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INFERTILITY CURATIVE PLANTS AS PLANT GROWTH INHIBITORY AGENTS

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Abstract

Long Term use of infertility curative plants is common in Sindh Pakistan. Continues intake of these plants may lead to cellular destruction based on toxic compounds in addition to fertility boosters. Assessment of non-cytotoxic dose to human can initially be tested in plant based assay. Therefore, three fertility enhancing plant parts viz. Sweet Flag Rhizome (S.F.R.), Peppermint Leaves (P.L.) and Red Cabbage Flower (R.C.F.) were compared for germination inhibition and mitotic index (M.I) inhibition as main parameters of phytotoxicity. Chickpea seeds were incubated for 15, 30, 45 and 60 minutes in filtered aqueous extracts (1, 3 and 5%). Un-treated seeds were used as negative control and 0.2% Ethyl methane sulphonate treated seeds as positive control. Chickpea seed germination exhibited highly significant variations at $p \leq 0.01$ (LSD) for all the doses of R.C.F. among tested plant parts. Highest germination inhibition was observed in S.F.R and lowest in R.C.F. Incubation time dependent decrease in germination was only observed for all the concentration of sweet flag and 5% of Peppermint. In case of M.I. highly significant variations were recorded by applying LSD analysis ($p \leq 0.01$) for all tested plant extracts except 1% S.F.R. Incubation time and concentration dependent M.I (%) was only observed in peppermint. Overall P.M. was least phytotoxic among tested plant extracts as compare to +ve control.

Key words: Phytotoxic, Incubation, LSD, EMS, Positive control, Negative control.

Introduction

Medicinal plants used as complementary medicine all over the world. In Pakistan more than 6000 plants cultivated indigenously and used as medicinal plants. Most of Sindhi villagers (80%) rely on herbal remedies to cure different disease (Rehman *et al*., 2011). Recently after revealing cytotoxic potential along with different health benefits of bioactive compounds of medicinal plants, safe dose estimation is the primary object of most pharmacologists. Many workers rely on plant based assay system before final screening on mouse and mammalian cell studies (Katabale *et al*., 2017; Xiaobang, 2019). Fertility defect are one of the major cause of marriage breaks in Pakistan (Ali *et al.*, 2011). Therefore to save marriage life both male and female try every method of cure from allopathic to ayurvedic. Sometimes they try multiple medicinal plants over the time even for years. Thus infertile people are more at risk of cytotoxic effects leading to oxidation of membrane components and finally DNA. Most commonly used fertility enhancers of Sindh Pakistan are;

1. Sweet flag: *(Acorus calamus*) also known as Kini kathi belongs to family Acoraceae. Whole plant used for the treatment of leucorrhea, irregular menstruation and infertility due to cold womb (Rehman *et al*., 2011). Beside with these sweet flag also used to treat itching, anxiety, rhematitis, asthma, schizophrenia etc (Raja *et al.*, 2009; Amit and Vandana, 2013). Asarone (α-asarone and β-asarone) is main plant chemical of S.F with acoradin, galagin, 2, 4, 5-trimethoxy benzaldehyde, 2, 5-dimethoxybenzoquinone, calamendiol, spathulenol and sitosterol (Zuba & Byrska, 2012; Amit & Vandana, 2013). Prime adverse effects associated with Sweet Flag rhizome are nervous system disorder (Bjornstad *et al.*, 2009 and Gur'ev *et al*., 2010), Immunosuppressive, hyposensitive and respiratory depressant effects caused (Singh *et al*., 2001).

2. Peppermint: (*Mentha piperita*) is a perennial plant, 50-60 cm (3-4 feet) high, is flowering member of the mint family Lamiaceae. Active chemical constituents are volatile oils, menthol, menthyl acetate, isomenthone, menthofuran, menthone, eucalyptol, limonene and polyphenols (Mainasara *et al*., 2018). Peppermint leaves used as multipurpose component, related with the treatment of inflammation of the mouth and throat, sinus and respiratory problems. People exercise mint leave as a fertility booster in solid, tea as well as powder form. It helps in the treatment of menstrual misbalance and pain, also acts as stimulator (WebMD). Its beneficial constituent includes vitamins A, C, B12, natural form of folic acid (essential for pregnancy) also found in peppermint tea (babyprepping.com). However, heavy metals and essential oil are linked with toxic effects. Some common reactions caused by peppermint are heart burn, vomiting and renal failure (Kiggler & Chaudhary, 2007).

3. Red cabbage: (*Brassica oleracea*) belongs to family Brassicaceae, known to prevent fibroids and endometriosis in women (FertilityHomeopath.com). Cabbage is a rich source of vitamin c which helps to prevent sperm clumping (agglutination) (Integramassage.wordpress.com). Nutrients like calcium, iron, magnesium, phosphorous, sulfur, silica, vitamins a, b, c, e and k, amino acids, such as s-methyl cysteine, and anthocyans are present in cabbage. The active amino acid ingredients found in cabbage have also shown to promote the production of carcinogen-fighting enzymes. Due to considerable heavy metals accumulation like mercury, lead, arsenic and cadmium (herbwisdom.com) it also possesses antimicrobial properties. Unnecessary use of red cabbage cause goiter enlargement in animals because of degradation products of glucosinolate (Mithen, 2001). Jumbled effects of fertility enhancing plants reported in plant, animals and human beings as made assessed for safe dose obligatory for betterment of living organisms. While

focusing hazards of infertility curative plants to humans a preliminary study of cellular injuries resulting in growth inhibition has been designed using chick pea as standard assay plant.

Materials and Methods

Chick pea Seeds were incubated for 15, 30, 45 and 60 minutes in 1%, 3% and 5% solutions of aqueous extract of fertility enhancing plants for phytotoxicity.

Aqueous extract preparation: Plant parts via roots of sweet flag, leaves of peppermint and flower of red cabbage were purchased from local bazaar and cleaned with cotton cloth to remove dust particles than powder was obtained by pestle and mortar and electric grinder. Powder of all the plants were soaked in distilled water for overnight followed by filtration with filter paper (Whatman No.1 (12.5cm)). Untreated seeds (distill water soaked) were used as negative control and 0.2% EMS (Ethyl methane sulphonate) treated seeds as positive control.

Seed soaking: Total 80 chickpea seeds per plant filtrate were placed in different concentrations and 20 seed per treatment were removed after 15, 30, 45 and 60 minutes. Before sowing seeds were washed with distilled water to stop the effect of plant extracts.

Germination: Incubated chickpea seeds were placed in sand pot up to 2 inches depth at 25C^{o} in green house for germination analysis. After 48 hours seeds were counted for root emergence (germination), procedure repeated until germination stopped.

Fixation of mitotic roots: Carnoys-1 solution (3:1 glacial acetic acid and alcohol) used to form fixative, approximately 2.5 cm roots were kept in fixative for 24 hours to capture phase of cell division then roots were shifted in 70% alcohol till the time of slide preparation.

Slide preparation and photography: Root tips were cut very carefully and spread by squash technique (Dille & King 1983; Dille *et al.*, 1986), slides were stained with acetocarmine solution (2% acetocarmine in 45% glacial acetic acid), cover with Petri dish for 15 to 20 minutes for healthier staining, eliminate extra mark with filter paper and fix slides with flame. Digital microscope (Olympus51x) at 400 magnifications was used for slide analysis.

Cytogenetic studies: Initially 6 slides for each treatment were used to calculate abnormal dividing, non-dividing and normal dividing cells to compile mitotic index.

Data analysis of phytotoxicity and genotoxicity assay

Germination percentage: To obtain germination percentage following formula used (El-Shaieny, 2015).

$$\text{Germination \%} = \frac{\text{No. of germinated seed}}{\text{Total no. of seed sown}} \times 100$$

Least square difference (LSD) test at $p \leq 0.01$ was applied through computer software statistics 8.1 to evaluate germination percentage and mitotic index as compare to both (negative and positive) controls.

8.2. Mitotic index (M.I): A ratio between the numbers of total dividing cells and total cells analyzed called mitotic index. It was calculated by following formula (Didla *et al*., 2015).

$$\text{M.I.} = \frac{\text{Total dividing cells}}{\text{Total cells analyzed}} \times 100$$

Results

Germination (%): LSD analysis of chickpea seed germination (%) exhibited significant variations at $p \leq 0.01$ for all the doses of tested fertility enhancing plants.

Approximately all incubations of S.F.R. and P.L. revealed highly significant variation as compare to control except 15 minutes incubation of 1% R.C.F.

Mean comparison of germination percentage: The mean comparison of germination percentage as affected by different concentrations and incubation time of S.F.R. P.L. and R.C.F. aqueous extracts are compiled and presented in (Table 1; Figs. 1, 2 and 3). Highest germination percentage (90) of sweet flag treated seed was recorded in 15 minutes of 3% concentration whereas lowest (55%) also found in 3% with 45 minutes incubation period (Fig. 6). P.L. uppermost (90%) germination proportion was found in 15 minutes incubation period of 5% concentration and lowermost (50%) was found in 60 minutes of 3% (Fig. 2). Higher germination percent (95) of chickpea seed treated by R.C.F. extracts was resulted in 45 minutes of 1% incubation and lower (70%) again found in 1 percent's 30 minute incubation period (Fig. 3).

Mitotic Index (%): Mitotic index is very important phytotoxicity parameter that provide solid prove of growth retardation. It was obtained by mean number of non-dividing, normal dividing and abnormal dividing cells for all the tested plant parts (Tables 2, 3, and 4). Highly significant ($p \geq 0.01$) differences were observed for all the applied concentrations and incubation time (Table 5; Figs. 4, 5 and 6). In S.F.R. extract treated roots highly significant variation for M.I. was recorded for all the incubation periods of 5% and non-significant for 1%. (What about 3%) Maximum M.I. (74.63%) was resulted in 1% and minimum (59.49%) in 5%. Decrease in M.I increased by increasing extract concentrations.

¾ of all concentrations of P.L. extract showed significant variation in M.I. as compare to control. Maximum M.I. (86.17%) was given by 3% and minimum (59.29%) by 1%. Non-dose dependent increase and decrease was induced by P.L.

Most of incubations of R.C.F. were highly significant variant as compare to control. Maximum M.I. (86.53%) was recorded in 1% 15 minutes incubation and minimum (69.89%) also found in 1% but 30 minutes incubation. Over all random mito-depressive affects were observed in R.C.F. treated roots.

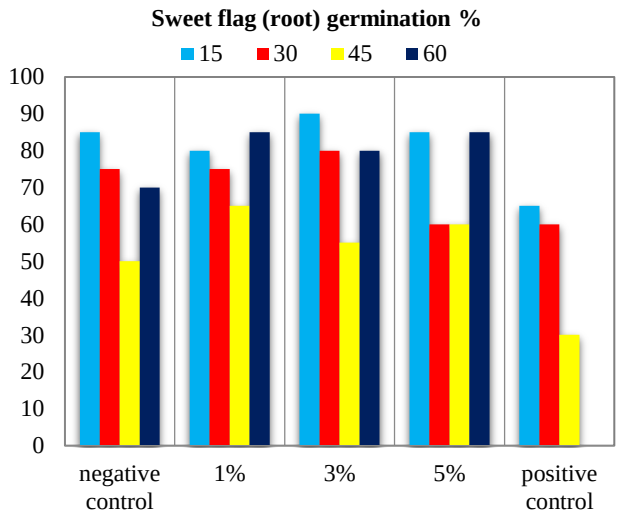

Fig. 1. Effect of different concentrations of S.F.R. aqueous extracts on germination (%).

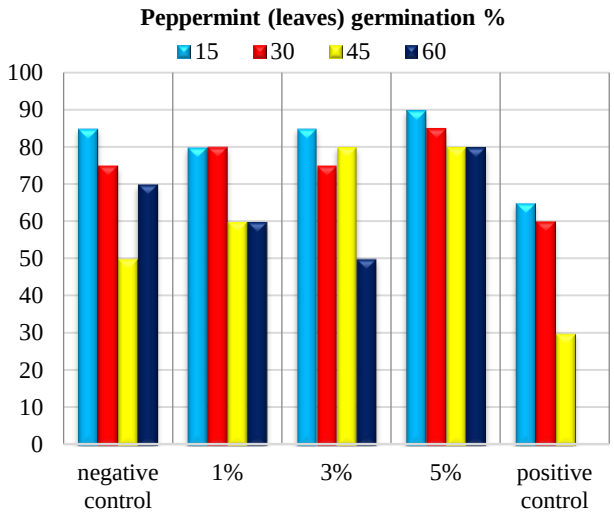

Fig. 2. Effect of different concentrations of P. L. aqueous extracts on germination (%).

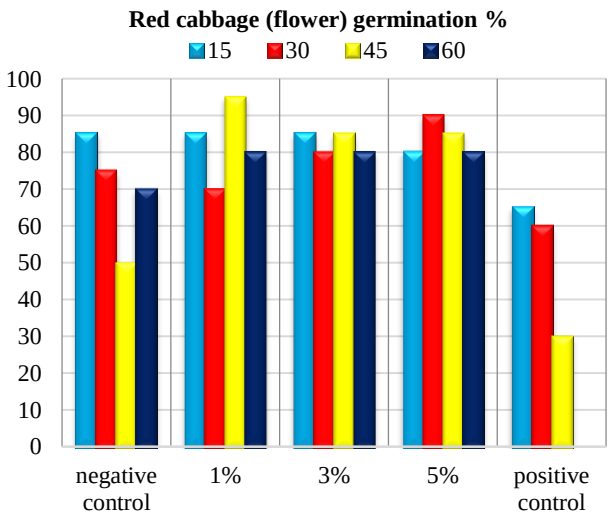

Fig. 3. Effect of different concentrations of R. C. F. aqueous extract of on germination.

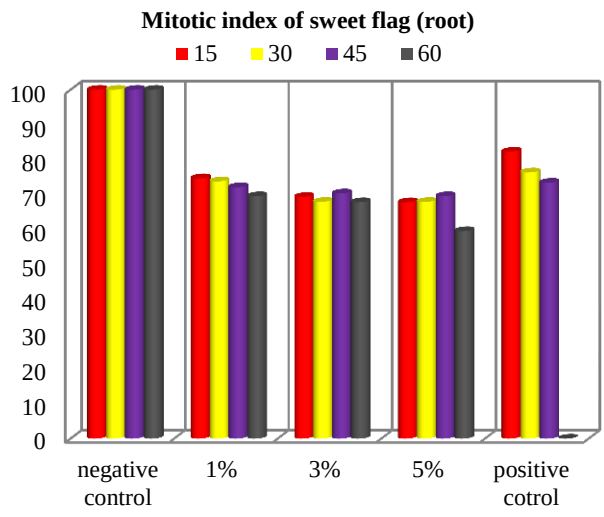

Fig. 4. Mitotic index given by different concentration and incubation of S.F.R.

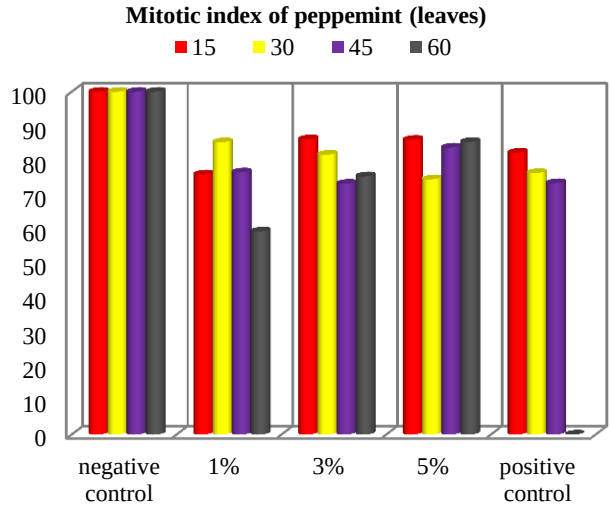

Fig. 5. Mitotic index given by different concentration and incubation of P.L.

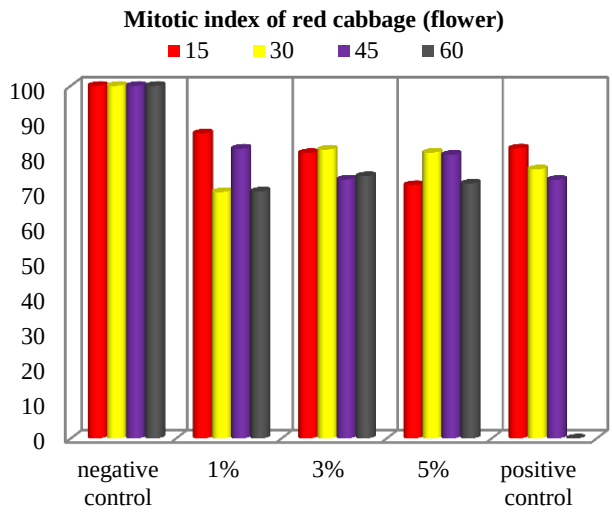

Fig. 6. Mitotic Index Given by different concentration and incubation of R.C.F.

Table 1. Mean germination percentage of chickpea seeds treated by S.F.R., P.L. and R.C.F. aqueous extracts (LSD $p \leq 0.01$).

Plant name	Germination percentage of treated plant (Chickpea) induced by infertility curative plants					
	Incubation time (minutes)	-ve control	+ve control	1%	3%	5%
Germination of Sweat flag	15	85^{b}	65^{f}	80^{c}	90^{a}	85^{b}
	30	75^{d}	60^{g}	75^{d}	70^{e}	60^{g}
	45	50^{i}	30^{j}	65^{f}	55^{h}	60^{g}
	60	70^{e}	00^{k}	85^{b}	80^{c}	85^{b}
Germination of Peppermint	15	85^{b}	65^{f}	80^{c}	85^{b}	90^{a}
	30	75^{d}	60^{g}	80^{c}	75^{d}	85^{b}
	45	50^{h}	30^{j}	60^{g}	80^{c}	80^{c}
	60	70^{e}	00^{k}	60^{g}	50^{h}	80^{c}
Germination of Red cabbage	15	85^{c}	65^{f}	85^{c}	85^{c}	80^{d}
	30	75^{e}	60^{g}	70^{f}	80^{d}	90^{b}
	45	50^{i}	30^{j}	95^{a}	85^{c}	85^{c}
	60	70^{f}	00^{k}	80^{d}	80^{d}	80^{d}

(Means with same alphabets are non-significantly different from each other and with different alphabets are significantly different at ($p \leq 0.01$))

Table 2. Number of normal dividing, non-dividing and abnormal dividing cells induced by different concentration of S.F.R. aqueous extract.

Treatment	Incubation (minutes)	Number of cells		
		Non-dividing	Normal dividing	Abnormal dividing
-ve control	15	0	485	5
	30	0	480	10
	45	0	485	08
	60	0	490	0
S.F.R. 1 %	15	181	111	95
	30	179	116	103
	45	124	132	177
	60	91	128	174
S.F.R. 3%	15	217	00	104
	30	267	19	190
	45	195	112	212
	60	203	167	260
S.F.R. 5%	15	129	16	109
	30	144	112	192
	45	123	114	254
	60	120	116	265
EMS 0.2 % (+ve control)	15	160	48	550
	30	180	30	468
	45	130	20	393
	60	-	-	-

Table 3. Number of normal dividing, non-dividing and abnormal dividing cells induced by different concentration of P.L. aqueous extract.

Treatment	Incubation (minutes)	Number of cells		
		Non-dividing	Normal dividing	Abnormal dividing
-ve control	15	0	485	5
	30	0	480	10
	45	0	485	08
	60	0	490	0
P.L. 1 %	15	67	35	179
	30	46	21	245
	45	139	19	432
	60	147	10	204
P.L. 3%	15	52	10	314
	30	64	11	274
	45	78	13	200
	60	74	34	192
P.L. 5%	15	46	21	262
	30	73	21	192
	45	40	20	186
	60	50	72	220
EMS 0.2 % (+ve control)	15	160	48	550
	30	180	30	468
	45	130	20	393
	60	-	-	-

Table 4. Number of normal dividing, non-dividing and abnormal dividing cells induced by different concentration of R.C.F. aqueous extract.

Treatment	Incubation (minutes)	Number of cells		
		Non-dividing	Normal dividing	Abnormal dividing
-ve control	15	0	485	5
	30	0	480	10
	45	0	485	08
	60	0	490	0
R.C.F. 1 %	15	33	7	205
	30	143	19	313
	45	48	7	215
	60	35	2	80
R.C.F. 3%	15	64	11	261
	30	65	7	288
	45	73	28	174
	60	108	102	212
R.C.F. 5%	15	130	77	255
	30	38	2	161
	45	79	13	313
	60	54	14	127
EMS 0.2 % (+ve control)	15	160	48	550
	30	180	30	468
	45	130	20	393
	60	-	-	-

Table 5. Mitotic index of chick pea root tip cells treated by different concentrations and incubations of S.F.R., P.L. and R.C.F. aqueous extract.

Type of index	Mitotic index of Chickpea root tip cells by infertility curative plants					
	Incubation time (minutes)	-ve Control	+ve Control	Concentration		
				1%	3%	5%
M.I. of Sweet flag	15	100^{a}	82.25^{b}	74.63^{cd}	69.29^{fg}	67.75^{g}
	30	100^{a}	76.32^{c}	73.75^{cd}	67.88^{g}	67.85^{g}
	45	100^{a}	73.35^{cde}	72.07^{def}	70.29^{efg}	63.47^{h}
	60	100^{a}	-	69.48^{fg}	67.77^{g}	59.49^{h}
M.I. of Peppermint	15	100^{a}	82.25^{cd}	75.88^{ef}	86.17^{b}	86.01^{b}
	30	100^{a}	76.32^{e}	85.29^{b}	81.66^{d}	74.47^{fg}
	45	100^{a}	73.35^{g}	76.49^{e}	73.19^{g}	83.73^{c}
	60	100^{a}	$-^{i}$	59.27^{h}	75.33^{ef}	85.38^{b}
M.I. of Red Cabbage	15	100^{a}	82.25^{c}	86.53^{b}	80.95^{cd}	71.86^{hi}
	30	100^{a}	76.32^{e}	69.89^{j}	81.94^{cd}	81.09^{cd}
	45	100^{a}	73.35^{gh}	82.22^{c}	73.45^{fg}	80.49^{d}
	60	100^{a}	$-^{k}$	70.08^{ij}	74.40^{ef}	72.30^{gh}

Discussion

Concentration and time dependent decrease in germination for 15, 30 and 45 minutes were observed for all applied incubations (concentration and time) of sweet flag and 5% of Peppermint. Similar findings were note down for *Satureja thymbra* L. and *Cassia fistula* extracts in different assay plants by a range of research workers previously (Masoud, 2018 and Muhammad, 2019). Whereas other incubations of rest of plants revealed that chick pea seeds were totally compromised irrespective used aqueous extracts. Recent results are although unusual but occasionally reported from Brazil (Gomes *et al*., 2017). On the contrary other scientists found concentration dependent germination inhibition as well as yield and growth inhibition mediated by different plant extracts.

Actually low to moderately toxic plants give concentration dependent toxicity, whereas highly toxic plants have lethal effects right from the lowest dose that fluctuates a little randomly. As metals are linked with many physiological and biochemical complications (Ackova, 2018) hence experimental germination inhibition can be correlated with remarkable amount of mercury, lead, cadmium and arsenic piled up in S.F.R. (Meena, 2010), lead, Manganese, iron, zinc and copper in P.L. (Bagdatlioglu *et al*., 2010) and mercury, cadmium, arsenic, and lead in R.C.F. It is reported by many researchers all over the world that chickpea and onion seed germination hang-up and plant root growth slow down by cadmium, lead and arsenic (Babatunde & Bakare, 2006; Bhattacharya *et al*., 2012; Mondal *et al*., 2013). Decrease in the length of wheat seed, membrane breakage and germination inhibition in pea seeds and embarrassment of root length as well as seed germination in zea mays recorded due to elevated quantities of mercury, cadmium, zinc and iron (Rahoui, *et al*., 2010; Pattanaik *et al*., 2011 and Rasafi *et al*., 2016).

Resulted decrease in mitotic index (%) by S.F.R aqueous extracts was directly proportional towards dose. Likewise Soliman (2001), Sobita and Bhagirath (2005), Lubini *et al*., (2008), Sousa *et al*., (2009 and 2010), Celik and Aslanturk (2010), Sousa & Viccni (2011) and Qureshi *et al*., (2015) reported mitotic index suppression by neem extract in onion, *Nerium odorum* and *Solanum indicum*

extract in *Viccia faba*, psychotoria extract in onion, aqueous extracts of *Lantana camara* L., *Lippia alba* (Mill) and *Cymbopogon citratus* (DC) in lettuce, *Lavandula stoech* in onion, *Achillea millefolium* in lettuce and by aqueous extracts of Thyme seed, Neem leaf, Neem seed and Eucalyptus leaf in chickpea root tip cells. β-asarone, α-asarone and Eugenol are main constituents of sweet flag, that might be causes of decrease in mitotic index supported by findings of earlier investigator like use of β-asarone and its oil for two years or more induce intestinal tumor in rats (Singh *et al*., 2001). α-asarone at high concentration reduce mitotic index (Cassani-Galindo *et al*., 2005). Stomatitis is caused by eugenol an active agent of sweet flag (Deshpande *et al.,* 2014).

Time dependent hit and miss in M.I was found in peppermint treated organism. Peppermint chemicals directly or indirectly affect on plant as well as other organisms, its cytotoxic effects reported in earlier researchers as resulted by Lazutka (2001), according his findings P.M essential oil having cytotoxic concern for human lymphocytes, it induces mutations in dose-independent manner.

The concentration and time dependent decrease in mitotic index was also observed by many researchers in aqueous extracts of *Vicia villosa*, *Rubus sancatus*, *Cinnamomum zeylanicum* (bark) and *Citrullus colocynthis* (leaves) by using *Allium cepa* and *Viccia faba* root tip cells (Soltys *et al*., 2011; Selmi *et al*., 2014; El-Ghamery & Basuoni, 2015; Sameer, 2016). The reduction of the mitotic index treated by silk dyeing industry waste on root tip cell of onion is the signal of the inhibition of DNA synthesis (Sudhakar *et al*., 2001).

Time dependent decrease and fluctuated curve may be due to random effect of phytochemical glucosinolates. Metabolites of glucosinolates (thiocyanates, thiourea and oxazolidithione) are liable cellular injuries in the form of hepatotoxicity and nephrotoxicity (Ahlin *et al*., 1994; Zang *et al*., 1999; Wallig *et al*., 2002; Tanii *et al*., 2004). Reduction and increase in mitotic index in zigzag mode because of deposition of secondary metabolites (alkaloids**,** tannins, terpenoids, steroids, glycosides, Phytosterol, Flavonoids, saponins) in red Cabbage. Inhibitory action against germination, seedling growth and mitotic index due to the presence of secondary metabolites were observed in seed of *Vigna radiata* by the effect of aqueous bark extract of *oroxylum indicum* L. (Chetry & Bharali, 2018). Similar findings were reported in phytotoxic profiling of *Ziziphus mauritiana* var. *spontanea* Edgew. and *Oenothera biennis* L. against *Rhizopertha dominica, Tribolium castaneum* and *Sitophilus oryzea* (Ambrin *et al*., 2020). Absorption of high measure of lead and mercury reduce mitotic index of root tip cells of *Cicer arietinum* (Cavusoglu *et al*., 2009). Cd and Pb reason the boost of cytogenetic disorder in wheat by industrial release of heavy metals (Yakymchuk & Valyuk, 2018). In addition to mito-depressive effects in plants by heavy metals earlier researchers notified human toxicity as presence of lead and cadmium results liver damage (cytotoxicity) increased blood enzyme levels and reduced protein synthesis is (both are molecular indicator of oncogenesis) (Yuan *et al*., 2014; El-Boshy *et al*., 2017). Heavy metal intake in large amount related with dysfunction of immunological defense (mainly leukemia and lymphoma) and neurological behavior (Korfali *et al*., 2013). Food products (cereals, vegetables, fruits, fish, and meat) possessing toxic metals Cd, Pb, Cr, As, and Hg cause trouble for human being if nearby in huge amount. These metals toxify bodies mechanism finally stimulate chronological disorders like nervous system destruction, deformity, renal tubular dysfunction or anemia skeletal damage resulted due to oxidation of membranes and heredity material (Chang, 2014; Liu, 2014 and Liang *et al*., 2019).

Conclusion

All tested plants were potentially phytotoxic to assay plant with little difference; root germination percentage was more altered by sweet flag followed by land calotrops and least by peppermint, whereas root of sweet flag were and red cabbage was among the three plant parts. Therefore only P.L. should be used as fertility booster in moderate amounts.

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