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RESEARCH PAPER

ANNUAL VARIATION IN CAMPTOTHECIN AND 9-METHOXY CAMPTOTHECIN ACCUMULATION AND ITS DETERMINATION IN DIFFERENT PARTS OF *NOTHAPODYTES NIMMONIANA* BY HPLC ANALYSIS

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Nothapodytes nimmoniana is a rich source of camptothecin (CPT) and 9-methoxycamptothecin (9-MCPT), a well known anticancer alkaloid. We investigated annual variation in the concentration of CPT and 9-MCPT in different parts of _N. nimmoniana_, collected during three consecutive year starting from 2008 to 2010. The CPT and 9-MCPT content in _N. nimmoniana_ extracts was determined by HPLC analysis. The maximum CPT and 9-MCPT accumulation in different parts of _N. nimmoniana_ was found during the year 2010, followed by year 2008 and 2009. The CPT and 9-MCPT accumulation in different parts of _N. nimmoniana_ collected during all the three years was in the following order root > fruit > stem > leaf. The root collected in the month of February 2010, showed higher accumulation of CPT (2.65%) and 9-MCPT (1.06%) than fruit, stem and leaf of _N. nimmoniana_. The root showed more than 2-fold accumulation of CPT and 9-MCPT than fruit, stem and leaves of _N. nimmoniana_. The months starting from October to February were characterized by high humidity, low air temperature and less evaporation rate which enhanced CPT and 9-MCPT accumulation in different parts of _N. nimmoniana_ during all the three years (2008 to 2010). Moreover the variations in CPT and 9-MCPT accumulation might be because of changes in seasonal patterns, weather events, temperature changes, biotic and abiotic stresses. These findings indicate that the accumulation of CPT and 9-MCPT in different parts of _N. nimmoniana_ vary annually.

Key words: Annual variation, Camptothecin, 9-Methoxycamptothecin, HPLC, *Nothapodytes nimmoniana*.

INTRODUCTION

Nothapodytes nimmoniana (J. Graham) Mabberly [formerly, *Nothapodytes foetida* (Wight) Sleumer] is a rich source of potent alkaloid camptothecin (CPT) and 9-methoxy camptothecin (9-MCPT) (Govindachari and Viswanathan, 1972; Fulzele *et al* 2001). In addition to anticancer properties exhibited by plants (Chowdhury *et al* 2012; Zia Uddin *et al* 2012), biological screenings have recognized that CPT and its derivative, 9-MCPT, have promising anti-cancer drug of twenty first century (Wu *et al* 1995). The cellular target of CPT is DNA topoisomerase 1 and its numerous analogs have been synthesized as potential therapeutic agents (Wall and Wani, 1995). CPT inhibits the replication of human immuno deficiency virus (HIV) *in vitro* and is also shown to be effective in the complete remission of breast, cervical, lung and uterine cancer (Priel *et al* 1991; Takeuchi *et al* 1991; Potmesil, 1994). CPT itself is not used clinically due to its

cytotoxicity, but its derivatives are most effective for the treatment of cancer throughout the world. Interest in CPT congeners was renewed when it was reported that water soluble derivatives of CPT (topotecan and irinotecan) are currently being used for the treatment of colorectal and ovarian cancer (Vladu *et al* 2000). These two semi-synthetic derivatives of CPT have the ability to decrease cytotoxicity and increase water solubility. Higher concentration of CPT alkaloid is present in *N. nimmoniana* than other plants *Camptotheca acuminata*, *Ervatamia heyneana* and some species of the genus *Ophiorrhiza*, and thus it represent the most convenient and attractive source for large-scale production of this pharmacologically interesting biologically active compound (Govindachari and Viswanathan, 1972). Some other pharmacological activities of *N. nimmoniana* have been also reported (Sheeja *et al* 2005; Namdeo *et al* 2010a; Khan *et al* 2012).

Primary metabolism in plants is essential for growth and development of cells, tissues and organs while secondary metabolism was generally regarded as a nonessential process that produce by-products or plant wastes regarded as secondary metabolites. However, there is evidence that secondary metabolites such as alkaloids, glycosides, flavonoids, phenolics and terpenoids may contribute to plant defense against attacks by animals as well as microorganisms (van der Meijden, 1996). In addition, the cytotoxic nature of some secondary metabolites has made them medicinally important. Examples include CPT and its derivatives from *N. nimmoniana*, the taxanes from Taxus species and the ginkgolides from *Ginkgo biloba* L (Heinstein and Chang, 1994). Numerous factors influence the concentration of active constituents present in the herbal drug. Some of the notable factors are time and period of collection, geographical origin and climatic conditions. Many times even the absence of active constituents may be observed with same plant collected from different region, which was difficult to assess earlier as evidenced by several research reports (Bilia, 2002).

Recent developments in analytical techniques made this task easier to identify and quantify the presence of active constituents in the herbal drugs, extracts and their formulations (Bilia, 2002). High performance liquid chromatography (HPLC) is a very powerful and versatile chromatographic technique for the separation, selective detection, quantification or general profiling of natural products. The method is widespread and has been adapted to the analysis of a broad range of natural products (Wolfender, 2009). Now a days, HPLC is becoming a routine analytical technique for herbal drug standardization due to its advantages such as simplicity, speed, need for minimum sample clean up, reproducibility, accuracy, and reliability (Wilson *et al* 2005).

We hypothesized that annual variation provoked changes in the content of biologically active secondary metabolites in a particular part of the plant could be of vital interest from the research point of view. The present study was aimed to determine annual variation in the accumulation of medicinally important alkaloid, camptothecin and 9-methoxycamptothecin and its determination in different parts of *N. nimmoniana* by HPLC analysis.

MATERIALS AND METHODS

Plant material and chemicals

The plant material of *N. nimmoniana* was collected from Mahabaleshwar region of Maharashtra state, India, and was authenticated by Chief Botanist, Botanical Survey of India (BSI) and voucher specimen (NNASP1) was kept at departmental herbarium of BSI. The plant material was collected starting from the month of January to December during three consecutive years (2008-2010). The chemicals used in the experiments were of analytical grade. Standard of CPT and 9-MCPT (purity 95% *w/w*) was purchased from Hi Media (Mumbai, India).

High performance liquid chromatography

Instrumentation and chromatographic conditions

The HPLC system used consist of a pump (Agilent 1200 series) with a sampler programmed at 20 μl capacity per injection. The UV detector operated at a wavelength of 360 nm. The column used was Germany and Eclipse XDB-C_{18} (250 mm × 4.6 mm, 5.0 μ) from Agilent Technologies, USA. The CPT and 9-MCPT content were determined using acetonitrile: water (45:55 *v/v*) as the optimized mobile phase at the flow rate of 1 ml min^{-1}. The mobile phase was degassed by ultrasonic vibrations prior to use.

Preparation of standard solutions and calibration curve of CPT and 9-MCPT

The stock solution of CPT was prepared by dissolving 3 mg of CPT in 10 ml methanol (300 μg/ml). The aliquots were prepared by dilution of the stock solution with methanol to reach the concentration range of 3-15 μg/ml. The stock solution of 9-MCPT was prepared by dissolving 5 mg of 9-MCPT in 50 ml of methanol (100 μg/ml).

The aliquots were prepared by dilution of the stock solution with methanol to reach the concentration range of 20-140 μg/ml. Triplicate 20 μl injections were made six times for each concentration and chromatographed under the conditions described above. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs (Namdeo *et al* 2010b).

Sample preparation and extraction of CPT and 9-MCPT from *N. nimmoniana*

The fruits of *N. nimmoniana* were collected, thoroughly washed and dried in shade. Dried fruits were powdered to pass 20 mesh sieves and stored in sealed plastic bags. The 500 mg of the various powdered material was mixed with 5 ml of methanol (MeOH) in a volumetric flask and vortexed for 2 min followed by sonication (44 KHz, Branson Ultrasonic Cleaner, USA) at room temperature for 5 min. The process was repeated thrice for complete extraction of CPT and 9-MCPT. After sonication, methanolic extracts were combined and evaporated to dryness *in vacuo* using a Rotary evaporator (RE111, Switzerland).

Quantitation of CPT and 9-MCPT from *N. nimmoniana*

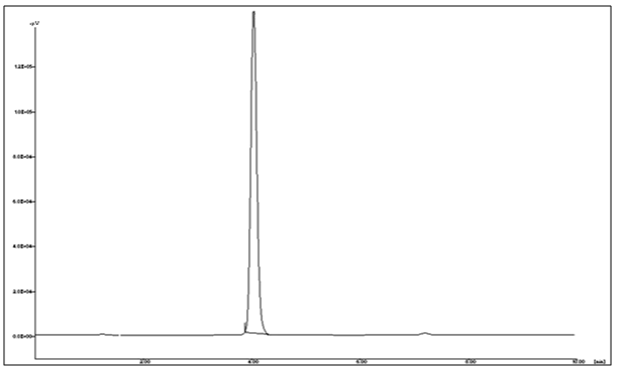
For determination of CPT and 9-MCPT content, the dried extract was transferred into polypropylene micro-centrifuge tubes, mixed with HPLC grade MeOH (1 ml), vortexed for 20s followed by centrifugation at 5000 rpm for 10 min. The extract solution was filtered through 0.45 μm syringe filter. The 20 μl of extract solution in the concentration range of 10 μg/ml was applied in triplicate to the column. The peak area was recorded at the wavelength of 360 nm. The amounts of CPT and 9-MCPT in the samples were calculated using the linear regression equation derived from the calibration curves.

Statistical analysis

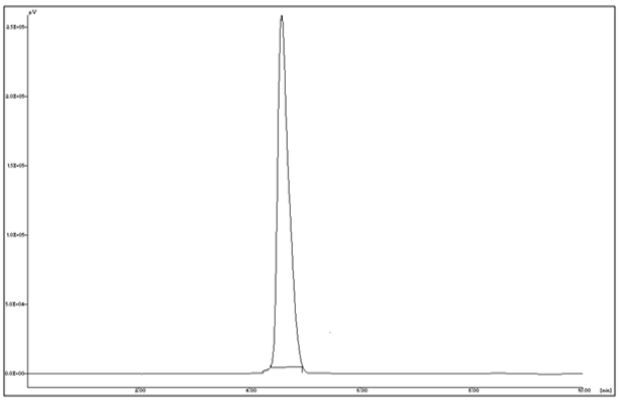
Analysis was carried out by using MS Office Excel 2007. All values are expressed as mean±SD (n=3).

RESULTS AND DISCUSSION

For the analysis of raw herbal materials and herbal formulations, HPLC is superior to other instrumental analytical techniques because it is simple, fast, sensitive and automated technique with higher degree of resolution and reproducibility. The HPLC technique is, therefore suggested for the determination of CPT and 9-MCPT in different parts of *N. nimmoniana*. The method was validated and the standard deviation proved that the accuracy and reproducibility was found to be satisfactory for the quantitative assay. Different mobile phases were tried and optimized as acetonitrile: water (45:55 *v/v*) in order to find the best separation of CPT and 9-MCPT with the retention time of 4.02 min and 4.57 min, respectively. The HPLC chromatogram of CPT, 9-MCPT and different extracts of *N. nimmoniana* is presented in **Figure 1, 2**. The concentration of CPT and 9-MCPT in methanolic extract of different parts of *N. nimmoniana* was calculated by the regression equation, y = 113140x + 453.5 and y = 1592.60x + 1876, respectively.



(a)



(b)

Fig. 1. HPLC chromatogram of camptothecin (a) and 9-methoxycamptothecin (b) authentic sample

Usually, chemical defense can be induced by biotic and abiotic factors. Plants produce more chemical defense agents to protect themselves under stress since growth is slower and biomass loss by damage becomes worse under these conditions than under regular conditions. Several factors such as worldwide changes in seasonal patterns, weather events, temperature changes, geographical location, biotic and abiotic stresses may affect the production of secondary metabolites in plants (Cavaliere, 2009; Namdeo *et al* 2010b; Szakiel *et al* 2011).

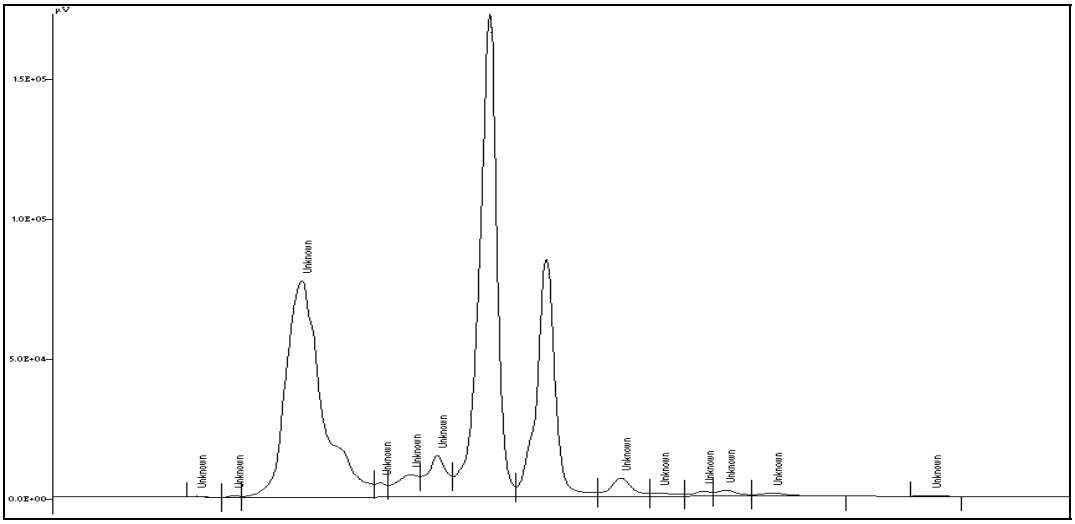


Fig. 2. HPLC chromatogram of *N. nimmoniana* root extract collected during year 2010 (February)

N. nimmoniana might also have an induction mechanism to produce CPT and 9-MCPT. In our study, annual variation in CPT and 9-MCPT accumulation was observed in different parts of *N. nimmoniana*. The results are presented in **Figure 3-8**.

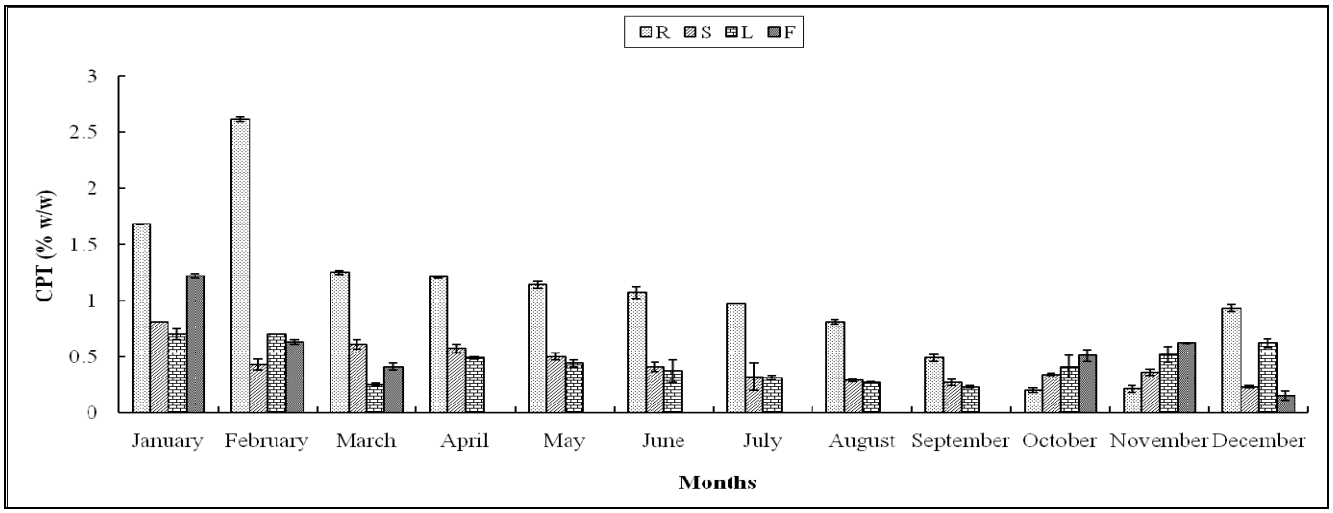


Fig. 3. Annual variation in CPT content of different parts of *N. nimmoniana* collected during year 2008
*Values are expressed as mean±SD of three parallel measurements

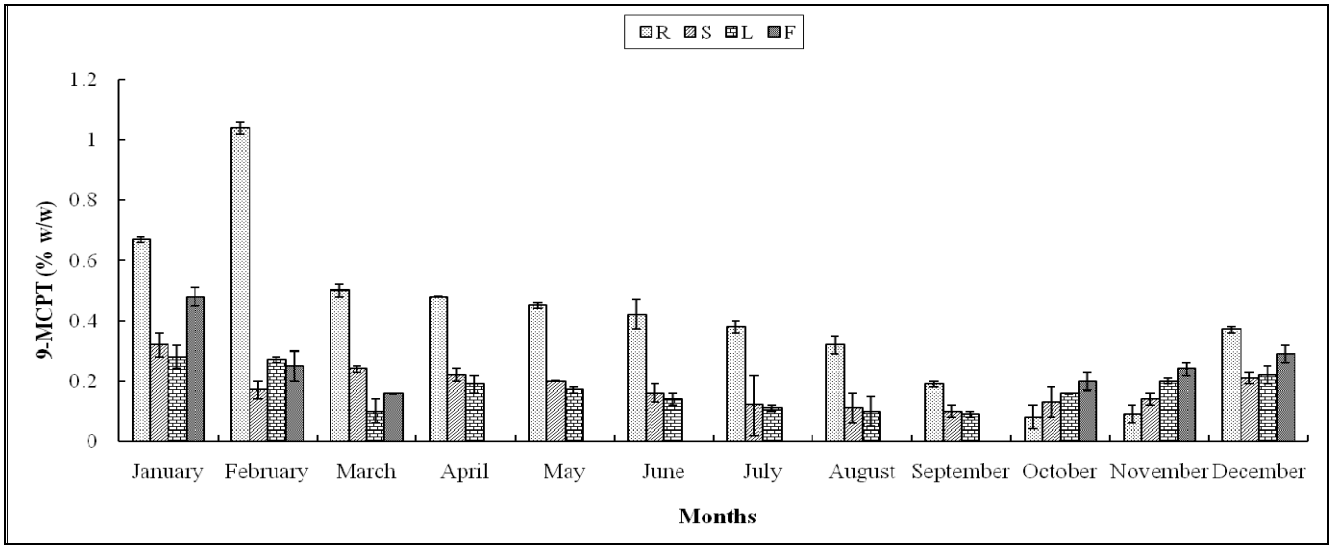


Fig. 4. Annual variation in 9-MCPT content of different parts of *N. nimmoniana* collected during year 2008
*Values are expressed as mean±SD of three parallel measurements

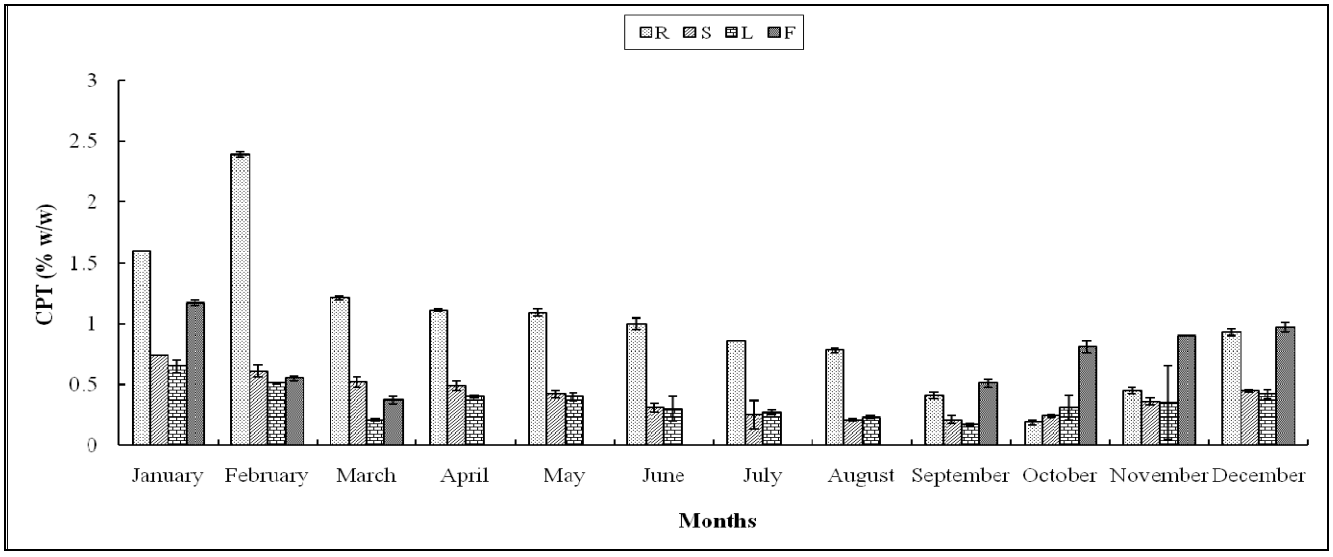


Fig. 5. Annual variation in CPT content of different parts of *N. nimmoniana* collected during year 2009
*Values are expressed as mean±SD of three parallel measurements

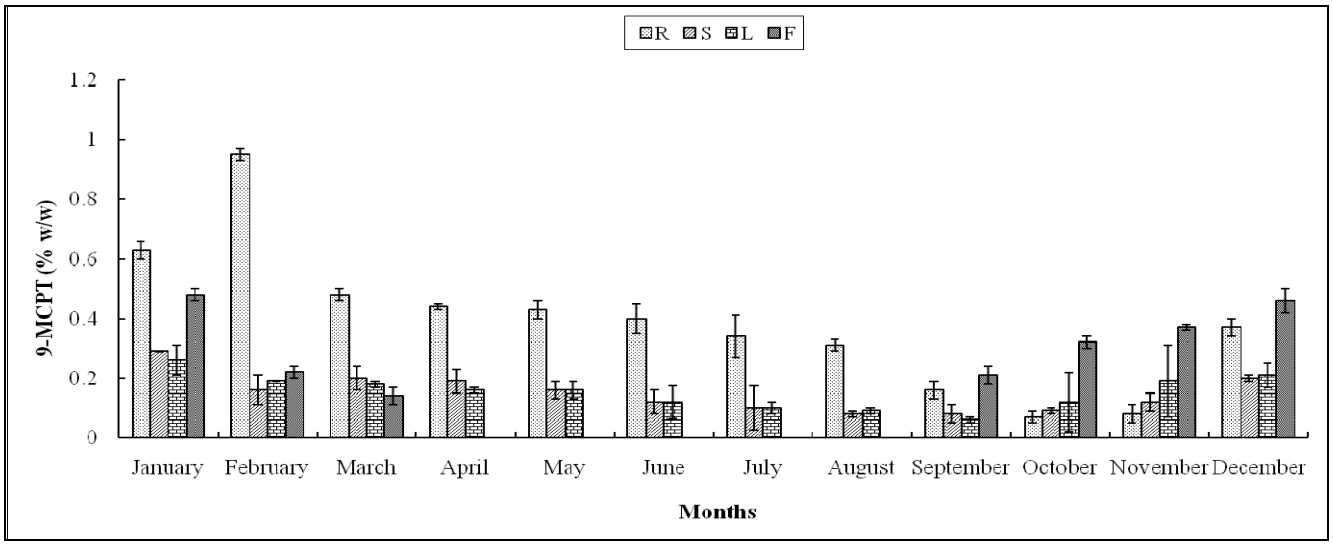


Fig. 6. Annual variation in 9-MCPT content of different parts of *N. nimmoniana* collected during year 2009
*Values are expressed as mean±SD of three parallel measurements

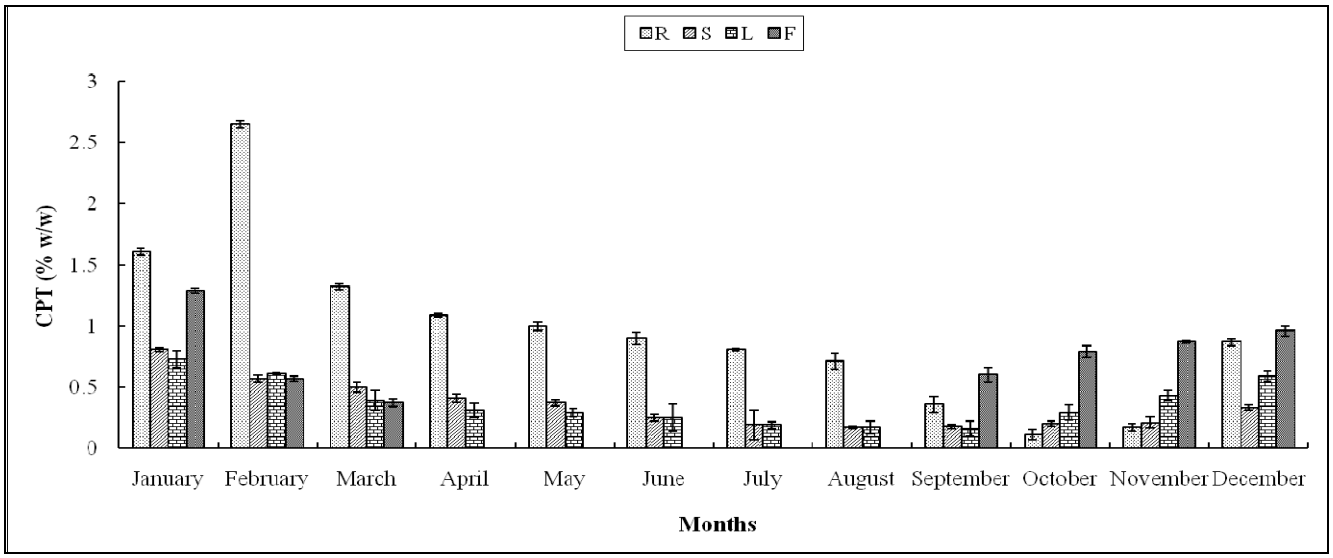


Fig. 7. Annual variation in CPT content of different parts of *N. nimmoniana* collected during year 2010
*Values are expressed as mean±SD of three parallel measurements

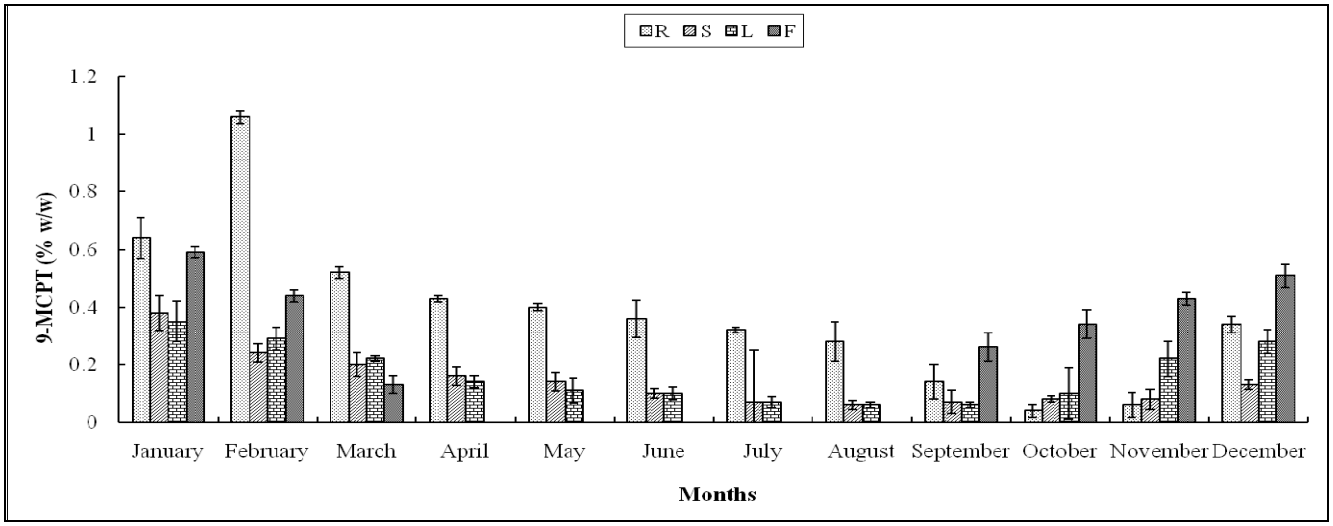


Fig. 8. Annual variation in 9-MCPT content of different parts of *N. nimmoniana* collected during year 2010
*Values are expressed as mean ± SD of three parallel measurements

The maximum CPT and 9-MCPT accumulation in different parts of *N. nimmoniana* was found during the year 2010, followed by year 2008 and 2009. The CPT and 9-MCPT accumulation in different parts of *N. nimmoniana* collected during all the three years was in the following order: root > fruit > stem > leaf.

The root collected in the month of February 2010, showed maximum accumulation of CPT (2.65%) and 9-MCPT (1.06%) than fruit, stem and leaf of *N. nimmoniana*. The root showed more than 2-fold accumulation of CPT and 9-MCPT than that of fruit, stem and leaves of *N. nimmoniana.* The months starting from October to February are characterized by high humidity, low air temperature and less evaporation rate enhanced CPT and 9-MCPT accumulation in different parts of *N. nimmoniana* during all the three years (2008 to 2010).

Moreover, year 2008 was characterized by delay fruiting (in the month of October) while year 2009 and 2010 showed early fruiting (in the month of September). The month April to August showed absence of fruiting during the year 2008 to 2010.

Distribution of CPT and 9-MCPT in *N. foetida* is reported in literature (Fulzele and Satdive, 2005). The bark of *C. acuminata* accumulated lower concentration of CPT, whereas leaves contained 2.5-fold higher CPT than that of the bark (Lopez-Meyer and Nessler, 1997). *N. nimmoniana* trees cultivated in north-western agro-climatic region of Jammu, India, accumulated 0.1% *w/w* of CPT in roots and seeds, whereas bark produced lower concentrations of CPT (Puri *et al* 1999). Also the geographical variation in CPT content of *N. nimmoniana* was reported previously (Namdeo and Sharma, 2012). The influence of climatic factors on CPT content in *Camptotheca acuminata* was reported (Yan *et al* 2003). The samples of five oat cultivars located in different parts of Norway during 1985-1990 were shown considerable differences in deoxynivalenol (DON) content. Moreover, the HPTLC densitometirc evaluation of CPT was also reported previously (Namdeo *et al* 2010c).

The mean DON concentration was found to be highest in 1988 and 1989. These years were both characterized by heavy rainfall. The lowest DON concentration was found in 1987 and 1990, two years with drought weather (Langseth *et al* 1995). Azadirachtin content in the seeds of *Azadirachta indica* varied from 200 to 16,000 ppm (*μ*g/g of the seed kernel). The highest content of azadirachtin was recorded in the neem tree populations growing in the southern part of India (Kaushik *et al* 2007). Also the seasonal variations of the antioxidant composition in ground bamboo *Sasa argentea striatus* leaves reported (Ni *et al* 2012). Our findings indicate that the accumulation of CPT and 9-MCPT in different parts of *N. nimmoniana* varied annually. The CPT and 9-MCPT accumulation in different parts of *N. nimmoniana* collected during the year 2010 was higher than the plant material collected during 2008 and 2009. The CPT and 9-MCPT content of *N. nimmoniana* root was more than fruit, stem and leaf. Lower to average air temperature and evaporation capacity, favored CPT and 9-MCPT production from the month October to February, showed that there was an induction mechanism to produce CPT and 9-MCPT in different parts of *N. nimmoniana*. The variations in CPT and 9-MCPT content might be because of changes in seasonal patterns, weather events, temperature changes, biotic and abiotic stresses. The results

of the present study showed annual variation in CPT and 9-MCPT content of different parts of *N. nimmoniana*.

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