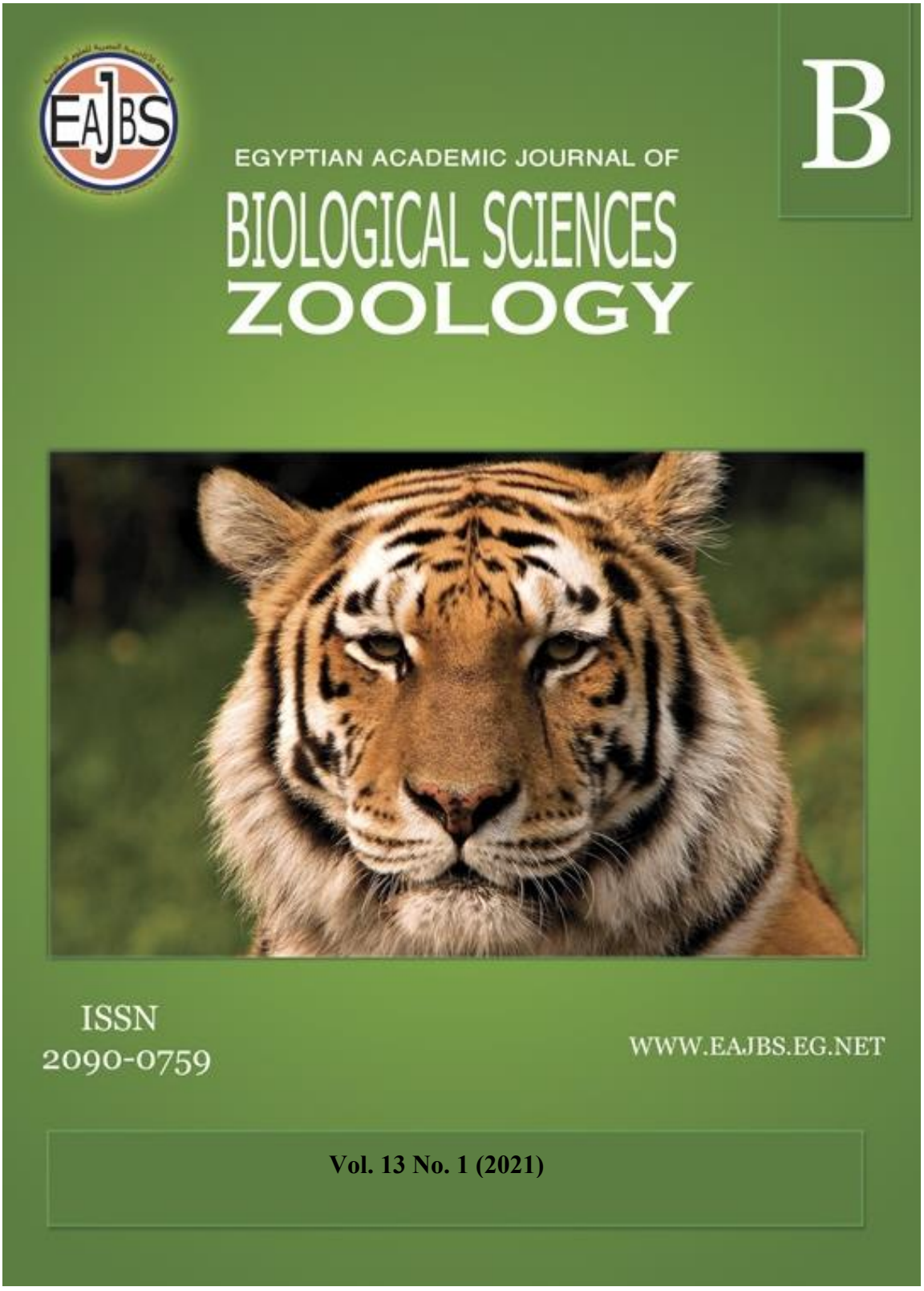

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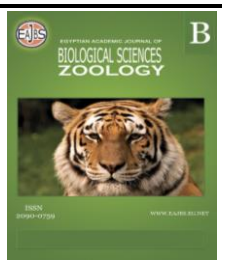

Testing the Repellent Effect of Clover Plant, *Trifollium alexandrium* Juice Against Black Rat, *Rattus rattus,* to Protect Stores

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ABSTRACT

Background: Rodent repellent substances are chemicals that have taste, odors, or both that will prevent rats from feeding or gnawing. Effects of fresh or fermented clover plant juice were studied as a repellent against both sex black rat, *Rattus rattus*, under laboratory and stores (fertilizers and equipment stores) conditions in Giza Governorate. Each fresh and fermented clover plant juice mixed with crushed maize at 23.8% (the appropriate concentration) was tested as bait using non and free choice feeding methods. **Results:** The results revealed that fermented juice was more repellent than fresh juice for rats as it achieved 92.6% repellency comparing with 38.7% for fresh juice after 4 days of treatment. The bodyweight of rats reduced after treated with fermented bait while it increased with fresh bait. The fermented and fresh clover juices were analyzed on Gas Chromatography-Mass Spectrometry (GCMS). The chemical compounds analysis names were: Erythro- 3- bromo -2- pentanol and other compounds while Thero -3- bromo -2- Pentanol compound was found only in fermented juice. The concentrations of the recorded compounds in fermented juice were higher than those in fresh juice. On the other side, the fermented clover plant juice bait achieved 85.7% efficiency against black rat, *R. rattus* as a repellent in stores after one week of treatment.

Conclusion: The fermented clover plant juice cussed repellent to the rodent. So, it can be used for protecting stores of different materials (equipment, electric cables and raw materials) from rodent attacks.

INTRODUCTION

Recently, scientists turned to the use of plant extracts containing the compounds to eliminate or repel agriculture pests instead of chemical pesticides that harm the environment. Rodents are considered one of the most important pests in Egypt and other countries. They cause great economic losses to growing crops, orchards, stored crops, poultry and animal farms (Singla and Babbar, 2010 and 2012).

Egyptian clover, or berseem, *Trifolium alexandrinum*, a very important crop, for fodder and soil fertility maintains, was domesticated in Egypt and is now widespread in irrigated cropping systems in the west and south Asia (Zayed *et al*., 2012). Rodent repellents are chemical substances that have taste or odor or both that prevent animals from feeding or

gnawing. Such substances are used for protecting an area from rodent infestation or for protecting packaged food, packing materials, electric cables, and other important raw materials. Several studies were carried out and published in several countries about using organic materials or plant extracts as a repellent against many pests. The potential of eucalyptus oil was evaluated as a repellent against *R. rattus,* a predominant rodent pest species (Thongsong *et al*., 2010; Thind and Mahal, 2014) revealed that Percent repellency was more when the oil was applied daily and alternatively as compared to when applied once a week indicating low persistence of the repellent effect due to volatile nature of the oil. Thongsong *et al.,* (2010) evaluated the efficacy of chilli, wintergreen oil, bergamot oil, peppermint oil, and geranium oil as repellents in the circular open field against adult male Westar rats. Werner *et al.* (2016) observed 24–37% repellency in black-tailed prairie dogs (*Cynomys ludovicianus*) offered corn seeds treated with 0.5–4.0% anthraquinone. Plants with strong smells act as repellents and can protect the crops nearby (Dubey, *et al.,* 2011). Single and Garg (2014) reported that Eucalyptus oil was caused repellant to house rat and *R. rattus* after used concentrations of 5, 10 and 20% of Eucalyptus oil for one week. Effect of *Plumeria rubra* (Apocynaceae) leaf extracts, a repellent of rice-field rats (*Rattus argentiventer*), on its metabolism and daily activity Bari (2020) found that Methanol extract of Plumeria leaves affects a repellent for the rice-field rat, *Rattus norvegcus*. The effect of *Trifollium* pretense on spermatogenesis and its acute toxicity (LD_{50}) in mice, Bakiral and Keles (2002) found that the LD_{50} was 4237 mg / kg and negative effects on sperm parameters. This plant has low toxicity on mice. *Trifolium* species (*Trifolium africanum, T. burchellianum, T. dubium, T. repens and T. pretense*) were found rich with nutrition elements (Ca, Mg, Fe, Mn, Zn, Se, Cu, Cr, Pb, Ni, Co, Cd, As, lipid, protein and carbohydrates. These types have the nutritional value of edible, Gounden and Janna (2018).
Fermentation is a metabolic process that produces chemical changes in organic substrates through the action of enzymes. In biochemistry, it is narrowly defined as the extraction of energy from carbohydrates in the absence of oxygen. Effects of feeding format-treated alfalfa silage or red clover silage on the production of lactating dairy cows (Broderick, *et al.,* 2010) recorded that cow fed on alfalfa silage 20% increased intake, yields of milk, fat correct milk and protein. Fermented clover plant (silage) was used as a protein source and effect of the production of lactating dairy cows. Effect of wilting and ensilage with or without additive on protein quality and fermentation of lucerne white clover mixture (Sousa *et al.,* 2019) rumen under gradable during reduction of lactating dairy cows ensilage, silages had lower true protein concentration than unwilted and wilted herbage, acid-treated silage had greater true protein concentration among the silage and acid-treated silage hah lowest dry losses among the silage.

The aim of this work to study the repellent effect of fresh and fermented clover plant juice as bait for protecting stores against black rat, *R. rattus*, attacks.

MATERIALS AND METHODS

Tested Plant:

Egyptian clover plant, *Trifollium alexandrium*, Fam. Leguminoseae (last cutting in may) was identified by Field Crops Research Institute, ARC., Giza Egypt. It was collected from the field of Shebin El-Kom district, Monufia Governorate. This plant safe as its LD_{50} is 4237 mg/kg for mice (Bakiral and Keles 2002).

Clover Plant Sample Preparation:

Four Kg of clover plants were cleaned from weeds and dirt. Two Kg were juiced and filtered as fresh clover juice and the other amount was fermented in plastic black sacks for ten days in the absence of oxygen until completely dissolved, squeezed and filtered

Tested Animals:

Both sexes of the black rats, *Rattus rattus*, were trapped from stores and houses in Giza Governorate. Animals were transferred to the laboratory of Plant Protection Research Institute, ARC., Giza Egypt, and caged individually in standard laboratory cages (50× 30×30) with sawdust before and after experiments. They were fed on a free crushed maize and water *ad libitum* at all the time including during the experiments. The animal was held at a 12 h light / dark cycle at 25°C keeping experimental conditions consistent at all times. Rodents were acclimatized to the experimental environment for at least two weeks. The unhealthy and pregnant animals were excluded. Twenty-five adult animals (180- 200g) were divided into groups (each of 5 rats).

Laboratory Experiments:

Serial concentrations of fresh and fermented clover juice were selected and tested as bait mixing with crushed maize to choose the most effective concentration. The most effective concentration was 23.8% of both.

1.Non-Choice Feeding Test:

Three groups of rats were used, two groups for each treatment and another one for control. One group fed on fresh clover juice bait and the other group fed on fermented juice bait at 23.8% for four successive days. The consumed amount of bait was estimated. Then the bait was removed and the animals fed on untreated crushed maize with observation for 28 days. During this period the mortality and body weight of rats were recorded.

2. Free Choice Feeding Method:

The free-choice feeding method is important to determine the acceptability of each tested juice bait comparing its consumption with the free crushed maize. One of each tested juice bait and free crushed maize were offered to each rat (50gm of each) in small separate dishes. Their position was daily altered to avoid feeding preference for a certain location. The consumed amount of treated and untreated bait was recorded daily for four successive days and the repellency was calculated using the following equation (Bullared *et al*., 1983)

$$\text{Repellent \%} = 100 - \frac{\text{Consumed amount of treated bait (g)}}{\text{Consumed (treated. + untreated) bait (g)}} \times 100$$

Another test was conducted with the same producer using crushed maize inside plastic sacks socked in fermented clover juice (Fatma Mostafa, 2005). After the study finished, the animals were euthanized.

3.Clover Plant Juice Analysis:

Fresh and fermented clover plant juices were analyzed using Gas Chromatography-Mass Spectrometry (GC/MS). The GC/MS system (Agilent Technologies) was equipped with gas chromatography (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. Headspace temperature program: oven temperature 80°C, loop temperature 120°C and transfer line temperature 140 °C with an equilibrium time of 20 min. The GC was equipped with an HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 μm film thickness). Analyses were carried out using hydrogen as the carrier gas at a flow rate of 1.0 ml/min at a split mode 50:1, injection volume of 1 µl and the following temperature program: 50 °C for 2 min; rising at 10 °C /min to 250 °C; rising at 15 °C/min to 300 °C and held for 10 min. The injector and detector were held at 280 °C and 220 °C, respectively. Mass spectra were obtained by Electron Ionization (EI) at 70 eV; using a spectral range of m/z 50-550. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Field Experiments:

Field evaluation of fermented clover juice at 23.8% was carried out under stores (fertilizer and equipment stores) conditions using crushed maize. The area which was

infested with black rat, *R. rattus*, in Abu-Rawash – Giza Governorate was divided into three replicate stores for treatment and another one was left without treatment as a check control. The population density of rats was estimated pre- and post-treatment using the food consumption method. Five kg of crushed maize were divided into small plastic sacks socked in fermented juice (50g of each) and distributed in and out of stores. The consumed amount of crushed maize was weighted daily and recorded for one week.

The efficiency of compound % was calculated as follow:

$$\text{The Compound efficiency \%} = \frac{\text{Average consumption of food (pre - post) treatment}}{\text{Average consumption of crushed maize pre-treatment.}} \times 100$$

Statistical Analysis:

The obtained results were statistically analyzed by one-way ANOVA and the Least significant difference (LSD) at ($P< 0.05$) Costate program (Cohort, 2005).

RESULTS

Laboratory Studies:

Effect of different concentrations of clover plant juice.

The effect of different concentrations of fresh and fermented clover plant juice bait using a non-choice feeding test was explained in Table (1). The results indicate that the fermented clover plant juice bait was more repellent than fresh juice bait. Whereas the consumption of the average bait for fresh juice bait was 25. 24. 25. 20. 20 and 12.85 g at 762.7, 381.3, 190.7, 95.3, 47,7 non-feeding consumption have occurred in case of fermented juice bait with the same concentrations, except 23.8% concentration which gave 1.75 g bait consumption with no mortality.

Table 1: Effect of different concentrations of fresh and fermented clover plant juice bait against black rat, *Rattus rattus,* using non choice feeding method for four days.

Concentrations %	Average Bait Consumption (g)			
	Fresh	Mortality%	fermented	Mortality%
	mean± SE	mean± SE	mean± SE	
762.7	25.0 ± 0.0	0.0	0.0	0.0
381.3	24.0 ± 0.6	0.0	0.0	0.0
190.7	25.0 ± 0.5	0.0	0.0	0.0
95.3	20.0 ± 0.2	0.0	0.0	0.0
47.7	20.0 ± 0.8	0.0	0.0	0.0
23.8	12.9 ± 0.7	0.0	1.75 ±0.2	0.0

1.Non-Choice Feeding Test:

Data in Table (2) tabulated the effect of fresh and fermented clover plant juice bait 23.8% on the bodyweight of the black rat, *R. rattus*, in a non-choice feeding test for four days. Data revealed that animals that fed on fresh juice bait were increased in their body weight between (20- 30 g) while the animals treated with fermented juice bait were reduced in their body weight between (10 -20 g).

Table 2: Effect of fresh and fermented clover plant juice bait 23.8% on the body weight of black rat, *Rattus rattus*, in non-choice feeding test for four days.

Rats No.	Body weight (g)					
	Weight before treatment	Control	After treated with 23.8%			
	Untreated		Fresh bait		Fermented bait	
			weight	Difference %	weight	Difference %
1	180	190	210	+16.6	170	-5.5
2	190	190	220	+15.7	170	-10.5
3	200	200	215	+7.5	180	-10
4	200	210	220	+10	190	-5
5	200	200	220	+10	180	-10

2.Free Choice Feeding Test:

The effect of fresh and fermented clover plant juice bait 23.8% against black rat, *R. rattus*, using free-feeding test were explained in Table (3). The results cleared that the fermented clover juice bait achieved a higher repellency percent (92.8 %) than fresh juice bait which gave 38.7% repellent percent. On the other hand, the fermented juice achieved 99.9 % repellency percent when using plastic sacks full of untreated crushed maize socked in the fermented juice.

Table 3: Effect of fresh and fermented clover plant juice bait 23.8% against black rat using free choice feeding test.

Treatments	Average of Consumption (g)			LSD	Repellent %
	Control	untreated	treated		
	Mean ± SE	Mean ± SE	Mean ± SE		
Fresh bait	$13.5^{b}\pm 0.7$	$11.2^{cC} \pm 0.4$	$17.1^{aA} \pm 0.4$	1.6	38.7
Fermented bait	$13.5^{b} \pm 0.7$	$16.2^{aA} \pm 0.3$	$1.3^{cB} \pm 0.2$	1.5	92.6
Sacks full of crushed maize	$15.0^{a} \pm 1.3$	$14.8^{aB} \pm 0.5$	$0.3^{bB}\pm 0.2$	2.5	99.9
LSD	2.9	1.4	0.8		

The mean of consumption showed a significantly different than control in all treatments (P value ≤ 0.05).
abc = Significant between the row.
ABC = significant between the column.

Chemical Analysis of Fresh and Fermented Juice:

Data in Table (4) and Figures (1& 2) explained the analysis of the chemical compounds in fermented clover juice. Twelve compounds were recorded with higher percentages in fermented than fresh clover juice except for the- 3- bromo- 2- pentanol was found in fermented juice with 57.6% concentration but it was absent in fresh juice. Also, the concentration of Erythro- 3- bromo-2- pentanol was 36.1% in fermented juice while it was 13.9% in fresh juice.

Table 4: Compounds analysis of fresh and fermented clover plant juice by Gas Chromatography/ Mass Spectrotrometry (GC/MS).

No.	Name of compounds	Probability%	
		Fresh clover juice	Fermented clover juice
1	Erythro-3-bromo-2-pentanol	-	57.6
2	Threo-3-bromo-2-pentanol	13.9	36.1
3	2-Fomylhistamine	0.98	2.19
4	Propanamide-2-hydroxy	0.14	0.93
5	Butanol-4-methoxy	0.41	0.78
6	Acetic acid, cyano	0.07	0.38
7	Ethylamine	0.01	0.28
8	Fluoroacetic acid	0.17	0.27
9	Topotecan	0.10	0.27
10	Ethanamine, 2-propoxy	0.02	0.21
11	2-Isopropoxyethylamine	0.01	0.16
12	Ethanamine, 2-propoxy	0.09	0.28

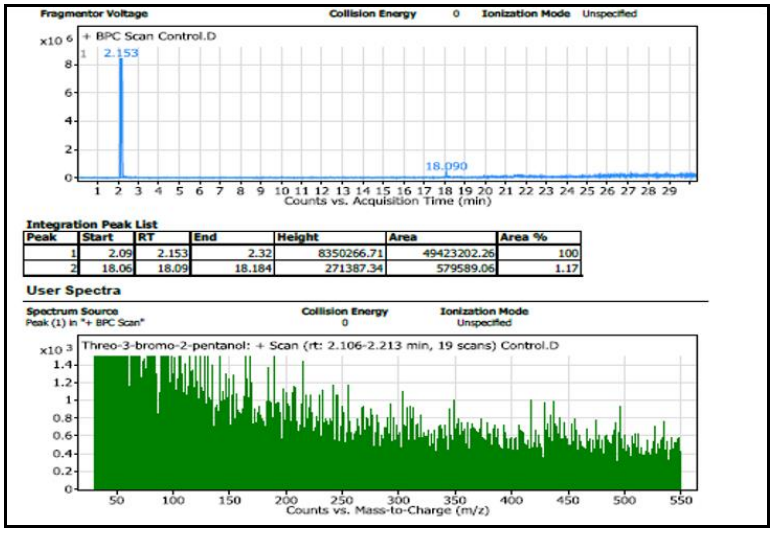

Fig. 1: Fresh clover juice analysis.

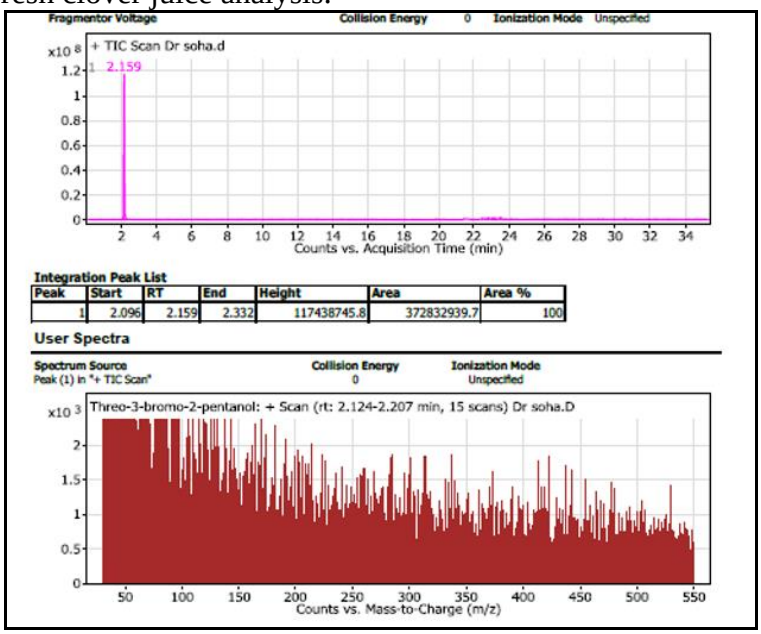

Fig. 2: Fermented juice analysis.

1.Field Studies*:*

Data in Table (5) showed the repellent effect of fermented clover juice bait (23.8%) for one week against black rat, *R. rattus*, under store conditions. Data cleared that the average consumption of crushed maize in pretreatment was 4200g from 5000g and the consumption of treated bait was 1250g while the average of post-treatment was 600g. Also, the results revealed that the fermented clover juice achieved 85.7% repellent efficacy.

Table 5: The repellent effect of fermented clover juice 23.8% against black rat, *Rattus rattus,* for one week to protect stores using socked plastic bags in juice.

Bait consumption (g)				Compound efficiency for repellent %
Control	Pre treatment	Treatment	Post treatment	
3800	4200	1250	600	85.7 %

DISCUSSION

Discussing the previous results, it could be found that the fermented clover plant juice bait was more repellent than fresh juice baits this result may be due to the odor of fermented juice bait is undesirable for rats, as it has smells like sewage. Coulston *et al.*, (1993) applied synthetic stoat odor (3- propyl- 1,2 dithiolane and 2-propylthietane) and faecal odor (2.5- dihydro- 2,4,5- trimethyl thiazoline) at various concentrations to chickpea (*Cicer arietimum*) seeds at sowing. They found the stoat odor at 10% concentration exerted a measure of predator odor, as did 1% fox odor against predation by laboratory mice. On the other hand, the fermented clover juice bait caused decreasing in the bodyweight of treated animals and repellent while vice versa occurred when treated with fresh juice which caused an increase in the body weight of treated animals and acceptance to bait this may be due to the fresh clover juice rich in protein, carbohydrates and many elements for health body this result confirms with Gouden *et al*., (2018) who recorded the elements analysis and nutritional value of edible some clover species were (Ca. Mg, Fe, Mn, Zn. Carbohydrates and proteins). Also, when fermented and fresh juice was analyzed, the results obtained that thero -3 bromo-2- pentanol was found in fermented juice while it was absent in fresh juice, and higher concentration of Erythro -3- bromo -2- pentanol in fermented than fresh juice. So, the odor which caused repellent to rats may be due to the presence of element bromine which has smelled like sewage odor. On the other side, the allergy suffocation and inflammation of the eyes may be due to the fluoro acetic acid which is found in fermented juice that causes respiratory irritation and severe irritation of the nose and throat (Safety Data Sheet). Regarding the repellent effect of fermented juice under in and out stores conditions the compound achieved satisfying results to repel the rodent faraway of stores may seem to the results occurred with Thongsong *et al.*, (2010) who investigated chili, peppermint oil, bergamot oil, and geranium oil caused a repellent effect in the field for rat comparing with control field.

Also, in the store's test the fermented clover juice was unacceptable to feed on it so it success to repel the rat and when spray the plastic which covered the cables, hungry rats couldn’t gnaw the cables. In addition, it should repeat the spray of plastic that covered the cables or the equipment with the fermented clover juice every week to keep the odor volatile and keep the rodent repellent effect. Thongsong *et al.*, (2010) recorded that when the rats were exposed to potential rat repellent, the number of visits to the listing core and the time spent in the inner zone was lower compared with control.

Conclusion

The stored materials such as equipment, electric cables and raw materials should be covered with plastic covers treated with fermented clover plant juice to protect it from rats' attacks. It can also be included in integrated rodent control materials.

Abbreviations:

MARL: Ministry of Agriculture and Reclaimed Land, LD_{50}**:** Half Lethal Dose, GC/MS: Gas Chromatography-Mass Spectrometry, EI: Electron Ionization, ANOVA: Analysis of Variance, (LSD): Least significant difference, No.: Number, ARC, Agriculture Research Center.

Ethics approval and consent to participate: Regarding animals ethics approval by Plant Protection Research Institute, ARC., Giza Egypt.

This article does not contain any studies with human participants performed by any of the authors.

Consent for publication: All authors consent for publication.

Availability of data and material:

The datasets used and/or analyzed during the current study are available in this article contains studies on animals (rats) performed by the authors.

Competing interests: The authors declare that they have no competing interests.

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