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Physical Stability and Bio-Efficacy Enhancement of Neem Kernel Aqueous Extract by Optimized Amount of Botanical Synergist for the Control of Early Stages of Mosquitoes

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The aim of present study is to enhance the stability of physico-chemical characteristics of neem kernel aqueous extract by botanical stabilizer system. There are variety of bioactive constituents are present in neem which give broad-spectrum of insecticidal activity. Neem aqueous extract is commonly used and found very effective in pest control applications without harming the environment. However, due to hydrolytically unstable characteristics of neem active ingredients which results its lesser bioactivity and limits its usage in aqueous form. To overcome this un-stability issue oil extracted botanical stabilizer (*Prosopis juliflora)* (Junglee kikar)) were used in various ratios. In 70-30 (NKP-KP) composition (NKP- Neem kernel powder; KP- Kiker powder), neem aqueous extract was found stable without any turbidity, pH change, and fungal growth. Active ingredient, Azadirachtin was found stable with very less degradation i.e only 20–30% degradation. This may possibly be due to inhibition of hydrolytic reactions. Bio-efficacy evaluation data also showed improved and stable mosquito larvae mortality per cent i.e 75–90% with 8 µg/g LD_{50} value. The approach used in this study could be very useful in long term stability of neem kernel extract in various geographical conditions without adding toxic solvents or chemical compositions.

Keywords: Aqueous extract, Azadirachtin, Botanical stabilizer, Kikar Powder (KP), Neem kernel powder (NKP), Oil extract

Introduction

The Neem tree (*Azadirachta indica* Juss.) belong to the Meliaceae family. Initially, neem was known for their medicinal properties and later on other properties like pesticidal, acaricidal, antifungal, antibacterial, antifeedent and growth inhibibitory properties are also reported by different scientist all over the world.[1] A variety of bioactive constituents are present in different parts of neem tree. Major bioactivity of neem is due to azadirachtin salannin, meliantriol, and nimbin constituent.[2] This constituent quantity is different in various parts of the tree. Neem seed has broad spectrum insecticidal property. About 200 insect species have been controlled by neem spray.[3]

Neem seed kernel extraction has been done by different solvents which are very costly and flammable. In addition to that another major limitation of solvent extraction is decomposition of different bioactive compounds and extract impurities also. So, solvent extraction should be replaced by aqueous extraction to remove the hazards and stability of neem bioactive constituents by adding botanical stabilizer. Neem aqueous extract is safe for non target organisms and environmental frientdly.[4] Neem aqueous extract is also used for mosquito control in household and in unrecognized ecosystems like stagnant water etc. many studies explored larvicidal properties of neem extracts.[5,6]

As neem is safe and green bio-pesticide with broad spectrum activity so, there is urgent need of convenient, scalable and cost efficient neem kernel extraction by using greener approaches. The main objective of this study is to stabilize the aqueous neem extract with enhanced bioactivity by adding botanical extract which act as stabilizing agent as well as synergist.

Materials and Methods

Neem kernel from Agrikendra, distilled water, botanical synergist collected from local Forest

Procedures

The neem kernels were grinded by a mixer grinder into a very fine powder and then sieved by 32 mesh size sieve to obtain uniform size powder. In the same

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way botanical synergist pods were grinded and sieved by the same sieve. After grinding and sieving, powdered form was coded as neem kernel powder (NKP) and kernel powder (KP).

Preparation of Oil Extract of KP Powder

Finely grinded 200 gm KP powder was weighed in 500 ml beaker. Subsequently, 300 ml soyabean oil was added in KP powder containing beaker. Sonicate the content for 10 minutes and then left the beaker on mechanized shaker for 24 hrs at ambient temperature. after 24 hrs, filter the oil extract by whatman filter paper. The filterate then used in subsequent experiments in various proportions.

Preparation of Aqueous Extract of NKP and KP

Then NKP and KP oil extract were mixed in different composition i.e. 100-0, 80-20, 70-30, 50-50 and 0-100. After that conical flask were left for shaking over shaker at 25°C for 24 hrs. After 24 hrs, filter the content by whatmen filter paper and store the filtrate for further testing of stability and bioactivity studies. Different proportions were stored in room temperature, 54°C, and sunlight for 14 days. After 14 days aqueous extract was prepared of different proportions.

Physical Stability Testing

Physical appearance of freshly prepared aqueous extract was transparent pale colored solution. Physical appearance was varied during storage in each proportion. After 24 hrs, sample Shows turbidity. The sequence of turbidity in different proportions was as 100-0>0-100>80-20>70-30>50-50. The turbidity was measured by turbidity meter. Each proportion aqueous extract was put in the sample holder of turbidity meter and record the value of turbidity. All measurement was done in triplicates.

Chemical stability

HPLC

HPLC Analysis by HPLC (model-Perkin Elmer Series 200 HPLC) as prescribed in BIS method 14299 : 1995.

Preparation of Standards: Two mg of azadirachtin standard of 95% purity was dissolved in methanol :water (90:10) and make up the volume 50 ml volumetric flask. 2ml of this solution was further diluted to 10 ml and use this as standard. 10-50 ppm standard solutions were prepared were prepared for calibration curve.

Operating Conditions: Stationary phase – C-18 Column, Mobile phase –Acetonitrile/water (35:65).Run time-60 minutes, flow rate 1.2 ml/min, injection volume-10 µl, detector wavelength -214 nm and volume of injection-10 µl.

Fourier-Transform Infrared Spectroscopy

The functional group was characterized by Fourier-transform infrared spectroscopy (**FTIR**).FTIR of neem oil and neem oil with adjuvant was recorded by the Perkin Elmer spectrum. FTIR spectra were recorded in the frequency range of 4000-500 cm^{-1}.

Estimating Fungal Growth in Aqueous Extract by Uv- Visible Spectroscopy

The color absorbance spectrum was recorded by using shimadzu UV-Visible. The samples were scanned in 200-600 nm wavelengths for identification of absorption maxima. Water was used as reference blank. The Aqueous extract of neem with synergist in various combinations i.e. 80-20, 70-30, 50-50 and 100-0 after 15 days of storage were screened for absorbance measurement. The absorbance measurements were used in calculating optical density by using following formula (Eq. 1) :

$$\text{OpticalDensity} = \frac{\text{Abs } 400 \text{ x dilution factor}}{\text{sample weight}} \quad \ldots (1)$$

Result and discussion

Neem has been using potentially in various agricultural applications. Various studies have proved that neem is very effective in irradicating various pests. However, unstability of bio-active constituents due to hydrolysis, photodegradation or fungal growth limits its usage for longer storage conditions.[7] Due to these limitations popularity of neem products turn down in farmers. Because during storage, the degraded products terminate the bio-efficiency of neem which creates dis-belief in consumers towards neem products. Various researchers made outstanding efforts to decipher this problem. Parmer and Kumar in 1999 effectively used chemical stabilizer anthraquinone or epichlorohydrin and found very effective.[8] It's a ray of hope to overcome the unstability problem of neem and regain the attention of farmers towards neem. After that Johanson *et al*., 2003 worked on phostostability of active constituents by using stabilizers such as ter. butyl-p-cresol, 8-hydroxy quinoline and ter. butyl hydroquinone in sunlight. The result shows that these stabilizers enhanced the half life of neem active constituent's upto 55 days.[9] There are several studies have explained various chemical stabilizers. The present study aim to, enhance the stability of neem in aqueous extract by using botanical

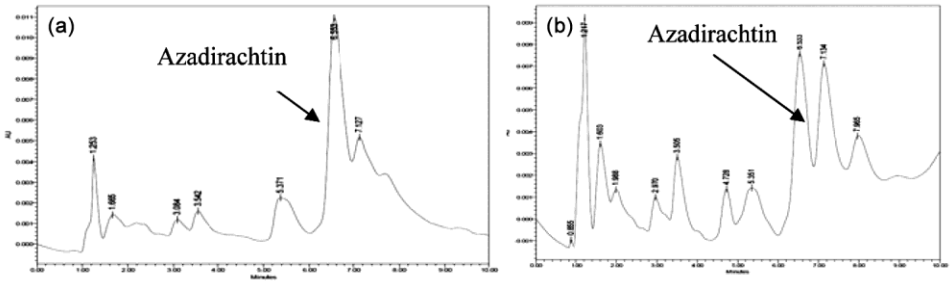

Fig. 1 — Chromatogram of Neem Azadirachtin (a) with or (b) without botanical synergist

Table 1 — Turbidity difference in different compositions of NKP - KP stored in various conditions

NKP-KP (Percentage)	Sunlight	Ambient temperature	Oven temperature	Freez temperature
100-0	Turbid 400 NTU	Less turbidity 300 NTU	Highly turbidity 500 NTU	No turbidity 50 NTU
0-100	No turbidity 20 NTU	No turbidity 15 NTU	No turbidity 20 NTU	No turbidity 16 NTU
80-20	Less turbidity 100 NTU	Very less turbidity 30 NTU	Turbid 300 NTU	No turbidity 40 NTU
70-30	No turbidity 40 NTU	No turbidity 30 NTU	Very less turbidity 100 NTU	No turbidity 30 NTU

Table 2 — Active ingredient concentration in different concentrations of NKP and KP after 15 day storage in various conditions

NKP -KP (Percentage)	Sunlight	Ambient temperature (25°C)	Oven temperature(45°C)	Freeze temperature (0–2°C)
100-0	500 ppm	1000 ppm	375 ppm	2000 ppm
80-20	2079 ppm	3004 ppm	2387 ppm	4345 ppm
70-30	3876 ppm	3678 ppm	3487 ppm	4890 ppm

originated stabilizer (Fig. 1).

Neem kernel powder and kikkar powder oil extract was taken in various composition i.e. 100-0, 80-20, 70-30, 50-50 and 0-100 and stored in various environmental conditions i.e. sunlight, ambient temperature and 54°C. In a study it has explained that active ingredient of neem is highly unstable in high temperature and less in freezing temperature.[10] So, the purpose of taking these combinations is to find the optimized ratio of botanical stabilizer for neem kernel powder for maximum stability. Botanical stabilizer optimized ratio was identified by studying physio-chemical analytical studies.

Physical appearance of NKP and KP oil extract is the preliminary indication of unstability. In pure NKP samples turbidity level was noticed very high but in 70-30 samples turbidity was found very less (Table 1). Turbidity of aqueous extract is directly linked with individual degraded products or microbial growth.[11] As per Farrell *et al*.[11] study, it was assumed that in 70-30 NKP and KP ratio microbial growth inhibited and active ingredients were also stabilized. The exact turbidity measurement was recorded by turbidity meter in NTU (Nephelometric Turbidity unit) as shown in Table 2. In presence of botanical stabilizer turbidity level was lowered from 400 to 40 NTU. Maximum reduction was found in 70-30 NKP and KP samples. It might be due the presence of 30% botanical stabilizer which is adequate for prevention of microbial growth and degradation of active constituents.

After physical appearance and turbidity check, active ingredient of neem kernel extract was analyzed in HPLC. The initial active constituents of neem kernel were found 6000 ppm, but after storage of 15 days in sunlight, ambient temperature, oven temperature and freez temperature it remains 500 ppm, 1000 ppm, 375 ppm and 2000 ppm respectively (Table 2 and Fig. 2). The stability of azadirachtin was found maximum in 30% botanical stabilizer i.e. only 40-50% degradation was recorded in these samples. It was found by Johanson *et al*., 2003[(9)] that exposure of azadirachtin in sunlight convert the (E)- 2-methylbuty-2-enoate ester group to(Z)-2-methylbut-2-enoate ester as shown in Fig. 1.

The half life of azadirachtin and other bioactive constituents of neem has recorded by various researchers is for few hours in sunlight and in

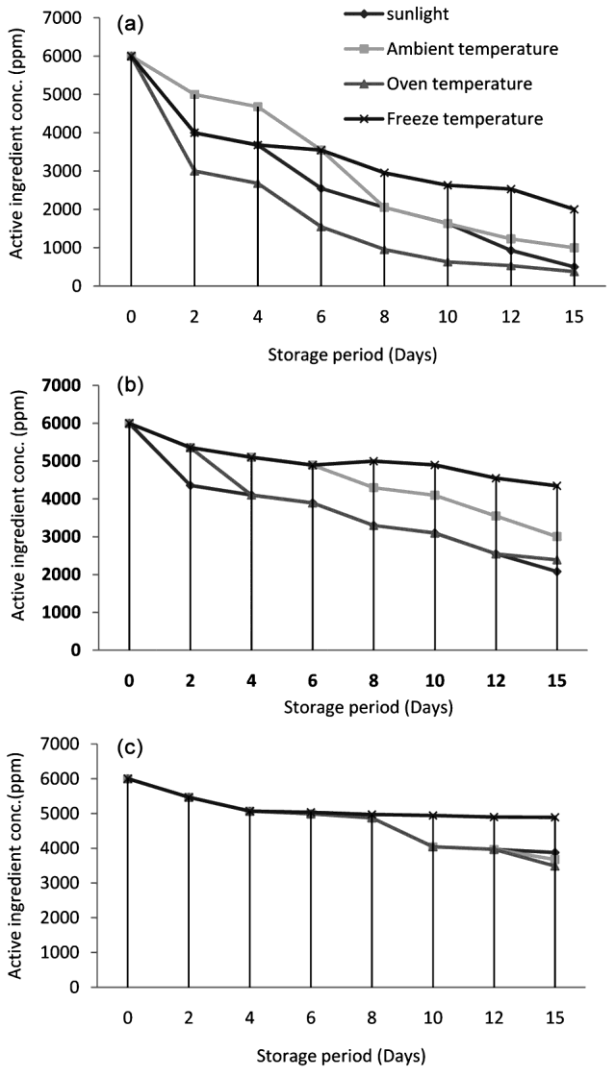

Fig. 2 — Concentration of Azadirachtin content in different interval of time

higher temperature half life is 5-6 days.[12,13] In presence of suitable stabilizer it might be enhanced to several days.

In water azadirachtin molecule undergoes rapid hydrolysis and hydrolyxation reactions due to presence of enol ether, acetal, hemiacetal, and tetra-substituted oxirane and varety of carboxylic esters.

The neem kernel compatability with botanical stabilizer was confirmed by FT-IR analysis. The FT-IR spectra conformed that no chemical reaction occurred in between the neem active constituents to botanical synergist active constituents. The function group peaks of neem and botanical were separate in FT-IR spectra. The FT-IT data confirms the compatibility of botanical adjuvants with neem oil in nanoemulsion formulation. Chemical constituents of *Prosopis juliflora* have been well studied by various researchers. It mainly constitutes fatty acids, oleic and linolinic acid, phenolic constituents.[14] The peaks appear in FT-IR of botanical spectra mainly occurs in 3000 cm^{-1} and 700 to 645 cm^{-1} region (Fig. 3).

In 100-0 NKP and KP, fungal growth was very prominent and increasing upto 15 days in sunlight, ambient, oven and freezing temperature. In 0-100 KP and NKP, optical density of aqeous extract slightly changed during different interval of time. In a study, it has been well defined that *Prosopis juliflora* has anti-microbial and anti- oxidative properties.[15]Several studies revealed that *P. juliflora* plant constituted several bioactive constituents which mainly include phenolics, tannin, alkaloid, terpenes, flavonoid, steroids etc.[16] Therefore, it is concluded in this study microbial growth in aqeous extract may be inhibited due to these constituents. In Fig. 3 (c) NKP and KP (80-20) showed less optical density as compare to 100-0, it shows that it provides antimicrobial property in neem aqeous extract. Similarly, in Fig. 4(c) NKP and KP (70-30) showed slight change in optical density in all temperature conditions. Hence, it is concluded that *P. juliflora* aqueous extract in 70-30 ratio is the optimized concentration for anti-microbial inhibition activity of neem aqueous extract.

Results of table showed that mortality percentage was found maximum in samples along with and without synergists i.e. 85–97% initially. After 15 days of storage, in sunlight, ambient temperature, oven temperature and freez temperature a wide variation could be found in mortality data. In sunlight stored samples with or without synergist, mortality was reduced from 95 to 67% with 12 mg/g LD_{50} value and in presence of synergist, mortality percentage is slightly reduced i.e. from 95 to 87% with LD_{50} value 7 mg/g. in ambient temperature mortality % reduced from 85% to 79% in presence of synergist and 67% in without synergist samples and LD_{50} value increased from 6 to 10 mg/g. In oven stored samples at 54° C mortality reduced from 86 to 75 and 67 % with and without synergist respectively. Similarly mortality % is also affected in freeze stored samples but with slight reduction in comparison to other storage conditions, here mortality % reduced from 97 to 79 and 87% in samples of with and without synergists. The results of Table 3 concluded that in presence of synergists % mortality is improved with slight variation in LD_{50} values. The obtained results indicated that synergist may reduce the active ingredient

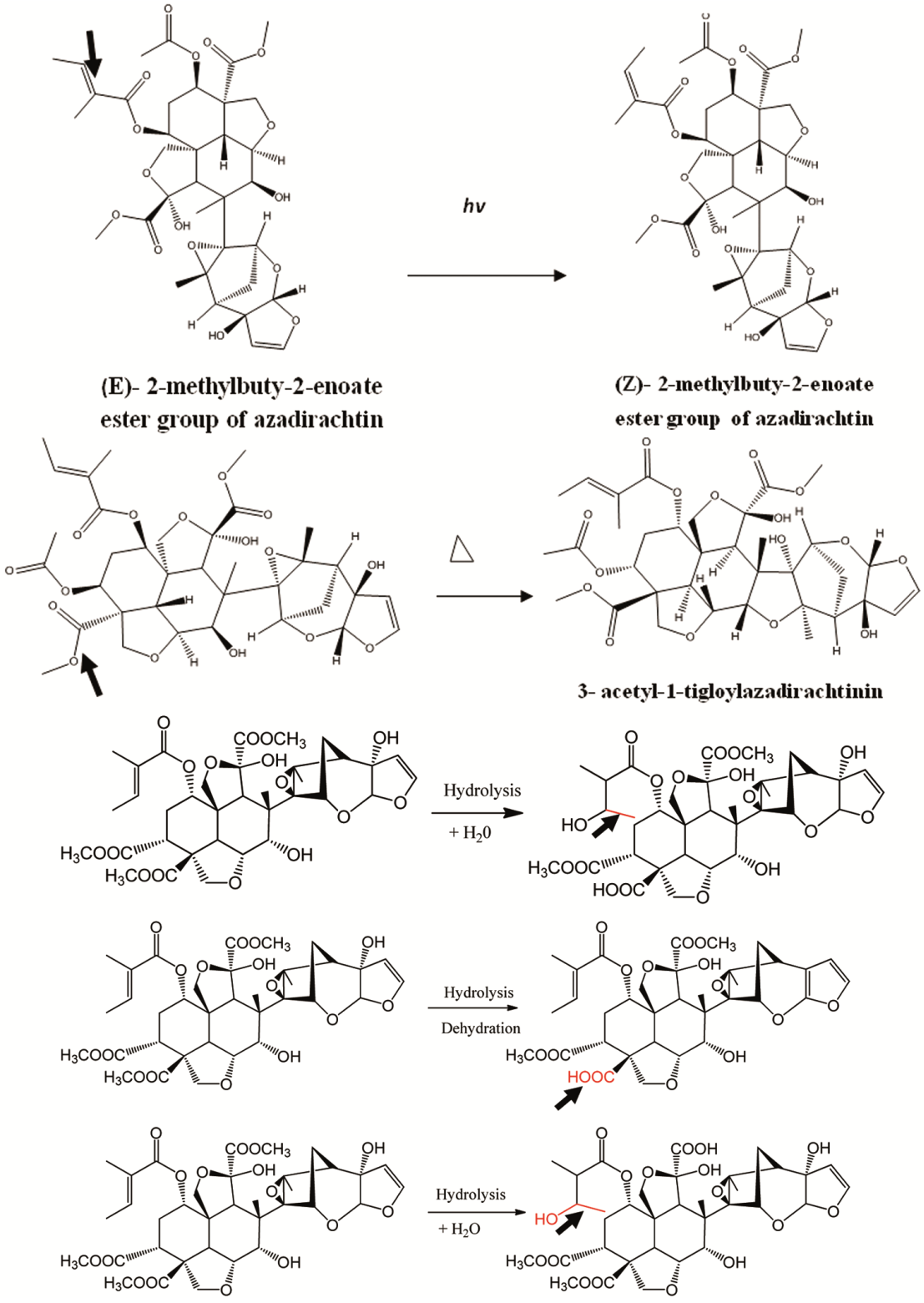

Fig. 3 — FT-IR spectra of neem aqueous extract with botanical stabilizer

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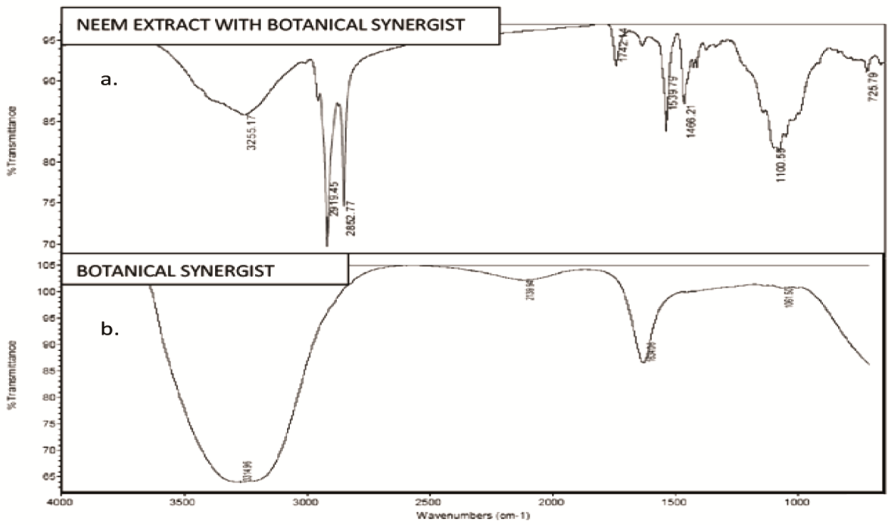

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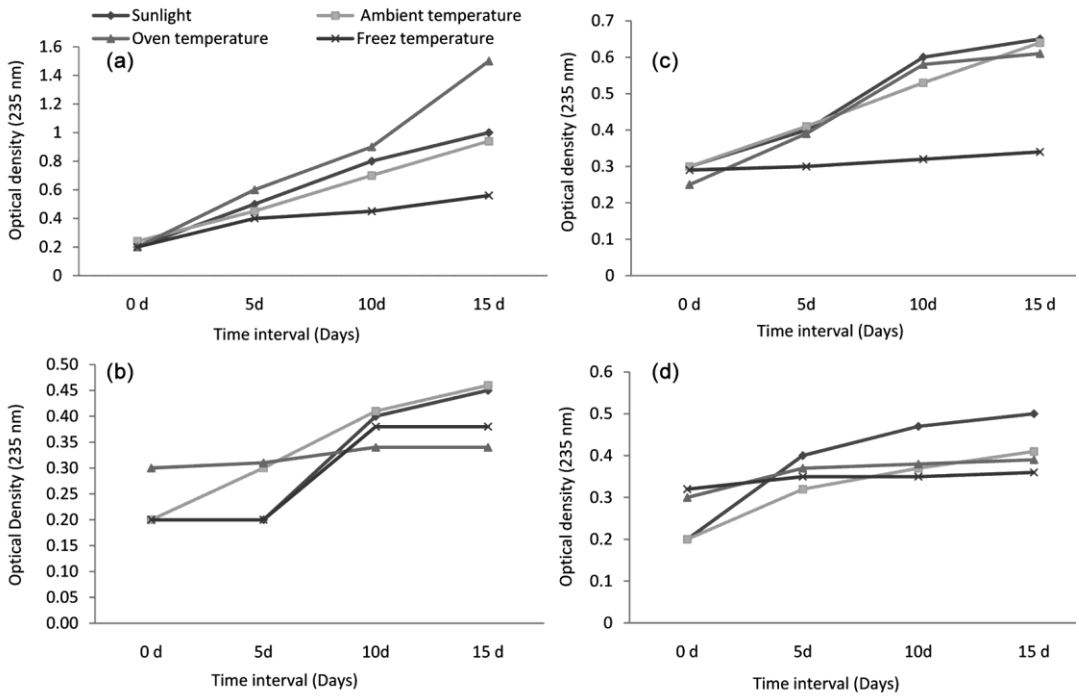

Fig. 4 — Estimation of fungal growth in neem aqueous extract by optical density calculation in different interval of time

Table 3 — Bio-efficacy evaluation of NKP aqueous extract with KP in treating mosquito breeding sites

	Initial 0 days		without synergist after 24 hrs		with synergist after 24 hrs	
	% Mortality	LD_{50}	% Mortality	LD_{50}	% Mortality	LD_{50}
Sunlight	95	6	67	12	87	7
Ambient temperature	85	6	68	10	79	7.5
Oven temperature	86	6	67	12	75	8
Freez temperature	97	5	79	9	87	8

degradation due to which mortality % is not altered in various temperature conditions.

Conclusions

The study elaborated the stabilization of neem active ingredient for long and effective utilization for common people in daily usage and agricultural applications. The botanical stabilizer used in this study is commonly available in rural areas. In presence of this botanical stabilizer, stability and efficacy of neem aqueous extract maintained for longer period of time. The approach used in this study maintains the faith of consumers on neem. Physiochemical analysis data showed that neem ingredient physical and chemical characteristics maintained during storage in various conditions. Bio-efficacy evaluation results against early stages of larvae were also found stable in presence of stabilizer. The present study gives a new ray of hope to preserve the neem physio-chemical and bioactive properties for extended period of time in various conditions.

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