As the aim of the present study is to evaluate genetic ill effects of a substance which is used by human beings by habit human subjects would be the automatic choice, it does not involve extrapolation of the findings. However, studies based on human lymphocytes or fibroblasts or various human cell lines exposed in vitro to the agent or its extract (as it was done earlier for cigarette smoke by exposing the cells in vitro to CSC) no doubt provide 'short term' test system but none of them can compete with the complexity of the in vivo exposure. Recent innovation of S9 mixture to simulate in vivo exposure has no doubt added a new dimension to in vitro testing; but this solution of liver enzymes cannot just mimic all the metabolic complexities of human system, particularly the aspects of absorption, distribution and excretion. Besides, factors like route of exposure, the target organ(s), detoxification, etc. should also be taken under consideration. For this reason in evaluating potential mutagenicity of an agent increasing emphasis is being laid to use in vivo systems. Further, genetic damage can also be detected in blood lymphocytes obtained from individuals exposed to the agents and taken in culture. This non-target human cell in vitro system may not provide a true picture particularly for smokeless tobacco preparation or chemical agent. It is very important to analyze the cells that are exposed to the agent directly. Keeping this in view a part of the proposed work was conducted on habitual users of gudakhu (vide infra).

Although such a study in habitual users is more relevant, data from carefully controlled experiments on whole mammals are important to evaluate human exposure. Laboratory mammals in which metabolism of an agent would be
may I more or less similar to those in man be employed to study the potential mutagenicity and then the results may be extrapolated to man. The Adhoc committee of the Environmental Mutagen Society (1972), Committee-17 (1975), WHO (1985), Vanparys and Marsboom (1985), Styles (1985), Das (1986), Heddle (1991) also recommended the use of in vivo mammalian systems for the purpose.

2.1 CHOICE OF ANIMAL

The present investigator has chosen the mouse as experimental animal. Though the murine chromosomes compared to those of other laboratory mammals are not suitable for cytogenetic work for their characteristic shape and size the plus point which is perhaps the most important is that most of our knowledge on the protocols adopted here has come from experiments conducted on mice. Besides, in comparison to other laboratory mammals, it is cheaper; rearing and handling are easier too. Over and above, this laboratory is engaged in environmental mutagenesis work since long and mostly mice are being used as experimental animal, which provides an extra advantage to the author.

2.2 PROTOCOLS ADOPTED

A single test system is not sufficient for testing genotoxic potential of an agent particularly in view of contradictory reports. For having a comprehensive idea use of a battery of test systems is always desirable. WHO (1971), Committee-17(1975), EPA (1978), UK Guidelines (1979), EEC (1979), FSC (1980), ICPEMC Report (1983), Ashby (1986), Carrano and Natarajan (1988) and others also suggest to adopt different protocols for the purpose. Although multiple testing approach which comprises a series of individuals assays should yield definitive information on the substance under investigation, the number of tests, specially the animal procedures, constitutes a substantial outlay in time and resources. Recently the "combined testing
protocol (CTP)" for whole animal experimentation has become very attractive (Legator and Harper, 1983). In the CTP different tissues for different protocols are provided by the same animal. This approach not only saves animals and time considerably but, since only a same treatment regime is employed, provides scope for comparison of the results of different end points also. We have also used a number of in vivo protocols for testing gudakhu induced toxicity, and in this section would like to introduce the various protocols adopted in the present study.

2.2.1 Chromosome Aberration Assay

Since chromosomes are the physical entity of the genetic system it is expected that any structural change in the chromosomes due to the action of an agent is a direct indication of the effect of the agent on the genetic system of the organism. Advancement in methodologies and their simplicity have made analysis of chromosome aberrations, particularly in somatic metaphases, very popular. Chromosome aberration analysis in somatic cells by itself no doubt fails to provide unassailable proof of concurrent gametic involvement but it appears likely that somatic chromosome aberrations may indicate a parallel gonosomal events of a similar nature. Metaphase chromosome analysis in somatic cells has, thus, become one of the important assay systems for recognizing potential genotoxicity and carcinogenicity. It is the oldest and the most widely used test system. Chromosome aberration assay in mammalian cells in culture is recommended as an efficient test to determine genotoxic potential in routine work (Ishidate, 1990). Ishidate (1988) made a review on chromosome aberration tests on 951 chemicals from over 250 published papers and showed a very good correlation between the clastogenicity test and other biological assays. The chromosome aberration assay, in vitro and in vivo, is included in the battery of test systems recommended by various organisations and countries.
to assay genetic hazards of an agent (see Ishidate, 1990; Ashby, 1986; Brusick, 1989). However, chromosome analysis for structural aberrations in vivo, compared to in vitro, is more relevant. In whole mammals bone marrow being haemopoietic provides a large number of dividing cells for scoring; it is easy to handle the bone marrow cells too. In addition, because of their proliferating nature they routinely pass through an S-phase, which is very important for the production of chromosome aberrations by a chemical agent. The bone marrow cells in mammals, therefore, constitute the unique material for recognizing genetic toxicity of an agent in vivo.

2.2.2 Micronucleus Test

The micronucleus test (MNT) was developed in the early 1970s by Schmid and his co-workers (Boller and Schmid, 1970; Matter and Schmid, 1971; Ledebur and Schmid, 1973; Schmid, 1973, 1976) and Heddle (1973) to screen environmental agents inducing chromosome aberrations. In principle, lagging acentric fragment(s) or whole chromosome(s) may be included into one or other daughter cell where it either fuses with the main nucleus or forms a micronucleus (MN). Dys-function of the spindle apparatus or chromosomal breakage results in the appearance of lagging chromatin material during cell division and subsequently formation of MN. In the former case micronucleus formed out of whole chromosome(s) is of big size, but the MN formed from acentric fragment originating from chromosomal breakage is, as a rule, small in size. Both clastogens and spindle poisons, thus, can induce MN.

In mammalian bone marrow during erythroblastosis the MN when formed may not follow the fate of the main nucleus and remains included in the erythrocytes (polychromatic) for some unknown reason(s). As a result scoring of MN (earlier referred to as Howell-Jolley-body) in enucleate erythrocytes becomes easier without confusion.
Since the development of this test system lot of works have been done with different environmental agents and the micronucleus test has now been proved to be a simple, quick and sensitive assay system for the detection of potential cytogenetic effect in vivo in mammalian polychromatic and normochromatic erythrocytes (Jensen and Ramel, 1980; Kliesch et al., 1981; Salamone et al., 1981; Heddle et al., 1983; 1984; Das and Kar, 1986; Roy and Das, 1990). The MNT has a number of advantages over the analysis of bone marrow chromosome aberrations: it is simpler and faster without loss of sensitivity, it can be conducted in any mammalian species regardless of its karyotype. Further, "no agent is known that can produce viable chromosome aberrations without also producing fragments that give rise to micronuclei" (Heddle et al., 1984). The recent reviews on the MNT (Mavournin et al., 1990; Heddle et al., 1991) indicate its high efficiency in detecting carcinogenesis with more than half being detected.

As mentioned earlier the MNT was developed originally in bone marrow system of rodents. During the last two decades research efforts have been directed towards development, validation, standardization and even automatization of the test (Schmid, 1976; Jenssen and Ramel, 1980; Das and Kar, 1980; Salamone et al., 1981; OECD, 1983; Heddle et al., 1984; Ashby and Mohammed, 1986; MacGregor et al., 1987; Collaboration Study for Micronucleus Test, 1986; 1988, 1989; Romagna and Staniforth, 1989; Melcion and Cordier, 1989). Owing to its usefulness the test has already gained application in several other tissue systems in different organisms including man. It has also been demonstrated that the micronucleated bone marrow erythrocytes survive well, come out in peripheral circulation and can be scored in blood smears of some laboratory mammals (Mac Gregor et al., 1980; Schlegel and Mac Gregor, 1982; Styles et al., 1983; Parida and Mohapatra, 1986) and fish (Hooftman and de Raat, 1982; Das and Nanda, 1986). With the accumulation of host of data on MN induced
by chemical and other environmental agents in cells of bone marrow and blood and with the sensitivity, simplicity and success of the test it was realized that it would be worthwhile to extend the test system in various other cell types. It has led to the development of MNT in hepatocytes (Cole et al., 1979, 1981, 1983; Tates et al., 1980, 1986; Das and Roy, 1988; Cliet et al., 1989a), male germ cells (Lahdetie and Parvinen, 1981; Lahdetie, 1983; Tates et al., 1983; Tates and de Boer, 1984), foetal liver cells (Cole et al., 1981; Balansky and Blagoeva, 1989), colon cells (Heddle et al., 1981), spleen cells (Shindo et al., 1983), pre-implanted mouse embryos (Molls et al., 1981), exfoliated cells of buccal mucosa (Stich et al., 1982 a,b; Stich and Ander, 1989) and even in plant cells (Ma et al., 1982; Panda et al., 1989). Recently Tates et al. (1990) reviewed nicely the importance of MNT in mammalian cells for clastogenicity and carcinogenicity studies. It has also been extended in cultured human lymphocytes of healthy and diseased individuals (Heddie et al., 1978; Hogstedt, 1984). The MNT is included as one of the important protocols in the battery recommended by most of the organizations and centres like the United States Environmental Protection Agency (USEPA), the United States Office of Pesticide Programs (USOPP), Health and Welfare, Canada, European Economic Community (EEC), Organization of economic Co-operation and Development (OECD), Ministry of Health and Welfare, Japan (see Heddie et al., 1991) for assessment of genotoxicity. Cliet et al. (1989b) compared sensitivity of MNT in bone marrow, testis and liver cells with six carcinogens and found positive effect for all six in testis and liver, but for two only in bone marrow cells. It indicates that one cannot simply rely on clastogenicity data obtained from bone marrow cells to make prediction about effects in cells from other organs like liver and testis.

The present study includes analysis of MN in, besides bone marrow erythrocytes, regenerated hepatocytes and peripheral erythrocytes of mice, and buccal mucosa cells
and peripheral erythrocytes of habitual users of gudakhu.

2.2.3 Sister Chromatid Exchange

Although sister chromatid exchange (SCE) was first demonstrated autoradiographically by Taylor (1958) long ago, its importance was not realized at that time. Sister chromatid differential fluorescence, when examined under fluorescence microscope by differential incorporation of 5-Bromodeoxyuridine (BrdU) and subsequent staining of slides in fluorochrome, Hoechst 33258, (Latt, 1973) enabled the cytogeneticists to score SCE more easily than the autoradiographic technique. However, this staining is not long lasting, and its examination needs fluorescence microscope which is beyond the reach of ordinary laboratories. Shortly after, Perry and Wolff (1974) simplified the technique by just staining the Hoechst treated slides in Giemsa, which made the preparation permanent and dark and light stained chromatids could be easily distinguished under ordinary microscope. This technique of Perry and Wolff (1974) which has revolutionized the field of SCE analysis, is popularly known as fluorescence plus Giemsa (FPG) technique. Modification was further made by directly staining the slides in alkaline Giemsa without using fluorochrome dye (Korenberg and Freedlender, 1974; Alves and Jonasson, 1978; Takayama and Sakanishi, 1979). The FPG and alkaline Giemsa techniques, compared to earlier ones, have very high resolution and are very simple and less time-consuming. The exchange points between two harlequin stained sister chromatids can be easily identified and scored by relatively inexperienced observer. SCE does not induce any overall change in morphology of the chromosome.

Originally the sister chromatid differential (SCD) staining technique with BrdU labeling was developed in mammalian cells in culture (Perry and Wolff, 1974; Latt,
Subsequently, realizing the importance of the technique attempts were made to apply it in whole animal model. Since most of BrdU is debrominated within 1h of intravenous administration (Kriss et al., 1963) in vivo BrdU labeling was achieved by either serial injections (Allen and Latt, 1976; Vogel and Bauknecht, 1976), intravenous infusion (Nakanishi and Schneider, 1979, Schneider et al., 1976), injection of BrdU adsorbed to charcoal (Ramirez, 1980) or subcutaneous implantation of a BrdU tablet (Allen et al., 1977). Sometimes fluorodeoxyuridine was injected hours before BrdU administration just to minimize endogeneous thymidine synthesis and thereby enhance incorporation of BrdU. This in vivo technique was extended to several non-mammalian vertebrates (Bloom and Hsu, 1975; Das and Pani, 1980; Chakrabarti et al., 1984; Kligerman and Bloom, 1976) and plants (Kihlman and Kronborg, 1975) also. Besides, there are minor modifications varying from laboratory to laboratory.

Although the molecular mechanism of SCE formation, as well as the biological significance of exchange remain unclear as yet, the exchange is known to involve DNA breakage and reunion. Induction of SCE is an S-phase dependent phenomenon. S-dependent agents such as alkylating agents, UV-rays, etc. are very efficient in inducing SCEs, where as S-independent agents like ionizing radiations and bleomycin are poor inducers of SCE. SCE thus seems to occur during replication of chromosomal DNA at 4 strand stage probably at the replication fork and involves DNA recombinational mechanism. But the DNA lesion that eventually results in SCE can be caused at any stage of the cell cycle (Wolff, 1982; Sasaki, 1982). Experimental evidences suggest that SCE results from unremoved adducts (Wolff, 1982). Its incidence is, therefore, taken as a measure of DNA damage and repair. As such, SCE can be considered as a qualitative indicator of potential mutagenic events and can possibly indicate a wider variety of DNA damage than is currently detectable using only mutational assays.
A host of reports have conclusively proved that mutagenic carcinogens nearly always induce SCEs often at doses well below those required to induce positive results in other mammalian test systems and the frequency of SCEs varies in a dose-dependent manner (Perry and Evans, 1975; Stetka and Wolff, 1976; Perry, 1980). Analysis of SCE is thus, considered to be a rapid, simple and very sensitive indicator for evaluation of potential genotoxicity of physical (such as UV-light) and chemical agents. A large number of factors are now also known to modulate the SCE frequency (Wolff and Morgan, 1982; Das, 1988).

2.2.4 Spermatocyte Chromosome Analysis

Analysis of germ line chromosomes deserves special attention as they are directly concerned with heredity and the aberrations induced in them may affect the progeny. In this regard male germ line cells constitute a nice system.

The test systems now available to analyze different end points for evaluation of chromosomal anomalies in germ line cells of rodents are dominant lethal test (Kar and Das, 1987), heritable translocation test (Generoso et al., 1980; ICPEMC Report, 1983), numerical sex chromosome anomaly (Russell, 1976; Russell and Matter, 1980), analysis of embryo chromosomes (Rohrborn et al., 1977; Generoso et al., 1979), analysis of MN in meiotic cells (Lahdetie, 1983; Tates et al., 1983) and cytogenetic analysis in germ line cells.

Some of the assay systems described above are laborious and time-consuming, some of them again need large scale housing and breeding facilities. Comparatively cytogenetic analysis in male germ line cells is easier and less time-consuming. Analysis of diakinesis-metaphase-I and spermatogonial chromosomes from treated males is extensively used in the study of male germ cell mutagenesis; mutagen sensitivities of different cell stages can also be evaluated. The germ line cell stages in which chromosomes
are studied can be divided into premeiotic (spermatogonial cells) and meiotic (spermatocytes) stages. Of these cell types spermatogonial stem cells are very important because they are present throughout the reproductive life span. Nevertheless analysis of meiotic (spermatocyte and oocyte) chromosomes represent an important protocol for evaluation of genetic damage. In females the investigation is, however, somewhat difficult (Brewen and Preston, 1982).

The ingenious methods developed by Evans et al. (1964) and later by Meredith (1969) and Hoo and Bowles (1971) for handling germ line chromosomes have enabled the present day animal cytogeneticists to study germinal cells in male laboratory mammals for induced mutagenesis with some ease. Adler (1982 a) has made a comprehensive review on the technical procedures for studying different stages of male germ cells and illustrated the importance of such studies.

Most of the cytogenetic studies involving male germ line cells have been performed in mice. Though the karyotype of the mouse which consists of 40 acrocentric chromosomes of almost equal size is not suitable for cytogenetic analysis of somatic cells, it is just that uniformity that makes meiotic chromosomes analysis fairly easy. It, however, requires a good number of high quality metaphases and trained observers to analyze chromosomes to have a clear cut decision. Here in the present study we have analyzed the spermatocyte (diakinesis metaphase-I) chromosomes to assess the effect of extracts of gudakhu on the germ line cells of mice.

2.2.5 Sperm Test

Since long semen analysis is a routine practice to diagnose testicular damage and infertility in humans and domestic animals. In several instances reduced fertility is found to be associated with high incidence of production of abnormal sperm and/or with large reduction in sperm number
or motility. Studies in domestic animals, laboratory rodents and man exposed to different chemicals and radiations have shown that sperm anomalies can be used as indicators of induced antispermatogenic effects (Wyrobek et al., 1983,b). In this regard attempts are being made, particularly in mammals, to use different characteristics of sperm like count (number of sperm per ml of ejaculate or per epididymis), motility, acrosome abnormality, and $F_1$ sperm morphology. Recently sperm unscheduled DNA synthesis assay to detect DNA lesions that require unscheduled DNA synthesis for repair by the cellular uptake of $^3$H thymidine has gained considerable recognition (Working and Butterworth, 1984; Ashby, 1985). Among the sperm tests mentioned above the 'sperm morphology' test is the most thoroughly worked out, while others have not been sufficiently tested. As the present work includes only sperm morphology and sperm count tests the author would like to restrict himself to these aspects only.

### 2.2.5.1 Sperm morphology assay

Sperm are the most accessible germ cells in mammals. Their morphology is species specific. However, a fraction of sperm population is always with abnormal morphology in normal healthy adult males. It is known that there is a host of non-genetic conditions by vitamin deficiency (Blair et al., 1968; Leathem, 1970), mumps orchitis (Bartak, 1973), hyperthermia (Van Demark and Free, 1970; Cairnie and Leach, 1980), dietary restriction (Komatsu et al., 1982) and early age (Krzanowska, 1981) which can elevate the incidence of mis-shapen sperm.

On the other hand, numerous physical and chemical mutagens and clastogens have been reported to enhance the frequency of mis-shapen sperm in several strains of mice and in humans according to time and dose of exposure (Wyrobek and Bruce, 1975; Bruce and Heddle, 1979; Wyrobek, 1979; Kar and Das, 1983; Pylkkanen and Lahdetie, 1984). In the EPA Gene
Tox report (Wyrobek et al., 1983a) it is reported that out of 154 chemical agents so far tested for their capacity to cause abnormality in sperm morphology in mouse system more than 25% compounds have been judged positive. Similarly out of 44 chemicals and their combinations 17 have been reported to cause adverse effect on human sperm (EPA Gene Tox report, Wyrobek et al., 1983b). The most interesting thing is that all mouse germ cell mutagens (as identified from dominant lethal test, heritable translocation test and specific locus mutation test) so far tested in the mouse sperm morphology test have yielded positive response; and germ cell non-mutagens have been identified as negative, but at 53-100% level (Bruce and Heddle, 1979; Topham, 1980; Wyrobek et al., 1983a, b). Their studies suggest that the sperm morphology test properly identifies almost all germ cell mutagens. A positive correlation between carcinogenic capacity and the capacity for production of morphologically abnormal sperm has also been marked for a large number of compounds (Wyrobek, 1984). Further, spontaneous levels and types of mis-shapen sperm though vary from strain to strain are remarkably consistent within and characteristic of strains (Krzanowska, 1972, 1981; Wyrobek, 1979).

Higher incidence of sperm abnormalities have also been noted in the male progeny of mutagen treated mice (Hugenholtz and Bruce, 1979; Sotomayer, 1979). Besides, several autosomal and sex linked genetic factors such as T-locus alleles (Bennett, 1975), hop sterile (Johnson and Hunt, 1971), quaking (Bennett et al., 1971), factors on chromosome 17 (Forejt, 1976), factors on the Y-chromosome (Krzanowska, 1972, 1976) and factors on the X chromosome (Hugenholtz and Bruce, 1977) are now known to control sperm shape in the mouse.

All these findings suggest that morphological characteristics of sperm might be under genetic control and an increased incidence of mis-shapen sperm might be indicative of exposure of male germ cells to a mutagenic
agent. However, there is no direct evidence in support of this possibility.

Leaving aside the genetic mechanism for their occurrence shape abnormalities in sperm may permit a simple quantitative assay for genetic damage in the male germ line. Bruce and Heddle (1979) conducted a comparative study to assess potential mutagenicity of 61 agents by using Salmonella/microsome test, micronucleus test and sperm morphology test, and established the usefulness and reliability of sperm morphology test.

2.2.5.2 Sperm count

For fertilization an egg needs one sperm only. But a moderate size of the sperm population is required for successful fertilization of an egg. However, we are still in dark about the level of sperm population at which failure of egg fertilization starts to occur. As mentioned before the test based on sperm count (quantitative evaluation of sperm number) to study germ cell damage is not sufficiently tested. The bulk of our knowledge on factors affecting sperm count has come from cases of human exposures which comprise occupational/environmental exposure, personal drug use, side effects of therapeutic agents and voluntary exposure. Comparatively very little is known from laboratory animals. Although it is generally agreed that low sperm count is linked with reduced fertility, there is no report as yet relating sperm count with the genetic alteration. But quantitative analysis of sperm is certainly relevant to assess germinal toxicity and fertility.

Prolonged or severe exposure to X-rays is known to cause death of spermotogenic cells leading to affect adversely the fertility in man as well as rodents (Hahn et al., 1982; Searle and Beechey, 1974; Meistrich, 1989). Pharmaceutical agents including anticancer drugs,
dibromochloropropane (DBCP) and lead are recognized testicular toxins which result oligospermia and in some cases aspermia. Similarly other environmental chemicals like methyl mercury, beryllium, polychlorinated biphenyls, benzene, chlorodecane, hexachlorobenzene, carbon monoxide, carbon disulphide, a number of pesticides, cadmium salts and alcoholic beverages have been reported to damage germ line cells leading to oligospermia in persons exposed to those agents (Barlow and Sullivan, 1981; Wyrobek et al., 1983 b). Effect of smoking reviewed by Vogt et al. (1984) seems to have no effect on spermatogenesis. Recently Meistrich (1989) has reviewed nicely the advantages and importance of sperm count assay in testing spermatogenic toxicity.
2.3 MATERIALS AND METHODS (GENERAL)

Animals: Inbred Swiss albino mice (Mus musculus L.) of both the sexes, if not otherwise mentioned, constituted the experimental animals in the present investigation. Healthy adult mice aged 8-10 weeks and having body weight about 20 gm were employed. For repeated treatment regimen (vide infra) these specifications (age group and body weight) of animals are applicable to them at the time of starting of the treatment. The animals were maintained in the animal house of the School at room temperature 30-39°C and with a relative humidity of 45-55%. The twelve hour light-dark cycle was regulated in the animal room. Standard rodent chow and tap water were made available to them ad-libitum.

Gudakhu and preparation of its extract: Gudakhu of "Samaleswari" brand purchased from the local market was tested. A known weight of it was homogenized thoroughly in a porcelain mortar and pestle for 20 min with acetone (EMerck, India, Ltd.) or distilled water and the extract was filtered under vacuum and evaporated to complete dryness through vacuum dessication. During evaporative preparation the extract was maintained at 4°C. Acetone extract of 1 gm of gudakhu yielded about 23 mg of the dry substance. Water extract of gudakhu (WEG) was also prepared in the same way, but here the extract was not evaporated. It was simply filtered and the filtrate was used. Water extract of 1 gm of gudakhu, yielded about 375 mg of dry substance. Usually a freshly prepared extract was used; however, when stored (particularly for acetone extract) the dry pellet was stored at -10°C.

Treatment: A dry pellet of known weight of acetone extract of gudakhu (AEG) was suspended in an appropriate amount of distilled water and the suspension was fed to mice by gastric intubation. For water extract, a mouse
received an appropriate amount of the filtrate. The volume of the suspension or the filtrate fed to a mouse was kept constant (0.25 ml). For dose-response study preparation of the suspension was adjusted to keep the volume of the suspension administered constant (0.25 ml). Details of the experimental design including treatment schedule, number of animals employed etc. for different test systems are presented in respective sections.

Control : In order to evaluate the effects of the extract of gudakhu and its ingredients the data of treated series presented in subsequent sections were compared with those of the respective negative control series. Since the treated animal received the extract in aqueous suspension or solution via oral route, age and sex matched untreated mice were kept as control (negative control). Some mice treated with cyclophosphamide (CP) or mitomycin C (MC) were used as positive control just to establish criteria for analysis of various aberration types (Details are given in Section 2.4).

The details of the methodological aspects in general for different protocols are described in the following sections.

2.3.1 Chromosome Aberration Assay in Bone Marrow Metaphases

Both time-response and dose-response analyses were performed following single treatment. Effect was also evaluated following long-term repeated treatment (once daily) for different periods. A dose of 4 mg/kg of colchicine (Ciba) in aqueous solution was injected intraperitoneally (ip) to both treated and control individuals one and half hours before killing. Cytological preparations were made following the conventional
hypotonic-acetic acid and methanol fixative (1:3)-flame-drying-Giemsa schedule (Saksela and Moorhead, 1962).

Scoring: Well spread and intact metaphases were examined under oil immersion lens of the Olympus Binocular Research microscope. From each of the control and treated animals 100 metaphases were scored from 2-4 slides taken at random. Some selected fields were photographed for presentation.

Analysis of aberrations: The structural aberrations of chromosomes were mostly of chromatid type and they were categorized under different heads. The criteria followed by us to recognize different types of structural aberrations are as follows:

1. Gap: Unstained part appearing like interruption of the chromatid continuity and smaller than the diameter of the chromatid was designated as gap.

2. Sub-chromatid-break: When a portion of a chromatid was found to be connected with the parent body by a comparatively thin connection (i.e. not completely dislocated) the aberration was called sub-chromatid break.

3. Chromatid break: Clear cut interruptions of chromatid continuity exceeding the diameter of a chromatid were designated as chromatid breaks. Any acentric chromatid fragment placed anywhere in the metaphase plate but of traceable origin was also included under this category.

4. Fragment: Tiny acentric chromatin fragments of untraceable origin were categorised as 'fragments'. Presumably they resulted from terminal deletions.
5. Iso-chromatid-break: Sometimes both the sister chromatids of a chromosome were involved in breakage at the same point. This type of aberration was referred to as iso-chromatid break. Because of its rarity in occurrence it was considered as iso-chromatid break rather than chromosome break.

6. Exchange: Interchanges involving two or more chromosomes were considered under this category. They were mostly of chromatid type and provided with or without acentric fragment(s).

7. Ring chromosome: A few metaphases were noted to contain centric "ring" chromosome(s). Sometimes they were associated with acentric fragment(s), but in some other cases the latter could not be traced. The "rings" referred to here were actually iso-chromatid breaks with sister chromatid union (intra-arm-intrachange).

The above criteria were followed as far as practicable for scoring different types of aberrations. But the distinction between gaps and breaks at times appeared to be confusing. Despite an attempt to give clear-cut definition for gaps and sub-chromatid breaks in the Chatham Bars Conference (1971) the controversies regarding their significance remain unsolved (Bender et al., 1974; Savage, 1975; Natarajan, 1976). Hsu (1982) is of the opinion that gaps, as they constitute a type of chromosome lesion and their incidence increases with the treatment of several environmental agents, should be considered along with breaks and other chromosome aberrations. However, WHO (1985) suggests not to consider gap as an indicator in genotoxic evaluation of an agent. In the present study quantitative analysis of the aberrations was done both excluding and including 'gaps' and 'sub-chromatid breaks'. As the aberrations under
categories 3-7 mentioned above result from breakage, for convenience in analysis, they were together referred to as "break type aberrations" in the entire text. However, categories 1-7 together constituted "total aberrations". Again, for convenience gaps and sub-chromatid breaks were taken together under the heading, "gaps". Although gaps were considered and mentioned in the Tables emphasis was laid on break type aberrations during discussion.

2.3.2 Micronucleus Test

Animals of either sex were employed for this protocol. The details of the treatment schedule have been described later in respective sections.

2.3.2.1 In bone marrow cells:

Collection and processing of material and preparation and staining of slides: The bone marrow cells were processed and the slides were prepared according to the schedule developed in this laboratory by Das and Kar (1980). The technique was basically the same as that of Schmid (1976) but instead of using fetal calf serum (FCS) they used 1% Sodium Citrate solution for preparation of smear and for staining only Giemsa stain was used instead of May-Grunwald and Giemsa. The details of the schedule are as follows:

1. The animals treated and control series were made unconscious under anaesthesia without colchicine pre-treatment. Two femora were dissected out quickly. The bones were cleared from muscles and washed thoroughly with sodium citrate solution (1%). Both the end joints of each bone were cut out.

2. The bone marrow cells were flushed out in a glass centrifuge tube by passing a jet of 1% sodium
citrate solution at 20°C from a syringe.

3. The material was aspirated gently with a pasteur pipette to make a homogeneous cell suspension and it was centrifused immediately at 1000 rpm for 3-4 minutes.

4. The supernatant was decanted off and a concentrated homogeneous suspension of bone marrow cells was prepared in the left-over drop of the citrate solution by placing the centrifuse tube on a revolving cyclomixer.

5. A small drop of the suspension was taken on a clean grease free slide and a smear was drawn by pulling the material behind the smooth edge of another slide or coverslip. The smear was allowed to dry in air.

6. Just before getting dried the smear was fixed in absolute methanol for 10-15 minutes, dried in air and one day old slides were stained in Giemsa diluted (1:10) with buffer (pH 6.8) for (15-20) min. The slides were rinsed in deionized water and air dried.

The entire process involving steps 3-5 was conducted at 20-25°C in a humid chamber and finished in about 7 minutes to avoid bursting of the cells.

**Scoring**: Well stained slides with good smears were screened under low magnification of the microscope. The final scoring was done under oil-immersion lens. For quantitative analysis of micronuclei (MN) polychromatic erythrocytes (PCE), normochromatic erythrocytes (NCE) and nucleated cells were examined. 2000 PCEs were scored from each animal; however, the number of NCEs and nucleated cells scored from each animal have been mentioned in respective Tables (vide infra). Poly- and normochromatic erythrocytes did not pose any difficulty for scoring.
However, nucleated cells sometimes lead to confusion due to characteristic lobulated nature of the nuclei in some cells. Such doubtful cases were not recorded and clear-cut cases only were considered. Young polychromatic erythrocytes were easily differentiable from mature normocytes by their characteristic staining behaviour. However, as the latter is derived from the former through differentiation; confusion regarding their identification sometimes arose in the transient phases, which was obvious. Such cases were recorded separately, and 50% of them were considered as PCEs and rest 50% as NCEs. Abnormal mitotic figures like anaphases with lagging chromosome(s) or fragment(s) and with bridges, and multipolar spindles whenever available in the preparation were also noted.

Analysis of data: Quantitative analysis was done separately for each category of cells (PCEs, NCEs and nucleated cells). Percentage of micronucleated cells (irrespective of the number of MN present in them) for each individual was first calculated; then mean percentage of 3-4 such values (from 3-4 animals) was obtained for each category of cells for a particular dose or time-response.

To find out any effect on the proliferation of the bone marrow cells attempts were made to calculate the ratio of polychromatic to normochromatic erythrocytes (PCE/NCE). As PCEs and NCEs were scored simultaneously from the same fields the ratio of total PCEs to total NCEs was used for the purpose.

2.3.2.2 In hepatocytes:

Hepatectomy: A longitudinal incision of about 1 cm was made just below the sternum of the animal under etherization and both the skin and the muscle layer were cut open. 2/5th of the liver, preferably the peripheral lobes, was surgically removed following the conventional
procedure. The muscle layer and the skin were stitched with the help of a fine curved surgical stitching needle using cat-gut (no.4). The wound was cleaned and dressed. The animal was allowed to recover from anaesthesia and kept in a clean paper padded sterilized cage.

The animals were kept on starvation during pre- and post-operation periods for 10 h (5+5). They were supplied with liquid diet (glucose and milk) only for one day following post-operation starvation period. For the next two days the animals were kept on diet with low carbohydrate content to minimize glycogen content and thereby vacuolated appearance in the hepatocytes. The survivality of the hepatectomized mice was more than 95%.

Isolation of hepatocytes and preparation and staining of slides: The animals were killed at 72 h post-hepatectomy, this timing was chosen on the basis of our part experience (Das and Roy, 1988). The technique of Das and Roy (1988) developed in this laboratory was followed for preparation of slides. It is simpler than that of Tates et al. (1980).

1. The animals were killed and the regenerated portions of the liver were cut out. The tissue was washed thoroughly in 1% sodium citrate solution. For further blanching it was cut into small pieces (1 mm³) and washed repeatedly with fresh citrate solution.

2. The small pieces were incubated in the citrate solution containing collagenase Type 1A (Sigma, final concentration 0.005% W/V) at 37°C for 10-15 minutes (Collagenase was added from a 0.05% stock solution prepared in 250 mM sucrose). The material was agitated gently from time to time by repeated aspiration.
3. The big pieces of the tissue were allowed to settle down. The upper part of the cell suspension was removed and centrifused at 1000 rpm for 2 minutes.

4. The supernatant was discarded. The residual cells were resuspended in an appropriate amount of citrate solution (0.25 ml) to make the suspension of required density. The suspension was made homogeneous by repeated aspiration.

5. A little amount of the final suspension was taken in a pasteur pipette having a narrow nozzle and a small drop was put on a clean grease-free slide by touching the nozzle of the pipette onto the slide. The drop was immediately drawn back into the pipette. Thus, the drop would leave only its mark on the slide with no extra fluid and reduce clumping. The process was repeated on the entire slide and a good number of such drop-marks could be accommodated on it.

6. Just before the drop-marks were dried the slides were transferred to absolute methanol and kept for 15 minutes for fixation.

7. The air dried slides were stained next day in buffered (pH 6.8) Giemsa (1:15) for 15 minutes, washed in deionized water and air-dried. Next day the slides were soaked in xylene and mounted with DPX.

The entire procedure of processing of the cells and preparation of the slides (steps 1-6) took 40-45 minutes. Drawing of a smear like a blood film as well as dropping of the suspension from a certain height and subsequent air-drying were avoided as they caused clumping and bursting of cells and/or nuclei to a great extent.
Scoring: Well stained slides with good number of intact cells with intact nuclei were screened under low power of the microscope. The scoring was done under oil immersion lens. Scoring was kept restricted only to hepatocytes which are very large in size and easily differentiable from other liver cells. Uni-, bi- and poly-nucleate hepatocytes were scored. For quantitative analysis micronucleated cells were taken under consideration irrespective of the number of micronuclei present in them. 1000 cells were scored per animal.

2.3.2.3 In peripheral erythrocytes:

Animals of the both the sexes were employed; after repeated treatment for different periods blood was collected from tail vein and smear was drawn. Air-dried smears were fixed in absolute methanol for 15 min and stained next day in Giemsa diluted (1: 10) with buffer (pH 6.8) for 10-15 min. Erythrocytes (both poly-and normo-) were examined for presence of MN and frequency of micronucleated erythrocytes were determined.

2.3.3 Sister Chromatid Exchange Analysis

Sister chromatid differential staining in vivo was performed by surgically implanting 5-bromodeoxyuridine (BrdU) tablet following the technique of Allen et al. (1977) with certain modifications (Dash et al., 1991). The details are as follows:

1. On the lateral side (left or right) of the neck region of the animal an incision (about 0.5 cm) was made on the skin below the ear, and one BrdU tablet (50 mg, Galenus Chemicals, Germany) was implanted sub-cutaneously.

2. The 'cut' was stitched with the help of a fine curved surgical stitching needle using cat-gut (no.4).
3. The wound was cleaned and dressed. The animal was allowed to recover from etherization and kept in a clean sterilized cage.

The animal was kept on starvation during pre-and post-operation periods for about 10 h (5+5).

24 h before killing both control and treated animals were injected ip with a dose of 4 mg/kg of colchicine (Ciba). 26 h after implantation of the tablet cytological preparation was made following the conventional hypotonic-acetic-alcohol (acetic acid and methanol, 1:3)-flame-drying schedule.

Staining: Sister chromatid differential staining was done mainly according to the FPG technique of Perry and Wolff (1974).

1. 4 days old slides were stained in 10 μg/ml Hoechst 33258 for 15 minutes.

2. Slides were rinsed in deionized water and allowed to dry in air.

3. Dried slides were mounted with cover glasses with a few drops of buffer solution of pH 6.8. and then exposed to UV light for 30 minutes. However, in the middle, i.e. 15 minutes after the starting of UV exposure, a few drops of buffer solution were put on the slides again just to avoid dryness.

4. The slides were rinsed in deionized water and incubated in 2 x SSC solution at 60°C for 1 h.

5. The slides were then stained in 4% Giemsa (diluted with buffer of pH 6.8) for 8 minutes, rinsed in deionized water and air-dried.
Scoring: Well spread intact second division metaphases were selected for quantitative analysis of SCEs. From each animal at least 30 such cells were scored at random from 2-3 slides. Observation was done under oil-immersion lens. Some selected fields were photographed for presentation. The exchange points were scored and number of SCEs per cell was recorded.

2.3.4 Spermatocyte Chromosome Analysis

Adult male mice were treated with different doses of the extract via oral route for different periods. Details of the treatment schedule have been mentioned in respective sections (vide infra). Both control and treated animals received ip a dose of 4 mg/kg of colchicine (Ciba) in aqueous solution two hours prior to killing. Cytological preparations of male germ cells were made following the technique of Evans et al. (1964) with certain modifications. The flame-dried slides were stained with Giemsa and scored for spermatocyte chromosomes.

Scoring: Well stained slides with a good number of dividing stages (diakinesis metaphase-I) were screened under low magnification. 50 such intact cells were examined from 3-4 slides taken at random from each animal under oil-immersion lens for analysis of structural, numerical and other abnormalities of meiotic chromosomes.

Analysis aberrations: For qualitative analysis of the effect on meiotic chromosomes, the aberrations were categorized under three different heads: numerical changes, structural changes and the effect on the pairing behaviour. Numerical changes included both polyploid and aneuploid cells. Under aneuploidy emphasis was laid on hyperdiploid cells because hypodiploid cells the occurrence of which was common might result from technical shortcomings. Breaks, fragments of untraceable origin and
exchanges constituted the structural aberrations. The breaks were of chromosome and chromatid types. For analysis of effect on pairing behaviour of chromosomes, univalent formation involving both autosomes and sex chromosomes was noted. The univalents while placed widely apart only were taken under consideration; homologues just started separation at late metaphase I as revealed by their close alignment along the same axis were excluded.

2.3.5 Sperm Test

The male mice of both control and treated series used for spermatocyte chromosome analysis provided the materials for both the sperm morphology and sperm count assays, no separate treatment was done for the purpose.

2.3.5.1 Sperm morphology assay

Mature vas deferential sperm constituted the material for the study of their morphological abnormalities.

1. A portion of the vas deferens from near the cauda epididymis was taken out; fat and blood vessels were cleared off from it and its content was squeezed out and smeared with a drop of sodium citrate solution (2.2%).

2. The smear was air-dried and fixed in absolute methanol for about 10-15 minutes.

3. Next day the slides were stained in 1% aqueous eosin-Y for 10-12 h rinsed in deionized water and air-dried.

Scoring: 1000 sperm heads were scored at random from each animal under oil-immersion lens. Any deviation from the normal shape and size of the head morphology was considered
as abnormal. Percentage of abnormal sperm morphology was
calculated for each individual, and then mean percentage for
each sampling period was calculated.

2.3.5.2  Sperm count assay

Soon after killing the animal the testis along
with epididymis was dissected out in 2.2% sodium citrate
solution. The testis was processed for meiotic chromosome
analysis and epididymis for sperm count. The two caput
epididymis, left and right, were dissected out as shown in
the diagram below, cut into smaller pieces and macerated with
the help of a knobbled glass rod on a sieve having mesh size

\[ 80 \mu m \]

and kept over a solid watch glass containing 1% sodium
citrate solution. The material on the sieve was washed
thoroughly with citrate solution. The suspension of sperm in
Na-citrate was taken in a graduated tube and the volume was
made up to 16 ml. The suspension was thoroughly mixed with a
Pasteur pipette.

Scoring: A small drop of the homogenous suspension of sperm
was taken on a Neubauer haemocytometer after placing the
cover glass firmly on it, and it was kept for 5 minutes as
such just to allow the sperm to settle. The number of sperm
heads present in each of the four large corner squares and in
and in the large central square of the haemocytometer was counted separately under low magnification.

For each animal sampling was done five times, every time taking 5 large squares. The mean of 25 (5x5) such values obtained from 25 squares was referred to here as 'mean sperm count'. represents the mean number of sperm present in one large square chamber of the haemocytometer. Total sperm count per epididymis one can obtain by multiplying the 'mean sperm count' with the dilution factor (8 X 10 ). As the dilution factor was kept constant in the entire study for convenience in statistical analysis simply the 'mean sperm counts' were considered. For a particular dose level or test week 3-4 animals were employed and a value referred to in a particular table represents the mean of 3-4 individual 'mean sperm count' values.

2.3.6 Statistical Analysis
The mean value with standard error (SE) for each point was calculated from values obtained from different individuals used for that point. Student's t-test and one-way analysis of variance (model I ANOVA, Sokal and Rohlf, 1973) were conducted to compare treated data with the respective negative control. One way ANOVA was also employed to verify if more than 2 treatment means were statistically different from each other. Data were subjected to correlation coefficient analysis and linear regression analysis to ascertain if the treatment data have any correlation with the time-course-of-treatment or-dose.
2.4 POSITIVE CONTROL

Since in the present investigation extract of gudakhu, its ingredients or saliva was administered to experimental animals (mice) via oral route through gavage. Negative control was run in parallel with age and sex matched untreated mice. Mice treated with cyclophosphamide (CP) or mitomycin C (MC) served the purpose of positive control. Our idea of conducting positive control experiment was mainly to standardize the test protocols and to fix up criteria for analysis of various aberration types. To simulate human exposure most of the experiments with gudakhu and its ingredients were conducted following chronic treatment; in several cases, however, single treatment was done. In contrast, in case of positive control mice received CP or MC once only. Further, in this investigation same type of experiments or assays were conducted with various agents. Obviously presentation of the positive control data side by side along with experimental ones in the same tables would result repetition. To avoid this repetition we would like to present the positive control data here in a separate chapter.

As mentioned above, two widely worked out potent genotoxic agents: CP and MC, were used for positive control. CP treated mice served the purpose for assays such as, chromosome aberration in bone marrow cells, SCE in bone marrow cells, MNT in bone marrow cells, sperm head abnormality test and sperm count, while MC was used for spermatocyte chromosome analysis.

2.4.1 Materials and Methods

Animals: As described in section 2.3.
Chemicals: CP (Khandelwal Lab. Ltd., Bombay, manufactured under agreement with ASTA, Germany) was purchased from a local medical store. It was diluted with sterile distilled water just before use and mice were treated with different doses via oral route. MC (Biochem Pharmaceutical Industries, Bombay, manufactured under agreement with Kyowa, Japan) was dissolved in sterile distilled water just before use. The animals were treated ip with a dose of 1, 2, or 4 mg/kg of the drug.

Details of dose, treatment schedule, number of animals used etc. are summarized in Table-4. Different tissues were collected from CP/MC treated mice for various assays at different sampling times and processed according to the procedures described earlier (vide 2.3.1, 2.3.2.1, 2.3.3, 2.3.4, and 2.3.5).

2.4.2 Results

As CP and MC are well worked out clastogen-mutagen the qualitative aspects are not dealt with here. However, we would like to point out that the criteria followed to identify a particular aberration type in CP or MC treated series were also used for the same purpose for all the agents tested and the negative control. The data obtained from CP and MC treated individuals were compared with those of untreated age and sex matched controls.

2.4.2.1 Chromosome aberrations in bone marrow metaphases

The data on the types and frequencies of chromosome aberrations induced by CP in the bone marrow cells are presented in Table-5. Both break-type aberrations and total aberrations (breaks+gaps) exhibited distinct dose-related increase (Fig.1a) and the incidences, compared to negative controls, were highly significant even at the lowest dose level.
## TABLE 5

DOSE-RESPONSE ANALYSIS OF STRUCTURAL CHROMOSOME ABERRATIONS IN BONE MARROW CELLS OF MICE TREATED ONCE WITH CYCLOPHOSPHAMIDE

The animals were killed at 24 h post-treatment.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Metaphases scored/animals</th>
<th>Break-type aberrations</th>
<th>Gaps</th>
<th>Total aberrations (mean% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chromatid</td>
<td>Isochromatid</td>
<td>Fragment</td>
</tr>
<tr>
<td>Control</td>
<td>1000/10</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2.5</td>
<td>300/3</td>
<td>20</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>5.0</td>
<td>300/3</td>
<td>53</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>10.0</td>
<td>400/4</td>
<td>191</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

Each of the iso-chromatid breaks, rings and exchanges was considered as two breaks.

Significantly different from the control ("t"-test and Anova test): c = p < 0.001.
2.4.2.2 Micronucleus test

Table-6 summarizes the data on the incidence of micronucleated cells in bone marrow following cyclophosphamide treatment. The frequencies of both polychromatic (PCE) and normochromatic (NCE) erythrocytes with micronuclei (MN) increased in a dose-dependent manner and significantly, over the control value, even with the lowest dose tested (5 mg/kg)(Fig.1d). The frequency of micronucleated PCEs (MNPCEs) for the highest dose level (20 mg/kg) was about 6 times the control value.

2.4.2.3 Sister chromatid exchange

Dose-response analysis of SCEs' exhibited a very good positive correlation between the doses tested and the incidences of SCEs (Table-7,Fig.1b). The incidence of SCEs in untreated mice was 4.70, and the highest dose tested (10 mg/kg) increased the incidence by about 7-fold.

2.4.2.4 Spermatocyte chromosome analysis

Dose-response analysis of chromosome aberrations in spermatocytes induced by MC also revealed significant increase, relative to untreated control of aberrations for all the dose levels (Table-8). The increases were significantly correlated with the doses tested (Fig.1c). Induction of ploidy remained in the control range.

2.4.2.5 Sperm test

Only one dose level (40 mg/kg) was tested and it raised the incidence of abnormal sperm to 16.5% from the control value 2.83%. CP also reduced the epididymal sperm count very significantly (Table-9).

2.4.3 Discussion

Cyclophosphamide (CP) is a cytostatic drug used in some cancer therapy. It is an indirectly acting agent
TABLE 6
INCIDENCE OF MICRONUCLEI IN BONE MARROW CELLS OF MICE TREATED WITH DIFFERENT DOSES OF CYCLOPHOSPHAMIDE

Animals were killed at 30 h post-treatment.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. of animals</th>
<th>PCEs with MN (MN/PCEs)</th>
<th>NCEs with MN (MN/NCEs)</th>
<th>PCEs+NCEs with MN</th>
<th>PCE/NCE ratio</th>
<th>Nucleated cells with MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>0.20±0.02 (16/8000)</td>
<td>0.21±0.02 (14/6887)</td>
<td>0.20±0.02</td>
<td>1.17±0.04</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.55±0.07b (44/8000)</td>
<td>0.51±0.08a (37/7207)</td>
<td>0.54±0.07b</td>
<td>1.11±0.07</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0.95±0.06c (57/6000)</td>
<td>0.79±0.07c (39/4918)</td>
<td>0.92±0.06b</td>
<td>1.22±0.05</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>1.21±0.05c (97/8000)</td>
<td>1.15±0.05c (79/6897)</td>
<td>1.19±0.05c</td>
<td>1.16±0.03</td>
<td>0.12±0.00</td>
</tr>
</tbody>
</table>

Values are mean of 100 cells ± SE.
Significantly different from the control; ('t'-test and Anova test): a = p<0.05; b = p<0.01; c = p<0.001.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Hour of treatment</th>
<th>II Metaphases scored/animal</th>
<th>Total SCE</th>
<th>SCE/Cell (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>--</td>
<td>293/10</td>
<td>1392</td>
<td>4.70 ± 0.22</td>
</tr>
<tr>
<td>CP</td>
<td>2.5</td>
<td>18 h</td>
<td>200/4</td>
<td>2356</td>
<td>11.78 ± 0.28 ***</td>
</tr>
<tr>
<td>CP</td>
<td>5.0</td>
<td>18 h</td>
<td>200/4</td>
<td>4130</td>
<td>20.66 ± 0.52 ***</td>
</tr>
<tr>
<td>CP</td>
<td>10.0</td>
<td>18 h</td>
<td>135/3</td>
<td>4955</td>
<td>36.70 ± 0.56 ***</td>
</tr>
</tbody>
</table>

*** p<0.001 compared to the negative control value (Anova and 't'-test).
TABLE 8
INCIDENCE OF STRUCTURAL ABERRATIONS AND UNIVALENT FORMATION IN SPERMATOCYTES OF MICE INDUCED BY MITOMYCIN C

The animals received the drug once and were killed after 11 days.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Diaki. scored/animals</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chrom. br.</td>
<td>Chromt. br.</td>
<td>Frag.</td>
</tr>
<tr>
<td>Control</td>
<td>500/10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(Pooled)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>200/4</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>200/4</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>150/3</td>
<td>35</td>
<td>7</td>
</tr>
</tbody>
</table>

Each exchange was considered as two breaks.

Significantly different from the control; ('t'-test and Anova test): c = p< 0.001.
TABLE 9

INCIDENCE OF MIS-SHAPEN SPERM AND EPIDIDYMAL SPERM COUNT IN MICE 35 DAYS AFTER A SINGLE TREATMENT OF CYCLOPHOSPHAMIDE

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Total sperm head scored/animals</th>
<th>Abnormal sperm head (mean% ± SE)</th>
<th>Animals analyzed</th>
<th>Mean count/epididymis (mean X 8 X 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10000/10</td>
<td>2.83 ± 0.21</td>
<td>10</td>
<td>103.97 ± 0.55</td>
</tr>
<tr>
<td>40</td>
<td>4000/4</td>
<td>16.50 ± 0.96\textsuperscript{c}</td>
<td>4</td>
<td>74.89 ± 0.89\textsuperscript{c}</td>
</tr>
</tbody>
</table>

Significantly different from the control; ('t'-test and Anova test): c = p < 0.001.
Mean % of Aberration  Mean * A of Chromosome break
53
70
50
r=0.9998 
- 0=0.46 
b=10.823 
p< 0.001
30-
10
©
Q ... 0.05
p<0.05 ® /
® //
/
/
s.
/
0
c
10 15 20
Dose (mg/Kg }
Mean % of Micronuclei
O p  0 o
fo b) oo o  " ro In

\[ r = 0.9996 \]
\[ a = -0.076 \]
\[ b = 6.391 \]
\[ p < 0.01 \]

\[ r = 0.99995 \]
\[ a = 4.32 \]
\[ b = 3.23 \]
\[ p = 0.001 \]

\[ r = 0.9998 \]
\[ a = 0.46 \]
\[ b = 10.823 \]
\[ p < 0.001 \]

\[ r = 0.96741 \]
\[ a = 0.29 \]
\[ b = 0.05 \]
\[ p < 0.05 \]
which means that metabolic activation is needed before the
drug can act as a bifunctional alkylating agent. In vivo
CP is a proven clastogen and its potency has been verified
earlier in various non-mammalian and mammalian species in
vitro and in vivo as exemplified by chromosome aberration
and micronucleus analyses (Schmid and Staiger, 1969; Bruce
and Heddle 1979; Renner, 1979; Yamamoto and Kikuchi, 1981;
Tinwell et al., 1990; Bhunya and Pati, 1990; Krishna et
al., 1991; see also Mavournin et al., 1990 and Heddle et
al., 1991). It induces chromosome aberration in meiotic
cells also (Generoso et al., 1978; Goetz et al., 1980).
Lahdetie (1988) has shown that it is highly effective in
pre-leptotene G\textsubscript{1} - S phase in inducing MN in meiotic cells.
It is also known to induce SCE in vivo and in vitro
(Renner, 1979; Perry, 1980; Allen et al., 1981; Rutten and
Wilmer, 1986; Arnsdorff-Roubicek and Targa, 1990) and
morphologically abnormal sperm in various animals (Wyrobek
et al., 1983a; Bhunya and Pati, 1990). Bruce and Heddle
(1979) demonstrated mutagenic capacity of CP in the Ames
test in presence of S-9 mixture. CP is now widely used as
a known clastogen in positive control experiment (Ishidate,

MC is also a well known mutagen-clastogen. Its
clastogenic effect has been proved in human and other
mammalian in vivo and in vitro systems (Cohen and Shaw,
1964; Cohen, 1969; Natarajan and Schmid, 1971; Adler, 1973,
1974; Michelmann et al.,1978; Shiriashi and Sandberg,
1979), in several non-mammalian (Krishnaja and Rege, 1982;
Das and Nanda, 1986) and plant (Merz, 1961) species. Its
mutagenic potency is also well proven (Ehling, 1974).
Clastogenic effect of MC as well as dose-related increase
of chromosome aberrations induced by MC were also
demonstrated by several other workers earlier by analyzing
somatic (Cohen and Shaw, 1964; Michelmann et al., 1978;
Shiriashi and Sandberg, 1979) and meiotic chromosomes
(Adler, 1973, 1974, 1976). The same trend was noted with
MC while assessing other end points like sister chromatid
exchange (Latt, 1974; Perry and Evans, 1975; Huttner and Ruddle, 1976; Shiriashi and Sandberg, 1978; Perry, 1980; Vijayalaxmi and Evans, 1982), micronucleus production in bone marrow cells (Matter and Grauwiler, 1974; Michelmann et al., 1978; Kliesch et al., 1981), and regenerated hepatocytes (Das and Roy, 1988), sperm head abnormality (Wyrobek and Bruce, 1975), dominant lethality (Ehling, 1971) and host-mediated assay (Maier et al., 1978).

Here for meiotic chromosomes no time-response study was performed. Animals were killed 11 days after the treatment, so the cells we observed at diakinesis-metaphase-I stage were expected to remain at pre-leptotene stage at the time of treatment (Oakberg, 1957). The aberrations, therefore, seemed to be produced at pre-leptotene stage. The high incidence of spermatocyte chromosome aberrations following MC treatment may be attributed to the fact that the effect of MC is pre-leptotene S-phase related as suggested by Adler (1982b).

In our experiment doses were selected on the basis of reports of earlier workers as well as on the basis of our preliminary experiments. Our data obtained with CP and MC are in good agreement with those of earlier workers. Slight variation noted in certain cases may be attributed to the strains of animals used, route of administration of the drug and/or time of sampling. Distinct dose related increase of chromosome aberrations, MN, SCEs and mis-shapen sperm in CP/MC treated animals as manifested in linear regression curves (Fig.1) confirms their genotoxic potential. It is reasonable to assume that the criteria followed here to recognize different aberration types were well standardized.
2.5 EFFECT OF ACETONE AND WATER EXTRACTS OF GUDAKHU

As mentioned earlier we tested the effect of both organic polar solvent (e.g. acetone) and inorganic polar solvent (water) extracts of gudakhu. Acetone extracts of gudakhu contains mainly tobacco extracts, other ingredients may not come in it. For that an attempt was made to study the effect of aqueous extract of gudakhu in mice. Molasses is soluble in water, and lime and red-soil are extractable in water to a great extent. The details of the working parameters followed for two types of extracts, are presented below.

Acetone Extract

I. Chromosome Aberration (CA) Assay in Bone Marrow Metaphases

A. Dose-response study .... Treated once with different doses and sampling was done at a particular interval.

B. Time-response study .... Treated once with a particular dose and sampling was done at different intervals.

C. Short term repeated treatment .... Treated twice or thrice at an interval of 4 h and sampling was done 24 h after the first treatment.

D. Long term repeated treatment .... Treated once daily for different time-course (4-16 weeks) and sampling was done 24h after the final treatment. Here two dose levels were tested.
II. Micronucleus Test

A. In bone marrow cells
   1. Dose-response study as in I-A.
   2. Long term treatment as in I-D.

B. In hepatocytes
   1. Long term repeated treatment as in I-D.

C. In peripheral blood
   1. Long term repeated treatment as in I-D, but here treatment was done for 1-4 weeks.

III. Sister Chromatid Exchange Analysis

A. Dose-response study as in I-A.
B. Long term repeated treatment as in I-D.

IV. Spermatocyte Chromosome Analysis

A. Long term repeated treatment as in I-D. Here two dose levels were tested.

V. Sperm Test

A. Sperm morphology assay: Long term repeated treatment as in I-D. Here two dose levels were tested.

B. Sperm count assay: Long term repeated treatment as in I-D. Here two dose levels were tested.
Water Extract

I. Chromosome Aberration Assay in Bone Marrow Metaphases

A. Single treatment
B. Long-term repeated treatment with a particular dose for various time-courses (4-16 weeks) and sampling was done 24 h after the final treatment.

II. Spermatocyte Chromosome Analysis

Chronic treatment time-course study as in I-B.

III. Sperm test

A. Sperm morphology assay: Chronic treatment time-course study as in I-B.

B. Sperm count assay: Chronic treatment time-course study as in I-B.

2.5.1 Materials and Methods

As mentioned earlier gudakhu of "Samaleswari" brand purchased from the local market was extracted in acetone (E. Merck) of analar grade or distilled water (vide 2.3). Aqueous suspension of the dried extract (in case of AEG) or the filtrate of the extract (in case of WEG) was fed to mice. The details of treatment schedule such as dose, period of treatment, time of sampling, number of animals employed, etc. for different protocols adopted here are presented in Tables-10 and -11 for acetone and water extracts respectively.

We would like to mention here that for sperm test animals were not treated separately, the animals used for
spermatocyte chromosome analysis also provided epididymes and vasa deferentia for sperm count and sperm morphology assays respectively. Details of collection and processing of different tissues for different protocols, and preparation and staining of the slides have been described in Materials and Methods (General) (vide 2.3).

2.5.2 Results

With regard to any particular end point (e.g. CA, MN, SCE or mis-shapen sperm) the aberrations or abnormalities did not vary qualitatively for two types of extracts (AEG and WEG) as well as for single and chronic treatment. For that in subsequent sections the qualitative aspects of any protocol have been dealt with in common.

2.5.2.1 Chromosome aberration analysis in bone marrow cells

Qualitative: The structural chromosome aberrations encountered were mainly of chromatid type. Among the break type aberrations chromatid breaks constituted the most frequently encountered aberrations (Figs. 2a-c,g), the acentric chromatid fragments were found to lie either near to its place of origin with little displacement (Figs.2a-c) or somewhere else in the metaphase plate (Fig.2g). The affected chromosome was usually found to have only one break. However, iso-chromatid gaps and breaks (Figs. 2i,j) were common. Small acentric fragments of untraceable origin were noted in a number of metaphases (Figs. 2b,e,h); in fact, they constituted the second category of break type aberrations to chromatid breaks so far their frequency of occurrence was concerned. Metaphases plates with centric "rings" accompanied with or without fragment were also recorded (Figs. 2k,l,m); the rings were assumed to have originated through sister chromatid reunion following terminal chromatid deletion. A number of exchanges were
noted; these exchanges included metacentric chromosomes and end to end chromatid inter-change involving two chromosomes leading to the formation of a dicentric chromosome with or without fragments (Figs. 2n,o,p). Gaps and sub-chromatid breaks together constituted very common type of aberration (Figs. 2a,d,f). Though in general the affected cells were found to contain one break type aberration in some cases cells were noted to contain a number of breaks (chromatid and iso-chromatid) and fragments (paired and unpaired) (Fig. 2q). Sometimes centromeric region of some chromosomes were seen to be extended (Fig. 2r).

The occurrence of polyploid cells though common did not bear any significance in their scoring. Although cells with hypo-diploid number of chromosomes were common only two hyper-diploid cells (each with 41 chromosomes) were recorded.

Quantitative-AEG: Types and frequencies of structural chromosome aberrations noted in bone marrow metaphases following treatment of AEG are summarized in Table-12. The frequencies of chromosome aberrations, excluding and including gaps, increased significantly over their corresponding control values in several sampling intervals following AEG treatment. The frequency of breaks increased greatly in a dose-dependent manner following single treatment (Fig. 3). Such a dose-effect relationship \( r = 0.950, b = 1.661, p < 0.05 \) was also documented when data from animals treated once, twice and thrice within 24 h were compared (Fig. 4a). The frequency of breaks, however, decreased with the increase of sampling interval (Fig. 4b). Repeated treatment for the dose of 300 mg/kg/day for 8, 12, and 16 weeks yielded significantly high breakage frequencies but the values showed almost no difference (Anova test); however, the value for week 4 did not exhibit significant increase. Similarly repeated treatment for the dose of 150 mg/kg/day for 8 and 12 weeks yielded significantly higher
Explanation for Fig. 2

Photomicrographs of mouse bone marrow metaphase plates showing various types of structural aberrations induced by gudakhu extracts.

a. One chromosome with two chromatid gaps and one chromatid break with undisplaced acentric fragment.

b. One chromatid break with undisplaced fragment and a fragment of untraceable origin.

c. One chromatid break with a little displacement of the acentric fragment.

d. A chromosome with a sub-chromatid break.

e. A fragment of untraceable origin.

f. Chromatid gap.
Explanation for Fig. 2 (Contd.)

g. Chromatid break, the acentric fragment placed widely apart.

h. A fragment of unknown origin.

i. Iso-chromatid breaks of untraceable origin.

j. Iso-chromatid break, fragment displaced.

k. Ring chromosome but without fragment.

l. Ring chromosome accompanied with a fragment.
Explanation for Fig. 2 (Contd.)

m. One ring chromosome but without fragment.

n. Exchange forming a metacentric chromosome.

o. Chromatid translocation forming a dicentric chromosome and a chromatid gap.

p. Chromatid exchange forming a dicentric chromosome, but no fragment.

q. A place showing a number of chromatid and isochromatid breaks, fragments and unequal chromatids.

r. Chromosomes showing centromeric stretching.
Each of the isochromatid breaks, rings and exchanges was considered as two breaks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Period of treatment</th>
<th>Metaphases scored/animals</th>
<th>Break-type aberrations</th>
<th>Gaps</th>
<th>Total aberrations (mean%±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>(Pooled)</td>
<td>3000/30</td>
<td>7</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>50.0</td>
<td>24h</td>
<td>400/4</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>150.0</td>
<td>24h</td>
<td>400/4</td>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>300.0</td>
<td>24h</td>
<td>400/4</td>
<td>7</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>300.0</td>
<td>48h</td>
<td>400/4</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>300.0</td>
<td>24h **</td>
<td>400/4</td>
<td>18</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>300.0</td>
<td>24h ***</td>
<td>400/4</td>
<td>19</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>150.0</td>
<td>4wk ***</td>
<td>500/5</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>150.0</td>
<td>8wk ***</td>
<td>500/5</td>
<td>7</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>150.0</td>
<td>12wk ***</td>
<td>500/5</td>
<td>12</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>300.0</td>
<td>4wk ***</td>
<td>500/5</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>300.0</td>
<td>8wk ***</td>
<td>400/4</td>
<td>16</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>300.0</td>
<td>12wk ***</td>
<td>500/5</td>
<td>16</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>300.0</td>
<td>16wk ***</td>
<td>500/5</td>
<td>14</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Each of the isochromatid breaks, rings and exchanges was considered as two breaks.

^a p<0.05, ^b p<0.01, ^c p<0.001 compared to the negative control value (ANOVA,'t'-test).

Treated twice (*) and thrice(**) at an interval of 4 h and killed at 24 h after the first treatment.

Treated once daily for different weeks(***).
Fig. 3 Dose response of the genotoxic effect of gudakhu extract as revealed by the micronucleus (0), chromosome aberration (□) and SCE (△) assays in bone marrow cells of mice. 'C' indicates the control value.

Fig. 4 Histogram analysis of metaphase chromosome aberrations in mice treated once (1), twice (2) or thrice (3) at an interval of 4 h (a), and showing the time-response effect following single treatment (b). 'C' stands for control.
incidences of breaks, but not for week 4. Further, in the repeated treatment regimen the frequencies of breaks for 150 mg/kg/day were comparatively lower than those for 300 mg/kg/day.

Quantitative-WEG: Following single treatment the frequency of breaks and total aberrations increased by about 8 and 3.5 folds respectively over the corresponding control values (Table-13), the increases were highly significant. Chronic treatment regimen also yielded significantly high incidences of chromosome aberrations, excluding and including gaps, for all the time-courses. The values for break type aberrations for weeks 8, 12, and 16 were very close to each other and also close to the value obtained following single treatment. The frequencies of breakages as well as total aberrations in chronic treatment regimen showed a tendency of gradual increase with the increase of time-course of treatment (Fig.5).

2.5.2.2 Micronucleus test in bone marrow cells

Qualitative: Micronuclei obtained in bone marrow erythrocytes were invariably round in shape, but varied in size (Figs 6a-i) suggesting loss of chromosome fragments of various sizes from the nucleus. The position of the micronuclei in the cell was also variable. The occurrence of more than one micronucleus in one erythrocyte was rare; in two instances erythrocytes were found to contain two MN. The MN recorded in the nucleated cells were in general bigger in size (Figs. 6j-l). The affected nucleated cells in most of the cases also had one micronucleus.

Quantitative: Analysis of MN in bone marrow erythrocytes revealed significantly high frequencies of micronucleated PCEs (MNPCEs), compared to control, for two higher dose levels in single treatment regimen, and also for all test weeks in repeated treatment regimen (Table-14). Dose-
Each of the iso-chromatid breaks, rings and exchanges was considered as two breaks.
* dry extract of gudakhu; ** treated once; *** treated once daily.

<table>
<thead>
<tr>
<th>Periods of treatment</th>
<th>Control</th>
<th>24h</th>
<th>4wk</th>
<th>6wk</th>
<th>12wk</th>
<th>16wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Pooled)</td>
<td>3000/30</td>
<td>400/14</td>
<td>600/16</td>
<td>600/16</td>
<td>600/16</td>
<td>600/16</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>Control</td>
<td>625</td>
<td>625</td>
<td>625</td>
<td>625</td>
<td>625</td>
</tr>
<tr>
<td>Gaps Total aberrations (mean±SE)</td>
<td>1.93±0.24</td>
<td>0.63±0.14</td>
<td>5.00±0.50</td>
<td>5.00±0.39</td>
<td>5.00±0.44</td>
<td>5.00±0.85</td>
</tr>
<tr>
<td>Fragments Total aberrations (mean±SE)</td>
<td>1.00±0.10</td>
<td>0.35</td>
<td>4.67±0.36</td>
<td>8.67±1.02</td>
<td>10.17±0.64</td>
<td>10.17±0.64</td>
</tr>
<tr>
<td>Isochromatid Exchange Total aberrations (mean±SE)</td>
<td>39</td>
<td>12</td>
<td>15</td>
<td>27</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>Metaphases scored/animals</td>
<td>3000/30</td>
<td>400/14</td>
<td>600/16</td>
<td>600/16</td>
<td>600/16</td>
<td>600/16</td>
</tr>
</tbody>
</table>

Significantly different from the control; (t-test and Anova test): c = p<0.001.
Fig. 5

Linear regression curves showing relation between weeks of chronic treatment of gudakhu and chromosome aberrations in bone marrow cells (A), micronucleated peripheral erythrocytes (B), Chromosome aberrations in spermatocytes(C), or mis-shapen sperm (D).

Fig 5A  □, ______ r = 0.957, a = -0.039, b = 0.458, p < 0.05
         ●, ----- r = 0.898, a = 0.884, b = 0.384, p = < 0.05
         △, ........ r = 0.976, a = 1.074, b = 0.315, p < 0.01

Fig 5B  ●, -------- r = 1.000, a = 0.150, b = 0.101, p < 0.001

Fig 5C  □, ______ r = 0.943, a = 0.870, b = 0.240, p < 0.05
         ●, -------- r = 0.758, a = 1.280, b = 0.138, p > 0.05
         △, ........ r = 0.956, a = 1.148, b = 0.271, p < 0.05

Fig 5D  □, ______ r = 0.993, a = 0.840, b = 0.575, p < 0.01
         ●, -------- r = 0.864, a = 3.240, b = 0.458, p > 0.05
         △, ........ r = 0.945, a = 3.358, b = 0.438, p < 0.05
Mean % of breaks in Bone marrow cells

Mean % of MN in Peripheral Erythrocytes

Mean % of breaks in Spermatocytes

Mean % of Mis-shapen sperm

150 mg/kg acetone extract of gudakhu
300 mg/kg acetone extract of gudakhu
625 mg/kg water extract of gudakhu

C- - indicates the control values.
Explanation for Fig. 6

Cut-out photomicrographs of bone marrow smears displaying micronucleated erythrocytes and nucleated cells.

a-h. Polychromatic erythrocytes showing one micronucleus each, size and location of MN vary.

i. Normochromatid erythrocyte showing one micronucleus.

j-l. Nucleated cells each containing a micronucleus. MN vary in size and location.
TABLE 14
INCIDENCE OF MICRONUCLEI IN BONE MARROW CELLS OF MICE TREATED WITH ACETONE EXTRACT OF GUDAKHU

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>No. of animals</th>
<th>Period of treatment</th>
<th>PCEs with MN (MN/PCEs)</th>
<th>NCEs with MN (MN/NCEs)</th>
<th>PCEs+NCEs with MN</th>
<th>PCE/NCE ratio</th>
<th>Nucleated cells with MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>--</td>
<td>0.20±0.02 (16/8000)</td>
<td>0.21±0.02 (14/6887)</td>
<td>0.20±0.02</td>
<td>1.17±0.04</td>
<td>0.06±0.01</td>
</tr>
<tr>
<td>50.0</td>
<td>3</td>
<td>30h</td>
<td>0.33±0.07 (10/6000)</td>
<td>0.10±0.00 (6/6000)</td>
<td>0.22±0.04</td>
<td>1.01±0.02</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td>150.0</td>
<td>4</td>
<td>30h</td>
<td>0.43±0.04b (34/8000)</td>
<td>0.18±0.02 (14/8000)</td>
<td>0.30±0.02a</td>
<td>1.16±0.02</td>
<td>1.00±0.01</td>
</tr>
<tr>
<td>300.0</td>
<td>4</td>
<td>30h</td>
<td>0.54±0.02c (43/8000)</td>
<td>0.10±0.02 (9/8000)</td>
<td>0.33±0.01b</td>
<td>1.24±0.09</td>
<td>0.12±0.03</td>
</tr>
<tr>
<td>300.0</td>
<td>3</td>
<td>8wk</td>
<td>0.55±0.04c (33/6000)</td>
<td>0.22±0.02 (10/4640)</td>
<td>0.40±0.02b</td>
<td>1.29±0.02</td>
<td>0.08±0.01</td>
</tr>
<tr>
<td>300.0</td>
<td>4</td>
<td>12wk</td>
<td>0.63±0.03c (51/8000)</td>
<td>0.27±0.02 (16/5852)</td>
<td>0.48±0.02c</td>
<td>1.37±0.04</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>300.0</td>
<td>4</td>
<td>16wk</td>
<td>0.61±0.06c (49/8000)</td>
<td>0.33±0.02b (20/6065)</td>
<td>0.49±0.03c</td>
<td>1.32±0.02</td>
<td>0.14±0.02</td>
</tr>
</tbody>
</table>

Values are mean of 100 cells ± SE.
Significantly different from the control; ('t'- test and Anova test) : a = p < 0.05; b = p < 0.01; c = p < 0.001.
response study exhibited a clear dose related increase in the incidence of MNPCEs (Fig.3). However, the data for repeated treatment for different weeks failed to show any significant difference among themselves (Anova test). The incidence of MN in NCEs remained in the control range except for week 16. In no case did the PCE/NCE ratio differ significantly from the control value, indicating little cytotoxic effect.

2.5.2.3 Micronucleus test in hepatocytes

Qualitative: Our study was restricted to the regenerated hepatocytes only. Other types of cells like Kupffer cells, endothelial cells and fat storing cells were not considered; they are much smaller in size, fewer in number and have a higher nucleus to cytoplasm ratio, and are, thus, easily differentiable from hepatocytes. Characteristic nucleus to cytoplasm ratio (1:4) of the hepatocytes helped greatly in identifying them and facilitated easy scoring of MN in them. The hepatocytes are known to vary greatly in their size (1-6 folds) and shape as well as in number and ploidy states of the nucleus (Elias and Sherrick, 1969). The cytoplasm of the hepatocytes sometimes exhibited a little vacuolation due to the presence of glycogen (Figs. 7a,c). As usual the bi-nucleate and uni-nucleate cells constituted the major bulk of the hepatocyte population, multi-nucleates (represented by trinucleates only) were very few.

The particles noted in the present study as MN looked like chromatin bodies in all respects. Interestingly in hepatocytes the MN exhibited the same degree of condensation as the main nucleus (Figs. 7a-d). The location of the main nucleus as well as the micronucleus, whenever present, in the cytoplasm varied greatly. The size of the MN showed a range of variation too (Figs. 7a-d). Most of the affected cells contained only one micronucleus, a few were found to contain two. Some of the tri- and bi-nucleate cells were found to have one nucleus smaller than the other(s)
Explanation for Fig. 7

Photomicrographs of regenerated hepatocytes with micronucleus induced by gudakhu extract.

a, c. Uninucleate hepatocytes containing one micronucleus each.

b, d. Binucleate hepatocytes, each with one micronucleus.
(≈ half); they were very few in number and categorized separately under nuclear anomalies but not considered for quantitative analysis. A few hepatocytes were noted to have nucleus as big as twice the size of normal nucleus.

Quantitative: The frequencies of regenerated hepatocytes with MN increased significantly over the control value in all the test weeks (Table-15), further, a distinct positive linear relationship was observed with the time-course of treatment (Fig.8).

2.5.2.4 Micronucleus test in peripheral erythrocytes

Qualitative: Micronuclei were invariably round in shape and were of varied size groups (Fig.9a-f). Never an erythrocyte was found to contain more than one MN. Both polychromatic and matured erythrocytes were found to contain MN.

Quantitative: Table-16 summarizes the data on the frequencies of MN in peripheral blood of mice treated repeatedly with a dose of 300 mg/kg/day for 1 to 4 weeks. The frequencies of MN increased significantly, compared to the control value, in all the test weeks, and the values were about 3-fold the control value for weeks 3 and 4. Further, the increase showed a good positive correlation with the time-course of chronic treatment (Fig.5b).

2.5.2.5 Sister chromatid exchanges

Qualitative: Harlequin stained second-division metaphases only were examined (Figs. 10a-f). SCE per cell was scored and it ranged from 2 to 29.

Quantitative: The incidence of SCE increased greatly, compared to the control value, in all the test weeks and for all the dose levels, but the values for single and repeated
### TABLE 15

INCIDENCE OF MICRONUCLEI IN REGENERATED HEPATOCYTES OF MICE TREATED CHRONICALLY WITH A DOSE OF 300 mg/kg OF ACETONE EXTRACT OF GUDAKHU FOR DIFFERENT PERIODS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weeks of treatment</th>
<th>Total cells score/animals</th>
<th>Uninucleate hepatocytes with MN</th>
<th>Binucleate hepatocytes with MN</th>
<th>Uninucleate &amp; binucleate hepatocytes with MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>4000/4</td>
<td>0.27 ± 0.03</td>
<td>0.40 ± 0.02</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>GE</td>
<td>8</td>
<td>3000/3</td>
<td>0.79 ± 0.10**</td>
<td>1.06 ± 0.13***</td>
<td>0.97 ± 0.11***</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4000/4</td>
<td>1.42 ± 0.06***</td>
<td>1.79 ± 0.15***</td>
<td>1.68 ± 0.11***</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>4000/4</td>
<td>1.30 ± 0.08***</td>
<td>2.22 ± 0.14***</td>
<td>1.78 ± 0.06***</td>
</tr>
</tbody>
</table>

Values are of means of 100 cells ± SE.

** p < 0.01, *** p < 0.001 compared to the negative control value (Anova and 't' test).
Fig. 8 Graph showing linear relations of the incidence of MN in regenerated hepatocytes with the time-course of chronic treatment. 'C' indicates the control value.
Explanation for Fig. 9

Photomicrographs of mouse peripheral blood smears showing micronucleated erythrocytes induced following treatment of gudakhu extract.

a-f. Peripheral erythrocytes containing one micronucleus each. MN vary in size.
TABLE 16
INCIDENCE OF MICRONUCLEATED ERYTHROCYTES IN PERIPHERAL BLOOD OF MICE TREATED WITH THE ACETONE EXTRACT OF GUDAKHU FOR DIFFERENT WEEKS

<table>
<thead>
<tr>
<th>Dose* (mg/kg)</th>
<th>Weeks of treatment</th>
<th>Total RBCs scored/animals</th>
<th>Micronucleated erythrocytes (mean%±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>35,000/7</td>
<td>0.17 ± 0.006</td>
</tr>
<tr>
<td>300</td>
<td>1</td>
<td>35,000/7</td>
<td>0.27 ± 0.02b</td>
</tr>
<tr>
<td>300</td>
<td>2</td>
<td>35,000/7</td>
<td>0.28 ± 0.11c</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>35,000/7</td>
<td>0.49 ± 0.10c</td>
</tr>
<tr>
<td>300</td>
<td>4</td>
<td>35,000/7</td>
<td>0.56 ± 0.07c</td>
</tr>
</tbody>
</table>

* The amount indicates dry extract.

Significantly different from the control; ('t'-test and Anova test); b = p<0.01; c = p<0.001.
Explanation for Fig. 10

Photomicrographs of bone marrow second-division metaphase showing sister chromatid exchanges induced by gudakhu extract.

a-f. Harlequin stained chromosomes of sister chromatid exchanges. Number of exchanges varies from cell to cell.
treatment for the dose 300 mg/kg exhibited no marked
difference. Lower dose, however, induced less effect
(Table-17, Fig.3).

2.5.2.6 Spermatocyte chromosome analysis

Qualitative: So far structural changes were concerned the
effects in the AEG and WEG series as well as in different
sampling intervals were the same except presence or absence
of certain aberration type(s) at certain sampling intervals.
The structural aberrations comprised chromatid and
chromosome type breaks, fragments of untraceable origin and
translocations. Chromatid type breaks involved both
autosomes and sex chromosomes (Fig.11a-c). In most of the
cases the fragments resulted from chromosome type breaks
were seen placed far away from their places of origin
(Fig.11f), in some cases of course the fragments exhibited a
little displacement only (Fig. 11g). In some instances the
X chromosome found to be involved in breakage (Fig.11g).
A bivalent in a cell was seen without a part (Fig.11h). A
few cells were found to contain some extra fragments smaller
than the smallest univalent (Figs. 11e,i,j), because of
their smallness their origin could not be traced.
Translocations were noted in a number of cases involving
autosomes as well as sex chromosomes. Chain of four
involving two autosomal bivalents was also recorded(Fig.11k).
Translocation multivalents involving sex bivalent and
autosomal bivalent were also available (Figs. 11 l,m).

Though cells with less than 20 bivalents were of
frequent occurrence cells with more than 20 bivalents were
very rare. Because of this rarity the latter was not
considered for quantitative analysis. No importance was
given to plates with less than 20 bivalents as they might
have been originated due to technical shortcomings, and as
such they are not presented. Though occurrence of polyploid
**INCIDENCE OF SCE IN BONE MARROW CELLS OF MICE TREATED WITH ACETONE EXTRACT OF GUDAKHU**

<table>
<thead>
<tr>
<th>Period of treatment</th>
<th>Total SCE</th>
<th>SCE/Cell (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>293/10</td>
<td>1392</td>
</tr>
<tr>
<td>AEG</td>
<td>4.70 ± 0.22</td>
<td>508</td>
</tr>
<tr>
<td></td>
<td>7.85 ± 0.21</td>
<td>1324</td>
</tr>
<tr>
<td></td>
<td>9.16 ± 0.22</td>
<td>1022</td>
</tr>
<tr>
<td></td>
<td>8.58 ± 0.33</td>
<td>1117</td>
</tr>
<tr>
<td></td>
<td>8.82 ± 0.33</td>
<td>989</td>
</tr>
<tr>
<td></td>
<td>9.12 ± 0.17</td>
<td>1046</td>
</tr>
</tbody>
</table>

**Dose (mg/kg)**

<table>
<thead>
<tr>
<th></th>
<th>Pooled</th>
<th>150.0</th>
<th>300.0</th>
<th>300.0</th>
<th>300.0</th>
<th>300.0</th>
<th>300.0</th>
</tr>
</thead>
</table>

**Metaphases scored/animal**

<table>
<thead>
<tr>
<th></th>
<th>24 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>16 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>150.0</td>
<td>65/2</td>
<td>146/4</td>
<td>102/3</td>
<td>129/3</td>
<td>115/3</td>
</tr>
<tr>
<td>300.0</td>
<td>1324</td>
<td>1022</td>
<td>1117</td>
<td>989</td>
<td>1046</td>
</tr>
</tbody>
</table>

---

**Table 17**

**INCIDENCE OF SCE IN BONE MARROW CELLS OF MICE TREATED WITH ACETONE EXTRACT OF GUDAKHU**

*P < 0.001 compared to the negative control value (Anova and 't'-test).*
Explanation for Fig. 11

Photomicrographs of diakinesis-metaphase I stages showing structural chromosome aberrations induced by gudakhu extract.

a. Chromatid break involving an autosome, the fragment placed apart.

b. Chromatid break involving an autosome, showing a large gap.

c. Chromatid breaks involving two autosomal bivalents and a fragment of untraceable origin.

d. A fragment of untraceable origin.

e. A fragment of untraceable origin and univalent formation involving an autosomal bivalent.

f. A fragment resulted from chromosome break placed widely apart. Another autosomal bivalent showing univalent formation.

g. Chromosome type break involving sex chromosome.

h. One homologue in an autosomal bivalent showing chromosome break and loss of the fragment.
i, j. Fragments of untraceable origin.

k. Two autosomal bivalents involved in translocation forming a chain of four.

l, m. X-Y and autosomal bivalence forming translocation multivalent.

n. X and Y chromosome lying widely apart.

o. Univalent formation: two autosomal homologues placed widely apart.
diakinesis metaphase-I cells was common, but the incidence did not vary remarkably from that of control.

Both autosomes and sex chromosomes were involved in univalent formation. In several cases X and Y were found to lie wide apart (Fig. 11n). Similar cases were also noted involving autosomes (Figs. 11e,f,o).

Quantitative-AEG : Data on the effect of AEG as revealed by spermatocyte chromosome analysis following chronic treatment are presented in Table-18. Data exhibited significantly higher incidence of structural chromosome aberrations over the control value, but the values for different weeks particularly at later weeks remained very close to each other; the data for 2 dose levels did not also vary (Fig. 5c).

So far univalent formation was concerned, higher incidence was noticed particularly for the higher dose (300 mg/kg/day) and the increase was marked for autosomal bivalents.

Quantitative-WEG : Analysis of meiotic chromosomes revealed significantly high incidences of breakages in all the sampling weeks (Table-19), and the incidences showed a linear relationship with the time-course of treatment (Fig. 5c). The highest incidence of breaks noted at week 16 was about 10 times the control value, while at week 4 it was about 5 times the control value.

With regard to univalent formation the incidence increased in the treated animals, and the increase was highly significant for X-Y bivalents particularly following longer time-course of treatment. The frequency of univalent formation for X-Y bivalent for both weeks 12 and 16 was about 4 times the control value. On the other hand, the highest incidence involving autosomes observed at week 16


**TABLE 18**

**FREQUENCY DISTRIBUTION OF DIFFERENT TYPES OF STRUCTURAL CHROMOSOME ABERRATIONS AND UNIVALENT FORMATION IN SPERMATOCYTE OF MICE INDUCED BY CHRONIC TREATMENT OF ACETONE EXTRACT OF GUDAKHU**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Weeks of treatment</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diak., Met.-I scored/animals</td>
<td>Chrom. Break</td>
</tr>
<tr>
<td>Control (Pooled)</td>
<td>1200/24</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>150.0</td>
<td>4</td>
<td>150/3</td>
<td>1</td>
</tr>
<tr>
<td>150.0</td>
<td>8</td>
<td>150/3</td>
<td>2</td>
</tr>
<tr>
<td>150.0</td>
<td>12</td>
<td>150/3</td>
<td>-</td>
</tr>
<tr>
<td>300.0</td>
<td>4</td>
<td>150/3</td>
<td>1</td>
</tr>
<tr>
<td>300.0</td>
<td>8</td>
<td>150/3</td>
<td>2</td>
</tr>
<tr>
<td>300.0</td>
<td>12</td>
<td>150/3</td>
<td>1</td>
</tr>
<tr>
<td>300.0</td>
<td>16</td>
<td>150/3</td>
<td>2</td>
</tr>
</tbody>
</table>

Each exchange was considered as two breaks.

* The amount indicates dry extract.

Significantly different from the control; ('t'-test and Anova test): <sup>a</sup> = p < 0.05; <sup>b</sup> = p < 0.01; <sup>c</sup> = p < 0.001.
<table>
<thead>
<tr>
<th>Dose* (mg/kg)</th>
<th>Weeks of treatment</th>
<th>Diaki. Meta.-I scored/animals</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chrom. br.</td>
<td>Chromt. br.</td>
</tr>
<tr>
<td>Control (Pooled)</td>
<td>1200/24</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>625</td>
<td>4</td>
<td>200/4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>625</td>
<td>8</td>
<td>200/4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>625</td>
<td>12</td>
<td>200/4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>625</td>
<td>16</td>
<td>200/4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Each exchange was considered as two breaks.
* Dry extract of gudakhu.

Significantly different from the control; ('t'-test and Anova test): c = p<0.001.
was about twice the respective control value.

2.5.2.7 Sperm morphology assay

Qualitative: A population of sperm in both control and treated individuals exhibited abnormal morphology in their head as well as tail structure. As the head abnormalities were easily recognizable and more common than tail abnormalities the author concentrated his studies on the former. It was not possible to group the abnormalities under some distinct categories. They were classed into some arbitrary 'types'. The 'types' like with flat base, with acrosome spine abnormalities, vacuolated and amorphous were very common in occurrence (Fig. 12). The sperm heads with acrosomal spine abnormalities exhibited a high degree of variation in the spine morphology ranging from a total absence to a long hook shaped one (Figs. 12q,r,n). In addition to the above types, triangular, rectangular, balloon shaped, umbrella shaped, reduced and giant size, sperm heads with bifurcated acrosomal spine and invagination were also available in both control and treated animals (Figs. 12e,f,h,i,r,u,v) though rare in the former. Sometimes, a particular sperm head exhibited a combination of two or more 'types' of abnormalities (Figs. 12g,q,r,w).

Quantitative-AEG: The incidence of mis-shapen sperm increased greatly over the control value following chronic treatment of AEG (Table-20), the highest being about 4 times the control value at week 12 for 300 mg/kg. Dose-dependent increase in the frequency of mis-shapen sperm is quite evident when week-wise data for two doses were compared. Again, for any dose level, frequency of abnormal sperm increases with the weeks of treatment, particularly up to a period of 12 weeks (Fig. 5d).

Quantitative-WEG: The frequency of sperm with abnormal head morphology increased significantly, compared to
Explanation for Fig. 12

Photomicrographs of vasa deferential sperm of mice showing abnormalities in head morphology induced by water or acetone extract of gudakhu.

a, c. Sperm containing a vacuole.
b. Sperm with flat base and an invagination.
d. A sperm without acrosomal spine, and twisted head, another sperm with rectangular head.
e. All sperm in the field showing abnormality: with flat base, with hook shaped or short acrosomal spine, gigantic size, triangular with a vacuole, etc.
f. An umbrella shaped sperm-head.
g. Triangular head with a vacuole.
h. Rectangular head; with flat base.
i. A sperm head with a vacuole, another with curved or hook shaped acrosomal spine.
Explanation for Fig. 12 (Contd.)

j. Sperm heads with flat base, sickle shape or amorphous shape.

k. Sperm heads with amorphous shape.

l. Balloon shaped, with bifurcated acrosomal spine with flat base.

m. Twisted head without acrosomal spine.

n. Hook shaped acrosomal spine.

o. Sickle shaped sperm head.

p. Sperm head with an invagination.
Explanation for Fig.12 (Contd.)

q. Sperm head showing various abnormalities with flat base, hook shaped spine, triangular shape, etc.

r. Rudimentary sperm head.

s. Amorphous shape.

t. Hook shape spine and amorphous type.

u. Triangular and rectangular sperm heads.

v. Cup-shaped sperm head.

w. Sperm head having a vacuole at the centre.
TABLE 20
EFFECT OF REPEATED TREATMENT OF ACETONE EXTRACT OF GUDAKHU ON SPERM HEAD ABNORMALITY AND SPERM PRODUCTION IN DIFFERENT WEEKS

<table>
<thead>
<tr>
<th>Dose <em>(mg/kg)</em></th>
<th>Weeks of treatment</th>
<th>Total sperm head scored/animals</th>
<th>Abnormal sperm head (mean%±SE)</th>
<th>Animals analyzed</th>
<th>Mean count/epididymis (mean X 8 X 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>24,000/24</td>
<td>2.64 ± 0.19</td>
<td>24</td>
<td>104.33 ± 3.89</td>
</tr>
<tr>
<td>150.0</td>
<td>4</td>
<td>3000/3</td>
<td>3.27 ± 0.65 c</td>
<td>3</td>
<td>101.51 ± 0.48</td>
</tr>
<tr>
<td>150.0</td>
<td>8</td>
<td>3000/3</td>
<td>7.63 ± 1.24 c</td>
<td>3</td>
<td>94.23 ± 0.93</td>
</tr>
<tr>
<td>150.0</td>
<td>12</td>
<td>3000/3</td>
<td>8.37 ± 0.66 c</td>
<td>3</td>
<td>90.85 ± 0.68 b</td>
</tr>
<tr>
<td>300.0</td>
<td>4</td>
<td>3000/3</td>
<td>4.27 ± 1.32 a</td>
<td>3</td>
<td>Not done</td>
</tr>
<tr>
<td>300.0</td>
<td>8</td>
<td>3000/3</td>
<td>8.33 ± 0.31 c</td>
<td>3</td>
<td>102.48 ± 2.44</td>
</tr>
<tr>
<td>300.0</td>
<td>12</td>
<td>3000/3</td>
<td>10.77 ± 0.31 c</td>
<td>3</td>
<td>94.22 ± 0.82 a</td>
</tr>
<tr>
<td>300.0</td>
<td>16</td>
<td>3000/3</td>
<td>8.53 ± 0.20 c</td>
<td>3</td>
<td>91.22 ± 1.00 b</td>
</tr>
</tbody>
</table>

* The amount indicate dry extract.

Significantly different from the control; (t-test and Anova test): a = p < 0.05; b = p < 0.01; c = p < 0.001.
controls, in all the test weeks (Table-21). The increase was highly marked particularly from week 8 onward. The values for week 8, 12, and 16 were close to each other. The maximum increase was noted at week 16. Here also a good positive correlation was noted between frequency of misshapen sperm and time-course of treatment (Fig.5d).

2.5.2.8 Sperm count

Quantitative: The mean sperm count for untreated control animals comes to 104.26. To obtain total sperm count per epididymis this value would be multiplied with a factor of $8 \times 10^4$ (Tables-20,21). Sperm count following AEG treatment showed a tendency of reduction with the increase of course of treatment, but the decrease was not remarkable. For any particular test week the data for two doses did not differ greatly. The same situation was noted with WEG; but here reduction in count with the increase of course of treatment was a little more, and appreciable at 12 and 16 weeks.

2.5.3 Discussion

Significantly high incidences of chromosomal aberrations in bone marrow, regenerated hepatocytes and peripheral erythrocytes, and SCEs clearly indicate a positive genotoxic potential of the organic solvent and water extracts of gudakhu. The extracts also induce abnormality in sperm head morphology. Our finding is in accordance with that of Stich and Anders (1989) who have reported higher incidence of MN in exfoliated cells of buccal mucosa of habitual users of gudakhu.

Qualitatively, almost all the aberrations induced in bone marrow metaphases by acetone extract (AEG) and water extract (WEG) of gudakhu are of chromatid type, even after weeks of chronic treatment. It seems the extracts like
TABLE 21
EFFECT OF REPEATED TREATMENT OF WATER EXTRACT OF GUDAKHU ON SPERM MORPHOLOGY AND SPERM PRODUCTION IN DIFFERENT WEEKS

<table>
<thead>
<tr>
<th>Dose* (mg/kg)</th>
<th>Weeks of treatment</th>
<th>Total sperm head scored/animals</th>
<th>Abnormal sperm head (mean% ± SE)</th>
<th>Animals analyzed</th>
<th>Mean count/epididymis (mean X 8 X 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>24000/24</td>
<td>2.64 ± 0.19</td>
<td>24</td>
<td>104.33 ± 3.89</td>
</tr>
<tr>
<td>625</td>
<td>4</td>
<td>4000/4</td>
<td>4.98 ± 0.45^c</td>
<td>4</td>
<td>94.28 ± 1.10^b</td>
</tr>
<tr>
<td>625</td>
<td>8</td>
<td>4000/4</td>
<td>8.28 ± 0.68^c</td>
<td>4</td>
<td>89.53 ± 0.28^c</td>
</tr>
<tr>
<td>625</td>
<td>12</td>
<td>4000/4</td>
<td>9.03 ± 0.54^c</td>
<td>4</td>
<td>86.17 ± 0.56^c</td>
</tr>
<tr>
<td>625</td>
<td>16</td>
<td>4000/4</td>
<td>9.38 ± 0.38^c</td>
<td>4</td>
<td>79.35 ± 1.44^c</td>
</tr>
</tbody>
</table>

* Dry extract of gudakhu.

Significantly different from the control; ('t'-test and Anova test): b = p < 0.01; c = p < 0.001.
great majority of chemical chromosome damaging agents require replicative DNA synthesis in order to have the lesion it induces translated into structural aberrations.

In AEG series dose-response analyses of chromosome aberrations in bone marrow metaphases, MN in bone marrow polychromatic erythrocytes, and SCEs clearly reveal positive correlation of the genotoxic efficiency of the extract with the doses. Such a positive correlation was also marked when data on chromosome aberrations in bone marrow metaphases for chronic treatment for two different doses, as well as for data obtained from animals treated once, twice and thrice spaced 4 h were compared. Decrease of effect with the increase of time between treatment and sampling as documented in chromosome aberration study in bone marrow metaphases is obvious. Bone marrow is a proliferative tissue, and the affected cells probably pass on to the next stage or are destroyed. Besides, there is a question of gradual elimination of the substance, thereby, decreasing its concentration in the body.

The pattern of incidences of chromosome breakages in different test weeks particularly in bone marrow cells following chronic treatment of WEG is similar to that obtained with AEG. In bone marrow cells for both the extracts the frequencies of breaks at weeks 8, 12 and 16 are close to each other. This is also probably due to proliferative nature of the bone marrow cells. However, the values for week 4 for both the extracts are much less, it seems to indicate some sort of accumulation of pre-clastogenic damages or of the clastogenic agent(s) in the body to a certain level.

Sensitivity, reliability as well as simplicity of the micronucleus test have already been illustrated. Its principle has also been described (vide 2.2.2). All these aspects as well as the aspects of standardization an
modification of protocols have recently been reviewed by Mavournin et al. (1990), Ashby et al. (1990) and Heddle et al. (1991). In the present investigation poly- and normochromatic erythrocytes as well as nucleated bone marrow cells were considered. However, during scoring of MN in the nucleated cells, as mentioned earlier, due to the presence of large nucleus and comparatively little cytoplasm in certain cells separate existence of MN became doubtful. Similarly confusion arose regarding the genuineness of MN in certain other cells with lobulated nucleus. Though those doubtful cases were not recorded, the data on nucleated cells were surely influenced by it. For that we do not want to put much importance on the incidence of MN in the nucleated cells. Higher incidence of MN, compared to controls, in erythrocyte line, particularly in PCEs in which incidence is more relevant (Ledebur and Schmid, 1973; Schmid, 1976) indicates positive effect of AEG.

Micronuclei result from a clastogenic as well as spindle poisoning effect. The absence of abnormal mitotic figures such as lagging chromosome(s), and large sized MN and PCEs in our micronucleus preparation suggest that MN have been induced by AEG due to its clastogenic, not spindle poisoning, effect. This can further be substantiated by the fact that no dose level tested here could enhance the frequency of MNNCEs at 30h post-treatment though MNPCEs increased remarkably for all the doses.

The carcinogens and mutagens present in tobacco have a broad distribution of probable target organs: N-nitrosamines (the most important tobacco specific clastogens and mutagens) for the liver, oesophagus and other organs (Lofroth, 1989). This prompted us to study hepatocytes. No positive control experiment was conducted for the MNT in hepatocytes. However, our past experience (Das and Roy, 1988; Roy and Das, 1990) was of great help in
analysing MN in regenerated hepatocytes. Since the liver has little proliferative activity repeated treatment for long periods probably leads to accumulation of more and more clastogens and/or pre-clastogenic damages in the hepatocytes. Pre-clastogenic damage was expressed by partial hepatectomy. This explains the linear relationship noted between the incidence of MN in regenerated hepatocytes and the time-course of treatment. A very high incidence of MN was also noted in hepatocytes of mouse foetuses when pregnant mothers were exposed to tobacco smoke for the last 6 days of gestation (Balansky and Blagoeva, 1989). Persistence of pre-clastogenic damage in hepatocytes of rats following single treatment with ethylnitrosourea, diethylnitrosamine and dimethylnitrosamine was noted earlier by Tates et al. (1986). Our results on regenerated hepatocytes indicate that the longer the time-course of gudakhu use the more the genotoxicity it induces. Our data on habitual users of gudakhu also support the view (vide infra).

Identical picture was also documented while analyzing MN in peripheral RBCs. In mice micronucleated RBCs are known to remain in circulation, and not removed by spleen (Schlegel and Mac Gregor, 1982, 1983, see Mavournin et al., 1990, Heddle et al., 1991). As a result micronucleated RBCs (MNRBCs) get accumulated in the circulation. Thus, peripheral blood in mice provides a very unique system to monitor genotoxic effect of an environmental agent following chronic exposure (treatment). It is very simple too. In the present study the same animals provided blood samples for control as well as treatment data for different weeks. Blood sampling was done by puncturing the caudal vein. Blood samples collected before the starting of treatment served the purpose of control; for week-wise data in treatment series sampling was done from the same animals at the end of each week. Here also we find a very good linear relationship
between the incidence of MN and time-course of treatment. The nature of the regression curve is identical to that for regenerating hepatocytes. Our study included both PCEs and NCEs. Thus, accumulation of MN-cells in the peripheral blood is quite evident. Recently Hayashi et al. (1990) reported equal incidences of micronucleated peripheral reticulocytes (MNRETs) and micronucleated bone marrow polychromatic erythrocytes (MNPCEs) in mice treated with mitomycin-C though the induction of MNRETs was delayed by about 12 h compared to that of MNPCEs. Elevated incidence of MNPCEs was also noted in peripheral blood of new-born mice the mother of which had been exposed to tobacco smoke daily for 1 h during the last 6 days of pregnancy (Balansky and Blagoeva, 1989) indicating free passage of tobacco smoke specific clastogen(s) through placenta.

Although the precise molecular mechanism for its formation is not yet clearly known, SCE is considered to be a rapid, simple and very sensitive cytogenetic end-point for evaluation of potential genotoxicity of chemicals and other environmental agents. SCE occurs during replication of chromosomal DNA at 4 strand stage and its incidence is taken as a measure of DNA damage and repair (Sasaki, 1982; Wolff, 1982). Dose-response data for SECs revealed a significant difference between the frequencies of SCEs for two doses. Similar dose effect relationship was documented for hundreds of chemicals and other environmental agents (Perry and Evans, 1975; Perry, 1980; Das, 1988).

Significantly higher breakage frequencies in all test weeks and for both the doses (in case of AEG) clearly demonstrate clastogenic capacity of the extracts. As noted in the case of bone marrow cells, the frequencies of breakages in spermatocytes following repeated treatment for different time-courses did not differ markedly. This is not at all unexpected for germ line cells which are proliferative in nature. Interestingly, two doses tested
here, for AEG yielded exactly the same result for any particular week. This was, however, not noted in bone marrow chromosome aberration study. WEG reveals a quite different pattern; gradual increase in aberration frequency is marked with the increase of course-of-treatment. But it is very unlikely that stem cells are affected. Very few chemicals are found to induce cytogenetic effects in stem cells that can be measured in meiotic cells. Adler (1982a,b) has discussed the probable reasons for this at length, but definite answer is yet to be known. Our study on spermatocyte chromosomes is based on limited data, and at this stage we are not in a position to put forward any explanation for it.

Oakberg (1957, 1960) and Ghosal and Mukherjee (1971) determined the time scale of different spermatogenic stages in mice; Adler (1982a) has reviewed it nicely. As in the present study daily treatment was done for different weeks (minimum for 4 weeks) it is not possible to pin point which stage is more susceptible to the extracts.

With regard to effect on pairing behaviour both auto-and sex-chromosomes exhibited some degree of susceptibility to univalent formation. In AEG series autosomal bivalents showed higher susceptibility than the X-Y bivalents, particularly with the higher doses, while in WEG series the sex chromosome bivalents seem to be more susceptible. As mentioned earlier it is not known if such a behaviour results either due to early break down of association or due to complete lack of it. As assumed by many workers (Brewen and Preston, 1978) method of slide preparation is largely responsible for such phenomenon and the incidence in untreated animals remains in the order of 10%. But here taking autosomes and sex chromosomes together the control value comes to less than 6%(consideraing both AEG and WEG series), nearly 3 times increase in the incidence of univalent formation following
the treatment of the extracts cannot simply be explained from preparational point of view. It is, therefore, not unlikely that the extracts impair the meiotic pairing of chromosomes. Antirabies vaccines and tetanus toxoid (Das and Nayak, 1988), chlordiazepoxide (a benzodiazepine tranquilizer, Kar and Das, 1987), 3-methyl 4-nitrophenol (Nehez et al., 1985) and MC (Chakrabarti et al., 1986), but not diazepam (Kar and Das, 1981), have been reported to induce such univalent formation in mice. Whatever may be the mechanism, univalent formation involving sex chromosomes (Beechey, 1973; Chandley et al., 1976; Chandley, 1981) as well as autosomes (Purnell, 1973) has earlier been shown to be associated with meiotic breakdown in male mammals.

Although the criteria of sperm morphology include different parts of a sperm analysis becomes easier if limited to head abnormalities only, since head shape is most insensitive to preparational damage. So far qualitative aspect is concerned treated animals compared to control ones produced a few more types of abnormal sperm heads, though their frequencies were low. Similarly, occurrence of some particular abnormal types specifically for certain chemicals were also noted earlier (Wyrobek and Bruce, 1975; Kar and Das, 1983). Significant increase in the incidence of mis-shapen sperm particularly in late sampling weeks clearly indicates positive toxic effect of both the types of extracts. Considering the frequency of abnormal sperm, the effects induce by WEG and the higher dose of AEG are almost the same. Although literature provides a number of reports on the frequencies of mis-shapen sperm in smokers (Evans et al., 1981; Evans, 1982; Wyrobek et al., 1983b) no such report is available with smokeless tobacco. The smokers, compared to non-smokers, are found to have a significantly higher percentage of abnormal sperm; however, data give no indication of any relation between the incidence of abnormal sperm and
numbers of cigarettes smoked per day (Evans et al., 1981; Evans, 1982). Here also we fail to note any correlation between the incidence of mis-shapen sperm and time-course of treatment.

56-days is the time required for the stem cells to become sperm in the mouse (Meistrich, 1989). Then there is question of coming of the sperm in vas deferens. So the stem cells which received the extract at the time of starting of treatment are expected to reach vas deferens after 8 weeks. The increase of frequency of abnormal sperm after 4 weeks of chronic treatment was marginal only and from 8 weeks onwards the increases were significant. This suggests that both the types of extracts affect the spermatogonial stem cells. However, this proposition should be taken with certain reservation, because the animals received repeated treatment.

In mice, as mentioned earlier, the shape of a normal sperm seems to be genetically controlled and induced abnormalities are suggested to be the result of genetic damage (Wyrobek and Bruce, 1975, 1978; Bruce and Heddle, 1979; Wyrobek et al., 1983a, b). Although there is no direct evidence in support of this possibility an increased incidence of mis-shapen sperm might be indicative of exposure of male germ cells to a mutagenic agent. Sensitivity of the sperm head abnormality assay for detecting deleterious effects of chemicals and other environmental agents has been demonstrated by several workers (Kar and Das, 1983; Wyrobek et al., 1983a,b, 1984; Bhunya and Pati, 1990).

An attempt was made to know if gudakhu extract has any adverse effect on sperm production. Although a reduction in epididymal sperm count is evidenced for both AEG and WEG the decline seems to have little significance with regard to reproductive efficiency. The maximum
suppression in the count is noted following 16 weeks of chronic treatment with the water extract and the count has reduced to about 77.5% of normal. Meistrich (1989) from a series of studies with physical and chemical agents has come to the conclusion that if the count is reduced to about 15% level of control then infertility may be seen. So, this reduction seems to have little significance from the point of fertility.

Sperm count provides a simple method for quantifying reproductive toxicity. It integrates all reproductive functions. It is simpler and more important than any other reproductive toxicology test like testicular histology or testis weights (Meistrich, 1989). In the mouse the stem cells take about 8 weeks to become sperm (Meistrich, 1989). Increase in suppression of the count particularly after 8 weeks seems to suggest that the stem cells are affected by the extracts, effect on other spermatogenic cell types however, cannot be ruled out.

Smokeless tobacco is an important ingredient of gudakhu. Polar extracts of smokeless tobacco have earlier been demonstrated to be genotoxic in the Ames test and in mammalian in vivo and in vitro assays as well (Shirname et al., 1984; Shah et al., 1985). Extracts of brown and black 'masheri', pyrolysed preparations of tobacco, have been shown to induce MN and CAs in mice and 8-azaguanine resistant mutations in V79 Chinese hamster cells (Kulkarni et al., 1987). Experimental findings with tobacco extracts are consistent with the observations on habitual chewers of tobacco. Stich and his co-workers (Stich et al., 1982; Brunnemann et al., 1987; Stich and Anders, 1989) have recorded a higher incidence of MN in buccal mucosa cells of habitual users of several tobacco preparations like 'Khaini', snuff, nass and betel quid with tobacco. Similarly a higher incidence of SCE has been noted in peripheral lymphocytes of persons chewing betel quid with
tobacco (Ghosh and Ghosh, 1984). That "Zarda", a chewing variety of tobacco preparation, induces mitostatic and clastogenic effect on the root meristems of Allium cepa is on record (Patnaik et al., 1984). Whong et al. (1987) and Shirname-More (1991) tested mutagenic potential of polar and non-polar solvent extracts of a number of brands of smokeless tobacco in Salmonella/microsome assay system. In contrast to our observation, and in contrast to the findings of Shirname et al. (1984) and Shah et al. (1985) mentioned above not a single extract was found mutagenic even in presence of metabolic activation; however, the extracts were mutagenic at acidic pH. Such a differential effect, i.e. negative mutagenic response of the extracts (without altering pH) in the Ames test and positive response in mammalian assays is not unknown in the field of chemical mutagenesis. Recently a host of substances and proven genotoxins have been reported to exhibit positive effect in mammalian test systems but negative in the Ames test (Adler, 1980; Ishidate, 1981; Zeiger and Tennant, 1986; Roy and Das, 1988; Ashby, 1989; Das and Roy, 1990). So, gudakhu, like other tobacco preparation, proves to be genotoxic.

Nicotine, the principal alkaloid of tobacco, has been shown to induce chromosome aberrations and SCEs in CHO cells in a dose-and duration-dependent manner following both pulse and continuous treatment (Trivedi et al., 1990). Peripheral lymphocytes of habitual chewers of 'Mava', a tobacco plus areca nut preparation, also exhibit an elevated incidence of chromosome aberrations and SCEs (Adhvaryu et al., 1991). An elevated frequency of micronucleated cells in exfoliated buccal mucosa of habitual chewers of 'Mava' has also been reported by Adhvaryu et al. (1991). However, in their study alkaloids of both tobacco and areca nut may be responsible for the effect.
A number of mutagens and clastogens have been identified in varieties of tobacco and tobacco products, and they include several nitrosamines, polycyclic aromatic hydrocarbons, polonium-210 and several other compounds (Hecht and Hoffmann, 1988; Nair et al., 1989; Lofroth, 1989; Claxton et al., 1989). Further, saliva of users of gudakhu as well as varieties of other tobacco preparations are known to contain a number of nitrosamines (Stich and Anders, 1989; Nair et al., 1985; Sipahimalani et al., 1984). Besides, tobacco extract is a complex mixture of a number of compounds. It contains a number of nitrostable precursors and nitrites. Those nitrostable substances may undergo nitrosation by nitrites in acidic medium of the stomach, thereby releasing more nitroso-compounds. Probably these tobacco specific nitrosamines and other substances are the causative agents for induction of clastogenic effects, SCE and abnormal sperm by gudakhu extract. However, the role of other ingredients of gudakahu cannot be ignored.

Bone marrow is a proliferative tissue. But very recently Hayashi et al. (1991) have shown with known clastogens that a multiple treatment schedule for some days provides additive accumulation of MNPCEs in mouse bone marrow, particularly when the chemicals produce little bone marrow depression. As evidenced from PCE/NCE ratios AEG has no demonstrable inhibitory effect on bone marrow proliferation. Earlier Yamamoto and Kikuchi (1981) also suggested a 5 times daily-treatment schedule for induction of MN in bone marrow cells of rodents. The International Collaborative Micronucleus Study (Ashby and Tinwell, 1990) also recommends multiple treatment. Again Ghosh and Ghosh (1984) have reported a significantly higher incidence of SCE in peripheral blood lymphocytes of persons who had chewed betel quid with tobacco for more than 10 years than in persons who had chewed for less than 10 years. The frequency of MNPCEs in mice has been reported to increase
greatly after 6 weeks of daily exposure (2 X 30 min daily) to tobacco smoke, compared to 1 day exposure (Balansky et al., 1988). All these studies tempted us to evaluate effects following prolonged treatment. Besides, the long term treatment schedule was followed to simulate human exposure. However, in the present study the data for different time-courses in the repeated treatment regimen in most of the assays followed here (except CA in bone marrow cells for AEG treatment) were close to each other and close to the values obtained following single treatment with the same dose. Multiple treatment for long period seems to have little significance here and this is probably due to the proliferative nature of the bone marrow cells.

It would be interesting to compare the data of AEG and WEG in relation to gudakhu intake. 1 g of gudakhu on being extracted in acetone gives about 23 mg of dry extract and the dose received by one mouse at 300 mg/kg dose level is equivalent to an extract of 250 mg of gudakhu. On the other hand, 1 g of gudakhu on water extraction yields about 375 mg of dry extract. At 625 mg/kg dose level of WEG, in fact, a mouse received an extract of only 42 mg of gudakhu. Mice could not tolerate any dose of WEG higher than 625 mg/kg, within 2-4 days of daily treatment they died. Now if we compare the data of chromosome breakages and total aberrations in bone marrow cells for AEG and WEG for 24 h following single treatment it is clear that water extract is a more effective clastogen. So far tobacco content is concerned, 250 mg of gudakhu (the extract of which was received by one mouse in acetone extract series for 300 mg/kg dose level) contains 25 mg of tobacco, while 42 mg of gudakhu (the extract of which was received by one mouse in water extract series) contains only 4.2 mg of tobacco. In acetone extract series for 300 mg/kg dose level a mouse, therefore, receives about 6 times the amount of tobacco received by a mouse in water extract series for 625 mg/kg dose. Yet, clastogenic
efficiency of the water extract is higher than that of the acetone extract. Higher effectiveness of WEG is clearly demonstrated if the data for CAs in bone marrow cells and spermatocytes, and sperm morphology following repeated treatment for four weeks are compared with those of AEG (Table 21A). But following repeated treatment for 8, 12 and 16 weeks the differences in frequencies of CAs in bone marrow cells and of mis-shapen sperm not discernible. However, if one considers the incidences in terms of gudakhu consumption WEG is found to be always more effective. This observation is very important from the point of human use in which case gudakhu extract(or gudakhu itself mixed with saliva is absorbed (or swallowed). In acetone tobacco portion was no doubt extracted, other ingredients do not probably come in solution. Acetone extract in solution does not show any colour of molasses or red soil. Higher efficiencies of WEG may be attributed to either of the following two possibilities or both:

i) Other ingredients of gudakhu which do not come in acetone extract contribute a lot for induction of chromosome aberrations.

ii) Water facilitates better extraction of tobacco specific clastogens.
### TABLE 21A
COMPARATIVE ANALYSIS OF THE DATA ON CHROMOSOME ABERRATIONS (BREAK TYPES ONLY) IN BONE MARROW CELLS AND SPERMATOCYTES, AND MIS-SHAPEN SPERM INDUCED BY ACETONE AND WATER EXTRACTS OF GUDAKHU

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BM/CA</th>
<th>SPC/CA</th>
<th>SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEG</td>
<td>3.25</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>WEG</td>
<td>5.00</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>AEG</td>
<td>2.00</td>
<td>2.50</td>
<td>--</td>
</tr>
<tr>
<td>WEG</td>
<td>3.33</td>
<td>4.00</td>
<td>--</td>
</tr>
<tr>
<td>AEG</td>
<td>6.00</td>
<td>4.50</td>
<td>--</td>
</tr>
<tr>
<td>WEG</td>
<td>5.75</td>
<td>4.17</td>
<td>--</td>
</tr>
<tr>
<td>AEG</td>
<td>6.00</td>
<td>5.00</td>
<td>--</td>
</tr>
<tr>
<td>WEG</td>
<td>8.53</td>
<td>5.67</td>
<td>--</td>
</tr>
<tr>
<td>AEG</td>
<td>9.38</td>
<td>8.28</td>
<td>--</td>
</tr>
<tr>
<td>WEG</td>
<td>8.28</td>
<td>9.38</td>
<td>--</td>
</tr>
<tr>
<td>Duration of treatment</td>
<td>24 h</td>
<td>4 wk</td>
<td>8 wk</td>
</tr>
<tr>
<td></td>
<td>1.40</td>
<td>5.75</td>
<td>6.00</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>4.17</td>
<td>5.00</td>
</tr>
<tr>
<td></td>
<td>3.33</td>
<td>4.00</td>
<td>4.50</td>
</tr>
<tr>
<td></td>
<td>5.75</td>
<td>5.00</td>
<td>8.33</td>
</tr>
<tr>
<td></td>
<td>8.53</td>
<td>9.38</td>
<td>10.77</td>
</tr>
</tbody>
</table>

Only mean values are presented.

For AEG (Acetone extract of gudakhu) data for dose 300 mg/kg and WEG (Water extract of gudakhu) data for dose 625 mg/kg were considered.
2.6 EFFECTS OF INGREDIENTS OF GUDAKHU

As mentioned earlier gudakhu is a complex tobacco preparation which contains tobacco, lime, molasses, red-soil and water. In an attempt we tried to find out if any particular ingredient or some ingredients are responsible in causing genotoxicity. For that, the ingredients: tobacco, lime, molasses and red-soil, were tested separately. Before going into the details of the tests we would like to mention here a few points about the ingredients. Lime used here is slaked lime in powder form, it is mixed with other ingredients before making the paste. Molasses used in gudakhu preparation is of raw quality. Before mixing with other ingredients this semiliquid raw substance is heated to make a thick blackish pyrolysed substance which is added to other ingredients. Red-soil is colloquially known as 'gerumati'. The factory workers refer it to as 'red-oxide'. The following tests were followed for each of the ingredients:

1. Chromosome aberration assay in bone marrow cells
2. Spermatocyte chromosome analysis
3. Sperm morphology assay
4. Sperm count analysis

In addition, for tobacco, SCE assay has also been conducted. For lime, molasses and red-soil water extract was used, for tobacco both water and acetone extracts were tested.

2.6.1 Materials And Methods

Regarding animals details have been mentioned in section 2.3. Lime, molasses and red-soil were procured from "Samaleswari gudakhu" factory, while the particular variety of tobacco used in gudakhu preparation was
purchased from the local market. Tobacco, lime, molasses or red-soil of known weight was homogenized in distilled water in a mortar and pestle for 20 min and then filtered. The filtrate was diluted with an appropriate amount of water and fed to mice by gastric intubation.

Acetone extract of tobacco was prepared in the same manner as described for gudakhu in section 2.3. The dry pellet of the extract of tobacco was suspended in an appropriate amount of distilled water and the suspension was fed to mice by gastric intubation. Animals of both the sexes received aqueous extracts (filtrate) or suspension (in case of acetone extract) of the ingredients via oral route once daily for different weeks (4-16) and were killed 24 h after the final dose. For chromosome aberration assay in bone marrow cells for all the ingredients single treatment regimen was also followed. In that case animals were fed with the extract or suspension once and killed at 24 h post-treatment. Details of the doses tested for different ingredients and animals employed have been mentioned in the respective tables (vide infra). Different tissues were collected and processed and slides were prepared and stained as per the general procedures mentioned earlier (vide 2.3.1, 2.3.3, 2.3.4 and 2.3.5).

2.6.2 Results

In a particular assay, qualitatively the aberration types encountered following treatment of different ingredients did not differ much. Hence, it would be worthwhile to discuss the qualitative aspects of aberrations for all ingredients in common for a particular assay.

2.6.2.1 Qualitative

Chromosome aberrations in bone marrow cells: Almost all the structural aberrations encountered were of chromatid
type. Although in some cases of chromatid breaks the broken acentric chromatid fragments remained in close proximity of their original position (Figs. 13a,d,e), in several instances they were greatly displaced (Figs. 13c,e,f). Fragments of unknown origin were of frequent occurrence (Figs. 13c,d,j). Secondary constriction regions were also involved in breakage (Figs. 13g,h). Iso-chromatid breaks were recorded (Figs. 13i,j). 'Rings' (formed from sister chromatid union) accompanied with or without acentric fragments were also available (Figs. 13k-m). In a few cells chromosomes were recorded to be involved in chromatid exchanges leading to formation of dicentric chromosomes, in some cases they were accompanied with acentric fragments (Fig. 13n). Exchanges in certain cases led to formation of bi-armed chromosomes (Figs. 13o,p). Gaps, both chromatid and iso-chromatid types, constituted the most common type of aberrations (Figs. 13b,q,r). Affected cells in several instances were found to contain two or more aberrations of same or different types (Figs. 13q,r). Numerical changes were of least significance. Though a number of metaphases with hypodiploid chromosome number were available hyperdiploid cells were seldom noticed.

Spermatocyte chromosome analysis: With regard to structural changes of chromosomes, the effects were manifested mainly in the form of chromatid breaks (Figs. 14a-d). A few chromosome type breaks were also noted (Figs. 14e,f), and in those cases the broken fragments were seen placed somewhere in the metaphase plate. A number of cells were found to contain some extra fragments smaller than the smallest chromosome (Fig. 14g); because of their smallness their origin could not be traced and were categorized as fragments of untraceable origin. A few cells were noted to contain translocation multivalents involving autosomal bivalents only, or autosomes and X-Y bi-valents, they were in the form of chain-of-four (Figs. 14h,i).
Explanation for Fig. 13

Photomicrographs of mouse bone marrow metaphase plates showing various types of structural aberrations treated with ingredient of gudakhu.

a. A chromatid break, fragment not displaced.
b. Two gaps and one sub-chromatid break.
c. A fragment of unknown origin, and a chromatid break, fragment well displaced.
d. One chromatid break and two fragments of untraceable origin.
e. Two chromatid breaks, one with displaced fragment.
f. A chromatid break fragment placed far apart.
g,h. Breaks at secondary constriction region.

i. A chromosome with an iso-chromatid break, and another chromosome with a gap.

j. Two chromatid breaks with well displaced acentrif fragments; a paired fragment probably originated from isochromatid break; and two small fragments of unknown origin.

k,l. Ring chromosomes without any fragment.
m. Ring chromosome without fragment.

n. Chromatid exchange forming a dicentric chromosome but without any extra acentric fragment.

o, p. Metacentric chromosome, but not accompanied with fragment.

q. A number of chromatid breaks and gaps involving 5 chromosomes.

r. A metaphase showing a number of chromatid breaks, gaps and fragments of unknown origin.
Explanation for Fig. 13 A

Photomicrographs of bone marrow second-division metaphase showing sister chromatid exchanges induced by tobacco extract.

a-f. Harlequin stained chromosomes of sister chromatid exchanges. Number of exchanges varies from cell to cell.
Explanation for Fig. 14

Photomicrographs of diakinesis-metaphase I cells showing structural chromosome aberrations and univalent formation.

a,b,c,d. Chromatid breaks involving autosomes.

e,f. Chromosome breaks involving autosomal bivalents, fragmented part placed widely apart.
Explanation for Fig.14(Contd.)

g. A small chromosomal fragment of unknown origin.

h-i. Exchange between two autosomal bivalents forming a chain of four.

j. X-Y chromosomes well displaced.

k. Univalent formation involving one autosomal bivalents.

l. Plate having 22 bivalents.
Both autosomes and sex chromosomes were involved in univalent formation (Figs. 14h,j,k), among autosomal bivalents the smaller one was more frequently involved.

Though the occurrence of polyploid diakinesis metaphase-I plates was common, the incidences did not vary remarkably from that of control. In mice the occurrence of such polyploid spermatocytes is common (Dym and Fawcett, 1971, Das and Nayak, 1988). Aneuploid cells were extremely rare (Fig. 14 I).

**Sperm morphology assay**: Qualitatively the abnormality induced in the head morphology of sperm following treatment of ingredients of gudakhu were of varied types. Among them, with flat base, amorphous, with acrosome spine abnormalities, with vacuoles, giant size, triangular were very common both in treated and control mice (Figs. 15a-u). Here also a high degree of variation in spine morphology was noted in sperm heads with acrosomal spine abnormalities, it ranged from total absence to a long hook shaped one (Figs. 15a,c,e,g,j,k,l,m,o and t). Abnormal types like giant size, triangular, rectangular, etc. were also available, though not so common, in both control and treated animals. No particular abnormal type was found specific for a particular ingredient. Varieties like 'balloon' shaped, tubular, 'mushroom', 'cashew fruit', and 'clove' shaped, twin etc. were recorded only in treated animals (Figs. 15a,b,c,d,f,g,i). Sometimes a particular sperm was found to exhibit a combination of two or more said arbitrary types of abnormalities in its head morphology (Figs. 15 l,q,r,s,t).

2.6.2.2 Quantitative

2.6.2.2.1 Tobacco

Chromosome aberration assay in bone marrow cells: Table-22 summarizes the data on chromosome aberrations in bone
Explanation for Fig. 15

Photomicrographs of vas differential sperm showing abnormality in head morphology induced in mice treated with different ingredients of gudakhu. Sperm heads with abnormal morphology were categorized into same arbitrary types.

a. Balloon shaped, without acrosomal spine, cashew fruit shaped and with flat base.

b. Tubular sperm head.

c. With flat base, amorphous type, without acrosomal spine, triangular, and balloon shaped.

d. Cashew-fruit type and triangular.

e. With flat base and without acrosomal spine.

f. One sperm head with two vacuoles and a twin sperm.

g. With flat base, without acrosomal spine and mushroom shaped.

h. Rectangular sperm head.

i. Clove shaped.
Explanation for Fig. 15 (Contd.)

j. 'L' shaped acrosomal spine.
k. Hook shaped acrosomal spine.
l. Rectangular head with small blunt spine.
m. Without acrosomal spine.
n. Amorphous type.
o. Without acrosomal spine, 'L' shaped acrosomal spine.
p. Sperm with a vacuole.
q. With flat base and a vacuole.
r. Rectangular head with a vacuole.
s. Giant size with flat base.
t. Triangular with a vacuole, flat base, without acrosomal spine, amorphous.
u. With flat base.
Each of the iso-chromatid breaks, rings and exchanges was considered as two breaks.

* Dry extract of tobacco; ** treated once; *** treated once daily.

Significantly difference from the control; ('t'-test and Anova test) : b = p < 0.01; c = p < 0.001.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose* (mg/kg)</th>
<th>Period of treatment</th>
<th>Meta. scored/animals</th>
<th>Break - type aberrations</th>
<th>Total (mean%+SE)</th>
<th>Gaps</th>
<th>Total aberrations (Mean%±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>--</td>
<td>3000/30</td>
<td>7 2 2 1 2</td>
<td>0.63 ± 0.14</td>
<td>39</td>
<td>1.93 ± 0.24</td>
</tr>
<tr>
<td>WET</td>
<td>8</td>
<td>24 h**</td>
<td>400/4</td>
<td>13 2 -- 2</td>
<td>3.75 ± 0.41^c</td>
<td>15</td>
<td>7.50 ± 0.56^c</td>
</tr>
<tr>
<td>AET</td>
<td>8</td>
<td>24 h**</td>
<td>400/4</td>
<td>12 -- 1 --</td>
<td>3.25 ± 0.22^c</td>
<td>10</td>
<td>5.75 ± 0.41^b</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4 wk***</td>
<td>500/5</td>
<td>17 1 -- 2</td>
<td>4.60 ± 1.08^c</td>
<td>21</td>
<td>8.80 ± 0.72^c</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8 wk***</td>
<td>500/5</td>
<td>16 4 5 2 6</td>
<td>9.00 ± 2.93^c</td>
<td>16</td>
<td>12.20 ± 1.18^c</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>12 wk***</td>
<td>500/5</td>
<td>23 -- 7 3 2</td>
<td>8.00 ± 0.40^c</td>
<td>25</td>
<td>13.00 ± 0.63^c</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16 wk***</td>
<td>500/5</td>
<td>21 1 9 5 2</td>
<td>9.20 ± 0.44^c</td>
<td>18</td>
<td>12.80 ± 0.82^c</td>
</tr>
</tbody>
</table>

Note: Each cell should contain numbers and not visible in the image.
marrow cells induced by acetone and water extracts of tobacco. Frequencies of chromosome aberrations, excluding and including gaps, in all the sampling intervals following treatment of water and acetone extracts of tobacco increased significantly over their respective control values. Chromatid breaks and fragments of untraceable origin constituted the major bulk of the break type aberrations. Water extract, compared to acetone extract, induced slightly higher frequency of breaks as well as total aberrations. The highest incidence of breaks was noted after 16 weeks of daily treatment; however, the values for weeks 8, 12 and 16 remained very close to each other though at week 4 the value was much less. The same trend was marked for total aberrations. Influence of course-of-treatment on 'breakage' and 'total aberration' frequencies was marginally significant only (Fig.16A). Numerical changes were not marked.

SCE analysis: The frequency of SCE per cell in untreated control animals was 4.7 (Table-23). Chronic daily treatment for different weeks (4-16) elevated the SCE frequencies by more than two folds. But the values for four test weeks were very close to each other; analysis of variance did not show any significant difference among them.

Spermatocyte chromosome analysis: Data on structural changes of chromosomes and univalent formation following repeated treatment of acetone extract of tobacco are presented in Table-24. The breakage frequencies increased significantly over the control value particularly in the last three test weeks and the values for the last 3 weeks were close to each other. The highest frequency obtained at week 16 was about 10 times the control value.
Explanation for Fig. 16

Linear regression curve showing relation between weeks of chronic treatment of water extract of tobacco (T), lime (L), molasses (M) or red-soil (RS) and chromosome aberrations in bone marrow cells (A), chromosome aberrations in spermatocytes (B), or mis-shapen sperm (C). The values are as follows:

Fig 16A

\[ r = 0.976, a = 1.074, b = 0.315, \quad p < 0.01 \]

\[ o, \quad r = 0.993, a = 0.212, b = 0.456, \quad p < 0.001 \]

\[ \bullet, \quad r = 0.970, a = 1.046, b = 0.314, \quad p < 0.01 \]

\[ \square, \quad r = 0.950, a = 0.030, b = 0.324, \quad p < 0.05 \]

Fig 16B

\[ r = 0.965, a = 0.614, b = 0.321, \quad p < 0.01 \]

\[ o, \quad r = 0.501, a = 1.444, b = 0.084, \quad p > 0.05 \]

\[ \bullet, \quad r = 0.992, a = 0.548, b = 0.291, \quad p < 0.05 \]

\[ \square, \quad r = 0.964, a = 0.216, b = 0.204, \quad p < 0.01 \]

Fig 16C

\[ r = 0.997, a = 2.512, b = 0.314, \quad p < 0.001 \]

\[ o, \quad r = 0.964, a = 2.552, b = 0.194, \quad p < 0.05 \]

\[ \bullet, \quad r = 0.889, a = 2.636, b = 0.291, \quad p < 0.05 \]

\[ \square, \quad r = 0.976, a = 2.742, b = 0.142, \quad p < 0.01 \]
Mean % of breaks in Spermatocytes

Mean % of breaks in Bone marrow cells

Mean % of Mis-shapen sperm

Weeks of treatment
TABLE 23

INCIDENCE OF SISTER CHROMATID EXCHANGES IN BONE MARROW CELLS OF MICE TREATED REPEATEDLY WITH ACETONE EXTRACT OF TOBACCO

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Weeks of treatment</th>
<th>Total II metaphases scored/animals</th>
<th>Total SCEs</th>
<th>SCE/Cell (mean% ± SE)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>293/10</td>
<td>1392</td>
<td>4.70 ± 0.22</td>
<td>0 - 22</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>116/3</td>
<td>1104</td>
<td>9.50 ± 0.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 - 22</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>108/3</td>
<td>1010</td>
<td>9.55 ± 0.58&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 - 19</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>120/3</td>
<td>1275</td>
<td>9.78 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 - 20</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>124/3</td>
<td>1312</td>
<td>10.62 ± 0.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2 - 32</td>
</tr>
</tbody>
</table>

* Dry extract of tobacco.

Significantly different from the control; ('t'-test and Anova test): c = p<0.001.
TABLE 24

INCIDENCE OF STRUCTURAL CHROMOSOME ABERRATIONS AND UNIVALENT FORMATION IN SPERMATOCYTES OF MICE INDUCED BY ACETONE EXTRACT OF TOBACCO FOLLOWING CHRONIC TREATMENT

<table>
<thead>
<tr>
<th>Dose (mg/kg) of treatment</th>
<th>Weeks of treatment</th>
<th>Diaki. Meta.-I scored/animals</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Pooled</td>
<td></td>
<td>1200/24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>150/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>150/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>150/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>150/3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each exchange was considered as two breaks.

* Dry extract of tobacco.

Significantly different from the control; ('t'-test and Anova test): c = p<0.001.
Univalent formation involving both autosomal and sex-chromosomal bivalents increased greatly, and the increase was more with the longer course-of-treatment.

Sperm morphology assay: The highest incidence of sperm with abnormal morphology was noted following 16 weeks of daily treatment and the value was nearly 3 times the control one (Table-25). Data indicated a gradual increase of frequencies of abnormal sperm with the increase of course-of-treatment.

Sperm count: Sperm count data failed to exhibit any difference between the control value and treated one for any test week.

2.6.2.2.2 Molasses

Chromosome aberration assay in bone marrow cells: Dose-response analysis of break type aberrations in bone marrow metaphases at 24 h post-treatment of water extract of molasses exhibited no difference in breakage frequency, though the total aberrations increased a little at the higher dose level (Table-26). The higher dose following repeated treatment induced more breakages, compared to the lower dose, particularly after 12 and 16 weeks of treatment. Total aberrations also showed the same trend. 'Rings' and exchange type aberrations were very few.

Spermatocyte chromosome analysis: The frequency of breaks increased with the increasing course-of-treatment (Table-27) and the frequencies were significantly higher than the control value at weeks 8, 12 and 16. The highest frequency obtained at week 16 was nearly 8 times the control value. No exchange type configuration was noted in molasses treated animals.
### TABLE 25

**EFFECT OF REPEATED TREATMENT OF ACETONE EXTRACT OF TOBACCO ON SPERM MORPHOLOGY AND SPERM PRODUCTION IN DIFFERENT WEEKS**

<table>
<thead>
<tr>
<th>Dose* (mg/kg)</th>
<th>Weeks of treatment</th>
<th>Total sperm head scored/animals</th>
<th>Abnormal sperm head (mean% ± SE)</th>
<th>Animals analyzed</th>
<th>Mean count/epididymis (mean X 8 X 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>24000/24</td>
<td>2.64 ± 0.19</td>
<td>24</td>
<td>104.33 ± 3.89</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>3000/3</td>
<td>3.70 ± 0.20^b</td>
<td>3</td>
<td>110.40 ± 1.49</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>3000/3</td>
<td>5.13 ± 0.03^c</td>
<td>3</td>
<td>100.70 ± 1.50</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>3000/3</td>
<td>6.60 ± 0.22^c</td>
<td>3</td>
<td>100.33 ± 0.95</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>3000/3</td>
<td>7.47 ± 0.53^c</td>
<td>3</td>
<td>99.27 ± 0.63^a</td>
</tr>
</tbody>
</table>

* Dry extract of tobacco.

Significantly different from the control; ('t'-test and Anova test): a = p < 0.05; b = p < 0.01; c = p < 0.001.
TABLE 26
INCIDENCE OF STRUCTURAL CHROMOSOME ABERRATIONS IN BONE MARROW CELLS OF MICE INDUCED BY AQUEOUS EXTRACT OF MOLASSES FOLLOWING SINGLE AND CHRONIC TREATMENT

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Period of treatment</th>
<th>Metaphases scored/animals</th>
<th>Break-type aberrations</th>
<th>Gaps</th>
<th>Total aberrations (mean%±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chromatid</td>
<td>Isochromatid</td>
<td>Fragment</td>
</tr>
<tr>
<td>Control</td>
<td>(Pooled) 3000/30</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>24h 400/4</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>24h 400/4</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>4wk 600/6</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>45</td>
<td>8wk 600/6</td>
<td>17</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>45</td>
<td>12wk 600/6</td>
<td>23</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>45</td>
<td>16wk 600/6</td>
<td>30</td>
<td>1</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>

Each of the iso-chromatid breaks, rings and exchanges was considered as two breaks.

* Aqueous extract of the amount received by mouse at a time.

Significantly different from the controls; ('t'-test and Anova test): b = p<0.01; c = p<0.001.
Each exchange was considered as two breaks.

* Aqueous extract of the amount received by a mouse at a time.

Significantly different from the control ; ('t'-test and Anova test): a = \( p < 0.05 \); b = \( p < 0.01 \); c = \( p < 0.001 \).
The incidence of univalent formation in molasses treated animals was not also highly marked. Taking autosomes and sex chromosomes together the maximal frequency of univalent formation was obtained at week 16 the absolute value for which was about twice the control value.

Sperm morphology and sperm count: Data on sperm test are presented in Table-28. Two to three-fold increase in the frequency of abnormal sperm heads, compared to the control value, was recorded in treated males, the increases were significant. The values for weeks 4, 8 and 12 were very close to each other. In no test week the sperm count data revealed any appreciable deviation from the control value.

2.6.2.2.3 Red-soil

Chromosome aberration assay in bone marrow cells: Aqueous extract of red-soil in quantity equivalent to that present in gudakhu extract also induced chromosome aberrations in bone marrow cells of mice (Table-29). The frequencies of both breaks and total aberrations increased significantly at 24 h following single treatment, as well as after 12 and 16 weeks of chronic treatment; the increases following 4 and 8 weeks of chronic treatment were not so appreciable.

Spermatocyte chromosome analysis: Break type aberrations encountered in spermatocytes comprised mainly of chromosome type breaks, no exchange type configuration was recorded (Table-30). The frequencies of breaks exhibited notable increase after 12 and 16 weeks of treatment only. The incidence of univalent formation also increased markedly after 12 and 16 weeks of treatment.

Sperm morphology and sperm count: There was a trend of gradual increase in the incidence of sperm with abnormal morphology with the increase of course-of-treatment
<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Weeks of treatment</th>
<th>Total sperm head scored/animals</th>
<th>Abnormal sperm head (mean%±SE)</th>
<th>Animals analyzed</th>
<th>Mean count/epididymis (mean X 8 X 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>24,000/24</td>
<td>2.64±0.19</td>
<td>24</td>
<td>104.33±3.89</td>
</tr>
<tr>
<td>45</td>
<td>4</td>
<td>4000/4</td>
<td>4.48±0.38c</td>
<td>4</td>
<td>101.92±3.83</td>
</tr>
<tr>
<td>45</td>
<td>8</td>
<td>4000/4</td>
<td>4.63±0.47c</td>
<td>4</td>
<td>98.51±2.27</td>
</tr>
<tr>
<td>45</td>
<td>12</td>
<td>4000/4</td>
<td>4.73±0.32c</td>
<td>4</td>
<td>98.74±1.44</td>
</tr>
<tr>
<td>45</td>
<td>16</td>
<td>4000/4</td>
<td>8.33±0.72c</td>
<td>4</td>
<td>100.32±1.68</td>
</tr>
</tbody>
</table>

* Aqueous extract of the amount received by a mouse at a time.

Significantly different from the control; (*t*-test and Anova test): c = p < 0.001.
<table>
<thead>
<tr>
<th>Dose* (mg)</th>
<th>Period of treatment</th>
<th>Metaphases scored/animals</th>
<th>Break-type aberrations</th>
<th>Gaps</th>
<th>Total aberrations (mean%±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(Pooled) 3000/30</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>24h</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>4wk</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>8wk</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>12wk</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>16wk</td>
<td>13</td>
<td>5</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Each of the iso-chromatid breaks, rings and exchanges was considered as two breaks.

* Aqueous extract of the amount received by a mouse at a time.

Significantly different from the control; ('t'-test and Anova test): b = \(p<0.01\); c = \(p<0.001\).
<table>
<thead>
<tr>
<th>Dose* (mg)</th>
<th>Weeks of treatment</th>
<th>Diaki. Meta.-I scored/animals</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chrom. Chromt. Frag. Exchange</td>
<td>Total (mean%±SE)</td>
</tr>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>1200/24</td>
<td>2 3 2 -</td>
<td>0.58±0.19</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>150/3</td>
<td>- - 1 -</td>
<td>0.67±0.54</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>200/4</td>
<td>- 3 - -</td>
<td>1.50±0.43</td>
</tr>
<tr>
<td>16</td>
<td>12</td>
<td>200/4</td>
<td>2 3 1 -</td>
<td>3.00±0.50</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>200/4</td>
<td>3 4 - -</td>
<td>3.50±0.43</td>
</tr>
</tbody>
</table>

Each exchange was considered as two breaks.

* Aqueous extract of the amount received by a mouse at a time.

Significantly different from the control; (*t*-test and Anova test): b=p<0.01; c=p<0.001.
(Table-31), however, the increases were remarkable only after longer period of treatment. The highest frequency obtained at week 16 was less than 2 times the control value.

With regard to the sperm count data, a tendency of decrease in the count was noticed (Table-31). Though the decrease was significant in certain test weeks in the case of maximal reduction the count reduce to about 85% of the normal count.

2.6.2.2.4 Lime

Chromosome aberration assay in bone marrow cells: Neither break type aberrations nor total aberrations increased significantly over the control for any of the doses tested following single treatment (Table-32). The higher dose which is three times the lower one, produced slightly higher frequency of aberrations. However, significantly elevated incidences were noted following chronic treatment particularly for longer treatment schedule. Both breaks and total aberrations exhibited a good positive correlation with the course-of-treatment.

Spermatocyte chromosome analysis: Long-term treatment of lime elevated the frequency of chromosome aberrations in spermatocytes also (Table-33). The increase was not so high particularly after 4 weeks of treatment, however, significant increase was noted after 8, 12 and 16 weeks of treatment. Similarly, the incidence of univalent formation showed a tendency of increase over the control value following longer chronic treatment schedule.

Sperm morphology and sperm count: Frequency of sperm with abnormal head morphology increased with the increase of course-of-treatment (Table-34). The highest value obtained at week 16 was about twice the control value.
### TABLE 31

**EFFECT OF REPEATED TREATMENT OF WATER EXTRACT OF RED-SOIL ON SPERM MORPHOLOGY AND SPERM PRODUCTION IN DIFFERENT WEEKS**

<table>
<thead>
<tr>
<th>Dose* (mg)</th>
<th>Weeks of treatment</th>
<th>Total sperm head scored/animals</th>
<th>Abnormal sperm head (mean% ± SE)</th>
<th>Animals analyzed</th>
<th>Mean count/epididymis (mean X 8 X 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>24000/24</td>
<td>2.64 ± 0.19</td>
<td>24</td>
<td>104.33 ± 3.89</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>3000/3</td>
<td>3.57 ± 0.26^b</td>
<td>3</td>
<td>100.27 ± 2.61</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>4000/4</td>
<td>3.63 ± 0.07^c</td>
<td>4</td>
<td>95.07 ± 2.51</td>
</tr>
<tr>
<td>16</td>
<td>12</td>
<td>4000/4</td>
<td>4.58 ± 0.96^c</td>
<td>4</td>
<td>93.65 ± 3.04^a</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>4000/4</td>
<td>4.98 ± 0.29^c</td>
<td>4</td>
<td>87.73 ± 1.10^c</td>
</tr>
</tbody>
</table>

* Aqueous extract of the amount received by a mouse at a time.

Significantly different from the control; ('t'-test and Anova test): a = p < 0.05; b = p < 0.01; c = p < 0.001.
TABLE 32

REQUENCY DISTRIBUTION OF STRUCTURAL CHROMOSOME ABERRATIONS INDUCED BY AQUEOUS EXTRACT OF LIME IN BONE MARROW CELLS OF MICE FOLLOWING SINGLE AND CHRONIC TREATMENT. ±N B°NE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose* (mg)</th>
<th>Period of treatment</th>
<th>Meta. scored/animals</th>
<th>Break-type aberrations</th>
<th>Chro-</th>
<th>Iso-</th>
<th>Frag-</th>
<th>Ring</th>
<th>Ex-</th>
<th>Change</th>
<th>Total (mean% ± SE)</th>
<th>Gaps</th>
<th>Total aberrations (mean% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>--</td>
<td>3000/30 7</td>
<td>2 2 1</td>
<td>2</td>
<td>0.63 ± 0.14</td>
<td>39</td>
<td>1.93 ± 0.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>24 h</td>
<td>400/4 4</td>
<td>--</td>
<td>-- 1 2</td>
<td>--</td>
<td>1.25 ± 0.21</td>
<td>8</td>
<td>3.25 ± 0.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>24 h</td>
<td>400/4 4</td>
<td>--</td>
<td>-- 2 2</td>
<td>--</td>
<td>1.50 ± 0.41</td>
<td>8</td>
<td>3.50 ± 0.74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4 wk</td>
<td>600/6 4</td>
<td>--</td>
<td>-- 1 2</td>
<td>--</td>
<td>1.67 ± 0.38</td>
<td>10</td>
<td>3.33 ± 0.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>8 wk</td>
<td>600/6 18</td>
<td>--</td>
<td>-- 1 2</td>
<td>--</td>
<td>3.67 ± 0.56&lt;sup&gt;C&lt;/sup&gt;</td>
<td>13</td>
<td>5.83 ± 0.55&lt;sup&gt;C&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>12 wk</td>
<td>600/6 19</td>
<td>3</td>
<td>2 2</td>
<td>2</td>
<td>5.50 ± 0.20&lt;sup&gt;C&lt;/sup&gt;</td>
<td>17</td>
<td>8.33 ± 0.39&lt;sup&gt;C&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>16 wk</td>
<td>600/6 30</td>
<td>2</td>
<td>2 1 4</td>
<td>--</td>
<td>7.83 ± 1.16&lt;sup&gt;C&lt;/sup&gt;</td>
<td>21</td>
<td>11.33 ± 1.58&lt;sup&gt;C&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Aqueous extract of the amount received by a mouse at a time.

significantly different from the control; ("t"-test and Anova test): c = p < 0.001.
TABLE 33

effect of aqueous extract of lime on structural chromosome aberrations and univalent formation in spermatocytes of mice following chronic treatment

<table>
<thead>
<tr>
<th>Dose* (mg)</th>
<th>Weeks of treatment</th>
<th>Diak. Meta.l scored/animals</th>
<th>Structural aberrations</th>
<th>Univalent formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chrom. Chromt. Frag. Ex-</td>
<td>Autosomal (mean%±SE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>change</td>
<td>Total (mean%±SE)</td>
</tr>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>1200/24</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>200/4</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>200/4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>200/4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>200/4</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Each exchange was considered as two breaks.

* Aqueous extract of the amount received by a mouse at a time.

Significantly different from the control; (‘t’- test and Anova test): a = p<0.05; c = p<0.001.
<table>
<thead>
<tr>
<th>Dose* (mg.)</th>
<th>Weeks of treatment</th>
<th>Total sperm head scored/animals</th>
<th>Abnormal sperm head (mean%±SE)</th>
<th>Animals analyzed</th>
<th>Mean count/epididymis (mean X 8 X 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pooled</td>
<td>24000/24</td>
<td>2.64 ± 0.19</td>
<td>24</td>
<td>104.33 ± 3.89</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>4000/4</td>
<td>3.48 ± 0.53</td>
<td>4</td>
<td>94.15 ± 2.98</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>4000/4</td>
<td>3.55 ± 0.36</td>
<td>4</td>
<td>93.48 ± 2.73</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>4000/4</td>
<td>5.20 ± 0.42&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>85.73 ± 3.05</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>4000/4</td>
<td>5.65 ± 0.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>84.02 ± 2.89&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Aqueous extract of the amount received by a mouse at a time.

Significantly different from the control; ('t'-test and Anova test); b = p<0.01, c = p<0.001.
Sperm count data revealed a tendency of decrease, the count came down nearly to 85% level of the control.

2.6.3 Discussion

2.6.3.1 Tobacco

Significantly high incidences of chromosome aberrations in bone marrow cells and spermatocytes and of SCEs clearly indicate genotoxic capacity of acetone extract of tobacco. Though it does not have any effect on the production of sperm it certainly elevates production of mis-shapen sperm. Analysis of sperm morphology is considered to provide information on toxic effect of an agent (Wyrobek et al., 1983a,b).

Kulkarni et al. (1987) while testing clastogenic ability of alcohol extract of tobacco in mouse by analyzing bone marrow metaphases used pyrolysed tobacco. Probably for that they obtained very high incidence of aberrations after single ip injection. It is well documented that during pyrolysis a number of carcinogens and mutagens are formed (Sugimura, 1985). Genotoxic potential of pyrolysed and non-pyrolysed chewing tobacco was also demonstrated by analyzing micronuclei in bone marrow cells of mice, 8-azaguanine resistance in V79 cells, and reverse mutation in Salmonella/ microsome assay (Shirname et al., 1984; Kulkarni et al., 1987), pyrolysed tobacco inducing higher effect. Clastogenic ability of varieties of smokeless tobacco in various assays as well as in habitual users has earlier been discussed at length (vide 2.5.3). Discussions have also been done on the probable tobacco specific factor or factors responsible for induction of chromosome damages.

In both bone marrow cells and spermatocytes effect is low after 4 weeks of treatment, but it increases greatly after 8 weeks of treatment, further increase of
time-course of treatment seems to have little effect. Probably, the clastogenic factor(s) needs more than 4 weeks time to reach to the maximum level with this dosage in mouse system. Again due to proliferative nature of those cells the incidence of aberrations does not exhibit any change with the increase of treatment course beyond 8 weeks. The same supposition, except that here 4-week period or less than that is sufficient to reach to the maximal level, seems to hold good for SCEs, in which case we do not find marked variation in the frequencies for different courses of treatment. On the contrary, Ghosh and Ghosh (1984) noted significantly higher incidence of SCEs in the lymphocytes of persons who chewed betel quid with tobacco for more than 10 years compared to those who chewed for less than 10 years. The reason for differential results remains unknown.

Although the results are inconclusive literature provides a number of reports on the effects of smoking on spermatogenesis (Vogt et al., 1984). But the present report seems to be the first to document effect of smokeless tobacco on meiotic chromosomes. As the animals were treated repeatedly for longer periods it is not possible on our part to pin-point the stage in spermatogenesis most susceptible to tobacco. With regard to the effect on pairing behaviour both autosomes and sex chromosomes exhibited susceptibility to univalent formation.

Comparative analysis of the data of AEG and AET on cytogenetic end points reveals some interesting facts (Figs. 17a-e). The tobacco tested here was of the same variety as used in gudakhu. In AEG series for 300 mg/kg dose a mouse received an extract of 250 mg of gudakhu which contains about 25 mg of tobacco. But in AET series for 8 mg/kg dose a mouse received an extract of 8 mg of dry tobacco. Thus, the mice treated with AET, compared to AEG
Fig. 17 a Graphs showing the comparative analysis of mean % of breaks in bone marrow cells of mice treated with acetone extract of gudakhu or tobacco. 'C' indicates the control value.
Fig. 17 b  Graph showing comparative analysis of SCEs in bone marrow cells of mice treated with acetone extract of gudakhu or tobacco. 'C' indicates the control value.
Fig. 17c Comparative analysis of the genotoxic effect of acetone extracts of gudakhu and tobacco as revealed by the spermatocytes chromosome analysis. 'C' indicates the control value.
Fig. 17 d Graphical representation of the mean % of univalent formation (autosomes + sex chromosomes) of acetone extracts of gudakhu and tobacco as revealed by the spermatocyte chromosome analysis. 'C' indicates the control value.
Fig. 17 e Graphs represent the comparative analysis of acetone extract of gudakhu and tobacco as revealed by the vas deference sperm head abnormality assay. 'C' indicates the control value.
treated mice, received about 1/3 the tobacco extract. But comparative analysis clearly indicates that tobacco is more effective than gudakhu in inducing clastogenic effect and SCEs. In both the cases the extract was fed identically (through gavage), so there is no question of any role of saliva for differential effect. Recently Whong et al. (1987) have reported that tobacco snuff extract under acidic condition is more mutagenic than at neutral or basic pH. They have shown experimentally that under acidic condition nitrosation by nitrite present in the tobacco is more leading to generation of more nitroso-compounds, and hence more mutagenicity. The ingredients of gudakhu are mixed thoroughly and marketed in tin packs. The lime present in gudakhu makes it alkaline (pH 8.5), probably the alkaline condition of gudakhu (due to soaking of tobacco in lime for long period) plays some great role in reducing nitroso-compound formation and thereby reducing cytogenetic effect.

Stich and Anders (1989) have made a comparative analysis on the incidence of micronucleated oral mucosa cells as well as on the nitrosamine contents in the saliva of habitual users from different parts of the world using various types of tobacco preparations (snuff, "khaini", gudakhu, nass, betel quid, etc.). The incidences of both micronucleated cells and nitrosamine content are found to be very low in gudakhu addicts compared to those in users of any other tobacco preparation, even snuff (only tobacco). This observation is at par with our findings and seems to support our assumption.

2.6.3.2 Molasses

Analysis of chromosome aberrations in bone marrow cells and spermatocytes documents clastogenic efficiency of molasses. It also induces mis-shapen sperm. Molasses is used as a food item by human beings. But the molasses used
in the preparation of gudakhu is of very low quality. Over and above, before use this semiliquid substance is pyrolysed by heating to obtain its particular consistency. The cytotoxic and genotoxic effect of molasses may well be attributed to its pyrolyzed products. Pyrolyzed products of meat as well as cooked food with charred surfaces have been reported to be mutagenic in the Ames test (Sugimura et al., 1977, 1981; Kikugawa et al., 1985) and carcinogenic in experimental animals (Takahashi et al., 1983; Takayama et al., 1984a, b; Sugimura, 1985). Pyrolyzed food products have been demonstrated to contain several mutagenic and carcinogenic principles like pyrolyzed amino acids, proteins and numerous other chemicals (Sugimura and Sato, 1983; Takahashi et al., 1983). Highly pyrolyzed tobacco has been shown to be more genotoxic than its less pyrolyzed variety (Kulkarni et al., 1987).

Molasses is soluble in water. The aqueous extract of 15 mg of molasses received by a mouse is equivalent to the amount received by a mouse with the aqueous extract of gudakhu for the dose of 625 mg/kg (this figure represents the dry weight of the aqueous extract of gudakhu; here each animal receives extract of about 15 mg of molasses with each dose of gudakhu). The higher dose tested here for chromosome aberrations in bone marrow cells was about 3 times the lower dose. However, in single treatment regimen the two doses failed to reveal any marked difference in the frequencies of chromosome aberrations. At this stage on the basis of the limited data it is not possible on our part to put forward any explanation for that. From gradual increase in the incidence of CAAs in both somatic and meiotic cells as well as of abnormal sperm with the increase of course-of-treatment, it seems the toxic factor(s) accumulates to a certain level, particularly after 12 weeks of treatment. It seems to have little influence on sperm productivity.
2.6.3.3 Red-soil

Red-soil, as mentioned earlier, is a type of soil containing number of metallic oxides of which Fe$_2$O$_3$ and Al$_2$O$_3$ constitute the major bulk. Single treatment with a dose (extract of 12 mg) equivalent to the amount ingested in a single dose of water extract of gudakhu (40 mg) is proved to be non-clastogenic in bone marrow cells after 24 hours. Chronic treatment of the extract, particularly after 12 weeks of treatment, however, induces clastogenicity both in somatic and meiotic cells, and sperm abnormality. The dose used for chronic treatment was slightly higher than that tested in single treatment regimen. About 8- and 6-fold increase in the incidence of chromosome aberrations in bone marrow cells and spermatocytes respectively cannot be attributed to the higher dose only; repeated treatment might have played an important role, specifically when gradual increase in the incidence is marked with the increase of treatment schedule. So far our knowledge goes, no work has been done earlier with this substance in this line.

These days increasing attention is being focused on metals and metallic compounds for their potential mutagenic properties and ability to cause cancer (Leonard, 1981). IARC (1987) has listed Cr, Ni, Be, Cd, Co, Pb, and Sb as human carcinogens and probable mutagens. Aluminium which constitutes the most abundant metal in the earth's crust, also poses great problem in biological systems. Al$^{3+}$ ions are considered to be highly toxic (Mac Donald and Martin, 1988); uptake and toxicity of it have been studied widely (Fageria et al., 1988; Roy et al., 1988). Al$^{3+}$ ions are known to be released exponentially below pH 4.5 (Bergkvist, 1987). So Al$^{3+}$ released at low pH inside the stomach of mice is assumed to be the probable factor for red-soil induced toxicity. Further, clastogenic activity of aluminium salts has earlier been observed in plant (Wojciechowska and Kocik, 1983) and animal (Manna and Das,
1972) systems. Fe$_2$O$_3$ is another major constituent of red-soil. We have little knowledge regarding chromosome damaging capacity of iron, but the role of this transition metal in modulating the clastogenic capacity cannot be neglected. Fe$^{3+}$ is known to enhance clastogenicity of saliva of betel nut chewers and to decrease clastogenicity of saliva of tobacco chewers in CHO cells (Stich and Stich, 1982). Tobacco specific phenolics in combination with the transition metal can be potent clastogen (Stich et al., 1981). Involvement of other metal ions and metallic compounds present in the red-soil is less understood. The metallic oxides present in it form hydroxides in presence of water; these alkaline solutions through ionic disbalance, as assumed in case of lime, may also cause toxicity. However, compared to Al, Fe and Ca are considered as less active elements with reference to their toxicity on living organisms. Sharma and Sobti (1989) have reviewed nicely the cytotoxic, mutagenic and clastogenic efficiency of various metals and their compounds.

2.6.3.4 Lime

It constitutes an important ingredient of betel-quid, gudakhu, 'Khaini' and several other tobacco preparations. Though number of reports are now available on the mutagenic and carcinogenic potential of tobacco preparations and betel-quid not many works have been done with lime only in this line. Lime causes irritation and hyperplasia of the oral mucosa and is considered to play an important role in the genesis of oral cancer (Dunham et al., 1966; Tanaka et al., 1983; Agrawal et al., 1986). One study (Stich and Rosin, 1984) with Wister rats had shown that moderate to severe hyperplasia associated with hyperkeratosis, cytoplasmic vacuolation and invagination of the rete pegs into the papillary layer resulted due to painting of the oral mucosa for 12 months with lime. However, no chromosome damaging effect was seen following the application of of lime to cultured human fibroblasts and CHO cells (Stich et al., 1985).
The results on chromosome aberrations in bone marrow cells obtained here following single treatment corroborate the earlier finding based on in vitro exposure of human fibroblasts and CHO cells to lime (Stich et al., 1985). But repeated treatment of the higher dose particularly after 8 weeks of treatment induced significantly high incidences of chromosome damages both in somatic and meiotic cells as well as mis-shapen sperm. We fail to explain mechanism leading to production of chromosome damages and abnormal sperm. Repeated ingestion of high doses of alkaline solution surely upset the pH balance of the stomach, which may lead to the production of certain genotoxin. Thus, it may affect secondarily. Low pH condition, not high, of the culture medium has recently been demonstrated to be highly clastogenic in CHO cells (Morita et al., 1989). It is supposed to be an indirect consequence. But there is no evidence of clastogenesis in rat lymphocytes exposed to low pH (Sinha et al., 1989). Thus not all cell types suffer from this problem. In vivo situation is certainly different. At alkaline pH tobacco specific phenolics are converted to potent clastogens (Rosin, 1984). Chromosome damage due to alteration of ionic condition, both low and high, was reported earlier in grasshoppers by Manna and Mukherjee (1966). Thus the possibility of ionic disbalance leading to induction of chromosome damage cannot be overruled.

2.6.3.5 General

All the ingredients in general exhibited gradual increase of chromosome aberrations both in bone marrow cells and spermatocytes and of mis-shapen sperm with the time-course of treatment (Figs.16A-C). The same trend was also seen in animals treated with the aqueous extract of gudakhu. Bone marrow cells and spermatocytes both are proliferative in nature, and such an incidence is not expected. These substances do not also cause inhibition of cell division as exemplified by availability of the divisional stages in the
in the preparation. No particular experiment was conducted to study this, but such a prolonged treatment would certainly reflect the inhibitory effect, if they have, in the preparation. The cause for such an effect remains unknown.

Some or all ingredient(s) of gudakhu other than tobacco also contribute to the induction of genotoxicity is evident from the comparative analysis of the data on chromosome aberrations in bone marrow cells induced by water extracts of gudakhu and tobacco (Fig.18). Water extracts of gudakhu induces much higher effect than the water extract of tobacco.

Earlier in connection with acetone extracts of tobacco and gudakhu it was found that tobacco extract induced much higher effect than the gudakhu extract and on the basis of that observation it was ascertained that toxic effect of tobacco was suppressed to certain degree by the preparation of gudakhu. An analysis of the data on CAs in bone marrow cells following treatment of aqueous extracts of gudakhu and its ingredients (Fig.19) reveals that gudakhu induces higher incidence of breaks than tobacco alone. But the incidence of breaks for gudakhu was significantly less than that of the additive value for all the ingredients the doses of which tested here were in equivalent preparation to gudakhu. This seems to indicate that the gudakhu preparation has some suppressive effect. Though here gudakhu induces higher incidence of breakage it does not go against our earlier proposition that gudakhu preparation suppresses the effect of tobacco. Since in the acetone extract of gudakhu only tobacco portion is extracted this proposition is clearly documented. While the water extract contains in addition to tobacco, all other ingredients which have their own clastogenic effect. Hence, suppression of the effect of tobacco here in the water extract is not discernible. However, the proposition is based on limited data, more work with single treatment regimen using other assay systems (particularly MNT) is needed for confirmation.
Fig. 18 Histogram showing incidences of chromosome aberrations in bone marrow cells induced by water extract of tobacco and water extract of gudakhu at 24 h post-treatment. Mice were treated once only.
Fig. 19 Histogram showing incidences of chromosome aberrations in bone marrow cells of mice treated once with water extract of gudakhu, tobacco, molasses, red soil or lime. Mice were killed at 24 h post-treatment.