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mRNA EXPRESSIONPROFILINGOF GLUTATHIONE PEROXIDASE (GPX GENE) USING FIVE MEDICINAL PLANTS ON *S. CEREVISIAE*

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Keywords:

Messenger RNA, Gene expression analysis, Medicinal plants, Phytochemicals, Glutathione Peroxidase, Real time polymerase chain reaction

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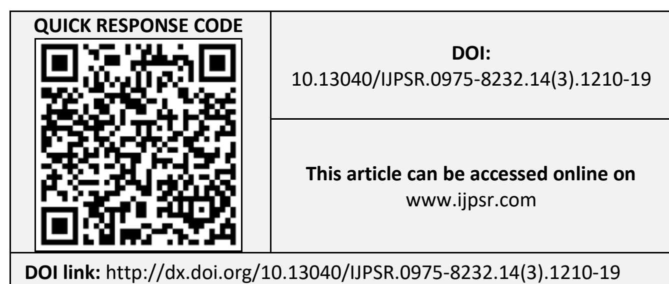
ABSTRACT: Glutathione peroxidase gene expression profiling has been determined using budding yeast, *S. cerevisiae*, as a model organism mainly used for extensively reliable microbial studies of various cellular processes evolved in evolutionarily distant species. Roots of five medicinal plants were used: *Withania somnifera*, *Terminalia arjuna*, *Ranunculus sceleratus*, *Bacopa monnieri* and *Acalypha indica.* The maceration method exhibited the extraction process, followed by *S. cerevisiae* culturing at 600nm. Classic Trizol method for RNA extraction of yeast cells. Further, evaluation of the quantity and quality of the isolated RNA before the CDNA synthesis, respectively. Polymerase Chain Reaction was performed using specific conditions, having $60^{O}C$ as GPx primers annealing temperature. Real-Time Polymerase Chain Reaction of samples was performed where Beta-actin primers as a housekeeping gene. High purity of RNA samples was yielded to be in the range of 1.8-2.0. The size of cDNA PCR products was found to be 118bp. The RT-qPCR results showed high over-expression, *i.e.,* fold change unit of GPx gene in all sample extracts compared to the control yeast. Ethanol and methanol extracts of *Acalypha indica,* ethanol extract of *Ranunculus sceleratus,* and methanol extract of *Terminaliya arjuna* showed high overexpression of GPx gene. The fold change of GPxgene profiling ranged from 17.638 ± 0.1-64.415 ± 0.18. One-way ANOVA was calculated, showing $p<0.05$ considerably as highly significant. Therefore, the bioactive components of five plant root extracts can potentially enhance the antioxidant GPX gene expression in *S. cerevisiae* at an extensively good level.

INTRODUCTION: Oxidative stress are the major cause of various harmful, life-threatening diseases leading to long-term illness. Free radicals playan important role in increasing oxidative stress levels due to the disruption between the quantities of free radical ions and antioxidant compounds. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in all living organisms as aerobic metabolism by-products [1].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The generation of reactive oxygen and nitrogen species is mainly enhanced when plants and animals are subjected to biotic or abiotic stresses. These are chemical species that are generated by incomplete reduction of oxygen, which includes hydroxyl radical (HO·), superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) *etc*. These are the free radicals having single electrons on their outer orbital shells, leading to unstable in nature.

These molecules tend to become stable by obtaining electrons from other important molecular components, making them unstable. ROS participate in a huge range of necessary cell signalling processes, including growth, cell cycle, development, stress acclimation and programmed

cell death [2]. Antioxidants stabilize these free radicals. ROS and RNS excessive production can lead to protein oxidation, lipid per-oxidation, metabolic malfunction, membrane disruption, DNA damage, cellular damage, and immune dysfunction [3-4]. For centuries, medicinal plants are being used worldwide. Plants are a high source of bioactive constituents, including therapeutic functions, e.g., insect inhibition activity, antiviral, antimicrobial, anti-inflammatory, antifungal activity, and various positive effects for humans and animals. Each herb's therapeutic activity is due to various phytochemicals groups showing biological medicinal values, such as polyphenols, tannins, natural colorants, coumarins, essential oils, flavonoids, mineral compounds, alkaloids, vitamins, *etc.* [5].

Humans use such traditional natural compounds for their better health, long lifespan, relieving from harmful diseases and infections [6]. The major bioactive compounds in medicinal herbs are polyphenols (specifically phenolic acids and flavonoids), showing high medicinal properties. These compounds show therapeutic biological activity, *e.g.,* anticancer, antioxidant, neuroprotective, antimicrobial, antidiabetic, antitumor, cardioprotective, *etc.* [7-8]. Among various enzymatic defense systems, glutathione peroxidase (GPx) is one of the important enzyme antioxidant families that contributes to the detoxification of several ROS [9].

GPxs are very important in the enzymatic antioxidant system as they protect all organisms from oxidative damage and scavenge peroxides, oxides, *etc*., generated inside the cells. They catalyze the H_2O_2 reduction and conversion of organic hydro-peroxides to water or corresponding alcohol glutathione compound (GSH), acting as an electron donor. Hence, they maintain a particular balance between the ROS levels and the antioxidants [10-12]. This enzyme a generally grouped into two sub-groups: one is selenium-dependent glutathione peroxidase (SeGPx) and the other is non-selenium glutathione peroxidase (non-SeGPx) mainly based on the presence of the cysteine (Cys, C) or selenocysteine (Sec, U) residues at their active sites [13-15]. This current research has been objected to determining the proper effectiveness of the various applications of the medicinal plant extracts for evaluating change mRNA expression of GPx genes present in *S. cerevisiae* under treatment with medicinal plant ethanol and methanol extracts. GPx family has been recognized as the evolutionary gene in mammals. Therefore, yeasts containing GPx play a great role as the first defense line against ROS. In many studies, it had been analysed that the antioxidant activity of GPx genes was much lower in yeast due to high oxidative stress production, which is the most important antioxidant enzyme. It is necessary to increase the activity; hence an external force is required to increase the antioxidant GPx gene activity. Therefore, it is important to analyze the changes in the gene expression of such important antioxidant enzymes (*i.e.,* the antioxidant activity of GPx gene against these oxidative stress) using the bioactive properties of medicinal plants, occurring in a wide range all around the world. It has been studied that plants' bioactive compounds (phenols & flavonoids) are majorly responsible for high antioxidant properties [14-15]. GPx has been researched to be evolutionarily gene acquired by all mammals. GPx comprises various proteins that are found in most living organisms [16]. GPx family is the key enzyme for ROS detoxification in living organisms. In response to ROS formation during temperature fluctuations, GPx gene expression is easily explained by simultaneous metabolic processes [17-18].

MATERIAL & METHODS:

Plant Material: Specimens of *Withania somnifera, Terminalia arjuna, Bacopa monnieri, Ranunculus sceleratus,* and *Acalypha indica* were utilized from the locations of Agrakhal, Uttarakhand.

Plants Authentication: Botanical authentication was performed by botanist Dr. Swapnil Sisodia, SS Jain Subodh PG College, Jaipur, Rajasthan, India.

Plant Root Extraction: The maceration technique was used for the extraction process [19]. Two solvents, methanol, and ethanol were used for the low to high-polarity bioactive component extraction. In 1:10 ratio, mixed, heated at 55 °C and shaken for 5 days at room temperature. The mixture was then collected by filtering through Whatman No.1 filter paper and dried at 50 °C. The crude root powder extracts were stored at 4 °C.

***S. cerevisiae* Bioassay:** Baker Yeast (Blue diamond) was purchased. 1g sugar was added in 100ml warm water with 0.5 g yeast in a glass beaker and incubated overnight[20]. The yeast was activated with froth formation as shown in **Fig. 2**. Activated yeast cells were inoculated in 50ml Potato Dextrose Agar (Himedia, India) pH5.6, plated for 24-48 hrs at 35°C. *S. cerevisiae* cells were isolated and prepared in Potato Dextrose Broth (Himedia, India), pH 5.6, incubated for 24-72 hrs at 35°C until an $O.D._{600\ nm}$ = 0.8-1.0 was measured using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA).

Extract Inoculation in Yeast Cells: 120ml of Fresh *S. cerevisiae* culture was prepared in Potato Dextrose Broth (Himedia, India), pH 5.6, and 10ml were transferred in each 12 flacon tubes of 15ml. One falcon tube containing the yeast culture was used as a positive control, and the other had only media as a negative control. The remaining 10 falcon tubes containing yeast were inoculated with powdered ethanol and methanol plant extracts as depicted in **Fig. 3**. The tubes were kept in shaker incubator at 35°C for 20 daysand $O.D._{600nm}$ measured around 0.8-1.0 in NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA)[20].

RNA Extraction: RNA was extracted using Trizol method, a monophasic solution containing phenol and guanidinium isothiocyanate [21]. 3ml yeast culture was centrifuged at 10,000 rpm for 10 min. 0.5 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) was added to the pelletand vortexed (2min), incubated at room temperature for 5min, and then 0.5 ml of chloroform-isoamylic alcohol (24:1) was added. The solution was invertedly mixed and again incubated for 5 min, centrifuged for 10 min at 10,000 rpm. The upper layer, (aqueous phase) was added with 0.5 ml of isopropanol and invertedly mixed. Lastly, 4°C refrigerated centrifuge was usedfor 10 min at 10,000 rpm. The pellet was washed with 1ml 70% ethanol. After drying for 10 min, the RNA pellets were dissolved in 70ul of pyrogen-free sterile water and stored at -20°C for further usage.

RNA Quality and Quantity Determination: The quality of the extracted RNA was determined by 0.8% gel electrophoresis method [22]. 3ul of ethidium bromide was used as fluorescent RNA binding dye. 7ul of samples were mixed with 2ul of 6X RNA loading dye and were loaded in the gel wells. The desired voltage was set up, and RNA bands were visualized under U.V. transilluminator. The quantity of RNA yielded, *i.e.,* purity and concentration, were determined by measuring the optical density (O.D.) at 260 nm and 280nm using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) [23].

Primer Design: Primers were designed in National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) for GPx gene region in (RefSeq mRNA genome databases search) *S. cerevisiae* and synthesized from GCC BiotchPvt. Lt, India. Primer Blast designing software algorithm was enabled for Glutathione Peroxidase primers, GPX1F (ACCACCTTCCCATTTCGGTC) and GPX1 R (GGCAAAAGCAAGATCCCGTC) and Beta Actin housekeeping primers, ACT1F (TCGTTCCAATTTACGCTGGTT) and ACT1 R (CGGCCAAATCGATTCTCAA) with particular parameters.

Complementary DNA Synthesis ($_{C}$DNA):1st strand cDNA was synthesized using ThermoFisher Scientific DyNAmo cDNA, U.S. Synthesis Kit [24]. 2µg of the RNA template, 2ul of Oligo (dt) 18 primer (1 µg), 5ul of nuclease-free water, vortexed, centrifuged, and kept at 65°C in a water bath for 5 min. The solution was centrifuged, and 4ul of 5X Reaction Buffer, 2ul of RiboLockRNase Inhibitor, 2ul of 10 mMdNTP mixes, and 2ul of Revert Aid Reverse Transcriptase (200 U/µl) were added, mixed gently, and again centrifuged, incubated for 60 min at 42°C. Lastly, reaction termination was done by adding 5ul of 0.5M EDTA (Ethylenediaminetetraacetic acid) and incubating for 15 min. The cDNA samples were stored at -20°C.

Polymerase Chain Reaction (PCR): Assay was carried out in MWG AG Biotech Primus 96 Plus PCR system (Outback Equipment Company, Gilroy, California, US). G- Bioscience, India PCR Chemical kit was used [25]. The PCR cocktail consisted of 5.0 ul nuclease-free water, 3.0 ul of cDNA samples, 2.0 ul of 20 pmol of each GPX1 F and R primer, 12.0 ul of PCR master mix (2.5mM

Deoxy-nucleoside triphosphate each, 25 mM Magnesium chloride and 500U/ml Taq DNA polymerase, Taq Buffer PCR) was set up having following conditions as shown in **Table 1**.

Real-Time Polymerase Chain Reaction (RT-PCR): It was performed in a 96-well plate on Applied Biosystems Real-Time Polymerase Chain Reaction Instruments, Sequence Detection System 7000 using SYBR^{TM} Green master mix (Thermo Fisher Scientific, U.S). The cocktail contained 25 µl of 2X SYBR Green Master mix, 5.0ul of DNA samples, 2 ul of each F,R GPX1, and ACT1 primers, having a total volume of 50 µl. The thermal cycling condition is shown in **Table 1**. Threshold values were obtained, and fold change was calculated [26].

Statistical Analysis: Each work has been performed thrice. The data were recorded and calculated as means ± standard deviations (SD). Standard equations in Excel (Microsoft) were used to conduct calculations. All samples P values were calculated, and one-way ANOVA was performed on all the statistical data; p <0.5 is taken to be significant.

RESULTS:

Plant Root Extraction: Fig. 1 shows the root extracts of all five plants with each of the two solvents performed by maceration technique, where A, known as *W. somnifera*, Ar-*T. arjuna*, B-*B. monnieri*, J-*R. sceleratus*, K-*A. indica*. A different range of colours was depicted in each of the root extracts of five plants with two different solvents, contributing to various polyphenolics and flavonoid compounds.

It also has some other phytochemical compounds in it. These bioactive compounds exhibiting various colours are the major cause of enhancing potential therapeutic effects against any illness or infection. It also contains various kinds of beneficial therapeutic activities.

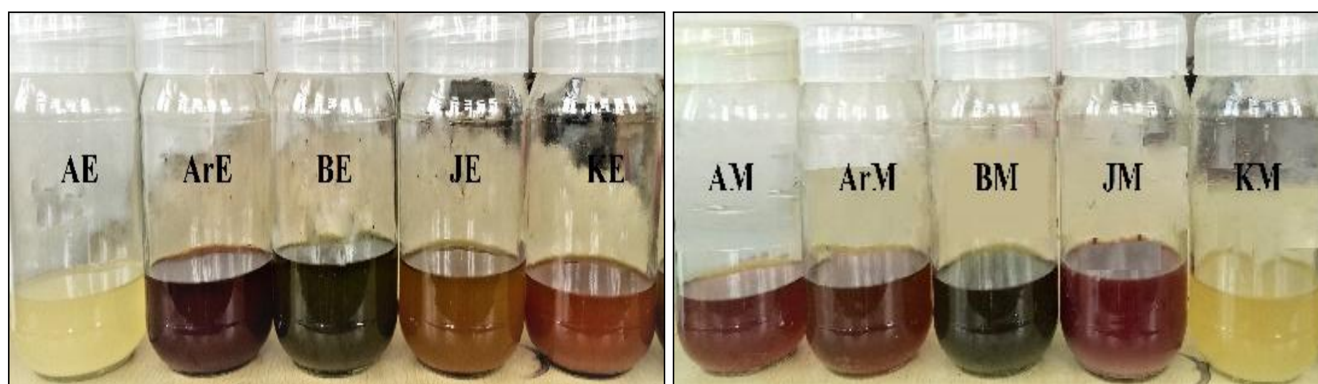

FIG. 1: ROOT PLANT EXTRACTION, WHERE E- ETHANOL, M-METHANOL

Fig. 2 shows yeast, *S. cerevisiae* activation *i.e.,* yeast growth within 8 hrs using sugar fermentation, and **Fig. 4** had been depicted as the yeast growth curve plotted with absorbance, days and biomass production. **Fig. 3** represented the incubation period, *i.e.,* inoculation of yeast cells with plants extracts along with a positive control for 7 days.

FIG. 2: YEAST ACTIVATION

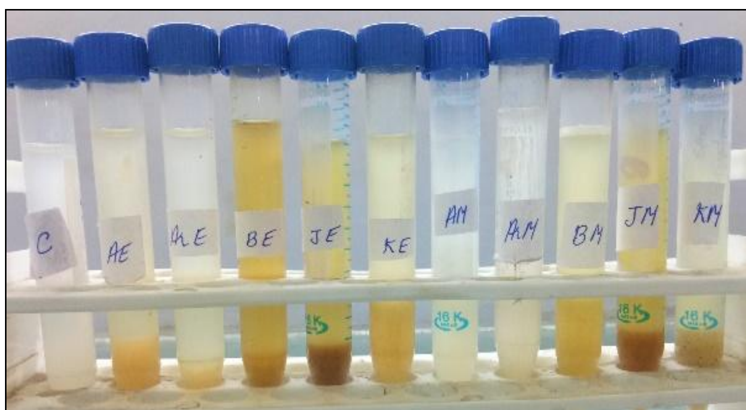

FIG. 3: EXTRACTS SEEDING IN *S. CEREVISIAE*CULTURE

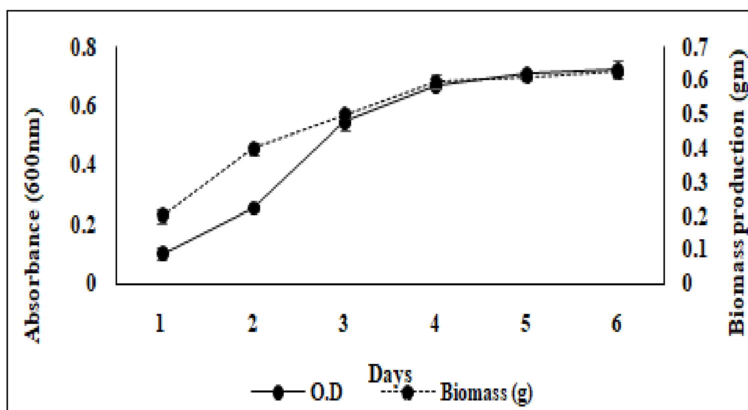

FIG. 4: *S. CEREVISIAE* GROWTH CURVE

RNA Determination And cDNA PCR Results: RNA Extraction bands have been represented in **Fig. 5**, showing RNA bands of all the 11 sample extracts with control using Trizol method. Wells has been labelled as L- Ladder, S1- Control yeast culture. Wells S2-S6- were the ethanol extracts of *W. somnifera, T. arjuna, B. monnieri, R. sceleratus,* and *A. indica,* respectively. Wells S7-S11 were the methanol extracts of the same plants, respectively. All the RNA bands are of the same base pairs, *i.e.,* more than 1,500 bp. It is estimated the amount ranges to be in between 5ug-10ug. Two RNA bands had been shown, firstly 28S (4000-5000bp) and 18S (1000-2000bp).

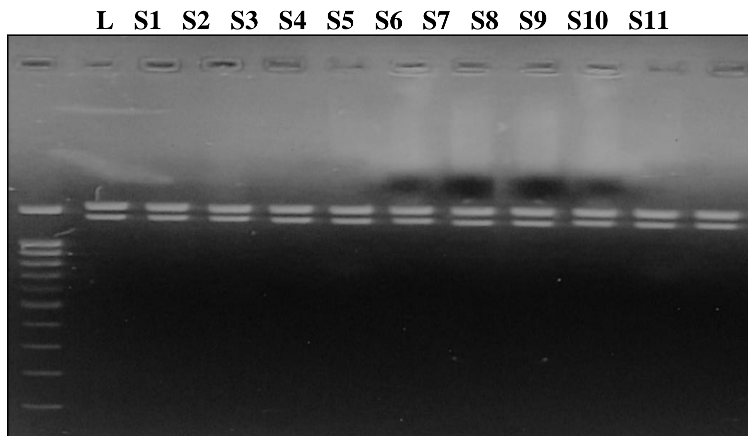

FIG. 5: RNA SAMPLES GEL ELECTROPHORESIS

Different concentrations of RNA samples in each extract were shown in **Fig. 6**. The ratio of all 11 samples A260/A280 for RNA was between 1.8-2.0, and around 10-40ng was obtained. It was estimated that the process extracted pure RNA without any contamination of proteins. The proper RNA ratio also revealed intact bands of RNA without any breakage or disruption. The RNA purification

process revealed that the total RNA of 11 sample cells showed O.D values between 1.8/1.9-2.0 indicating proper, well-structured RNA. The control containing only yeast culture has 1.92±0.00015 as the lowest concentration as compared to other sample extracts. It has been seen that ethanol extracts of *R. sceleratus* 6.37 ± 0.001 and *A. indica* 5.42 ± 0.001 have more RNA sample concentration.

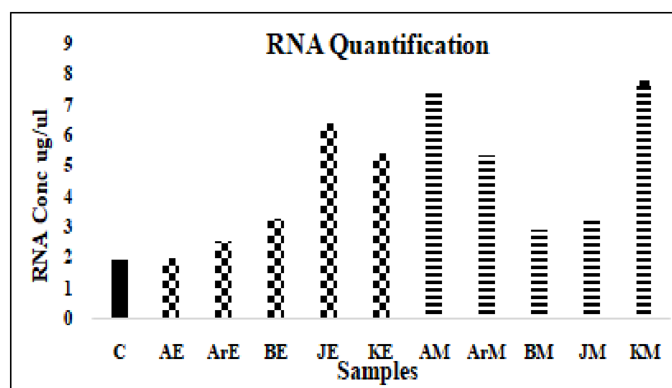

FIG. 6: RNA SAMPLES CONCENTRATION

Similarly, methanol extracts of *W. somnifera* 7.49 ± 0.015, *T. arjuna* 5.33 ± 0.003, and *A. indica* 7.65 ± 0.012 has more RNA sample concentration. Comparing control and the root extract seeded yeast culture shows a huge difference in RNA concentration. Therefore, the extract enhances the amount of RNA present in *S. cerevisiae*. Thus, these five medicinal plants had the capability to increase the functional/coding region of microorganisms at a potentially higher genetic level.

Fig. 7 represented cDNA PCR product bands of all the 11 sample extracts with control using glutathione peroxidase primers, GPX1 F and GPX1 R. The DNA bands size were reported as 118bp, i.e., 40ng product amount. Wells has been labelled as L- Ladder, S1- Control yeast culture. Wells S2-S6 and S7-S11 were the ethanol and methanol extracts of *W. somnifera, T. arjuna, B. monnieri, R. sceleratus* and *A. indica,* respectively.

The primer-independent cDNA sequence amplification curve was maximally achieved when RT-PCR was performed at 60°C. However, at this temperature, the efficiency of the specific primer-binding sites initiated the quantitative PCR process. After normalization, the annealing temperature was set at 60°C for 30 sec. Various previous studies have suggested that primer-independent cDNA synthesis mainly takes place commonly in RT-PCR assays, contributing to a highly significant part of the final amplification products.

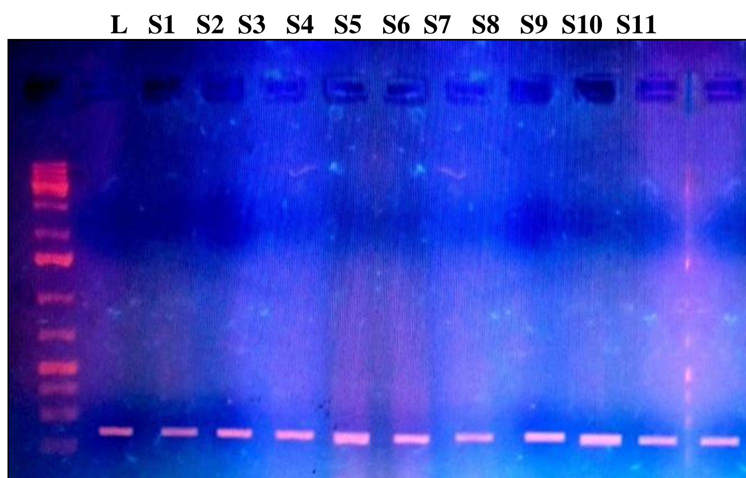

FIG. 7: GPXCDNA-PCR AMPLIFICATION B AND GEL ELECTROPHORESIS

GPxGene Expression Analysis: The fold change of different sample extracts and control has been shown in **Fig. 8**. It was seen that the root extracts of methanol and ethanol samples are showing over-expression, i.e., up-regulation of GPx gene in the yeast model in response to the the plant extracts as compared to control. **Table 1**, has depicted the threshold limit, *i.e.,* mean Ct values of beta-actin gene (ACT 1) and Glutathione peroxidase gene (GPx 1). RT-PCR programming has been shown in **Table 1**. The annealing temperature was normalized at 60°C for 1 min.

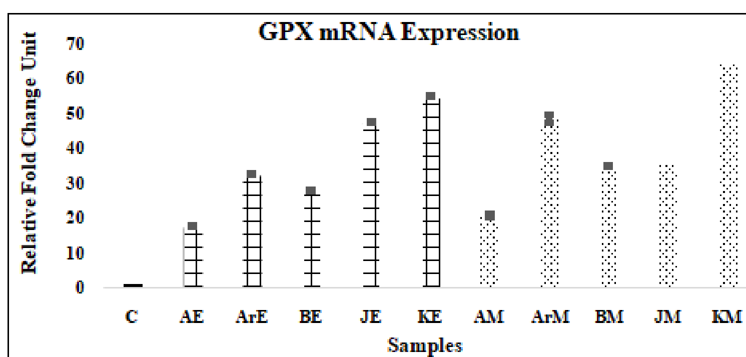

FIG. 8: GPx GENE EXPRESSION PROFILINGUSINGPLANT EXTRACTS ON *S. CEREVISIAE*

The data has been calculated in Mean ± Standard deviation form. Ethanol plant extracts of *T. arjuna, R. sceleratus,* and *A. indica* have shown potential GPx gene over-expression. Similarly, methanol extracts of *T. arjuna, A. indica* has resulted in high GPx gene expression. *W.somnifera* plant extracts showed a bit lower over-expression as compared to the other extracts but higher than the control. Hence, the root extracts were able to increase the GPx gene level of yeast when incubated. The root of all these five plants contains high polyphenolic compounds which were responsible for such potential effects, minimizing the oxidative-related stress and problems. These are polyphenolic-rich extracts protecting from various illnesses and diseases. The results also suggested that these natural compounds can be actively collected in discrete cell structures displaying pleiotropic action with antioxidant effects on cells.

DISCUSSION: Natural products are the major origin of pharmacologically active constituents, which are highly effective with zero side effects [27]. Major secondary metabolites like phenols, and flavonoids are potent water-soluble antioxidants which are free radicals' scavengers preventing cell oxidative damage [28]. Natural products containing therapeutic agents act as completely safe and economical medications [29]. Understanding their roles is the main criteria for methodological and economic biological structures. Plant compounds phenols and flavonoids are highly powerful antioxidant compounds. They can minimize the dangerous ROS production (oxidative stress) in yeast cells. Plants with high flavonoid phenols concentration tremendously deal with oxidative stress and adverse environmental state due to their high antioxidant potential. It is also reported that high concentration of such compounds in plants leads to the over-expression of antioxidant genes, hence scavenging oxidizing involved in free radicals' productions, minimizing all the negative effects of oxidative stress in biological systems [30-31].

Maceration techniques have been termed the most reliable, easy, applicable, convenient, and less expensive method compared to other modern extraction techniques [1]. This method has acquired crude extracts containing huge mixtures of different metabolites, having high purity [32]. This method is highly recommended for small-molecule compounds like polyphenolic compounds, *e.g.,* various phenolic acids (containing gallic acid and ellagic acid), flavonoids, tannins, quinones, isoflavones, alkaloids as these molecules are highly stable under heating conditions up to 60°C-65°C.

It has been reported that analyzation of the mRNA molecules profile of relative abundance expressed in treatment response has become a key standard tool for comprehensive analysis of high-throughput gene expression techniques. It is evaluated that the mRNA population assessed for either single or compared between different conditions; it is more essential that these mRNAs be *in-vivo* population representative. Therefore, significant considerations must be provided to the applications of the proper RNA extraction protocols to minimize errors [33]. Fungal cells, along with the yeasts, *S. cerevisiae* cells are mainly surrounded by a high rigid cell wall varying in composition and thickness, depending on the various growth conditions, which has been the main barrier in the extraction of various cellular contents. Sonicating cells with heat treatment functionally and effectively removes the cell wall without damaging the cellular components; hence generating readily

lysed cells are highly seen as the most potential method [34].

RNA nucleic acid concentrations derived from calculation counts was contributed to the high RNA pools. Normally, microbial RNA accounted for an average of 150%-174% RNA concentrations spectrophotometrically. It is reported to be a highly sensitive and precise technique as the lability nature of the RNA molecules. In certain samples, the microbial RNA concentration estimation greatly exceeded the total RNA concentrations measured spectrophotometrically, which means 80% recovery of the RNA, indicating no major loss or destruction of RNA samples occurred during the extraction process [35].

This study determined the changes in GPx gene expressions in *S. cerevisiae*. The relative mRNA gene expression levels were analysed through the most advanced RT-PCR. The fold increase in the yeast cells' expression of the GPx genes was evaluated; relative quantification equals one. Few studies on genome-wide Identification and characterization of whole antioxidant GPX family proteins had been performed [36]. It has been reported that the plant GPX family has multiple GPXs with particular sub-cellular localisations and functions and exhibits patterns of differential tissue-specific expression, coordination functioning, immune responses, and responses to environmental stress against reactive species. Therefore, the depicted outcome also highlights the basic need of GPX whole genome-wide level study [36]. GPx over-expression is also influenced by RNA quality used in expression profiling by RT-qPCR [37]. The Overall homology (identity) of Human GPx homologs and yeast GPx homologs was much higher, about 58% to that mammalian. Yeasts containing GPx and this glutathione play a very crucial role in the defence line of action against the reactive oxygen and nitrogen species [38]. It has been researched that methanol extracts of *E. platycarpa, E. punctata, E. subcoriacea*, used in diabetes treatment in Mexico, protected homogenate rat pancreatic cells and induced high levels of GPx activities [39]. Increased in GPx levels in the young leaves Of *N. tabacum* were recorded against various induced abiotic stresses [40]. GPxs have a very low level of substrate specificity, resulting in high conductibility in reducing a huge wide spectrum of peroxides with H_2O_2 [41]. As per a report, plant GPxs compounds are very important for plants using numerous metabolic pathways in response to various stresses.

In the future development, the exact GPx gene activation mechanism using these medicinal plant extracts should be explorated in a broader prospect at a cellular level. It is also suggested that the potential effective bioactive substance of these extracts acting on GPx gene expression analysis should be properly identified. Further, deep experimentation on other animal models, including mice, rats, *Homo sapiens,* in particular by these particular natural drugs of these medicinal plants enhancing GPx antioxidant activity, was also strongly recommended, thus studying the effective antioxidant functions in terms of proper health.

CONCLUSION: The work revealed that the root extracts of medicinal plants *W. somnifera*, *T. arjuna*, *B. monnieri*, *R. sceleratus*, *A. indica* exhibited high levels of GPx gene expression in *S.cerevisiae*. The increased expression of GPx antioxidative enzyme seeded with different plant extracts experimentally proved to serve as one of the crucial components of increasing the antioxidant defense mechanism of plants in combating various oxidative injuries and diseases. High polyphenolic-rich medicinal plant root extracts are the main compounds in the elevation and activation of GPx antioxidant enzyme gene. Therefore, gene overexpression property is caused by various phenolic compounds.

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CONFLICTS OF INTEREST: The authors had declared no conflict of interest in this conducted research work.

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