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Long-term impact of Artificial Sweetener on *Caenorhabditis elegnas*

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Abstract: **To explore the effect of different ratio of Phenylalanine and Aspartic acid on longetivity and also quantify its effect at cellular and genetic level of C.elegans. Different ratio of phenylalanine and aspartic acid in ratio of 2:1, 1:2 and 1:1 were assessed. Treated C.elegans were compared with the negative control that was established by culturing normal C.elegans alone. For studying the behavioral assays, different parameters like brooding assay, lifespan assay and lethality assay were evaluated. Cellular toxicities was calculated by MTT assay. Genotoxicity assay indicated the impact on DNA fragmentation. Compared to the normal group, treated group exhibited reduced fecundity rate when treated with 1:2 ratio of Phe:Asp followed by 2:1 and 1:1 ratio. Moreover, these results were in consonance with the results obtained from longetevity assay, whereas the results obtained from lethality assay and MTT assay exhibited that 2:1 ratio of Phe:Asp was more toxic than 1:2 ratio followed 1:1 ratio. DNA fragmentation was maximum in 2:1 ratio of Phe:Asp treated group. Aspartame exhibited apoptotic effect on C.elegans. Aspartic acid component of aspartame reduced the fecundity rate, whereas phenyalalanine component showed enhanced toxicity when compared to aspartic acid in lethality, MTT and DNA fragmentation assay. Results indicated that prolonged use of aspartame as an artificial sweetener may be genotoxic causing deleterious effects on the users.**

Keywords: **Aspartame, Phenylalanine, Aspartic acid, MTT, DNA fragmentation.**

1. INTRODUCTION

Artificial sweeteners are commercially synthesized substitutes of sugar used in various low calorific products. Commonly used artificial sweeteners include saccharin, sucralose, neotame, aspartame and acesulfame. Aspartame, especially has a considerably high rate of usage in beverages and as foods additive worldwide, preferably by the diabetic patients. It is a non-carbohydrate sweetener synthesized from the methyl ester of the dipeptide of the naturally occurring amino acids, phenylalanine and aspartic acid ($C_{14}H_{18}N_20_5$).

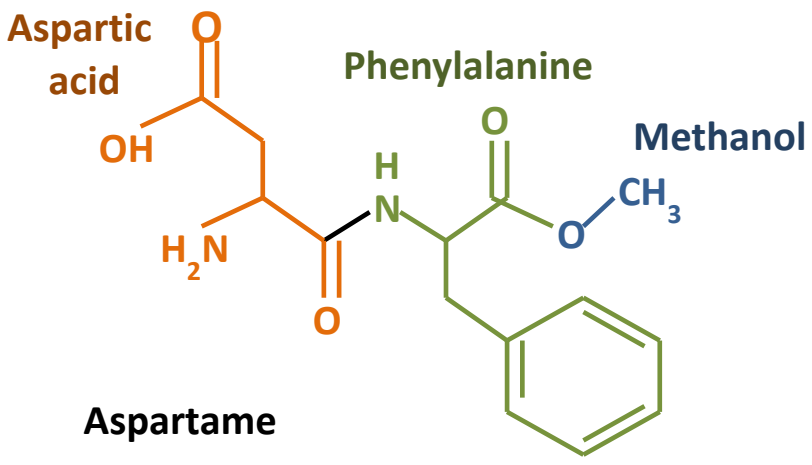

Fig 1: Molecular structure of aspartame.

Prolonged use of aspartame is harmful as it reportedly elevates the level of phenylalanine in the brain and blood (Stegink *et al.*, 1977), that may interfere with the synthesis of neurotransmitters, thereby downmodulating the generation of catecholamines and serotonin (Maher and Wurtman, 1987). Accumulation of phenylalanine may further result in the formation of fibrils, that have been associated with the development of phenylketonuria, an autosomal recessive mental disorder (Adler-Abramovich *et al*., 2012). Higher concentrations of prenatal phenylalanine exposure, above 360 μmol/l, have an impact on the developing fetus, especially in their cognitive outcomes (Widaman and Azen, 2003). Meanwhile, aspartic acid or aspartate ($C_4H_7NO_4$) is a non-essential amino acid with a molecular weight of 133.103 g/mol. It is associated with the citric acid, urea cycle and functions as a neurotransmitter**.** Young rodents subjected to elevated dosage of aspartic acid have been reported to develop retinal damage (Grant, 1986). Aspartic acid has a potential involvement in postsynaptic membrane depolarization, inducing neuroendocrine instability (Olney, 1975). Excess Consumption of aspartame has been further associated with seizures (Maher and Wurtman, 1987), depression, anxiety, loss of memory (Mehl-Madrona, 2005), neurodegeneration and oxidative stress (Bowen and Evangelista, 2002). In view of the popular use of aspartame coupled with the literature survey of the innumerable and deleterious side effects of the excess usage, we proposed to conduct the lethality and toxicity assays in an *C. elegans* as an ideal model.

C. elegans, a nematode was used as the model, as it is free-living, non-parasitic nematode, a multi-cellular eukaryote with its entire genome sequenced (*C. elegans* sequencing consortium, 1998). With 18,452 protein coding genes, the nematode genome has ~83% homology with human genes (Lai et al., 2000) and ~65% similarity with human disease genes (McKay and Davis, 2002). Its short life span, small size, large brood size, genetic amenity, easy maintenance and cost efficiency in the laboratory can drastically reduce he cost of research.this is a sensitive for high throughput screening programmes for bioactive compounds.

The genotoxicity and cytotoxicity of Aspartame and its various constituents were evaluated in *C. elegans.* The impact on the brood size and lethality asay were assessed too.

2. METHODS AND MATERIALS

Material: Phenylalanine, Aspartic acid and bactopeptone were procured from CDH; KH_2PO_4, $MgSO_4$, $CaCl_2$, $NaOCl_2$ were purchased from Merck; Bactoagar and Na_2HPO_4 were purchased from Loba Chem (India); NaCl were purchased from Fisher Scientific; Edvotek Bactobeads *E.coli* OP50 were obtained from Fisher Scientific (USA).

Maintenance of *C. elegans* strains: The wild type (N2) Bristol strains were maintained in accordance with the guidelines provided by Brenner, 1974. They were grown in Nematode Growth Medium (NGM) agar plates seeded with *Escherichia coli* strain, OP50 at 20˚C. **Synchronization of nematodes –** NGM plates with gravid adults and eggs were washed with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2PO_4, 85.5 mM NaCl in distilled water, sterilized and 1 mM $MgSO_4$ added the next day) and pelleted at 3000 rpm for 2 mins at 20˚C. The supernatant was discarded and the pellet was treated with 5% hypochlorite solution for 10-15 mins and pelleted at 3000 rpm for 2 mins at 20°C in order to dissolve the nematode body and isolate the eggs (Emmons *et al.*, 1979). The supernatant was discarded and the pellet was dissolved in M9 buffer, further pelleted at 3000 rpm for 2 mins at 20°C. This step was repeated 3-4 times until the odour of the hypochlorite solution was undetectable. Finally, the pellet was dissolved in 9 ml M9 buffer and kept overnight on a rotor at 20°C. The following day, all the worms were synchronized to L1 stage.

Compound preparation: Phenylalanine and aspartic acid were dissolved in double distilled water (DDW) at a ratio of 1:1, 2:1 and 1:2 to a final concentration of 5 µM and 10 µM.

Behavioral assay: Lethality - Synchronized L4 larvae (n=20) were treated with different ratio (1:1, 2:1 and 1:2) of phenylalanine and aspartic acid compounds (10 µM) for 40 hours at 20˚C. The survival of the nematodes was determined for each parameter with an interval of 24 hours using a dissecting microscope. **Brood size assay -** Synchronized L4 larvae (n=20) were exposed to defined ratios (1:1, 2:1 and 1:2) of phenylalanine and aspartic acid compounds (10 µM) for 40 hours at 20˚C. Gravid adults were treated with 20% hypochlorite solution. The bleach dissolved the body and exposed the eggs. Under a dissecting microscope, the number of eggs per individual nematode was recorded. **Life cycle assay -** 96-well microtitre plates were prepared with NGM seeded with OP50 with a final concentration of 10 µM of phenylalanine and aspartic acid solutions (1:1, 2:1 and 1:2). Synchronized L1 larvae were maintained in the microtitre plates at 20˚C. With the usage of a dissecting microscope, the nematodes were transferred every 24 hours to fresh wells to isolate it from the progenies. Individual developmental stage of the nematodes were observed and recorded for each experimental condition.

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Genotoxic assay: Synchronized L1 larvae (n=1000) were exposed to different preparations (1:1, 2:1 and 1:2) of the phenylalanine and aspartic acid compounds (10 µM) for 40 hours at 20˚C. The L4 staged nematodes were washed with M9 buffer and pelleted at 3000 rpm for 2 min at 22˚C. Osmolarity and pH was maintained by the addition of Glucose (34 mM)-Tris HCl (50 mM)-EDTA (10 mM) mix and incubated for 5 min at room temperature. This was followed by the addition of 34 mM SDS that breaks the cell wall, nuclear membrane and removes the histone proteins. After incubation for 5 min, NaCl (5 M) was added and further incubated for 5 min to maintain the osmotic balance. A solution of chloroform-isoprophyl alcohol (1:24) was added and centrifuged for 10 min to separate the proteins from the nucleic acids into two distinct layers. The upper layer comprising of the nucleic acid was carefully extracted. The DNA was precipitated with alcohol and separated on a 1% agarose gel. H_2O_2 was used as a positive control and negative control was treated with PBS only.

Cytotoxic assay: Synchronized L1 larvae (n=500) were exposed to different ratio (1:1, 2:1 and 1:2) of phenylalanine and aspartic acid compounds at a final concentrations of 5 µM, 10 µM and 20 µM for 24 hours, 48 hours and 72 hours at 20˚C; positive control (H_2O_2) and negative control (PBS). Nematodes were washed with M9 buffer and pelleted at 3000 rpm for 2 min at 22˚C. Triplicates of uniform number of nematodes in 96-well microtitre plate were treated with 10 mg/ml of 3-(4,5-Dimethylthiazol-2-YI)-2,5-Diphenyltetrazolium Bromide (MTT) and incubated for 4 hours at 19˚C. To dissolve the crystals, 50 µl of dimethyl sulfoxide (DMSO) was added and the viability of cells was recorded using an Elisa reader at 540 nm.

3. RESULTS

In the brood size assay, the number of eggs retained in the uterus, were observed after being subjected with the constituents of Aspartame (Phenylalanine and Aspartic acid) in the ratio of 1:1, 1:2 and 2:1 (10 µM) for 40 hours.

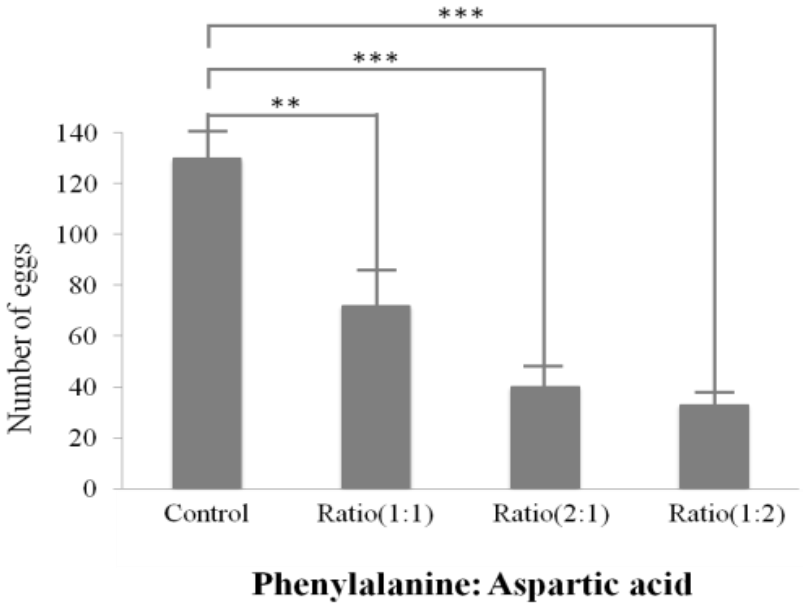

Fig 2: Brood-size assay on the *C. elegans* on being treated with the constituents of Aspartame.The graph depicts the number of eggs recorded per individual nematodes per condition. Under a dissecting microscope, gravid N2 nematodes were subjected to 10% bleaching solution until their body was dissolved and eggs were counted in control, 1:1, 1:2 and 2:1 (phenylalanine : aspartic acid) with a final concentration of 10 µM. The readings recorded were for the different parameters where n= 20, Tukey's Multiple comparison Test using One-way ANOVA, ** $p < 0.0051$, for ratio 1:2, *** $p < 0.0001$ and ratio 2:1, *** $p < 0.0001$.

The number of eggs detected was highest in the untreated nematodes with a fecundity rate of 100%. In case of 1:1 ratio, the number of eggs reduced by half with a fecundity rate is 55.38%. With increase in the concentrations of the amino acids, the fecundity rate reduces to 30.71% and 25.28% for 2:1 and 1:2 ratios, respectively.

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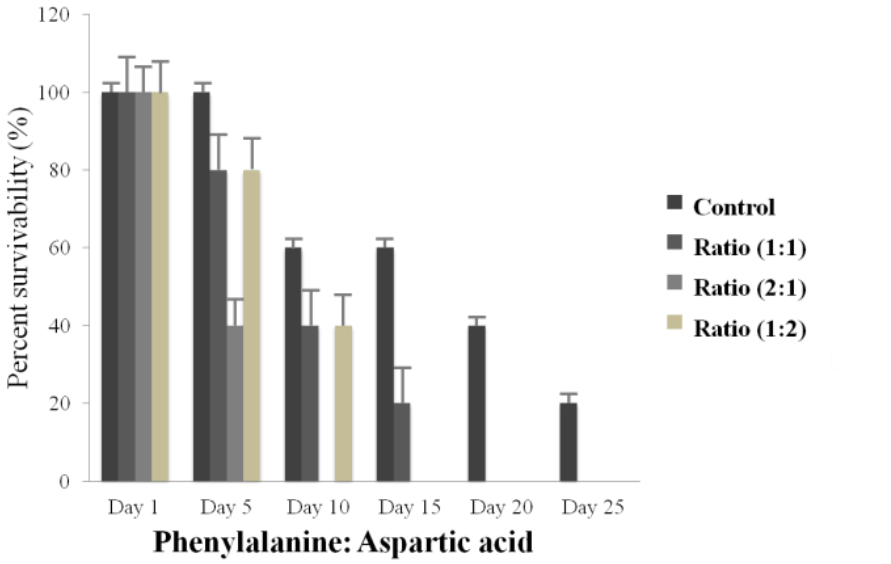

Fig 3: Lethality assay of the *C. elegans* on treatment with the constituents of Aspartame.L4 stage synchronized N2 nematodes were grown on OP50 inoculated NGM plates with a final concentration of 10 µM in different parameters of phenylalanine and aspartic acid (1:1, 1:2 and 2:1); and control. Under a dissecting microscope, with an interval of 24 hours, the survivability of the nematodes was recorded.

In the lethality assay, the four parameters tested were untreated nematodes, nematodes treated with 1:1, 1:2 and 2:1 ratios of phenylalanine and aspartic acid. On day 5th, 100% of the nematodes survive whereas 80% of the nematodes treated with 1:1 and 1:2 ratios survive. The survival of the nematodes exposed to 2:1 ratio drop to 40%. More than half of the untreated nematodes (60%) survive till day 10. 40% of the nematodes treated with 1:1 and 1:2 ratios survive. However, 100% mortality was detected when treated with 2:1 ratio. By day 15th, all the nematodes treated with 1:2 ratio were dead. 20% of the 1:1 ratio treated nematodes and 60% of the untreated nematodes survived. By day 20th, all the treated nematodes were dead whereas 20% of the untreated nematodes still survived till day 25th.

TABLE 1: Observations recorded for life-span assay on treatment of the *C. elegans* with the constituents of Aspartame.

Number of hours	**Control**	**Phenylalanine : Aspartic acid**		
		1:1	2:1	1:2
0 hours	L1 larvae	L1 larvae	L1 larvae	L1 larvae
48 hours	L4 larvae	L3/L4 larvae	L3/L4 larvae	L2/L3 larvae
96 hours	Transferred to fresh well (Eggs detected)	L4 larvae	L4 larvae	L4 larvae (abnormal size)
144 hours	Transferred to fresh well (Eggs detected)	Gravid adult	Gravid adult	Gravid adult
192 hours	Transferred to fresh well (Eggs detected)	Transferred to fresh well (Eggs detected)	Transferred to fresh well (Few eggs detected)	Transferred to fresh well (Very few eggs detected)
240 hours	Adult	Transferred to fresh well (Eggs detected)	Adult (slow locomotion)	Adult
288 hours	Adult	Adult	No nematodes alive	Adult (slow locomotion)
336 hours	Adult	Adult (slow locomotion)	-	No nematodes alive
384 hours	Adult	Adult (slow locomotion)	-	-
432 hours	Adult	No nematodes alive	-	-

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480 hours	Adult (slow locomotion)	-	-	-
528 hours	Adult (slow locomotion)	-	-	-
576 hours	Adult (slow locomotion)	-	-	-
624 hours	No nematodes alive	-	-	-

In the life cycle assay, all the different stages of the nematode were observed with time interval of 24 hours. In the control, the nematodes have a normal developmental rate such that L1 larvae develops into L4 stage in 48 hours and by 96 hours, eggs are laid. The adult nematode under observation was transferred to fresh wells to avoid confusion with the progenies. The life-span of the nematode lasts till 624 hours (26 days) at 20˚C. On exposure to aspartame, the developmental rate of the nematode was altered. Growth from L1 to L4 stage was delayed by 48 hours. In 1:1 ratio of phenylalanine and aspartic acid, there was slight reduction in the number of eggs detected and in the life-span to 432 hours (18 days). In 2:1 case, the life span of the nematode was drastically reduced to 288 hours (12 days). In 1:2 case, not is the life span of the nematodes reduced to 336 hours (14 days), but the amino acids had a further impact such that although the nematode grew into abnormal sizes, the number of eggs laid were reduced.

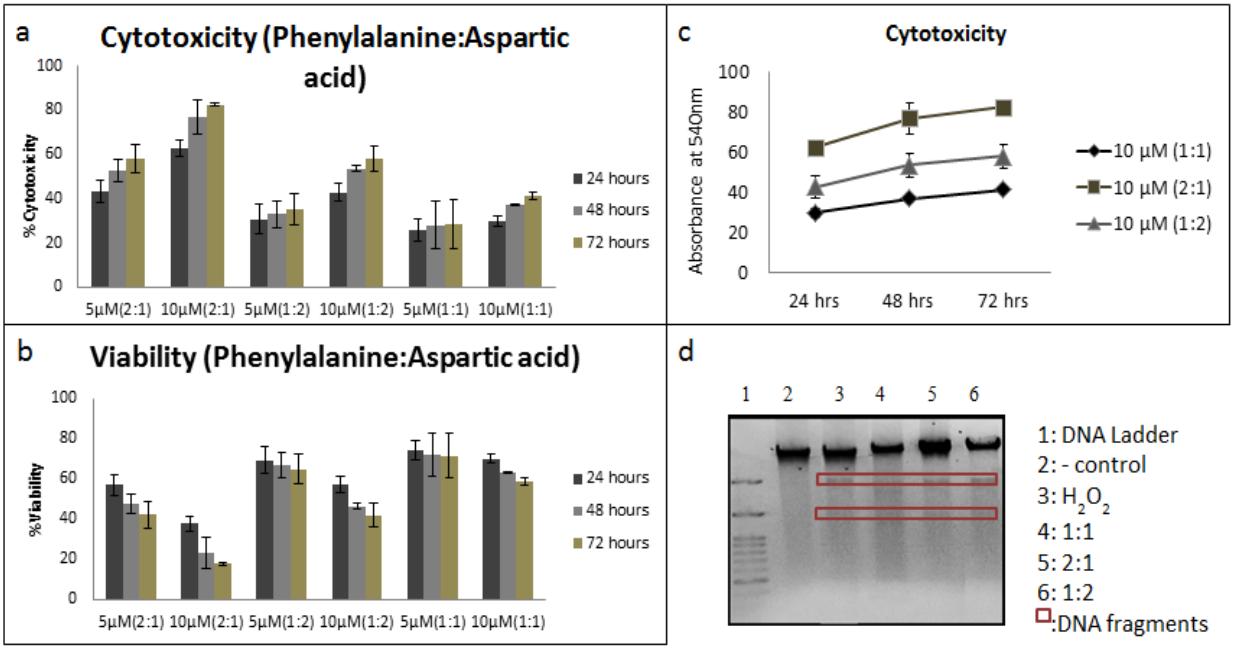

Fig 4: Determination of genomic and cellular toxicity on *C. elegans* on exposure to the two constituents of Aspartame. See methods and materials for description of toxicity assays. a.) The graph represents the percent cytotoxicity on the N2 nematodes at 5 µM and 10 µM concentrations of the constituents of aspartame at different time intervals (24, 48 and 72 hours). b.) The graph represents the percent viability. c.) Graphical representation of the time-dependent cytotoxicity on the *C. elegans* on exposure to the constituents of aspartame (10 µM). d.) DNA fragmentation assay of the exposed nematodes to different ratios of phenylalanine and aspartic acid (1:1, 2:1 and 1:2) visualized on 1% agarose gel.

In the MTT assay, the *C. elegans* were treated with different concentrations (5 µM and 10 µM) of the constituents (phenylalanine and aspartic acid) of aspartame in the ratio 1:1, 1:2 and 2:1. Another parameter used in this experimental set-up were the three different time points (24 hours, 48 hours and 72 hours) to record the level of toxicity. In the positive control, the nematodes were subjected to H_2O_2 treatment for 30 mins, where the level of cytotoxicity was detected to be 97.92%. The negative control was without treatment and the level of cytotoxicty recorded is 0%. Now, in case of 5 µM, in 1:1 ratio, the toxicity levels calculated is 25.69% in 24 hours, 27.91% in 48 hours and 28. 56% in 72 hours post treatment. In case of 1:2 ratio, 30.604%, 32.84% and 35.13% is detected at 24 hours, 48 hours and 72 hours of incubation, respectively. In the set-up with 2:1 ratio, the level of toxicity was recorded to be 43.18%, 52.52% and 57.99% at 24 hours, 48 hours and 72 hours, respectively. On increasing the final concentration to 10 µM, the over-all toxicity level increased when compared to the toxicity level of 5 µM. In the 1:1 ratio, the level of cytoxicity was 29.88% in 24 hours post

treatment, 37%.18% in 48 hours and 41.42% in 72 hours. When the aspartic acid concentration was doubled, the level of toxicity was 42.82%, 53.63% and 58.12% in 24 hours, 48 hours and 72 hours respectively. In 2:1 ratio, the toxicity percent calculated was 29.88%, 37.18% and 58.57% at 24 hours, 48 hours and 72 hours post treatment respectively. In this study, the genotoxic results clearly indicated nuclear DNA fragmentation for all the three combinations of phenylalanine and aspartic acid (Figure 5d).

4. DISCUSSION

Using *C.elegans* model is ideal as it is an intermediate prior to the confirmation in a rodent model and further for clinical trials. The compact yet simple *C. elegans* system allows the conduction of various toxicity assays with ease. Williams and Dusenbery, in 1988, showed that the toxicity studies in the nematode were consistent with the LD_{so} ranking in rat and oral mouse model. Due to the presence of conserved pathways, several laboratories utilize *C. elegans* to perform toxicity screens. The toxic behavior of manufactured ZnO-NPs has been identified using the nematode (Ma *et al*., 2009). In a similar study, the toxicity of manufactured oxide nanoparticles such as ZnO, AL2O3 and TiO2 were determined using *C. elegans* (Wang *et al*., 2008). The brood size assay was the determination of the fecundity rate of the nematodes on treatment with our compound. In 1:1, 2:1 and 1:2 ratios, the percent fecundity was 55.38%, 30.71% and 25.28%. These indicated a significant effect of aspartic acid on the egg production ability of the nematode, ultimately causing a reduction in the population growth rate. A continuous exposure to the compounds might completely erase the nematode population. In the lethality assay, the survival percent was lowest, 40%, in the nematode cultures treated with increased concentration of phenylalanine (2:1). When the amount of aspartic acid was increased, the survival rate slightly increases to 60%. In 1:1 ratio of the amino acids, representing aspartame, the rate of survival was 80%. In this case, the toxic nature of phenylalanine was more prominent in the nematode.

The life cycle assay marks the recording of the development of the nematode from the larval stage 1 to its demise. Our results indicated that consumption of the phenylalanine and aspartic acid in the three combinations of 1:1, 2:1 and 1:2 and with a concentration of 10 µM, renders a severe impact on the development of the nematode. It considerably lowered the rate of development such that it takes 96 hours (4 days) for the nematodes to grow from L1 stage to L4 stage whereas the normal growth rate from L1 to L4 stage is 40 hours (1.6 days), which has been recorded for the control nematodes (Table 1). In the set-up with increased concentration of aspartic acid, the number of eggs detected on the microtiter plates, were very few which corresponded with the brood size assay that we conducted. The nematodes developed into abnormal sizes, the movement of the adult was quite slow and by 336 hours (14 days) no live nematodes were detected. In the ratio 2:1, where the concentration of phenylalanine was doubled, the eggs detected are less and by 288 hours (12 days), no live nematodes were observed. In 1:1 ratio, there was reduction in the number of eggs laid and decreased life-span to 432 hours (18 days). Therefore, it can be inferred that aspartic acid influences the reproductive ability of the nematodes, whereas phenylalanine was significantly toxic as observed by the shortened its life-span. Overall, aspartame consumption has a considerable impact on the developmental rate of *C. elegans*.

MTT forms formazan crystals with the mitochondria of live worms, hence was directly proportional to the number of live nematodes, and was a reliable parameter to determined the cytotoxity in any model. In the cytotoxic assay, the highest rate of toxicity was observed in the case of 2:1 ratio and a final concentration of 10 µM. Time dependent toxicity was observed with 62.46%in 24 hrs, 76.74% in 48 hrs and 82.48% in 72 hrs. The above data convincingly suggested that there was a time-dependent increase in toxicity (Figure 5c), thereby, establishing that continued consumption of aspartame might have a long-term deleterious effect on health.

The compiled data indicated that the cells were undergoing apoptosis on treatment with the constituents of Aspartame. In the *C. elegans*, programmed cell death takes place in three distinct phases: initiation, activation and execution (Horvitz, 1999). Our studies indicated that in the initiation phase, the cells were triggered by the presence of aspartame and it admissions the activation phase. On the surface of the mitochondria, CED-9 and CED-4 proteins co-localize such that CED-4 is dependent on CED-9 (Chen *et al*., 2000). The gene, *ced-9*, and the human ortholog, *bcl-2*, encode proteins that function in inhibition of apoptosis (Hengartner and Horvitz, 1994). Various transcriptional regulators such as Zn finger DNA-binding proteins, TRA-1 (Zarkower and Hodgkin, 1992) and CES-1 (Thellmann *et al*., 2003), control the expression of *egl-1*. Binding of EGL-1 to CED-9, causes the CED-9 protein to undergo conformational changes resulting in the breakdown of the CED-9/CED-4 complex and translocation of CED-4 from the mitochondria to perinuclear membranes (Conradt and Horvitz, 1998; del Peso *et al*., 1998; Parrish *et al*., 2000). Self-oligomerization of the CED-4

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protein interacts with CED-3 and proteolytically activates it (Yang *et al*., 1998), generating an active protease (Alnemri *et al*., 1996; Xue *et al*., 1996). A proenzyme, *ced-3* (human ortholog, caspase-3) on activation commences the execution phase, which comprises of degradation of the nuclear DNA, followed by engulfment of the cells.

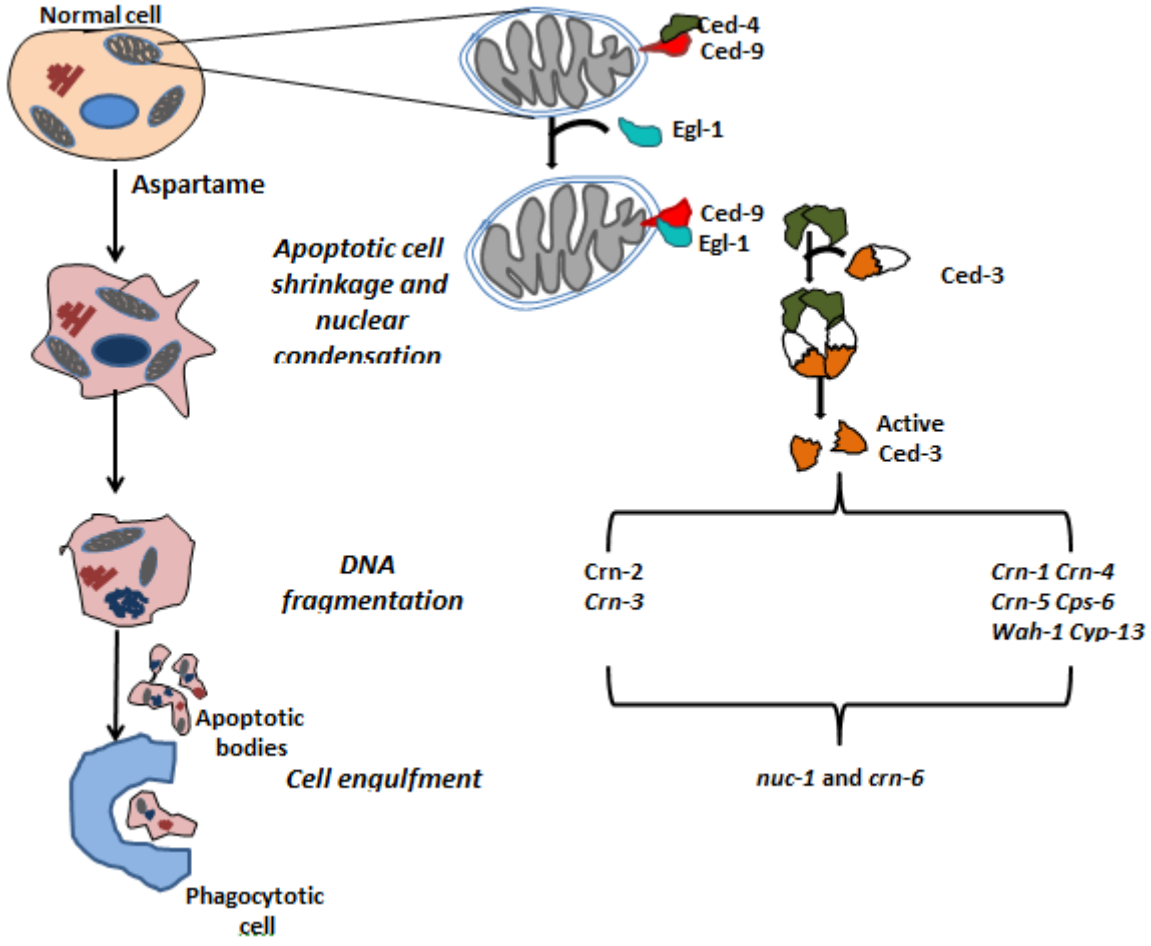

Fig 5: Induction of apoptosis on consumption of Aspartame in *C. elegans*.

Conradt and Xue, 2005 described a set of genes that were associated with DNA fragmentation, including *crn-1*, *crn*-2, *crn*-3, crn-*4*, *crn-5*, *crn*-6 (cell death related nucleases), cyp-*13* (cyclophilins), *cps-6* (protease suppressors), *nuc-1* (nuclease defective) and *wah-1* (nematode apoptosis inducing factor homologue). Down-regulation or loss of function of any of these genes, with the exception of *nuc-1* and *crn-6*, causes reduced cell deaths, rendering their significance in conduction of apoptosis. Aspartame might further influence any of these genes causing the DNA to deteriorate because the genes, *crn-1*, crn-*4*, *crn-5*, cyp-*13*, *cps-6* and *wah-1* function together whereas the genes, *crn*-2 and *crn*-6 coordinate to regulate DNA fragmentation. Interaction of the genes, *crn-1*, crn-*4*, *crn-5*, cyp-*13* and *cps-6*, possibly result in the formation of a complex that participates in DNA degradation. The mitochondrial protein, WAH-1(human ortholog, apoptosis inducing factor) interacts and increases the endonuclease activity of CPS-6 (human ortholog, mitochondrial endonuclease G). After DNA is degraded, Type II acidic DNases are encoded by *nuc-1* and *crn-6*, which are involved in cell engulfment, the final step in nematode apoptosis (Parrish and Xue, 2003). The observation of DNA fragmentation clearly indicated that aspartame initiates cell apoptosis.

5. CONCLUSION

The study conclusively indicated that the constituents of aspartame, phenylalanine and aspartic acid, have a significant long-term toxic effect on *C. elegans*. In fact, accumulation of the individual amino acids in the body could further result in damages both at the genomic and cellular level, leading to several severe health issues. Therefore, it is of outmost importance to extend the study at molecular level to sensitize the mankind and help the reduction or minimalistic use of aspartame.

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