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Full Length Research Paper

Free radical scavenging potential of some Indian medicinal plants

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Indian medicinal herbs and plants are used since ancient times to treat different diseases and ailments as these natural products exert broad-spectrum actions. The present study was aimed to explore the hydrogen donating and hydroxyl radical scavenging potential of methanol extract of 10 medicinal plants belonging to six families including mimosaceae, Apocynaceae, moraceae, sapindaceae, rutaceae and meliaceae using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and plasmid nicking assay, respectively. The total phenol and flavonoid content of these extracts was also estimated using Folin-Ciocalteu and colorimetric assay respectively. It was found that methanol leaf extract of *Koelreutaria paniculata*, *Acacia catechu* and *Mimusops hexandra* showed strong inhibitory activity whereas that of *Hamelia patens* exhibited moderate DPPH radical scavenging activity at concentration of 200 µg/ml. However, methanol extract of *Swietenia mahogoni*, *Murraya exotica*, *Murraya koenigii*, *Alstonia scholaris*, *Ficus benjamina* and *Sapindus trifoliatus* exhibited weak hydrogen donating potential in DPPH assay. The methanol extract of these plants was effective in plasmid nicking assay and the activity was found to be correlated to the phenolic and flavonoid content in these fractions. These results emphasized the benefit of the phenolic compounds rich plant extracts and thus augmented the urge of *in vivo* studies to further confirm the beneficial effect of these extracts.

Key words: Medicinal plants, flavonoid content, phenolic content, hydrogen donation, hydroxyl radical scavenging.

INTRODUCTION

In developing countries, like India where poverty and malnutrition is rampant and people are unable to bear the cost of medical treatment for the diseases arising due to environmental pollution, the knowledge of plant derived antioxidants could reduce the cost of health care. The antioxidants are used to protect human beings from the ill-effects of oxidative stress that is exerted by enhanced production of reactive oxygen species (ROS) as a result of exposure to pollutants. The body has several mechanisms to counteract oxidative stress by producing antioxidants either naturally generated *in situ* (endogenous antioxidants) or externally supplied through foods (exogenous antioxidants) (Halliwell and Gutteridge, 2007).

Medicinal plants have been used to treat human diseases in India for centuries. In recent years, there has been a great interest in finding natural antioxidants from plants (Barla et al., 2007; Bektas and Ozturk, 2007). The search for safe and effective natural antioxidants is now focused on edible plants, especially spices and herbs (Nakatani, 1997). People are becoming interested in medicinal herbs because of their low toxicity and good therapeutic performance. Instead, many Indian plants have been used as flavors, pigments and food (Ali et al., 2008; Bhattacharya et al., 2009). Traditionally, Indian medicinal herbs are boiled in water (decoction method) and used to treat different diseases. Although, these extraction methods have been used by Indians for thousands of years, but there is an increased quest to

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Abbreviations: ROS, Reactive oxygen species; **DPPH,** 2,2'-diphenyl-1-picrylhydrazyl radical; **ddH_2O,** double distilled water; **TBE,** tris-boric acid- EDTA.

obtain natural antioxidants with broad-spectrum actions. The majority of active antioxidant compounds are phenols, flavonoids, isoflavones, flavones and anthocyanins etc. A direct relationship between antioxidant activity and phenolic content of plant extracts has also been reported (Sharififar et al., 2009; Gollucke et al., 2008; Du et al., 2009; Conforti et al., 2009; Chirinos et al., 2008). Keeping the significance of plant based antioxidants in mind, the present study was planned to investigate and compare the antioxidant activities of medicinal plants that are predominant in northern region of India. In this study, methanol extracts of leaves of 10 medicinal plants (*Acacia catechu*, *Hamelia patens, Alstonia scholaris, Ficus benjamina, Koelreutaria paniculata, Mimusops hexandra, Murraya koenigii, Murraya exotica*, *Sapindus trifoliatus, Swietenia mahogoni*) belonging to families mimosaceae, Apocynaceae, moraceae, sapindaceae, rutaceae and meliaceae is prepared separately using maceration method and these extracts are further studied for their antioxidant activity through 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and DNA nicking assays. These plants have ethnobotanical value and are used by human beings to treat various ailments. The medicinal properties of these plants along with their common name and part used is mentioned in Table 1.

MATERIALS AND METHODS

Chemicals

DPPH and ethidium bromide were obtained from Himedia Laboratories Pvt. Ltd., Mumbai. Supercoiled plasmid pBR 322 DNA and agarose was obtained from Genei, Bangalore. Bromophenol blue, ethylenediaminetetraacetic acid (EDTA), L-ascorbic acid, Tris (hydroxymethyl) aminomethane, Folin–Ciocalteu reagent, sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), ferric chloride ($FeCl_3$), hydrogen peroxide (H_2O_2), rutin, quercetin and gallic acid were of analytical grade.

Preparation of extract

The leaves of plants were washed with tap water twice and then air dried at room temperature. Air dried leaves were finally powdered and three successive extractions with 80% methanol were carried out at room temperature for 24 h. The extracts were filtered using Whatman No. 1 sheet. The filtrates obtained were concentrated under vacuum on a rotary evaporator at 40°C and the concentrated solution was then lyophilized.

Phytochemical analysis

Determination of total phenolic content

The concentration of total phenols in plant extract was estimated by procedure given by Yu et al. (2002). In this procedure, 100 µl of extract fraction (100 µg/ml) was mixed with 900 µl of double distilled water to make final volume 1000 µl. To this solution 1.5 ml of 20% sodium carbonate solution and 0.5 ml of 1:1 Folin-Ciocalteu reagent was added. By addition of distilled water, the volume was raised to 5 ml and the mixture was then incubated for 2 h at room temperature. The absorbance of mixture was measured at 765 nm using UV-VIS spectrophotometer. The total phenolic content of samples were expressed in terms of gallic acid equivalents (GAE) which reflected the phenolic content as the amount of gallic acid (mg) in 1 g of dry material. Quantification was carried out on the basis of standard curve of gallic acid and described by equation $y = 0.000\ x + 0.039$ ($R^2 = 0.987$) where y = absorbance and x = concentration.

Determination of total flavonoid content

The method given by Kim et al. (2003) was used for analyzing total flavonoid content (TFC) employing rutin as a standard. In this procedure, 1 ml extract (each of 100 µg/ml concentrations) was added to 4 ml of ddH_2O, 300 µl of $NaNO_3$ and 300 µl of $AlCl_3$. The mixture was then incubated at room temperature for 5 min. After incubation, 2 ml of sodium hydroxide (1 M) was added. Then final volume of solution was raised to 10 ml by further addition of distilled water. The absorbance of sample and blank were taken at 510 nm by UV-VIS spectrophotometer. The total flavonoid content was then expressed in terms of mg rutin equivalents (RE) / g of dry sample. For rutin, the curve absorbance versus concentration is described by the equation:

$y = 0.0011\ x + 0.0409$ ($R^2 = 0.9892$)

Where y = concentration; x = absorbance.

Antioxidant assays

DPPH free radical scavenging assay

The hydrogen atom donating ability of the different plants extracts was determined from the decolorization of a purple colored methanol solution of DPPH following the method of Blois (1958) with minor modifications. DPPH is stable nitrogen centered radical. The odd electrons in the DPPH free radical give a strong absorption maximum at 517 nm. In this assay, 200 µl of extract solution (concentrations ranging from 20 – 200 µg/ml) was mixed with 3 ml of DPPH (0.1 mM) in methanol solution. The absorbance of reaction mixture at 517 nm was taken. The decrease in absorption was correlated with the scavenging action of the test compound. Gallic acid being a phenolic compound was used as a positive control. The radical scavenging activities were expressed as percentage of inhibition and calculated according to the following equation.

Percentage of DPPH inhibition= $[(A_C-A_S)/A_C] \times 100$

Where A_C = absorbance of control and A_S= absorbance of sample.

A percent inhibition versus concentration curve was plotted and the concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value.

Plasmid nicking assay

The ability of different plant extracts to protect supercoiled pBR322 DNA from devastating effects of hydroxyl radicals generated by Fenton's reagent was assessed by the DNA nicking assay described by Lee et al. (2002) with slight modifications. In this case, phenolic compound ellagic acid was used as positive control. The reaction mixture was initiated by 0.5 µl of plasmid DNA (pBR 322) in a micro centrifuge tube with 10 µl of Fenton reagent (30 mM

Table 1. Botanical name, family, vernacular name, parts used and traditional use of studied medicinal plants.

Botanical name	Family	Vernacular name	Parts used	Traditional use to cure	References
A. catechu (Linn.f.) Willd	Mimosaceae	Cutch tree	Leaves	Asthma, cancer, gonorrhea, dysentery, Bronchitis	Rahm, Rahman et al., 1986
A. scholaris R.Br.	Apocyanaceae	Satwina, saptparini	Leaves	Expectorant, cancer especially against lung cancer, cytotoxicity, antimalarial.	Gupta et al., 2002; Shah et al., 2010
F. benjamina Roxb.	Moraceae	Weeping fig	Leaves	Fever, ulcer, vomiting, leprosy, malaria, stomachic, hypotensive and against dysentry	Almahy et al., 2003
H. patens Wild.	Rubiaceae	Fire bush	Leaves	Cuts and eruptions of the skin, astringent, malaria and as antiseptic	Gomez-Beloz et al., 2003; Duke, 2007
K. paniculata Laxm.	Sapindaceae	Golden raintree	Leaves	Ophthalmic, conjunctivitis, have antitumor, antioxidation and antibiosis activities	Lin et al., 2002
M. hexandra Roxb.	Sapotaceae	Ceylon wood	Leaves, bark, fruits	Fever, colic, helmenthiasis, hyperdipsia, as appetizer, leprosy, opacity of cornea, ulcers, as tonic and astringent	Pullaiah, 2006; Warrier et al., 1995.
M. koenigii Linn.	Rutaceae	Curry-leaf tree	Leaves	Purgative, flavouring agents, as tonic, dysentery, against beta cell damage and antioxidant defence system of plasma and pancreas in streptozotacin induced diabetes in rats	Yadav et al., 2002; Arulselvan and Subramanian, 2007.
M. exotica Linn.	Rutaceae	Satin wood	Leaves	Diarrhea, dysentery, cuts, joint pain, stomachic, taenicide, fever, cough, influenza	Duke, 2007
S. trifoliatus Linn.	Sapindaceae	Ritthaa	Leaves	Expectorant, eczema, aphrodisiac, abortifacient, migraine, psoriasis and freckles, inflammation	Arulmozhi et al., 2005a, 2005b
S. mahogoni A. Juss.	Meliaceae	Rohan	Leaves	Astringent, malaria, fever, diarrhea, hypertension, cancer, diabetes, coughs and intestinal parasitism	Sahgal et al., 2009

H_2O_2 + 50 µM ascorbic acid and 80 µM $FeCl_3$). To this mixture plant extract (200 µg/ml) was added and final volume of mixture was brought up to 20 µl by using ddH_2O. The mixture was then incubated for 30 min. at 37°C followed by addition of 2 - 5 µl of loading buffer (0.25% bromophenol blue, 50% glycerol) and then centrifuged for 10 s. Quercetin (200 µg/ml) a known phenolic compound was taken as a positive control. DNA were analyzed using Gel Doc XR system (Bio-Rad, USA) after agarose gel electrophoresis, using 1% agarose gel, in TBE buffer, at 50V (1.5 – 2 V/cm) for 4 h and reagents used in Fenton reaction were prepared fresh, for each reaction.

Statistical analysis

Results are depicted as the mean ± SE. The data was analyzed for

Table 2. Total phenol content in terms of mg gallic acid equivalents (GAE) / gram dry weight of extract and flavonoid content in terms of mg rutin equivalents (RE) /gram dry weight of extract.

Plant name	Total phenolic content in mg GAE/g dry weight of extract	Total flavonoid content in mg RE/g dry weight of extract
A. catechu	810	346.3
H. patens	420	246.3
Mi. hexandra	370	73.6
M. koenigii	320	Nil
M. exotica	510	55.4
A. scholaris	80	Nil
S. mahogoni	260	82.7
K. paniculata	600	210
F. benjamina	10	Nil
S. trifoliatus	60	Nil

statistical significance using one way ANOVA (Analysis of Variance) and the difference among means was compared by Honestly Significant Differences (HSD) calculated by. The significance of results was tested at $p \leq 0.05$.

RESULTS

Table 2 depicts the total phenolic content of methanolic extract of 10 medicinal plants. Among all the plants studied, it was observed that *A. catechu* showed the highest amount of polyphenols *viz*. 810 mg GAE/g dry wt. of extract and the extract of *F. benjamina* showed least phenolic content that is 10 mg GAE/g dry weight. The phenolic content of different plants was observed to be in the following order: *A. catechu* (810) > *K. paniculata* (600) > *M. exotica* (510) > *H. patens* (420) > *M. hexandra* (370) > *M. koenigii* (320) > *S. mahogoni* (260) > *A. scholaris* (80) > *S. trifoliatus* (60) > *F. benjamina* (10). Flavones and flavonols are flavonoid compounds sometimes referred to, as "Super Antioxidants" was also observed to be present in some plant extracts. Table 1 depict the total flavonoid content of methanol extract of medicinally important plants in mg RE/g dry weight of extract. It was observed that extract of *A. catechu* (346.3) showed the highest TFC, where as in other plants the flavonoid content was found in the following order: *H. patens* (246.3) > *K. paniculata* (210) > *S. mahogoni* (82.7) > *M. hexandra* (73.6) > *M. exotica* (55.4). It was noticed that some plants *viz*. *M. koenigii*, *A. scholaris*, *S. trifoliatus* and *F. benjamina* did not show the flavonoid contents in detectable amount.

The antioxidant activity of above mentioned plants was determined by employing DPPH and DNA nicking assay. Table 3 represents the IC_{50} values of plants as observed in DPPH assay whereas Figures 1 and 2 depict the inhibition (%) by methanol extract of these plants in comparison to gallic acid which was used as natural antioxidant compound. The results of these plants with different assay systems are thus described.

As clear from Figures 1 and 2, it was found that methanol leaf extract of *K. paniculata* exhibited a remarkable inhibition of 83.40% at highest concentration tested that is 200 µg/ml followed by *A. catechu* that showed 76.94% inhibition at 200 µg/ml concentration whereas methanol leaf extract of *M. hexandra* showed 72.11% scavenging of DPPH radical at the same concentration. Methanol extract of *H. patens* exhibited moderate DPPH radical scavenging activity with maximum inhibition of 42.21% at concentration of 200 µg/ml. However, methanol extract of other plants that is *S. mahogoni*, *M. exotica*, *M. koenigii*, *A. scholaris*, *F. benjamina* and *S. trifoliatus* exhibited weak hydrogen donating potential in DPPH assay with inhibitory effect of 14.31, 13.12, 9.54, 7.23, 6.20 and 5.13% respectively, at the highest tested concentration that is 200 µg/ml. It was found that the results obtained for different plant extracts in DPPH assay were statistically significant at $p \leq 0.05$. In DNA nicking assay, it has been found that the addition of Fenton's reaction mixture (Fe^{3+}+H_2O_2+ ascorbic acid) to plasmid DNA resulted in time dependent increase of single stranded (ss) and double stranded (ds) nicked and linear forms of DNA (Form II and Form III respectively) due to the attack of $^{\cdot}OH$ radicals generated in reaction mixture, on the nitrogenous bases or the deoxyribosyl backbone of DNA (positive control). However, addition of methanol extract of different medicinal plants to reaction mixture reduced the hydroxyl radical mediated strand breaking and conversion of supercoiled DNA to Form II and III DNA. Figure 3 shows that methanol extract of *S. trifoliatus* leaves (200 µg/ml concentration) showed potent effect again $^{\cdot}OH$ radicals mediated damage as this extract maintained the integrity of supercoiled DNA in a manner just equivalent to quercetin (known phenolic compound). The methanol extract of *A. catechu*, *M. koenigii*, *M. exotica* and *H. patens* showed weak effect in plasmid nicking assay. The intensity of supercoiled band was relatively less as much of this DNA got nicked by $^{\cdot}OH$ radicals and converted into Form II (linear form).

The conclusion could be drawn from Figure 4 that

Table 3. IC_{50} values for methanol extract of different medicinal plants used in the study. These values were calculated from the regression equation obtained using MS excel program.

Plant name	IC_{50} value
A. catechu	133
H. patens	229
M. hexandra	130.73
M. koenigii	1001
M. exotica	830
A. scholaris	1308.9
S. mahogoni	1514
K. paniculata	107
S. trifoliatus	3152.0993
F. benjamina	1575.24

methanol extract of *K. paniculata* showed good ˙OH radicals scavenging potential as its leaf extract at 200 µg/ml concentrations, was effective in protecting DNA from the damaging effect of ˙OH radicals and thus maintained the supercoiled DNA in its respective form. It is pertinent to mention here that same extract of *A. scholaris*, *F. benjamina* at 200 µg/ml concentration also showed remarkable ˙OH radical scavenging potential as a result of maintaining the integrity of Form I DNA. However, *M. hexandra* that acted as potent hydrogen donator in DPPH assay did not show a very good effect even at highest concentration tested that is 200 µg/ml whereas methanolic extract of *S. mahogoni* that exhibited weak hydrogen donating potential, showed a remarkable effect in protecting the conversion of supercoiled DNA to single stranded form (Figure 4).

DISCUSSION

For health conscious consumer, the words “free radicals and antioxidants” have become very important. Antioxidants help the organisms to deal with oxidative stress, caused by free radical damage. It is possible to reduce the risk of chronic diseases and prevent their progression by either enhancing the body’s natural antioxidant defense or by supplementing with proven dietary antioxidants (Stanner et al.. 2004). Cancer chemoprevention by using antioxidant approaches has been suggested to offer a good potential in providing important fundamental benefits to public health, and is now considered by many clinicians and researchers as a key strategy for inhibiting, delaying, or even reversal of the process of carcinogenesis (Shureiqui et al., 2000; Tsao et al., 2004).

Extracts of medicinal herbs and spices are the most studied natural antioxidants (Yanishlieva et al., 2006). Curcumin, main component present in the rhizomes of *Curcuma longa*, is reported to act as antioxidant due to its ability to scavenge free radicals generated *in vitro* including $O_2^{\cdot-}$, ˙OH radicals (Maheshwari et al., 2006). The potential of ethanolic extract of *Juglans regia* to reduce the hydroxyl radical induced degradation of deoxyribose in a concentration dependent manner was reported by Almeida and co-workers (2008). They furthermore concluded that protective effect of *J. regia* against ˙OH was related to the presence of flavonoids (quercetin-3-galactoside, quercetin-3-rhamnoside and quercetin) in the extract (Almeida et al., 2008). In another study, Benherlal and Arumughan (2008) reported the protective effect of standard compounds including vitamin C, gallic acid, catechin, apigenin, naringenin, naringin and extracts of medicinal plants (*Hippophae rhamnoides* kernel (HRK), *Syzygium cumini* kernel (SCK), *Punica granatum* pericarp (PGP)) on the DNA damage in Fenton’s system.

Literature survey has revealed a direct relationship between antioxidant activity and total phenolic content. It has been reported by Halliwell (1990) that for a polyphenol to be defined as an antioxidant it must satisfy two basic conditions: first, when present in low concentrations relative to the substrate to be oxidized it can delay, retard, or prevent the autoxidation of free radical mediated oxidation; second the resulting radicals formed after scavenging must be stable through intramolecular hydrogen bonding on further oxidation (Shahidi et al., 1992). In general, free radical scavenging and antioxidant activity of phenolics (e.g. flavonoids , phenolic acids) mainly depends upon the number and position of hydrogen donating hydroxyl groups on aromatic rings of the phenolic molecules, and is also affected by other factors, such as glycosylation of glycones, other H- donating groups (-NH, -SH) etc. For example flavonolaglycones such as quercetin, myricetin and kaemperol, containing multiple hydroxyl groups had higher antioxidant activity than their glycosides such as rutin, astragalin. It is believed that thousands of phenolic compounds occur in medicinal herbs. For instance, more than 4000 kinds of flavonoids and hundreds of coumarins and lignans have been reported as naturally occurring compounds (Iwashina, 2000; Xiao et al., 2000).

As seen in Table 1, total phenolic and flavonoid contents of the methanolic extracts vary in different plant species. *A. catechu* belonging to family mimosaceae has highest total phenolic content that is 810 mg GAE/ dry weight of extract. It is often difficult to decide in a screening for antioxidants from natural sources that plant species studied can be considered the best one, as each of them exhibited different antioxidants and scavenging activities. The antioxidant capacities of the plant extracts largely depend upon the composition of the extracts and condition of test system. The antioxidant activities of 10 plants were measured using an improved DPPH radical decolourization assay, one of the methods most commonly employed to measure antioxidant capacity, which actually measures the ability of compound to donate hydrogen ion. From percent inhibition and IC_{50}

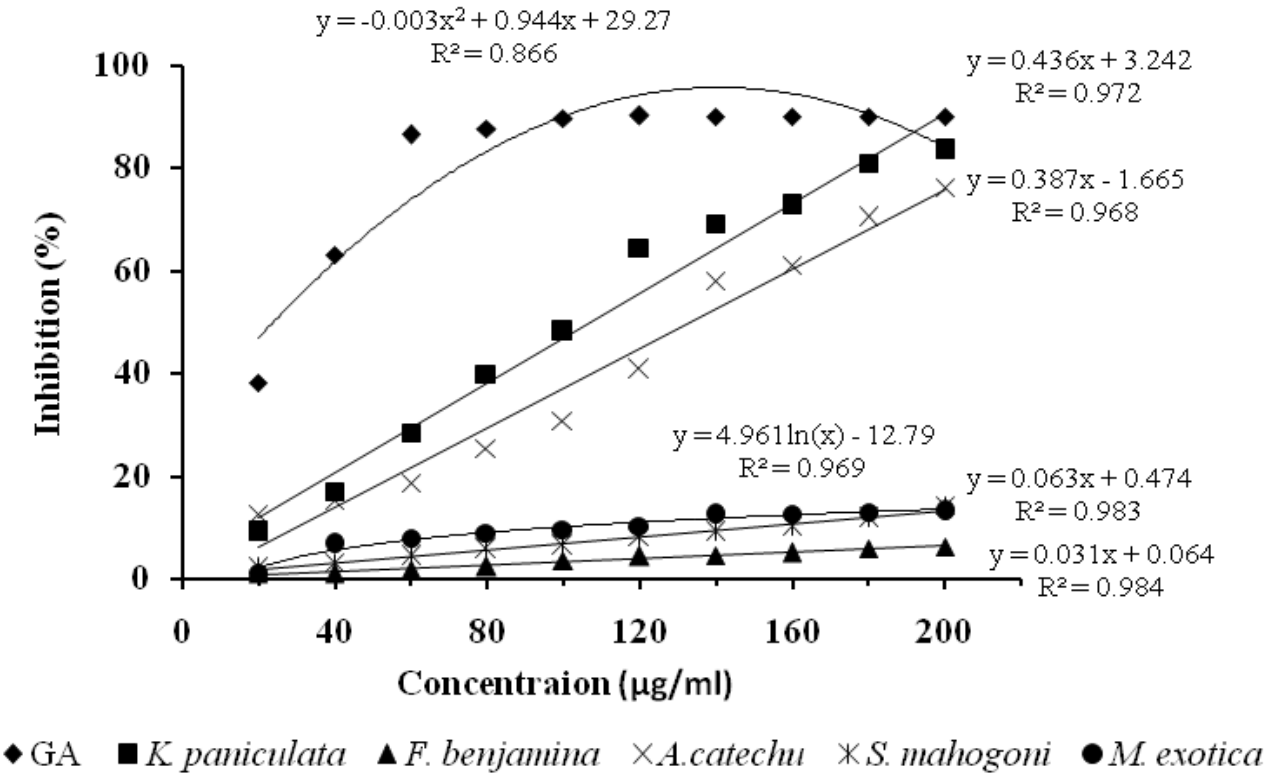

Figure 1. depicts the hydrogen donating ability of methanol extract of *K. paniculata*, *F. benjamina*, *A. catechu*, *S. mahogoni* and *M. exotica* in comparison to natural antioxidant (gallic acid).

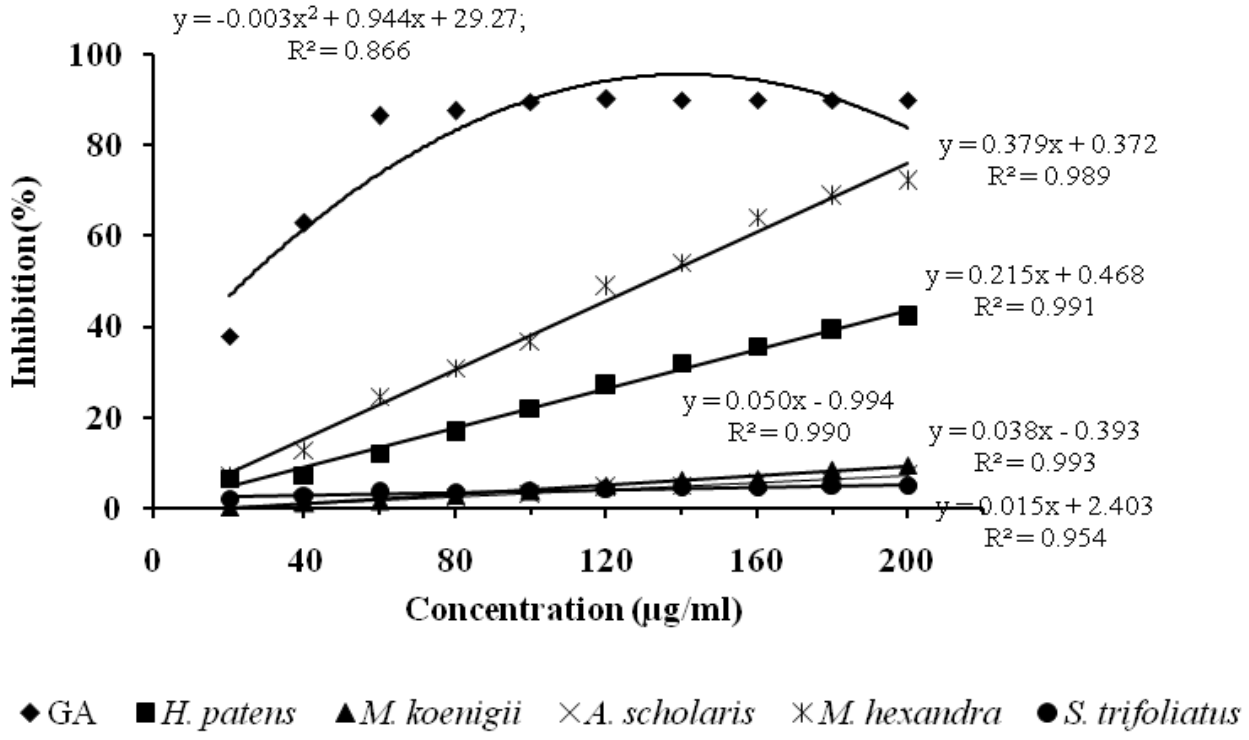

Figure 2. depicts the hydrogen donating ability of methanol extract of *H. patens*, *M. koenigii*, *A. scholaris*, *M. hexandra* and *S. trifoliatus* in comparison to natural antioxidant (gallic acid).

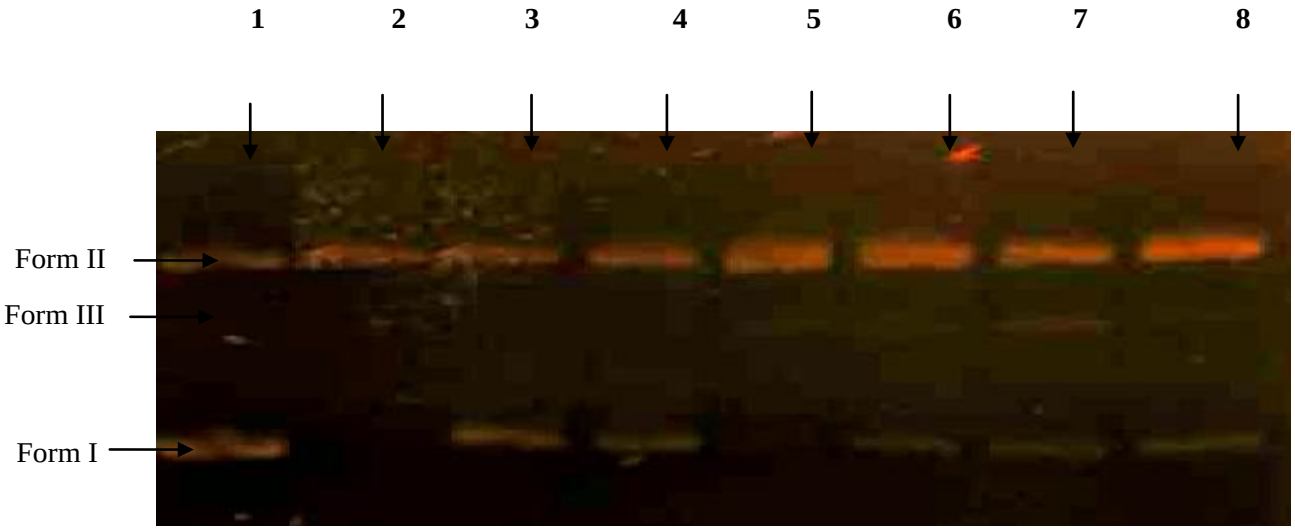

Figure 3. Show the effect of methanolic extracts of different plants on the integrity of pBR322 plasmid DNA in the presence of Fenton's reagents. Lane 1: pBR322 DNA + H_2O; Lane 2: pBR322 DNA + FR; Lane 3: standard antioxidant compound (quercetin) in the presence of FR; Lane 4: FR + *S. trifoliatus* (200 µg/ml); Lane 5: FR + *H. patens* (200 µg/ml); Lane 6: FR + *M. koenigii* (200 µg/ml); Lane 7: FR + *M. exotica* (200 µg/ml); Lane 8: FR + *A. catechu* extract (200 µg/ml). Here, FR = Fenton's Reagent (H_2O_2 + Fe^{3+} + Ascorbic acid), Form I = Supercoiled; Form II = Linear; Form II = Single strand nicked DNA.

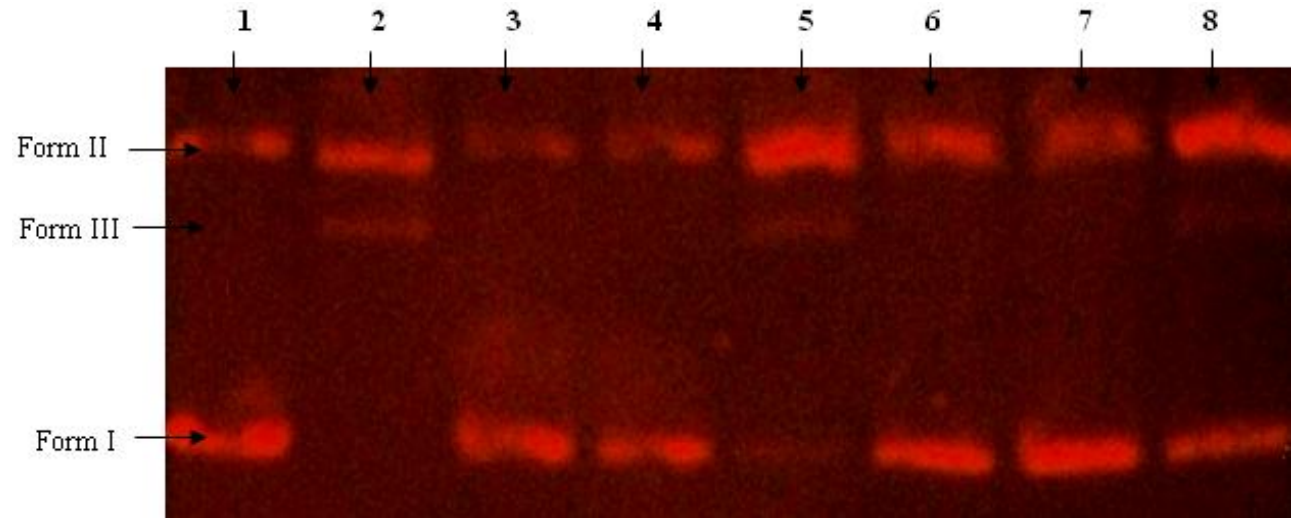

Figure 4. show the effect of methanolic extracts of different plants on the integrity of pBR322 plasmid DNA in the presence of Fenton's reagents. Lane 1: pBR322 DNA + H_2O; Lane 2: pBR322 DNA + FR; Lane 3: standard antioxidant compound (quercetin) in the presence of FR; Lane 4: FR + *K. paniculata* (200 µg/ml); Lane 5: FR + *M. hexandra* (200 µg/ml); Lane 6: FR + *A. scholaris* (200 µg/ml); Lane 7: FR + *F. benjamina* (200 µg/ml); Lane 8: FR + *S. mahogoni* extract (200 µg/ml). Here, FR = Fenton's Reagent (H_2O_2 + Fe^{3+} + Ascorbic acid), Form I = Supercoiled; Form II = Linear; Form III = Single strand nicked DNA.

values of methanol extract of different plants, it has been observed that there existed a significant correlation between antioxidant potency and phenolic content (r = 0.771) and antioxidant potency and flavonoid content (r = 0.7818) at $p \leq 0.01$. The plants showed dose dependent inhibition in DPPH assay. The present investigation also involved DNA protecting activity of plant extracts against the damage caused by hydroxyl radicals in plasmid nicking assay. It was observed that the some of the plant extracts provide good protection against the damage caused by $^{\cdot}OH$ radicals *viz*. *K. paniculata*, *S. trifoliatus*, *A. scholaris* and *F. benjamina*. Very little or undetected antioxidant activities were observed with the plant extracts from *H. patens*, *S. mahogoni*, *M. exotica*, *M. koenigii*, *M. hexandra* and *A. catechu*. The results of this study are corroborated by the findings in literature

(Wang et al., 2002; Kumar and Chattopadhyay, 2007; Benherlal and Arumughan, 2008). Several studies are focused on the relationship between antioxidant activities of phenolic compounds, as hydrogen donating free radical scavengers, and their chemical structures. Although, the interactions between reactive oxygen species and putative antioxidants depend on many biological, physical and chemical parameters, these preliminary results on the *in vitro* DPPH radical scavenging capacity and protection of plasmid DNA against oxidative stress emphasized the benefit of the phenolic compounds of medicinal plant extracts. *In vivo* studies are now needed to further confirm the beneficial effect of these plant extracts.

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