \qquad (35)$$

where, Nc is the number of insects in the untreated half paper and Nt is the number of insects in the treated half.

Bioefficacy test of VNO NE

Same methods were followed for studying the contact, fumigant and repellent toxicity of VNO NE as described in VNO bioefficacy test. Doses of VNO NE were taken based on LD_{50} values of contact, fumigant toxicity of VNO. For contact toxicity against *S. oryzae* treatment, doses of VNO NE were 1.05, 1.3, 1.55, 1.8 µL cm^{-2} and control, against *T. castaneum* doses were 4, 4.5, 5, 5.5 µL cm^{-2} and control. In case of fumigant toxicity, treatment doses of VNO NE were 325, 375, 425, 475 µL L^{-1} and control for *S. oryzae*. Lethal doses were calculated in all cases. For repellency study, treatment of one half filter paper was VNO NEs (150 µL volume of VNO NEs with different oil concentrations of 1.5, 4.5 and 7.5 µL) and % repellency was calculated.

Glutathione-S-transferase (GSTs) assay

Enzyme extraction

S. oryzae and *T. castaneum* adults were exposed under different treatments of contact toxicity (LD_{50} dose of VNO, LD_{50} dose of VNO NE and control) at different exposure times (1, 6 and 24 h). Extraction of the enzymes done (36). Before exposing the adults to bioassay, all the beetles were pre-weighed and the weight of the insects were 3.62 mg and 6.32 mg (per 3 insects) for *S. oryzae* and *T. castaneum* respectively. After the exposure time, both the treated and untreated samples were kept at -80 °C and then crushed with 1 mL Dulbecco's phosphate-buffered saline (DPBS) in eppendorf tube. The crushed sample were centrifuged at 10000 rpm and the supernetants were collected in the fresh tube and kept for further analysis.

Enzyme assay

The glutathione S-transferase (GSTs) enzyme activities were measured by using the commercially available GST assay kit (CS0410, Sigma- Aldrich, Merck, India). GSTs activities were recorded in the microplate reader (Epoch™2 microplate reader, Agilent, United States) at 340 mm and 1-min intervals according to the manufacturer instructions. Change in absorbance was calculated at 5 min. Total GST activities were calculated from the CDNB (1-chloro, 2,4-dinitrobenzene) extinction coefficient (0.0096). The reaction solution had 4 µL of enzyme solution, 196 µL of substrate solution (192.08 µL of DPBS, pH 7.2, 200 mM Glutathione reduced and 1.96 µL of 100 mM CDNB (1-chloro-2,4-dinitrobenzene).

Statistical analysis

The Probit regression analysis was done using 1.5 EPA Probit Analysis Program software (37). Lethal doses (LD_{10}, LD_{50} and LD_{90}) were estimated. GSTs data were obtained using GEN5 absorbance microplate software and analysed using one-way ANOVA at P<0.05 in Microsoft excel version 2016.

Results and discussion

Chemical constituents of VNO

Recovery of oil from dried leaves of *V. negundo* (L.) was 0.65 % which was pale-yellow in colour. According to earlier reports *V. negundo* leaves produced 0.4% oil (38) and 0.5% essential oil by hydro-distillation method (28). The variation in amount of oil yield might be due to the genetic make-up of the *V. negundo* plant population as well as extraction methods, seasonal variations, environmental, soil and climate conditions (18).

From gas chromatography–mass spectrometry (GC–MS) technique a total of 40 chemical compounds were identified constituting almost 70% of oil. Major compounds of oil were found to be Aromandendrene (13.21%), β-caryophyllene (7.79%), Squalene (5.48%), 3-octen-5-yne,2,7-dimethyl-,(E)-(5.43%),5-(1-isopropenyl-4,5-dimethylbicyclo[4.3.0]nonan-5-yl)-3methyl-2-pentenol acetate (3.59%), Farnesyl bromide (3.51%), 4-terpeneol (2.97%) and Elemol (2.18%) (Table 1). Many chemical components from our results such as α-Pinene, β-pinene, 3-octanone, p-cymene, 1,8-cineole, γ -terpinene, Linalool, 4-terpeneol**,** α-terpineol, β-elemene, β-caryophyllene, Elemol, Nerolidol, Caryophyllene oxide, epi-α-cadinol, β-eudesmol corroborated the earlier studies (27, 38, 39). Also some other compounds Humulane-1,6-dien-3-ol, Sclareol, Nerolidol, 4-Terpinenol, β-caryophyllene and β-eudesmol were identified previously which are similar with our result (28). β- caryophyllene found to be one of the major compound of *V. negundo* oil (40). Geographical and climatic factors can sometimes be the primary causes of variations in chemical composition, both in terms of quality and quantity (27).

Preparation and characterization VNO NE

Keeping VNO concentration at 5% with constant ratio of VNO: surfactant (1:2) several combinations of emulsions were formulated in order to comprehend the homogenization time and homogenizer rotation speed (Table 2). To achieve the best combining ratio and the maximum loading capacity of the oil, *V. negundo* oil and surfactant were combined in various ratios at a given time (15 min) and rotation speed (20000 rpm) (Table 3). Varying combining ratios and oil concentrations led to distinct colored VNO NE (Fig. 3). Milky white color nanoemulsion was produced from 2.5% oil concentration and 1:1 (oil, surfactant ratio) and at ratio 1:2 with same oil concentration milky VNO NE resulted. Transparent white and super white coloured emulsions were obtained at 1:2 and 1:1, VNO and surfactant ratio at 5% oil concentration respectively. At 7.5% VNO concentration, super white emulsion was observed at a ratio 1:1 of oil to surfactant and turbid emulsion was formed at a ratio of 1:2.

VNO and surfactant at 1:2 and 1:1 ratio of 5% VNO concentration were found to be more stable nanoemulsions and passed the stress tests as no phase separation was observed when centrifuged. Phase separation noticed when loading capacity of VNO was increased to 7.5% (Table 2). These unstable nanoemulsions may be occurred due to larger droplets formed by Ostwald ripening and coalescence of oil (41).

VNO and tween80 at 1:1 and 1:2 ratios of 5% oil concentration were further characterized for hydrodynamic diameter, PDI and ζ-potential (Table 4). Factors like droplet size, poly dispersity index and zeta potential are crucial for the stability of nano-formulations as well as their biological activity (42). The droplet sizes of VNO: surfactant at 1:1 and 1:2 of 5% oil concentration were recorded 185.41 and 166.62 nm respectively. This is within the previously reported range of nanoemulsion droplet sizes, i.e between 20 to 200 nm (43). Tween 80 sterically stabilises nanoemulsion droplets due to its high hydrophilic and lipophilic balancing (44). Smallest droplets might be produced because of high power energy generated by the homogenizer (45). An increased stirring speed led to decrease in particle size (46). Particle size decreased as a result of the decrease in interfacial free energy caused by the rise in surfactant concentration, which could serve as a mechanical barrier to coalescence.

Table 1. Chemical composition of *Vitex negundo* essential oil as identified by gas chromatography coupled with mass spectroscopy.

Retention time	Chemical name	Area	Area Percentage	SI	*RI(Li)	*RI(Cal)
6.905	β-thujene	19776541	0.18	94	873	930
7.085	α-pinene	40657279	0.37	95	909	937
8.3	3-octen-5-yne, 2,7-dimethyl-, (E)-	590952890	5.43	86	912	979
8.36	β-pinene	52738955	0.48	96	943	981
8.485	Vinyl hexanol	106960214	0.98	93	961	985
8.675	3-octanone	12867508	0.12	95	965	991
8.785	Myrcene	68674820	0.63	95	984	996
8.945	3-octanol	21802761	0.2	95	990	1001
9.515	δ-carene	75026451	0.69	95	1001	1020
9.755	p-cymene	8334998	0.08	94	1008	1029
9.875	D-limonene	68991116	0.63	92	1011	1032
9.955	1,8-cineole	28525768	0.26	96	1016	1035
10.475	β-ocimene	47491778	0.44	96	1026	1053
10.785	γ -terpinene	125885231	1.16	93	1040	1063
11.665	Isoterpinolene	36158415	0.33	96	1082	1093
12.025	Linalool	35519846	0.33	96	1083	1105
12.17	Isopentyl 3-methylbutanoate	16799896	0.15	92	1094	1110
14.335	4-terpeneol	323167077	2.97	91	1161	1185
14.71	α-terpineol	26608713	0.24	95	1179	1197
20.225	β-elemene	20825391	0.19	95	1385	1401
21.025	Aromandendrene	1437590039	13.21	90	1440	1433
21.85	β-caryophyllene	847257487	7.79	90	1449	1466
24.15	Elemol	237257608	2.18	95	1538	1562
24.405	Nerolidol	38813853	0.36	90	1546	1572
24.625	3-hexen-1-ol, benzoate, (Z)-	19649946	0.18	96	1553	1581
24.985	Caryophyllene oxide	84793572	0.78	93	1566	1597
26.09	γ-eudesmol	82972462	0.76	92	1596	1646
26.285	epi-α-cadinol	59907995	0.55	94	1625	1654
26.52	β-eudesmol	54231287	0.5	92	1629	1665
26.585	α-eudesmol	44454785	0.41	89	1635	1668
31.435	Farnesyl_bromide	381514220	3.51	80	1764	1899
32.010	Verticillol	42265135	0.39	83	2036	1929
32.815	Cycloeucalenol acetate	272433913	2.5	82	2074	1972
33.26	Humulane-1,6-dien-3-ol	116294413	1.07	86	2080	1996
33.495	Phytol	282736248	2.6	90	2125	2009
33.945	Sclareol	193612163	1.78	91	2228	2037
34.33	5-(1-isopropenyl-4,5-dimethylbicyclo[4.3.0]nonan-5-yl)-3-methyl-2-pentenol acetate	390435014	3.59	81	2265	2061
34.835	Kolavenol acetate	201479113	1.85	92	2290	2092
35.29	cis-3,14-clerodadien-13-ol	18409197	1.70	93	2411	2126
42.085	Squalene	8172815	5.48	95	2833	2848

***SI**: Similarity index, **RI** (**Li**): Retention index literature, **RI** (**Cal**): Retention index calculated.

The attraction forces were weaker and the nanoemulsions were more stable due to these down sized droplets (47).

Polydispersity index evaluated the non-uniformity of the formulation's size distribution (48). A PDI value less than 0.6 showed that the emulsion was more homogenous and stable (42). VNO: tween 80 at 1:1 and 1:2 of 5% oil concentration displayed uniformity with low PDI values of 0.281 and 0.263 respectively (Table 4). Homogeneity of droplet size distribution increased with decreased polydispersity value (49). ζ-potential of VNO: tween 80 at 1:1 and 1:2 ratio were -4.3 and -3.4 mV respectively which prevents quick phase separation. Negative zeta potential caused more repulsion between droplets thus stabilizes the

Table 2. Thermodynamic parameters of nanoemulsion (5% *V. negundo* oil concentration and *V. negundo* oil: surfactant :: 1:2) with respect to different time and rotation speed.

Rotation speed (rpm)	Time (Min.)	Colour of Original Emulsion	Centrifugation	Heating and cooling cycle	Freezing and thawing cycle
10,000	15	Transparent white	No phase separation	Transparent white	Transparent white
15,000	15	Transparent white	No phase separation	Transparent white	Transparent white
20,000	15	Transparent white	No phase separation	Transparent white	Transparent white
20,000	10	Transparent white	No phase separation	Transparent white	Transparent white
20,000	20	Transparent white	No phase separation	Transparent white	Transparent white

***rpm**: rotations per minute, **Min.**: minute.

Table 3. Thermodynamic characterization of different *V. negundo* nanoemulsion formulations at fixed time (15 min) and rotation speed (20000 rpm).

Oil Conc.	*V. negundo* oil: Surfactant	Colour of Original Emulsion	Centrifugation	Heating and cooling cycle	Freezing and thawing cycle
2.5%	1:1	Milky white	No phase separation	Milky white	Milky white
2.5%	1:2	Milky	No phase separation	Translucent	Translucent
5%	1:1	Super white	No phase separation	Super white	Super white
5%	1:2	Transparent white	No phase separation	Transparent white	Transparent white
5%	1:3	Milky white	Phase separation	Suspended particle	Milky white
7.5%	1:1	Super white	Phase separation	Suspended particle	Suspended particle
7.5%	1:2	Turbid	Phase separation	Suspended particles	Suspended particle

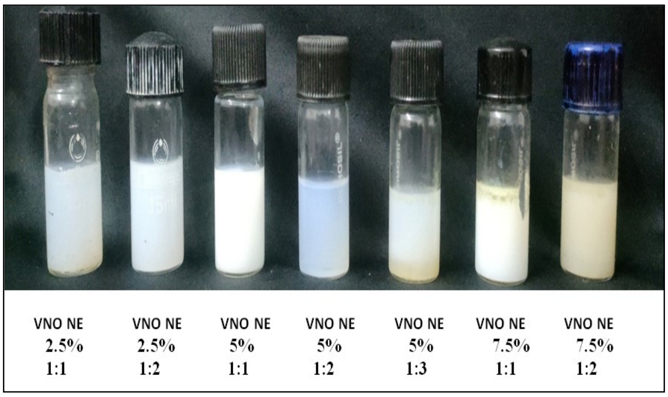

Fig. 3. Different *V. negundo* nanoemulsions (VNO NEs) (rotation speed : 20000 rpm, duration : 15 min).

Table 4. Particle size, polydispersity index and zeta potential of *Vitex negundo* oil nano-emulsions (VNO NE).

	***Nanoemulsion 1**	***Nanoemulsion 2**
Hydrodynamic diameter (nm)	185.41nm	166.62 nm
Polydispersity index	0.281	0.263
Mean zeta potential (mV)	-4.3 mV	-3.4 mV

*Nanoemulsion 1: Contain 5% VNO and VNO :surfactant at 1:1 ratio,
*Nanoemulsion 2: Contain 5% VNO and VNO: surfactant at 1:2 ratio

formulation (50).

The most crucial method for studying microstructures is transmission electron microscopy because it directly generates high-resolution pictures and can record any coexisting structures and microstructure changes (51). The droplets of VNO: tween 80 at 1:1 and 1:2 of 5% oil content were found to be spherical when determined by transmission electron microscopy (TEM) and particle size ranged between 100 to 200 nm (Fig. 4). Centre of droplets were appeared dark, while the surroundings were bright. Our results from the dynamic light scattering (DLS) technique's finding of particle size were supported well by data

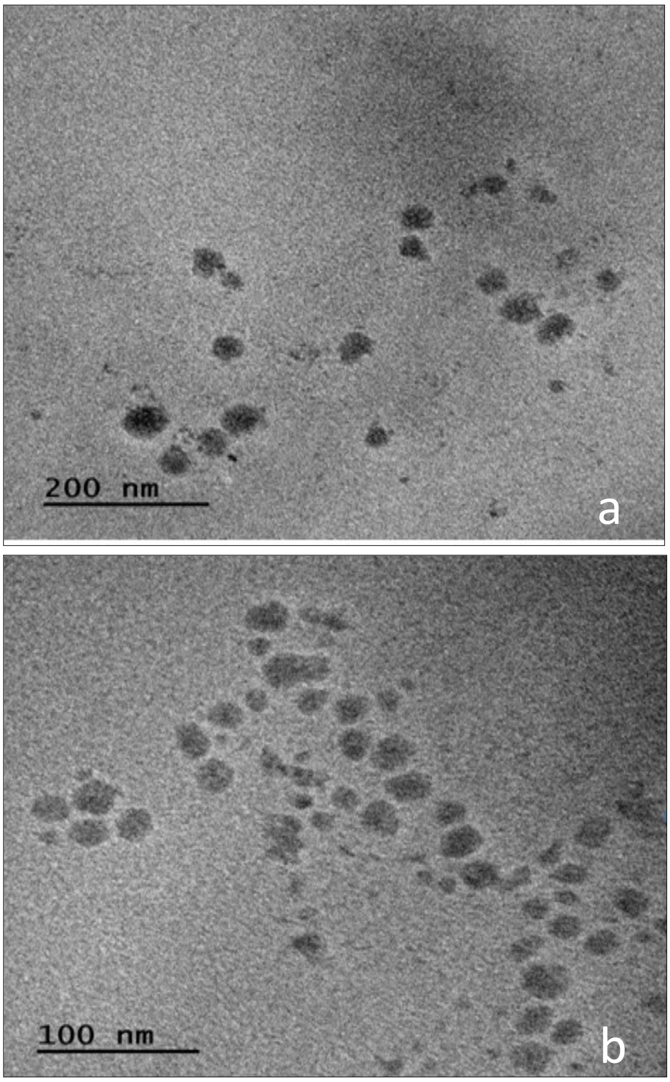

Fig. 4. Transmission electron microscopic view of *V. negundo* oil nanoemulsions. **(a).** *V. negundo* oil nanoemulsion (5%, 1:1), **(b)** *V. negundo* oil nanoemulsion (5%, 1:2).

got from TEM. Particle size from TEM view of lime oil nanoemulsion had size more or less similar to our results i.e ranged between 20-200 nm (52). Because of the extraordinary small size of nanoemulsion, Brownian motion may have been more prominent and gravitational force less effective, limiting sedimentation or flocculation, which may have contributed towards its stability (53).

Fig. 5. Death of insects due to bio-efficacy. **(a).** Dead individuals of *S. oryzae,* **(b).** Dead individuals of *T. castaneum.*

Bioefficacy study

Bioefficacy test conducted against *S. oryzae* and *T. castaneum* and motalities were recorded (Fig. 5). The efficacy of VNO NE (VNO: tween 80 at 1:2) and bulk oil were compared. At 24 HAT, the LD_{50} values for contact toxicity of VNO NE (a.i.) against *S. oryzae* and *T. castaneum* were 0.755 and 3.666 µL cm^{-2} which were 41.60 and 29.88% less respectively, compared to VNO. However the values of LD_{50} values of VNO NE (a.i.) at 48 HAT were 0.704 and 3.480 µL cm^{-2} against *S. oryzae* and *T. castaneum* respectively which were 18.14 and 17.12% less respectively than crude oil (Table 5). Results depict that secondary metabolites of essential oils like terpenoids and sesquiterpenoids were responsible for contact, fumigant and ingestion toxicity of essential oil (54). Terpenes, notably monoterpenes, were assumed to be the principal components responsible for essential oils' effectiveness against insect pests (55). Thus, it can be said that several chemicals, such as β-caryophyllene, Terpinen-4-ol, linalool, α-humulene and 1,8-cineole, may be responsible for contact toxicity towards test insects. An increase in efficacy of formulated nanoemulsions over eucalyptus oil against *S. oryzae* and *T. castaneum* was observed (56). Superior larvicidal activities of VNO NE than VNO against *Aedes aegypti* L. were reported previously (28). Also it was reported that nanoemulsion of *P. anisum* showed toxicity against *T. castaneum* (57). Nanoparticles had increased surface area and mobility, allowing them to penetrate the insect cuticle more efficiently, resulting in higher insecticidal efficacy (24). Due to their small size in comparison to bulk oil,

Table 5. Contact toxicity of *Vitex negundo* oil and its nanoemulsion against important stored grain pests of rice.

Time (HAT)	No. of insects tested	Lethal dose 50 (LD_{50}) (a.i.) (µL/Cm^2)	95% fiducial limits		Lethal dose 10 (LD_{10}) (a.i.) (µL/Cm^2)	Lethal dose 90 (LD_{90}) (a.i.) (µL/Cm^2)	Slope	Standard error	X2 calculated	df	P value
			Lower	Upper							
***Bulk VNO**											
***Sitophilus oryzae* (Rice weevil)**											
24	180	1.327	0.717	2.455	0.124	14.181	1.248	0.136	0.997	4	0.910251
48	180	0.860	0.420	1.763	0.050	14.833	1.037	0.159	0.998	4	0.910099
***Tribolium castaneum* (Red flour beetle)**											
24	180	5.228	3.014	9.067	0.753	36.309	1.525	0.122	0.994	4	0.910704
48	180	4.199	2.383	7.398	0.518	34.025	1.410	0.125	0.996	4	0.910402
***VNO NE**											
***Sitophilus oryzae* (Rice weevil)**											
24	180	0.775	0.606	0.992	0.425	1.415	4.890	0.055	0.995	2	0.608049
48	180	0.704	0.538	0.921	0.388	1.280	4.997	0.059	0.918	2	0.631915
***Tribolium castaneum* (Red flour beetle)**											
24	180	3.666	3.131	4.319	2.237	6.010	5.988	0.036	0.996	2	0.607745
48	180	3.480	2.953	4.101	2.10	5.581	6.246	0.036	0.994	2	0.608353

***Bulk VNO**: *Vitex negundo* oil, **VNO NE**: Nanoemulsion of *Vitex negundo* oil (5% VNO; VNO and surfactant were mixed at 1:2 ratio), **HAT**: Hours after treatment active components in nanoemulsions distribute and penetrate well in the target site (26).

In terms of fumigant toxicity, the LD_{50} value of VNO NE (a.i.) for *S. oryzae* was 322.28, µL L^{-1}, which was 26% less than that of crude oil (Table 6). Terpinen-4-ol had major role in fumigation against stored grain pests (58). Smaller particle size of nanoemulsion has a significant impact on pesticide activity by accelerating insecticide penetration through the insect cuticle (59). In our result *T. castaneum* demonstrated resistance to both VNO and VNO NE fumigant toxicity which may be due to it's strong exoskeleton (60).

Highest repellency of VNO NE was increased by 33.33 and 30.14% for *S. oryzae* and *T. castaneum* respectively than VNO (Table 7). Common monoterpenoids present in VNO like linalool and terpinen-4-ol had been proven to have repellent properties (61). The essential oil's potent repellent properties were connected to the presence of α-pinene and limonene (50). Our results demonstrated the higher repellency of nanoemulsions over bulk oil which is similar to the earlier studies that, *Citrus sinensis* (sweet orange) oil nanoemulsion was more poisonous and repellent than regular oil against targeted pests (34). Our results are in conformity with previous results, that essential oil nanoemulsions were widely employed as an effective insect repellent, alternative to

Table 6. Fumigant toxicity of *Vitex negundo* oil and its nanoemulsion against important stored grain pests of rice

Time (DAT)	No. of insects tested	Lethal dose 50 (LD_{50}) (µL/L)	95% fiducial limits		Lethal dose 10 (LD_{10}) ((µL/L)	Lethal dose 90 (LD_{90}) (µL/L)	Slope	Standard error	χ^2 calculated	df	P value
			Lower	Upper							
***Bulk VNO**											
***Sitophilus oryzae* (Rice weevil)**											
5	120	435.55	317.67	597	156.69	1210.69	2.888	0.070	0.983	2	0.611708
***VNO NE**											
***Sitophilus oryzae* (Rice weevil)**											
5	120	322.28	284.79	364.71	224.75	462.14	8.253	0.027	0.988	2	0.610181

***Bulk VNO**: *Vitex negundo* oil, **VNO NE**: Nanoemulsion of *Vitex negundo* oil (5% VNO; VNO and surfactant were mixed at 1:2 ratio), **DAT**: Days after treatment

Table 7. Percentage repellency of *Vitex negundo* oil and its nanoemulsion against important stored grain pests of rice.

Concentration (µL/64 Cm^2)	2 HAT	6 HAT	12 HAT
***Bulk VNO**			
Sitophilus oryzae			
1.5	30.00	18.57	12.86
4.5	47.14	35.71	25.71
7.5	57.14	45.71	28.57
S.E(m) ±	4.07	4.90	3.56
CD (0.05)	12.09	14.57	10.58
Tribolium castaneum			
1.5	20.00	14.29	11.43
4.5	32.86	21.43	17.14
7.5	41.43	27.14	20.00
S.E(m) ±	3.12	3.33	2.86
CD (0.05)	9.28	9.90	8.49
***VNO NE**			
Sitophilus oryzae			
1.5	41.43	32.86	24.29
4.5	60.00	48.57	37.14
7.5	75.71	60.00	48.57
S.E(m) ±	4.59	4.39	4.36
CD (0.05)	13.64	13.04	12.97
Tribolium castaneum			
1.5	31.43	24.29	18.57
4.5	42.86	35.71	20.57
7.5	55.71	51.43	40.00
S.E(m) ±	5.35	5.06	3.53
CD (0.05)	15.88	15.04	10.49

***Bulk VNO**: *Vitex negundo* oil, **VNO NE**: Nanoemulsion of *Vitex negundo* oil (5% VNO; VNO and surfactant were mixed at 1:2 ratio), **HAT**: Hours after treatment.

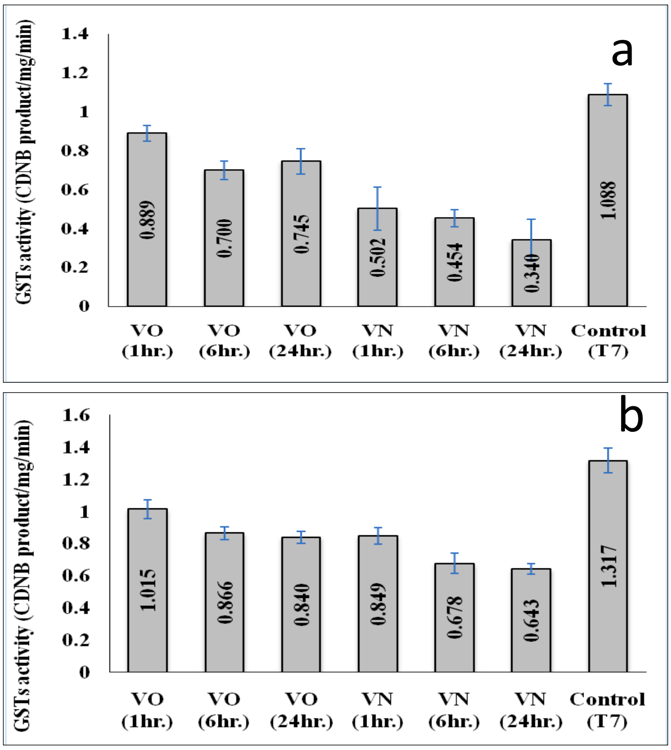

Fig. 6. GSTs inhibition activities of *V. negundo* oil and its nanoemulsion (**VO**: Treated with *Vitex negundo* oil; **VN**: Treated with *Vitex negundo* oil nanoemulsion) in test species. (**a**). CDNB product/mg/min) in *S. oryzae* adults, (**b**). CDNB product/mg/min) in *T. castaneum* adults.

synthetic chemical pesticides (62).

Biochemical assay

The inhibitory activities of GSTs demonstrated that GSTs levels in nanoemulsion treated beetles were significantly lower than the treated beetles of bulk oil and untreated beetles (Fig. 6). After exposed to plant extracts, some detoxifying enzymes in target insects' tissues and organs were eliminated or inhibited (63). Earlier, it has been demonstrated that essential oil nanoemulsions prevent GSTs activity in stored grain pests (64).

Conclusion

Vitex negundo oil contains major compounds like Aromandendrene, β-caryophyllene, Squalene, 3-octen-5-yne,2,7-dimethyl-, (E)-, Farnesyl bromide, 4-terpeneol and Elemol which could be responsible for insecticidal properties of the oil. Optimum VNO NE had 5% bulk oil mixed at a 1:2 (w/w) ratio with surfactant and prepared using a high-speed homogeniser at 20000 rpm for 15 min. VNO NE was more effective than VNO against *Sitophilus oryzae* and *Tribolium castaneum* in terms of insecticidal activities as it has enhanced contact toxicity, fumigant toxicity and repellency. Significant reduction in GSTs activities observed in VNO NE treated insects as compared to control. In future nanoformulation of essential oil will be emerged as a novel alternative to synthetic pesticides which could protect stored grains from pests more efficiently and with low doses.

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Authors' contributions

NB, PR and TA conceptualised and supervised the research design and experimental planning. PP carried out the experiment and analysis. GP participated in the GSTs enzyme assay. BG, PG, PC and SD participated in the statistical analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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