ACTION OF ARECANUT (*ARECA CATECHU* L.) AND ITS CHEWING FORMS ON LABORATORY ANIMALS AND ITS IMPLICATION ON HUMAN CARCINOGENESIS – AN ASSESSMENT

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Areca palm, *Areca catechu* L., is widely cultivated in several South Asian and Southeast Asian countries including India, China, Bangladesh, Indonesia, Myanmar, Thailand, Malaysia, the Philippines, Vietnam, etc. (Balasimha and Rajagopal, 2004). Its fruit or seed is called as arecanut (misnamed as betelnut in some places as it is generally chewed along with the leaves of *Piper betle* L), or *supari*. It is a fibrous, ovoid to round drupe with a central ruminant endosperm covered by pericarp (husk) which is green in colour when unripe and orange-yellow when ripe. The nut has a characteristic astringent and slightly bitter taste (Ananda, 2004). Some minimum processing of areca fruit is done in most parts of India for its marketing. One type of arecanut, called as 'red supari' is obtained by dehusking tender or unripe arecanut at different stages of its maturity, slicing or without slicing the endosperm, boiling, coating with *kali* (the concentrated liquid obtained after boiling unripe arecanut) and drying. Another type, called as 'white supari' is obtained by properly drying ripe nut and dehusking it later on before marketing as whole nut (Selvan *et al.*, 2004).In India, the antiquity of chewing arecanut goes back to 650 BC as mentioned in the work of Magha in '*Sisupala Vadha*' (Rao,1982). In other countries such as Vietnam, the antiquity of arecanut chewing goes back to the Bronze age (Oxenham *et al.*, 2002).

Since time immemorial, arecanut is being used for chewing as it is believed to have lots of medicinal properties (Aman, 1969). It has an important place in the ancient Indian system of medicine such as Ayurveda, Unani and Homeopathy (Krishnamurthy, 2008). The seeds of arecanut have been widely used in clinical practices in China and other south and southeast Asian countries (Arjungi, 1976; Li Shizhen, 2003; Pardo De Tavera, 1901; Rahmatullah *et al.*, 2009). WHO (2009) has listed out as many as 25 different beneficial effects of *A. catechu* on mankind. Chewing arecanut sweetens the breath, removes bad taste from the mouth, strengthens the gums and checks perspiration (Badanaje, 2008). It has potent antioxidant, antiulcer, antidiabetic and neuroprotective properties (Amudhan, 2011; Chempakam, 1993; Zhang *et al.*, 2009). It is also traditionally used in a number of ailments for its laxative, digestive, carminative, antiulser, antidiarrhoeal, anthelmintic, antimalarial, antihypertension, diuretic, prohealing, antibacterial, hypoglycaemic, antiheartburn properties

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(Jaiswal *et al.*, 2011). The therapeutic values and pharmacological uses of arecanut have been reviewed in detail by several workers (Amudan *et al.*, 2012; Mujumdar *et al.*, 1979; Patil *et al.*, 2009; Peng *et al.*, 2015a; Rashid *et al.*, 2015). All the seven alkaloids (arecoline, arecaidine, guvacine, guvacoline, isoguvacine, arecolidine and homoarecoline) present in arecanut possess drug-like properties (Peng *et al.*, 2015b).

The major constituents of arecanut (both green and ripe) on dry weight basis are 17.3–25.7% of polysaccharides, 11.1–29.8% of polyphenols (including flavonoids and tannins), 8.2–15.4% of fibres, 8.1–15.1% of fats, 6.2–9.4% of proteins, 1.1–2.5% of ash and 0.11–0.24% of arecoline (Shivashankar *et al.*,1976). Apart from arecoline, other minor alkaloid contents of arecanut are arecaidine, guvacine, guvacoline, isoguvacoline and arecolidine (Annamalai *et al.*, 2004). Arecoline is the main alkaloid and physiologically the most active one and has a stimulating effect on the central nervous system (Bhat, 2008). In mature arecanuts there are distinct white and brown portions and as the nut matures, arecoline, arecadine and guvacoline get segregated in the brown portion, whereas guvacine gets to the white portion of the nut (Srimany *et al.*, 2016). Polyphenols decrease with maturity, whereas polysaccharides, alkaloids, fats and fibres increase with maturity of the nut (Mathew *et al.*, 1964). All the major chemical constituents of arecanut, including arecoline decrease significantly while drying and storing with husk as whole nuts compared to fresh mature nuts (Chempakam and Saraswathy, 1985) and also while roasting, soaking and boiling (Awang, 1988).

In spite of the beneficial effects of arecanut, lots of controversies are reported on its adverse effects, including carcinogenesis (Chaturvedi *et al.*, 2014; IARC, 2004). Major contributions on the knowledge on the adverse effects of arecanut were from animal studies especially on laboratory bred rodents including rats, mice and hamsters. In most of such experiments, the animals were exposed to very high doses of arecanut or its chewing forms and correlated the data for human conditions (Sumanth, 2008). In the present report an attempt has been made to review and assess the results of such studies carried out on different rodent species and how best they could be applied on human beings. For evaluation purpose the average body weight of an adult human being is taken as 60kg.

2. Studies using arecanut

Suri *et al.* (1971) studied the carcinogenic effect of arecanut on golden hamster buccal mucosa by painting the paste of arecanut mixed with dimethyl sulphoxide (DMSO) on the mucosa. The paste was prepared by using 30g of dried arecanut powder with 20 ml of DMSO and painted the mucosa three times a week for 21 weeks. In this study 38% of hamsters received arecanut extract mixed with DMSO developed tumors, whereas none developed tumors in the control group which received only DMSO. However, in this experiment, the authors did not mention or reveal the quantity of the paste they used for painting the buccal mucosa. Without knowing the quantity, it is not possible to find out the correct dosage of arecanut actually applied by them.

In another study when 0.1ml of such extract was painted to the buccal pouch of golden hamster at tri-weekly intervals

throughout the life of the animal, no tumor was developed either in the place of painting or in any of the internal organs (Ranadive *et al.*, 1976).If we calculate the quantity of arecanut used (0.1ml extract of 30g of arecanut in 20ml DMSO), it comes to about 0.15g of arecanut per animal. When this dose is converted to per kg basis, the figure is 1.5g/kg bw for hamster (the average body weight of hamster is 100g). It was reported that in human being the habitual chewers chew arecanut along with other ingredients 5-20 times a day and in each time they take about 1.5g of arecanut for chewing or 0.125 to 0.5g/kg bw (Rao and Das, 1989). This quantity is much on the lower side than observed as safe for hamsters.

Similar studies were also carried out on C17 mice, by painting 0.1ml of such extract on the interscapular region at tri-weekly intervals throughout the life of the animal. Control groups were maintained by painting with DMSO only. The results showed that at this concentration, no tumor was developed either in the place of painting or in any of the internal organs of the treated mice during their entire life, though one mouse in the control group which received only DMSO showed pancreatic tumor (Ranadive *et al.*, 1976).If we calculate the quantity of arecanut used (0.1ml extract of 30g of arecanut in 20ml DMSO), it comes to about 0.15g of arecanut per animal. When this dose is converted to per kg body weight, the figure is 5g/kg bw for mouse (the average body weight of a mouse is about 30g). It was reported that in human being the habitual chewers chew 0.125 to 0.5g of arecanut/kg bw (Rao and Das, 1989). This dose is nearly 10 times lower than the safe dose observed for mice.

In another study, it was observed that the application of both acetone and dimethyl sulphoxide extracts of arecanut on the skin of laboratory mice (both in normal and immune suppressed conditions) for nearly two years did not exhibit any carcinogenic activities (Kumari, *et al.*, 1974). The extracts were neither found to initiate nor promote tumor growth. Significant observation in their study was that the arecanut extracts even exhibited a retarding and / or inhibitory effect on the development and growth of tumors induced by a known chemical carcinogen 3:4, benzpyrene (BP). When the mice skin were applied with 5µg of BP alone, all the animals showed tumors within 39 weeks of exposure, whereas, none of the BP + arecanut extract exposed mice showed tumors during that period. At the end of 45th week, when all BP exposed animals showed large tumors, only 25-33% of the BP + arecanut extract treated mice showed tumors, and significantly they were smaller and fewer in numbers. The extract of *A. catechu* seeds was found to arrest the growth and multiplication of several human cancer cells such as MCP-7 breast cancer cells (Anajwala *et al.*, 2010), SGC-7901 gastric cancer cells and SMMC-7721 liver cancer cells (Xing *et al.*, 2010).

Ranadive *et al.* (1976) also studied the carcinogenic potency of arecanut on Swiss mice by injecting the extract subcutaneously. The aqueous extracts of arecanut were prepared using 50g of arecanut in 100ml distilled water and injected subcutaneously at the rate of 0.2ml for six days per week to experimental animals. In this study it was noticed that nearly 60% of the animals showed tumor at the site of injection, but not in any of the internal organs which were found totally free of tumors. On

calculation, it looked as if the dose selected for injection by the authors was much on the higher side. They prepared the extract using 50g of arecanut in 100ml of water and injected 0.2ml of such extract. This equals to the extracts made out of 100mg of arecanut. When this is injected to a mouse (average body weight 30g) the quantity comes to 3.33g of arecanut /kg. At this rate the normal human adult is to be injected subcutaneously with the extract obtained from nearly 200g of arecanut or 25 mature dry arecanuts – a very high dose indeed. Further, the study carried out by injecting the arecanut extract to mice is not at all relevant for human health point of view as no human being is injecting himself with the extract of arecanut for any reason whatsoever.

Rao and Das (1989) evaluated the carcinogenicity of five commonly used arecanut types (processed in different ways) on Swiss mice by oral feeding. They tried ripe unprocessed sundried (R-UP-SD), ripe processed sundried (R-P-SD), unripe processed sundried (UR-P-SD), ripe unprocessed sundried water soaked (R-UP-SD-WS) and ripe unprocessed undried water soaked (R-UP-UD-WS) arecanuts on these animals by oral feeding continuously for 12 months. Two experiments were conducted. In one experiment the powdered arecanut was mixed with the normal diet at a concentration of 0.25%, 0.5% and 1.0% of the feed and fed to them. In another experiment the arecanut was administered orally in the form of paste at 0.25g, 0.5g and 1.0g/kg bw twice daily. In both the trials, the animals were free from any type of tumors before the commencement of experiment. In the first group of experiments where the mice were fed with the diet containing different types of arecanuts, both the concentrations of 0.25% and 0.5% of arecanuts were found safe for R-UP-SD (White chali, common type in Mangaluru region), R-P-SD (Red whole nuts, common in Shivamogga region) and UR-P-SD (Red split nuts, again very common in Shivamogga region) types. Similarly, in the second group of experiments where the mice were given arecanut by oral administration in the form of paste, both the doses of 0.25gx2/kg bw/day and 0.5gx2/kg bw/day were found safe for R-UP-SD and UR-P-SD types. Further, there was no significant change in weight gain by all these animals when compared to control. From these experiments it is clear that white chali and red split types (the most common arecanut types in Indian market) were safe at a dose of 0.5gx2/kg bw/day (=1.0g/kg bw/day) for these animals. In the same paper, it was also reported that in human being the habitual chewer masticate 0.125 to 0.5g of arecanut/kg bw per day.

The study conducted to identify the toxicity effect of arecanut extract to Sprague-dawley rats revealed that the extract was not at all toxic to these rats. The LD50 value of arecanut extract to these rats was found to be ≥15.000mg / kg bw (Sari *et al.*, 2014). The authors even suggested that the extract of arecanut could be used in pharmaceutical formulations.

3. Studies using arecoline, arecaidine and tannin of arecanut

Arecoline and arecaidine are the two important alkaloids of arecanut and tannin is its major polyphenol. Boiland and Nery (1969) studied the lethal doses of arecoline and

arecaidine to Chester Beatty rats by giving single intraperitoneal injections to adult male rats in laboratory. They conducted these experiments by using synthesized compounds purchased from the reputed Chemical laboratories. From these experiments they recorded an LD50 value of arecoline to these rats as 40mg/kg bw. As per the arecoline content of arecanut which is about 0.24% (Shivashankar *et al.*, 1976), the dose of 40mg of arecoline/kg bw comes to about 16.70g of arecanut /kg bw. At this rate for 60kg body weight (average weight of an adult human being) the figure goes beyond 1000g of arecanut. The LD50 for the other alkaloid, arecaidine was still higher and reported to be 0.80g/kg bw for these rats.

Bhide *et al.* (1979) studied the carcinogenicity of arecoline extracted from arecanut against two mice, C17 and Swiss mice by oral feeding. Both the mice were fed with arecanut extract containing 1.5mg of arecoline daily by intragastric tube for five days in a week for the entire life of the animal starting from 8-10 week of age. They found that the extract induced tumors in 25% of C17 mice and 58% of Swiss mice in internal organs. Here too the dosage tried looked to be much on the higher side. To get 1.5mg of arecoline one has to use 625mg of arecanut. This comes to about 20g of arecanut/kg bw. Again, this is a very high dose when compared to a habitual chewer of arecanut, who masticate around 0.125 to 0.5g of arecanut/kg bw per day (Rao and Das, 1989).

Panigrahi and Rao (1982) studied the chromosome-breaking ability of arecoline in mice by injecting 0.25, 0.5, 1 and 2mg of arecoline for different days ranging from 10 to 30. They reported weak chromosome-damaging effects of arecoline in test animals. To get 0.25mg of arecoline we should have at least 100mg of arecanut. If we consider the weight of a mouse as 30g, 100g of arecanut per mouse is equal to 3.3g of arecanut/kg bw. Accordingly, for the other doses such as 0.5, 1 and 2mg the quantity of arecanut comes to 6.7g, 13.3g and 26.7g/kg bw. To get these doses, an adult human being should be injected with the arecoline extracted from 198g to 1,602g of arecanut, indeed a very high dose. Further, as stated earlier, these results are not at all relevant for human being as no one is injecting himself with the extract of arecanut for any reason whatsoever.

Bhide (1985) studied the carcinogenicity of arecoline extracted from arecanut on Swiss mice by injecting as well as by gavage feeding. One ml of arecanut extract containing 1.5mg of arecoline was injected to these mice subcutaneously as well as intraperitonially (ip) for five days a week until they are moribund. The results showed that the mice treated with ip injections did not develop any tumors, whereas 60% of the mice received subcutaneous injections developed tumors at the site of injections. When the mice were given the same dose of arecanut extract by gavage feeding five days a week, 47.4% of animals showed tumors in internal organs. Again, the dose of 1.5mg of arecoline per mouse (works out to 20g of arecanut/kg bw) is very high as in the case of his earlier studies mentioned above. Even in such high dose of arecoline, the reason for not getting any tumor in animals exposed to ip injection is not explained by the author.

In order to provide experimental evidence for a safe clinical dose of arecoline, Wei *et al.* (2015) studied the toxicity of arecoline

hydrobromide to Wistar rats by administering three different doses of arecoline, 1000mg/kg, 500mg/kg and 100mg/kg bw by gastric lavage for 14 consecutive days and observed that the arecoline at a concentration of 100mg/kg bw/day was safe to these rats. As per the arecoline content of arecanut which is about 0.24%, this dose comes to be around 40g of arecanut /kg body weight.

Similarly, the carcinogenicity of tannin fraction of arecanut was also studied both by injections as well as by gavage feeding (Bhide, 1985). When the mice were injected with 0.1ml of arecanut extract containing 1.9mg of tannic acid by subcutaneously, 80% of mice developed tumors at the site of injections, but those received tannic acid through ip injections none of them developed any tumor. Similarly, when the mice were fed with arecanut extract containing 1.9mg of tannic acid by gavage feeding no tumor developed in any of the internal organs. These observations clearly indicate that carcinogenicity is a complex mechanism involving several factors most of them are still unknown to us which are actually responsible for such actions.

4. Studies using betel quid

Betel quid is a mixture of arecanut, betel leaf, slaked lime and certain condiments. In certain areas such as Papua New Guinea, Taiwan, etc., betel inflorescence, betel fruits or betel stem are used instead of betel leaf (Lin *et al.*, 1997). The betel quid is either chewed as such or chewed by mixing with tobacco as per the preference (Gandhi *et al.* 2005).

Dunham and Herrold (1962) studied the effects of betel quid without tobacco on the buccal epithelium of 375 golden hamsters by incorporating all the component substances of betel quid used in several geographical areas of Maryland of United States in pellets of bees wax and inserting it into their cheek pouches. No malignant tumor was developed in the epithelium of treated hamsters cheek pouches. However, in the control experiments using two carcinogenic hydrocarbons: (7, 12 – dimethylbenz (a) anthracene and 3-methylcholanthrene) incorporated in bees wax pellets, malignant tumors were developed in the cheek pouches. This showed that betel quid was not dangerous on hamsters buccal epithelium.

Similar results were also observed by Kumari, *et al.* (1974) on laboratory mice when they were applied with the betel quid extracts on their skin. The betel quid included 100g of betel leaves, 50g of cured arecanut and 4g of lime. The extract was applied thrice a week for a period of two years, but neither any lesion nor any tumor developed in the treated area. One interesting observation was that even after applying an initiator such as croton oil for 16 times, twice a week, the betel quid treated mice did not get any tumor.

In a study on the carcinogenic effect of Taiwan betel quid consisting of fresh green arecanut sandwiched with an unripe betel fruit and slaked lime on the buccal pouch of hamster, it was found that even after painting the buccal pouch of hamsters for 14 weeks with the extracts of betel quid purchased from the local market at Taiwan, no visible tumors were observed (Lin *et al.*, 1997). In their experiment, a mixture of fresh green arecanut (450g), unripe betel fruit (120g) and slaked lime (50g) were ground together into a paste and extract was obtained

using 300ml DMSO. In other treatments, one using the known carcinogenic hydrocarbon DMBA alone, and another with betel quid + DMBA, both developed tumors. This showed that the betel quid prepared and marketed for chewing purpose in Taiwan was safe on hamsters buccal pouch.

5. Studies using pan masala

Like betel quid, pan masala is another form of chewing product containing arecanut. But it is in dry form and available in the market in different brand names in sachets. Apart from arecanut, it also contains lime, catechu, condiments and certain flavoring agents (IARC, 2004). Generally pan masala does not contain tobacco. When tobacco is mixed with pan masala it is called as *gutka* or *khaini* (Gandhi *et al.*, 2005).

Adhvaryu *et al.* (1989) carried out several *in vitro* studies on the genotoxicity of pan masala (without tobacco) against the ovary of hamster. The results were quite interesting and debatable. The ovary cells of hamster were cultured and maintained in standard growth medium. They were exposed to three different doses, 10 µl (equivalent to the soluble constituents of 1.11mg of pan masala), 20 µl and 50 µl of aqueous extract of pan masala along with one control. Data were obtained on sister chromatid exchanges (SCE) and chromosome aberrations (CA) and they concluded that the extract of pan masala was genotoxic at these doses to hamsters ovary. In the case of SCE, the results showed that in the group treated with 10 µl/ml extract the value was 9.64. Even in the control group without any pan masala extract, the value was as high as 6.76. Similar results were noticed in CA trial also. The treatment with 10 µl/ml extract showed 11% aberrations, whereas the control group without any pan masala extract showed 7% aberrations. From these studies it was clear that even the standard growth medium (control group) was quite genotoxic. The authors could not give any clear explanation for this sort of observation. In such conditions it was not appropriate to draw any solid conclusion. Further, these studies were conducted by applying the extract of pan masala directly on the cells. Generally, in the case of human beings, the pan masala is chewed and either spit out or swallowed but definitely not injected or applied to cells. Further, the metabolism of human digestion is quite complicated and one has to take into consideration all such aspects before suggesting to restrict pan masala chewing by humans.

Ramchandani *et al.* (1998) studied the carcinogenic activity of pan masala on mouse skin by topical application and on internal organs by administering by gavage. In both the experiments, three concentrations (12.5mg, 25mg and 50mg) of the ethanol extract of pan masala were tested. In the experiment by topical application on bare skin, there was no sign of toxicity or change in body weight in the treated mice when compared to control. Even multiple applications of 25mg or 50mg of pan masala extract for 40 weeks did not induce any tumor. Similarly, administration of pan masala extract to mice continuously for 6 months also did not induce any tumor in all the three dosages. On calculation, 50mg of pan masala per mouse is equal to a dose of 1.67g of pan masala/kg bw. This dose comes to be around 100g of pan masala for an adult human being. The maximum per day consumption of pan masala by one

individual in urban areas of Punjab was calculated to be around 30g (Gandhi *et al.*, 2005). However, slight variations might occur from place to place.

Bhisey *et al.* (1999) evaluated the carcinogenicity of pan masala by feeding a mixture of food containing 2.5% and 5.0% of pan masala to albino mice for their entire life span and reported that it was carcinogenic to experimental animals at both these doses. They further reported that the experimental animals had consumed 3.8g of pan masala mixed food. This means that an adult mouse got a daily meal of 0.1g to 0.2g of pan masala at 2.5% and 5.0% concentration, respectively. This comes to be around 3.33gand 6.66g of pan masala/kg bw, respectively at the above two concentrations. In order to get these dosages, the average adult human being should consume daily about 198g to 396g of pan masala. Ramchandani *et al.* (1998) had already arrived at a safe dose of 1.67g of pan masala/kg bw per day for mouse. This comes to be around 100g of pan masala for 60kg body weight.

Nigam *et al.* (2001) evaluated the toxicity of pan masala in Swiss mice by feeding normal diet containing 2% pan masala for nearly 80weeks and found that as many as 8.3% of animals in the control group and 33% of animals in pan masala group had developed tumors after 56 weeks of exposure. The actual quantity of food consumed both by experimental and control group of mice was not mentioned in the report. Without that it is not fair to arrive at any definite conclusion. Further, the authors did not explain the reasons for getting 8.3% tumor in the control group as well.

6. Conclusion

Arecanut is reported to have lots of medicinal and pharmacological properties. Studies conducted on certain rodents such as albino rats, mice and hamsters have confirmed that the arecanut was safe to these animals at a normal dose of 1.5 to 5g/kg bw per day. This comes to 90 to 300g of arecanut for 60kg bw per day. Even the betel quid and pan masala were also found safe to rodents in normal doses. The safe dose of pan masala (without tobacco) to these rodents was found to be 1.67g /kg bw per day. At this rate the safe dose for 60kg body weight comes to around 100g of pan masala per day. Arecanut and its chewing products caused problems in experimental animals only in higher doses. For that matter 'any medicine if taken in large dose can be poison and poison taken in small dose can be medicine'.

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