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GENETIC VARIABILITY INDUCTION, UPR, ISSR AND GC-MS IDENTIFICATION THROUGH EMS MUTAGENESIS OF *DATURA STRAMONIUM* L.

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Abstract

This experiment was carried out at the fields of Medicinal and Aromatic plants research unit, College of Agriculture Engineering Sciences, the University of Baghdad within two spring seasons of 2017 and 2018. Randomized complete block design by factorial arrangement was applied with three replicates to study the effect of EMS (Ethyl methane sulfonate) chemical mutant on *Datura stramonium*. The treatments were included the first factor as presoaking of Datura seeds in four EMS different concentrations 12.5, 25, 50, 100 µM with untreated seeds 0 µM (control) during two incubation times 24 and 48h as the second factor. This project aims to evaluate the possibility of producing phenotypic and genetic variations to increase the yield of active alkaloids by increasing of their concentration in the leaves and /or increase plant leaves area, in addition to improve some of growth traits such as plant height, branching which may be induced by of EMS to obtain a range of genetic variation in mutant population to be used for further studies including screening for various traits. The resulting of M_1 selfing plants population was planted in the field at the next following year to get M_2 and M_3 for phenotype, phytochemicals, and genetically analysis by using (URP and ISSR) techniques as molecular genetic fingerprints. At the beginning of capsule maturation stage of each season, plant traits including leaves area, plant height, branching no fresh and dry weight in addition to phytochemical compounds including some of alkaloids were measured by GC-MS. This EMS mutant's population will be used for induced the variations and further studies, including screening for various traits such useful agronomic traits, total alkaloids. Also, the beneficial traits from these mutants' plants can be exploited for future Datura breeding program. The vegetative growth of M_2 generation showed that treatment 50 ìM of EMS with 48 hours seeds soaking it get the highest in plant leaves area it reached 15999.80cm^2 / plant, the highest fresh weight it reached 386.59 gm, The highest dry weight it reached 76.79gm and the highest percentage of total alkaloids with11.05%. And the lowest 5.32% was in control. The phytochemical analysis of Datura leaves extract revealed the presence of alkaloids, tannins, flavonoids, glycosides, saponins, quinone and triterpene. Also the GC-MS fraction analysis results indicated to the ethanolic extracts of Datura plant treated with 100 mM EMS with seeds soaking of 48h were contained forty-two of the identified phytochemical compounds, Majority of these new compounds were 1-(4-Methoxyphenyl) piperazine and 5-Azacytidine of 11.67%, 15.47% area sum respectively, which have been reported to be highly of therapeutic importance. Piperazine belongs to the family of medicines called anthelmintic. These two treatments with EMS showed genetic variations and considered a useful gene mutation.

Key words: *Datura stramonium* L, Genetic Variability, EMS, URP, ISSR, GC-MS Analysis, Photochemical Compounds, Total Alkaloids

Introduction

Datura crop is one of the most important medicinal plants belonging to the Solanaceae family. It has nine species of wild and cultivated spread around the world, including *D.innoxia*, *D.Stamonuim*, *D.Metel*, *D.ferox* and others (Ioannis *et al.,* 2014). The chemical analysis of Datura demonstrated that the leaves especially were rich in alkaloids, including atropine, hyoscyamine, and scopolamine, in order to demonstrate the medicinal importance of Datura sp., Neeraj *et al.,* (2013) mentioned that Datura contains more than 30 types of alkaloids,

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mainly Scopolamine, hyoscyamine and Tropine, which have different medical uses such as antibacterial, anti-inflammatory, antibacterial, antitumor, antidiabetic, hepatoprotective. Kunio *et al.,* (1996) found that the content of scopolamine in the Datura plant varies according to plant parts and age, and varies according to cultivated species and environmental conditions, ranging from 0.29-0.69% (dry weight). The developing commercial status of secondary metabolites in recent years has resulted in a great interest in secondary metabolism, different strategies have been extensively studied to improve the production of secondary metabolites in medicinal plants, one of these strategies were use the mutations (Jamwal *et al.,* 2018). Mutations have played a great role in increasing world food security (Kharkwal and Shu, 2009). Mutation induction offers the possibility of inducing desired attributes that either cannot be found in nature or have been listed during evaluation. Treatment with mutagens alters genes or breaks chromosomes. Gene mutations occur naturally as errors in DNA replication.

Consequently, genetic variation appears rather limited, and breeders have to resort to mutation induction (Novak and Brunner, 1992). Chemical mutagenesis was regarded as a useful and important tool in improving the yield of many crops and quality characters. In general, alkylating agents are very active mutagens in higher plants. The role of mutation breeding is increasing the genetic variability for quantitative traits in various crop plants have been reported by a number of scientists (Rachovska and Dimova, 2000; Khan and Wani, 2009; Khan and Goyal, 2009; Marcelina *et al.,* 2011; Kozgar *et al*., 2011; Singh, *et al.,* 2019; Jeevi and Mullainathan, 2019).

For medicinal plants, mutations are usually more valuable because they exhibit increased biomass and content of active compounds. A number of chemical compounds have been studied for mutations on the basis of their effectiveness in the possibility of increasing the active substances and / or causing genetic variations in the Datura plant or increasing their content of the active substance, especially in obtaining plants multiplying the number of Ploidy (4n) of the treatment of normal plants (2n) Taking into consideration its universally and researchers common use and its relatively low-risk and relatively non-toxic human condition (Hassan, 2012). Several studies were conducted in the early 1950s and 1960s to investigate the genetic behaviour and the succession of alkaloids and to maximize the content of active substances in Datura through hybridization and selection (Lubis, 1967; Amiri *et al.,* 2010**).** Al-Taweel *et al.,* (2019) got a tetraploid plant in *Datura Stramonium* by treating seeds with 1% Colchicine for 48h which lead to improve the vegetative growth and increased the useful a tropane alkaloids percent in the Datura plant leaves.

Ethyl methane sulfonate (EMS) is a chemical mutagen which is frequently used for seeds mutation because it is effective and induces high frequency point mutations, some of which lead to a novel stop codon for different genes (Arisha, *et al.,* 2015). Mallick and Sasmal (2000) presoaked Datura stramonium seeds in 1, 2% EMS, then noted that all studied traits such as branching and fruiting traits were decreased except plant height but they not measured leaves area and valued Alkaloids content in treated individual plants. Saha, (2019) founded that EMS-induced genetic variability for yield, plant height, number of primary and total branches, capsule/plant, seeds/capsule and total seeds/plant in M_2 and M_3 generations of *Sesamum indicum* L. and the treatment with 1% for 4h (M_3) is found most promising for induction positive genetic variation. More studies is required to investigate the mutagenic effectiveness and efficiency of EMS and especially the metabolite, more knowledge about the effect of time, PH value, temperature, seed soaking time and various concentrations are required (Khan and Tyagi, 2009). There are several criteria by which genetic variability can be evaluated, including DNA analysis. DNA markers rely on the analysis of the DNA molecule directly, allowing for a more precise assessment of the genetic group to be studied. These indicators allow for the study of the entire genome and the detection of genetic differences between the individuals comparing them with each other and by revealing the largest number of genetic sites. These indicators express the natural variations inherited from the nucleotide sequence in the DNA of the individuals under study, which is the result of deletion, addition, replacement or for any reason whatsoever. Random amplified polymorphic DNA (URP) markers and inter - simple sequence repeats (ISSR) markers are two molecular approaches that have been used to investigate variation among plants treated with EMS. Each method has been used to identify and determine the changes that may happen in plants genomes (Raina *et al.,* 2001: Martins *et al.,* 2003). These methods are widely applicable because they are inexpensive, rapid, simple to perform, do not require pre-knowledge of DNA sequence and require very little amount of DNA template (Esselman *et al.,* 1999). ISSR markers are reproducible markers, because ISS primers designed to anneal to a microsatellite sequence (Goulao and Oliveira, 2001), To identify some genotypes of Henbane *Hyoscyamus muticus* L. from Solanaceous family the same family of Datura. URP and ISSR techniques were used as molecular genetic

fingerprints (EL- Shawaf *et al.*, 2009) URP primers referred to universal rice primer, were developed from a repetitive sequence of the rice genome. The generality of URP was indicated by applying it to 15 cultivars from five rice species. PCR approach using URP primers will be useful for detecting DNA dissimilarity of most eukaryotic or prokaryotic genomes (Kang, *et al.*, 2001). URP primers were employed for the first time in Datura species genetic fingerprinting and proved to be successful in amplification of polymorphic fragments that are sufficient to discriminate their phylogenetic origin (Tsialtas *et al.*, 2014). That study have provided evidence to induced genetic variability can effectively be exploited for evolving mutant strains possessing desirable attributes and for rectification of simply inherited morphological deficiencies in wheat crop.

The aim of this study is investigating the effect of various concentrations of EMS mutants, seeds soaking time on the possibility of useful genetic variation by using (URP and ISSR) techniques as molecular genetic fingerprints. Also, the results of the combination between the URP and ISSR techniques analysis revealing of Datura under study, for developing useful gene mutations and effect on the level of genetic mutations of nitrogen bases by deletion, addition or replacement. Also, this study aims to identify the phytochemical compounds and yield of active medically alkaloids in *Datura stramonium* treatments which effected by EMS with a chance to improve the vegetative traits and useful alkaloids content of Datura plant by plant to raw selection programs within the following generations M_2-M_5.

Materials and methods

Experimental Design and Plant Materials:

A factorial experiment was carried out in the fields of medicinal plants research unit, Faculty of Agriculture engineering sciences, University of Baghdad-Iraq, for two growing seasons, 2017-2018. The Datura seeds were obtained from medicinal and aromatic research unit in faculty of agricultural engineering sciences - Baghdad and kept in the refrigerator until the next planting season The cleaned homogenous seeds were soaking in tap water for 24h, then the 250 separated seed were treated in five levels of EMS (0, 12.5, 25, 50,100 µM) symbol as (C_0, C_1, C_2, C3, C_4) respectively for two incubation time 24h and 48h symbol as T_1, T_2 respectively. The researchers selected EMS chemical mutant that are processed and processed as high-quality analytical materials from Sigma-Aldrich. The seeds of the M_0 breeder mixed with the distilled water and changed for 24 hours and then placed in special dishes for 48 hours at the temperature of the refrigerator to encourage germination. At the beginning of the emergence of the stalk length of 2 mm selected 250 seeds of each dish and for 24h, 48h hours for an ethyl sulfonate ethylene in PH = 7 and at room temperature (Hassan, 2012). After the treatment time, the seeds of the first M_1 baby embryo are washed well with distilled water for later cultivation in special flint dishes. The vigorous seedlings are then selected for active growth to be transferred to the plastic house. The appropriate plastic house is not heated when it is allocated, the suitable soil and the water source for the experiment or to be planted in pots with a diameter of 30 cm (plant / pot) when it reaches the stage of transplant appropriate to the age of 2-3 real leaves, The treatment's plantlets from treated seeds and control were planted at the middle of February 2017, the plantlets transfer to the open field to be planted at the rate of 10 plants per unit experimental and thus the number of treatment coefficients 8 (4 EMS conc. × 2 times) treatments and three replicates and 24 experimental units and a total of 240 plants and of *Datura stramonium* under this study. Crop management has been operated by various services of the crop of fertilization and weeding and irrigation recommendations**.** and the number of leaves of the plant taking into account the height of the plant and the plant and free of diseases and insects' field and then superior in content of total alkaloids. The Statistical Analysis System- SAS (2012) program was used with least significant difference test (LSD) to significant compare between means values.

Physiological and Biochemical Properties:

Seeds Germination %:

The present measurement was initiated to find out the effect of EMS on germination percentage and to get an idea on survival plantlet for *D. stramonium*. Two hundred healthy seeds were taken for each treatment. Presoaked seeds were treated in three EMS different concentrations 25, 50, 100 µM with presoaked seeds in tap water for EMS 0 µM (control) treatment during two incubation times 24 and 48h. After washing with running water for three hours, they were planted to germinate in 15cm pots filled with washed sand soil in greenhouse. The germinated seeds number was recorded at an interval of seven days and the count was continued up to four weeks when the germination was stopped within three continues days. Plantlets survival was counted at the end of this experiment (Mallick and Sasmal, 1997). The final germination percent was measured according to (AOSA, 1988).

Vegetative Treaties:

Individual phenotypic selfing and selection on the first

and second generation plants at the beginning of the flowering stage, labelling and plants selection during M_2, M_3 generation have been done at the maturity of the capsules to diagnose the different plants and superior appearance in the strength of growth. Leaves fresh weight, plant height, branches numbers, leaves area, to the measurement of dry leaf weight, the cleaned leaves were placed in the oven at 50 C for 3 days. The collected data analysed by SAS software based on LSD ranged test.

Phytochemical Analysis:

The leaves methanolic extract tested for the presence of various bio-active compounds. The phytochemical identification in Table 2 was determined using chemical methods and by adopting standard protocols to identify the constituent's substances as described by (Harbone, 1973). The GC-MS component's fraction analysis in Table 5 was carried out at Feed and Regional Food Center – Agriculture Researches Center-Egypt by using GC-MS (Agilent Technologies 7890A), (Santana, *et al.,* 2013; Taweel, *et al.,* 2019).

Total Alkaloids:

Alkaloids content was calculated and expressed as a percentage of weight of sample analyzed according to (Ijarotimi *et al*., 2013; Al-Taweel *et. al*., 2019).

Genetic Properties:

DNA isolation:

Total DNA was isolated from the young leaves of ten treated *Datura stramonium* samples according to (Dellaporta *et al.,* 1984).

URPs Analysis:

Using URP primers table 1 with the Polymorphic Chain Reaction (PCR), The amplification was performed for 42 cycles as follows; initial denaturation at 94°C for 3 min, one cycle, denaturation at 94°C for 1min., annealing at 60-62°C (depending on the GC content of the primer, Table 1) for 1min., extension at 72°C for 1 min., (35 cycles) and final extension at 72°C for 7 min., (one cycle). The product resolved on agarose gel (1.2%) in TBE buffer. A 100-bp DNA ladder (Biotools) was used to estimate the approximate molecular weight of DNA bands for each PCR product. The run was performed for about 1h at 82 V in a mini gel agarose electrophoresis apparatus (Biorad) and stained with ethidium bromide. The products visualized by using the U.V. light. The PCR products were photographed by gel electrophoresis system (Gel Doc. BIORAD 2000) under Transilluminatorr.

Table 1: URP and ISSR primers code, their sequences used for detection of banding patterns in Datura s, treated with EMS.

Sequence (5 → 3)	Primer code	Primer Number
URP PRIMERS		
ATCCAAGGTCCGAGACAACC	URP1F	1
CCCAGCAACTGATCGCACAC	URP2R	2
GGCAAGCTGGTGGGAGGTAC	URP6R	3
ISSR PRIMERS		
GAGAGAGAGAGAGC	A_{98}	1
CACACACACACAGT	HB_{13}	2

ISSRs analysis:

The primers used in PCR amplification of Inter-simple sequence repeat regions (Table 1) synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems) at AGERI, ARC. Amplification performed as URP but with 55°C for annealing instead of 60-62°C.

Results and Discussion

Seeds Germination %:

Fig. 1 shows a gradual reduction of germination percent with the increase of EMS doses. Similar observations were reported by Mallick and Sasmal, (1997). *Datura stramonium* seeds showed maximum germination in EMS 0 µM (control) treatment in both presoaking times 24, 48h of 92%, 80% respectively after four weeks followed with gradual reduction of germination with EMS 25, 50 and 100 µM to 46, 35 and 5% in 48h soaking time respectively. The delay in germination following mutagens was also reported by Sen and Ghosh (1978) and by Mallick (1996) in *Datura stramonium*.

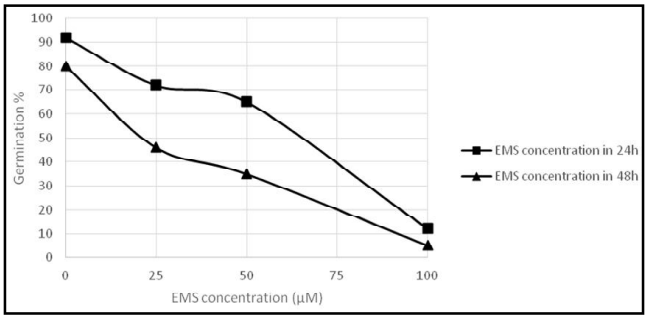

Fig. 1: Final Germination percent in response to EMS concentration and soaking time.

Vegetative Traits:

Table 2 showed the highest number of branches/Plant was found 9.55 with $C_3T_1G_2$ and the lowest number of branches was 5.66 in control. Highest plant height of 129.87cm showed in $C_2T_2G_2$ and the lowest 101.56 cm was in $C_4T_2G_2$. Highest plant leaves area cm^2 /Plant of 15999.8 cm^2 showed in $C_3T_2G_2$ and the lowest 6680.8

Table 2: Vegetative traits in *D. stramonium* leaves extract as effect by EMS and soaking time.

Treatment		Generation ***	No. of Branches /Plant	Plant height (cm)	Leaves Areacm² /plant	Fresh weight(gm)	Dry weight(gm)
*EMS	**Time						
C_0	----	----	5.66 ± 0.27	113.33 ± 7.82	6680.8 ± 148.3	143.33 ± 10.57	30.88 ± 1.88
C_1	T_1	M_2	5.77± 0.27	121.70 ± 9.66	7363.88 ± 170.39	199.77 ± 18.39	31.77 ± 2.74
		M_3	6.90 ± 0.31	125.77 ± 8.57	7668.88 ± 175.39	220.79 ± 18.08	39.89 ± 2.66
	T_2	M_2	6.10 ± 0.35	123.70 ± 9.06	7999.99 ± 218.44	273.39 ± 17.5	38.80 ± 2.92
		M_3	7.02 ± 0.50	119.77 ± 6.57	6966.90 ± 105.57	203.35 ± 14.54	40.66 ± 3.05
C_2	T_1	M_2	8.40 ± 0.63	123.70 ± 9.06	6980.77 ± 172.4	197.77 ± 17.39	33.77 ± 2.74
		M_3	7.66 ± 0.35	126.88 ± 9.46	6866.90 ± 103.57	225.78 ± 19.08	39.89 ± 2.66
	T_2	M_2	6.90 ± 0.29	129.87 ± 8.91	7879.99 ± 208.44	203.35 ± 16.54	38.80 ± 2.92
		M_3	6.10 ± 0.33	118.77 ± 6.57	7568.88 ± 174.39	210.22 ± 13.98	40.66 ± 3.05
C_3	T_1	M_2	9.55 ± 0.72	129.66 ± 0.09	8977.90 ± 215.77	280.50 ± 21.76	45.88 ± 3.41
		M_3	8.40 ± 0.58	119.86 ± 8.36	9705.88 ± 239.02	290.98 ± 24.05	50.66 ± 3.58
	T_2	M_2	7.60 ± 0.41	108.90 ± 5.38	15999.8 ± 268.36	386.59 ± 28.61	76.79 ± 4.02
		M_3	7.01 ± 0.35	100.88 ± 4.23	14777.9 ± 217.08	310.89 ± 27.88	70.05 ± 3.60
C_4	T_1	M_2	7.99 ± 0.44	108.16 ± 7.52	11087.90 ± 173.49	196.66 ± 15.48	49.78 ± 2.87
		M_3	7.01 ± 0.27	105.97 ± 6.28	9987.95 ± 182.46	185.10 ± 13.51	45.88 ± 2.17
	T_2	M_2	7.66 ± 0.33	101.56 ± 4.92	11447.92 ± 151.02	280.94 ± 22.08	57.75 ± 2.63
		M_3	6.35 ± 0.24	108.88 ± 6.15	12566.8 ± 237.61	230.76 ± 17.31	48.90 ± 2.09
LSD value			1.772 *	11.094 *	1372.82 *	42.75 *	5.63 *

*Concertation: C_1=0 µM, C_2=25 µM, C_3=50 µM, C_4=100 µM **Seeds soaking time: T_1=24h, T_2=48h ***M_2=2nd Mutant Generation, M_3=3rd Mutant Generation.

cm² was in control., Highest Fresh weight 386.59 gm. Showed in $C_3T_2G_2$ and the lowest 143.33 was in control. Highest Dry weight 76.79 gm. Showed in $C_3T_2G_2$ and the lowest 30.88 was in control. The plant branches and, plant height, increased by treating with EMS same results were obtained (Mallick and Sasmal, 2000). The treatment $C_3T_2G_2$ (50% concentration of EMS with 48 hours in the second generations) had highest leaves area cm²/plant, fresh weight (gm.) dry weight (gm.), The plant contained of alkaloids increased by treating with EMS similar results were obtained (Lubis,1967; Amiri *et al.*, 2010) all these increased are significant on level 0.05. The leaves area was doubled as effect genetically by EMS 50 µM treatment which consider important in medical plants. These indicators are characterized by the large numbers and speed of access to them and not affected by the environment and the type of fabric and age of the object under study, and can detect the variations in parts of the

Table 3: Total Alkaloids in *D. Stramonium* ethanolic leaves extract as effect by EMS and soaking time.

Treatment			Gener-ation	Total Alkaloids %
Treatment No.	EMS	Time		
1	C_0	-----	-----	5.32 ± 0.46
2	C_1	T1	M_2	6.09 ± 0.62
			M_3	6.09 ± 0.62
3		T2	M_2	7.55 ± 0.59
			M_3	8.94 ± 0.80
4	C_2	T_1	M_2	6.09 ± 0.62
			M_3	6.55 ± 0.49
5		T_2	M_2	8.54 ± 0.71
			M_3	9.80 ± 0.83
6	C_3	T_1	M_2	9.99 ± 0.77
			M_3	10.87 ± 0.62
7		T_2	M_2	11.05 ± 0.93
			M_3	10.55 ± 0.73
8	C_4	T_1	M_2	9.59 ± 0.81
			M_3	8.95 ± 0.75
9		T_2	M_2	8.78 ± 0.57
			M_3	7.88 ± 0.62
LSD value				2.079 *

*Concertation: C_1 = 0 µM, C_2 = 25 µM, C_3 = 50 µM, C_4 = 100 µM **Seeds soaking time: T1 = 24h, T2 = 48h ***M_2 = 2nd Mutant Generation, M_3 = 3rd Mutant Generation.

Table 4: Phytochemicals scanning in ethanolic extract of *Datura stramonium* leaves.

Phytochemicals substance	
Saponins	+*
Flavonoids	+
Tannins	-
Glycosides	+
Phenols	+
Alkaloids	+
Steroids	-
Terpenoids	-

* -: absent, +: present

DNA is also encoded, which constitute (50%-90%) of the size of the genome in high organisms and beyond the effects of overlapping samples They are much more efficient than phenotypic and enzymatic (Hussein, 2008). Mutation is one of the important factors that affect on the all or some vegetative traits and on the growth capacity of these plants to be high or rapid levels of genetic variation which lead to high levels of phenotypic variation, high

Table 5: GC-MS Phytochemical compounds identification in ethanolic extract of Datura leaves as affected by EMS treatments and soaking time.

No.	RT (min.)	Name	Area Sum%	Area Sum %
			*C_0	C_4
1	4.272	2 - Amino - 4 - methylbenzothiazole	0.72	0.55
2	5.311	Salsoline	0.72	0.78
3	8.55	1 - (4 - Methoxyphenyl) piperazine	0.81	11.67
4	10.391	Colchicine	0.47	1.59
5	10.874	Isolupanine	1.08	7.19
6	11.821	Pyrimido{1, 2 - a} azepine, 2, 3, 4, 6, 7, 8, 9, 10 - octahyroxy	5.50	0.10
7	12.509	Pimonidazole	0.45	3.29
8	12.896	2 - Acetyl - 5 - (tetrahydroxybutyl) imidazole	13.63	3.97
9	13.051	Isophytol	4.91	1.90
10	13.173	Dihydropinene	7.01	2.32
11	13.315	Terpineol	0.27	0.32
12	13.409	Nalorphine	0.55	0.27
13	13.597	Swainsonine	0.46	0.41
14	13.698	5 - Azacytidine	0.29	15.41
15	13.768	Vitexin	-----	0.78
16	13.837	6, 4 - Dimethoxy - 7 - hydroxyisoflavone	-----	1.37
17	13.923	Quinine	2.97	3.08
18	14.212	Thebaine	0.54	0.24
19	14.465	Oxazepam	0.57	1.06
20	14.709	Baicalein trimethyl ether	0.60	0.38
21	14.815	1, 6 - Dihydrocarveol	11.19	2.27
22	14.868	Isolongifolol	10.89	-----
23	15.063	Quercetin 3, 5, 7, 3, 4 - pentamrthyl ether	1.59	0.89
24	15.361	Farnesol	0.84	2.87
25	15.418	Zolpidem	0.62	1.54
26	15.479	Citronellal	1.02	2.49
27	15.703	Valeranone	9.52	-----
28	15.911	Warfarin	3.17	0.26
29	16.237	Propyl gallate	-----	1.86
30	16.563	Flavone, 3, 5, 7 - trimethoxy	2.16	2.92
31	16.836	3, 4 - Dihydrocoumarin	2.78	1.91
32	17.565	7, 8, 3, 4 - Tetramethoxyflavone	2.66	1.31
33	18.441	Pyridoxine	-----	3.14
34	19.272	3 - Hydroxy - 2, 4, 4, 6 - tetramethoxychalcone	1.11	7.62
35	20.393	6, 7, 3, 4 - Tetramethoxyisoflavone	1.98	2.46
36	20.62	3, 2, 4, 5 - Tetramethoxyflavone	1.35	1.11
37	20.939	Nobiletin	-----	2.71
38	21.37	Acetylcodeine	4.06	1.00
39	22.475	Herbacetin	0.65	2.31
40	22.723	3 - {3, 4 - Dimethoxyphenyl) - 4 - methylcoumarin	0.48	3.90
41	22.906	Squalane	1.14	0.73
42	23.093	3 - Hydroxy - 7, 8, 2 - trimethoxyflavone	0.52	0.28

*C_0 = Untreated seeds (control), C_4 = EMS 100 µM

levels variation of chemical content include the active secondary substances, high relative growth rate, a wide environmental tolerance, highly competitive ability and/ or avoidance of genetic bottlenecks following founder effects (Baker, 1965; Levin, 2000). These potential variations in vegetative traits of the treated plants may lead to an increase in the concentration or appear the new ones of some beneficial bioactive substances in these plants (Table 2). In general, the M_1 generation indicated a highly reduction for the all quantitative characters in several other crops with increasing because the treated seeds were suffering from growth inhibition du to mutagenic stress agents (Jeevi and Mullainathan, 2019), but this reduction effect were disappearing step by step in the following next generation M_2-M_5.

The comparative assessment of polygenic variability released at M_2 in different treatments than control for the studied traits is found to be shifted in both positive as well as in negative directions (Table 1) reduction in mean values in different treatments of different variables in relation to control is in agreement with the hypothesis due to mutagenic treatment mean is shifted to a direction opposite to selection (Bhatia and Swaminathan, 1962). Scossiroli (1965) opined that decrease in mean is due to detrimental mutations occurring more frequently than the favorable ones. Induce polygenic variability by EMS considering highlights than selection specially in M_2 offspring to induce mean in desirable direction at M_3 mostly for different variables thereby offering scope of raising superior micro mutants' lines in subsequent generations. (Saha,2019).

Total Alkaloids:

In Table 3, the highest percentage of total alkaloids 11.05% showed in $C_3T_2G_2$ and the lowest 5.32 was in control. In general, alkaloid content and composition in various Datura species may vary depending on the species, the phenological stage.

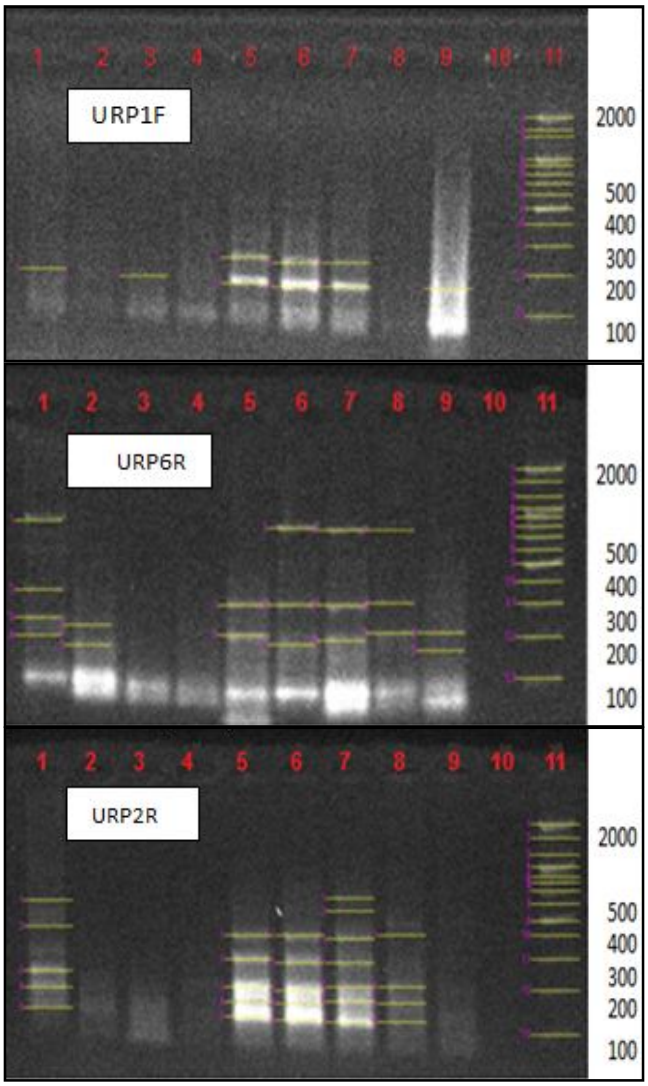

Fig. 2: URP primers banding patterns in nine Datura samples treated with EMS, Lane 11 =100-bp ladder.

Photochemical Analysis:

Datura species are a potential source of a many of useful secondary metabolites. The study of these secondary metabolites which isolated from medicinal plants can open new possibilities to find bioactive alternatives to synthetic chemicals. A number of alkaloids compounds including Hyoscine, Hyoscyamine, Meteloidine, Scopolamine, etc. have been noted from Datura species (Monira, *et al.*, 2012). Some of these alkaloids have such as Hyoscine and Hyoscyamine found application in human health care. Many researchers try to increase these useful alkaloids in Datura plant by following the breeding methods (Lavania,1986; Al-Taweel *et al.*, 2019). Some of these bioactive compounds

Table 6: The total number of amplified and polymorphic bands, percentage of polymorphism in nine Datura treated EMS using URP and ISSR markers.

Polymor-phism %	Monomorphic Bands	polymorphic Bands	No. of Total Bands	Primers	Primer Number
URP					
66.6	3	6	9	URP1F	1
47.3	9	10	19	URP2R	2
70.3	8	19	27	URP6R	3
61.4	20	35	55	Total	
ISSR					
35.13	24	13	37	A98	1
29.58	31	13	44	HB_{13}	2
32.35	55	26	81	Total	

or fractions of phytochemical substances of *D. stramonium* summarized were summaries in Table 5. The phytochemical analysis of Datura leaves ethanolic extract revealed the presence of alkaloids, tannins, flavonoids, saponins, glycosides, quinone and triterpenes (Table 4). This finding results in Table 2 is in agreement with (Akharaiyi, 2011). *Datura stramonium* L. is highly interested by the workers; it was considered a great resource of botanical tropane alkaloids. These alkaloids (*i.e.* atropine, hyoscyamine and scopolamine) are one of the important products with it contain is 26% in the Datura

Table 7: Molecular weights of polymorphic bands produced by URP primers of nine Datura samples treated with EMS.

9	8	7	6	5	4	3	2	1	MW. (bp of TAB	No. of polymo-rphic bands	URP Primer	Pri-mer No.
+		+	+	+					176	6	URP1F	1
						+			186			
									215			
				+					263			
+									179	10	URP2R	2
			+				+		190			
		+							195			
	+								300			
								+	380			
	+	+	+						762			
								+	922			
	+	+							133	19	URP6R	3
			+	+					140			
	+	+	+	+					205			
								+	210			
								+	252			
		+	+						300			
				+					304			
		+							400			
	+		+	+					407			
								+	503			
		+							558			

Table 8: Molecular weights of polymorphic bands produced by ISSR primers of nine Datura samples treated with EMS.

9	8	7	6	5	4	3	2	1	MW of polym-orphic brands	No. of polymo-rphic bands	ISSR Primer	Pri-mer No.
+									328	13	A98	1
			+						335			
		+							343			
								+	366			
						+			435			
							+		445			
							+		495			
						+			566			
								+	616			
								+	719			
	+								758			
								+	956			
				+					385			
						+			400	13	HB13	2
							+		410			
+									439			
					+				471			
							+		494			
	+								539			
				+					551			
+									613			
					+				641			
							+		670			
+									724			
						+			774			
								+	1346			

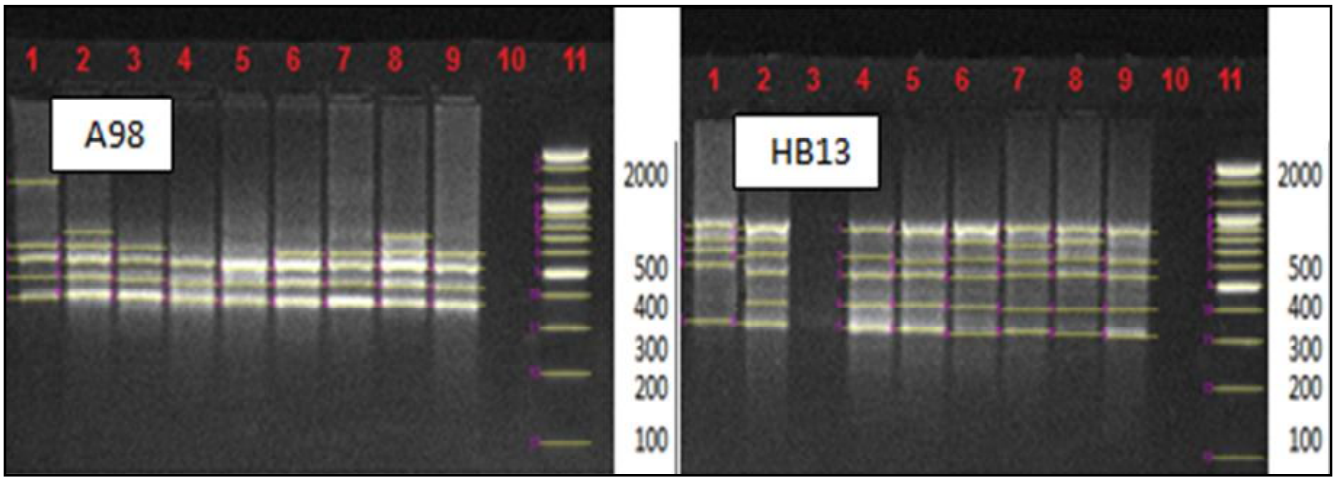

Fig 3: ISSR primers banding patterns in nine Datura samples treated with EMS, Lane 11 = 100 bp ladder.

leaves and considered form the major part of medicinal compounds (Gaire and Subedi, 2013, (Samier, *et al.,* 2015, Al-Taweel, *et al.,* 2019). Datura treated plants with EMS 100 ìM appeared an increasing in the Isolupanine valued alkaloids contain from 1.08% to 7.19% area sum in GC-MS analysis (Table 5).

Medicinal plants contain some bioactive substances included alkaloids, tannins, terpenoids, steroids and flavonoids. These compounds are synthesized in plant tissue by primary or rather secondary metabolism pathways. For more screening details and searching on a new valued compound, Table 5 highlights on the phytochemical substance's fractions of the ethanol extracts of plant leaves. Main while, the GC-MS results indicated to the ethanolic extracts of Datura plant treated with EMS 100¼M with seeds soaking of 48h contained forty-two of the identified phytochemical compounds, some of them w appeared as new substances as affected by EMS mutant treatment on the secondary metabolize pathways. Majority of these new compounds were 1-(4-Methoxyphenyl) piperazine and 5-Azacytidine of 11.67%, 15.47% area sum respectively, which have been reported to be highly of therapeutic importance. Piperazine belongs to the family of medicines called anthelmintic. Anthelmintic used in the treatment of worm infections Piperazine derivatives exhibit activities toward the central nervous system, such as anti-anxiety and anti-convulsive activity. It is also known that a certain kind of piperazine derivatives possess calmodulin inhibitory activity. Götzeet *et al.,* (2009) indicate to the role of azacytidine in the management of myelodysplastic syndromes (MDS) Myelodysplastic syndromes (MDS) are a group of common bone marrow disorders characterized by ineffective hematopoiesis, peripheral cytopenia's. The recent approval of the demethylating agent azacytidine represents a significant advance in the treatment of MDS. The secondary metabolites pathways may be modifying by EMS genetically effect, which leads to appear or disappear some substances in mutagenic plants. The promising result obtained has subjected this plant extract to further analyses in the following generation to screen for its more medicinal and their qualitative and quantitative stability and side effect for possible perfect therapeutic value.

Molecular Analysis:

The researchers Amiri *et al.,* (2018) using ISSR molecular markers to evaluate of genetic diversity of styrian pumpkin populations. CS Analyzer program (ATTO- JAPAN) depended to analyze the data. Total of 55 bands generated from URP primers 35 were polymorphic bands and 20 monomorphic bands and the Primer efficiency of URP6R was 70.3%, URP1F was 66.6% and URP2R was 47.3%, the polymorphism percentage of all URP primers were 61.4% Table 6.

Total of 81 bands generated from ISSR primers 26 bands were Polymorphic and 55 bands were monomorphic and the Primer efficiency of primer A 98 and primer HB_{13} was 35.13%, 29.58% respectively, the polymorphism percentage of all ISSR primers were 32.35% (Table 6).

Table 7 shown the URP primers produced multiple bands, varying in size from 179 to 922bp as Fig, 2 Table 7, URP amplification showed that treatment C_3T_2 produced a unique band with molecular weight 195bp, 400bp and 558bp and treatment C_4T_2 produced a single unique band with molecular weight 179bp.

The ISSR primers produced multiple bands with differences in size from 328bp to 1346bp Fig. 3 Table 8.

ISSR amplification revealed that treatment C_3T_2 in second generation produced a single unique band with molecular weight 343bp, treatment C_4T_2 also produced a unique band with molecular weight 228bp, 439bp, 613bp and 724bp as shown in Table 8. Genetic variation estimated by using URP primers, which have been proved valuable in genomic fingerprinting of Datura plant (Kang, *et al.,* 2001). ISSR techniques were useful as molecular genetic fingerprints (EL-Shawaf *et al.,* 2009) We realized there were many genomic changes occur with treatments C_3T_2 and C_4T_2 and these changes were a useful for medicals plants as phenotype, phytochemicals analysis detected, these changes may the result of deletion, addition, replacement which occurred by using EMS. URP and ISSR markers are two molecular approaches which have been used to detect variation among plants and express the variations inherited from the nucleotide sequence in the DNA of the treated samples under This study may be useful in identifying the threshold dose of a EMS mutagen that would increase the genetic variation and number of economically useful mutants to improve Datura plant traits with increasing in the active medicinal compounds specially in the following segregating generations, which might be further utilization in crops improvement programs.

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