The phytochemical screening showed that the hydroalcoholic extracts of *M. spinosa* and *O. indicum* contained tannins, reducing sugars, sterols and alkaloids. The pharmacological properties are based on the presence of these biologically active phytochemicals such as triterpenes, flavonoids, alkaloids, steroids, tannins and glycosides in various plant extracts (Agarwal and Rangari, 2003; Liu et al., 1996; Mbagwu et al., 2007; Narendrahakannan et al., 2007; Singh et al., 2002). Alkaloids are low molecular weight, nitrogen-containing substances. They are major class of plant secondary metabolites used both in modern and traditional medicine. For instance, vincristine and taxol are widely used as anticancer drugs and morphine (in some countries) is an indispensable analgesic in clinical medicine (Otani et al., 2005). Alkaloidal compounds isolated from different parts of several medicinal plants have been reported to be responsible for some pharmacological properties (Duwiejua et al., 2002; Whitehouse et al., 1994). Sterols, specifically phytosterols helps to reduce cholesterol in human body by blocking cholesterol absorption sites in the human intestine (Ostlund et al., 2003). Reducing sugars, with aldehyde or ketone group, in solution is able to act as a reducing agent. Presence or absence of specific phytochemicals in a plant can explain the benefits or dangers the plant contributes when ingested. The presence of sterols, reducing sugars, tannins and alkaloids may confer several pharmacological activities on the stem bark of the plant which may explain the effect of the hydroalcoholic extracts observed in traditional medicine.

In animals, the effects of DON vary on the basis of severity, exposure duration and species involved (Rotter and Prelusky, 1996). Histopathological findings from 14 days acute oral toxicity studies in mice suggested that DON severely affects liver and kidneys. It had already been established that acute exposure of mice to lethal DON dose causes histopathological effects ranging from haemorrhage/necrosis of the intestinal tract, necrosis in bone marrow and lymphoid tissues, to kidney and cardiac lesions (Pestka et al., 1994; Yoshizawa and Morooka, 1977). Cardiac lesions were also observed in balb/c mice fed with 10-20 mg DON/kg diet for a period of few weeks (Robbana-Barnat et al., 1988). But here the major interesting finding is hepatotoxicity by DON consumption that can induce phase I and phase II liver biotransformation enzymes (Gouze et al., 2005). However, DON is conjugated to glucuronides in liver and
resultant metabolites found in animal tissue and excreta (Gareis et al., 1987). DON was found mostly as, glucuronide conjugate in plasma within 20 min of feeding (Eriksen et al., 2003). Although the mechanism of action of DON is largely studied in kidney, it is also well documented that the elimination of this toxin is also though liver, either as metabolites or as it is (Gauvreau, 1991). DON was primarily excreted in urine in free and conjugated forms with smaller amounts in faeces as de-epoxidated and free form. Danicke et al. (2004) similarly found in pigs that DON is eliminated as metabolites in urine when administered orally. Thus, DON exposure was detectable after 14 days of treatment period in kidney. DON effects were significantly observed in tested organs (liver and kidney). Taken together, acute exposure of DON dose caused marked tissue injury in experimental animals.

Cytotoxicity is an important parameter for assessing chemical agents for toxicity and health risks. In genotoxicity testing, cytotoxicity analysis is a prior step because the cytotoxic effect of chemicals may lead to false interpretation of genotoxicity (Goyary et al., 2014). At the cellular level, protein synthesis is inhibited at the ribosome during the elongation-termination step. DON can enhance transformation of BALB/3T3 mouse embryo cells in vitro (Sheu et al., 1988). In the present study, it was observed that hepatocytes in the control and vehicle treated groups showed no significant cytotoxic effect (>90% viable cells) after 12 h incubation. However, DON treated groups showed a potent cytotoxic effect (<70%; P<0.05) in hepatocytes. It was also interesting to note that DON possesses cytotoxicity effect on hepatocytes. Flow cytometric analysis results of apoptosis when correlated with the morphological studies, it showed that the frequency of early apoptotic cells reached a peak level after 12 h of treatment. While, it takes time by the late apoptotic cells to gradually increase its frequency and reach at a maximum level. The level of correlation was observed when compared the comet assay results (the significantly increased level of DNA single strand breaks) with flow cytometric data (the significantly increased frequency of late apoptotic cells). Moreover, it was also seen that the frequency of late apoptotic cells gradually increased and reached a maximum level after a certain period of M. spinosa treatment at highest dose (1600 mg/kg body weight). Further comparison of the comet assay results (the significantly increased level of DNA single strand breaks) with flow cytometric data (the significantly increased frequency of late apoptotic cells) similar correlation was
observed. Hence, it may be expected that *M. spinosa* treated group leads to break DNA single strand that are related to genotoxicity and cytotoxicity as well. But, the molecular mechanism behind cellular apoptosis is still not clear and needs to be investigated further.

Although, DON is a mycotoxin, thus the main aim of the present study was to determine the DNA damaging properties of DON. Therefore, the present study evaluated the genotoxic effect of DON (cytotoxic and non-cytotoxic) to draw conclusions about its relevance in hepatocyte toxicity using the established comet assay. The comet assay has become one of the standard methods for assessing DNA damage due to its simplicity, sensitivity, versatility, speed and economy (Goyary *et al*., 2014). The alkaline (pH >13) assay detects single-strand breaks, crosslinks, incomplete excision repair sites as well as apurinic or apyrimidinic sites, which are alkali labile (Singh *et al*., 1988). In the present study, no significant increase in the total comet score was detected between the control and vehicle treated group, however, DON elevated the comet score.

This is due to the fact that DON increases the production of reactive oxygen species, induces oxidative stress and causes lipid peroxidation. Therefore, the present findings suggested an indirect genotoxic effect in which the oxidative stress preceded cytotoxicity and DNA damage, leading to DON hepatotoxicity.

The frequencies of MNPCE in micronuclei (evaluated on the basis of the MN frequencies scored on Giemsa stained slides) was also determined and the results revealed that the frequencies of MNPCE was significantly increased in DON treated group as compared to the control group. These results provide the first evidence that DON is effective in producing lagging chromosomes but not chromosome fragments. The data presented here do not explain the mechanisms of aneuploidy caused by induction of DON. It can be inferred that the production of lagging chromosomes might result from the translational inhibition in the 60S ribosomal subunit, which is important for the functional integrity of the cytoskeleton, as it participates in modulation of cell differentiation and division. DON transduce a signal to RNA activated protein kinase (PKR) and hemoitopoetic cell kinase (Hck) by entering and binding with the active ribosomes of the cell. Subsequent phosphorylation of mitogen-actived protein kinases
(MAP) drives transcription factor (TF) activation apoptosis and resultant chronic and immunotoxic effects (Pestka et al., 2004).

A series of safety studies were performed systematically to investigate the safety of the stem bark extract of O. indicum and M. spinosa leaves extract in detail. The results of the present study were consistent by using the tester strains TA98, TA100, TA1535 and TA1538 to calculate the mutagenicity of extracts by the Ames assay. Mutagenicity was induced only by M. spinosa extracts in a concentration-dependent manner with and without metabolic activation in S. typhimurium tester strains. In the present study, tester strains TA100 and TA1535 were used to detect base-pair substitution mutations whereas, tester strains TA98 and TA1538 were used to detect frameshift mutations. Different investigators have used different tester strains to investigate the mutagenicity of test materials (Banerjee et al., 2013). Even though many investigators have sometimes used just two strains to determine the mutagenic potential of materials, but it is recommended to use at least four tester strains by Mortelmans and Zeiger (2000) to get more definite result. Because it is, noted that every biomaterials may or may not show mutagenic effect to each and every tester strain.

Since, M. spinosa extract reveals cytotoxin which is highly toxic in nature. In this context the DNA damaging properties of M. spinosa was investigated. Therefore, the present study evaluated the genotoxic effect of M. spinosa extract (cytotoxic and non-cytotoxic) to draw conclusions about its relevance in cellular toxicity using the established comet assay. The comet assay has considered as one of the standard methods due to its simplicity, sensitivity, versatility, speed and economy for assessing DNA damage. The alkaline (pH >13) assay detects single-strand breaks, cross-links, incomplete excision repair sites as well as apurinic or apyrimidinic sites, which are alkali labile (Mortelmans and Zeiger, 2000). Finally, in the present study no significant increase in the total comet score was detected between the control, vehicle and O. indicum treated group, but M. spinosa elevated the comet score at elevated doses. This might be due to the excessive production of reactive oxygen species, which induces oxidative stress and may cause lipid peroxidation. Therefore, the present findings
suggested an indirect genotoxic effect in which the oxidative stress preceded cytotoxicity and DNA damage, leading to carcinogenicity.

The result of the present investigation has generated detailed information on content of polyphenols and flavonoids in successive stem bark extracts of *O. indicum* and their antioxidant leading to hepatoprotective activities and for which the TPC and TFC were determined. The total content of phenols was highest in hydroalcoholic extract followed by methanol, chloroform and acetone extract. It was reported that solubility of the phenolic compounds increases with polarity of extracts (Sidduraju and Becker, 2003; Sultana et al., 2007) and polarity of hydro-methanolic mixture is the highest among the rest solvent extracts used in this study. Methanol is a highly preferred solvent for the extraction of phenols (Yen et al., 1996). Similarly, total flavonoid content was also found to be highest in methanol and aqueous extract as well. An earlier report showed increase in yield of flavonoid from *Mirabilis jalapa* L. tubers due to extraction using solvents of increasing polarity (Hajji et al., 2010). Thus, in the present study, hydroalcoholic stem bark extract of *O. indicum* exhibited highest level of antioxidant activity tested by various models, suggesting that the hydroalcoholic stem bark extract of *O. indicum* is a potential natural antioxidant. Further, this leads to hypothesize that polar antioxidants are stronger antioxidants than non polar antioxidants. Kalaivani and Mathew (2009) observed high antioxidant potential of ethanol stem bark extract of *O. indicum* in β-carotene bleaching assay and chloroform extract in reducing power assay. They attributed variation among antioxidant potential to the phytochemical diversity in the extracts. Again, several authors have reported association of high total phenolic content and high antioxidant activity in several plants (Anesini et al., 2008; El Babili et al., 2010; Carvalho et al., 2010).

Hence, the present study was undertaken with the aim to carry out a systematic comparative antioxidant study on different solvent extracts of *O. indicum* stem bark using different models, and to find any correlation between the antioxidant activity and total phenolic, total flavonoid contents of the extracts. The presence of phenolic and flavonoid compounds, were reported to be responsible for the natural sources antioxidative effectiveness. Polyphenolic compounds have received extensive attention because of their beneficial physiological role, including antioxidant, antimutagenic, and
for other diseases caused by oxidative stress. The phenolic compounds show scavenging activity because of presence of hydroxyl groups and hence, considered as an important plant constituents. Flavonoids are a large group of pervasive molecules having antioxidant activities. Their planar structure, number and position of their hydroxyl groups, as well as the presence of $\text{C}_2\text{C}_3$ double bond, play a important role for metal chelation, free-radical scavenger capacities, and the inhibition of free radical producing enzymes (Shirwaikar et al., 2011; Sen and Chakraborty, 2011; Bhagat et al., 2011, Philips et al., 2010; Atmani et al., 2009; Ozsoy et al., 2008).

DPPH is a stable free radical, when antioxidant reacts with DPPH the electron is paired off and the DPPH solution is decolorized. The scavenging activity of the antioxidant or the bleaching of the colour depend stochiometrically on the number of electrons taken up (Shirwaikar et al., 2011; Bhagat et al., 2011). The strong scavenging capacity of the hydroalcoholic stem bark extracts of *O. indicum* on DPPH was may be due to the presence of hydrogen donating ability of the polyphenolic compounds present in the extracts.

Hydrogen peroxide is a non-free radical species and usually by the oxidation of essential thiol groups it can directly inactivate different enzymes. $\text{H}_2\text{O}_2$ is able to cross cell membranes quickly and may slowly oxidize a number of cell compounds. $\text{H}_2\text{O}_2$ probably can react to form hydroxyl radicals with metals ions like $\text{Fe}^{2+}$, $\text{Cu}^{2+}$ and this may create toxic effects (Nagulendran et al., 2007; Bozin et al., 2008; Hazra et al., 2008). Thus, the abolition of hydrogen peroxide by the extracts is significant for protection of human health as well as of pharmaceutical and food system.

The reducing power capacity of the extracts, impart noteworthy indication about the potential antioxidant capacity of the plant. The reducing properties are generally connected with the presence of reductones. The antioxidant action of reductones depends on the breakdown of the free radical chain by reacting with certain precursors of peroxide to prevent peroxide formation and by donating a hydrogen atom (Nagulendran et al., 2007; Liu et al., 2007; Gulcin et al., 2010). In the present study, the hydroalcoholic stem bark extracts of *O. indicum* showed a promising result in this assay.

In agreement with earlier reports, in the present study a significant inverse correlation was obtained between TPC and DPPH radical scavenging model and also
between TFC and DPPH radical scavenging model suggesting that the high antioxidant activity is due to the high polyphenol and flavonoid content in the hydroalcoholic stem bark extract of *O. indicum* Antioxidant activity of plant extracts containing polyphenol components is due to their capacity to be donors of hydrogen atoms or electrons and to capture free radicals (Shon *et al.*, 2003). Sankara and Nair (1972a, 1972b) reported that stem bark and leaves of *O. indicum* contain flavonoids such as baicalein, chrysin and scutellarin is shown to possess antioxidant activity (Ng *et al.*, 2000) which reduces oxidative stress induced by free radicals. Oxidative stress has been implicated in the causation of diseases such as diabetes, cardiovascular diseases, liver cirrhosis nephrotoxicity, cancer and aging, etc. A correlation between the polyphenolic content and the antioxidant activity is observed by the present study, where the methanol extract containing the highest phenolic and flavonoid content shows higher antioxidant activity.

Similarly, in the present study, the ability of *O. indicum* stem bark extract to prevent DON-induced hepatotoxicity was investigated in mice. A single toxic dose of DON was used to induce liver damage. The possible protective effect of *O. indicum* against DON was investigated. Since hepatic enzymes are restricted (especially SPT/ALT and SGOT/AST) in periportal hepatocytes, where they are involved in amino acid metabolism, they are good biochemical markers for early acute hepatic damage (Adedara *et al.*, 2010b). This is because these transamination reactions and their serum activities apparently increase as a result of cellular membrane damage and leakage (Das *et al.*, 2010). Treatment of animals with a single dose of DON caused increase in serum transaminases (ALT and AST). The observed increase in serum enzymes may have been caused by a leakage of these enzymes from the periportal regions. Thus, the results obtained in toxin treated group showed that DON treated mice shows tremendous hepato-cellular injury, when there is a rise in marker hepatic enzymes in serum like AST and ALT levels as compared to the control rats. It is known that DON is a dynamic hepato-toxin in rat, mouse and human liver cells concurrently (Sahu *et al.*, 2010) which produces free radicals to persuade oxidative stress and mitochondrial dysfunction in hepatic cells and as a result stimulates acute liver damage. In an earlier study it is also reported that oxidative stress and mitochondrial dysfunction were used as the endpoints of toxicity for evaluating the hepato-toxic potential of DON. DON toxicity mechanism is related to oxidative stress investigated by generation of intracellular reactive oxygen
species (ROS). Their previous observation reported that DON significant induced oxidative stress on rat liver Clone-9 cells (Sahu et al., 2008). The oxidative stress leads to liver toxicity which is generated by the food-borne mycotoxins, aflatoxin B₁ (Shen et al., 1995) and fumonisin B₁ (Abel and Gelderblom, 1998).

Pretreatment with O. indicum extract inhibited the negative effect of the DON on the liver. The increase in serum transaminases levels was significantly attenuated. Compared to the control, there was a very low toxic effect of the O. indicum stem bark extract. Once the injury to the hepatocytes is healed, the leakage is stopped. This may be the case of the animals treated with the extract, as serum enzymes were reduced to an appreciable level compared to the control. The possibility of DON to generate ROS during metabolism has been earlier postulated (Sahu et al., 2008). The ability of O. indicum extract to attenuate the hepatotoxic effect of DON could be due to the antioxidant property these compounds may have since DON, is known to exert its toxicity through the induction of free radicals (Rizzo et al., 1994). Polyphenols for example are known to be soluble chain-breaking inhibitors of the peroxidation process, acting as scavengers of intermediate peroxyl and alkoxy radicals and chelating metal ions (Domitrovic et al., 2009). Prevention of DNA oxidation is also achieved by these polyphenols chiefly by modulating biometabolism enzymes and quenching free radicals (Angeli et al., 2010).