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Evaluate The Genotoxicity of Two Different Doses of Methanolic Extract of Vitex Negundo (Leaf) On Bone Marrow, Spleen and Peripheral Blood Cells in Mice

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Abstract

Vitex negundo belongs to family Verbenaceae. All parts of the plant contain numbers of secondary metabolites such as alkaloids, phenols, flavonoids, glycosidic irridoids, tannins and terpenes. Vitex negundo possess a number of therapeutic uses; antimicrobial, anti-inflammatory, CNS-depressant, diuretic, anticancer and hepatoprotective etc. In the present study, the proliferation of bone marrow and spleen cells of mice was evaluated after extraction of vitex negundo with methanol. Two doses of the extract (100mg/kg and 200/kg body weight) were given to the mice for seven successive days. On day eight, mice were sacrificed and Mitotic index, structural chromosomal aberration, micronucleus appearance index and bloods cells have been measured to evaluate fraction effects. the results were compared with that of methotrexate at a dose 20mg/kg (positive control) and dimethylsulphoxide (DMSO) (negative control). The results showed that the dose 100 and 200 mg/kg body weight of vitex negundo extract caused significant increase in mitotic index in both bone marrow and spleen cells of mice when compared with negative control.

Keywords

Vitex Negundo, Bone Marrow Cells, Spleen Cells, Chromosomal Aberration, Methotrexate, Mitotic Index, Micronucleus Index.

Medicinal plants have been used worldwide as an alternative and/or acomplementary medicines. The use of herbal medicine has vastly increased due to their efficacy, availability and safety claims. Medicinal plants are rich in a variety of important phytochemicals such as alkaloids, flavonoids, terpenoids, saponins and many others. These compounds possess antioxidant properties that are important for pharmaceuticals and drug development, as well as direct use as therapeutic agents.[1]

Vitex negundo L. (Verbenaceae) commonly known as five leave chase tree, and nirgundi in India. The genus Vitex has around 270 known species, ranging from shrubs to trees in the tropical, sub-tropical regions and temperate zones . The Vitex is used as a folk medicine in Bangladesh, India, China, Indo-China, Indonesia, Nepal, Pakistan, Philippines, and Sri Lanka [2]

V. negundo is a component of various commercially available herbal formulations and had shown beneficial effects in various ailments [3,4]. V. negundo is reported to possess various pharmacological activities, including antioxidant [5,7], analgesic [6], anti-inflammatory [8], anti-convulsant and central nervous system (CNS) depressant activity [9]. V. negundo is reported to contain terpenoids [10], flavonoids [110.11], iridoids [12], and lignans [13,14]

Since chromosomes are a key component of the genetic code , any damage to a healthy cell's chromosome could have disastrous results, such as the

development of cancer or a variety of heritable diseases, if the damage is not repaired . abnormalities involving the structure or number of chromosomes can be found within populations During normal course of life cycle, structural rearrangements in chromosomes do occur but less frequently but, under the effect of mutagenic agents such as radiations and conditions of stress like very high temperature, chromosomes are more prone to these changes[15]

Mitotic index is a measure for the proliferation status of a cell population, and defined as the ratio between the number of cells in mitosis and the total number of cells. The mitotic index can be worked out from a slide, even with light microscopy[10]. There is a direct relationship between cancer and value of mitotic index; cancer cells have high mitotic index and they grow uncontrollably and divide fast[16]

A micronucleus assay is an assay used in toxicological screening for potential genotoxic

compounds. The assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens . There are two major versions of this test, one in vivo and the other in vitro . The in vivo test normally uses mouse bone marrow or mouse peripheral

blood. When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise nucleated cytoplasm .[17]

this study designed to evaluate the genotoxicy of methanol fraction of vitex negundo extract administered orally, in two different doses [100mg/kg and 200mg/kg] on both bone marrow and spleen cells in mice for seven successive days, and comparing their effects with methotrexate (positive control) and dimethylsulfoxide [DMSO](negative control as well as measurement of the malonaldehyde and superoxide dismutase enzyme in each group .

Materials And Methods

Plant material

The plant had been collected from Baghdad in April, from Alzawra public garden , washed thoroughly, chapped into bits, and allowed to dry under shade. The dried plant was blended into fine powder using electric blender.

Preparation of extract

Five hundred grams of the powdered plant was defatted by maceration in 1500 ml of hexane for 24 hours with occasional agitation then filtered. The defatted plant materials were dried introduced in a thimble and extracted using soxhlet apparatus using 1500ml of methanol (B.p.63 °C) for 15 hours then cooled, filtered and evaporated under reduced pressure at 40 °C using rotary evaporator [18].

The yield values for methanol fraction have been obtained.

Experimental model

Twenty four Albino Swiss mice (Mus musculs) were used for each experiment. They were supplied by collage of pharmacy –university of baghdad. Their weights were 20-25 gram. They were divided into four groups; each was kept in a separate plastic cage. The animals were maintained at a temperature of 23 – 25°C, and they had free excess to food (standard pellets) and water.

The animal divided into 4 groups as below

Group1: six mice were treated with dimethylsulfoxide (DMSO). This group was served as negative control the dose was given (I.P.) daily for seven successive days.

Group2: six mice were treated with a single dose (20mg/kg) of methotrexate (MTX). This group was served as positive control.

Group3: six mice were treated (oral) with (100mg/kg) of methanol extract of vitex negundo for seven successive days.

Group4: six mice were treated (oral) with (200mg/kg) of methanol extract of vitex negundo for seven successive days.

Mice were sacrificed by (spinal dislocation). Samples of bone marrow cells and spleen cells were taken and genotoxic analyses were carried out as described latter.

Phytochemical Investigation

Preliminary phytochemical investigation was carried out for methanol fraction using, 5%

KOH Test/flavonoids ,1 % Lead actate test/tannins , Dragendorff Test/alkaloids ,Vaniline/H2SO4 Test/steroidal, Ferric chloride Test/phenolic compound Benedict test/ glycoside

Chemical Test	Methanol Fraction
5% KOH Test/flavonoids	Positive+
1 % Lead actate test/tannins	Positive++
Dragendorff Test/alkaloids	Positive+
Vaniline/H2SO4 Test/steroidal	Positive+
Ferric chloride Test/phenolic compound	Positive++
Benedict test glycoside	Positive +

Evaluation of mitotic index in Bone marrow cells and spleen cells

After seven days of treatment, all animals were

injected intraperitoneally with 1mg/kg colchicines, and then two hours later they are scarified by cervical dislocation. Bone marrow samples was aspirated from the femur bone and spleen cells have been collected processed using aseptic technique for evaluation of mitotic index as previously reported elsewhere[19].

Evaluation of micronucleus assay in Bone marrow cells

After chemical treatment, mice were killed and femoral marrow cells were smeared on clean glass slides, fixed with methanol for 5 min at room temperature, and stained with Giemsa [20]

Measurement of the malonaldehyde

Malondialdehyde (MDA) concentration measured by the ELISA reader by using the commercial assay kits according to manufacturer's protocols (Sunlong Biotech Co., LTD) . This ELISA kit uses the Sandwich-ELISA as a method. The Micro Elisa strip plate that provided in this kit was precoated with an antibody specific to the MDA. Standards or samples added to appropriate Micro Elisa strip plate of the wells and then combined to specific antibody. Then a Horseradish Peroxidase (HRP) conjugated antibodies specific for the MDA added to each of the Micro Elisa strip plate well and then incubated. Free components washed away. The Tetramethylbenzidine (TMB) the substrate solution added to each well. Only those wells contain the MDA and HRP conjugated the MDA antibodies will appear blue in the color and then turn to the yellow after addition of stop solution. The optical density (OD) spectrophotometrically measured at the wavelength of 450 nm. The OD value proportional to concentration of the MDA, calculated in samples by comparing OD of samples to standard curve.[44]

Measurement Of Superoxide dismutase Enzyme

THE concentration measured by the ELISA reader by using the commercial assay kits according to manufacturer's protocols(Sunlong Biotech Co., LTD) [45]

Statistical Analysis

Data are expressed as Mean ± SD; unless otherwise indicated, statistical analyses were performed using unpaired t-test. If the overall F value was found statistically significant ($P<0.05$), further comparisons among groups were made according to post hoc Tukey's test. All statistical analyses were performed using SPSS GraphPad InStat 3 (GraphPad Software Inc., La Jolla, CA, USA) software.

Results And Discussion

Phytochemical Investigations

Phytochemical investigations revealed the presence flavonoids ,tannins , alkaloids ,steroidal, phenolic compound and glycoside compounds. The yield value was (76 gram) of methanol extract

1- Mitotic index and micronuclei index

A- Mitotic index and micronucleus appearance of different concentrations of methanol fraction of vitex negundo

In table 1 shows that, methanol extract of vitex negundo at both doses 100mg/kg and 200mg/kg caused increase of mitotic index in both bone marrow cells and spleen cells when compared to negative control (DMSO) ($P<0.05$), which is a parameter that give indication about cell division when compared to negative [DMSO], as well as , these two doses show increase in mitotic index in both bone marrow cells and spleen cells when compared to positive control ($P<0.05$). Methotrexate caused significant decrease ($P<0.05$) of mitotic index compare to negative control and extract in bone marrow and spleen cell.

Table 1: Incidence of mitotic index in bone marrow and spleen cells of albino mice treated with different doses of the methanol extract of vitex negundo compared to methotrexate and dimethylsulfoxide

Treatment Groups	Mitotic Index	
	Bone Marrow Cells	Spleen Cells
Dimethylsulfoxide (DMSO) (Negative control)	5.884±0.578	4.354±0.488
Methotrexate (MTX) (positive control) 20mg/kg	2.6±0.36*a	1.897±0.36*a
Methanol extarct 100mg/kg	6.575± 0.344*Ab	5.163±0.341*Ab
Methanol extract 200mg/kg	6.953±0.397*Ab	5.35±0.385*Ab

B- In Table 2, methanol fraction of vitex negundo at a dose 100mg/kg and 200mg/kg caused non - significant reduction of micronucleus appearance in comparison to DMSO (negative control) ($p>0.05$), meanwhile methotrexate at a dose (20mg/kg) caused a significant elevation of micronucleus appearance in comparison with DMSO ($p<0.05$). also both doses of methanol

fraction of vitex negundo showed to be significantly lower in micronucleus appearance in comparison to methotrexate ($p<0.05$).

Table 2: Incidence of micronucleus appearance in bone marrow cells of albino mice treated with different doses of the methanol extract of vitex negundo compared to methotrexate and dimethylsulfoxide figure 1

Micronucleous appearance	
Treatment group	Bone Marrow Cells
Dimethylsulfoxide (DMSO) (Negative control)	6.454±0.437
Methotrexate (MTX) (positive control) 20mg/kg	28.75±3.37*a
Methanol extract 100mg/kg	5.3132± 0.539Ab
Methanol extract 200mg/kg	4.998±0.66Ab

For table 1 and table 2 Data are expressed as mean±S.D; n=6 animals in each group; -*significantly different compared to DMSO (negative control) ($P<0.05$); -Values with non-identical small letters superscripts (a,b) consider significant different when compared between groups ($P<0.05$); - Values with non-identical capital letters superscripts (A,B) consider significant different when compared among tests doses ($P<0.05$).

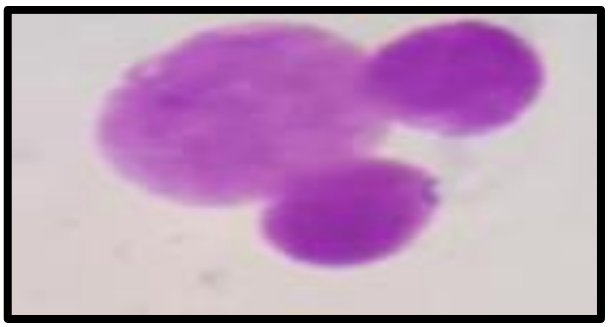

Figure 1: Micronucleus appeared after treatment with methanol extract of vitex negundo

In Tables 1 and 2 indicate that, methotrexate effects on mitotic index cells was significantly lower when compared to methanol extract in both doses 100mg/kg and 200 mg/kg. Meanwhile they showed significant increase in micronucleus appearance in both doses as compare to extract.

Chromosomal aberration

In bone marrow

Table 3 chromosomal aberration in bone marrow cell

Parameters / BM / Groups	Deletion % (mean±SD)	Dicentric % (mean±SD)	Acentric % (mean±SD)	Ring % (mean±SD)	Chromosome break (mean±SD)	Chromatid break(mean±SD)	Chromosome Gap (mean±SD)	Chromated Gap (mean±SD)	Total % (mean±SD)
Control	AB 0.2335±0.1415	AB 0.2553±0.1201	AB 0.2489±0.0982	BC 0.2594±0.1289	AB 0.1462±0.1174	AB 0.1423±0.0921	B 0.1721±0.1263	A 0.1721±0.1263	B 1.5672±0.2117
MTX	A 0.3127±0.1309	A 0.3279±0.0997	A 0.3442±0.1421	A 0.4551±0.1312	A 0.2721±0.1172	A 0.2751±0.1252	A 0.3397±0.1465	A 0.1856±0.1411	A 2.387±0.229
Methanol 100	B 0.1603±0.1186	AB 0.2256±0.0914	C 0.0958±0.0876	BC 0.2258±0.0923	AB 0.1943±0.0801	AB 0.1598±0.1091	B 0.1894±0.1305	A 0.1894±0.1305	B 1.381±0.228
Methanol 200	B 0.1316±0.0737	B 0.1305±0.1373	BC 0.1638±0.1138	C 0.1638±0.1138	AB 0.1996±0.0789	AB 0.1657±0.1205	B 0.1644±0.1163	A 0.1644±0.1163	BC 1.218±0.428

From table 3 we found the methotrexate significant increase in the total chromosomal aberration in compare to the control group and to treatment group.

The dose 100 and 200 mg /kg decrease the total chromosomal aberration non significantly in comparing to the control group and the value of 200 mg /kg is less that the dose 100 mg/kg.

in spleen

Table -4 chromosomal aberration in spleen cell

Parameters / Spleen / Groups	Deletion % (mean±SD)	Dicentric % (mean±SD)	Acentric % (mean±SD)	Ring % (mean±SD)	Chromosome break (mean±SD)	Chromatid break(mean±SD)	Chromosome Gap (mean±SD)	Chromated Gap (mean±SD)	Total % (mean±SD)
Control	AB 0.1439±0.1049	AB 0.199±0.0726	AB 0.195±0.1279	B 0.2284±0.0759	AB 0.1731±0.069	B 0.0814±0.0746	ABC 0.2322±0.0897	AB 0.2322±0.0897	B 1.367±0.323
MTX	A 0.2715±0.1063	A 0.3352±0.1176	A 0.3057±0.1013	A 0.4289±0.1154	A 0.3128±0.1131	A 0.2768±0.126	A 0.3371±0.1141	A 0.3371±0.1141	A 2.545±0.242
Methanol 100	B 0.1284±0.1333	AB 0.1956±0.1359	B 0.1594±0.1124	B 0.195±0.1369	B 0.1279±0.0717	B 0.0987±0.1495	BC 0.1926±0.1368	B 0.1926±0.1368	B 1.257±0.46
Methanol 200	B 0.1019±0.093	B 0.1005±0.0918	AB 0.2016±0.0798	B 0.1986±0.137	AB 0.2006±0.1423	AB 0.131±0.1362	C 0.0999±0.0913	C 0.0999±0.0913	C 1.136±0.321

From table 4 we found that the methotrexate significant increase in the total chromosomal aberration in compare to the control group and to treatment group.

The dose 100 and 200 mg /kg decrease the total chromosomal aberration significantly in comparing to the methotrexate group and the dose 200 mg/kg significantly decrease the total chromosomal aberration in comparing to the methotreaxate and control group

Hematological Effect of vitex negundo

Table 5 Hematological Effect of vitex negundo

Parameters / Groups	Total WBC $\times 10^3$ (mean±SD)	RBC$\times 10^6$/mm^3 (mean±SD)	Hb gm/dl (mean±SD)	PCV % (mean±SD)
Control	B 11.13±0.821	C 7.568±0.567	B 14.814±1.046	B 41.52±4.28
MTX	C 8.48±0.783	D 7.008±0.2213	B 14.698±1.265	C 32.32±2.58
Methanol 100	A 11.85±0.1696	ABC 7.918±0.283	AB 15.882±0.821	B 42.15±2.36
Methanol 200	AB 11.59±0.571	A 8.244±0.1764	A 16.628±1.353	B 43.31±2.44

From table 5 we found methotrexate significantly decrease all blood parameters except hemoglobin non-significant decrease in comparing to the control group . also, methanol 100 mg increase the total wbc significalntly, RBC,HB,PCV increased non significantly in comparing to the control group ,the dose 200 mg/kg methanol group increase RBC,HB ,significantly in comparing to the control group

measurement of malondialdehyde (MDA) and super oxide dismutase enzyme (SOD)

Parameters / Groups	SOD ng/ml (mean±SD)	MDA pg/ml (mean±SD)
Control	B 72.31±4.31	B 402.3±38.8
MTX	C 31.64±3.36	A 840.6±58.6
Methanol 100	AB 75.64±5.03	BC 383.14±15.3
Methanol 200	AB 79.47±8.28	C 356.57±14.69

Table (6) the effect of methanolic extract of vitex negundo on MDA and SOD

From table 6 we find that methotrexate group significantly decrease the sod comparing to the control group(DMSO) group and increase the MDA significantly comparing to the control group also we find methanol group (100,200 mg/kg) increase the level of SOD significantly comparing to the methotrexate group also decrease the level of MDA IN group (100 and 200 mg/kg) methanol extract significantly comparing to the methotrexate group

Discussion

Medicinal plants are the principal sources of polyphenols and phytosterols. Briefly, plant polyphenols are the natural compounds which decrease the catalytic action of enzymes which are responsible for reactive oxygen species (ROS) generation, reduce the production of nitric oxide synthases (NOS), modulate other metabolizing enzymes including cyclooxygenase (COX), lipoxygenase, downregulate the transcription factor NF-κB, and expression of some inflammatory cytokines such as IL-1β, TNF-α, and IL-10 involved in inflammatory response[21,43]

The main antioxidant compounds in plants are phenols, which have an aromatic ring that enables the unpaired electrons in their arrangement to be stabilized and relocated, allowing electrons and hydrogen atoms to be donated from their hydroxyl groups[22]

Regarding to the results obtained from the chemical investigation methanolic fraction of vitex negundo contain poly phenolic compounds (simple phenol flavonoids), tannins and steroids.

The basic monomer in polyphenols is phenolic ring and generally these are classified as phenolic acids and phenolic alcohols. Depending upon the strength of phenolic ring, polyphenols can be classified in many classes, but the main classes in the polyphenols are phenolic acids, flavonoids, stiblins, phenolic alcohols, and lignans. [23].

the methanolic extract of vitex negundo contain many type of phenolic compound as simple polyphenols as benzoic acid aspartic acid,gallic acid,dihydrobenzoic acid vannillic acid ,ferulic acid ,p-hydroxy benzoic acid ,cinnamic acid and chlorogenic acid .or flavonoids as flavanones naringnenin,quercetin,apigenin,kaempferol,luteolin ,catechin ,rutin ,iso flavones and genistein Phenolic contents of methanolic showed significant correlation with free radical scavenging, Fe+3-reduction and cyto-protective potential against H2O2-induced toxicity [24,25]. also the methanolic extract has stronge inhibitory effect on two type of proinflammatory cytokins(IL-1B,IL-6) and

enhance the release of anti-inflammatory cytokinsas IL-10.[25].

The protective effect observed in many studies could be due, in part, to the presence of phenolic and/or non-phenolic constituents that have ability to act as antioxidant by free radical scavenging and chelating metal ions [26]. They can also act as indirect antioxidant by increasing levels of antioxidants such as glutathione (GSH) and/or by increasing the activity of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) [27,28.29]

One of the most important role of phenolic acids is their antioxidant activity, which depends primarily on the number of hydroxyl and methoxy groups attached to the phenyl ring . Ferulic acid is considered to be a superior antioxidant. The antioxidant action mechanism of ferulic acid is complex can be sumerized as below[30]

1- inhibition of the formation of reactive oxygen species (ROS) or nitrogen,and neuterilzed it .
2- chelating protonated metal ions, such as Cu(II) or Fe(II)
3-inhibitor of enzymes that catalyze free radical generation and an enhancer of scavenger enzyme activity. It is directly related to its chemical structure
4-have the ability to form stable phenoxyl radicals, by the reaction of the radical molecule with the molecule of antioxidant. This makes it difficult to initiate a complex reaction cascade leading to the generation of free radicals.
5-This compound may also act as hydrogen donor, giving atoms directly to the radicals.
So it protect cell membrane ,lipid acids from undesired autoxidation processes.[27]

Also ferfulic acid prevents UV-induced cell cycle alterations and DNA damage and regulates the expression of DNA repair genes.[31]

Flavonoids as luteolin , catechin ,rutin and epicatechin showed antigenotoxic effects against oxidative DNA damage in several cell models[32] .Rosmarinic acid (RA), reduced the frequency of micronuclei and the extent of DNA damage induced by doxorubicin. Human blood lymphocytes irradiated with UVB (280-320) after pretreatment with caffeic acid exhibited lower levels of lipid peroxidation markers such as thiobarbituric acid reactive substance (TBARS) and lipid hydroperoxide (LPH) and also a decrease of UVB induced[33]

also the mehanolic fraction of vitex negundo contain orirntin which is flavonoid they found that orientin led to a significant decrease in the percentages of cells in the sub-G phase, which represent the population of apoptotic cells. This finding signifies that pre-treatment with orientin may directly or indirectly reduce the incidence of apoptosis caused by exposure to H2O2, indicating neuroprotective properties.[34]

luteolin and luteolin-7-glucoside increased rejoining of strand breaks after treatment with H2O2[35] . Quercetin also increased rejoining of strand breaks induced by t-BHP in HepG2[36,37]

also the methanolic extract of vitex negundo contain negunodoside(NG)which is irridoid glycoside compound have hepatoprotective effect where NG strongly inhibited lipid peroxidation promoted by H2O2+Fe in microsomes and CCl4 in HuH-7 cells . It has been reported that glycosides, such as NG, are potent cytoprotective agents against oxidative stress induced cytotoxicity ,the effectiveness of NG in protecting against the CCl4-induced LPO in HuH-7 cells may involve its capability to prevent lipid peroxidation chain reactions as a consequence of scavenging free radicals or chelating iron[38] .

NG restored the calcium and cAMP to normal levels, inhibited lipid peroxidation, activated cPLA2 levels were inhibited and cytotoxicity was reversed without altering CYP2E1 levels. Also maintainance of glutathione by. NG also acts as a very potent membrane stabilizer, it is suggested that NG, may be acting as amphipathic substance, localizing near the membrane surface, trapping any free radical The main mechanism involved in the cytoprotection of NG seems to be its ability to protect the mitochondria against depletion in its membrane potential, an event that is very critical in the loss of cell viability[38] .

In another in vitro study with adipose derived MSCs, the antioxidant supplements not only synergistically decreased oxidative stress, but also increased proliferation and cell number in the S phase of the cell cycle . The suggested mechanisms behind these effects involved downregulation of CDKs inhibitors and upregulation of CDK2, CDK4, and CDC2 expression.[39]

also the methanolic extract contain the phytoeterol (b-sitosterol −stigmasterol) these compound have antioxidant activity and can prevent the mutagenesis by scavenging free radical and modulation of varies antioxidant enzyme with hydrogen peroxide production as well as nitric oxide.betasitosterol BS, in doses from 200 to 1,000 mg/kg, was able to significantly reduce the frequency of sister chromatid exchanges induced by 10 mg/kg of doxorubicin (DX) in bone marrow cells. The same range of BS doses also gave rise to a strong reduction in the rate of micronucleated, polychromatic erythrocytes induced by DX[39,40] .In a previous study they found that in doses up to 1,000 mg/kg, BS was unable to induce micronucleated polychromatic erythrocytes (MNPE) and sister chromatid exchanges (SCE) in mouse .[41]

Antioxidant has effect on the Hematopoietic stem cells as increase cellular proliferation ,genomic stability, SOD cellular concentration ,genoprotective ,increase the survival of hematopiotic stem cell from bone marrow .antioxidant decrease in the percentage of aberrant cells and the number of chromatid as well as chromosome breaks [42]

Conclusion

The methanolic fraction of vitex negundo have antioxidant effect and has no genotoxicity in bone marrow and spleen cells in mice

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