International Journal of Natural Medicine and Health Sciences
ISSN (Online):2790-2471 ISSN (Print): 2790-2463
Volume 2(1) December 2022
Journal homepage: https://journals.iub.edu.pk/index.php/ijnms

Original Research

In vitro evaluation of antimicrobial and cytotoxic potential of *Epimedium grandiflorum* hydroethanolic extract as natural medicine

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Article Info.

Received: 28-09-2022
Revised: 31-10-2022
Accepted: 10-12-2022
Online: 31-12-2022

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Keywords: Antimicrobial, Cytotoxic, Safety, Therapeutic agent, Nontoxic, Natural medicine

Abstract

Medicinal plants are used as fundamental and low-cost source for remedy of numbers of infectious and metabolic diseases in developing and developed countries. Current research work was planned to evaluate the antibacterial, antifungal, and cytotoxic potential of hydroethanolic extract of E. grandiflorum. It was found that selected natural medicinal herb have significant ($p<0.05$) antibacterial activities tested against *Bacillus subtilis, Staphylococcus aureus, Pasteurella multocida, Escherichia coli, Klebsiella pneumoniae, Acinetobacter species, Pseudomonas Species and Salmonella Species.* The results of bacterial biofilm inhibition also explored that selected natural herb has significant ($p<0.05$) capacity to prevent the microbial biofilm particularly at higher dose. The results of antifungal activities showed that selected medicinal plant has significant ($p<0.05$) antifungal potential evaluated against *Aspergillus flavus, Aspergillus niger, Aspergillus terreus, Fusarium solani, Alternata alternaria, and Schizophyllum species*. Moreover, the results of mutagenicity test and DNA damage preventive test explored that selective medicinal plant has significant ($p<0.05$) DNA protective capacity or in other words it is non-mutagenic or cytotoxic in nature. It could be concluded that *E. grandiflorum* could be a potential candidate as therapeutic agent to manage infectious diseases especial bacterial and fungal infections with non-toxic nature.

Citation: Munir N, Mahmood Z, Riaz M, Tahir IM, Shah SMA, Bacha U, Mehmood R, Hussain S. *In vitro* evaluation of antimicrobial and cytotoxic potential of *Epimedium grandiflorum* hydroethanolic extract as Natural medicine. IJNMS. 2022; 2(1)25-31.

Introduction: Medicinal plants are used as fundamental and low-cost source for remedy of numbers of infectious and metabolic diseases in under developing and developing counties. Most medicines under used have recently been derived either directly or indirectly from different medicinal herbs and shrubs. The conventional treatment approach of herbal medicines has been reported to be commonly used by various socioeconomic populations [1]. Various benefits have been achieved through herbal drug usage including improved tolerability, rejuvenation function, minimum side effects, natural basic structure, reduced allergic activity, degree of effectiveness and harmony for the normal physiology of the body [2]. Plants resources are simplest available treatment options due to naturally existence of more than half million plants of various species around the world. The most therapeutic properties of herbal plants have not yet discovered, and many are under investigation at various level to explore their therapeutic characteristics, and then evaluation of treatment potential against various diseases [3].

Antimicrobial drugs are types of medicines with tremendous curing properties for bacterial, fungal, and viral infections with wide range of side effects. Phytoconstituents are well known for their anti-microbial activity from different medicinal plants [4]. *E. grandiflorum* is a type of flowering plant in the family of Berberidaceae, found in Japan, China, and Korea, a large flowered barrenwort or bishop's cap, which have many common names, such as Rowdy Lamb Plant, Horny Goat Weed, Fairy Wings, Xianlinpi, Barrenwort, Yangheye, Yin Yang Huo or Bishops Cap etc., [5]. *Epimedium* contains a wide range of phytoconstituents like saponins, phenolics, glycosides, fats, lignans, sesquiterpenes, basic petrol and other biochemical substances that contain potential helpful properties, with traces of epimedosides, epimedines, quercetin and magnflorins. However, flavonoids mainly icariin is predominantly present have health-promoting activity [6]. The active ingredients in *Epimedium* have noticeable therapeutic effects, including as enhancing immune system function, antimicrobial, liver protective effects, anti-oxidation, lipid lowering, anti-cancer, anti-aging, and depress releasing effects [7].

It was reported that flavonoids present in the medicinal plants could inhibit the growth of *Micrococcus pyogenes* var. albus, *Diplococcus pharyngis* communis, *Staphylococcus aureus*, *Micrococcus catarrhalis* as well as *Haemophilus influenzae*. Moreover, it was also found that compounds from *Epimedium* and radix *Morindae officinalis* (0.5 mL/Kg) have significant antiviral potential in asthma young children [8]. Anti-bacterial and anti-fungal activities against *E. coli*, *S. aureus*, *B. subtilis*, *Penicillium sp*., *Hansenula sp*. and *Aspergillus sp*, of Icariin, a major 8-isoamyleneflavonol glycoside in the genus *Epimedium* was reported in food products [9]. Therapeutic potentials like antimicrobial, anti-inflammatory, anti-viral and anti-fungal of selected medicinal herb might be new hope for pharmacological industries in drug development. Therefore, this research work was planned to evaluate the antimicrobial including antibacterial and antifungal potential as well as to evaluate the cytotoxic activities of *E. grandiflorum* hydroethanolic extract.

Material and Methods: *E. grandiflorum* leaves and arial parts were used after taxonomically identification and confirmation from Department of Botany, Government College University, Faisalabad-Pakistan to prepare the hydroethanolic (30:70) extract protocol described in Munir *et al*., [10].

Phytochemical Analysis: Qualitative determination of flavonoids, glycosides, tannins, alkaloids, saponins, triterpenoids, and steroids was performed in hydroethanolic extract of selected plant by following the reference methods [11,12].

Anti-bacterial and Anti-fungal potential of *E. grandiflorum*: Antibacterial and antifungal activities using selective microbial strains by well diffusion method were investigated. All of the bacterial strains used for the screening of antimicrobial activities, including *B. subtilis, S. aureus, P. multocida, E. coli, K. pneumoniae, Acinetobacter* species, *Pseudomonas* species, and *Salmonella* species; and fungal strains, including *A. flavus, A. niger, A. terreus, F. solani, A. alternaria,* and *Schizophyllum* species, were obtained from the Department of Microbiology, GC University, Faisalabad-Pakistan and from Department of Biochemistry, University of Agriculture Faisalabad-Pakistan.

Anti-bacterial activity by Well Diffusion method: The nutrient agar (Oxoid) was prepared for the antibacterial potential and after mixing homogenously autoclave was used to sterilize the medium at 121ºC for 15 min. Then on cooling the medium 100 µL/100 mL of homogenous bacterial inoculum was mixed and then poured into sterile culture plates. After solidification of inoculated agar plates sterilized well borer machine was used to make wells by punching. Then streptomycin/ciprofloxacin as positive control and plants extracts (0.001 g/mL DMSO) were added in respectively labelled wells on each petri plate and then placed for 24 h at 37 ºC. Zone reader was used to observe the growth inhibition zone (mm) around the wells as clear zone for each plants extracts and positive controls [13].

Biofilm formation inhibition assay: Di Ciccio *et al*., [14] procedure to determine the biofilm formation inhibition activity of selected extract was used against two gram-positive bacteria and two gram-negative bacteria. Then calculation of the Biofilm inhibition (%) was done following the O'Toole, [15] protocol and then by following formula:

Biofilm reduction % = (OD control − OD sample)/ OD control x 100

Well diffusion method for antifungal activity: Sabouraud's dextrose agar (SDA) media was used for the fungal growth under sterilized conditions. SDA medium was prepared separately for all fungal strains then 100 µL/ 100 mL of homogenous fungal inoculum was dispensed under sterilized conditions. After mixing gently growth medium was poured into sterile petri-plates and borer machine was used to form well in semi-solidified culture media. Then after respectively labelling positive control (terbinafine) and extracts of selected medicinal plants were dispensed into wells. Then all the plates were kept at 28°C for 48 h in temperature-controlled incubator. The inhibition zone measured in mm using Zone reader represent the antifungal activity of extracts [16].

Cytotoxic evaluation: Mutagenicity/ Genotoxicity Assay

(AMES test): Bacterial Reverse Mutation Test (Ames test) was recommended by the Food and Drug Administration, the Organization for Economic Co-operation and Development, and the International Conference on Harmonization as a simple and reasonably inexpensive screening method for drugs due to its potential to prevent genotoxic activity. Selected bacterial strains (auxotrophic) to evaluate the genotoxic behaviour of a substance are commonly used through reverse mutations (base-pair substitutions or frameshift mutations) and *Salmonella typhimurium* (histidine –) or *Escherichia coli* (tryptophan –) are the commonly used bacterial strains. For the current study the selected medicinal plant extract was tested for genotoxicity using TA98 and TA100 bacterial strains in 96 well microplate for incubating up to 5 days and number of revertant were compared with controls [17].

DNA Damage Prevention test: To investigate the geno-protective potential of the aqueous ethanolic extract, a DNA damage protection experiment was carried out using Tian and Hua's [18] technique with a few changes. Calf thymus DNA (Ct DNA) (0.5 μg/3 μl) was used for current study and medicinal plant extract (100 μg/mL) in the presence of DNA damaging ages as Fenton reagent (30% (v/v) was evaluated. Agarose gel electrophoresis was used and then to visualize the DNA ethidium bromide as stain was used. Gel documentation was done using Syngene GeneGenius Gel Light Imaging System.

Statistical Analysis: The obtained data was represented as Mean ± SD and student t test, one way ANOVA and probability test as statistical tests were used to evaluate the results statistically using Minitab 17 statistical software (Trial version).

Results and Discussion: Phytochemical constituents: The hydroethanolic extracts of the chosen medicinal plant contained flavonoids, tannins, alkaloids, saponins, steroids, glycosides, and triterpenoids, according to the findings of several phytoconstituent analyses shown in Figure 1. It was widely known that the alkaloids found in or produced from medicinal plants were responsible for the therapeutic uses, such as analgesic, antibacterial, and antispasmodic [19] and phenolics contents might be responsible for the significant antioxidant potential [20]. The most significant natural phenolic agents include flavonoids, which act as natural antioxidants. Results showed that these natural flavonoids, which also have the potential to prevent the oxidation of low-density lipoproteins, are responsible for the reactive oxygen species detoxifying capacity [20]. Our results are also in agreement of Zulfiqar et al., [21] who investigated that more than thirty chemical compounds like flavonoids, lignans, terpenoids, and phenyl alkanes with or without sugar(s) are present in the *E. grandiflorum* methanolic extract.

Antimicrobial activities of selected hydroethanolic extract: *Antibacterial activity by Gel diffusion*: Antibacterial activity evaluated by gel diffusion method of selected medicinal herb hydroethanolic extract results explored that it possessed significant ($p<0.05$) potential to inhibit the growth of selected pathogenic bacteria. Antibacterial potential against *B. subtilis* and *S. aureus* (Gram-positive bacteria); and *P. multocida, E. coli, Klebsiella pneumoniae, Acinetobacter species, Pseudomonas species,* and *Salmonella species* (Gram-negative bacteria) were investigated, respectively. Antibiotics streptomycin (1 mg/mL) and ciprofloxacin (1 mg/mL) as positive controls were also added. The growth inhibition zones measured in mm were given in the Table 1. It was cleared from the results that significant ($p< 0.05$) clear inhibition zones were produced against tested bacterial strains using *E. grandiflorum*.

Ethnomedicine is becoming an emerging field in the development of therapeutic modalities by identifying the efficient and active phytochemical constituents with minimal or no side effects. Moreover, drugs for the treatment and prevention of infectious disorders might be made from medicinal plants with significant ($p<0.05$) antibacterial activity [22]. It was reported that *Epimedium* species different types of extracts including aqueous, methanolic and ethanolic have significant activities to inhibit the growth of *S. aureus* ATCC 43300 (methicillin-resistant), *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853 and *K. pneumoniae* RSKK 574 [23].

Antibacterial activity by Biofilm formation inhibition assay: One of the main ways that various bacterial strains resist current antibiotics is through the production of biofilms, which also regulate the ability of transmission and raise the possibility for reversibility [23,24], and moreover induce resistance against antimicrobial agents. It was reported that formation of biofilm decreases the penetration of antibiotic, alter microbial microenvironment, and produces persistent cells which modulate the life cycle of bacterial strains. The development of biofilm may lead to the development of different chronic disorders including cystic fibrosis, pneumonia, male, female genital problems, and periodontitis.

The ability of the chosen medicinal herb to suppress biofilm development and prevent adhesion was tested *in vitro*; the findings are shown in Table 2. Percent biofilm inhibition was used to determine the findings in accordance with the technique outlined by Sandasi *et al*., [25]. According to these results between 0 to 100% represent the biofilm inhibition potential of a drug. The results having more than 50% inhibition are considering as good and values between 0 and 49% represents the poor activities to inhibit the biofilm formation. Tested plant has varying potential in the prevention of growth when incubated with selected bacterial strains and all hydroethanolic extracts have good biofilm prevention potential (>50%) against *E. coli*, *S. aureus* and *B. subtilis*. Moreover, results represent that *E. grandiflorum* had inhibition potential significantly ($p<0.05$) more than 50% and considered as good inhibitors of biofilm formation (Table 2).

It was also well reported that different herbal preparations have potential to prevent the bacterial biofilm formation [26-30]. So, with the passage of time multidrug-resistant strains against wide range of effective antibiotics, safe and effectual alternatives medicines are becoming the choice of treatment for different types of infectious diseases [31]. According to literature review biofilm inhibition determination of selected medicinal plant were performed first time against the selected bacterial strains.

Antifungal activity by Gel diffusion method: It was reported that > 90% of systemic mycoses are due to some

specific types of fungi including *Candida spp., Cryptococcus spp., Coccidioides spp.,* and *Aspergillus spp*. Urethritis, ulcers, orchitis, pseudotumor, balanoposthitis, and prostatitis are the male reproductive tract disorders caused due to fungus infections [32,33]. It was considered that *candida spp.* causes the only sexually transmitted fungus infection particularly due to *Candida albicans* and *C. glabrata* [34], which is highly prevalent in females than in males, but males might be acted as reservoir of infection [34,35]. The metabolites of *C. albicans* or its presence in semen alters quality of spermatozoa which can occur in the reproductive tract of male before ejaculation or can this occur in the reproductive tract of female after ejaculation or sperm capacitation [36,37]. It was reported that the metabolites from fungi influenced the activity and viability of spermatozoa, DNA fragmentation of sperms increased, and a change in the membrane of mitochondria was also reported during in vitro studies. Such affects caused the male infertility and increased the agglutination of spermatozoa [36,37]. Moreover, Tian *et al*., [37] investigated the effects of *C. albicans* infection on the activity and damaging effects on the ultrastructure of spermatozoa. So, it becomes very important to explore the natural medicinal plants for antifungal activities as alternative medicines for the management of both male and female reproductive problems.

Well diffusion method was used to determine the antifungal potential of selected medicinal plant and different six fungal strains including *A. flavus, A. niger, A. terreus, F. solani, A. alternaria* and *Schizophyllum species*. The results revealed that *E. grandiflorum* showed significantly ($p<0.05$) high antifungal activity (Table 3). It was reported that *Epimedium spp.* have potential to inhibit the growth of *C. albicans* ATCC 10231 [23].

Mutagenicity evaluation (Ames test): The Ames test (fluctuation method) was used for the investigation of mutagenicity activity of selected medicinal plant [38]. *S. typhimurium* TA98 and TA100 mutant bacterial strains were used for this purpose. The blank plate's purple colour (which had no bacterial strain) demonstrated that the method was genuine and uncontaminated. Figure 2 and Table 4 exhibit the results of the examination of the medicinal plant's mutagenicity against TA98 and TA100, respectively. The Ames test findings revealed that *E. grandiflorum* is naturally non-mutagenic, meaning that it did not display mutagenic activity. According to the Fold Rule, there must be twice as many positive wells as positive wells on the background plate for positive findings (mutagenic behavior of substance) [39]. For statistical analysis, probability was calculated [17]. The results revealed that *E. grandiflorum* plate showed minimum reversion both of *S. typhi* TA98 and TA100 (Figure 2 and Table 4).

Medicinal plants and their preparations are being consumed for the treatment of wide range of diseases as well as nutraceuticals from ancient time with wide range of applications with not well reported side effects [40]. This was the first time that selected medicinal herbs were evaluated for mutagenic potential, according to the literature study.

DNA Damage Prevention test: To investigate potential risks and because only pharmaceutical chemicals are tested for mutagenic activity, it is becoming more vital to evaluate herbs for genotoxicity tests. The mutagenic molecules might be present in some medicinal plants which could be the risk of carcinogenic hazards for their consumers as medication or foods supplement for long-term usages. The genetic toxicity of medicinal plants must be declared, and they must be labelled as dangerous. So, it becomes vital to identify the genotoxic herbs and describe that such medicinal plants should be used with caution [41]. To further establish the chemo-preventive nature of medicinal plant and their therapeutic potential for usage in clinical settings, it is crucial to evaluate the antimutagenic actions of their medicinal qualities. Antimutagens compounds work to stop mutagenicity by neutralizing the mutagen or stopping the interaction between the mutagen and DNA [42].

A Fenton reaction-based *in vitro* study was conducted to assess the medicinal plant' ability to prevent DNA damage. DNA damage preventive potential of *E. grandiflorum* was found when compared to the controls. Figure 3 shows the electrophoresis pattern of calf thymus (Ct) DNA after treatment with Fenton reagent and extract of *E. grandiflorum* for the investigation of DNA-damage prevention capacity against oxidative DNA damage. The agarose gel was used to run the electrophoresis and the patterns of DNA represented the relative band intensity of Ct DNA (Figure 3). The electrophoresis pattern showed that *E. grandiflorum* had a protective effect against DNA damage. It was demonstrated that medicinal plant extract plays an important role in the protection of DNA damage by reversing the effects of oxidative stress induced by a mutagen and preventing mutagenicity in mice [19]. Potential of a particular medicinal plant to protect against DNA damage Our research on *E. grandiflorum* is being done for the first time, to the best of our knowledge, based on earlier literature reviews.

Conclusion: Medicinal plants are used as fundamental and low-cost source for remedy of numbers of infectious and metabolic diseases in under developing and developing counties. The phytochemicals results explored the presence of different biologically active constituents in *E. grandiflorum* hydroethanolic extract. It was found that selected natural medicinal herb have significant ($p<0.05$) antibacterial and anti-fungal activities tested against selected bacterial and fungal strains, respectively. Furthermore, the results of mutagenicity test and DNA damage preventive test explored that selective medicinal plant has significant ($p<0.05$) DNA protective capacity or in other words it is non-mutagenic or cytotoxic in nature. I could be concluded that *E. grandiflorum* could be a potential candidate as therapeutic agent to manage infectious diseases especial bacterial and fungal infections with non-toxic nature.

Acknowledgement: The Pakistan Science Foundation (PSF) funded the study through PSF research project # PSF/NSLP/P-GCUF(710), which the author(s) sincerely appreciate.

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Table. 1. Examined medicinal plant extract's antibacterial effectiveness against a number of different bacterial strains.

Bacterial Strains	Anti-bacterial potential Zones of Inhibition (mm)		
	E. grandiflorum	**Streptomycin**	**Ciprofloxacin**
S. aureus	21 ± 2.40^{B}	36 ± 2.70^{A}	
B. subtilis	15 ± 1.90^{B}	35 ± 2.10^{A}	
E. coli	29 ± 1.50^{B}		34 ± 2.00^{A}
P. multocida	22 ± 1.80^{B}		32 ± 1.90^{A}
Acinetobacter species	27 ± 1.93^{B}		37 ± 2.10^{A}
Pseudomonas Species	19 ± 1.02^{B}		33 ± 1.54^{A}
Salmonella Species	14 ± 0.92^{B}		35 ± 1.71^{A}
K. pneumoniae	12 ± 0.82^{B}		28 ± 1.39^{A}

The results are Mean ± SD (standard deviation) of the multiple determinations. Different alphabets at within same rows represent significantly different ($p<0.05$).

Table. 2. Biofilm inhibition potential of the *E. grandiflorum* hydroethanolic extract against selected bacterial strains.

Plant Extract/ Bacterial Strains		Biofilm formation inhibition (%)			
		S. aureus	***B. subtilis***	***E. coli***	***P. multocida***
E. grandiflorum	10 mg/mL	49.09 ± 0.68^{c}	**79.66 ±1.40^{c}**	**66.74 ± 1.22^{c}**	32.76 ± 1.40^{c}
	20 mg/mL	**72.40 ± 1.24^{b}**	**85.31 ± 1.31^{b}**	**77.05 ± 1.27^{b}**	36.77 ± 1.33^{c}
Streptomycin		85.69 ± 1.44^{a}	94.32 ±1.34a	80.30 ± 1.67a	51.82 ± 2.00^{b}
Ciprofloxacin		77.64 ± 0.99b	91.90 ± 2.46^{a}	76.00 ± 1.22b	59.35 ± 1.24^{a}

The results as Mean ± SD (standard deviation) of multiple investigation. Different alphabets at within same column represent significantly different ($p<0.05$). low activity = numbers > 0% ≥ 50% show and show high activity= > 50% (in bold).

Table. 3. Anti-fungal potential of the *E. grandiflorum* hydroethanolic extract against selected fungal strains.

Bacterial Strains	Antifungal activity Zones of Inhibition (mm)	
	E. grandiflorum	**Terbenafin**
Schizophyllum species	8 ± 0.60	22 ± 1.91
F. solani	6 ± 0.33b	11 ± 1.1a
A. Alternaria	11 ± 0.51b	26 ± 2.2a
A. flavus	12 ± 0.38b	29 ± 1.3a
A. niger	9 ± 0.61b	28 ± 2.0a
A. terreus	14 ± 1.26b	31 ± 1.99a

The results as Mean ± SD (standard deviation) of multiple investigation. Different alphabets at within same rows represent significantly different ($p<0.05$).

Table. 4. *E. grandiflorum* hydroethanolic extract' mutagenicity evaluation by Ames test using S. typhi TA 98 and TA 100.

Extract and Bacteria	Positive wells/Total wells	Results
Mutagenic activity against TA98		
a) Background	20/96	-
b) Standard (K2Cr2O7)	80/96	+
c) E. grandiflorum	4/96	-
Mutagenic activity against TA100		
a) Background	25/96	-
b) Standard (NaN3)	86/96	+
c) E. grandiflorum	3/96	-

+, Significant increase in the number of positive wells compared to the related control ($p<0.05$). -, Non-significant ($p>0.05$) effect observed.

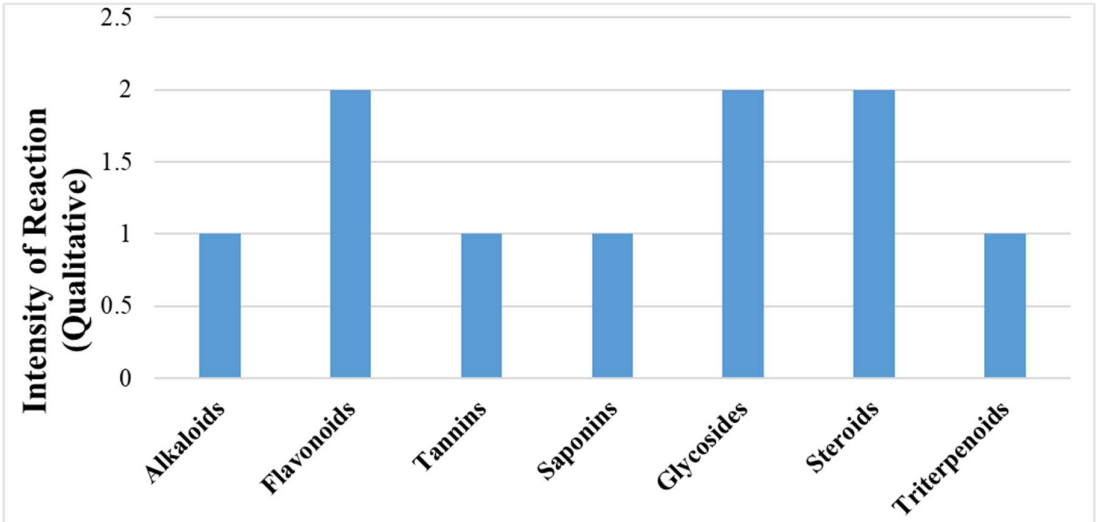

Fig. 1. Graphical representation of qualitative phytochemical constituents present in hydroethanolic extract of *E. grandiflorum*. (+= 1) presence of phytoconstituent, (++= 2) Moderate level of phytoconstituent, (+++= 3) High level of phytoconstituent, (- = 0) indicates non detection of phytoconstituent present

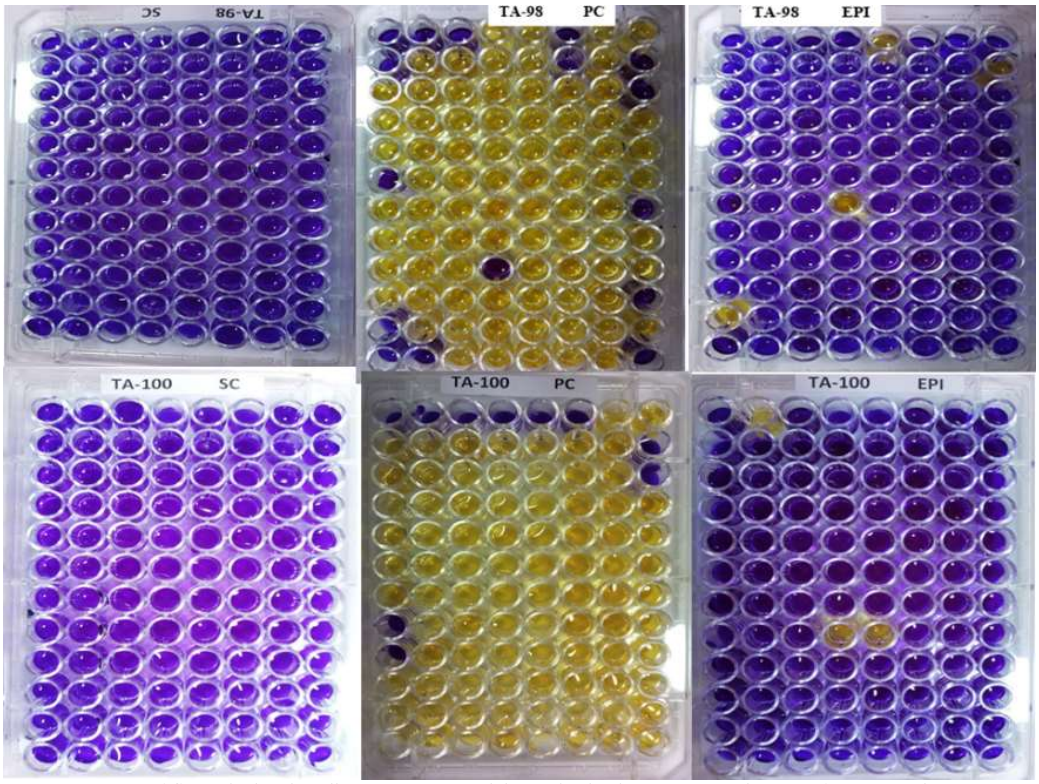

Fig. 2.*E. grandiflorum* hydroethanolic extract' mutagenicity evaluation by Ames test using *S. typhi* TA 98 and TA 100.
SC= sterility control, PC= positive control, EPI= *E. grandiflorum*; blue colour indicates no bacterial revertant (no bacterial growth) and yellow colour indicates bacterial revertant (bacterial growth).

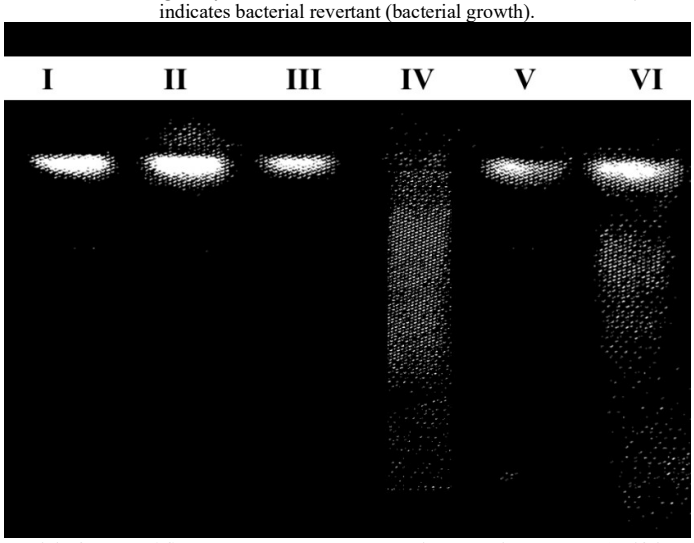

Fig. 3. Potential of *E. grandiflorum extract* to prevent DNA damage using Ct DNA (Calf thymus DNA)
Where; Lane I = Untreated DNA, Lane II= 2mM $FeSO_4$, 30% H_2O_2 + DNA + 1mM Quercetin, Lane III = 30% H_2O_2 + DNA, Lane IV = 2mM $FeSO_4$, 30% H_2O_2 + DNA, Lane V = 2mM $FeSO_4$ + DNA, Lane VI =2mM $FeSO_4$, 30% H_2O_2 + DNA + *E. grandiflorum*.