Estimation of the fern toxin, ptaquiloside, in certain Indian ferns other than bracken

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Ptaquiloside (Pta) concentration was determined in 40 non-bracken fern samples collected mostly from the northern mountaineous State of Uttaranchal (Ut), India. Of these, only *Onychium contiguum* contained high levels; 499 and 595 mg/kg of Pta on dry matter basis in the two samples collected. A few samples of *Diplazium esculentum*, *Polystichum squarrosum* and *Dryopteris juxtaposita* showed moderate levels (19 to 31 mg/kg), but most samples had no detectable Pta present. Other ferns species such as *Cheilanthes farinosa* and *Christella dentata* contained nil to a very low level of Pta (0.4 mg/kg), while *Adiantum incisum* and *Pteris stanophylla* had no detectable Pta present. Samples of *O. contiguum* were collected from high-altitude areas of the Himalayas (District Chamoli and Uttarkashi), where enzootic bovine haematuria is not uncommon. This fern was reported in trials with guinea pigs to induce ileac, urinary bladder and mammary tumours on prolonged feeding, although in experimental rats it failed to induce any mortality and malignancy. The present study indicates that a few non-bracken fern species can contain high levels of the fern toxin, Pta, which may induce hazardous effects for animals and men, either alone or in combination with bracken fern.

Keywords: Bracken and non-bracken fern, enzootic bovine haematuria, ptaquiloside, *O. contiguum*.

ENZOOTIC bovine haematuria (EBH) is an economically important chronic disease of hill cattle in India and elsewhere. It is characterized by intermittent haematuria, chronic cystitis and a variety of benign and malignant neoplastic conditions of the urinary bladder[1]. Pteridophyte bracken fern, which contains ptaquiloside (Pta) and a number of other toxins, is considered to cause this disease. Pta is a nor-sesquiterpenoid glycoside and it is clastogenic, mutagenic and carcinogenic. It undergoes a series of reactions and produces a reactive aglycone dienone intermediate, inactive pterosin-B and DNA adducts. The dienone is the ultimate carcinogen. This is activated at alkaline pH, which is considered as the reason for the location of tumours in the urinary bladder of ruminants and the ileum of rats[2].

The level of Pta in ferns has been estimated in different countries, including Australia and New Zealand. These ferns were found to contain 0 to 9776 (mean 1257) mg/kg levels of Pta[2]. In India, Lal Krishna and Dawra[3] were the first to report Pta in *Pteris quadriaurita* and *Onychium contiguum*, besides bracken fern by TLC method. In our laboratory Pta was estimated in bracken fern from different mountainous regions of India, with levels varying from 0 to 1026 (mean 149) mg/kg[4]. The level of fern toxin had positive correlation with altitude and latitude, and finally with the prevalence of EBH in these areas[5].

From abroad also workers[6] reported the distribution of Pta and Pta-like compounds in Pteridaceae by chemical assay (TLC) and the modified Ames test. They observed the widespread occurrence of such compounds in a variety of ferns, including *Cheilanthes myriophylla*, *Cibotium harometz*, *Dennstaedtia scabra*, *Histiopteris incisa*, *Pityrogramma calomelanos*, *Pteris cretica*, *P. nipponica*, *P. oshimensis*, *P. tremula* and *P. wallichiana*. Smith and associates[7] analysed Pta in *Cheilanthes sieberi* samples collected from New Zealand and Australia using reversed-phase HPLC and indicated that this fern contained Pta and other potentially carcinogenic pterosin B precursors. Similarly, earlier workers[8] also reported the presence of Pta and related substances in rock fern, *C. sieberi*.

Experimental attempts were made to induce toxicity/malignancy in laboratory animals using non-bracken ferns. Only *O. contiguum* was able to induce ileac, urinary bladder and mammary cancers in guinea pigs after prolonged feeding[1], while in rats it failed to induce any mortality or malignancy on long-term feeding[9,10]. It is evident that under field conditions, cattle may consume a variety of ferns at one time while grazing or compelling situation under stall-feeding. This necessitated us to investigate the levels of Pta in different ferns from various regions of Uttaranchal (Ut), a Himalayan State of northern India where EBH is prevalent. The results of these investigations are reported in this communication.

Fern samples were collected from different places at Ut (long. E–W 81–77°; lat. N–S 32–29°, altitude 500–5000 m asl). These were sent for identification either to the Department of Botany, Kumaon University, Nainital or Punjabi University, Patiala. Samples of both young and mature fern were collected in thin polythene bags and freeze-dried in Edwards Modulyo Freezedrier, UK for 2–3 days using P_2O_5 and/or shade-dried in room temperature under a fan for two days. Each sample was powdered using in a laboratory mill and stored in dry plastic bags. Concentrations of the fern toxin, Pta were estimated in 40 samples using a reversed-phase HPLC method[8], either at the Biochemistry Laboratory, Regional Research Station,

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IVRI, Palampur, or at HortResearch, Ruakura Research Centre (Hort), Hamilton, New Zealand. The samples were extracted and extracts were partially purified for analysis by the procedure of Olerich *et al.*[43].

For the extraction of pterosin-B, 2 g fern powder sample was taken in a 100 ml clean conical flask and mixed thoroughly with 10 ml of HPLC-graded methanol by a magnetic bar for 30 min. This mixture was filtered using Whatman No. 1 filter paper and the filtrate was collected. Likewise, the filtrate was made two more times with the same residues. This was vacuum-dried using vacuum-drier at 42°C. To this, 10 ml of distilled water was added and made into suspension. Further, 10 ml of extra pure ethylacetate was also added and mixed thoroughly by gentle shaking. The mixture was filled in separating funnels and aqueous layer was removed. This step was repeated twice. Pooled aqueous fractions were vacuum dried. The residue thus obtained was ready for HPLC injection.

For the extraction of Pta 2 g fern powder sample was treated with 10 ml methanol and was filtered using Whatman No. 1 filter paper after intermittent shaking. The filtrate was collected from the suspension. The same procedure was repeated twice again with methanol and the filtrates were pooled. This was vacuum-dried at 42°C. The final residue was reconstituted with 1.5 ml of distilled water. After centrifuging at 10,000 rpm for 2 min, 0.75 ml of supernatant was added to 0.250 g of methanol-washed polyamide powder for the clean-up procedure. It was vortexed for thorough mixing and kept for 20 min. This was then centrifuged at 10,000 rpm for 2 min. The supernatant was removed and final volume was adjusted to 1 ml with distilled water in a clean vial. To this product, 75 μl of 1 N NaOH was added and kept for 1 h at 40°C. Then, 0.76 μl of 5N HCl was also added for a few seconds. This was centrifuged at 10,000 rpm for 10 min. Clear amber coloured supernatant thus obtained was ready for HPLC injection.

The toxins were subjected to reversed-phase HPLC (Waters HPLC system) for analysis[8], with 510 and 515 binary pumping system, rheodyne injector, 490 E multichannel detector and C_{18} reverse phase column (4.6×250 nm). The mobile phase was acetonitrile–water gradient (20 : 80 for the first 20 min followed by increase to 100% acetonitrile in next 20 min). Before injection, the supernatant collected was filtered by 0.22 μm filter paper and 20 μl of sample was injected into the HPLC system for 60 min. Elution at 29.50 min (standard Pta/pterosin-B usually elutes at this time) was detected at 220 and 260 nm using a computerized spectrophotometer. Then toxin concentration was calculated. Since Pta is a highly unstable compound containing glucose moiety and is soluble in water, it was necessary to calculate both pterosin-B (Pta is converted into pterosin-B by acid-base conversion) and Pta (pterosin-B is removed by polyamide clean-up). Pterosin-B estimation is the indirect way of calculating the concentration of Pta. For expressing the concentration of toxin in terms of dry weight basis, moisture content of bracken fern was estimated keeping known quantity of fern in an hot-air oven at 100°C overnight. The result was expressed as mg/kg.

During 2004, samples were tested by HortResearch, and all estimations were confirmed by liquid chromatography–mass spectrometry (LC–MS) to give unequivocal confirmation of the presence of Pta in the positive samples.

A total of 40 non-bracken fern samples were tested and results are presented in Table 1.

There were some differences in the HPLC techniques used at HortResearch[8] and IVRI (R. K. Dawra, unpublished). The HPLC column used at IVRI was C_{18} as compared to C_8 at HortResearch. The Horticulture Laboratory used methanol–H_2O as elution solvent compared to acetonitrile–H_2O gradient system at IVRI. Use of these modifications improved resolution and increased sensitivity. There were certain other modifications in the extraction procedure. At IVRI, estimation was started with more amount of fern powder, extraction was done with chloroform and the residue was extracted with methanol. The methanol extract was dried, residue dissolved in H_2O, treated with polyamide to remove any free pterosin B and treated solution was hydrolysed with alkali. Pterosin B was extracted from hydrolysate, and dissolved in suitable aliquot of solvent for HPLC analysis. This helped in quantitation of comparative lower level of toxins (R. K. Dwara, unpublished).

The results obtained from aqueous extracts of different non-bracken fern species indicated varying concentrations of Pta in different species of ferns from various places and different altitudes. Highest concentrations were seen in *O. contiguum*, 449 and 595 mg/kg in two samples collected. *O. contiguum* is a common fern in grazing areas in the Northern Indian States of the Himalayan region, where EBH is enzootic or sporadic. Interestingly, long-term experimental exposure to guinea pigs with *O. contiguum* collected from near Palampur, Himachal Pradesh caused tumours of the ileum, urinary bladder and mammary glands[1]. However, we[9] failed to induce specific lesions or ileal or urinary bladder tumours in laboratory rats by feeding *O. contiguum* collected from Mukteswar (30% w/w) in the feed mixture for a period of 180 days. *O. contiguum* fern collected from Mukteswar was found to contain 0.68 mg Pta/kg fern. In the present work, fern which was collected from high altitude areas of Joshimath, Dist. Chamoli and Dist. Bhatwari Uttarkashi contained much higher levels of Pta. In these areas, a high prevalence of EBH is known to occur and bracken fern was also found to contain high levels of Pta[4]. The role of *O. contiguum* in the causation of EBH requires more investigation.

Besides *O. contiguum*, the presence of Pta in other fern samples was not common. A few samples of *Diplazium esculentum*, *Polystichum squarrosum*, *Dryopteris juxtaposita*, *Chelianthes farinosa* and *Christella dentata* contained very low to moderate levels (0.4 to 30.5 mg/kg), while single samples of *Adiantum incisum* and *Pteris stanophylla* tested failed to show any Pta. Indian researchers[11] ana-

Table 1. Concentration (mg/kg) of ptaquiloside (Pta) in 40 fern samples collected from different sites in Uttaranchal

Fern	Area	Place	Altitude	Pta concentration	Testing laboratory/ year
Diplazium esculentum					
Young frond (*linguda*)	Enzootic	Joshimath–Chamoli	H.A.	n.d.	Hort, 2002
Mature	Enzootic	Joshimath–Chamoli	H.A.	n.d.	Hort, 2002
Mature	Enzootic	Jhala, Uttarkashi	H.A.	n.d.	Hort, 2003
Young frond (*linguda*)	Non-enzootic	Garampani, NT	L.A.	n.d.	IVRI, 2004
Young frond (*linguda*)	Non-enzootic	Kainchi, NT	L.A.	n.d.	IVRI, 2004
Young frond (*linguda*)	Non-enzootic	Saliyakund, NT	L.A.	n.d.	IVRI, 2004
Young frond/*linguda* (freeze-dried)	Non-enzootic	Garampani, NT	L.A.	n.d.	IVRI, 2004
Young frond/*linguda* (freeze-dried)	Non-enzootic	Kainchi, NT	L.A.	n.d.	IVRI, 2004
Young frond/*linguda* (freeze-dried)	Non-enzootic	Saliyakund, NT	L.A.	n.d.	IVRI, 2004
Mature	Enzootic	Kharsali, Jankichatti, Uttarkashi	H.A.	n.d.*	Hort, 2004
Young frond/*linguda*	Enzootic	Harsil, Gangotri, Uttarkashi	H.A.	18.9*	Hort, 2004
Polystichum squarrosum					
Green	Sporadic	Mukteswar, NT	M.A.	n.d.	Hort, 1997
Brown	Sporadic	Mukteswar, NT	M.A.	n.d.	Hort, 1997
Mature	Sporadic	Mukteswar, NT	M.A.	n.d. (Figures 1	Hort, 1997
Young frond (stage-I)	Sporadic	Mukteswar, NT	M.A.	n.d. and 2)	IVRI, 2002
Young frond (stage-I)	Sporadic	Mukteswar, NT	M.A.	n.d.	IVRI, 2002
Young frond (stage-I)	Sporadic	Mukteswar, NT	M.A.	n.d.	IVRI, 2002
Young frond (freeze-dried stage-I)	Sporadic	Mukteswar, NT	M.A.	n.d.	IVRI, 2002
Young frond (freeze-dried-stage-I)	Enzootic	Hartola, NT	M.A.	3.42	IVRI, 2002
Mature	Enzootic	Augustyamuni, Rudraprayag	H.A.	n.d.	IVRI, 2003
Young frond (stage-I)	Enzootic	Dharali, Uttarkashi	H.A.	n.d.	IVRI, 2004
Mature	Enzootic	Bhatwari, Uttarkashi	H.A.	30.5*	Hort, 2004
Mature	Enzootic	Hanumanchatti, Uttarkashi	H.A.	n.d.*	Hort, 2004
Christella dentata					
Mature	Non-enzootic	Izatnagar, Bareilly	S.L.	n.d.	IVRI, 2002
Mature	Non-enzootic	Izatnagar, Bareilly	S.L.	n.d.	IVRI, 2002
Mature (freeze-dried)	Non-enzootic	Izatnagar, Bareilly	S.L.	n.d.	IVRI, 2002
Mature (freeze-dried)	Non-enzootic	Izatnagar, Bareilly	S.L.	0.40	IVRI, 2003
Mature	Non-enzootic	Izatnagar, Bareilly	S.L.	n.d.	IVRI, 2003
Mature	Non-enzootic	Izatnagar, Bareilly	S.L.	n.d.*	Hort, 2004
Dryopteris juxtaposita					
Mature	Sporadic	Pauri, Uttarkashi	M.A.	n.d.	Hort, 2001
Mature	Enzootic	Harsil, Gangotri, Uttarkashi	H.A.	n.d.	IVRI, 2004
Mature	Enzootic	Hanumanchatti, Uttarkashi	H.A.	n.d.*	Hort, 2004
Mature	Enzootic	Harsil, Gangotri, Uttarkashi	H.A.	18.9*	Hort, 2004
Onychium contiguum					
Young frond	Enzootic	Joshimath, Chamoli	H.A.	498.5	Hort, 2002
Mature	Enzootic	Bhatwari, Uttarkashi	H.A.	594.7*	Hort, 2004
Cheilanthes farinosa					
Mature	Sporadic	Nainital (NT)	M.A.	n.d.	Hort, 1997
Mature	Enzootic	Tyoni, Dehradun	M.A.	0.40	IVRI, 2003
Mature	Enzootic	Barkot, Uttarkashi	H.A.	n.d.*	Hort, 2004
Adiantum incisum					
Mature	Sporadic	Pauri–Garhwal	M.A.	n.d.	Hort, 2002
Pteris stanophylla					
Mature	Enzootic	Phata, Rudraprayag	H.A.	n.d.	IVRI, 2003

Categories of area are enzootic, sporadic and non-enzootic for EBH. In some places sporadic cases occur. H.A., High altitude (>3000 m); MA, Medium altitude (> 2000 m); LA, Low altitude (> 1000 m) and S.L., sea level (< 100 m).
n.d., Pta not detected; *Results confirmed by LC–MS.

lysed aqueous extracts of *D. juxtaposita* and *P. squarrosum*[11] and detected the presence of 20.30 and 18.13 mg Pta/kg fern on a dry matter basis respectively. *P. squarrosum* and *D. juxtaposita* are prevalent in the Himalayan region and constitute the bulk of vegetation in grazing areas. The long-term toxicity of these ferns was tested in laboratory animals and cattle[12–18] but these ferns failed to induce carcinogenicity in these experimental animals.

C. farinosa collected for testing was found to contain very low to negligible levels of Pta. In contrast, other researchers[19] detected 26.3 mg Pta/kg in *C. farinosa*. This fern is found in abundance on rocks, generally shaded by certain other plants, which helps to retain moisture. On experimental feeding to rats and guinea pigs, *C. farinosa* also failed to induce mortality and carcinogenicity[9,10,20,21]. The genus *Cheilanthes* has been associated with either mutagenesis or carcinogenicity. *C. myriophylla* was shown[6] to have mutagenic properties using a modified Ames test, and to have components with similar or identical, mutogenic properties using TLC. *C. sieberi* has been shown to cause both the acute toxicity[22] and urinary bladder cancer[23] typical of bracken fern and has also been shown to contain significant quantities of Pta[7]. These findings together with the demonstration of Pta in *C. myriophylla*, albeit in small quantities, suggest that further analysis of members of this genus should be carried out.

The young fronds of *D. esculentum* are popularly known as fiddlehead, *linguda* or *kathura* and consumed as a seasonal vegetable during monsoon season in most Himalayan regions in northern India. Most shade- and freeze-dried samples of these ferns failed to show any Pta, but one sample collected from high-altitude area of Harsil–Gangotri had 19 mg/kg. Earlier studies have indicated that *linguda* is moderately toxic in guinea pigs and rabbits, while rats showed little adverse effect[24–26].

C. dentata was the only fern collected from tropical areas of Bareilly, Uttar Pradesh. Generally, this fern was found not to contain Pta. However, experimental feeding with fresh green fern induced mortality, acute toxic effects, including haematuria and haemorrhagic urinary bladder. Compared to fresh green fern, shade-dried fern was found to be more toxic[27–29]. It induced proliferative urocystica and adenoma only in one animal[30]. *C. dentata* was found to be comparatively less toxic to laboratory rabbits but it induced certain toxic and adverse immunologic effects in these animals[31].

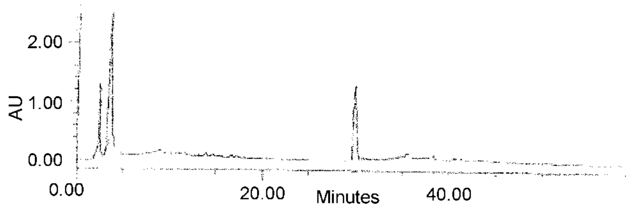

Figure 1. Standard Pterosin-B at 220 nm.

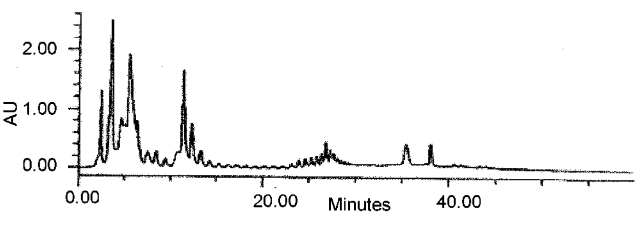

Figure 2. Absence of ptaquiloside in *P. squarrosum* fern at 220 nm.

On reviewing the subject of Pta occurrence in fern species, Smith *et al.*[32] provided evidence and opined that the difference in concentration of Pta appears to be related to the fern genotype, although other factors such as soil, season, site and stage of growth appear to have some influence. There also appears to be a relationship between the morphological appearance of ferns and different Pta concentrations. Pta levels were highest in those plants emerging in early spring. The method of drying, storage and extraction can also influence the concentration of Pta. The potential rapid breakdown of Pta in collected plant material necessitates controlled and timely drying procedures.

The toxicity and carcinogenicity of bracken fern for livestock, especially cattle and various species of laboratory animals have been of particular interest. Research has demonstrated that consumption of the plant caused both acute bracken poisoning and enzootic haematuria in cattle. Subsequently, its oncogenicity has been reported in various species of laboratory animals, namely mice, rats, guinea pigs, etc. Man also consumes bracken either directly as crosiers or the rhizomes, or its toxin indirectly through the consumption of milk from animals grazing on bracken fern[5].

Sources of bracken carcinogens in human diet and risk for bracken poisoning have been discussed by earlier researchers[33]. The use of bracken as a human food has been described over many centuries. In Europe, this has generally occurred only in times of food shortage. In marked contrast, in Canada, parts of USA, Siberia, China and especially, Japan and its neighbourhood, bracken fern is grown commercially for human use, as a food or herbal remedy. It has been reported[34] that 13,000 tonnes of bracken fern is imported annually into Japan, all in salted form. Young bracken fern is the most popular edible wild plant in Japan. In India also, fronds of *D. esculentum* (*linguda*) are consumed by local inhabitants in mountainous regions[26].

The results of a case control study[35] examining the factors responsible for higher frequency of oesophageal cancer in Nara, Wakayama and Mie prefectures of central Japan indicate that daily intake of bracken fern was found to elevate the risk of oesophageal cancer (relative risks for males and females, 2.10 and 3.67 respectively). Overall, the relative risk was 2.68 for individuals consuming bracken fern daily and 1.53 for those occasionally eating this product. When subjects were stratified by intake of *chagayu* (hot tea gruel), smoking and intake of meat and fruit, the increase in the risk of oesophageal cancer associated with consumption of bracken fern appeared to be confined to those who also took *chagayu* (5.2 and 4.7 for males and females respectively) and was enhanced (relative risks 9.2 and 6.4) amongst smokers.

It is important, however, to realize that indirect exposure to bracken toxicants may also occur. It is possible that rain might leach carcinogen fronds (fresh and dried) and dried rhizome with cold water for 14 days. Earlier workers[36] have carried out an examination of the influence of environmental factors on the pattern of mortality from gastric cancer in Gwynedd (North Wales). No positive correlation was obtained between such deaths and the percentage of bracken ferns in water catchments in the areas studied.

A more probable risk would appear to be associated with the passage of the carcinogens into animal products. It is clear that such compounds are readily transferred to milk[37–39]. In addition, they can cross the placenta in mice. Thus, exposure could occur in humans consuming contaminated milk and dairy products, the risk being greatest when bulk dairy processing (and thereby dilution) is not employed. Under such circumstances, infants may be at an additional tertiary risk as a result of passage of carcinogens into their mother's milk[40].

Epidemiological studies conducted in Costa Rica[41] suggest a high correlation between the consumption of milk potentially contaminated with bracken (*P. aquilinum,* L. var. *caudatum*) carcinogens and human gastric carcinoma. A similar situation is likely to exist in Colombia and Venezuela. Furthermore, a possible risk to human health by bracken in causing gastric cancer was also reported[42].

Bracken fern is categorized in 2B group by International Agency for Research on Cancer (IARC 1998). This group contains agents, mixture and exposure circumstances for which there is limited evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals. The aforementioned information is neither available nor worked out for non-bracken ferns.

From this study it can be concluded that few non-bracken ferns consistently contained high levels of the fern toxin, Pta, which may induce hazardous effects for animals and man, either alone or in combination with bracken fern. However, many species show the possibility of Pta occurrence in some samples. This means that there is a need for selection of non-Pta genotypes for human consumption, and that Pta analysis of fern species is important in areas where animals are regularly grazing ferns. Such studies are required in other mountainous States of India. For research, Pta analysis of fern feedstock should be an integral part of animal feeding studies in order to relate toxic observations to Pta concentrations.

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Mapping and evaluation of urban sprawling in the Mehadrigedda watershed in Visakhapatnam metropolitan region using remote sensing and GIS

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Urban sprawl detection and monitoring can be facilitated through the multispectral high resolution Indian Remote sensing Satellite (IRS-P6) images of LISS III and LISS IV sensors. The present study makes an attempt to analyse the dominant changes that occurred in land-use/land-cover features of Mehadrigedda watershed during the past three decades (1975–2005). It is found that the phenomenal increase in built-up area from the watershed is spreading haphazardly into the fringe of the Visakhapatnam metropolis.

Keywords: GIS, Mehadrigedda watershed, remote sensing, urban sprawling.

THE rapid growth and development of urban areas has been a topic of concern to planners all over the world. At present, with rapid urbanization and industrialization, there is an increasing pressure on land, water and environment in the cities of the country. As a consequence, the cities are expanding in all directions resulting in large-scale urban sprawl and changes in watershed areas. The spatial patterns of such changes are clearly noticed on the city peripherals. This is resulting in haphazard urban extension and development trends, changes in the spatial watershed land-use patterns, loss of productive agricultural lands, forest cover, loss in surface water bodies and depletion in groundwater levels due to increasing built-up area. There is an urgent need to constantly monitor such changes and take corrective measures towards planning such as urban utilities and infrastructure, traffic planning, water supply and sanitation, cadastral and real estates, vacant lands and urban land-use zoning[1–5].

Visakhapatnam is the second largest city in Andhra Pradesh (AP) with a population of around 22 lakhs. It is a sprawling industrial city and one of the emerging metropolis. The city has the advantage of a natural harbour, contrast in geological and topographical features with narrow coastal strip along the Bay of Bengal. Till 1970 development was confined to the port area, a focal point in the growth of the city in terms of industrial development. Subsequently, the establishment of Hindustan Shipyard, Indian Navy and further progress of rapid industrializa-

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