Indian Journal of Natural Products and Resources
Vol. 1(3), September 2010, pp. 322-327

Demonstration of the potential of *Hibiscus cannabinus* Linn. flowers to manage oxidative stress, bone related disorders and free-radical induced DNA damage

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Received 11 August 2009; Accepted 28 October 2009

Hibiscus cannabinus Linn. flowers (HCF) were investigated for free-radical scavenging properties *in vitro*, capacity to protect DNA from oxidative damage and inhibiting gelatinolytic activity of collagenase type I and II. For this purpose, flowers extracted with methanol and water and subjected to DPPH free radical scavenging assay, calculation of IC_{50} values, using vitamin C as a standard, reducing power assay, DNA damage protection assay and inhibition of gelatinolytic activity of collagenase type I and II. The DPPH free radical scavenging activity ranged from 440 to 700µg/ml for different extracts vis-à-vis 39 µg/ml for standard vitamin C. A similar trend was visible in reducing power activity. Both activities reflected a strong anti-oxidant potential of HCF and in turn against stress. Further, both extracts at 100 µg/ml (i) were efficient in protecting DNA against oxidative damage and (ii) exhibited inhibition of gelatinolytic activity of collagenase type I up to 87% and type II up to 65%. Cumulatively, the present data on *H. cannabinus* flower extracts indicated for the first time that they can be used as a (i) potent functional food to control oxidative stress, (ii) free radical-induced DNA damage and (iii) bone related disorders like osteoarthritis.

Keywords: *Hibiscus cannabinus*, Collagenase inhibition, Free radicals, Reactive Oxygen Species, Oxidative, DNA damage.

IPC code: Int. cl.[8]—A61K 36/00, A61P 17/18, A61P 19/00

Introduction

Traditional medicinal treatment in India, *Ayurved*, used several herbs in the form of powder, mixture, tablets, extracts, syrups, *kadha* (concentrated extract) and *asavas* (fermented extracts)[1]. These preparations/ formulations addressed several medical problems in reproducible manner, rapidly or at times slowly, presumably due to differences in their amount of bioactive molecules. These observations using several herbal preparations for several ailments prompted us to look into their concentration of bioactive molecules or devise marker assays which could validate reliability of their performance. One such tropical medicinal plant *Hibiscus cannabinus* Linn., commonly known as kenaf, (locally known as *Ambadi* in Marathi or *Ambashtha* in Sanskrit)[1], belonging to family Malvaceae is used in Cameroon, India and many other countries. Its leaves are eaten as vegetable, dried seeds after roasting and powdering used as a beverage in a similar way as coffee; dry flowers are used by *Pawra* and *Bhill* tribes of Maharashtra state to make syrup and vegetables[3-4] and the same have been reported to be used as medicine in Ayurveda[1]. On this background, present study aims to evaluate *H. cannabinus* flowers (HCF) for their antioxidant properties with a special reference to alleviate oxidative damage to DNA and gelatinolytic inhibition potential for addressing collagenase-induced problems in osteoarthritis (OA).

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Materials and Methods

Plant material and chemicals

Entire fresh flowers of *H. cannabinus* were collected from the plants during March 2008 from Western Ghat region of India, air dried (in shade) at ambient temperature and blended into fine powder. All chemicals used were of analytical grade. Standard pUC18 plasmid DNA (Genei, Bangalore) was utilized for the DNA protection assay.

Extraction

Methanolic extract in Soxhlet apparatus was prepared using 40 g fine powder of HCF, in 200 ml AR grade methanol at 55 ± 1ºC over 8 h, washed thrice with hexane to remove fatty matter and centrifuged (10000 rpm, 15 min, 27ºC) to obtain a clear supernatant. Similarly, an aqueous extract was prepared using HCF powder in 200 ml distilled water

under comparable experimental conditions. The extract was passed through Whatman filter paper no. 1 to remove sizable amount of residue. It was divided into two aliquots, using one aliquot without any treatment, while washing another aliquot with hexane to remove fatty matter, prior to use. Both aliquots were centrifuged (10000 rpm, 15 min, 27ºC) and used for assay. Both extracts, methanolic and aqueous were mixed to attain five following combinations to test if there is any additive effect of the bioactive molecules: (i) methanolic extract, (ii) aqueous extract without hexane wash, (iii) aqueous extract with hexane washing, (iv) methanolic extract + aqueous extract without hexane wash (1:1, v/v) and (v) methanolic extract + aqueous extract with hexane washing (1:1, v/v).

Biochemical assays (antioxidant activity)

In vitro antioxidant assays like (i) DPPH- free radical scavenging assay was carried out as described by Brand-Williams *et al* (1995)[5] and (ii) reducing power assays were carried out as described by Oyaizu (1986)[6] in triplicates.

Inhibition of gelatinolytic activity of collagenase type I and type II

Inhibition of gelatinolytic activity of collagenase type I and II were measured as per optimized protocol according to Moore and Stein (1948)[7]. Average values of all the above assays were considered.

DNA cleavage protection assay

Methanolic extract and aqueous extract of HCF washed with hexane were added at various concentrations (100 to 500 µg/ml) to the plasmid DNA (approximately 700 ng/µl of pUC 18 plasmid) for examining their DNA cleavage protection property in 10 mM Tris-HCl-1 mM EDTA (*p*H 7.6) according to Russo *et al.* (2001)[8]. The DNA subjected to various treatments (i-v) was electrophoresed on 1% agarose gel and visualized using Alphamager EC gel documentation system.

High performance thin layer chromatography (HPTLC)

Both, methanolic and aqueous, extracts of HCF were analyzed by HPTLC, using solvent system chloroform:methanol (9:1, v/v) as per Pawar *et al.* 2008[9]. The spots were exposed to iodine vapors and respective bands scraped out for anti-oxidant assays.

Results

Antioxidant activity

In methanolic extract of HCF (extract i), DPPH-free radical scavenging activity was nearly 30% at 100 µg/ml concentration and increased with the increasing concentration of extract to 65% at 1000 µg/ml, with the corresponding IC_{50} value for the extract 600 µg/ml. Similarly, the aqueous extract without and with hexane washing (extracts ii and iii) showed 30% and 24% free radical scavenging activity, respectively, at 100 µg/ml. It increased to 71 and 75%, respectively at 1000 µg/ml, with corresponding IC_{50} value for these extracts 500 µg/ml.

In methanolic extract mixed with aqueous extract without hexane wash (extract iv), scavenging activity significantly reduced to 9% at 100 µg/ml and 61% at 1000 µg/ml. However, scavenging activity elevated to 78% in mixed extract containing methanolic extract and aqueous extract with hexane washing (extract v) at 1000 µg/ml. The IC_{50} values for these extracts (iv and v) were 800 and 500 µg/ml, respectively (Figure 1). Since vitamin C is a strong antioxidant, its scavenging activity was carried out simultaneously to serve as a standard for comparison. It was 15% at 10 µg/ml and 84% at 100 µg/ml and IC_{50} value was 39 µg/ml.

Reducing power activity

Since reducing power of a compound serves as a significant indicator of its antioxidant activity[10], extracts of HCF were assayed for the reducing power activity. Except extract (v), all (i-iv) extracts showed nearly 0.05 absorbance at 100 µg/ml concentration and at 1000µg/ml, they showed an increase in the absorbance to 0.34, 0.24, 0.25, and 0.33, respectively. In this case also, like that of DPPH assay, mixture of two extracts (viz. combined extract v) has shown 0.078 absorbance at 100 µg/ml and absorbance 0.35 at

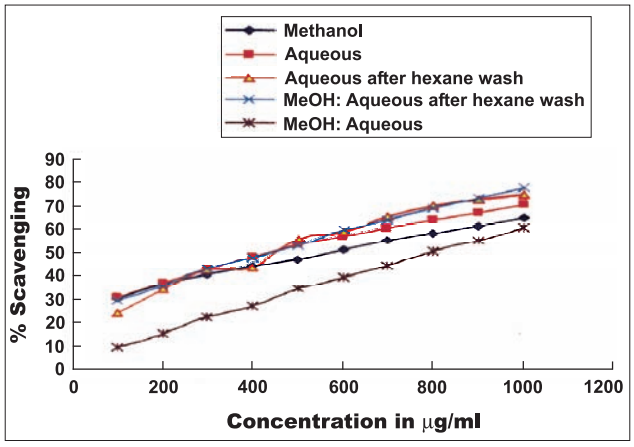

Fig. 1— Comparative DPPH scavenging activities of various extracts of HCF

1000 µg/ml concentration (Figure 2). An increase in absorbance by extracts indicated its increased reducing power[10]. Against these values, standard vitamin C showed absorbance of 0.03 at 10 µg/ml and 0.21 at 100 µg/ml.

Inhibition of gelatinolytic activity of collagenase type I and II

The aqueous extract of HCF showed inhibitory activity for both, type I and II collagenases. At 20 µg/ml concentration, it showed 58% inhibitory activity with type I collagenase, which increased to 87% at 100 µg/ml concentration. Similarly, type II collagenase showed 30% activity at 20 µg/ml, increasing to 65% at 100 µg/ml (Figure 3).

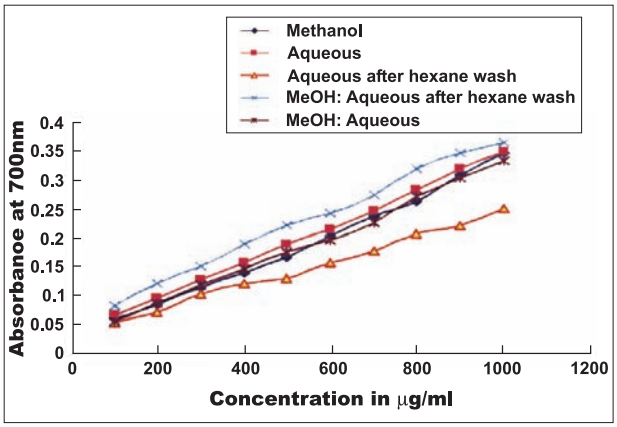

Fig. 2— Comparative reducing power activities of various extracts of HCF

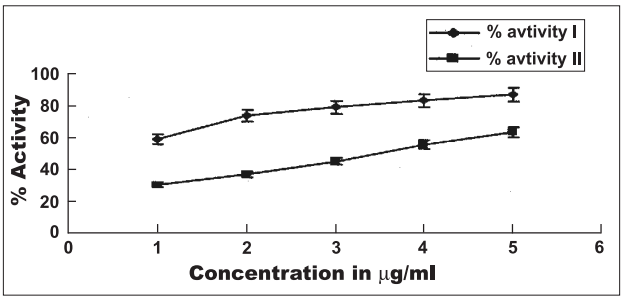

Fig. 3— Inhibition of gelatinolytic activity of collagenase type I and II with crude aqueous extract of HCF.

DNA cleavage protection assay

DNA cleavage, which is inducible by free radicals donated by hydrogen peroxide in the presence of UV light, was significantly protected by various concentrations (100 to 500 µg/ml) of the HCF extracts (Figure 4). At concentrations starting from 100 to 500 µg/ml, formation of open circular DNA in the presence of UV light by the free radicals donated by H_2O_2 are suppressed. However, the phenomenon of DNA protection was not dose-dependent, as in all concentrations, similar protective property of HCF extracts was observed.

HPTLC

HPTLC of all HCF extracts was performed to determine the spectrum of molecules contributing to their free radical scavenging activity. Chloroform: methanol (9:1, v/v) provided efficient separation of compounds. The separated bands from the HPTLC were exposed to iodine vapors, eluted separately after scraping and individual bands were subjected to assaying DPPH-free radical scavenging and reducing power activity. HPTLC plates pertaining to aqueous extracts upon scanning revealed 7 peaks (bands) with the corresponding R_f values ranging from 0.04 to 0.66 (Figure 5). In contrast, methanolic extract showed total 9 bands, with 2 new bands of R_f 0.18 and 0.22 values (Figure 5). Out of seven bands from aqueous and methanolic extracts on the HPTLC plates, only two bands showed reduced DPPH-free radical scavenging and reducing power activity, while other common five bands from both extracts had no activity at all (data not shown).

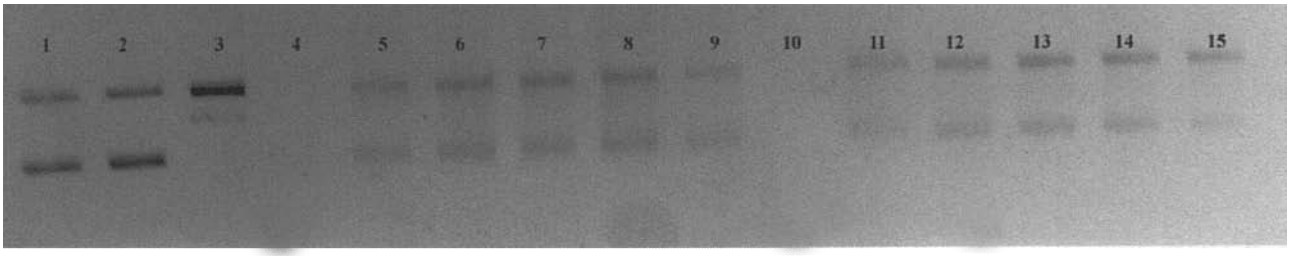

Fig.4— Photograph of gel showing DNA cleavage protection by HCF extracts

Lane: **1:** Control DNA, where faster moving band corresponds to circular-DNA and slow moving band corresponds to linear-DNA; **2:** UV-treated DNA; in the absence of free-radicals, it is not degraded; **3:** DNA+ H_2O_2+ H_2O, UV generated free radicals have cleaved the circular-DNA into linear DNA and open circular DNA; **4:** H_2O_2+ H_2O; control; **5-9:** DNA+ H_2O_2+ Crude aqueous extract (100 to 500 µg), here formation of open circular DNA was suppressed; **10:** H_2O_2+ MeOH, control; **11-15:** DNA+ H_2O_2+ Crude MeOH extract (100 to 500 µg), where also formation of open circular DNA was suppressed.

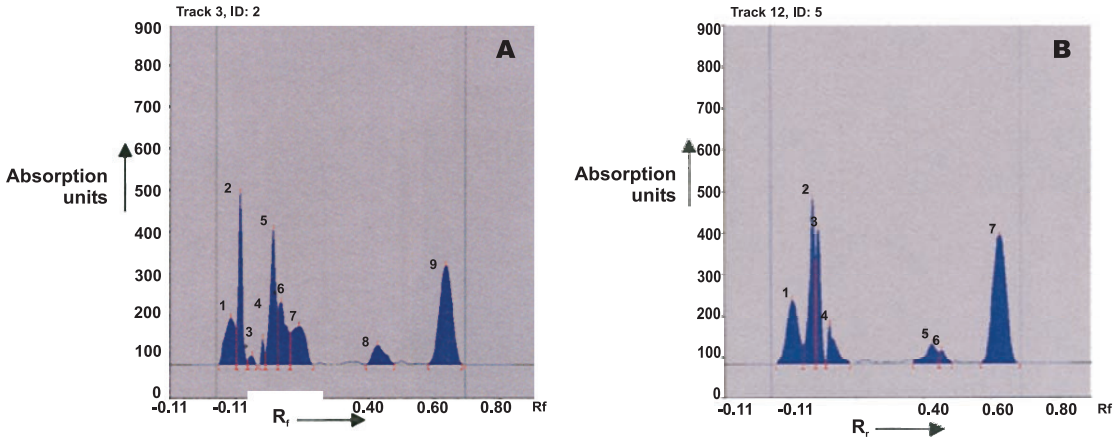

Fig. 5— HPTL Chromatogram of HCF. A: Crude aqueous extract after hexane washing; B: Crude methanolic extract as a function of Rf.

Discussion

In living organisms, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are common free radicals known to damage lipids, proteins, enzymes, and DNA[11-12]. Damages caused by such free radicals (like hydroxyl radicals, super-oxide anions, hydrogen peroxide and nitric oxides) lead to cell or tissue injury, ageing and a wide range of degenerative diseases, including asthma, ulcer, cancer and Parkinson's disease[10,12]. Harman in 1965 has proposed free radical theory, as a result of accumulation of oxidatively damaged macromolecules and consequently cells or tissues due to aerobic metabolism to which individuals are continuously exposed, giving rise to aging in human beings and other animals[13-14].

The herbs in the *Ayurved* are broadly classified into two groups: Food and Drugs. Accordingly, more then 2000 plant species have been identified, which are used successfully as medicines. Extracts of parts of thousands of plants like stem, leaves, flowers, fruits, seeds, tubers, roots, and bark have always been utilized by human beings in traditional medicinal practices. Their medicinal benefits typically result from the constituent secondary metabolites present in them[15]. Among the commonly available secondary products of a plant, alkaloids, flavonoids, phenolics, carotenoids and vitamins are major groups of compounds with antioxidant properties[15-16]. As plants produce huge amount of antioxidants, they can represent a potential source of new compounds with antioxidant activities[12, 17]. For this purpose, *in vitro* antioxidant activities like DPPH-free radical scavenging and reducing power were evaluated. Simultaneously the extracts were also assessed for the inhibition of gelatinolytic activity of collagenase type I and II enzymes. Gelatinolytic activity of collagenase type I and II was evaluated, since matrix metalloproteinases (MMPs) express collagenase activities which degrade cartilage matrix[18-22].

Since the dried HC flowers are utilized by the tribes from Satpura area of Maharashtra[4], to make syrup and vegetable preparations for consumption on a regular basis and cherished with a belief that it imparted them vigor and vitality, it may be inferred that their consumption would be useful to the consumer for managing the oxidative stress. Since aqueous extract exhibited more free radical scavenging activity than found in methanolic extracts, our data validates traditional usage of the dried flowers of HC by the tribal population. Regular supplement of HCF nutrition in the diet may provide requisite amount of antioxidants essential for reducing stress.

In the case of reducing power ability also, the crude aqueous extract without hexane washing showed comparatively low activity even at 1000 µg/ml concentration. Although differences in the reducing power amongst the extracts tested are not significantly high, they show considerable reducing capacity, *albeit* low as compared to the standard ascorbic acid. As indicated earlier, the standard ascorbic acid (at 10 µg/ml) has shown an absorbance of 0.031 vis-à-vis 0.05 to 0.078 by the extracts (at 100 µg/ml). When methanolic extract was mixed with the aqueous extract washed with hexane in equal quantity,

reducing capacity increased, as seen in DPPH-free radical scavenging activity. The free radical scavenging ability of two HCF extracts was validated by their protection against DNA strand scission (Figure 4). Elevated DNA damage may be reflection of either increased oxidative stress or decreased antioxidant ability[23].

Oxidative damage at cellular level is the major cause for the inflammation in various organs, which in turn may be the root cause of several diseases. Several MMPs are implicated in alveolar inflammation and finally disruption of alveolar wall[22]. In fact, oxidative damage causing inflammation and leading to osteoarthritis (OA) and various respiratory disorders is established. It is also found that increased levels of ROS/RNS and pro-inflammatory cytokines, such as interleukin-1 (IL-1) induce oxidative DNA damage in OA articular cartilage as compared to non-OA articular cartilage in pigs[24]. To address such inflammation-based breathing problems, plant-derived inhibitors of collagenase type II include curcumin, quercetin and aristolochic acid[19], hyaluronidase includes poly-phenols of blackberry[25] and gelatinase include green tea poly-phenols[20-21,26], useful in prescribed anti-arthritic drugs. For example, anti-inflammatory drugs, indomethacin and dexamethasone (0.1-0.2 mM), are moderate inhibitors of hyaluronidase[27]; doxycycline inhibited collagenase and gelatinase in cartilage *in vitro*[28]. Immuno-reactivity for MMP-2 and MMP-9 has been enhanced in lung from emphysema-related disorders caused by chronic inflammation[22]; green tea-derived epigallocatechin-3-gallate (EGCG) is a potent inhibitor of the gelatinases [matrix metalloprotease MMP-2 and MMP-9]; enzymes associated with invasive potential are frequently over-expressed in inflammation and cancer[20-21]. Gelatinase A (MMP-2) and gelatinase B (MMP-9) are reportedly involved in alveolar inflammation and disruption of alveolar wall[22]. In this context, gelatinase (MMP-2 and MMP-9) inhibitory activities of HCF aqueous extracts are promising to open an avenue for further study.

Combined effect of HCF extracts

The aqueous extract washed with hexane has shown comparatively higher DPPH free-radical scavenging activity than that of the aqueous extract, which was not washed with hexane, indicating the presence of inhibitors of fatty nature, soluble in hexane. This suggested that the compounds imparting the activity seem to be present in less proportion in methanolic extract or methanol alone might not be efficient in extracting the required compound(s), responsible for the activity as revealed in the HPTLC profile (Figure 5). DPPH free radical scavenging activity increased further to 78%, when extracts were mixed (e.g. extract v). The highest DPPH-free radical scavenging activity shown by the individual compounds isolated by HPTLC ranged from 30% to 54% and their IC_{50} value was 776 µg/ml. Thus, individual compounds either in extract or after separation by HPTLC, showed less effect. Since separated two molecules showed maximum activity up to 58%, and total activity in the extract is up to 78%, there is a distinct possibility of the presence of few more compounds with activity, which the present solvent system could not separate. Their activity increased by the presence of co-extractives. A detailed study regarding purification of individual compounds and their assay will throw more light on this phenomenon.

Although all HCF extracts showed lower free radical scavenging activity than the standard antioxidant like vitamin C (10 to100 µg/ml standard ascorbic acid showed 15.9 to 84.0% activity), HCF extracts exhibited increase in the antioxidant activity with an increasing concentration. This is evident from the IC_{50} value for the standard (39 µg/ml), while that of all extracts was ten times more, in the range of 400 to 700 µg/ml. This finding is partially supportive to the previous findings, where pre-treatment of experimental animals with *H. cannabinus* leaf extract prevented alteration of the membrane fluidity, with a decrease in the cholesterol:phospholipid ratio and role in per-oxidation by inhibiting the free radical attack on bio-membranes[29]. From the IC_{50} value, flowers of *H. cannabinus* appeared consumable as a source of antioxidants.

Conclusion

In conclusion, this study correlates *H. cannabinus* (HCF) active molecules with anti-oxidant/reducing power activity, gelatinolytic inhibition and anti-inflammation as practiced in traditional *Ayurved* and validates HCF as a potent candidate for preventing or fighting inflammation through managing ROS and RNS.

Acknowledgement

The authors are grateful to Dr. S. S. Kadam (Vice-Chancellor, BVU) for supporting this work, Dr. P. K. Ranjekar (Director, IRSHA) for providing facilities, and Professor S. P. Mahadik for invaluable discussions.

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